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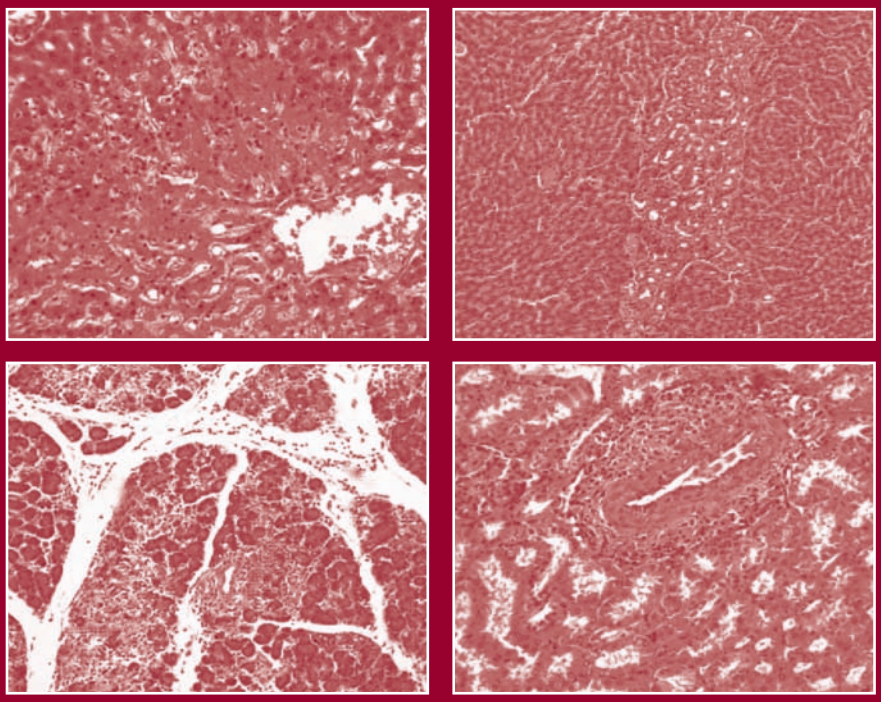

edited by
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Preclinical Drug Development

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PREFACE

As editors of the second edition of *Preclinical Drug Development*, we are pleased to present this updated text on the science of safely moving therapeutic candidates into, and through, clinical development. In the few years since we published the first edition, additional understanding and establishment of new technologies for preclinical evaluation have occurred. For this reason, this edition has been published. Readers of this edition will find updated and expanded chapters covering key content areas that include pharmaceutical profiling and lead molecule selection, interspecies differences in physiology and pharmacology affecting extrapolation to humans, pharmacokinetics (ADME) of both small and large molecules, pharmacokinetic-pharmacodynamic modeling and simulation of preclinical data, role of membrane transporters on drug disposition, toxicity evaluations, application of pathology in safety assessment, and utilization of preclinical data to support clinical trials. This edition also has limited emphasis on transgenic animals. While these genetically altered animals still provide tremendous insight into drug candidate selection and understanding of therapeutic potential, they have stalled with respect to becoming a mainstream technology for assessing safety (e.g., carcinogenicity) or pharmacokinetics (e.g., drug metabolism). On the other hand, the text provides greater detail and discussion on formulation and production strategies to improve bioavailability, as overcoming poor solubility can be essential for achieving optimal therapeutic outcomes. In short, the reader will find that this new edition provides updated information on core preclinical development topics with insight into key evaluation issues and strategies.

Like the original edition of *Preclinical Drug Development*, we have kept the content of this edition at a level that gives the reader a basic understanding of the elements that constitute a preclinical database. Many excellent textbooks are available that focus on these specific areas in significant depth and detail. We encourage the reader to use those resources for their specific area of responsibility and interest.

We believe this textbook will serve as a solid foundation for the reader to understand the basic science and order of therapeutic compound development. The scope covers all important elements that provide insight on in vitro and in vivo safety assessments, pharmacokinetic and toxicokinetic evaluations, and the bridging exercises that permit extrapolation between nonhuman and human species.

Our work in preclinical drug development is neither benign nor routine. For each new molecule a fundamental understanding of its physical/chemical properties must be known. There must be a reasonable understanding of the physiological pathway that is intended to be affected by the molecule. Understanding off-target binding and consequences of that binding must be known. Every unique molecule will have its own absorption and disposition characteristics—Where does the molecule go when it leaves the circulation? Does it get metabolized during residence in a specific organ or tissue? How long does it reside in a given tissue? How is the drug candidate and its metabolites eliminated from the body? Without a database that contains these elements, an informed first in human dose cannot be estimated nor can a safe human dose escalation or progression through clinical trials be achieved.

For the purposes of human therapeutic development, it is imperative to define the safety database as knowledge of what is known and also what is *not* known (the safety margin). The safety margin is proportional to the lack of knowledge on the molecule, its activity, and pharmacokinetic properties. While large safety margins may offset the lack of knowledge on the molecule, those same margins also potentially engender inefficiency in wasted time and financial resources during human development. It is in the best interest of a drug development

team to properly assess the safety and pharmacokinetic characteristics of a molecule early since human drug development is invariably much more expensive than nonhuman development. Notwithstanding cost and time issues, an ethical consideration must also predicate each decision on the need for an animal study versus directly gaining knowledge in humans. We hope this text will give you the appreciation necessary for safe and efficient drug development practices.

Creating this second edition has been challenging and time-consuming. We are indebted to each of the chapter authors; our appreciation for their efforts cannot be adequately expressed. We are also indebted to our families for their support and patience. Time is precious and the efforts to publish this book on schedule have competed with family priorities.

We hope that each of you, the readers of this book, will find it valuable. If we have achieved our intended goal, the text will bring insight and avenues to meet your own development programs.

Mark C. Rogge, Ph.D.
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1 The Scope of Preclinical Drug Development: An Introduction and Framework

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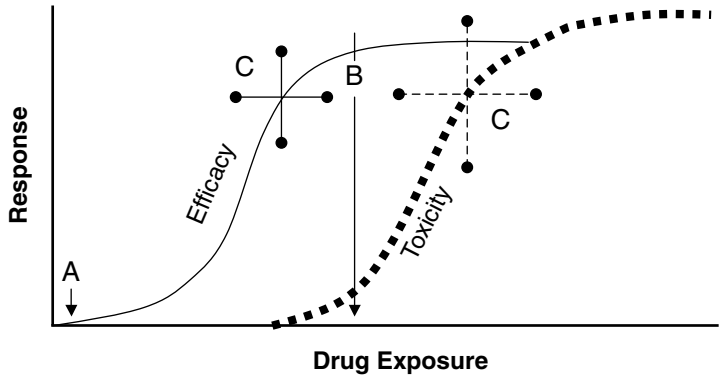
The science of preclinical drug development is a risk-based exercise that extrapolates nonhuman safety and efficacy information to a potential human outcome. In fact, the preclinical development program for many novel therapies is an exercise in predicting clinical results when little data support the use of the animal model under study. In the end, human studies validate the nonhuman models. Yet, understanding preclinical drug response-–pharmacologic and toxic—with respect to dose, frequency, and route enables the clinical scientist to initiate and continue human trials under rational and ethical conditions. These conditions include a starting dose and a dose frequency that produces an intended level of pharmacologic response, a safe dose escalation scheme that permits differentiation of response as a function of drug exposure, and an understanding of when potential toxicity may outweigh potential additional pharmacologic benefit (Fig. 1). Of similar significance but often underappreciated is an understanding of pharmacokinetics (PK) and response *variability*-–sources, type, and magnitude. While we report and often predict a PK outcome or pharmacologic effect, there is rarely a patient with an average PK exposure or response outcomes. Hence, there is little likelihood that an average pharmacologic response and adverse event profile will occur in any single patient. Rather, it is often diverse populations of individuals that receive pharmaceutical therapies. Phenotype and lifestyle variability affect body composition and mass, drug transport and metabolism, and sensitivity to pharmacologic as well as toxic drug effects. Understanding the sources of variability and the magnitude of that variability early in the development process permits clinical trial conduct that is most efficient and less likely to be encumbered with unanticipated events.

The realm of preclinical drug development can be compartmentalized into three disciplines, which work in parallel from the stage of late research through clinical development. Two of these disciplines, PK and pharmacology/toxicology, are the subject of this text. The third discipline, bioanalytical research and development, is outside the scope of this text and the reader will find many excellent publications elsewhere that are devoted to state of the art in bioanalytical technology.

Before discussing the elements of a preclinical development program, some comments on the regulatory environment should be considered. The fundamental mandate of regulatory agencies is to ensure that clinical trials are conducted in a safe manner and that only drug candidates shown to be safe and effective are approved for commercial use. Early, scientifically rigorous interactions between a regulatory agency and industrial scientists will ensure that all concerns are addressed and that common objectives are achieved.

The U.S. Food and Drug Administration (FDA) operates an active program designed to understand and utilize preclinical models as predictors of human xenobiotic exposure. The organization conducting this research is the Division of Applied Pharmacology Research and it resides within the office of Testing and Research (www.fda.gov/cder/offices/otr/APRdefault.htm). Nonprofit organizations outside of FDA, such as the Health and Environmental Sciences Institute (HESI, www.hesiglobal.org), also contribute to the development of more predictive and alternate models of safety assessment. One of the most notable and active preclinical assessment initiatives is the National Center for the Replacement, Refinement and Reduction of Animals in Research (www.nc3rs.org.uk).

During the FDA's drug review process, members of the pharmacology/toxicology staff within the office of New Drugs and members from the office of Clinical Pharmacology and Biopharmaceutics serve as the preclinical experts throughout a drug development program.



A: Starting dose, dose frequency, route of administration.

B: Toxicity risk outweighs benefit of increased efficacy.

C: Variability in response may have endogenous and exogenous sources. Either source can affect exposure or sensitivity of response for a given dose, frequency, or route.

Figure 1 Exposure to drug product increases the likelihood of efficacy but also the potential for toxicity. For any given exposure, a probability distribution will exist for both efficacy and toxicity outcomes.

High-quality, efficient interactions with regulatory authorities are requisite for the development process to proceed smoothly. These interactions occur when the sponsor understands the regulatory requirements necessary to progress a drug candidate to the next stage of development. The sponsor must also bring knowledge of established (validated) technology related to the drug candidate's development and must understand the state-of-the art technology that may also bring value to the drug candidate's development. While not necessarily validated, this technology may offer substantial value to the development of the therapeutic candidate (Fig. 2).

The established technologies and study designs carry the value of being validated, generally well-controlled, and having reference to historical databases. New technologies are typically not validated and, by their definition, do not have a relevant historical database for reference. Also, new technologies carry an inherent risk in their value. Certain new technologies may accurately and precisely measure a cellular event such as signal transduction, mRNA expression, or protein expression. However, until it is confirmed that these events robustly correlate with a therapeutic or toxic outcome, the technology carries a high-risk value. Interpretation of data

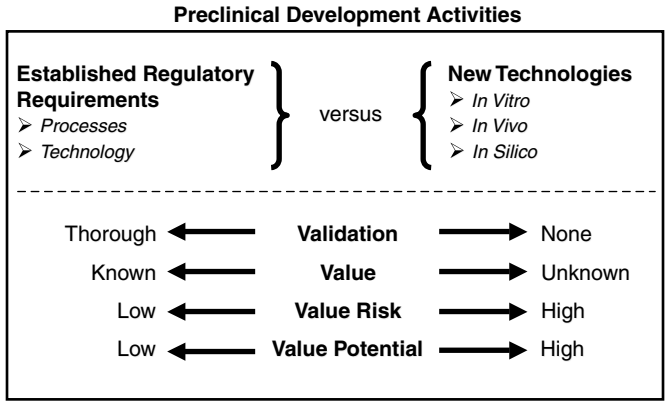


Figure 2 Novel therapies and next generation therapies are predicated on the use of established as well as new, but not yet validated, technologies. The inherent risk in new technology is unknown and may be high.

obtained from these technologies must be limited and not overweighted when making human dose regimen decisions.

Nonetheless, the potential value of new technologies must be recognized and their utilization should be considered. Pharmacogenomics and toxicogenomics are in early stages of assessment and each has a considerable potential value. This potential value will materialize when these technologies develop validated standards and their output can be correlated with a reasonable probability to clinically meaningful outcomes.

The International Congress on Harmonization (ICH) has established a basic repertoire of guidelines that outline the technical requirements of acceptable preclinical drug development (www.ich.org). Also, the Center for Drug Evaluation and Research (CDER) has compiled a series of guidelines to assist the innovator with development issues; these guidelines may be found at the FDA website (www.fda.gov).

With the ongoing implementation and refinement of guidelines from ICH, the geographic regions of the United States, Europe, and Japan have standardized approaches to the drug development process. However, while these guidelines provide a flexible and innovative basis for preclinical drug evaluation, they serve as a minimum requirement for achieving drug approval. It is generally accepted that additional studies may be required during the development process and that regulatory authorities among the three regions may have different scientific opinions on an acceptable preclinical development program.

The reader is cautioned that the ICH guidelines constitute only a minimum requirement and rarely encompass a development program that will be acceptable to the innovator company or all regulatory agencies. While the reader is encouraged to review and utilize these guidelines, a rational preclinical information database is fundamentally focused on minimizing clinical risk. The preclinical database serves to predict (*i*) drug absorption and disposition and (*ii*) the physiological outcome from exposure to that drug and its metabolites.

Figure 3 represents a temporal schematic of issues that are commonly addressed throughout the preclinical development program. In this example, the drug candidate might treat a chronic illness in a diverse patient population. A drug intended for acute or intermittent use or a drug intended for a narrower patient population might have fewer issues to consider and thus fewer studies in the program.

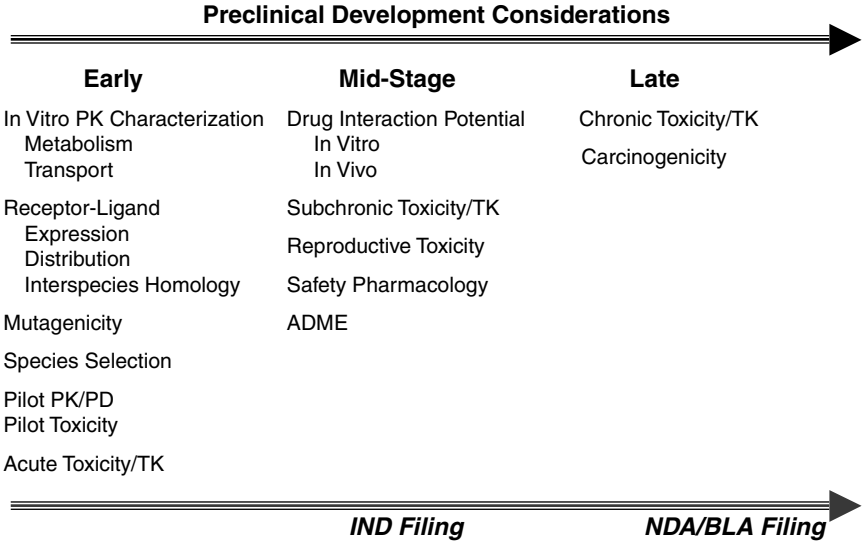


Figure 3 Preclinical development programs begin prior to investigational new drug (IND)–enabling work and extend through the clinical development stage. Each program is unique and is dependent on the intended therapeutic use, the potential patient population, and historical reference. The following program might be acceptable for treatment of a chronic illness in a diverse population.

Figure 3 also illustrates that understanding the similarities and differences between nonhuman and human physiological systems is vital to obtain quality information from the program. Virtually every study and every decision to be made on the development of a drug candidate will be predicated on the assumption that preclinical models are a predictor of human exposure.

Shapiro addressed the issue of animal models that mimic human disease states and his thoughts can apply to the broader scope of this text (1). Quantitative validity of an animal model may have less value than the productive generativity of a model. While it is unlikely that anyone will ever validate a nonhuman model in a true or absolute sense, the nonhuman model will generate a body of evidence and confidence that the drug candidate is worthy of further development or should be terminated from the development pipeline.

Conversely, a thorough understanding of any nonhuman model is fundamentally important so that drug-related outcomes can be separated from normal, endogenous variability or other processes unrelated to the drug. Rodents, canines, and nonhuman primates have become common preclinical models, not always because of their strong direct relevance to potential human outcome but because of the established understanding of these animals and their underlying physiology (2–4).

In the following chapters, preclinical drug development will be reviewed in a sequence consistent with the current rational and efficient practices. The reader will be introduced to animal models, species selection, and then to chapters on definitive PK, pharmacodynamic, and toxicology evaluations. Other chapters describe formulation impacts, alternative technologies, and the relationship between preclinical findings and the clinical setting.

Looking into the future, the scientist who is engaged in preclinical drug development will more than ever factor innovation into the balance of risk versus benefit (5,6). Even after rigorous preclinical and clinical evaluation, the potential for drug toxicity can be profound. For example, U.S. drug R&D expenditures for 1995 were $15.2 billion and had nearly doubled to $30.5 billion in 2001. Yet, in the United States alone over 100,000 patients die each year as a result of drug side effects (7, 8). Furthermore, an additional two million patients require hospitalization or extension of existing hospitalization each year to treat drug side effects. While current preclinical safety assessments generally identify drug candidates with systematic and high probability safety concerns, they remain insensitive to nonsystematic toxicity or to conditions that increase the risk of known toxicity.

There are limitations on how safe and efficacious a drug candidate can be made based on formulation, route of administration, and dose regimen. Hence, the best opportunity for achieving success lies with drug candidate selection. This is common sense but not often appreciated. Intelligent drug candidate selection incorporates but is not limited to knowledge of a molecule's (*i)* absorption, distribution, and metabolism properties; (*ii)* binding affinity to the intended pharmacologic receptor(s); and (*iii)* toxicity potential. Indeed, a 10-fold reduction in binding affinity may be more than offset by a bioavailability that has been improved by only twofold to threefold, since increasing bioavailability reduces variability in absorption. For example, a drug with just 10% bioavailability has intrinsically poor absorption properties that may include poor solubility, dissolution rate, permeability, or metabolic instability such as first pass metabolism. Consequently, dose-to-dose bioavailability may range between 5% and 20% (and likely more). In this case, the fourfold fluctuation may give rise to subtherapeutic or toxic target tissue concentrations in some or all of the patient population and could likely lead to treatment failure. It is intuitive that variability in serum drug concentrations has less magnitude when absorption approaches 100%, and therefore, high bioavailability plays a very significant role in determining the best lead candidate. In turn, it can be anticipated that as intrinsic bioavailability increases, the impact of food, age, and other factors on absorption will decrease. Clearly, in the quest for more potent and target-specific drugs, a similar effort must be exerted to achieve greatest bioavailability.

With respect to screening for drug clearance, numerous validated technologies are available to assess the potential for metabolism and likely routes of elimination (9–11). Greater utilization of human recombinant enzymes, cells, and tissues will accelerate our insight into appropriate selection of lead candidates for preclinical and clinical development. Likewise, isolated perfused organs can provide valuable insight into potential sites and mechanisms for drug metabolism and excretion.

Together, these technologies offer significant value in generating rank order information on lead drug candidates. In addition, they provide an early understanding of potential variables that may impact absorption or elimination.

With a lead drug candidate in hand, a more thorough assessment of drug disposition and elimination is undertaken. Tissue accumulation, sequestration, and metabolism strongly influence the profile of pharmacologic effect and also give early indication on sites of potential toxicity.

Most promising in the advancement of PK and toxicology are the technologies that enable greater quantitative information to be gained on drug disposition and toxicity while using fewer animals. Advanced physiologically based pharmacokinetics (PBPK) and mixed effects modeling offer insight into drug disposition that can provide immediate value to the toxicologist and can also be extrapolated to potential human exposure (12,13).

Mahmood and others have published extensively on interspecies scaling techniques (14,15). The prediction of drug distribution volume, clearance, and half-life provides a rational basis for prospective preclinical and clinical study designs. While providing significant value to the development team, these predictions also carry uncertainty and the scientist using the information must respect that caveat. Profound differences in anatomy and physiology between the preclinical species and humans can challenge the relevance of allometric scaling and, for that matter, all preclinical work. While rats have a lifespan that would not likely exceed 2 to 3 years, the lifespan of a human can exceed 90 years. While rats have heart rates of approximately 360 beats per minute, the human heart rate is approximately 65 beats per minute. Respiratory metabolism, measured as O_2 consumption, is approximately 0.84 and 0.20 mL/hr/g in rats and humans, respectively. Similarly, there are also substantial differences in various organ blood flows, relative organ weights, and tissue architectures (16). Simple cross-species extrapolation of doses, dose frequencies, or distribution of drug into tissues is likely to generate data with little predictive value.

In parallel, recent advances in identifying and quantifying gene expression and signaling processes permit mechanistic insight into drug activity and toxicity (17,18). Validation of new in vitro methods for toxicity assessment will further reduce animal use and increase the likelihood of a molecule entering clinical trials (19,20).

In summary, the understanding of preclinical drug disposition—distribution, metabolism, and excretion-–coupled with an understanding of cell- or tissue-specific activity/toxicity completes the knowledge base for a drug candidate to move into and through clinical evaluation. This understanding is achieved by generation of a clinical strategy that is then used to draft the initial preclinical plan.

Few, if any, preclinical plans remain intact throughout their lifespan. It should be anticipated that as studies are completed and observations are confirmed, ongoing and future studies are likely to require modification.

Throughout all development programs, it is imperative that the preclinical scientist assesses each study prior to implementation. What questions must be answered by the study? Do those questions warrant animal use or can in vitro methods be utilized? Does the proposed study have a high likelihood of answering those questions? If so, will the answers affect the subsequent clinical development? No study should ever be conducted unless there is clarity in the study goals and expectations on how much risk is being eliminated from the clinical program by conducting the study.

A scientifically sound preclinical program permits efficient, safe clinical development. The absence of such a program will promote poor decision making and potentially serious clinical consequences. In this era of the public demanding more efficacious and safer medications at less cost, the preclinical scientist oversees a vital responsibility.

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2 Lead Molecule Selection: Pharmaceutical Profiling and Toxicity Assessments

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INTRODUCTION

This chapter will focus on the techniques currently used in the selection of new pharmaceutical compounds according to their drug-like properties. These methods have applications early in drug discovery during which accurate and timely information can improve the quality of lead compounds and reduce the risk of subsequent, costly failures in preclinical and clinical testing. Many of the procedures discussed herein are based on methods previously used for many years during preclinical testing. In the past, lead compounds were selected for development on the basis of general pharmacodynamic and pharmacokinetic behavior in animal models, because a certain level of potency is a prerequisite for selecting a lead compound or series. As potency increases, more delivery routes become reasonable options. Unfortunately, selecting for high potency often selects out favorable pharmaceutical properties (e.g., solubility and intestinal permeability). Historically, this strategy has failed to reliably identify potent ligands that are also well-tolerated, efficacious medicines.

During the last two decades, there has been a marked increase in the demand for new and novel medicines exhibiting improved efficacy and selectivity. Recently, there has been an increase in the number of pharmacological targets as a result of sequencing the human genome. There are also business-related expectations that new drugs can be developed more rapidly and inexpensively. These expectations exert immense pressure to accelerate drug discovery and development and to increase success rates. In response to these demands, a strategy using parallel organic synthesis was widely adopted to prepare new and larger libraries of pharmaceutical compounds. This was followed by the necessary modification of in vitro binding and inhibition assays for primary screening of these large collections for appropriate biological activity and potency against a specific target (e.g., receptor, enzyme, second messenger). This primary pharmacological screening, using relatively impure material, is used to identify new high-affinity ligands, so-called hits, from among hundreds or thousands of new molecular entities (NME). This strategy frequently identifies many active compounds requiring further investigation after resynthesis to improve purity. These compounds are frequently also subjected to secondary screening against secondary targets (e.g., target subtypes, related targets) to eliminate the most promiscuous compounds—those more likely to manifest toxicity resulting from poor selectivity. However, information accrued from primary and secondary screening is frequently insufficient to estimate critical pharmacokinetic variables (e.g., clearance, bioavailability) and thereby select the NME most likely to become preclinical candidates and perhaps marketable medicines. Pharmaceutical profiling assays are frequently on the critical path of a discovery screening algorithm.

The majority of medicines developed for North America are intended for oral administration. Therefore, a tertiary profiling method, pharmaceutical profiling, has been adopted to help select promising lead candidates from among a number of potent and selective hits and to investigate important chemical and biological properties that confer a drug-like behavior. These assessments are frequently conducted in parallel with secondary profiling in order to further minimize the time required before nomination as preclinical candidates. The information generated by such pharmaceutical profiling experiments has been used to select and compare lead compounds according to their physicochemical properties, disposition, and toxicity. The results of pharmaceutical profiling have the potential to minimize the losses of attrition due to the failure of lead compounds in preclinical studies or human trials, reduce the time to market, and thereby help control the overall cost of drug development. It is possible that selecting lead

compounds in this way will result in the approval of efficacious and potent medications that tend to have fewer adverse side effects. This chapter describes protocols and applications of in vitro methods used in the evaluation of pharmaceutical properties.

THE EMERGENCE OF LEAD SELECTION IN DRUG DISCOVERY

The process of pharmaceutical innovation is complex, and measures of productivity are important in understanding the trends in drug development (1). The recent history of pharmaceutical industry performance suggests that despite the investment of billions of dollars in excellent human resources and state-of-the-art instrumentation, the development of new medicines remains a very risky business, one that has a record of many failures for each success. Several indices of success have been proposed including the number of NME synthesized and the number of patents issued. However, time to market and the number of new medicines approved in a given period of time may be the most practical measures of success. For example, between 1960 and 1980, the development time of new medicines, from synthesis to market, almost quadrupled and has remained relatively unchanged since 1980, with a current interval of 9 to 13 years (2). This has been accompanied by a corresponding decrease in the effective patent life from an average of nearly 13 to 6.5 years (1). Typically, from the day of the discovery of new targets and new lead structures until the decision to proceed with full-scale development, five to six years have passed (3). It has been estimated that in order to discover new lead structures, an average of 50,000 to 100,000 NME will be screened. It has been estimated that a proficient medicinal chemist is capable of synthesizing 200 to 300 NME per year (2).

The failure of drug candidates in phase I and phase II clinical trials is a major source of scientific and economic difficulty. In Britain, over a 17-year period from 1964 to 1980, a total of 197 NME were evaluated in humans for the first time (1). However, 137 were withdrawn from development and 35 (18%) continued through development; the ratio of drugs innovated to those marketed was 5.6:1 (1). This is similar to the 5.8:1 ratio reported for the United States and less than the 13.5:1 reported for Swiss companies (1). The major reasons for withdrawal included inappropriate pharmacokinetics in human subjects (39.4%) and a lack of clinical efficacy (29.3%). The incidence of unexpected toxicity or more subtle adverse effects was the third most common reason for termination (10%). Every excess year of drug development is an unnecessary use of resources that could be applied elsewhere. Thus, time also becomes a very important factor in drug development (3). A rough calculation of daily revenues lost on a new medicine with an average market potential suggests that each day of delay in getting NME to market is approximately US$2 million. Thus, the increasing cost of drug development is associated with prolonged development time and increasing regulatory requirements. In 1987, expenses for the development of NME averaged US$300 million to US$400 million. Currently, in the United States, the costs of drug research and development have increased between US$800 million and US$1 billion per approved medicine (4).

As indicated above, typically a large number of NME enter preclinical development for each one emerging as a new medicine (5). Furthermore, most of the escalating costs of drug development are associated with late-stage development. Therefore, poor candidates must be identified as early as possible and immediately terminated from development. In many cases, a sponsor's success depends on how the attrition of these compounds is managed. In order to manage attrition properly, however, the reasons why compound development slows down must be examined. The drain of valuable resources by slowdowns can be much more damaging to overall success than that arising from outright terminations. Some of the reasons for slowdowns are strategic in nature, others are strictly attributes of the individual NME, and some are attributable to how an organization makes these decisions. Poor pharmaceutical properties contribute significantly to both failures and slowdowns in development. In fact, poor drug-like characteristics can lead to consumption of large human and dollar resources throughout the development timeline. In the past, an NME with excellent potency and selectivity, but with poor pharmaceutical properties, was rarely disqualified from entering the development pipeline.

Given the record above, it is perhaps not surprising that screening a very large number of NME appears to be one logical strategy to improve the registration of new medicines. There is a historical precedent for this approach. The strategy of synthesis and screening of rationally designed NME was pioneered shortly after World War I. Typical molecular scaffolds

were systematically combined with many other groups that occur repeatedly among biologically active compounds. A battery of receptor binding systems, utilizing tissue homogenates, radiolabeled ligands, and animal models, helped in the characterization and optimization of biological activity. More than 50 new drugs, among them analgesics, antihistamines, neuroleptics, and antidepressants, resulted from this discovery strategy (3). However, mass screening in drug research essentially started with the testing of thousands of different sulfonamides for their antibacterial activities. However, from about 1970, large screening programs became less and less important (3), because the yield of new leads from random screening was considered to be too low compared to the necessary effort. Between 1970 and 1990, the structures of potential drug candidates were more rationally planned and the synthesized compounds were commonly tested in just one or two selected models before lead selection occurred.

The integration of automated laboratory systems, new rapid and sensitive analytical instruments, modified experimental protocols, and improved data management tools have permitted the combinatorial synthesis and testing of a substantial number of structurally distinct compounds by using similar reaction conditions (6). Many large global innovator companies have established extensive molecular libraries. The most positive consequence of using combinatorial methods to synthesize NME libraries is that several drug candidates can then be developed in parallel in order to avoid the failure of a whole program if a single compound yields negative results in its first administration to humans. Combinatorial and parallel chemistry and the enormous capacity of high-throughput screening systems allowed the synthesis and testing of thousands of compounds or mixtures per week. It has been estimated that new synthetic techniques contributed to reducing the time required to discover drug candidates by 18 to 24 months. However, the obvious disadvantage to this approach was that the number of drug candidates entering the pipeline soon overwhelmed development resources. Although it is sometimes claimed that combinatorial libraries are valuable also for lead structure optimization, this claim needs to be questioned because of the lack of appropriate starting materials for their synthesis. In general, there continues to be questions as to whether this combinatorial approach will actually deliver new and better medicines more rapidly than in the past (7).

LEAD SELECTION BY PHARMACEUTICAL PROFILING

Lead selection that employs pharmaceutical profiling has become an important bridge between medicinal chemistry/pharmacology and the nomination of high-quality lead candidates. Pharmaceutical properties are those that help us understand the barriers to appropriate bioavailability for each compound of interest. Many of the dispositional properties should be specified in a preliminary product profile developed at the beginning of a discovery program. Most of the experimental procedures discussed in this chapter are conducted in vitro in order to maximize their capacity and minimize costs. This constraint has forced the modification of existing techniques and the development of new models (some commercial products) and protocols, including the improvement and miniaturization of cell-based and cell-free assays.

Physical and Chemical Properties

The behavior of NME in biological solutions can markedly influence their success as orally administered medicines. The early determination of physicochemical characteristics (e.g., aqueous solubility, lipophilicity, and plasma-free fraction) provides useful information concerning drug disposition. More importantly, these criteria may be useful in identifying compounds in drug discovery that could be potentially difficult and expensive to take through the entire development process.

With the broad implementation of new parallel synthetic protocols, the sources of new compounds have changed significantly. Previously, lead sources included natural products, clinical observations of side effects, presentations at scientific meetings, published reports in scientific journals, and collaborations with external investigators. Typically, the most poorly behaved compounds were eliminated first, leaving a lead that possessed characteristics consistent with previous experience in discovering orally active compounds. However, this process has changed significantly since 1989, with the implementation of high-throughput screening (8).

Aqueous Solubility

There is a paucity of published reports on the determination of aqueous solubility of NME during drug discovery. However, information on the aqueous solubility of NME is valuable in selecting lead series and individual compounds. Low aqueous solubility is frequently associated with poor intestinal absorption, since permeability is proportional to concentration gradient. Formulation of poorly soluble compounds may also be difficult and time consuming, because the aqueous solubility of a drug influences its dissolution rate and therefore its rate of absorption. Furthermore, the preparation and selection of salts as a strategy for improving the solubility of the active pharmaceutical ingredient typically results in only a modest increase in solubility. The determination of aqueous solubility rarely precedes the other assessments in pharmaceutical profiling, as solubility information is of somewhat less importance when organic solvents are used to deliver NME to in vitro assays. However, poor solubility alone rarely terminates compound progression, although it does result in slower development and a lower probability of success.

It appears that two factors affect the physicochemical properties of hits arising from library preparation and primary screening. First, combinatorial and parallel methods of synthesizing NME tend to yield larger, more hydrophobic NME, and typical primary screening assays are biased toward selecting less soluble compounds (7). The latter artifact arises, in part, from the use of dimethyl sulfoxide (DMSO) as a universal water-miscible solvent in drug discovery, because the focus in discovery is on keeping NME in solution rather than measuring the actual aqueous solubility. This method alters the kinetics of solubilization and the result is that NME, added to aqueous media in DMSO, are transferred in a relatively high-energy state. Therefore, the "apparent" solubility in primary screening assays is almost always higher than the thermodynamic solubility measured by equilibration of a well-characterized solid in aqueous medium. The net result of these changes is that in vitro activity is reliably detected in NME with relatively poor actual aqueous solubility. Therefore, it is possible that lower potency hits with a more favorable pharmaceutical profile may be discarded. Solubility effects can be further complicated by the fact that products of parallel organic synthesis may differ substantially in their physical form than the purified, soluble salts available for preclinical testing. Typically, solution spectra, high-performance liquid chromatography (HPLC) purity data, and mass spectral analysis are sufficient to support the synthesis of compounds for primary and secondary screening. In most cases, NME submitted for pharmaceutical profiling are screened for aqueous solubility, because the final concentration of NME used in a typical panel of pharmaceutical profiling assays, as described in this chapter, varies considerably (1–50 μM).

The determination of aqueous solubility in pharmaceutical profiling is commonly conducted by one of the two methods, both of which may be considered to have relatively high throughput. One method is via turbidimetric measurement, a technique frequently labeled as a kinetic solubility (KS) measurement that ignores the traditional pharmaceutical precepts of thermodynamic solubility. As currently practiced (7), a concentrated stock solution (e.g., 10 μg/μL or 20–30 μM) in DMSO is added dropwise to a small volume of isotonic, buffered saline (e.g., 2.5 mL) while the absorbance at 600 to 820 nm is monitored, or the degree of light scattering by undissolved test compound is measured. An extended observation time is used in order to avoid missing slow precipitation that could affect the outcome of a biochemical experiment. Precipitation is identified with a diode array UV detector by an increase in absorbance due to light scattering by particulate matter. The method is quite sensitive to the juxtaposition of the cuvette and the detector, and intensely colored NME can cause false positive results. However, measurement of light scattering does not depend on the presence of a chromophore in the molecule. In order to maintain reasonable throughput in this assay (40–50 samples per day), the precipitation point is simply calculated from a curve fit to the absorbance versus volume of stock solution and the NME concentration is expressed as micrograms of NME per milliliter of buffer. The functional range of this assay is 5 to 65 μg/mL and the final concentration of DMSO remains below 0.7%.

Typical for assays included in pharmaceutical profiling, a set of reference compounds, normally existing medicinal products, is also screened with each assay. For example, among approximately 350 medicines selected from the Derwent World Drug Index, 87% exhibited turbidimetric solubility greater than 65 μg/mL and only 7% had solubility of 20 μg/mL or less.

Table 1 Measurement of the Kinetic Solubility of Marketed Medicines

Solubility category	Compound
Acceptable (KS $>$ 50 μM)	Verapamil
	Metoprolol
	Naproxen
	Dapsone
	Clozapine
	Amitryptiline
Marginal (KS $=$ 10–50 μM)	Terfenadine
	Estradiol
	Haloperidol
	Nicardipine
	Ellipticine
	Reserpine
Unacceptable (KS $<$ 10 μM)	Astemizole
	Amiodarone
	Paclitaxel
	Miconazole

Abbreviation: KS, kinetic solubility.

One interpretation of these results is that if turbidimetric solubility is less than 20 μg/mL, the probability of useful oral activity is quite low, unless the compound is very potent or unusually permeable or unless it is a substrate for an intestinal transporter. Furthermore, if aqueous solubility is greater than 65 μg/mL, poor pharmacological activity in vivo is probably not related to solubility (7). Table 1 provides examples of the KS of several existing active pharmaceutical ingredients as measured in a KS assay. Compounds were evaluated in duplicate in a 96-well plate by adding 1.5 μL of a concentrated DMSO solution to 300 μL of potassium phosphate buffer, pH 7.4, at room temperature. The light scattering was measured with a nephelometer. The NME were tested at 10 and 50 μM and the compounds were ranked as unacceptable (KS $<$ 10 μM), marginal (KS $=$ 10–50 μM), or acceptable (KS $>$ 50 μM).

An alternate method of determining aqueous solubility and purity has been developed. It incorporates aspects of a typical thermodynamic solubilization procedure and of the turbidimetric procedure discussed above. The assay is conducted in 96-well plates, requires only a very small amount of starting material, and it is capable of evaluating 80 NME per plate. A very small aliquot of a concentrated NME stock solution (10 mM in DMSO) is added to a small volume of phosphate-buffered saline. This solution is shaken overnight, after which the plate is centrifuged to separate the phases and an aliquot of the supernatant is injected for analysis by HPLC. A set of reference medicines, spanning the solubility range of this technique (1–100 μM), are also prepared in the same way and included in each plate. A quality control sample of each reference compound, prepared in methanol/water, is also analyzed. The throughput of this assay is supported primarily by rapid chromatography. The standard method is limited to evaluating compounds that contain a chromophore, but NME that do not absorb UV may be analyzed by HPLC/LC-MS (liquid chromatography–mass spectrometry), as long as the compound will accept a charge. The information can be used to determine which NME are soluble enough to be accurately assessed in other pharmaceutical profiling assays.

Lipophilicity

One of the most reliable methods used by medicinal chemists to change pharmacological activity in vitro is to incorporate properly positioned lipophilic groups to alter the hydrophobic interactions between a target and its ligand. Therefore, an assessment of lipid solubility is frequently included in an evaluation of physicochemical properties because lipophilicity has been associated closely with biological effect in structure–activity analyses (9,10). Furthermore,

lipophilicity appears to be proportional to molecular weight, and high molecular weight and high lipophilicity have been associated with poor intestinal permeability (11).

For nearly 50 years, the standard technique to determine relative lipophilicity has been the measurement of octanol/water distribution coefficient, using the widely accepted shake-flask method and calculation of the logarithm of the partition coefficient (log P or log D). This has been considered a surrogate for the biologically relevant membrane partition coefficient (k_m). The value of log P may be estimated by chromatographic partitioning techniques. Alternately, the degree of liposome partitioning of NME may also be used as a surrogate for the membrane partition coefficient (K_m). Although measurement of lipophilicity is preferred, attempts have been made to increase the throughput of lipophilicity determinations by predicting, rather than measuring, lipophilicity. More than 40 different approaches have been developed to predict lipophilicity by calculating the contribution of the molecular characteristics of compounds. The added value of octanol/water partitioning information for new NME lies, in large part, in the continuity it provides with historical determinations of lipophilicity of successful medicines.

Originally, lipophilicity was determined in drug discovery on a small scale by measuring the octanol/water partition coefficient (log P) or the apparent octanol/buffer partition coefficient (log D) experimentally with the well-established shake-flask procedure (12). Octanol, with its polar head and flexible, nonpolar tail, has hydrogen-bonding characteristics similar to those of membrane phospholipids. The octanol/water partition coefficient is also thought to model the hydrophobic interactions between xenobiotics and biological membranes. However, this method is laborious and time consuming, and there are serious issues associated with the chromatography of NME dissolved in octanol. In addition, the measurement of partition coefficient in this way may have only limited reproducibility. Unfortunately, at this time, analytical challenges remain to be addressed.

As expected, attempts have been made to improve the reproducibility and throughput of octanol/water partition coefficient measurements. Despite problems with standard HPLC measurement of partition coefficient, it has been suggested that HPLC retention data may prove to correlate with biological activity as well as, or better than octanol/water partition coefficient (13). The potential usefulness of this technique was demonstrated on a group of xenobiotics ($n = 52$) among which the log P values, as measured by shake-flask method, ranged from -0.28 to $+5.01$ (14). The results of this study suggested that the value for log P, estimated via a column-corrected chromatographic method, correlated very well with log P measured via the shake-flask method ($r = 0.999$). However, for maximal accuracy and minimal variability, numerous elution time measurements are required, which reduce its applicability to pharmaceutical profiling.

Partitioning of NME into liposomes has also been used to accurately approximate lipophilicity by measuring the membrane partition coefficient. Liposome partitioning can model both polar and nonpolar interactions between solute and membrane (15). However, liposome partitioning experiments are also labor intensive, requiring the preparation of liposomes, adequate solute equilibration time, measurement of free solute in the presence of liposomes, and corrections for the amount of solute that partitions into the aqueous phase. Although this technique may be unsuitable in lead selection, the approach appears to be a valid one, because it has been shown that liposome partition coefficient correlates well with partition coefficient in biological membranes (16).

Lipophilicity continues to represent one of the most informative pharmaceutical characteristics available during drug discovery. Because of the implementation of combinatorial and parallel synthesis and high-throughput biological screening, the motivation to develop new models to estimate lipophilicity more rapidly was increased significantly after 1991. Immobilized artificial membrane (IAM) chromatography columns utilize a solid phase membrane model in which phospholipids (e.g., phosphatidylcholine) are covalently bound to solid support at membrane densities. This technology had been used to purify membrane proteins, immobilize enzymes, and characterize enzyme–ligand interactions. Recently, these columns have been used in order to measure solute capacity factor as a surrogate for membrane partition coefficient (17,18). The technique combines the advantages of using membrane phospholipids with the high-throughput advantage of chromatographic partitioning. It has been found that k_{IAM} correlates well with membrane partition coefficient ($r = 0.907$) and structural differences. However, this relationship does not exist between log k_{IAM} or log k_m and log P ($r = 0.419$ and

0.483, respectively). It appears that when nonpolar interactions between solutes and membranes dominate membrane interactions, both log k_{IAM} and log K_m correlate well with log P (16). Conversely, when hydrophobic interactions dominate membrane interactions, liposome partition methods give the same results as IAM methods ($r = 0.985$). These qualifications suggest that this technique may be applied most reliably to a series of structurally similar NME (19) and that it should probably not be used to screen structurally diverse compounds for relative lipophilicity.

The pressure to screen several hundred NME per day subsequently yielded an excellent example of a high-throughput drug discovery assay via modification of conventional IAM chromatography technique described above using artificial membranes in a 96-well plate format (20). The major objective of creating this technique was to model human intestinal absorption, but it yielded an index of lipophilicity that was comparable to log P. IAM chromatography was a significant development for drug discovery, permitting rapid assessment of interactions between NME and the phospholipid component of biological membranes.

The number of new therapeutic targets and potential lead compounds continues to increase, while improvements in the techniques used to measure lipophilicity are dwindling. Therefore, a great deal of effort has been expended in finding algorithms to predict lipophilicity by calculating log P. This topic was recently reviewed comprehensively (21), so the discussion of these predictions here will remain general. The existing methods of predicting log P fall into three categories. Group contribution methods (e.g., C log P) dissect molecules into predefined fragments and their corresponding contributions are summed to obtain a calculated log P value. This method incorporates a number of correction factors, because a molecule is not just the sum of its fragments. Similarly, atomic contribution methods (e.g., M log P) of predicting log P do so by considering the individual contribution of single atoms. Like group contribution methods, this reductionist approach is based on the contributions of predefined fragments, which are determined by multiple regressions on a database of experimental log P values. Molecular methods of predicting log P (e.g., B log P) incorporate a description of each molecule as a whole, not just as a collection of fragments put together and consider the interaction between solute and solvents (octanol and water). These methods use quantum and geometric descriptors (e.g., surface area or volume) or conformational analysis. All three types of predictive methods require complex calculations. As a matter of practical application to drug discovery, current opinion suggests that M log P is the most useful method for tracking the physicochemical properties of a library of NME, because it covers a larger variety of molecular structures with acceptable accuracy. On the other hand, C log P is most applicable to characterizing analog series, because it emphasizes accuracy of predictions and it is applicable to less diverse collections of compounds (7).

Plasma Protein Binding

The behavior of NME in biological solutions (e.g., plasma) influences the activity and disposition of many medications. One important aspect of this behavior is plasma protein binding, which may be considered a pharmaceutical property of new compounds. In the past, precise but experimentally laborious methods were developed to evaluate plasma-free fraction. These include techniques based on equilibrium dialysis and ultrafiltration, which still play a valuable role in drug development (22,23). Table 2 illustrates the differences we have observed between

Table 2 Plasma Protein Binding of Existing Medications: A Limited Comparison of Species and Methods

	In vitro plasma protein binding (%)				
	Equilibrium dialysis		Ultrafiltration		Reported human plasma protein binding (%)[a]
Compound	Human	Rat	Human	Rat	
Warfarin	99.4 ± 0.4	99.7 ± 0.2	98.9 ± 0.2	99.5 ± 0.1	99 ± 1
Verapamil	90.5 ± 2.1	93.2 ± 0.5	84.8 ± 0.5	80.0 ± 2.2	90 ± 2
Propranolol	88.1 ± 0.5	91.0 ± 1.1	86.2 ± 2.1	78.3 ± 3.0	87 ± 6
Naltrexone	61.7 ± 3.1	73.9 ± 0.7	66.1 ± 3.1	48.9 ± 2.1	20

Values are mean ± SEM.

[a]Values were taken from the Physician's Desk Reference.

the values for plasma protein binding of several existing medications by using ultrafiltration and how these compare to values reported by the original innovator company. NME were evaluated in duplicate in 96-well plates at an initial concentration of 10 μM. Unfortunately, these methods continue to suffer from poor recovery due to the adsorption of test compound onto labware. However, chromatographic methods to measure serum albumin binding can also be used as rapid screening tools for investigating drug binding in drug discovery (24). Affinity chromatography generally uses retention time on a serum albumin stationary phase as the parameter that correlates with the degree of protein binding. These methods can be extended to the analysis of enantiomers (25–27). When compared to the results obtained by ultrafiltration, this method yields thermodynamically valid binding measurements.

Affinity capillary electrophoresis, combined with a variety of detection methods, has also been used to screen drug–albumin interactions. Several versions of this basic technique have been developed (28). Frontal and vacancy peak analysis use UV detection to measure unbound drug, and the Hummel–Dryer technique uses UV absorbance to measure bound drug. In affinity capillary electrophoresis, binding parameters are calculated from the change in electrophoretic mobility of the drug upon binding.

Permeability and Intestinal Absorption

One of the major influences on the success of orally administered medicines as therapeutic products is the rate and extent of their intestinal absorption. This complex process is a function of the physicochemical properties of the drug and the permeability of the intestinal barrier that determines drug absorption. Transcellular movement of solutes occurs via passive diffusion through the enterocyte and is the most significant absorptive mechanism for the majority of drugs. Membrane permeability typically depends on three interdependent properties, including lipophilicity, hydrogen-bonding potential, and molecular size (29). Paracellular movement of solutes also occurs via passive diffusion through pores, the enterocyte membrane, and at cellular junctions. It has much less significance in the intestinal uptake of most drugs compared to the transcellular pathway.

Physicochemical Properties

As discussed previously, there is value in evaluating the role of physicochemical properties in intestinal membrane permeation. Membrane permeability (P_m) is a function of the membrane diffusion coefficient (D_m), membrane partition coefficient, and the thickness of the membrane (17). Intestinal membrane thickness is equivalent to the enterocyte apical membrane, a lipid bilayer according to the fluid-mosaic model. However, the intestinal barrier in situ also includes an unstirred water layer that may have differential effects on drug absorption. Two of these three factors, D_m and K_m, are strongly influenced by the lipophilicity of the solute. The ability of NME to permeate cell membranes by passive diffusion is initially dependent on its partitioning into the apical membrane. The most frequently used index for predicting membrane permeability is log P, but the correlation between P_m and log P has yielded mixed results for diverse molecular structures. In general, more lipophilic compounds have higher membrane permeability coefficients (30). In fact, the relationship between permeability and lipophilicity is steeply sigmoidal when plotted for compounds grouped by molecular weight (31). A permeability plateau observed at high lipophilicity values is likely due to a stagnant diffusion layer, where permeation is rapid but diffusion through the unstirred layer is rate limiting. This relationship has been demonstrated in vivo, where it was observed that the rate of disappearance of drugs from a rat intestinal loop preparations correlated well with lipophilicity, up to a log P value of 3.0 (32). This relationship has been confirmed in vitro, where the plateau occurs when log P values exceed 3.5 (33). Conversely, the tailing effect appearing at low lipophilicity values is probably due to the uptake of small hydrophilic compounds via the paracellular pathway, which has a much lower capacity due to size exclusion effects. The intervening steep part of the curve represents the critical range for oral absorption (28).

As described previously, IAM chromatography has been used reasonably successfully to evaluate lipophilicity by measuring k_{IAM} as a surrogate for the membrane partition coefficient K_m. This method has also been used as a simple and high-throughput method for predicting membrane permeability. Although the correlation between k_{IAM} and log P is only moderately good for structurally diverse compounds ($r = 0.520$), it is slightly better for fraction absorbed

Table 3 Ranking of Existing Medicines for Transcellular Permeability with the Parallel Artificial Membrane Permeability Assay in 96-Well Format

Compound	Permeability
Atenolol	0.5 ± 0.7
Cimetidine	3.2 ± 0.2
Nadolol	3.3 ± 0.3
Doxorubicin	12.4 ± 3.7
Erythromycin	40.4 ± 6.4
Metoprolol	50.3 ± 7.1
Propranolol	84 ± 18
Verapamil	100.3 ± 4.6
Imipramine	100.3 ± 3.1

Values are mean percent flux $\pm$ SEM.

in the perfused rat intestinal model ($r = 0.791$) and for the apparent permeability (P_{app}) determined in enterocyte monolayers. In an excellent example of the rapid evolution of new higher throughput assays, Kansy et al. (20) markedly increased the throughput of the standard IAM chromatography by developing the parallel artificial membrane permeability assay. Their objective was to classify NME with respect to their lipophilicity as an index of the extent of intestinal absorption (% flux). Using commercially available active pharmaceutical ingredient (API), they reported that well-absorbed compounds ($F = 70$–100%) exhibited a flux of 23% to 100%, moderately absorbed compounds ($F = 30$–70%) exhibited 5% to 25% flux, and poorly absorbed compounds ($F = 1$–30%) exhibited $< 5\%$ flux. Approximately 80% of the compounds would be correctly predicted with respect to human intestinal absorption by this method. Table 3 shows the results obtained in our laboratory with this assay. The final test concentration was 50 μM, the incubation time was 18 hours, and the analysis was done by LC-MS/TOF.

The role of aqueous solubility in drug absorption was somewhat overlooked until a drug classification system, based on permeability and solubility, was proposed as a basis for establishing in vitro–in vivo correlations and bioequivalence (34). Essentially, there is a good correlation between the extent of absorption in humans and enterocyte permeability in vitro (see below), but the strength of the association is limited by aqueous solubility. This relationship was originally confirmed with a group of nine medicinal products, among which was represented a wide range of values for aqueous solubility (0.03–465 mg/mL), lipophilicity ($\log P = -0.8$ to 3.0), and enterocyte monolayer permeability coefficients ($P_{app} = 10^{-7}$ to 4×10^{-5} cm/sec) (35).

The molecular size of NME also affects their membrane permeability. Molecular size is a component of lipophilicity and the diffusion coefficient D_m in biological membranes and through membrane pores. Molecular size is frequently described by molecular weight (28). Therefore, two molecular size effects exist: The larger the molecular size of a compound, the smaller becomes its permeability coefficient through the membrane pores and the smaller the diffusion coefficient through the lipoid part of biological membranes (30). Finally, an excessive number of hydrogen bond donor groups included in a new medicinal compound impair the membrane permeability (7,36). This influence is accounted for quite well in the measurement of log P because of the similarities in hydrogen bonding between lipid membranes and water-saturated octanol. The combination of high molecular weight and high log P is observed in very few existing medications ($\sim$1%), but these characteristics appear to be enhanced in the leads from high-throughput screening (7). Lipinski et al. developed and published a practical method to predict the permeability of NME across the intestinal membrane (7) and flag unsuitable compounds. It incorporates many of the physicochemical factors described previously in this chapter. It is commonly called the "rule of five," and it states that poor absorption or permeation is more likely when

- there are more than five hydrogen bond donors (the sum of OHs and NHs),
- the molecular weight is greater than 500,
- the log P is over 5, and
- there are more than 10 hydrogen bond acceptors (the sum of Ns and Os).

Compound classes that are substrates for biological transporters are exceptions to this rule.

Cell Monolayers

The measurement of permeability alone is difficult to conduct in vivo. Oral absorption data are frequently difficult to interpret because of numerous factors that affect the overall process. However, two low-throughput models were originally developed for intestinal absorption studies. One in situ model was based on in situ isolation of intestinal loops in which the disappearance of drug from the loop or appearance in the blood is monitored. This is now primarily a research tool. Alternately, an intestinal segment is isolated and mounted in an Ussing chamber. The segment is placed between donor and receiver compartments. These have been used to characterize several factors that determine the transepithelial movement of drugs (37), and the results from each method correlate well with one another and with the fraction absorbed in human subjects (38). However, neither of these methods is suitable for modern lead selection.

The increasing pressure to screen many NME for intestinal permeability motivated the search for new in vitro models. Caco-2, a human colorectal carcinoma cell line, was first used to study glycogen metabolism (39). Shortly thereafter, it was noted that Caco-2 cells were unique among many similar cell lines (e.g., HT-29 cells). After they reach confluence in culture, Caco-2 cells spontaneously differentiate into polarized, columnar cells that are more representative of the small intestine. They exhibit well-developed microvilli and a polarized distribution of brush-border enzymes, and their electrical properties resemble colonic crypt cells (40–42). However, it was not until 1989 that a report was published suggesting that Caco-2 cell monolayers could be used as a model to predict intestinal permeability and absorption (43). Similarities in uptake and barrier properties between this system and the small intestine epithelial layer were observed. Almost immediately, a series of six well-known β-blocking drugs were tested with Caco-2 monolayers for permeability. The absorption rates for four of the six compounds were similar in the Caco-2 model and in a rat intestinal loop model. In a rapid follow-up study, 20 well-known drugs ($\log D = -4.5$ to $+ 3.48$) with different structural properties were tested in Caco-2 monolayers (44). The investigators concluded that when a drug was completely absorbed in humans, the apparent permeability coefficient (P_{app}) exceeded 2×10^{-6} cm/sec, and when less than 100% of the drug was absorbed in humans, $P_{app} < 0.1$ to 1×10^{-6} cm/sec. In fact, the Caco-2 model has been used with increasing frequency during the past 15 years as an in vitro surrogate for human intestinal permeability. Since 1991, the fundamental relationship between the fraction absorbed (F_a) and P_{app} has been clarified by a series of small studies. Cocultures of absorbing Caco-2 cells and mucus-secreting HT29-MTX cells have been used to simulate the unstirred water layer. A good prediction of F_a in humans was attained by separating the passively transported drugs ($n = 15$) into two groups—well-absorbed compounds ($P_{app} > 1 \times 10^{-6}$ cm/sec) and drugs that exhibit 40% to 70% absorption ($P_{app} < 10^{-6}$ cm/sec) (45). A strong correlation was observed between human absorption in vivo and P_{app} for a heterogenous collection of existing drugs ($r = 0.950$, $n = 35$). The authors observed that if F_a was 0% to 20%, P_{app} was less than 1×10^{-6} cm/sec; if F_a was 20% to 70%, then P_{app} fell between 1×10^{-7} and 1×10^{-6}; and if F_a was 70% to 100%, then P_{app} exceeded 1×10^{-6}. In this group, the range of $M \log P$ values was -4.91 to $+ 3.88$ (46).

The Caco-2 model appears to be a reasonable and reliable method to predict the fraction of intestinal absorption in humans and attempts to improve it continues. One subclone of Caco-2, TC-7, has been identified by higher levels of expression of the glucose transporter and increased taurocholic acid transport compared to the parental Caco- 2 cell line. The activity of phase II enzymes (UDP-glucuronosyltransferase and glutathione transferase) appears to be similar to human jejunum and higher than that in Caco-2 cells (47). In addition, TC-7 is more homogenous in terms of cell size and confluence is achieved earlier than Caco-2 cells because of a shorter doubling time (26 vs. 30 hours). Furthermore, P-glycoprotein (P-gp)-mediated cyclosporine efflux was less strongly expressed in TC-7 cells than in Caco-2, thereby allowing less complicated measurement of permeability. A threshold for absorption in humans exists, 2×10^{-4} cm/sec, above which 100% oral absorption is very nearly equivalent to a P_{app} value observed in Caco-2 monolayers of 2×10^{-6} cm/s (48). These studies have demonstrated the importance of analyzing the permeability during lead selection that is relative to a set of several

reference compounds exhibiting a large range of permeability and for which the value of F_a is known (e.g., propranolol).

The major reason for employing several reference compounds in these assays is the large variation in P_{app} values among test sites, which is primarily a result of differences in experimental protocols. A relatively hydrophilic reference standard is included as an index of monolayer integrity (e.g., Lucifer yellow). Despite recent advances in this model, Caco-2 studies are laborious and therefore not best suited to measurements of permeability during lead selection. The Caco-2 assay remains a relatively low-throughput method, due in part to the limitations of its 21-day growth period and regular maintenance feeding requirements. Proprietary culture conditions that accelerate differentiation to three days become costly for the purpose of screening large series of compounds (49,50,51). Until recently, the functional lower limit on the area of cell monolayers has restricted this assay to 6-, 12-, or 24-well Transwell plates, in order to accommodate low-permeability compounds. Typically, medicinal compounds are tested at an initial test concentration of 10 to 50 μM. Table 4 summarizes the results of evaluating the apical-to-basal permeability of existing medications, tested in duplicate, with Caco-2 monolayers in a 96-well format. The value of percent recovery is the method used to determine the validity of an experiment, that is, data from an experiment with a recovery of less than 50% are considered unreliable. In our laboratory, a compound is considered to be highly permeable (well-absorbed) if the value of P_{app} is greater than 1×10^{-6} cm/s. The initial test concentration was 30 μM and analysis was conducted with LC-MS. A caveat to using this method of evaluating permeability/absorption is that there is no unified cell culture or experimental protocol. Therefore, the criteria to distinguish well-absorbed compounds from poorly absorbed compounds need to be established at every location where the assay is conducted.

In an attempt to further reduce time, cost, and effort, monolayers of the Madin–Darby Canine Kidney (MDCK) epithelial cell line have also been investigated as an in vitro model to measure the relative permeability of NME. This approach was suggested by Cho et al. (53) and MDCK monolayers were first tested on antimicrobials (54). MDCK cells reach confluence after three days because they can be seeded at high density (650,000 cells/cm^2). Like Caco-2 cells, MDCK cells differentiate into columnar epithelium after reaching confluence and they form tight junctions on semipermeable membranes. This manipulation does not work to reduce culture time for Caco-2, because when these cells are seeded at high density, they display high permeability for Lucifer yellow by the third day, typical of poor tight junction integrity. Irvine et al. (55) tested 55 compounds, with known permeability values, in Caco-2 and MDCK monolayers. Their results suggested that P_{app} values measured in MDCK monolayers correlated well with P_{app} values from parallel Caco-2 experiments ($r^2 = 0.79$). In addition, Spearman's rank correlation coefficient for MDCK-derived P_{app} values and human absorption was 0.58 compared with 0.54 for Caco-2 P_{app} and human absorption. These results suggest that, under certain conditions, MDCK monolayers may be another useful tool in lead selection.

Another approach to increasing the throughput of permeability screening is the use of a single enterocyte monolayer to screen a mixture of NME. Taylor et al. (56) screened six arbitrary mixtures of 24 physicochemically diverse, N-substituted glycine peptoids. They used this technique to study structure–transport relationships. They added a unique methodological twist by analyzing the donor and receiver compartments for permeability and the receiver compartment for pharmacological activity. This process of coupling screens for permeability and therapeutic activity is very representative of the type of innovation possible. A major challenge for measuring permeability of libraries is the need for sensitive quantitative analytical techniques. Sensitivity is dictated by the solubility in the apical donor medium and the achievable concentration of transported compounds in the basolateral receiver compartment. It has been estimated that the application of LC-MS in single-ion mode to these permeability assays improves detection 1000-fold over HPLC and enhances selectivity over HPLC/UV that is extremely important in analyzing mixtures (57). Most recently, a report was published detailing the permeability screening of a combinatorial library containing 375,000 peptides (58). This mammoth task was accomplished testing a series of 150 pools, each containing 2500 tripeptide sequences. The NME in the receiver compartment were separated by capillary HPLC and analyzed by LC-MS/MS to identify structures. To discriminate between isobaric structures, several compounds were resynthesized and retested individually.

Table 4 The Permeability of Existing Medicinal Compounds in Caco-2 Monolayers

	A–B				B–A					Literature values			
	P_{app}		Recovery (%)		P_{app}		Recovery (%)		B–A/A–B				Human absorption
Compound	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Ratio	A–B	B–A	B–A/A–B	(%)
Ranitidine	4	1	88	5	3	0	99	9	0.83	0.49[a], 1.4[b]	1.95[b]	1.35[b]	55[a]
Erythromycin	4	1	91	3	10	1	110	10	2.52	3.73[a]			35[e]
Doxorubicin	5	1	81	16	5	2	87	12	0.95	0.16[a], 1.5[b]	0.97[b]	0.67[b]	5[a]
Atenolol	6	2	88	3	2	1	110	10	0.37	1.2[c], 3[h]			40–70[c]
Nadolol	7	1	85	4	4	1	98	6	0.58	3.88[a], 2.2[h]			32[a], 15[h]
Cimetidine	8	3	83	2	9	1	97	4	1.11	0.74[a], 3[f]			62[i]
Verapamil	27	4	101	6	66	11	105	11	2.46	59[b]	68.7[b]	1.16[b]	
Imipramine	32	8	92	5	55	8	90	12	1.74	14[a]			99[a]
Propranolol	37	6	90	5	52	3	94	8	1.39	41.9[a], 49.6[b], 34.4[c], 37.6[d], 86[f]	51.4[b]	1.04[b]	93[a]
Metoprolol	47	4	124	6	80	12	112	6	1.71	23.7[a], 18[c], 23.6[d]			95[a]

Well absorbed, $P_{app} > 10 \times 10^{-6}$ cm/sec.
Poorly absorbed, $P_{app} < 10 \times 10^{-6}$ cm/sec.
P-gp substrate, ratio > 2.
Not a P-gp substrate, ratio < 2.
[a] Ref. 50.
[b] Ref. 51.
[c] Ref. 48.
[d] Ref. 52.
[e] Ref. 46.
[f] Ref. 53.
[g] Ref. 54.
[h] Ref. 55.
[i] Ref. 35.

The Role of P-glycoprotein in Drug Absorption

The development of very potent and selective medications has implications on the dose size and dosing frequency. Ideally, medicines should require small and infrequent doses. Under these circumstances, the role of the ATP-binding cassette anti-porter P-gp may become very important in determining drug disposition. As detailed in another chapter in this volume, this protein is expressed in the intestinal epithelium, liver, kidney, testes, placenta, and the blood–brain barrier, and it is capable of restricting the passage of drugs across these cellular barriers and influencing the disposition of many drugs. It has a very broad substrate specificity that overlaps with that of cytochrome P4503A4 in many instances. Therefore, it has become important to determine very early on if the disposition of compounds are influenced by P-gp. Until recently, the investigation of interactions between NME and P-gp was typically conducted during preclinical testing, if at all. It focused on the use of a low-throughput Caco-2 cell monolayer model to determine the mucosal-to-serosal (apical-to-basal) efflux of individual candidates relative to existing medications, which are typically used as positive and/or negative controls, or in the presence or absence of widely used P-gp inhibitors such as verapamil (59). However, new models for higher-throughput assays have been developed to provide information on P-gp interactions during lead selection. One of these monitors the NME-stimulated ATPase activity of P-gp in isolated cell membranes, measuring the appearance of inorganic phosphate by a colorimetric reaction (60–62). Figures 1 to 3 illustrate the differences we have observed in the concentration-dependant ATPase activity. The ATPase activity of ritonavir in a membrane-based assay for P-gp interaction is shown in Figure 1. Membranes isolated from cells expressing P-gp (250 μg/mL) were incubated with ritonavir in Tris–MES buffer (final volume = 60 μL) for 20 minutes at 37°C. Note the 1000-fold difference between ritonavir and verapamil with respect to the concentration at which the maximum effect was measured.

Unfortunately, some compounds identified as substrates in the ATPase assay do not appear to undergo significant transmembrane movement in Caco-2 monolayers. This is true of midazolam that has a high passive permeability (63), leading to rapid transcellular flux that may overcome P-gp–mediated efflux. Conversely, some medicines previously identified as substrates in the Caco-2 model (e.g., vincristine, colchicine) fail to stimulate ATPase activity. A cell-based assay developed for this purpose involves the use of fluorescent substrates (e.g., Calcein AM or rhodamine 123), where intracellular accumulation of the parent compound, or a fluorescent metabolite, is caused by the inhibition of P-gp by NME (64). The ATPase activity of verapamil in a membrane-based assay for P-gp interaction is shown in Figure 2. This result is then compared to a standard positive control, such as nicardipine. These new assays appear to be suitable for high-throughput screening during lead selection, but they may be used to determine

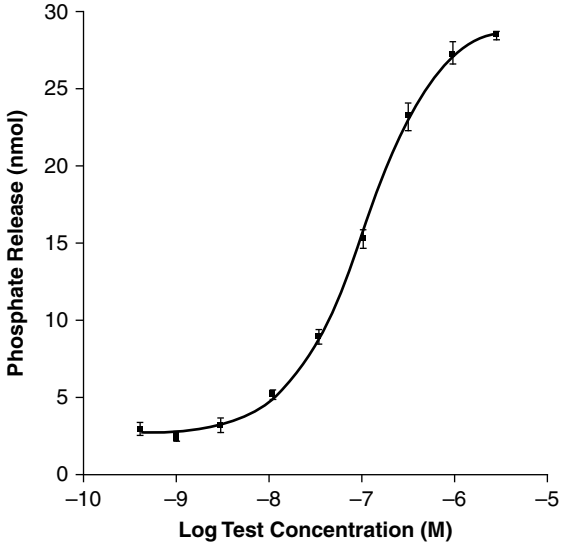

Figure 1 The ATPase activity of ritonavir in a membrane-based assay for P-glycoprotein interaction. Membranes isolated from cells expressing P-glycoprotein (250 μg/mL) were incubated with ritonavir in Tris-MES buffer (final volume = 60 μL) for 20 minutes at 37°C.

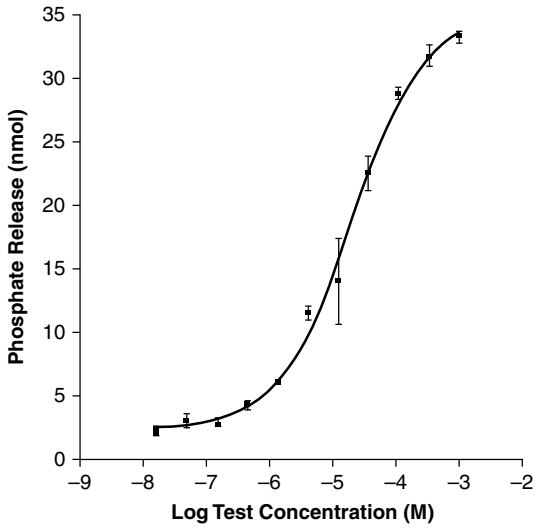

Figure 2 The ATPase activity of verapamil in a membrane-based assay for P-glycoprotein interaction. Membranes isolated from cells expressing P-glycoprotein (250 μg/mL) were incubated with verapamil in Tris-MES buffer (final volume = 60 μL) for 20 minutes at 37°C.

only if NME interact with P-gp, not whether they are inhibitors or substrates. Therefore, neither of these assays should be used alone during lead selection, when minor differences between structurally similar NME may become critical. In fact, the use of all three assays has resulted in the classification of verapamil as a nonsubstrate, a substrate, and an inhibitor (65). Therefore, it may be very difficult to classify new compounds as inhibitors, nontransported substrates, or substrates with a single assay, because different models/assays and test conditions frequently yield different results. It is becoming clear that efflux or inhibition data from P-gp interaction studies conducted in Caco-2 monolayers or other cultured cells expressing P-gp (e.g., human renal proximal tubule epithelial cells) depends on the substrate selected. In fact, the particular cell type chosen for screening may influence the kinetic properties of P-gp. Disparities arise not only from differences in assay conditions, but also classification criteria and nomenclature (62). Furthermore, assay reproducibility may be poor as typical test concentrations (20–50 μM) frequently exceed the solubility of many NME (66) in cell culture media. Therefore, it has been recommended that high-throughput screening for P-gp interactions using membrane- or cell-based assays during lead selection should be combined with an assay that can distinguish between substrates and inhibitors, even if the results from a fluorescent assay are negative (67).

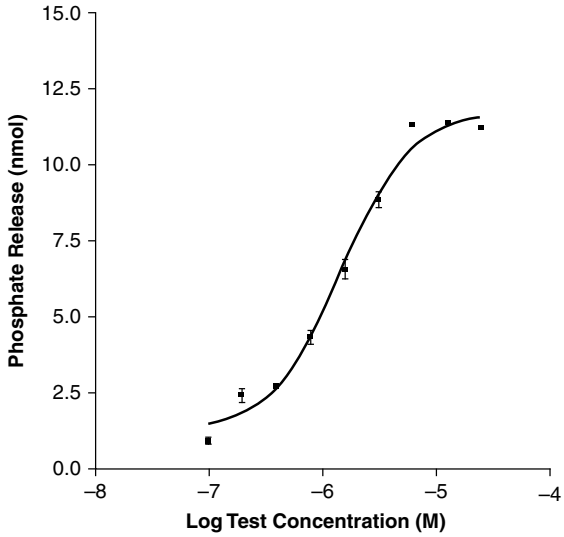

Figure 3 The ATPase activity of nicardipine in a membrane-based assay for P-glycoprotein interaction. Membranes isolated from cells expressing P-glycoprotein (250 μg/mL) were incubated with nicardipine in Tris-MES buffer (final volume = 60 μL) for 20 minutes at 37°C.

In an attempt to clarify these confounding observations, Polli et al. (65) have developed a rather complex classification system, based on the results of screening a variety of medicinal compounds in Caco-2, ATPase, and Calcein AM inhibition assays. Category I compounds (possible inhibitors) are not effluxed in standard Caco-2 assay, nor do they stimulate ATPase activity (e.g., testosterone). However, they cause an accumulation of Calcein AM in a cell-based assay by inhibiting P-gp. Compounds placed in category IIA (nontransported substrates) are not subject to efflux in Caco-2 monolayers, but they test positive in the ATPase and Calcein AM assays (e.g., verapamil). Compounds falling into category IIB are considered transported substrates. Category IIB1 compounds test positive in the efflux assay but negative in the ATPase and Calcein AM assay (e.g., vincristine). Category IIB2 compounds are effluxed in Caco-2 and are positive in the ATPase assay, but they are negative in the Calcein AM assay (e.g., erythromycin). Category IIB3 compounds are effluxed in the Caco-2 assay and are positive in the Calcein AM assay, but they do not stimulate ATPase activity (e.g., cyclosporine). Compounds in category IIB2 and IIB3 are considered transported substrates. Table 4 also illustrates the use of Caco-2 monolayers to determine if a compound interacts with P-gp. Typically, a compound should be considered as a P-gp substrate when the value of *P*app in the basal-to-apical assessing (B–A) direction exceeds the value of P_{app} in the apical-to-basal (A–B) direction by a factor of 2 or more (Dr. Ron Borchardt, personal communication, 2003).

Presystemic Metabolism

In Vitro Metabolic Stability and Intrinsic Hepatic Clearance

As a key component of lead seletion, the evolution of drug metabolism science during the past century has already been broadly documented very well from a first-hand perspective (68). This followed much more technical and less philosophical reviews of the progress in developing new biological tools and their application to specific investigations during lead selection (69,70). Historically, the comprehensive investigation of dispositional factors affecting the clinical success of a new drug have been delayed well beyond lead selection. However, these factors can have a profound impact on the duration and intensity of pharmacological effects by altering the bioavailability of medicinal compounds. Short duration of action renders it impossible to provide a patient with a convenient dosage regimen that encourages compliance. So estimates or predictions of human pharmacokinetic parameters are being shifted from preclinical development to discovery. It is generally desirable to design a drug that undergoes predictable metabolic inactivation or undergoes little or no hepatic metabolism. This simplifies the pharmacokinetics due to a lack of interindividual variation observed when hepatic drug–metabolizing enzymes are involved, particularly microsomal cytochrome P450 enzymes. In addition, drugs like terfenadine and cisapride that undergo extensive presystemic metabolism are potentially susceptible to clinically significant drug interactions (71). Although metabolically inert compounds are highly desirable lead candidates, the versatility of hepatic drug–metabolizing enzymes presents quite a challenge to achieving this goal (72). Examples of poorly metabolized drugs include the angiotensin-converting enzyme (ACE) inhibitor lisinopril and the β-blocker atenolol. This characteristic is attributable to their relatively low lipophilicity. The advent of combinatorial and parallel chemistry presents a formidable challenge to metabolism scientists to devise reliable, higher throughput methods of assessing presystemic metabolism and potential metabolic drug interactions. In a practical sense, the objective is to prevent drug metabolism studies from becoming a bottleneck in drug discovery. The target capacity for these drug metabolism screens is in the order of dozens to hundreds of compounds per week (73).

The search for systems that can meet these requirements has focused on automation and miniaturization of existing methods, but any improvements in throughput are worthless unless they are supported by rigorous and continuing validation of overall performance. In vitro models for the study of drug metabolism are probably the best established of the lead selection assessments discussed in this chapter. They have been used for two decades in preclinical metabolism studies to supplement pharmacokinetic and safety assessments in vivo. Liver S9 fraction and microsomes are the most widely used models for these experiments, but human and nonhuman hepatocytes and liver slices have become readily available for this purpose. Hepatic metabolism continues to be a major factor affecting the progression of potential lead compounds through preclinical and human clinical studies. During drug discovery,

measurement of relative metabolic stability in vitro provides a rapid means of ranking a series of molecules in the absence of factors such as absorption and plasma protein binding. In the experimentally simplest procedure, the extent of metabolism is determined from the ratio of parent compound remaining in the test sample to that in the control. Alternately, NME can be ranked by the initial rate of the disappearance of the parent compound (V_0), the in vitro half-life ($t_{1/2}$) or the intrinsic clearance (Cl_{int}) (74). These protocols generally require a larger number of samples per compound and consequently more bioanalytical and data management resources. In vitro metabolism studies now conducted in drug discovery may also be used to predict certain pharmacokinetic variables, because frequently the failure of candidate compounds in the clinic is associated with poor pharmacokinetic behavior. However, these predictions are based on relatively elaborate experiments that are not easily adapted to rapid lead selection. Well before high-throughput profiling was introduced, it was observed that, in rats under first-order conditions, the contribution of hepatic drug–metabolizing enzymes may be estimated by the ratio of the Michaelis–Menten kinetic constants V_{max} and K_M, normalized to the amount of microsomal protein and scaled up to reflect liver microsomal protein content. This determination is equivalent to the intrinsic hepatic clearance of the drug (Cl_{int}). This value was then used to predict the extraction ratio (E_h). The value of E_h is related to another very important pharmacokinetic characteristic of orally administered medicines, bioavailability (F_a), where $F_a = (1 - E_h)$. A comparison between the predicted ratio, based on a microsomal model of hepatic elimination, and that determined directly in the isolated perfused liver suggested good agreement between the predicted and the observed hepatic extraction ratios (75). At that point in time, these results were probably considered of academic interest, but the metabolic screening of libraries of medicinal compounds renewed the interest in pharmacokinetic predictions based on simple in vitro protocols (74,76,77). In one comprehensive analysis of predictive human models, 12 methods were assessed for their utility in predicting Cl_{int}. The most useful methods in which in vitro metabolism data from human liver microsomes were scaled to in vivo clearance values yielded predicted clearance values that were, on an average, within 70% to 80% of actual values. However, differences in Cl_{int} in vitro and Cl_{int} in vivo values greater than fivefold have been observed (68). Furthermore, it appears that there are probably significant differences in the values obtained for Cl_{int} and that these differences are frequently related to the model selected for the evaluation (78). An important assumption in initial studies of predictive models was that drug binding to incubation constituents would not have a significant impact on the scale-up of in vitro clearance data to in vivo clearance because of typically low protein concentrations in microsomal incubations compared to concentrations of protein in plasma. However, the degree of nonspecific binding of NME to microsomal protein and partitioning into microsomal lipids during incubations recently has been shown to influence the results of liver microsomal metabolic stability screening (79–82). If this phenomenon exists for even a small proportion of medicinal compounds screened each year, it could have a widespread impact on drug discovery, because liver microsomal studies have retrospective importance for drug metabolism investigations in vitro. However, it is still not known if nonspecific binding to microsomes and constituents of other in vitro models is characteristic of a particular subset of compounds or unique to each compound, or how the binding of specific drugs varies between in vitro models. When the Michaelis–Menten constants are used to estimate Cl_{int}, it appears that if nonspecific binding reduces unbound drug significantly, K_M values are overestimated, because they are based on the nominal substrate concentration added to the incubation and not the free substrate available to bind to the enzyme. It appears that the fraction unbound in the incubation matrix is highly dependent on the microsomal protein concentration. In one report, in vitro methods generally under-predicted intrinsic clearance in vivo, but these compounds were highly bound to plasma protein and all were lipophilic amines (72). Initial reports using in vitro metabolism data for the prediction of pharmacokinetic behavior have been followed by a tide of very revealing reports describing direct comparisons between rat liver microsomes and isolated rat hepatocytes (83–85), investigating metabolism with rat liver slices (86–88) and detailing comparisons of all the three models (69,89). Most recently, these inquiries have focused on the contribution of some of the principal microsomal cytochrome P450 enzymes involved in drug metabolism (90,91). These studies have revealed some model-specific and drug-related artifacts that are probably responsible for the kinetic differences observed between liver microsomes, isolated hepatocytes, and liver slices.

Model-specific differences have been reported for a small set of reference compounds, including tolbutamide, phenytoin, caffeine, diazepam, ethoxycoumarin, and dextromethorphan. These mature medicines have been well-characterized with respect to their pharmacokinetic behavior in vivo. However, significant compound-related effects on predictions have also been demonstrated in these models (74). For example, studies have consistently shown that, after appropriate consideration of experimental conditions (79), predictions of intrinsic clearance from isolated hepatocytes are closer to in vivo values than those from microsomal studies for phenytoin, but not for tolbutamide (74). This phenomenon may be rationalized by either end-product inhibition in microsomal incubations or differences in nonspecific binding to microsomal components. Furthermore, significant differences have been observed between microsomes and hepatocytes with respect to metabolite profile that are unrelated to differences in nominal drug concentration (75). Liver slices appear to under-predict V_{max}, overestimate K_M, and, therefore, underestimate intrinsic clearance relative to isolated hepatocytes (77,78). This effect may be attributed to poor diffusion of the substrate into all cells in a slice or restricted oxygenation leading to compromised metabolic function. Finally, the metabolism of a number of compounds by CYP3A4 in liver microsomes and hepatocytes does not exhibit classic Michaelis–Menten kinetics but displays sigmoidal kinetics (81). Consequently, intrinsic clearance cannot be calculated for these drugs because of the lack of a first-order region in their kinetic profiles. A suitable method has yet to be identified to allow these results to be scaled to predict in vivo clearance. The effect of this circumstance could be enormous, considering the large proportion of existing medications that are metabolized by CYP3A4. Figures 4 to 7 illustrate the determination of intrinsic clearance in rat or human liver microsomal incubations and the species differences that frequently occur. The value of intrinsic clearance, Cl_{int}, is proportional to the slope of the regression line.

The limited results of model-comparison studies may not be entirely applicable to new medicinal compounds arising from a combinatorial library and selected with primary screening against a pharmacological target. However, liver microsomes are the favored model for mainly practical reasons and can be applied to ranking one or multiple series of compounds by $t_{1/2}$ or Cl_{int} (92) or to flag NME having disadvantageous metabolic characteristics (analogous to Lipinski's "rule of five" used for intestinal absorption assessments). Experimental constraints,

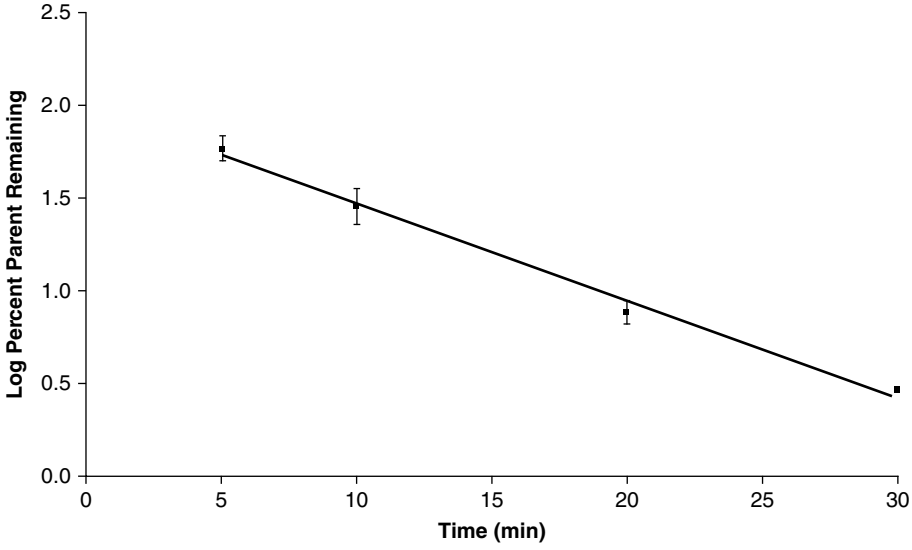

Figure 4 The intrinsic clearance of trazodone, a high clearance compound, determined in pooled rat liver microsomal incubations. Rat liver microsomes (1 mg/mL) were incubated with trazodone (10 μM) and NADPH (10 mg/mL) in potassium phosphate buffer (100 mM, pH 7.4) for 30 minutes at 37°C. Aliquots (45 μL) were taken at 0, 5, 10, 20, and 30 minutes and reactions were stopped with 100 μL of cold acetonitrile. $Cl_{in} = 54.3 \pm 7.5$ mL/min/kg (mean ± SE).

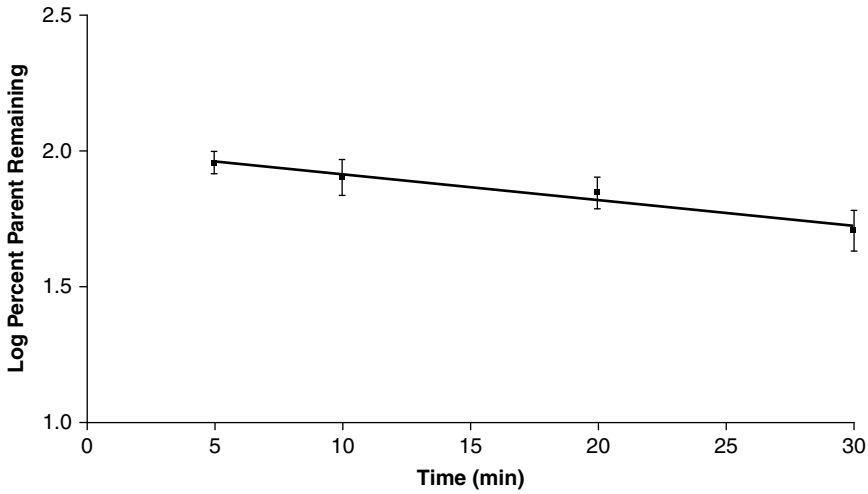

Figure 5 The intrinsic clearance of desipramine, a low clearance compound, determined in pooled rat liver microsomal incubations. Rat liver microsomes (1 mg/mL) were incubated with desipramine (10 μM) and NADPH (10 mg/mL) in potassium phosphate buffer (100 mM, pH 7.4) for 30 minutes at 37°C. Aliquots (45 μL) were taken at 0, 5, 10, 20, and 30 minutes and reactions were stopped with 100 μL of cold acetonitrile. $Cl_{in} = 7.5 \pm 3.2$ mL/min/kg (mean ± SE).

such as the preparation and culture of hepatocytes and slices, and the associated analytical and informatics requirements limit the usefulness of these methods in primary screening, but they can be adapted to 48- and 96-well plates or to a flow-through system. For example, rat liver microsomes have been used to determine the extent of metabolism and to identify the major oxidative metabolites of imipramine (93). Regardless of the biological model and the experimental protocol selected for rapid metabolic screening, limitations on analytical resources to support metabolism screening can create a potential bottleneck in the lead selection process. HPLC/UV is probably adequate to detect most compounds in these assays (94), but the selectivity of LC-MS is generally preferred on the basis of sensitivity (95). Practical experience has shown that

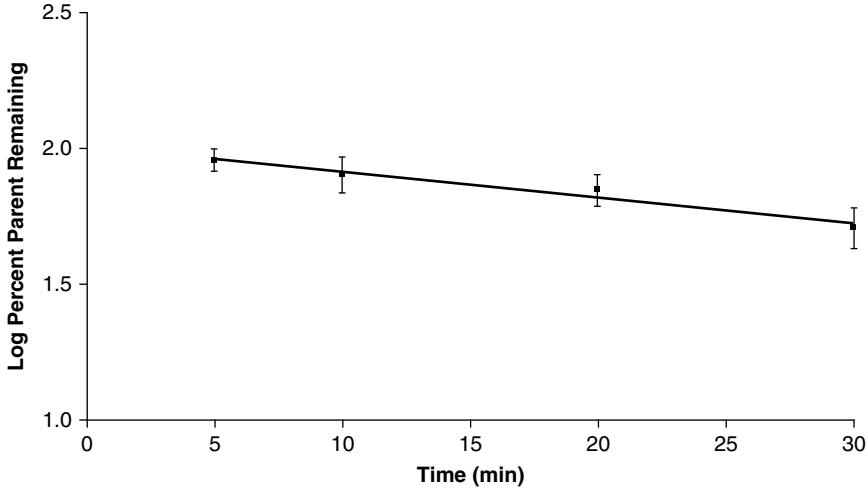

Figure 6 The intrinsic clearance of trazodone, a high clearance compound, determined in pooled human liver microsomal incubations. Human liver microsomes (1 mg/mL) were incubated with desipramine (10 μM) and NADPH (10 mg/mL) in potassium phosphate buffer (100 mM, pH 7.4) for 30 minutes at 37°C. Aliquots (45 μL) were taken at 0, 5, 10, 20, and 30 minutes and reactions were stopped with 100 μL of cold acetonitrile. $Cl_{in} = 8.7 \pm 1.2$ mL/min/kg (mean ± SE).

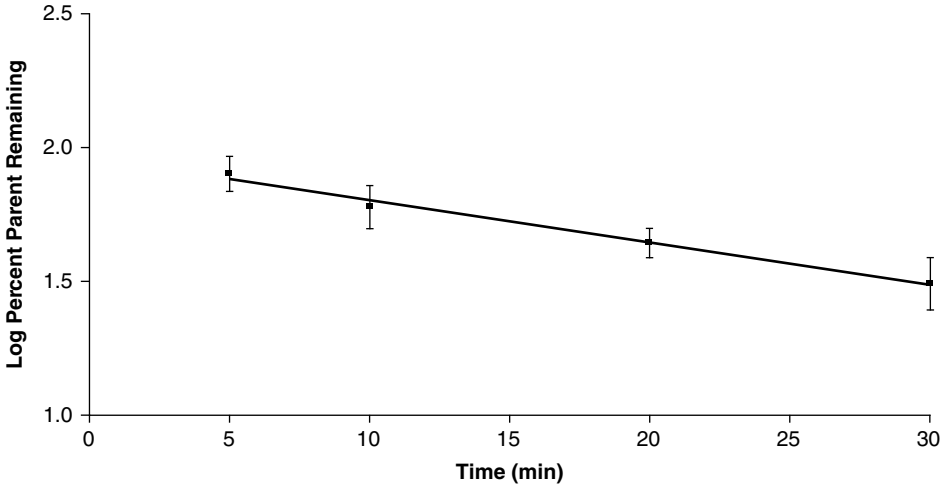

Figure 7 The intrinsic clearance of carbamazepine, a low clearance compound, determined in pooled human liver microsomal incubations. Human liver microsomes (1 mg/mL) were incubated with desipramine (10 μM) and NADPH (10 mg/mL) in potassium phosphate buffer (100 mM, pH 7.4) for 30 minutes at 37°C. Aliquots (45 μL) were taken at 0, 5, 10, 20, and 30 minutes and reactions were stopped with 100 μL of cold acetonitrile. Cl_{in} = 1.7 ± 0.7 mL/min/kg (mean ± SE).

miniaturization of in vitro assays is relatively straightforward if incubation conditions remain homogenous throughout the experiment. Typically, NME are included in incubations at a final concentration of 1 to 10 μM, and the final protein concentration is minimized to reduce nonspecific binding (e.g., 0.5–1.0 mg/mL). Incubations are normally conducted in duplicate and several reference substrates of varying metabolic stability are included (e.g., labetalol, verapamil, and terfenadine). Methods of ranking NME by metabolic behavior are widely used, but accurate and rapid prediction of intrinsic hepatic clearance and other pharmacokinetic parameters remains difficult and somewhat controversial.

Drug Interactions and Identification of Major Cytochrome P450 Enzymes

In the United States, the frequency of serious adverse reactions to drugs was recently estimated to be in the order of two million per year, of which 100,000 were fatal. A significant proportion of these incidents were observed in patients receiving multiple drugs. In fact, the morbidity and mortality resulting from a serious metabolic drug interaction between terfenadine and ketoconazole (96) caused scientists at the FDA and in the pharmaceutical industry to pay much more attention to the inhibition of hepatic drug–metabolizing enzymes (97). The problem of clinically relevant drug interactions arises from disturbances in pharmacokinetic behavior that raise the plasma concentration of one of the drugs above intended therapeutic levels. As the concentration of the parent drug rises, side effects appear as the selectivity of pharmacological action disappears. In the extreme situation, for example, when the medicine exhibits a relatively low therapeutic index, serious adverse effects may appear with only modest or moderate changes in exposure. Therefore, metabolic drug interactions are primarily an issue of drug safety. Both intensity and duration of drug action can be affected by these interactions. Medicines that are extensively metabolized tend to be involved in metabolic drug interactions more frequently and medications that are metabolized by several hepatic enzymes are less likely to cause clinically significant clinical interactions than drugs that are metabolized by a single enzyme. Furthermore, medicines metabolized extensively only by one of the polymorphically expressed microsomal P450 enzymes (e.g., CYP2C9, CYP2C19, CYP2D6) are also associated with a higher risk for drug-related toxicity, particularly in poor metabolizers. Because of the high cost of clinical investigations, there is a practical limit to the number and scope of clinical drug interaction studies that can be performed. Inevitably, some significant interactions could remain untested before a drug is in widespread use.

In human liver, several microsomal cytochrome P450 (CYP450) enzymes act as principal drug–metabolizing enzymes (e.g., CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP2B6). Although each enzyme exhibits a degree of selectivity, members of the hepatic microsomal CYP450 superfamily generally exhibit broad and overlapping substrate specificity for a very wide variety of xenobiotics. However, the identity of which CYP450 enzyme(s) are involved in oxidative metabolism can be determined by several methods. These protocols take advantage of the rapid commercialization of human liver products (e.g., liver microsomes, cryopreserved hepatocytes, recombinant enzymes, anti-P450 antibodies) during the past decade. In fact, by the end of the last century CYP450 identification (reaction phenotyping) studies became routine during the preclinical period because of the close association between drug interactions, altered pharmacokinetic behavior, and safety.

One direct approach to characterizing the inhibitory potential of a lead compound has been to determine and rank in vitro IC_{50} or K_i values for compounds against enzyme-selective substrates, utilizing pooled human liver microsomes or recombinant CYP450 enzyme preparations (98). Most frequently, during lead selection NME are tested at one concentration in several CYP450 inhibition assays to obtain a profile. In its most streamlined conformation in 96-well microtiter plates, this method facilitates the identification of NME that have a high potential for metabolic drug interactions (99) as well as the principal P450 enzymes involved in the metabolism of certain series. The assay is typically used to screen a large number of compounds in duplicate at a single concentration (1–10 μM) against a concentration of substrate equivalent to approximately twice the NME's K_M. Each microtiter plate contains buffer controls, solvent controls, and several wells used to establish an IC_{50} curve for the reference inhibitor. Statistically, if one assumes that these assays exhibit a background inhibition of 10%, then inhibition becomes significant ($p < 0.05$) at 25%. Although criteria vary between laboratories, NME that cause 80% to 100% inhibition at 10 μM are commonly retested to determine their IC_{50}. Inhibitory behavior is evaluated in this way by using six to eight concentrations of NME over at least two orders of magnitude, and the IC_{50} is calculated with nonlinear regression of the average value at each NME concentration. Experience has shown that the IC_{50} values determined with human liver microsomes and those derived with recombinant enzymes are rarely the same, primarily because there are virtually no CYP450 enzyme-specific substrates. Screening for CYP450 inhibition requires the application of potent and specific chemical inhibitors for each enzyme. The selectivity and potency of CYP450 enzyme inhibitors have been investigated in rat and human liver microsomes and microsomes containing a single human recombinant CYP450 enzyme. The information has been extremely useful in the effort to develop high-throughput CYP450 inhibition assays (100–102). Currently, there are a wide variety of fluorescent substrates and protocols used to determine if NME inhibit cytochrome P450. Table 5 illustrates data generated on a selection of reference compounds in our lab with recombinant proteins. The final testing concentration in these assays is 1 μM, using the substrates BFC (CYP3A4) or AMMC (CYP2D6). Reference compounds are included every time one of these assays is conducted. In addition, inhibition also suggests at a very early point in time, the involvement of a specific cytochrome P450 in the metabolism of a new chemical entity, an information that is valuable to preclinical investigators. High-throughput microsomal metabolic stability assays and, in particular, the recombinant CYP450 inhibition assays are very sensitive to the presence of organic solvents (e.g.,

Table 5 Inhibition of Cytochrome P450 Enzymes by Reference Compounds

CYP3A4			CYP2D6		
Compound	**Mean**	**SEM**	**Compound**	**Mean**	**SEM**
Verapamil	31	0.7	Thioridazine	26	0.4
Terfenadine	35	1.2	Chlorpromazine	29	0.5
Cyclosporin A	52	1.4	Promethazine	47	0.9
Astemizole	62	1.3	Terfenadine	56	1.2
Buspirone	91	1.0	Propranolol	74	0.8
Triazolam	94	1.3	Timolol	80	0.7

Results are expressed as percent of activity in solvent controls.

Table 6 The Biopharmaceutics Classification System (BCS)

BCS class	Solubility	Permeability
I	High	High
II	Low	High
III	High	Low
IV	Low	Low

DMSO, acetonitrile, methanol) in which the NME are frequently dissolved (92,103–105). DMSO appears to be a universal solvent used in pharmacological profiling, but it is very detrimental to the activity of many recombinant CYP450 enzymes. However, all of these common solvents adversely affect activity to some degree, and the total concentration of all organic solvents in microsomal incubations should be minimized (e.g., <0.5%). Recently, drug metabolism scientists employed in the pharmaceutical industry have gone on record concerning their view of high-throughput pharmaceutical profiling and its place between discovery and preclinical assessments of pharmacokinetics and safety (106,107). Furthermore, examples of the entire process, from initial synthesis of NME through preclinical evaluation, have been published (108,109).

Ideally, most medicines intended for oral administration should be reasonably soluble, readily absorbed, and relatively metabolically stable in order to reach their target at a sufficient concentration. Optimization of oral delivery requires careful lead selection and subsequent optimization of the pharmaceutical properties discussed here. In fact, a new mechanistic standard for evaluating bioavailability/bioequivalence (110) is based on a biopharmaceutics classification system (BCS). The criteria include the determination of three factors. The first is dissolution number, which is related to aqueous solubility at pH 6.8 and determined by mean intestinal residence time/drug dissolution time. The second factor is dose number, which is calculated as intestinal (drug)/aqueous solubility. The third factor is absorption number. This strategy facilitates the construction of four possible classes of medicinal compounds (Table 6) based on specific data, some of which can be obtained in vitro before preclinical studies begin.

Toxicity Assessments

As mentioned in another chapter of this volume, the assessment of drug safety is prescribed by federal regulations. This process normally follows elaborate, standardized procedures, but there has been a consistent effort to develop in vitro models that are capable of generating data on cellular toxicity before as early as lead selection. Cytotoxicity in vitro is poorly correlated with LD_{50}, but good correlations have been obtained between toxicity in vitro and in vivo, using systems in which the toxic endpoint reflects the probable mechanisms of acute toxicity (111). Generally, there is agreement that in vitro data might eventually make a significant contribution to, and perhaps improve, the determination of human risk. However, remaining objections relate to the extent to which in vitro toxicity data should be used to judge potential human safety. Clearly, it is important to use batteries of tests capable of evaluating a variety of potential toxic endpoints (112).

In vitro methods may be of doubtful value for broad-spectrum toxicity screening of single chemical entities or for priority selection of unrelated chemicals. However, they can be of value for priority selection of homologous lead series with a known, specific effect (113). However, without other information about the compounds to be tested and compared (e.g., physicochemical properties), interpretation of test results and subsequent comparisons become difficult (e.g., comparing lipophilicity rather than toxicity). Under these circumstances, in vitro data may not contribute to meaningful risk–benefit assessment and decisions required for medicines (114). Despite some reluctance to incorporate in vitro determinations of cellular toxicity into established drug safety programs, a strategy that incorporates toxicology early in the selection of a lead compound could help reduce risk and prove cost effective. Scientific value may be gained through toxicity studies that identify the mechanism of toxicity. A shift in emphasis is occurring for toxicity testing, with many companies beginning to move investigative toxicity screening from preclinical development to drug discovery. Toward this end, the use of exploratory or nonroutine studies of the potential of mechanisms of toxicity is becoming more

widely adopted in discovery. In the screening mode, in vitro assays of cellular injury can be used to screen several new compounds belonging to similar therapeutic classes so as to rank order the potential for known toxic effects. The results are frequently used to identify and eliminate toxic liabilities much earlier than in the past (115).

The development of appropriate in vitro models to evaluate the toxicity of xenobiotics in drug discovery has become increasingly important as post–market failures continue to appear for otherwise efficacious medicines (e.g., troglitazone). In vitro systems enhance our understanding of the mechanisms of drug-induced toxicity. The use of reference compounds that are known to be toxic and others that have been shown to be nontoxic is very important in this process. As for other in vitro assays described in this chapter, the effects of known toxicants can be compared with the toxicity profile of the unknown agents. By using a battery of cytotoxic endpoints and measurements of cellular function, general cytotoxic characteristics of the compounds can be determined. Therefore, during the past decade, the testing of in vitro models has expanded greatly in an attempt to reduce the time required by traditional animal testing models (116). One strategy that has been widely adopted in this regard is the use of biomarkers in toxicity screening. Biomarkers of exposure (e.g., GSH depletion) or effect (e.g., enzyme induction) may be used to rank NME (117). At this point of time, the primary difficulties associated with the application of these techniques to drug discovery are the vast amount of information generated by a single experiment and the interpretation of the results in the absence of information on the biological and pharmacological significance of observed changes. Therefore, attempts are underway to use simpler systems to detect biomarkers of exposure and effect as indicators of perturbations in normal cellular physiology. The underlying assumption is that these perturbations could lead to toxic events. In many cases, hepatocytes and hepatoma cell lines (e.g., HepG2 cells) may be used to test NME for these effects. For this purpose, non–liver-specific end-points for toxicity are used, including plasma membrane integrity (dye uptake, intracellular enzyme or ion leakage), lysosomal integrity, mitochondrial activity, and metabolic competence (protein and DNA synthesis, GSH content, lipid peroxidation) (106,118). One example of this technique is the application of fluorescence microscopy to evaluate the integrity of electron transport pathways via rhodamine reduction (119). However, the routine application of these assays in drug discovery is in its infancy and a great deal of work remains before the value of these determinations can be measured.

During the past decade, issues related to safety pharmacology in the cardiovascular system have become increasingly important and have come under regulatory oversight. Whereas successful development of in vitro systems related to respiratory and central nervous systems for the purpose of lead selection has been generally unsuccessful, an increase in the number of patients experiencing life-threatening arrhythmias after taking some nonsedating antihistamines and GI prokinetic agents in combination with certain antimicrobials (120) eventually prompted the successful development of in vitro models in order to understand the basis of this phenomenon. Two cell culture models dominate this effort including stably transfected *Xenopus* oocytes (121,122), which have become readily available, and transfected human embryonic kidney cells (123,124), which were initially more difficult to obtain in sufficient numbers. The basis of this adverse effect is the inhibition, by many xenobiotics, of the inward flow of K^+ ions through the hERG channel that permits delayed repolarization of cardiomyocytes, causing the onset of the characteristic irregular heartbeat known as *toursade de pointes* by prolonging the QT interval. These cells form the basis for two different methods of predicting the interaction of new compounds with the hERG channel. The first method is typically used during lead selection and measures the degree of displacement of a high-affinity hERG ligand such as dofetilide (124) or E4031 (125,126) by the test compound, using membranes isolated from the transfected cells. Dofetilide and E4031 are class III antiarrhythmics. The former is a successful drug and the latter is a sotalol derivative that did not make it to the market. The second method is more frequently used during lead optimization and measures the suppression of the hERG tail current by using a whole-cell patch-clamp recoding technique, which has been used for decades to study the function of many ion channels. Table 7 compares the inhibition of the hERG tail current by orally administered first- and second-generation antihistamines, a GI prokinetic drug, a common antifungal medicine, an opioid analgesic drug, and a class III antiarrhythmic drug. Clearly, the first-generation antihistamines are less active in this assay compared to the second-generation

Table 7 The Inhibition of the Potassium Tail Current by First- and Second-Generation Antihistamines and Other Drugs at 250 ng/mL in HEK293 Cells Transfected With hEGR cDNA

Test compound	Inhibition of tail current (%)
Diphenhydramine	23.9
Pyrilamine	32.4
Chlorpheniramine	46.3
Astemizole	78.4
Terfenadine	85.0
Cisapride	85.8
Ketoconazole	8.1
Codeine	54.0
Sotalol	17.4

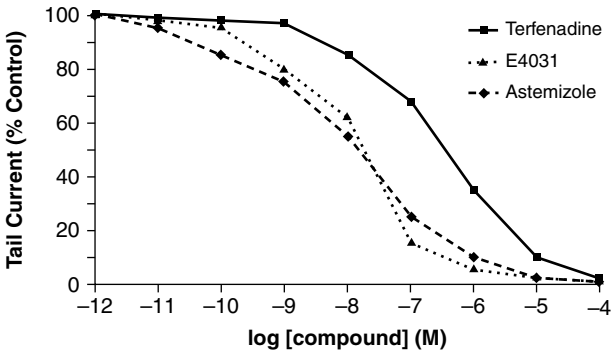

Figure 8 Dose–response curves assessing drug inhibition of hERG tail current. The graph compares the effects of the class III antiarrhythmic drug E4031 and two nonsedating antihistamines, terfenadine and astemizole.

antihistamines at the same concentration. Both methods may be used to determine the effect of a single concentration of the test compound or to characterize an IC_{50} value for hERG inhibition. Figure 8 illustrates the similarity in the dose-response curves between the failed class III antiarrhythmic drug E4031 and the two failed nonsedating antihistamines, terfenadine and astemizole, that should have had little or no effect on the hERG tail current. The results of these methods are highly influenced by the solubility of the compounds in question, although the influence of solubility on the outcome of binding method is less than that of the patch-clamp method, as the former employs serum proteins as a component of the culture medium and the latter does not.

CONCLUSION

This chapter was originally written while pharmaceutical profiling of NME during lead selection was first practiced widely. There are still debates about the value of this strategy and the extent to which one should profile the pharmaceutical properties before selecting a lead series or compound. The inclusion of toxicity assays is still very controversial. Published materials on the process are relatively scarce and the proof-of-principle for pharmaceutical profiling is still being tested. However, the most current research reports on this subject include that of Kerns and Di (127) and Eddershaw et al. (128). Several generalizations may be made at this time. First, many of the procedures described here are not necessarily applicable across diverse molecular structures or therapeutic areas. Instead, these assays appear to function most reliably when homologous series of NME are tested and compared. Many of the models mentioned here have been validated with small sets of existing medicines and the generalization of these results to new medicinal compounds arising from combinatorial synthesis may not be a valid procedure. Finally, all of these procedures require ongoing validation with the inclusion of a set of suitable reference compounds each time an experiment is conducted.

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3 Interspecies Differences in Physiology and Pharmacology: Extrapolating Preclinical Data to Human Populations

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OVERVIEW

Preclinical animal data are an integral component of the product development process, being used for predicting the potential for drug toxicity and for estimating first-time doses in humans. These extrapolations are based upon an assumption of a correlation between the exposure–response relationship in animals and man. Unfortunately, there is no single animal species that can serve as the "perfect" surrogate for human subjects, and the appropriate surrogate species needs to be evaluated for each situation (1).

Selection of the animal species to be used for toxicity testing should factor the potential interspecies differences that can influence systemic drug exposure and target cell sensitivity. These include potential differences in drug absorption, clearance, distribution, and metabolism. These factors can determine whether or not a species will exhibit toxicity or drug carcinogenicity (2).

In this chapter, the impact of study design, as well as interspecies differences in physiology and drug metabolism, will be explored from the perspective of the influence of these variables on the relationship between dose, drug exposure, and response.

TOXICOLOGY TESTS: POINTS TO CONSIDER

Several offices within the U.S. Food and Drug Administration (FDA) have Pre-Investigational New Drug Application Consultation Programs. These programs are designed to foster early communications between sponsors and the agency. Advice may be requested for any aspect of drug development. All such communications are considered "informal" under 21CFR 10.90(b)(9) and do not obligate the agency or the sponsor.

As defined in CFR 312.23(a)(8), a sponsor filing an investigational new drug (IND) application must provide the results of pharmacological and toxicological studies of the drug involving laboratory animals and/or in vitro tests, which can provide the basis for the conclusion that it is reasonably safe to conduct the proposed clinical investigations. As drug development proceeds, the sponsor is required to submit informational amendments, as appropriate, with additional information pertinent to safety.

As per the regulation [CFR 312.23(a)(8)], the IND filing must include the following:

(i) *Pharmacology and Drug Disposition*: A section describing the pharmacological effects and mechanism(s) of action of the drug in animals and information on the absorption, distribution, metabolism, and excretion of the drug, if known.
(ii) *Toxicology*: An integrated summary of the toxicological effects of the drug in animals and in vitro. Depending on the nature of the drug and the phase of the investigation, the description is to include the results of acute, subacute, and chronic toxicity tests; tests of the drug's effects on reproduction and the developing fetus; genetic toxicity testing; any special toxicity test related to the drug's particular mode of administration or conditions of use (e.g., inhalation, dermal, or ocular toxicology); and any in vitro studies intended to evaluate drug toxicity.

It is not unusual for the agency to request draft/final study reports for the pivotal studies conducted in support of the initial IND, especially for new molecular entities.

Regulatory pharmacology and toxicology guidances involving animal models published by the International Committee on Harmonization (ICH) and/or the U.S. Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER) include the following http://www.fda.gov/cder/PharmTox/guidances.htm):

Pharmacology

- Pharmacokinetics: *Guidance for repeated dose tissue distribution studies; ICH-S3B* (March 1995)
- Toxicokinetics: *The assessment of systemic exposure in toxicity studies; ICH-S3A* (March 1995)
- Safety pharmacology: *Guidance for industry: Safety pharmacology studies for human pharmaceuticals; ICH S7A* (July 2001)

Toxicology

- *Single and repeat dose toxicity: Nonclinical safety studies for the conduct of human clinical trials for pharmaceuticals; ICH-M3* (July 1997)
- *Single dose acute toxicity testing for pharmaceuticals; PT1* (August 1996)

Reproductive Toxicity

- *Detection of toxicity to reproduction for medicinal products; ICH-S5A* (September 1994)
- *Detection of toxicity to reproduction for medicinal products: Addendum on toxicity to male fertility; ICH-S5B* (April 1996)

Pediatric Drugs

- Draft Guidance for Industry: *Nonclinical safety evaluation of pediatric drug products* (February 2003)

Throughout the various ICH/CDER guidances, we see the same basic points to consider when selecting an appropriate animal model. These include

- similarity in toxicological/pharmacodynamic responsiveness,
- pharmacokinetic profiles similar to those seen in humans, and
- similar metabolic profile.

TOXICOLOGICAL ENDPOINTS

Observations generated in a toxicity study represent discrete protocol driven points on the dose–effect profile. Therefore, the outputs from these tests serve only as experimentwise approximations of the true continuous relationship between exposure and the biological effect. Nevertheless, these points serve as valuable information upon which to base first-time-in-human dosages of new chemical entities. Pivotal terms used to describe the results of toxicological investigations include the following (3):

NOEL (no effect level): The highest exposure level at which there is no drug-related adverse or nonadverse effect observed in the target population.

NOAEL (no adverse effect level): The highest exposure level at which there are no statistically or biologically significant increases in the frequency or severity of an adverse effect between the exposed population and the corresponding control group.

LOAEL (lowest adverse effect level): The lowest exposure level at which there are statistically or biologically significant increases in the frequency or severity of adverse effects between the exposed versus control populations.

MTD (maximal tolerated dose or minimally tolerated dose, depending on the implication): This is the dose at which biologically significant effects, directly/indirectly due to test compound administration, are found to have an appreciable effect on the quality and length of the life span of the animal. This may include a variety of outcomes, such as a lack of feed intake due to unpalatability of the test article, direct effect on the cardiovascular system, cecal dilatation, and torsion due to changes in cecal flora in rodents.

Table 1 Species-Specific Toxic Effects

Type of toxicity	Structure	Sensitive species	Mechanism of toxicity
Ocular	Retina	Dog	Zinc chelation
Ocular	Retina	Any with pigmented retinas	Melanin binding
Stimulated basal metabolism	Thyroid	Dog	Competition for plasma binding
Tubular necrosis	Kidney	Male rats	Androgen-enhanced sensitivity
Urolithiasis	Kidney and bladder	Rats and mice	Uricase inhibition
Teratogenesis: fetal mortality	Fetus	Rats and mice	Uricase inhibition
Cardiovascular	Heart	Rabbits	Sensitivity to microvascular constriction

Source: From Ref. 4.

Within this framework, an adverse effect is a biochemical, morphological, or physiological change that contributes to or is responsible for adversely affecting the performance (e.g., life span, health, well-being, growth) of the organism. Alternatively, it may reflect a reduced ability of the organism to respond to its environment. A biologically significant effect is a response that is considered to have a substantial or noteworthy positive or negative effect on the well-being of the biological system. This is contrasted with a statistically significant effect that may not be meaningful to the state of health of the organism (3).

Some adverse reactions are also reflective of physiologic idiosyncrasies associated with a particular species. These do not correlate with exposure–response relationships in humans. Several of these peculiarities are summarized in Table 1 (4).

In some cases, biological effects may reflect adaptive responses that are not related to the inherent toxicity of the test substance itself. An example of such a response is the histological change that may occur as an adaptive reaction to the inhalation of a compound (5). These include mucus cell hyperplasia induced by dehydration of the nasal epithelium because of inhalation of aerosols, macrophage accumulation in the lung after exposure to low solubility materials (in the absence of any other signs of an inflammatory reaction), and replacement of alveolar epithelium by ciliated epithelial cells as an adaptive response to high concentrations of exogenous materials.

Despite our best efforts, there will continue to be cases where toxicity in man could not be predicted from animal data. For example, fenclozic acid, which was a potential anti-inflammatory compound, was found to be without any adverse effects in an array of animal species including mouse, rat, dog, rhesus monkey, patas monkey, rabbit, guinea pig, ferret, cat, pig, cow, and horse. However, it caused acute cholestatic jaundice in people (6).

FACTORS THAT CAN INFLUENCE STUDY RESULTS

Variables that can affect the outcome of studies intended to examine preclinical exposure–response relationships include the following (7):

- *Weight*: Animals of the same weight may have differences in lean tissue mass.
- *Age*: Age does affect sensitivity for some drugs in some species, including humans.
- *Sex*: Females of some species can exhibit more (or less) frequent toxic effects as compared to males.
- *Time of Administration*: Considerations include period of fasting, gastric emptying rate, and diurnal rhythms.
- *Temperament*: Stressors may cause a constriction of the splanchnic visceral blood vessels, which can affect drug metabolism and the proportion of the total cardiac output reaching the peripheral tissues.

Animal age is an important consideration when conducting toxicological studies to support drug use in human pediatric populations. The age of the animal used as the toxicological test species should be consistent with the intended age of the targeted human recipients because of potential differences in drug disposition and action, metabolism, body composition, receptor expression, and organ function that may occur in juveniles versus adults. This issue is discussed in detail later in this chapter.

Formulation may also influence drug effects. For example, in mice, both the lethal dose (expressed as LD_{50}) and the ability to achieve some pharmacodynamic endpoint (e.g., righting reflex) for a fast-acting compound (sodium pentobarbital) was significantly different when administered as an intraperitoneal injection of an aqueous solution or in a 1% carboxymethylcellulose solution. The decrease in pharmacological response with the 1% carboxymethylcellulose solution was attributed to an increase in product viscosity, which in turn retarded drug uptake (8). This simple example underscores the influence of formulation in preclinical studies. Additional insight into potential species-specific considerations in drug formulations has been published elsewhere (9).

Excipients used in preclinical drug formulations can markedly affect the level of drug exposure. Permeability enhancers such as the bile salt sodium deoxycholate (10), fatty acids such as sodium caprate (10), and surfactants (11) such as polysorbate 80 (12), Cremophor EL (13), and vitamin E (14) can alter P-glycoprotein (P-gp) activity. P-gp is a membrane transporter protein that can affect the first-pass drug loss of many compounds. The role of P-gp in determining drug oral bioavailability is discussed later in this chapter and in Chapter 8.

Differences have been observed in the ability of the various animal species to express toxic reactions similar to that in humans (15). In a survey of the 20 chemical entities for which preclinical and clinical toxicity information was available, monkeys, rats, and mice appear to exhibit the greatest similarity to humans in adverse events. Dogs are associated with a more frequent occurrence of false-positive reactions (Table 2).

Similarly, in comparing the accuracy of the predictions of human drug toxicity generated in dogs and monkeys, Schein et al. (16) observed that bone marrow depression, gastrointestinal (GI) disturbances, and hepatotoxicity tend to be correctly predicted in monkeys and dogs. However, these same species present with a high percentage of false positives. Of the 25 anticancer drugs investigated, dogs exhibited a particularly high rate of false positives for pathology of the stomach, small and large intestine, liver (including increases in alkaline phosphatase), and kidney (including proteinuria). The rate of false positives in monkeys was slightly less than that of dogs. Cases where neither species expressed toxic reactions seen in humans were rare, although examples of renal, cardiovascular, and neuromuscular toxicity do exist.

With regard to rats, basic human–rat differences in physiology may affect study outcome. Unlike humans, rats can synthesize ascorbic acid, have no gall bladder, are coprophagous, are obligate nose breathers, and have important differences in their lung function and morphology (17). Moreover, when rodents are treated with antimicrobial agents, they frequently develop cecal dilation and torsion due to alterations in their intestinal flora. This finding may preclude their use as models for development of these drugs. Monroe and Mordenti (17) have summarized the physiological, anatomical, and biochemical factors that can be considered in preclinical studies when analyzing data from studies that employ rats as the target species. Their summary is reproduced in Table 3.

Strain of animal may also affect study outcome. For example, there is greater tobramycin toxicity observed in Fischer rats as compared to Sprague Dawley rats (18). Differences in toxic

Table 2 Correlation of Toxicity to That Observed in Humans

	Rat	Mouse	Dog	Monkey
Number of comparisons with humans	14	11	11	6
Similar to human (+ or −)	71%	73%	45%	83%
False positive	21%	—	36%	—
False negative	7%	27%	18%	17%

Source: From Ref. 15.

Table 3 Factors That May Influence the Ability to Extrapolate Toxicity and Carcinogenicity Data from Rats to Humans Parameter

	Rat	Human	Comment
Body weight (kg)	0.35	70	Humans have hundreds of more cellular (DNA) targets for carcinogenic attack
Surface area (m^2)	0.05	1.75	
Life span (hr)	2.5	70	Humans can be exposed much longer, but the aging and carcinogenesis processes are interrelated
Food consumption (dry) g/kg BW/day	50	10	High intake of lipid and protein leads to cumulative oxidative damage that contributes to aging and cancer
Basal metabolism (kcal/kg/day)	109	26	High metabolic rate correlates with DNA oxidative damage
Anatomical			
Forestomach, Zymbal's gland, Harderian gland, preputial gland, clitoral gland	Present	Absent or rudimentary	Difficult to interpret tumors in organs present in one species but not the other
Bronchial glands	Absent	Present	
Emetic reflex	Absent	Present	May retain some toxicant that humans would not
Liver weight (% BW)	5%	2.20%	Rates of organ growth and cell turnover may contribute to carcinogenic susceptibility
Physiological			
Reproductive cycle	Estrus	Menstrual	Difficult patterns and roles for estrogen and progesterone may affect susceptibility to certain cancers
Parity	High	Low	Pregnancy protects against some cancers
Prolactin—role in mammary gland activity	High	Questionable	Modulations of prolactin secretion will have different consequences for mammary carcinogenesis
α-2μ-globulin	Present, especially in males		Protein necessary for some renal and perhaps bladder cancers
Stomach pH	4–5	1–2	Can affect activation/deactivation of some xenobiotics that undergo enterohepatic cycling

(Continued)

Table 3 Factors That May Influence the Ability to Extrapolate Toxicity and Carcinogenicity Data from Rats to Humans Parameter *(Continued)*

	Rat	Human	Comment
Bacterial flora	Numerous	Few	
Thermoregulation			Key controlling tissue in rats is brown fat; in human it is the dermal vasculature
Hematology (expressed relative to human values)			
GSH peroxidase	10.2	1	Enzymes important in countering oxidative damage to cells
GSH reductase	0.2	1	
Superoxide dismutase	1.7	1	
DNA repair			
Excision repair	Low		May be a factor in determining life span and in defense against DNA alterations
Hepatic O^6-alkyl guanine transferase	1	10	Key enzyme in detoxifying a common type of DNA–carcinogen adduct
Xenobiotic metabolism			
Epoxide hydrolase (liver)	Low	High	Enzyme important in detoxifying epoxides
Phase I and II enzymes			Difficult to predict transformation pathways in the two species

Source: From Ref. 17.

responses between species of monkeys are also evident. Stump-tailed macaques exhibit the same thrombocytopenia as that seen in humans with compound BL-4162. However, neither the rhesus monkey, cynomolgus monkey, squirrel monkey nor the chimpanzee exhibits that same toxic effect (15).

Conditions associated with animal care, such as crowding, isolation, temperature, food or water restriction, alteration of light–dark cycle, immobilization, handling, and drug administration procedures, can result in physiological changes that are not drug-related. Each of these conditions can alter the release of hormones such as adrenal corticotrophic hormone, thyroid hormone, insulin, and many of the pituitary hormones. In turn, the latter can modify responses to the various toxicants (4,19).

The dose–effect relationship can also be influenced by normal diurnal rhythms. For example, both hepatic and renal functions exhibit diurnal variation in mice. Metabolism is higher during the active dark phase as compared to the light phase (20). Significant circadian-related fluctuations in drug pharmacokinetics have been observed for a wide variety of drugs including antimicrobial compounds, neurological and psychiatric drugs, anti-inflammatory drugs, and cardiovascular agents (21). There can be marked diurnal variability in disease expression and drug therapeutic activity (22–24). Similarly, the magnitude of drug toxicity may vary as a function of administration time (25–27). In some cases, circadian variability in drug toxicity has been attributable to fluctuations in the activity of certain metabolic pathways (27), and these variations may not be equally expressed in males and females (28).

Fasting can alter drug pharmacokinetics. In addition to the relationship between prandial state and factors such as gastric emptying, drug dissolution, enterocyte permeability, and hepatic blood flow (29,30), fasting itself can significantly affect the level of several metabolizing enzymes. In some cases, cycles in eating activity are responsible for the apparent circadian variability in drug pharmacokinetics (31). Partial dietary restriction was found to exert a protective effect against certain types of carcinogens. When fed 75% of ad libitum intake, rats were found to

have a significant reduction in certain types of tumors (i.e., pituitary adenomas, hepatic foci) as compared to animals provided food ad libitum (32).

The composition of the animal diet can also influence the response to toxic agents. Rats fed diets deficient in vitamin A or β-carotene showed significantly higher rates of malignant tumor formation in response to exposure to aflatoxin B1. However, diets containing 10 times the normal levels of vitamin A did not result in a protective effect above that observed under control conditions (32).

Dietary fats themselves may affect drug pharmacokinetics (33,34). This variable may influence the results of toxicological studies, since prior to oral administration in rats, lipophilic compounds are frequently dissolved in dietary vehicles such as corn oil, olive oil, or sesame oil. While these vehicles do not appear to significantly alter drug metabolism when administered in an amount consistent with that used during experimental dosing, significant changes were found to occur in the levels of certain microsomal enzymes (e.g., increased CYP3A and decreased CYP2C11). Accordingly, the possibility that these dietary oils may influence hepatic CYP-mediated drug metabolism or exacerbate certain CYP-mediated drug–drug interactions cannot be discounted (34).

In response to concerns regarding the influence of dietary fats on the outcome of toxicological studies when used as gavage vehicles, the National Institutes of Health sponsored a study comparing the toxic effects of corn oil, safflower oil, and tricaprylin (35). Each gavage dose was administered at volumes of 2.5, 5, or 10 mL/kg daily for five days per week for a total of two years. Observed effects of these oils included hyperplasia and adenoma of the exocrine pancreas, a decrease in the incidence of mononuclear cell leukemia, and a reduction in the incidence or severity of nephropathology in male rats. There was also an increase in the incidence of squamous cell papillomas of the forestomach of rats receiving 10 mL/kg of tricaprylin. For the most part, this investigation demonstrated that all three oils were capable of causing dose-related toxicities and that it is the level of fat rather than the degree of saturation that is the most important consideration in this regard.

In a two-year study where a 500 mg total dose of dichloromethane was administered with corn oil (2.5, 5, or 10 mL/kg) to male rats, the use of a corn oil vehicle substantially reduced the highly toxic effects associated with dichloromethane. When administered without corn oil, the dichloromethane group exhibited severe toxic reactions. However, rats survived the two-year study period when administered this compound along with corn oil. Pathological findings were consistent both with the dose-related toxic and protective effects of the corn oil itself as well as toxic effects of the dichloromethane (35). While this investigation substantiated that oily vehicles can influence the results of a toxicity study, a note of caution was raised with regard to the interpretation of some published investigations. In particular, several reported relationships between oil intake and carcinogenicity were found to use control groups administered diets deficient in vitamins, essential amino acids, or energy. Such deficiencies can inhibit the growth of neoplasms.

Sex differences themselves can be species specific. For example, in male rats, the rate of microsomal metabolism tends to be higher than that in females (36). This may lead to sex-related differences in the level of the parent compound or an active metabolite. In evaluating 98 pesticides, Gaines (37) observed that the majority of orally administered drugs were more toxic in females as compared to males. The reverse was true for only 9 out of 98 compounds. However, similar sex-related differences were not observed in dogs (38). Studies involving other P450 systems likewise support the premise that rats tend to express sex-related differences more frequently than do other animal species (38,39). In part, sex differences in the daily rhythm of rat hepatic enzymes have been linked to sex differences in the pattern of growth hormone secretion (28).

In humans, there is a statistically significant higher level of plasma cholinesterase in young healthy males as compared to females—the activity in females is estimated to equal 64% to 74% of that of males. This difference disappears in geriatric individuals (40). In contrast, there are no significant sex-related differences in erythrocyte cholinesterase (41) or in brain cholinesterase (42). Therefore, toxic effects associated with anticholinesterase agents in humans may or may not exhibit sex-related dependencies, depending upon the age of the recipient, the drug's site of action, and its relative affinities for the various forms of cholinesterase.

Although this chapter focuses on exposure to xenobiotics via the oral or parenteral routes, it is important to note that additional concerns may arise when examining exposure by other routes such as inhalation and dermal (4). For example, while rabbits are often used for testing the toxicity of dermal exposure, it is the pig that most closely resembles the dermal absorption characteristics of humans. With regard to inhalation exposure, the following differences need to be considered when extrapolating between rats and humans:

- There is a significant difference in the filtration size of particles that are inhaled via the nose (3 μm filtration) versus that by mouth (10 μm filtration). This will impact the nature of particulate drug exposure in humans (which are nose and mouth breathers) versus rats (which are obligate nose breathers).
- The number of daughter generations of the air passage in humans is 35, while there are fewer than 25 generations in the rat.
- The total lung volume of rats is only 10% of that of humans.

Furthermore, in contrast to convention, the species most closely resembling humans with regard to respiratory system structure and function are the horse and donkey.

In general, when using multiple species for assessing the risk of drug toxicity in humans, the probability of an inappropriate conclusion is generally low (43). Boxenbaum and Di Lea (44) estimated these risks in an effort to predict the likelihood of a serious adverse event when a drug is administered as a first-time dose to healthy human subjects:

- Sum of observed occasions when rat and other nonprimate species exhibited a "good" or "fair" model for human drug toxicity is 0.92.
- Frequency of an adverse event that is predicted incorrectly is 8% of total tests. This may be attributable to an adverse reaction seen in animals that does not occur in humans, or an adverse event in humans that was not predicted in animal studies.
- Assuming a 5% risk of failure to predict an adverse event in humans and given the safety factors built into the estimate of the first-time dose in humans, it was found that only 1% of these unpredicted events are serious. Accordingly, the risk of a serious adverse event associated with studies involving first-time dose in humans is $0.05 \times 0.01 = 0.0005$. In other words, in only 0.05% of the times do we anticipate that an unexpected serious adverse event will occur when a drug is administered for the first time to human subjects when there is an appropriate and adequate preclinical toxicity profile on which the assumptions are based.

During a workshop of the International Life Science Institute in which the toxicity of pharmaceuticals in humans and laboratory animals were compared (45), it was concluded that an interspecies difference in parent drug exposure was an unlikely cause for differences in adverse reactions. Rather, interspecies differences in target tissue response and drug metabolism were concluded to be the more likely reason for many of these discrepancies.

INTERSPECIES PHARMACOKINETIC DIFFERENCES

Drug Absorption

A host of physiological variables may contribute to interspecies differences in drug absorption and bioavailability. These variables include drug product dissolution, gastric transit time, intestinal permeability, first-pass drug loss, and food effects. Interspecies differences in GI physiology and the impact of these differences on drug absorption have been reviewed elsewhere (9,29).

Much of the interspecies diversity in GI anatomy and function reflects differences in primary sources of dietary constituents (46–49). For example, carnivores (e.g., dogs, cats) possess a relatively simple colon but a well-developed small intestine (long villi). This is consistent with a diet that is low in fiber but high in fat and protein. Omnivores (e.g., rats, pigs) possess a well-developed small intestine but have a more complex lower intestine to compensate for their more diverse diet. The lower intestine of pigs is differentiated enough to allow for dietary fiber fermentation. Herbivores are either foregut (e.g., sheep) or hindgut fermenters (e.g., horse) and rely upon fermentation processes for nutritional intake. Intestinal villi vary in length from 0.5 to 1.0 mm, depending on region and species. They are generally long and slender in carnivores,

and short and wide in ruminants. Rats, mice, and horses lack an emetic reflex (6,48), and rats and horses lack a gallbladder (6).

The digestive systems of ruminants differ markedly from those of monogastric species, and these differences can significantly alter drug absorption (48). In the case of ruminants, the forestomach (rumen, reticulum, and omasum) is a large volume compartment lined with stratified squamous epithelium. This site of microbial fermentation will both catabolize cellulose-containing materials and degrade drugs. The capacity of this is 10 to 24 L in sheep and goats. The pH values range from 5.5 to 6.5 because of a large volume of alkaline saliva (pH 8–8.4) that is secreted to buffer organic acid production in the rumen. Although gastric juices are not secreted in the forestomach, the rumen has a large capacity for drug absorption, particularly for weak acids. The abomasum, the fourth chamber, is the true stomach and secretes digestive juices. The generally larger hepatic capacity of herbivores tends to result in greater metabolism of lipophilic compounds.

Interspecies differences in GI transit time can markedly affect the extent of drug absorption and consequently, dose–effect relationships. An illustration of this is the failure of beagle dogs to adequately model the human bioavailability testing of acetaminophen sustained-release tablets (50), griseofulvin tablets (51), valproic acid (52), and ampicillin (53). When comparing the gastric emptying of fasted humans, dogs, and minipigs, the order in the rate of gastric emptying is dogs > humans > minipigs (54). These differences are observed both with tablets (enteric-coated aspirin, diameter = 5.8 mm, 1.24 g/cm^3; and barium sulfate tablets, diameter = 6.0 mm, 1.52 g/cm^3) and granules (diameters = 0.1 mm, density = 1.17 and 1.34 g/cm^3 respectively). Tablets empty more rapidly than granules in dogs, but are cleared at a similar rate in humans. In contrast, granules tend to clear slightly faster in pigs, as evidenced by the time required to move 50% of the tablets versus 50% of the granules through the swine stomach.

Despite the faster gastric emptying observed in dogs versus humans under fasted conditions, food induces a substantially greater delay in the emptying of large particles (tablets) and pellets in dogs as compared to humans (55). For example, in dogs, gastric emptying of pyridoxal phosphate enteric-coated tablets continued to occur for more than 10 hours in fed dogs. In contrast, gastric emptying of these tablets in humans did not extend beyond five hours after postprandial administration.

Marked interspecies differences are also observed in intestinal transit times. When fluid or particulate markers were administered intragastrically, the percent of dose excreted in the feces from 0 to 24 hours in dogs versus mature swine were 55% and 7%, respectively, for the fluid markers. For particulate markers, 24-hour fecal excretion was 40% and 2% in dogs and swine, respectively (56). Sustained-release preparations (eroding matrix) of the lipophilic compound propylthiouracil demonstrates very poor bioavailability in dogs, because the rapid GI transit does not provide the time needed for complete product dissolution. Generally, the product will reach the canine colon (two to three hours) before having an opportunity to dissolve (57).

Depending upon the pK_a of the drug in question, differences between human and canine gastric pH can lead to differences in the extent of drug dissolution. Accordingly, interspecies deviations in gastric pH have been implicated as a cause for dissimilarities in the bioavailability of indomethacin (58), metronidazole (59), and cinnarizine (60). However, differences in gastric pH are confounded by further interspecies differences in the food effects. Generally, the gastric pH of fasted dogs is highly variable, ranging between 3 and 8 (61). Following a meal, gastric acid secretion rates in dogs exceed those of humans and swine. The postprandial gut pH in humans tends to exceed that observed in dogs because of the strong buffering action of the diet, but human gastric pH values return to baseline values within approximately one hour (62).

Interspecies variation in intestinal absorptive surfaces can result from dissimilarity in the size and shape of the intestinal villi (62). Differences in surface area for paracellular absorption could influence the relative bioavailability of small hydrophilic (low permeability, high solubility) compounds. Conversely, highly permeable drugs are generally absorbed upon contact with the intestinal membrane, with the majority of absorption occurring at the villus tip (63). Minimal (if any) differences in the absorption of highly permeable compounds across animal species are anticipated.

To date, little information is available with regard to interspecies differences in lymphatic uptake. In part, this may be due to bias associated with the types of methods used to assess

lymphatic drug uptake in animals (64). Nevertheless, the comparative extent of drug uptake into the lymphatic system across species may by expected to be influenced both by the characteristics of lymph flow to various absorption sites and by the mechanism through which the lymphatic absorption occurs. Since lipid digestion may be expected to differ between herbivores and carnivores, it would be reasonable to expect better lymphatic uptake in carnivores or omnivores as compared to herbivores. This may also be attributable to diet-related differences in bile salt composition and its corresponding impact on lipid solubilization (65). Furthermore, since the density of intestinal lymphoid tissue shows species-related differences, we may expect dissimilarities in lymphatic entry into specialized tissues such as Peyer's patches (66).

The comparative estimates of oral bioavailability are often linked to species-specific differences in drug metabolism occurring in the gut and/or the liver. The principle intestinal biotransformation enzymes in humans include the cytochrome P450 (CYP) subfamily, glucosyltransferases, sulfotransferases, *N*-acetyltransferase, glutathione *S*-transferase, esterases, epoxide hydrolase, and alcohol dehydrogenase (67). Within the gut wall, differences in site-specific drug metabolism are known to occur across animal species. For example, esterase activity, while present in the order of duodenum > jejunum > ileum > colon, is greater in rats as compared to pigs and man. The esterase activity of humans is somewhat greater than that of swine (68).

The relationship between drug absorption, gut metabolism, liver metabolism, and drug bioavailability is described by the following relationship (69):

$$F = f_{\text{abs}} \times (1 - f_{\text{g}}) \times (1 - f_{\text{h}})$$

where F = the absolute bioavailability of the drug,
f_{abs} = the fraction of the dose absorbed from the GI lumen,
f_{g} = the fraction of drug metabolized by the gut wall,
f_{h} = the fraction of drug metabolized by the liver.

Since the permeability of molecules across the gut wall tends to be similar across species, the predominant cause of dissimilar bioavailability across animal species is related to corresponding values of f_{g} and f_{h}.

The importance of first-pass metabolism is seen with indinavir. Differences in oral bioavailability (72% in dogs, 24% in rats, and 19% in monkeys) are attributable to species-specific variations in the extent of hepatic first-pass extraction (approximately 68% in rats, 65% in monkeys, and 17% in dogs) (70). In human subjects, the oral bioavailability of indinavir is approximately 60% (71). In a survey conducted by Chiou and colleagues (72,73), the oral bioavailability of most drugs tended to be substantially lower in dogs as compared to rats and humans, largely because of the greater first-pass drug loss seen in dogs. In contrast, drug bioavailability in rats and humans tends to be highly correlated.

The small intestine is a potential site of drug metabolism, and substantial drug loss can occur via intestinal efflux mechanisms, gut wall metabolism (both phase I and phase II), and degradation within the gut lumen (69,74,75). While the total amount of P450 in the human intestine is much less than that in the liver (20 pmol/mg microsomal protein vs. 300 pmol/mg microsomal protein, respectively), the intestinal enzymes are strategically situated to maximize exposure to the intestinal contents. P450 concentrations tend to be greatest in the villus tips of the upper and middle third of the intestine (76).

Of particular importance is the synergy between P-gp and CYP3A4, which together are responsible for the active extrusion and subsequent metabolism of a wide variety of compounds (77). P-gp is located on the apical surfaces of many organs including the bladder, kidney, brain, liver, lungs, pancreas, stomach, spleen, esophagus, and the large and small intestines (78,79), and interspecies differences in the tissue of expression of drug transporters have been observed (80). In the intestine, the ratio of fluxes from basolateral to apical versus apical to basolateral direction ranges from 1.4 to 19.8, depending upon location within the GI tract (81). Evidence suggests that P-gp substrate affinity may vary as a function of intestinal site (82).

The importance of P-gp may be most clearly seen in bioavailability studies conducted with mice expressing the *mdr1a*(−/−) genotype ("knockout" mice). This strain exhibits a total absence of gut P-gp activity. These mice were used to examine P-gp role in limiting the intestinal

absorption of paclitaxel (83). Paclitaxel area under the curve (AUC) values after oral administration in wild-type mice [*mdr1a*(+/+)] versus knockout mice [*mdr1a*(−/−)] were 11% and 35%, respectively. Intestinal secretion following intravenous administration was practically eliminated in knockout mice, even though 40% of the dose underwent intestinal secretion in the wild-type mice. Similar differences in wild type versus knockout mice bioavailability were observed for compounds such as vinblastine, digoxin, indinavir, and talinolol (84). An effect corresponding to the *mdr1a*(−/−) genetic variant of mice was observed in humans, where certain variations in the *MDR1* gene have been shown to alter both the gut expression of P-gp and the oral bioavailability of P-gp substrates (85).

Compounds may also be extensively metabolized in the gut lumen by digestive enzymes or by activity of the gut microflora. The colon contains the largest population of microorganisms in the monogastric GI tract and is the major site of production and absorption of volatile fatty acids in the pig, rabbit, rat, dog, and human (62). An excellent example of the potential negative impact of microbial metabolism is the species-by-route differences in blood concentrations achieved when chloramphenicol is administered to goats, pigs, dogs, cats, and horses. Despite high levels achieved in the goat after intramuscular administration, the oral bioavailability of this compound in goats was negligible because of microbial degradation in the gut. Similar problems did not occur when this compound was orally administered to monogastric species (86). Conversely, the presence of gut microflora may enhance drug bioavailability by promoting biliary recycling of compounds such as ouabain, digoxin, and steroid hormones (87). In these cases, the bacteria remove the polar moiety from the derivatized conjugates, rendering them available for intestinal absorption (74).

In contrast to the oxidative and conjugative metabolism of the liver and intestinal mucosa, bacterial metabolic reactions are largely degradative, hydrolytic, and reductive. As such, they are involved in the enterohepatic recirculation of many compounds. Drugs conjugated with polar groups in the liver prior to their secretion into the bile are hydrolyzed within the upper and lower intestine. β-glucuronidase, sulfatase, and glycosidases are all bacterial enzymes found in the gut of human and domestic animal species (88,89).

An example of how bacterial flora can impact drug toxicity is seen with chenodeoxycholate (CDCA), a compound used to facilitate the dissolution of gallstones in man. It was found to produce toxic effects in rats, hamsters, rabbits, dogs, rhesus monkeys, and baboons but found not to be toxic to the squirrel money, chimpanzee, or humans (90,91). This species-specific sensitivity has been correlated with the ability of their respective intestinal flora to produce a toxic (sulfated) metabolite of chenodeoxycholate.

Drug Metabolism

Drug metabolism can be considered from the perspective of its influence on systemic exposure to the parent compound (i.e., clearance processes) or on the formation of potentially toxic metabolites. Accordingly, confounding the interpretation of in vivo toxicity study data are both the qualitative and quantitative interspecies differences in drug metabolism. Such differences are not uncommon, and an understanding of these factors can contribute to the interpretation of toxicity study data (92).

Benzidine is an example of where interspecies differences in drug metabolism lead to species-specific toxic reactions (93). In dogs, hepatic N1-glucuronidation of benzidine forms an acid-labile conjugate that is transported in the blood while bound to plasma proteins. Upon being filtered by the kidney, the drug accumulates in the urine whereupon acid hydrolysis releases the amine. The amine is subsequently activated by bladder enzymes, thereby initiating the carcinogenic process. In rats, liver rather than bladder cancer is the endpoint, presumably due to the low capacity of rat liver UDP-glycosyltransferases (UGT) to conjugate the benzidine.

The Laboratory of Clinical Pharmacology of the FDA provided other examples demonstrating the importance of understanding interspecies differences in drug metabolism when assessing preclinical study data (94):

- Paclitaxel is used in a polytherapy regimen. This may include its use in combination with other anticancer drugs or its coadministration with agents intended to minimize allergic reactions. In humans, the primary mechanism of drug elimination is via CYP2C8. However,

negligible amounts of this enzyme are present in rat microsomes. Therefore, rats cannot be used for examining drug–drug interactions in humans. In contrast, since paclitaxel itself is the primary agent of interest from both a toxicological and effectiveness perspective, the rat is an appropriate model for toxicity studies.

- Because of the rapid glucuronidation of zidovudine in humans (70–80%), the terminal elimination half-life in humans was much shorter than that expected based upon animal model data (dogs and rats). To maintain efficacious levels in humans, the frequency of dosing needed to be increased from bid, which was predicted on the basis of animal studies, to q4h.
- Iododeoxydoxorubicin is a drug for which there exists large quantitative interspecies differences in drug metabolism. This renders preclinical study data to be of questionable relevance to humans. While in rats the parent drug is the predominant circulating moiety, there is a 10-fold greater exposure to metabolites as compared to the parent compound in humans.

Variations in biotransformation generally occur in one of the following three forms (95):

- Species-specific deficiency in a particular metabolic reaction.
- Species-specific limitations in particular metabolic reactions.
- Variations in the activities of competing metabolic reactions.

When similar enzymes are involved in drug elimination, the (weight adjusted) intrinsic clearance of the compound generally tends to be greater in the smaller as compared to the larger mammalian species (96). However, exceptions to this pattern have been observed (97).

Generally, metabolic processes are classified as either phase I or phase II reactions. Phase I reactions are typically oxidative and add or expose polar functional groups on a lipophilic substrate. Phase II metabolic reactions are typically conjugative, reacting molecular functional groups (be it associated with the parent compound or a product of phase I metabolism) with an endogenous substrate to yield a metabolite that is readily excreted. Generally, the phase II metabolites are inactive, although certain compound classes, such as the reactive acyl glucuronides of xenobiotic carboxylic acids, do present with clinically relevant toxicities (92). Whether phase I metabolites result in toxicity or detoxification may depend upon the presence or absence of subsequent phase II metabolism.

Certain metabolic reactions appear to be negligible or even totally lacking in certain animal species. Examples are as follows (95,98):

- *Rat*: deficiency in the N-hydroxylation of aliphatic amines
- *Dog*: inability to acetylate compounds
- *Guinea pig*: deficiency in N-acetylation and unable to form *N*-acetylate S-substituted cysteines
- *Cat*: deficiency of glucuronidation reactions
- *Pig*: deficiency in most sulfation reactions

In other cases, there are drug-specific metabolic reactions that appear to occur in only certain animal species. An example includes the N-glucuronidation of sulfadimethoxine and other methoxysulfonamides, which appear to be limited to man and certain primates (95).

Numerous examples of metabolic divergence across animal species have been reported. Intestinal phase II biotransformation activities, which are carried out by UDP-glycosyltransferases (UGT) and sulfotransferases, are found to be higher in the rabbit than in the rat (99). Cultured hepatocytes from goats, sheep, cattle, and rats show similarities in glucuronidation and sulfation. However, while the enzymatic activities associated within goat liver cells showed higher activity in females versus males, the opposite gender effect was observed in rats (100). Metabolic idiosyncrasies also can be correlated with animal diet: Herbivores tend to be far more efficient than other species with regard to oxidative reactions (101).

In the case of the β-blocker acebutolol, the drug is hydrolyzed to an aromatic amine in man and then subsequently acetylated to the active metabolite, diacetolol. The latter not only has a very potent antihypertensive activity but also exhibits a markedly longer elimination half-life (8–13 hours) as compared to acebutolol (3–4 hours). In contrast, dogs are unable to form diacetolol because of their deficiency of the enzyme arylamine acetyltransferase. Accordingly, markedly different pharmacological activities and toxicological profiles can be expected in dogs versus humans (102).

When the metabolite profiles are qualitatively similar across species, what factors can lead either to differences in the intrinsic clearance of that compound or to differences in drug–drug interactions? Proposed factors to consider include the following (97):

- A metabolic pathway may be catalyzed by different enzyme isoforms in different species.
- Different inhibitory sites may be present, even if the same enzyme subfamily is involved in that drug's metabolism.
- Species differences in enzyme-specific ratios may lead to variability in the activity of metabolic inhibitors. For example, the ratio of CYP1A2 and CYP1A1 is 4–20:1 in most mammalian species but is 0.14–0.67:1 in rats.
- Slight differences in the enzyme's amino acid sequence may lead to marked differences in substrate specificity and enzyme activity.

Of particular interest is the cytochrome P450 family (particularly CYP1A1 and CYP1A2), since these are implicated in the carcinogenic activation of numerous xenobiotics (103). In man, the three major forms of cytochrome P450 (CYP) are CYP2D6, CYP2C9, and CYP3A4. While CYP1A2, CYP2C19, and CYP2E1 are also important, their involvement tends to be far less extensive than that associated with the former three isoforms (104).

Caffeine is often used as a metabolic probe for the activity of this family of enzymes. Using hepatic microsomes from humans, monkeys (*Macaca fascicularis*), rats, rabbits, and mice, three dimethylxanthines were formed, resulting from N-demethylation (theobromine, paraxanthine, and theophylline) and one compound resulting from oxidation at the C-8 position (trimethyloric acid) (105). Despite qualitative similarities, the relative proportion of the metabolites was markedly different across animal species. The ratio of N-demethylated metabolites versus the C-8 oxidative metabolite ranged from 0.52 in the rat to 10 in the monkey. The ratio in humans was 2.78. N-3 demethylation was the major pathway in humans and rabbits (involving CYP1A2), while N-7 demethylation predominated in monkeys (not mediated by CYP1A1 or CYP1A2). Moreover, unlike that seen in the other species, rats and mice exhibit dose-dependent in vivo caffeine metabolism. In humans, mice, rabbits, and rats, the CYP1A2 isoform predominated over CYP1A1, although the ratios of these enzymes differed across these species (with negligible amounts of CYP1A1 detected in humans and mice). In monkeys, no CYP1A isoform was detected. These findings are consistent with the substantial discrepancy noted in the major P450 enzymes across the four major toxicological test species: dog, rat, rabbit, and mouse (106).

Soucek and Gut (107) have summarized the DNA sequence homologies between various rat and human P450 isoforms. For numerous P450s, sequence homology of >75% was observed between rat and man. However, the potential for a difference in enzyme activity when a change in even one amino acid occurs should be considered when predicting the kinetic consequences of these similarities. The authors also noted upon a review of the literature that gene expression in rats is highly dependent upon such variables as gender (2A1, 2A2, 2C7, 2C11, 2C12, 2C13, 2D1, and 2A2), age (2A1, 2A2, 2B1, 2B2, 2C6, 2C7, 2C11, 2C12, 2C13, 2D, 2E1, and 3A2), strain (2B1, 2C13, and 2D1), circulating levels of growth hormone, and the physiological status of the animal (e.g., the effect of starvation, blood pressure, and diabetes). (As a note of caution, it should be recognized that this information is provided as a starting point for further consideration but that there is currently no universal agreement as to which specific isozyme is affected at any particular point in time).

Monkeys appear to express a higher proportion of reduction reactions associated with aldehyde oxidase as compared to that seen in other mammalian species. Aldehyde oxidase, an enzyme closely related to xanthine oxidase, is involved in the reduction of sulindac to sulindac sulfoxide and the reduction of imipramine N-oxide to the active parent drug, imipramine. In the presence of electron donors, it also mediates the reduction of sulfoxides, N-oxides, nitrosamines, azo dyes, oximes, epoxides, hydroxyamic acids, aromatic nitro compounds, and 1,2-benzisoxazole derivatives (108). In their study, Kitamura et al. observed that the aldehyde oxidase activity of cynomolgus monkeys was at least threefold greater than that of guinea pigs, rabbits, and rats. This enzyme was absent in dogs. Accordingly, it was concluded that unlike that seen in other mammalian species, the aldehyde oxidase in monkeys functions as the primary reductase enzyme for many compounds and that the reductase activity of the P450 system has a minor role in this species.

Despite the evolutionary proximity of humans and monkeys, large differences in phases I and II enzymatic reactions exist (109). Using human and rhesus monkey liver microsomes, the P450 content of the monkey microsomes was approximately threefold greater than that seen with human samples. Six in vitro phase I activities were markedly higher in the rhesus monkey as compared to humans. These included reactions involving erythromycin and benzphetamine N-demethylation (primarily CYP3A3 and CYP3A4), pentoxyresorufin O-dealkylation, ethoxyresorufin O-deethylation (CYP1A1/1A2), ethoxycoumarin O-deethylation (CYP2E1), and chlorpromazine S-oxygenation. Although ethoxycoumarin O-deethylase activity was significantly higher in the rhesus monkey as compared to human microsomal samples (which would suggest differences in CYP2E1 activity), there was no difference in the 2E1-catalyzed *N*-nitrosodimethylamine N-demethylation. Coumarin 7-hydroxylase activity was the only phase I reaction that was higher in humans as compared to monkeys (109) and is consistent with other reports of humans having higher coumarin 7-hydroxylase activity as compared to mice, rabbits, and guinea pigs (110). Rat liver microsomes do not appear to express the activity of this enzyme (111).

The studies by Stevens et al. (109) included an evaluation of the flavin-containing monooxygenases (FMO). In rhesus monkeys, significantly higher rates of cimetidine S-oxygenation and chlorpromazine N-oxygenation suggested that S- and N-oxide formation via flavin-containing monooxygenases constitutes a greater portion of drug oxidations in rhesus monkeys as compared to humans. For the phase II metabolic reactions, UDPGT activity (uridine diphosphate glucuronosyltransferase) was almost seven times higher in rhesus monkey microsomes as compared to human. Sulfation reactions showed no differences with regard to 17α-ethinylestradiol (EE) sulfotransferase, but cytosolic acetaminophen sulfotransferase was fourfold higher in the rhesus monkey. Glutathione (GSH) conjugation (which is important in the detoxification of electrophilic alkylating agents) also tended to be higher in monkeys than humans. In contrast, hepatic *S*-methyltransferase activity (which is important in the metabolism of thiopurines) tends to be significantly higher in humans as compared to the rhesus monkey.

Subsequent studies from that laboratory were expanded to include dog and cynomolgus monkeys (112). Interspecies differences were again observed (Fig. 1). The investigators note that even when a particular pathway is present in multiple animal species, interspecies differences in K_m and V_{max} need to be considered (Fig. 2). Although substrates used were known markers for the human isoforms, these results underscore the vastly different metabolic profiles that should be anticipated across species and the potential for these differences to result in species-specific drug effects.

Variations in enzyme kinetics (K_m and V_{max}) can result in marked interspecies differences in drug clearance and associated drug–drug interactions. For example, in the case of

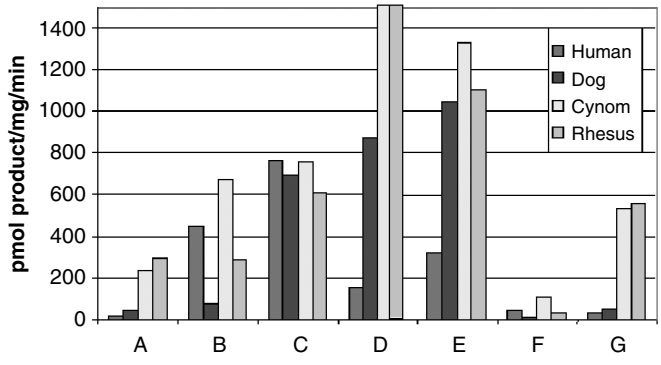

Figure 1 Interspecies differences in the relative activity of the various P450 enzymes. *Abbreviations*: A, ethoxyresofurin *O*-deethylase; B, coumarin 7-hydroxylase; C, *N*-nitrosodimethylamine *N*-demethylase; D, erythromycin *N*-demethylase; E, midazolam 1′-hydroxylase; F, *S*-mephenytoin 4′-hydroxylase; G, bufuralol 1′-hydroxylase. (Note that values for D in the two monkey species extend beyond the graph and have been truncated for the sake of illustration. Actual mean values in cynomolgus and rhesus monkeys are 2949 and 1997 pmol product/mg/min, respectively). *Source*: From Ref. 112.

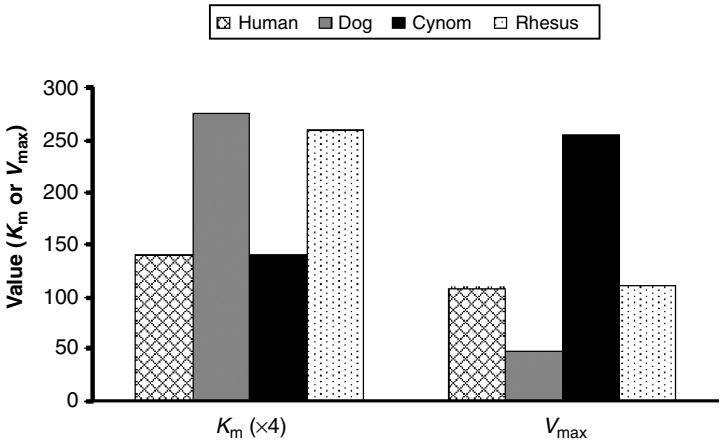

Figure 2 Interspecies difference in K_m and V_{max} for *S*-mephenytoin 4′-hydroxylase (K_m = micromoles, V_{max} = pmol product/mg/min). Note that for graphic purposes, all K_m values were multiplied by a factor of four. *Source*: From Ref. 112.

5,6-dimethylxanthenone-4-acetic acid (DMXAA), mice and rats form the same metabolites as humans and, from a qualitative perspective, would be considered appropriate preclinical species for this compound (97). However, based upon the results of an in vitro microsomal preparation, no one species could consistently predict the extent to which specific inhibitors reduced the rate of glucuronidation and hydroxylation of 5,6-dimethylxanthenone-4-acetic acid in humans (Fig. 3). Moreover, since these reactions exhibited Michaelis–Menton kinetics, the relative interspecies difference varied as a function of inhibitor concentration.

Glucuronidation is the most common conjugation pathway in mammals (113). These reactions are classified on the basis of the atom to which the glucuronic acid moiety is transferred: O-, S-, N-, and C-. The enzyme involved is UDP-glucuronosyltransferase.While N-glucuronidation

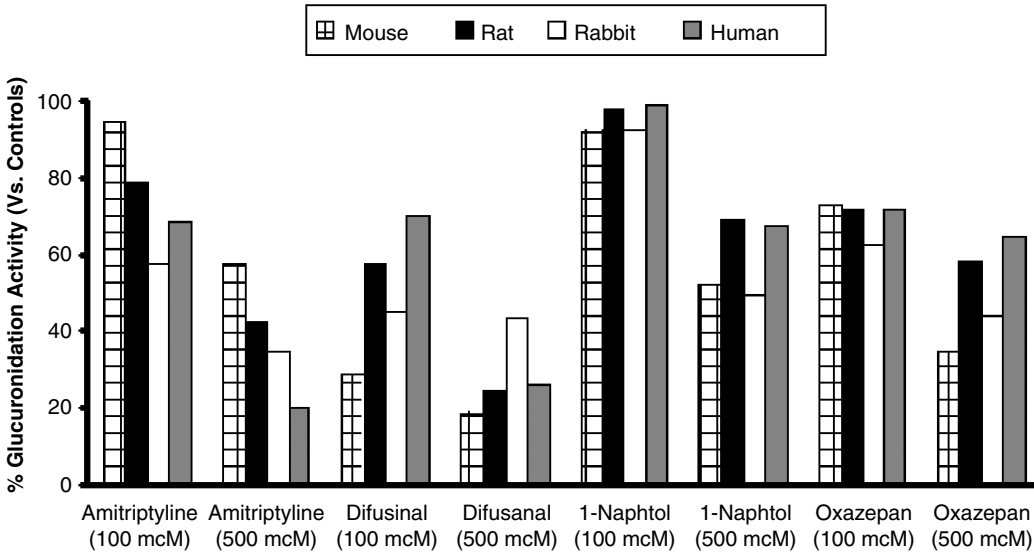

Figure 3 Interspecies differences in drug–drug interactions as demonstrated by the inhibitory effects of various compounds on the glucuronidation of 5,6-dimethylxanthenone-4-acetic acid. Data are expressed as the mean percentage of glucuronidation activity remaining in Brij 58-activated pooled microsomes from humans ($n = 3$), rat ($n = 6$), mouse ($n = 15$), and rabbits ($n = 2$) at 100 or 500 μM inhibitor concentration. *Source*: From Ref. 97.

generally is involved in detoxification reactions, in some cases (e.g., arylamines), this metabolite is believed to mediate the toxic effect of the parent compound (93).

Substrates for N-glucuronidation fall into one of the two categories: compounds that form nonquaternary N-conjugates (e.g., sulfonamides, arylamines and alicyclic, cyclic and heterocyclic amines) and those that form the quaternary conjugates (e.g., tertiary amines such as the tricyclic antidepressants and antihistamine drugs). For the nonquaternary conjugation reactions, there is no laboratory animal species that exhibits a deficiency when all of the substrates are considered. However, the ability of a species to form these conjugates is compound dependent, and the rabbit and guinea pig appear to exhibit the highest capacity for this reaction among the various preclinical species including the rat, mouse, dog, and nonhuman primate. For the tertiary amines, N-glucuronidation is commonly observed in nonhuman primates and man. N-glucuronides can be excreted in both the urine and bile of animal species (93).

When examining substrates possessing sites for both O- and N-glucuronidation, only the *N*-glucuronide metabolite was formed in human and canine microsomes, while both *O*- and *N*-glucuronides were formed in microsomes of monkeys and rats. This suggests the involvement of different UDP-glucuronosyltransferase isoenzymes in these reactions, and accordingly, differences in these isoenzymes across animal species (114).

There is much interest in the use of in vitro metabolism data to support the selection of animal species used in preclinical tests of a particular drug candidate (92). One of the problems when using in vitro test methods is the potential for interspecies difference in the conditions that optimize in vitro drug metabolism. For example, the optimal pH for N-glucuronidation reactions is 5.0 for the liver microsomes of monkeys and humans and 6.2 for the microsomes from dogs and rats. Another potential problem is that the microsomal preparation may not adequately reflect the in vivo substrate competition for a single metabolic pathway (114).

Age-Dependent Changes That Can Affect Drug Pharmacokinetics

Unlike the other sections in this chapter, this particular section focuses largely on information obtained in humans. The reason for this diversion is due to both the scarcity of information on the impact of maturation on the pharmacokinetics in preclinical animal species and the importance in recognizing the many ways in which adult animal or human data will fail to reflect the markedly different physiology and metabolism of juveniles.

On December 13, 1994, the FDA published a final rule encouraging manufacturers to provide information in product labeling that support the safe and effective drug use in the pediatric population (59 FR 64240). According to the 1994 Proposed Pediatric Rule (59 FR 64240), pediatric populations are defined as follows:

- Neonate: birth to 1 month
- Infant: 1 month to 2 years
- Children: 2 years to 12 years
- Adolescent: 12 years to <16 years
- Adult: ≥ 16 years

To date, the majority of preclinical safety information associated with pediatric indications has been based upon studies conducted in healthy animals. However, as our knowledge base evolves, it is becoming increasingly evident that such studies may not be appropriate for identifying the potential drug toxicities associated with drug use in pediatric populations. Certain adverse effects may be relatively rare events that may be difficult to detect in clinical trials or during routine postmarketing surveillance. In other cases, the expression of a pharmacological insult may not be apparent until several years after drug use. For this reason, CDER recommends the use of juvenile animals for preclinical toxicity assessments of drugs intended for use in pediatric populations (115). A comparison of human to animal developmental stages across various organ systems and animal species are provided in the CDER draft guidance titled "Nonclinical Safety Evaluation of Pediatric Drug Products" (115).

At least in part, age-related differences in drug response may be attributable to the influence of maturation on drug absorption, distribution, and metabolism. In his survey of

age-related differences in the pharmacokinetics of a wide range of compounds, Renwick (116) observed the following general trends:

- Children tend to eliminate drugs more rapidly than do adults.
- For renally cleared compounds, elimination is markedly slower in neonates as compared to other age groups.
- There are some drugs that show age-related shifts in body weight adjusted clearance (such as amrinone, meropenen, midazolam, and cefotaxime). However, other drugs reach adult-like clearance values after the first few months of life (such as zidovudine, amikacin, and ketamine).

Clark University, in cooperation with the Connecticut Department of Public Health, created an extensive pediatric database containing published information across a wide range of pharmaceutical substances (117). The database categorizes information in accordance with clearance pathways and specific age groups. Information can be downloaded into Excel spreadsheets for further examination. Along with allowing for the determination of specific trends within an age group, this database was constructed to facilitate an age-related comparison of the magnitude of inter-subject variability associated with drug pharmacokinetics. This is particularly important, given the variability in growth and maturation rates across individuals. For individuals interested in surveying an extensive comparative child/adult pharmacokinetic database, this information can be downloaded from http://www2.clarku.edu/faculty/dhattis/#Child/Adult Database.

On the basis of information contained within this database and consistent with the findings of Renwick (116), Ginsberg et al. (118) draws the following conclusions:

- Premature and full-term neonates tend to have a three- to ninefold longer terminal elimination half-life as compared to that of adults. This difference generally disappears by two to six months of age.
- Across a variety of compounds (reflecting different degrees of extravascular drug distribution and clearance pathways), there is a trend toward a shorter terminal elimination half-life within the six-month to two-year age group as compared to adults. This difference seems to be related to enhanced drug clearance (weight corrected) in infants.
- Across a range of P450 substrates, the terminal elimination half-life of neonates and infants up to two months of age tends to be significantly longer than that associated with adults. Conversely, for numerous compounds, the elimination half-life tends to be significantly shorter than in adults within the six-month to two-year age bracket. Since the latter age group tends to have a significantly larger (not smaller) volume of distribution, it would appear that this difference in half-life reflects a higher level of phase I drug metabolism. However, it was noted that despite this general trend, the magnitude of these differences is highly compound-specific.

The overall activity of the P450 system tends to be 50% higher in adults as compared to the neonate. In general, enzyme activity reaches levels equal to or greater than that in adults within 6 to 12 months of age, with the total hepatic P450 content approaching adult levels during the first 10 years of life. In children aged 6 months to 12 years, the activity of certain enzymes may be even higher than that seen with adults. This is believed to be linked to their inherently higher metabolic rate as compared to that of adults (119).

As a note of caution, it should be recognized that there are substantial differences in the age-related change in gene expression among the various enzyme systems. Moreover, there are minimal amounts of information regarding the factors involved in the activation of many of these systems. Accordingly, it is important to consider the differences in the rate of maturation for each of the various isoenzymes. For example, CYP3A7 is responsible for up to 85% of the total P450 activity in the fetal liver but declines to adult levels by 12 months of age. Conversely, CYP3A4, which is not present in the fetal liver, becomes the major P450 isozyme shortly after birth and remains such for the remaining lifetime (120,121).

The impact of the maturation processes on the activity of phase II metabolic pathways has resulted in dissimilarities in the handling of drugs such as acetaminophen. Approximately 50% of the administered acetaminophen dose is eliminated as the sulfate conjugate in children up to

12 years of age, while 50% of the administered dose is eliminated as the glucuronide conjugate in adults (122). This underscores the importance of considering the specific isoenzymes when predicting age-related changes in drug disposition. For example, there are 16 different UDP-glycuronosyltransferases, each with slightly different substrate affinities (123). The individual isoenzymes do not necessarily attain adult levels at same rate. Glucuronidation of simple substrates is higher at birth and subsequently decreases to adult levels by the seventh day. Conversely, the glucuronidation of bulkier substrates (such as chloramphenicol) is low at birth, and subsequently increases to adult levels by the twentieth day (116).

In humans, differences in body water, serum protein composition, and the affinity/capacity of hepatic biotransformation are observed between adults and pediatric patients (119,124). Many of these differences are particularly apparent when comparing adults versus neonates. A summary of some of the differences influencing drug pharmacokinetics is provided in Table 4 [based upon information contained in de Zwart et al. (119), Clewell et al. (121), and Kearns and Reed (124), unless otherwise noted].

Developmental changes in the renal function of humans and rats appear to be similar. For example, the glomerular filtration rate in 10-day-old rats is 50% to that of the adult. Filtration rate remains low for several weeks. By seven weeks of age, renal blood flow and glomerular filtration rate reach adult values (116). Relative kidney weight also changes dramatically with age (121). At birth, the ratio of kidney weight to body mass (1%) is twice that of the adult (0.5%). From birth through adolescence, kidney weight (expressed as a fraction of total body weight) declines. Change in kidney weight scales to the three-fourth power of body weight. Interestingly, this is the scaling relationship that many argue is appropriate for converting body mass to surface area (see section on allometric scaling).

With regard to volume of distribution, the largest changes occur within the first 12 months of life. Infants and neonates tend to have approximately 1.3- to 2.8-fold larger distribution volumes (per unit body weight) as compared to adults. After one to two years of age, the volume of distribution of most compounds tends to be similar to that of adults (119,121). This trend is seen both with lipophilic and hydrophilic compounds, corresponding to the higher Total Body Water (TBW) and lower serum protein binding seen in the very young.

The small intestinal villi of neonates tend to be broad leaf-shaped projections, rather than the elongated projections observed in adults. The length and diameter of the small intestine also increase from birth through adulthood, with up to a 40-fold increase in absorptive surface area. For the most part, maturation of the GI tract occurs within six months after birth, after which most of the absorption processes are similar to that of the adult (119).

There tends to be a prolonged residence of a compound in the stomach of infants and neonates. From 0 to 3 months of age, there also tends to be a higher gastric pH, and the nearly continuous presence of milk can both increase and decrease the bioavailability of compounds normally absorbed by the stomach. The slower rate of gastric emptying observed in neonates also decreases the rate of absorption from compounds absorbed in the small intestines, although there is generally little difference in the extent of absorption for most compounds (exceptions to this are described in the following paragraph). There also appears to be slower intestinal motility of young infants and neonates as compared to adults. For this reason, there is generally a slower oral absorption of compounds in neonates and young infants as compared to children and adults (119).

Despite age-related differences in small intestinal surface area, there are occasions when the extent of absorption of a substance in children exceeds that observed in adults. This is particularly true for compounds that are actively transported, such as calcium and iron (119). Differences in oral absorption of lead are also known to occur, with four to five times higher bioavailability seen in neonates than adults and three- to fourfold higher bioavailability in children aged two to six years as compared to adults. The mechanism for this difference is unknown (121). While it may, in part, reflect active transport via enterocyte receptors involved in the absorption of iron and calcium, there has been some suggestion of enhanced pinocytotic activity in early stages of development (125).

In neonates, the absorption of highly lipophilic molecules (including lipid soluble vitamins) tends to be substantially lower than that observed in adults. This is attributable to a deficiency in the secretion of both pancreatic lipases and bile salts. For neonates and infants

Table 4 Physiological Changes Associated with Maturation in Humans

Gastric volume (fasted)	2.5 mL for neonates, 8.8 mL for children, and 50 mL for adults. Volume can increase approximately 50-fold after feeding.
Gastric acid secretion	Neutral pH at birth but falls to between 1.5 and 3.0 within hours. Gastric acid secretion (corrected for body weight) approaches adult values by 3 months of age.
Gastric emptying	During the neonatal period, peristalsis is variable and unpredictable, with prolonged cycles relative to that of adult. Gastric emptying time approaches adult values within 6–8 months of age.
Interdigestive motor activity	Shorter in children than adults.
Exocrine pancreas	Low enzyme activity during neonatal period, but during infancy, secretion gradually approaches levels seen in adults.
Bile acid production	Less production in neonates than adults. However, infants are capable of efficiently absorbing fats within the first year of life.
Total body water (TBW)	Highest at birth, decreases steadily through the first year, plateaus between 1 and 10 years. An increase in the difference between the TBW of males and females occur at adolescence when the female TBW declines at a faster rate than that of the male. The TBW of the female is consistently less than that of males. Adult males and females have similar rates of decline, although TBS is consistently lower in females than males.
Total body fat (TBF)	Adipose tissue of the neonate may contain as much as 57% water and 35% lipids, whereas in adults, adipose tissue contains 26.5% water and 71.7% lipids. Total body fat increases up to 9 months of age, remains relatively constant from infancy through childhood (about 25–30%), and then dips during adolescence. The decline seen in adolescent males (about 15% decline in males, 5% decline in females). During adulthood, TBF increases in both males and females at the same overall rate (approaching 30% in geriatric males, 40% in geriatric females), but males consistently maintain a lower total body fat as compared to females.
Serum albumin and total protein	Less than adult values during neonatal period and early infancy, but approaches adult values by approximately 1 year of age. The serum protein of neonates differ from those of adults in several ways including • the presence of fetal albumin (absent by 1 month of age), • lower levels of plasma globulins (equivalent by early childhood), • presence of unconjugated bilirubin (equivalent to adult by 1 month), • free fatty acids are higher in neonates and are at adult levels in 1 month, and • α-1-glycoptroetins are lower in neonates but normal levels by 1 year of age.
Blood pH	Lower in neonates than adults, 7.26–7.29 vs. 7.35–7.45 for neonates and adults, respectively.
Hepatic phase I reactions	There tends to be lower alcohol dehydrogenase activity, carboxylesterase activity, and P450 activity in children and neonates as compared to adults.
Phase II reactions	Several not at adult level until 5 years of age. Children and neonates tend to show lower glutathione-*S*-transferase activity, glucuronyl transferase activity, but higher sulfotransferase activity. Neonates are typically slow acetylators. However, by 12 months of age, approximately 62% of individuals are fast acetylators. By 3–4 years of age, European and white and black children of the United States show NAT2 phenotypic characteristics equivalent to that of adults.
Renal function	There tends to be lower glomerular filtration and tubular section in neonates, infants, and young children as compared to adults.

fed with breast milk, the necessary enzymes for lipid digestion are derived from both lingual lipases and from those lipases contained within the breast milk itself (119).

Interspecies comparisons between humans and preclinical species are generally based upon in vitro metabolism data (116). The observed inclination is for the relationship between postnatal age and drug metabolism to exhibit trends similar to that observed in humans,

such as the generally lower glucuronidation occurring in the very young. A summary of changes in P450 isoenzymes in humans and animals can be found at http://www.icgeb.trieste.it/~p450srv/P450_ageing.html.

Even when we are able to accurately predict differences in drug pharmacokinetics that may exist between a pediatric versus adult population, unexpected toxicities have been known to occur. There may be critical windows of organ sensitivity that would not be evident in toxicity testing conducted in adult animals, and the dynamic processes of growth and development may result in the manifestation of toxicities that are not evident until a later stage of growth and maturation (119). These challenges underscore the importance of conducting toxicity testing in developmentally age-matched animals.

Protein-Binding Characteristics

Drugs can potentially bind to a variety of serum proteins including albumin, α1-acid glycoproteins, lipoproteins, sex hormone binding proteins, and immunoglobulins. They may also enter and bind to erythrocytes. Basic (cationic) drugs such as many β-adrenergic antagonists and macrolide antimicrobial agents are bound primarily to the α-glycoproteins. Conversely, acidic (anionic) drugs such as furosemide, β-lactams, salicylate, and phenylbutazone tend to bind to serum albumin (126).

Since it is predominantly the free drug concentrations that are responsible for the physiologic effects of a compound, failure to identify interspecies differences in total versus free drug concentrations may bias the interpretation of interspecies deviations in exposure/response relationships. For highly bound drugs, small differences in percent protein binding (e.g., 95% vs. 99%) can result in very large discrepancies in free fraction (e.g., a fivefold greater free fraction found with 95% binding as compared to 99% protein binding). Moreover, free drug fraction can affect drug clearance. While variations in protein binding are expected to have minimal effect on the clearance of drugs associated with a high extraction ratio, the clearance of low–extraction-ratio drugs are likely to be diminished in the presence of high-level plasma protein binding (126–128).

Examples of marked interspecies differences in free fraction are provided in Table 5 [based upon Cayen (95) and Mahmood (129)]. As evidenced below, protein binding tends to be highest in humans and lowest in mice.

Marked interspecies differences in plasma protein composition have been observed (130). Using a combination of the Biuret method for total protein measurement, the bromocresol sulfophthalein technique for quantifying blood levels of albumin, and electrophoresis to obtain estimates of the relative content of various plasma proteins, comparisons have been made across eight mammalian species. These differences are summarized in Table 6.

Again, there is a note of caution that the relative amounts of the various plasma proteins are not necessarily predictive of the relative extent to which a drug will bind to the plasma proteins of a particular animal species. However, knowing the interspecies difference in the extent of plasma protein binding for a particular compound may help explain some of theapparent

Table 5 Free Fraction of Drugs Across Blood of Various Species

Drug	Mouse	Rat	Dog	Monkey	Human
Cefpiramide	0.56	0.54	0.70	0.068	0.037
Cefoperazone	0.854	0.744	0.744	0.161	0.176
Cefmetazole	0.65	0.56	0.75	0.19	0.15
Diazepam		0.137	0.04		0.032
Quinidine	0.363	0.324			0.13
Valproic acid	0.881	0.366	0.215		0.052
Meloxicam	0.04	0.003			0.004
CIPB	0.65	0.25	0.15	0.05	0.03
Etodolac	0.052	0.007	0.017	0.012	0.008
Tolrestat	0.04	0.017	0.02	0.014	0.007
Pelrinone	0.78	0.28	0.20	0.21	0.11
Benoxaprofen	0.011	0.007	0.008	0.004	0.002

Table 6 Range of Plasma Protein Values of Eight Mammalian Species[a,b]

Parameter	Units	Mouse	Rat	Rabbit	Dog	Sheep	Man	Cow	Horse
Albumin	g/dL	3.5	2.1	3.9–4.3	3.2–3.8	3.1–3.5	4.6–4.9	2.2–2.4	2.7–3.1
Total protein	g/dL	6.0	6.5	6.7–7.4	5.6–5.9	6.7–7.6	7.2–8.4	5.8–6.8	6.0–7.8
$\alpha 1$	%		7	4–10	2–4	4–5	1–2		2–3
$\alpha 2$	%		5	3–15	8–10	9–11	6–8		7–10
β	%	12	12	14–16	20–23	21–26	10–11	30–37	13–18
γ	%	7	7	15–21	4–7	15–18	15–22	13–24	24–29
Albumin	%	59	68	55–60	58–64	46–49	59–65	32–41	40–50

[a]Mouse and rat blood represent pooled samples.
[b]$n = 4$ per species. Each animal's samples were run in triplicate.
Source: From Ref. 130.

differences in drugpharmacokinetics across animal species. For example, the interspecies differences in diazepam total plasma clearance and terminal elimination rate constant were highly correlated with free fraction [Figs. 4(A) and 4(B)]. Within human subjects, a similar correlation between plasma clearance and free fraction was also noted. No correlation between free fraction and the volume of distribution was observed for either humans or animals [Fig. 4(C)]. When converting plasma clearance to total body clearance and subsequently to extraction ratio (total body clearance/hepatic blood flow), man was found to have a low extraction ratio (E) for this compound while dogs, rabbits, and rats were found to have high extraction ratios. In some cases, values of E exceeded 1.0, indicating the presence of extrahepatic elimination processes (131). A high E value also suggests that hepatic clearance will be affected by variables that can alter hepatic blood flow (e.g., food) but not by changes in plasma protein binding. In man, the value of $E < 0.2$ is consistent with an elimination process that is highly dependent upon free fraction, not hepatic blood flow. Accordingly, a linear correlation was observed between clearance and free fraction across the human subjects.

Interspecies differences in protein binding may not reflect differences in the drug–protein interaction but may rather be attributable to the presence of other substances that compete for the protein-binding site. Alendronate (an inhibitor of osteoclast-mediated bone reabsorption) binds to both plasma proteins and bone. Irreversible binding to bone constitutes the primary mechanism of drug elimination (132). Relatively low plasma protein binding was seen in dogs, but high protein binding was observed in rats. This interspecies difference was, at least in part, attributable to the apparent presence of displacer(s) in dog but not rat plasma (based upon in vitro experiments). The addition of calcium to the dog plasma sample diminished the effect of the displacer(s).

Alternatively, interspecies differences in protein binding may reflect differences in protein-binding affinity. The affinity of fatty acid–acylated insulin for serum albumin of humans, pigs, and rabbits (expressed relative to the binding affinity to human albumin) varied from 1:1.5:35, respectively. As a result of the much higher binding affinity of this compound in rabbits, the fatty acid–acylated insulin exhibited a diminished but prolonged effect in rabbits as compared to pigs (133).

Interspecies differences in Michaelis–Menton binding characteristics have also been observed. For example, the basic compound propafenone was found to have at least a twofold higher free fraction in rabbits as compared to that of other species. There were also marked differences in the dose-dependency of protein binding across species. As the concentration of propafenone increased from 250 to 2000 ng/mL (in vitro test procedure), nonlinear protein binding was observed in horses (twofold change), mouse (threefold change), man (twofold change) and sheep (fivefold change). However, dose-independent protein binding was observed in rats, rabbits, dogs, and cattle (130).

Biliary Excretion

Interspecies differences in biliary excretion can lead to pronounced differences in drug exposure, particularly when the drug undergoes enterohepatic recirculation. In general, the extent of

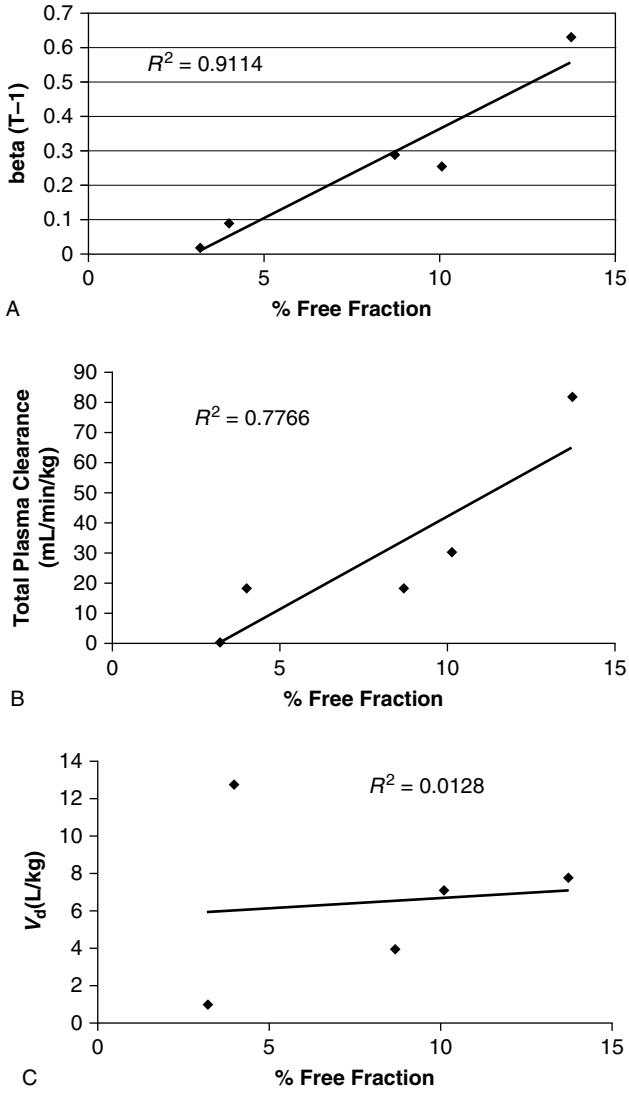

Figure 4 Interspecies relationship among pharmacokinetics parameters. (A) Free fraction versus terminal elimination rate constant. (B) Free fraction and total plasma clearance. (C) Free fraction and volume of distribution. *Source*: Plots are based upon data reported by Klotz et al. (Ref. 131).

biliary excretion tends to be much higher in dogs and rats as compared to pigs, monkeys, and humans. The mouse falls somewhere in between these two groups (95).

The marked interspecies differences in mean bile flow and composition can also affect drug solubilization and therefore drug absorption (134,135). The differences in bile flow across target animal species are summarized in Table 7. Although rats and horses have no gall bladders, both species synthesize bile salts and bile entry to the intestine occurs in a more or less continuous manner.

Interspecies differences in drug metabolism may also influence the extent of enterohepatic recirculation for a particular compound. In the case of oxaprozin, an anti-inflammatory agent, it was found to undergo both oxidative metabolism as well as glucuronidation. The glucuronide is not formed in rats, is excreted primarily in the urine of humans, is found in both the urine and bile of the rhesus monkey, and is eliminated almost exclusively in the bile of dogs. Upon elimination in the bile, oxaprozin glucuronide is deconjugated in the small intestine by intestinal glucuronidases. The parent drug is thereby regenerated and available to be reabsorbed (102).

Table 7 Mean Bile Flow

Species	Bile flow (μL/min/kg BW)
Cat	11
Chicken	20
Dog	4–10
Guinea pig	200
Hamster	50
Human	1.5–15
Monkey	10
Mouse	78
Pig	9
Pony	19
Rabbit	90
Rat	30–150
Sheep	9.4

Source: From Ref. 29.

Thus, human intestinal exposure to this drug would be underestimated on the basis of rat data but overestimated on the basis of dog data.

The extent of biliary excretion, if followed by enterohepatic circulation, can be an important factor contributing to the risk of drug toxicity. This point is clearly demonstrated with indomethacin, where there is a distinct relationship between enterohepatic recycling, intestinal drug exposure, and toxic dose. When indomethacin was administered, marked interspecies differences in cumulative intestinal exposure were observed, where dog > rat > rhesus monkey > guinea pig > rabbit > man. The corresponding toxic dose in these species was related to the magnitude of their intestinal indomethacin exposure. Therefore, while the toxic dose was only 0.5 mg/kg/day in dogs, it was as high as 20 mg/kg/day in rabbits (136). Similarly, unusually high bile/plasma concentration ratios of the sulfasalazine analogue, susalimod, were observed in dogs (ratio = 3400) as compared to monkey (ratio = 300) and rat (ratio = 50). This difference in bile concentrations correlated with the long-term hepatobiliary toxicity observed in dogs but not in the other two species (137).

Since presence or absence of a gall bladder also impacts the characteristics of bile release into the intestine, we anticipate that the presence of a gall bladder and the pattern of bile release in the intestine will influence the rate and extent of biliary drug recycling. In species with gall bladders, the discharge of bile into the duodenum occurs during phase II of the migrating motor complex (138). The latter is a myoelectric cycle, originating in the stomach and propagating throughout the intestine (139). Since rats lack a gall bladder, these fluctuations are not observed. Rather, bile flow appears to follow a circadian pattern, with secondary (superimposed) variation occurring as a result of food intake (140).

Efficient biliary excretion of a compound is a function of the molecular weight, chemical nature, and target animal species. The molecular weight threshold for the biliary excretion of acidic compounds is approximately 300 to 350 in dogs and rats but greater than 500 in humans. Similar molecular weight considerations apply to most neutral compounds (102).

ALLOMETRY

Allometry serves as a black-box approach for interspecies scaling of drug concentrations within some biological matrix (generally blood). While there are numerous examples of its successful application (141,142), there are also examples of where allometry fails to accurately predict drug pharmacokinetics across species.

The variable generally considered to be the most highly predictive factor for interspecies scaling is total body surface area. This is because pharmacokinetic elimination processes are affected by the size and function of the eliminating organ, which in turn, reflects the organisms' metabolic demands. In turn, metabolic rate appears to be related to total body surface (44,143). Therefore, it is not surprising that many of the pharmacokinetic elimination processes scale in accordance with total body surface area.

This leads to the issue of how to obtain an estimate of body surface area across the various animal species. To address this point, West and colleagues (144) suggested that the biological commonality supporting interspecies scaling is founded upon certain general principles:

- Living things are sustained by the transport of materials.
- Transport occurs through linear networks that branch to supply the various parts of the organism.
- This network can be characterized as a space-filled fractal-like branching system.
- The final branch of this system (e.g., the capillary) is a size-invariant unit.
- The energy required to distribute resources are minimized in all living creatures.

These authors suggest that an outcome of these principles is an inherent scaling relationship between mass and surface area.

From a naïve approach, the belief is that the relationship between mass and surface area is related to the need to release body heat through the surface area of the organism. In other words, the fundamental relationship is that of metabolic rate, body mass, and surface area. By convention, surface area is assumed to scale in accordance with $V^{2/3}$, where V = volume of the organism. In turn, V is considered to be proportional to mass, under the conditions of a constant body density (145). Subsequent reports, however, have suggested that the power relationship between mass and metabolic rate is that of 0.72 to 0.73 rather than of 0.67. West et al. (144,146) provide several theoretical and mathematical arguments supporting the use of a three-fourth rather than two-third power for converting mass to surface area.

In an attempt to reconcile this debate, Dodds et al. (145) examined the theoretical attempts to connect metabolic rate to mass, as described by the equation:

$$B = cM^{\alpha}$$

where B = the basal metabolic rate,
M = mass of the organism,
α = the allometric exponent,
c = a constant.

They examined several mathematical models to cover such theoretical approaches as dimensional analysis, four-dimensional biology, and nutrient supply networks. They concluded that none of these theories convincingly support a three-fourth rather than a two-third scaling relationship. Interestingly, they also examined the work of West et al. (144) and raised concerns regarding the assumptions and mathematical accuracy of West's arguments supporting the conclusion that surface area scales to mass by the three-fourth power.

Dodds et al. (145) also examined empirical data for metabolic rates for homeotherms. They observed that based upon the actual metabolic data obtained from almost 800 species (including birds and mammals), they could not find statistical support for rejecting $\alpha = 2/3$. However, they also observed an apparent shift in α as body mass increases. Basically, below 10 kg, the allometric exponent of $\alpha = 2/3$ appears to fit well. As body mass increases above 10 kg, there is a greater-than-predicted increase in metabolic rate, and α appears to scale better to a factor of $3/4$ rather than $2/3$. They hypothesize that this shift may reflect a change in body shape with increasing size, and therefore, a change in the surface area to mass relationship. In this regard, they note that the relationship between mammalian head-and-body length and mass is better fit by two rather than one scaling law and suggest that a higher metabolic rate might provide an evolutionary advantage to support larger brain sizes. (It is interesting to contemplate the relationship between these suggestions and the other potential use of additional normalization factors, such as brain weight, as discussed later in this section.) They also note that α values shift across species of birds with different normal core temperatures (differing by 1–2°C) when metabolic rates are grouped to different seasonal measurements. On the foundation of these evaluations, they concluded that while a single allometric relationship may be useful for obtaining rough estimates of interspecies predictions, the assumption of a single allometric relationship across a wide range of weights may not be justifiable.

Similar to the equation relating basal metabolic rate to mass, the general form of the allometric equation used in scaling pharmacokinetic parameters across animals is as follows (147):

$$Y = a\,\mathrm{BW}^b$$

where Y = the parameter of interest,
BW = the body weight,
a = the allometric coefficient [the value of the physiological variable (y) at one unit of body weight],
b = the allometric exponent that defines the proportionality between BW, body weight, and Y.

When $b = 1$, there is a direct correlation between body weight and the parameter, Y. When the constant equals 0.67 or 0.75, Y is said to scale in accordance with body surface area (148).

Conversions of body weight (kg) to body surface area (m^2) are provided in Table 8 [based upon Morris (20) and CDER guidance on first-time dose in man (149)].

To explore the impact of data variability on the ability to distinguish between $b = 2/3$ or 3/4, Hu and Hayton (142) examined the allometric relationship for 115 compounds. They found that 91 of the surveyed drugs exhibited a statistically significant allometric relationship. Estimated values of b ranged from 0.29 to 1.1. For drugs whose elimination included metabolism, the estimated values of b did not differ significantly from 0.75. Only in the case of drugs that are cleared solely by renal elimination were the allometric exponents significantly lower than 0.75 (mean = 0.65, 95% confidence interval = 0.62–0.69). Given the shape of the distribution of these estimates, the authors suggest that for all drugs except those cleared solely by renal elimination, reported differences in the estimates of the allometric exponents are more a function of population variability and experimental noise than of real differences in scaling factors.

These authors further examined the issue of using $b = 0.67$ versus $b = 0.75$ through the Monte Carlo simulation of 10 experimental scenarios. Each scenario differed with respect to the selection of sampling times, number of animal species, and coefficients of variation (CV). Under various simulated experimental conditions, they examined the impact of study design and random error on the estimated allometric exponent. They noted that the resulting estimates of b followed a normal distribution similar to that observed with 115 actual datasets (discussed above). They further noted that with a 30% CV, it was impossible to determine whether the true value of b was 0.67 or 0.75 (simulations were based upon an assumed value of $b = 0.75$). Accordingly, they conclude that for most compounds, it will not be feasible to assume that one can establish a conclusive relationship based upon conventional experimental data and study designs. Using Monte Carlo methods, similar conclusions were reached by Watanabe et al. (150).

Table 8 Conversion of Body Weight to Surface Area Across Species and Age Groups

Species	Body weight (kg)	Surface area (m^2)
Human, adult	60	1.6
Human, child	20	0.8
Mouse	0.02	0.007
Rat	0.15	0.025
Cat	3	0.24
Dog	16	0.65
Sheep/goat	50	1.1
Pig	75	1.5
Nonhuman primates		
Marmoset	350	0.06
Squirrel monkey	600	0.09
Baboon	12	0.06

For any given parameter, there can be several versions of the allometric equation that is said to best fit the specified parameter. For example, two versions of the equation for estimating cardiac output (expressed in mL/min/kg) are $166 \times BW^{0.79}$ (141) and $15 \times BW^{0.74}$ (151). Reasons for these differences can include such factors as variability within each species, breed of animal selected, number of animals per species, and sampling times.

Species-specific idiosyncrasies in absorption, distribution, and metabolism often confound the use of interspecies extrapolation to predict appropriate dosages (amount and frequency) for use in humans or other animals. Factors such as interspecies differences in protein binding and metabolic pathway can result in failed attempts to accurately predict an appropriate dose from animals to humans (152). Therefore, allometric scaling tends to work best for those compounds that are eliminated primarily by physical transport processes such as biliary or renal excretion (153). Accordingly, allometric scaling tends to fail for those compounds that present with the following characteristics (20,153):

- Low extraction ratio ($E < 0.2$), where hepatic clearance is much less than hepatic blood flow.
- The presence of interspecies differences in drug metabolism.
- Nonlinear pharmacokinetics.
- High protein binding (plasma and tissue).
- Renal tubular reabsorption. It should also be noted that the urine pH of herbivores tends to be alkaline while that of carnivores tends to be acidic, which may affect the renal clearance of certain compounds.

To determine whether or not drug physicochemical properties influence interspecies pharmacokinetic relationships, the accuracy of extrapolating terminal elimination half-lives between rats and humans were considered with respect to drug lipophilicity (154). The question was not one of interspecies differences in membrane solubilization for specific compounds, since a chemical's ability to be solubilized within tissues is assumed to be approximately constant across animal species (155). Rather, this question was raised in an attempt to address the substantially greater percentage of adipose tissue in humans (23% of total body weight) versus rats (7% of total body weight). These investigators found that with the exception of a slightly higher prediction error when the human half-life was estimated for very highly lipophilic compounds (e.g., $\log P > 6.5$), only minor differences in prediction error occurred between models with or without the inclusion of $\log P$ as a factor in the regression equations.

The following physiological parameters tend to scale in accordance with body weight [i.e., the value of b tends toward unity (148,156)]:

- Organ volumes: blood volume, $b = 1.02$
- Organ weight

  Kidney weight, $b = 0.85$
  Heart weight, $b = 0.98$
  Liver weight, $b = 0.87$
  Stomach and intestines weight, $b = 0.94$
  Blood weight, $b = 0.99$

In contrast, the following physiological parameters appear to be more closely linked to metabolic rate [i.e., approximately 0.75 (148,156)].

- Cardiac output, $b = 0.75$
- Alveolar ventilation, $b = 0.75$
- Creatinine clearance, $b = 0.69$
- Inulin clearance, $b = 0.77$
- Para-aminohippuric acid (PAH) clearance, $b = 0.80$
- Basal O_2 consumption, $b = 0.72$
- O_2 consumption by liver slices, $b = 0.85$

Despite differences in absolute amount of blood flow across species, as seen in Table 9, regional blood flow distribution, expressed as a mean percent of the cardiac output, was very similar across these four species.

Table 9 Regional Blood Flow Distribution Expressed as Percent Cardiac Output in Unanesthetized Animals[a]

Tissue	Mouse	Rat	Dog	Human
Adipose		7.0		5.2
Adrenals		0.3	0.2	
Bone		12.2		4.2
Brain	3.3	2.0	2.0	11.4
Heart	6.6	5.1	4.6	4.0
Kidneys	9.1	14.1	17.3	17.5
Hepatic artery	2.0	2.1	4.6	
Hepatic vein	14.4	15.3	25.1	18.1
Lung	0.5	2.1	8.8	
Muscle	15.9	27.8	21.7	19.1
Skin	5.8	2.8	6.0	5.8

[a]Based upon a compilation of data from studies employing a radiolabeled microsphere technique.
Source: From Ref. 151.

If the allometric exponent for intrinsic clearance is the same as that for the blood flow of the eliminating organ, then E will be nearly identical across animal species (157). Accordingly, E bears no relationship to body weight or body surface area. An example of this is propranolol, where the hepatic E was estimated to exceed 90% in mice, dogs, and humans.

Marked prediction errors can occur if differences in drug metabolism are not adequately considered. A case in point is a drug that was shown to be toxic to the gonads of several animal species. It was originally considered safe for use in humans, because on the basis of surface area equivalents (allometry), it was determined that the animals would be exposed to seven times the level of drug expected for humans. However, when human pharmacokinetic data became available, it was found that the exposure ratio was not a factor of seven but rather a factor of two (158). Unfortunately, knowledge of the P450 isoenzyme responsible for drug metabolism provides neither a guide as to the appropriate allometric exponent to use nor it is indicative of the overall ability to use allometric methods to predict human drug clearance (159).

In addition to the use of total body surface area to predict allometric relationships for drug pharmacokinetics, differences between chronological versus physiological time may also be considered when predicting interspecies differences in exposure–response relationships. For example, cellular division rates in smaller animals are significantly faster than those in large animals. This results in the former having a shorter latency for the proliferation of an immune response or the expression of an adverse cellular event. On the other hand, the larger animal species have a longer life span, resulting in a much longer time for the development of an adverse event (4).

Variables such as the duration of a single breath, heartbeat duration, longevity, pulse time, breathing rates, and blood flow are approximately constant across species when scaled to physiological time. In general, smaller, short-lived animal species clear drugs more rapidly (chronological time) than do larger, longer-lived animals. Since life duration tends to be related to body weight, the latter can be used to scale for differences in physiological time (141). The relationship between chronological time (t') versus physiological time (t) has been expressed as follows (147,148):

$$t' = t/BW^{0.25}$$

Dedrick et al. (160) were the first authors to suggest that interspecies scaling can be based on the concept of equivalent time. They proposed that drug elimination could be correlated between species if an intrinsic biological property such as creatinine clearance, heartbeat duration, longevity, breath rate, and duration or blood circulation velocity were used as an interspecies scaling factor. In other words, two apparently different rates of an event, when

based upon chronological time, may in fact be comparable if adjusted to a species' physiological time.

This difference in physiological time can impart substantial influence on the toxic or therapeutic response to a drug. For example, the total blood volume in the mouse is 2 mL (161) and its cardiac output equals approximately 15 to 20 mL/min (162,163). Consequently, in mice, tissues are exposed to the entire blood volume several times each minute. In contrast, the cardiac output of the human is 1/20th of its total blood volume and it takes five minutes for the entire system to be exposed once to the total blood volume (4). Therefore, mice are likely to exhibit more rapid acute responses to toxic substances as compared to humans.

In general, the time for one complete systemic exposure to the entire blood volume of any species can be scaled as $Y = 0.35 \times \mathrm{BW}^{0.21}$ (164). In this regard, Mordenti (164) notes that the blood volume turnover time for inulin can be scaled as $Y = 6.51 \times \mathrm{BW}^{0.27}$.

Heartbeat time is said to equal $0.2961 \times B^{0.28}$ (where B is body mass in kilograms)(165). Hence, a 30-kg mouse has one heartbeat every 0.111 seconds, while a 70-kg human has one every 0.973 seconds. Similarly, breaths per second (breath time) scales as $1.169 \times B^{0.28}$. These averages should be considered from the perspective of the wide range of factors that can influence these values such as exercise, gender differences, environmental temperature, posture (supine vs. standing), age, and the effects of a meal (151).

Boxenbaum (165) argues that when pharmacokinetic processes are similar across species, a pharmacokinetic parameter can be scaled to physiological time, thereby obtaining a time-invariant measure. This produces, in his terms, pharmacokinetic time. For example, the terminal elimination half-life for hexobarbital, based upon chronological time, can be described as

$$T_{1/2} = 80 \times B^{0.348}.$$

The equation for normalizing for differences in physiological time [in this case, using a term coined gut-beat duration (G, min)] is

$$G = 0.0475 \times B^{0.31}$$

By dividing $T_{1/2}\ /G$, one obtains a time-invariant terminal elimination half-life for hexobarbital that is approximately 1684. The importance of estimating a time-invariant terminal elimination half-life is that the value can then be evaluated from the perspective of the rates associated with other physiological events occurring within that animal species.

Along similar lines, Boxenbaum and Ronfeld (166) introduced the concept of the kallynochron ($=t/W^{1-b}$), where one kallynochron defines the time within which species have cleared a specified volume of plasma per kg of body weight. Failure of the kallynochron to scale chlordiazepoxide pharmacokinetic data from dogs to humans lead these authors to further develop a term coined the apolysichron. The latter is defined as follows:

$$t/W^{b'-b}$$

where b' and b are the algometric exponents relating volume of distribution and clearance to body weight.

To illustrate how physiological time can scale the rate of a response, consider two pendulum clocks, each identical in form but one being 64 times larger than the other (165). The duration of one cycle (swing) of the pendulum (T) can be defined as

$$T = 2\pi(L/g)^{1/2}$$

where L is the pendulum length and g is the acceleration of the pendulum due to gravity.

The 64-fold increase in L produces only an 8-fold increase in T (i.e., $64^{1/2} = 8$), causing the larger clock to produce fewer ticks per minute. To compensate for this difference, the larger clock will need to have an eightfold greater belt drive ratio to enable both the smaller and larger clocks to move through identical arcs per minute (representing chronological time). When this

example is considered from a biological perspective, these differences in T (without adjustment from a "belt drive") result in differences in life span and rates of physiological processes, as measured from the perspective of chronological time. However, with appropriate mathematical transformations, T can be expressed in a time invariant value.

When testing substances for potential carcinogenicity, the rate of carcinogenesis appears to relate to the species' basal metabolic rate. For example, the onset of cancer often occurs within approximately 1 year in rodents but may take 10 to 20 years to be expressed in humans (17). On the other hand, upon relating this finding to physiological rather than chronological time, Dedrick and Morrison (167) observed that interspecies differences in the daily dose and AUC values associated with the development of cancer largely disappeared when adjustments were made for total lifetime exposure.

Ultimately, there is any number of covariates that may be incorporated into an allometric equation to improve its predictive properties when scaling from animals to humans. To reduce some of the uncertainty associated with these allometric procedures, Mahmood and Balian (168) suggested a classification method for predicting the appropriate allometric exponent. Based upon additional scaling factors suggested by Boxenbaum et al. (44,165), Mahmood and Balian considered the impact of including maximum life potential and brain weight on the allometric fit associated with interspecies datasets obtained from literature surveys. Based upon regression analysis conducted on 40 compounds, they developed the following conditions for determining the appropriate scaling method:

- If the exponent of the simple allometric equation lies between 0.55 and 0.70, a simple allometric equation can predict the clearance reasonably well. In this case, total body clearance (CL) would be estimated as follows:

$$\mathrm{CL} = a(W)^b$$

where W = body weight.

- If the exponent of the simple allometric equation lies between 0.71 and 1.0, a prediction based upon the simple allometric equation will substantially overestimate the predicted clearance. In this situation, accounting for differences in maximum life span potential (MLP) appears to improve the fit. For this situation, CL would be estimated as follows:

$$\mathrm{CL} = \frac{a\,(\mathrm{MLP} \times \mathrm{CL})^b}{\mathrm{MLP\ of\ humans}}$$

where $\mathrm{MLP} = 185.4\ (\mathrm{BW})^{0.636}\ (W)^{-0.225}$,
BnW = brain weight,
MLP of humans $= 8.18 \times 10^5$.

- If the exponent of the simple allometric equation is greater than 1.0, the product of CL and BW can be used to predict human CL with reasonable accuracy. For this situation, CL would be estimated as follows:

$$\mathrm{CL} \times \mathrm{BnW} = a\,W^b$$

- In cases where $b > 1.3$ or < 0.55, neither of these three methods could adequately predict the CL of humans.

Under experimental conditions where drug pharmacokinetics can be examined across a wide spectrum of animal species, there is the luxury of being able to examine residual errors in order to determine the covariates that optimize the fit of the regression line. In so doing, the investigator can minimize the error in predicted versus observed parameter values in humans

(169). However, what happens when one attempts to estimate a human equivalent dose (HED) on the basis of the no adverse effect level (NOAEL) associated with the animal species of interest? In that situation, the fundamental objective is to ensure that the dose administered will result in negligible toxicity. This point brings us back to the debate described in the beginning of this section: Is it more appropriate to scale to the power of 0.75 or 0.67? To that end, the use of an exponent of 0.75 rather than 0.67 will result in a far larger estimated starting dose in humans (e.g., a nearly twofold greater estimate when scaled on the basis of data derived from smaller rodent species, such as mice). Accordingly, the use of 0.75 could result in a higher and potentially more dangerous starting dose in humans. For this reason, the human equivalent dose calculation is often based upon $b = 0.67$ (149), thereby increasing the probability that the drug will be safe when administered for the first time in healthy human volunteers.

CONCLUDING THOUGHTS

While this chapter focused on animal models, comparative anatomy and physiology, and the extrapolation of preclinical data to humans, a far more complex question is whether or not preclinical data can also predict toxicities that may be associated with a specific patient population. Numerous physiological changes can occur during disease conditions, and these changes can impact drug distribution, protein binding, clearance, drug metabolism, and tissue sensitivity. While we raise this question, we recognize that this point in and of itself can be the subject of an entire textbook. Nevertheless, it is a point worth considering as we use preclinical data to predict appropriate drug dosages in humans.

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4 Pharmacokinetics/ADME of Small Molecules

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INTRODUCTION

Medication efficacy and safety are the primary aims of drug development. The safety and efficacy of a drug depends on its pharmacokinetic (PK) and pharmacologic properties. Pharmacokinetics is the study of the time course of drug absorption, distribution, metabolism, and excretion (ADME).

Poor PK properties are the most common reason for early development failures, accounting for 40% of attrition in phase 1 clinical trials (1–3). To overcome this challenge, lead optimization, a process by which the pharmacologic and PK properties of most promising compounds are improved, is employed. Although pharmacokinetics is not the only determinant of safety and efficacy of a new chemical entity, it plays a major role in the lead optimization process.

The primary purpose of preclinical PK studies is to ensure that compounds do not fail in human studies due to ADME reasons. Through a combination of in vitro and in vivo studies, preclinical ADME screening facilitates early elimination of weak candidates and directs the focus of the drug development program toward fewer potential lead candidates (4).

This chapter describes the pharmacokinetic mechanisms (ADME) involved in the disposition of small molecules. It begins with a general overview of PK principles and parameters. Next, the individual ADME processes are presented, along with the factors that influence these processes. The chapter concludes with a discussion of relevant issues for drug development.

PHARMACOKINETICS: GENERAL OVERVIEW

Pharmacokinetic Parameters

Pharmacokinetics governs the relationship between dose and systemic exposure of drug in the body—this is assessed from a concentration–time profile, which describes the amount of drug in the blood (or plasma) over a time period following drug administration. From this profile (Fig. 1), several PK parameters are commonly measured including

- C_{max}: The maximum concentration of drug in the plasma
- T_{max}: The time at which the maximal concentration is observed observed
- $T_{1/2}$: Elimination half-life, a measure of how quickly a drug is eliminated from the body
- AUC: Area under the curve, area of the plasma concentration–time profile from time 0 (when dose is administered) to time ∞ (when dose is completely eliminated).
- V_D: Volume of distribution, an indicator of the extent of distribution of a drug in tissue
- Cl: Clearance, the proportionality between the rate at which a drug is removed from the body and plasma concentration

Linear vs. Nonlinear Pharmacokinetics

For most medications, PK parameters (Cl, V_D, $t_{1/2}$) do not change when a dose is increased, decreased, or when the drug is given via other routes of administration. Accordingly, the pharmacokinetics of these drugs is referred to as dose-independent; that is, the drug can be described by linear pharmacokinetics (Fig. 2).

The underlying assumption of linear pharmacokinetics is first-order elimination, where the rate of drug elimination from the body is proportional to the plasma concentration. Accordingly, $t_{1/2}$ is constant (dose-independent) and plasma concentrations and AUC are proportional to dose (since V_D and Cl are also assumed to be constant). Linear pharmacokinetics predicts that there is a linear relationship between plasma concentration and dose.

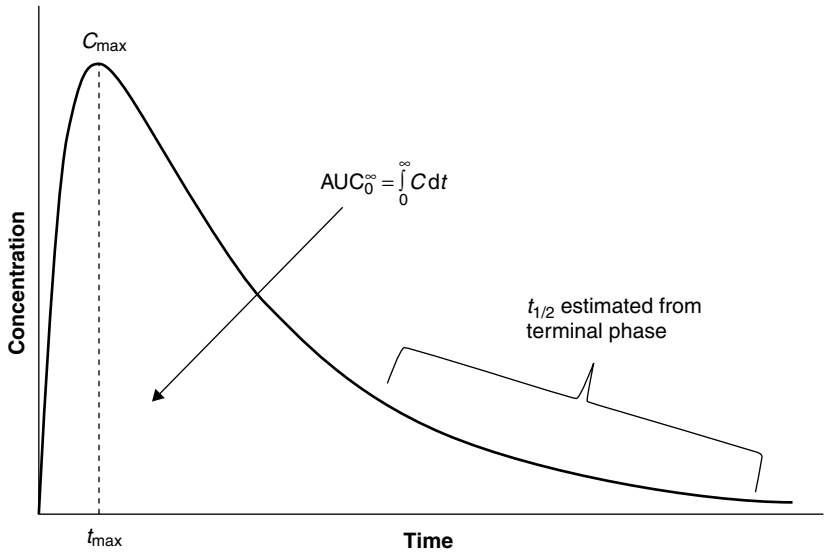

Figure 1 Schematic representation of a plasma concentration versus time profile following extravascular dosing. Depicted in the graph are pharmacokinetic markers of drug exposure including maximum plasma concentration (C_{max}), time to reach C_{max} (t_{max}), and area under the curve (AUC). Elimination half-life ($t_{1/2}$) is estimated from the terminal phase of the graph.

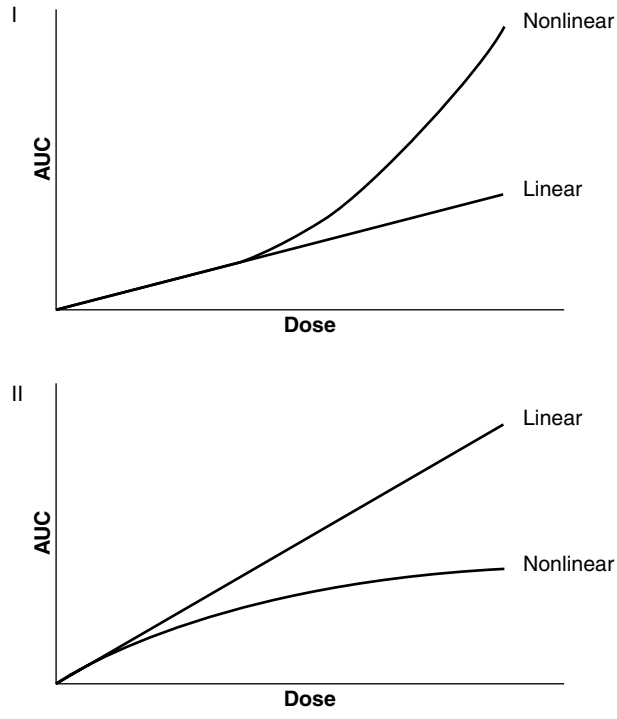

Figure 2 Plot of plasma AUC vs. dose: assessment of linear pharmacokinetics. Linear pharmacokinetics predicts a straight-line relationship between AUC and dose (dose-linearity). I. Nonlinear pharmacokinetics due to saturable metabolism (AUC increases disproportionately with dose). II. Nonlinear pharmacokinetics due to saturable absorption process (AUC shows less than proportional increase with dose).

While linear pharmacokinetics can be applied in most therapeutic situations, most drug disposition mechanisms (e.g., active membrane transport, drug metabolism) are saturable. The potential exists, therefore, for pharmacokinetics to be nonlinear; that is, increases in dose result in disproportionate changes in concentration. Dose-dependent pharmacokinetics may be due to transient saturation of enzymes or a carrier-mediated transport process, as described by the Michaelis–Menten equation:

$$\text{Rate} = \frac{V_{\max} \times C}{K_M + C} \tag{1}$$

This equation describes the rate of elimination as a function of concentration. $V_{\max}$ is the maximum elimination rate and K_M is the Michaelis constant. The relative magnitude of K_M and concentration determine the order of the elimination process.

Clearance

The most important PK parameter is clearance. Clearance is the critical connection between the administered dose and drug exposure (AUC). Since clearance is ultimately the link between the dose that a patient receives and the plasma level that is achieved, alterations in drug clearance due to disease, drug interactions, genetics, and other factors can have a direct impact on clinical outcomes. Mechanisms of drug clearance (metabolism and excretion) are discussed later in this chapter.

MECHANISMS OF SMALL MOLECULE ABSORPTION

When a compound is administered intravenously, the dose is delivered directly into the systemic circulation. All other routes of administration are collectively termed extravascular routes (e.g., oral, buccal, rectal, sublingual, topical, parental). Following extravascular administration, drug must be absorbed into the bloodstream across one of more membrane barriers before it is available to distribute to its site of action. Drug may cross these membranes by passive diffusion, facilitated passive diffusion, or active transport. Absorption is determined from the drug's physicochemical properties, the type of formulation administered, and the route of administration.

Bioavailability is a measure of the extent of therapeutically active drug reaching the systemic circulation and of the amount of drug available at the site of action. Bioavailability is a very important issue for drug development, particularly for orally administered medications. Both the physicochemical properties of the drug and the performance of the delivery system influence drug absorption. The impact of formulation and route of administration on drug absorption is the focus of Chapter 6. Presented in this chapter are general mechanisms of drug absorption, with particular focus on oral drug delivery.

Passive Absorption

The traditional view of oral drug absorption is that it occurs primarily from the small intestine and proceeds via a passive transcellular process. The small intestine represents the primary site of absorption in the GI tract because of the functional specialization of the intestinal cells (creating a large surface area for absorption) combined with the prolonged intestinal transit time. Drug diffuses across cell membrane from a region of higher concentration (e.g., GI fluids) to low concentration (blood) described by Fick's Law, with the driving force being the concentration gradient across the membrane (5).

Passive absorption is governed by several physicochemical properties including solubility, permeability, pKa, lipophilicity, and stability, each of which can influence drug absorption and pharmacokinetics (6–10). Lipinski et al. established the "Rule of 5" (8), which identifies the following ideal properties for drug absorption: (1) molecular weight <500, (2) Log P <5, (3) sum of hydrogen bond donors <5, and (4) a sum of hydrogen bond acceptors (as a sum of N and O) <10. If two of these criteria are not met, poor absorption or permeability is predicted.

Table 1 Examples of Food Effects on Drug Absorption

Mechanism	Explanation	Examples	Effect on absorption
Increased GI degradation (acid labile compounds)	Food increases drug retention in stomach (delayed gastric emptying), resulting in increased degradation	Penicillin, omeprazole	↓ Absorption
Increased dissolution (poorly soluble compounds)	Meals increase the absorption of drugs that are primarily absorbed in whole small intestine due to their increased solubility by gastric contents, biliary secretions, and fat in the food	Carbamazepine, diazepam; Griseofulvin, phenytoin; Tocopherol; Diltiazem, nicardipine; Sumatripan	↑ Absorption
Chelation	Calcium in dairy products chelates compound	Tetracycline	↓ Absorption
Reduced presystemic metabolism	Grapefruit juice inhibits intestinal CYP3A4		↑ Absorption
Medications with a "window of absorption"	Food increases contact time with absorption sites		↑ Absorption
Reduced "first-pass metabolism by liver following high protein meal	Food increases splanchic blood flow, resulting in transient reduction in hepatic extraction and reduced first-pass effect	Propranolol	↑ Absorption

Source: From Refs. 13–18.

As noted above, passive drug absorption is assumed to occur primarily in the small intestine. Accordingly, the rate-limiting step to oral drug absorption can be disintegration, dissolution, or the absorption process itself. For a compound with good membrane permeability, dissolution is generally the rate-limiting step. Here, attempts are made to establish in vitro and in vivo correlations between dissolution testing in the laboratory and clinical observations (i.e., in man).

Among the physiologic factors that influence oral drug absorption, gastric emptying is perhaps the most important. Gastric emptying time (GET) is the time it takes for the stomach contents to empty into the intestine. The phenomenon of GI motility has been extensively studied, and a number of factors including physiological (e.g., stomach content, pH, viscosity, temperature) and nonphysiological (exercise, body position, medications, age) affect this process (11,12). Perhaps the most important determinant of gastric emptying is food (Table 1). In the fasting state, gastric emptying is a rapid process (repeat cycles of 1–3 hours). However, in the presence of food, gastric emptying slows down significantly (resulting in ↑ GET up to nine hours). Therefore, GET can, in certain cases, be the rate-limiting step to absorption. For this reason, clinical studies of oral drug absorption must control the food intake of subjects because the presence of food, the type of food (hot vs. cold meal, liquid vs. solid), and the amount of food can affect GET.

In some cases, a delay in drug absorption in the presence of food may be disadvantageous due to the resultant delayed onset of therapeutic effect, as when a medication is administered with a meal. As described in Table 1, food can also affect drug bioavailability by increasing or decreasing drug degradation in the GI tract, increasing solubility, or reducing first pass hepatic metabolism (13–18). An additional concern, however, is the dose-dumping phenomenon noted with extended-release formulations of compounds when given concomitantly with food. This is particularly important for medications with a narrow therapeutic index (e.g., theophylline) (19). In these cases, food may result in an unintended rapid release of the drug. The dose-dumping effect has important implications for label claims for dose administration.

Solubility and Permeability

The two most important physicochemical determinants for drug absorption are solubility and permeability. In 1995, the Biopharmaceutics Classification System (BCS) was introduced (20–23). According to the BCS, compounds are grouped into the following categories, based on solubility and permeability properties:

Class I: High Solubility and High Permeability
Class II: Low Solubility and High Permeability
Class III: High Solubility and Low Permeability
Class IV: Low Solubility and Low Permeability

Since its introduction, regulatory agencies such as the FDA have formally recognized the value of the BCS. Most notably, the pharmaceutical industry is allowed to utilize in vitro dissolution data (as opposed to costly in vivo studies) to establish bioequivalence for highly soluble, highly permeable compounds (class I). This application of the BCS has resulted in an estimated cost savings of $35 million per year (24). Moreover, as shown in Figure 3, the BCS can be used to assess performance of new formulations in drug development. For class II compounds, for example, where dissolution is the rate-limiting step to absorption, dissolution data can be used to predict in vivo performance of formulation by establishing in vitro and in vivo correlations. Additionally, there has been increased interest in extending the provision for waivers of in vivo bioavailability and bioequivalence (BA–BE) studies to pharmaceutical products containing class III drugs (25).

Although the BCS classification is very useful during drug discovery in predicting drug absorption and bioavailability, several other physicochemical factors including crystallinity, particle size, and ionization state may influence these predictions (26). The aqueous solubility of highly crystalline drugs varies with crystal form and for the most stable crystal form the aqueous solubility is generally low. The formation of high energy, low-melting crystal or amorphous solids frequently yields more rapidly dissolving and higher solubility forms of the drug. Amorphous forms are generally used during drug discovery and therefore the solubility determinations using these forms may result in an erroneous BCS classification for the compound. Similarly, early PK studies conducted in drug discovery may result in high bioavailability estimates, which later may prove to be much lower when a highly crystalline form of the compound is used during drug development (27). Therefore, the determination of melting points and energy states could also be an important factor during screening in drug discovery.

Another factor that influences the solubility of drug is its particle size. In general, reducing the particle sizes can enhance the solubility and, consequently, enhance absorption. For

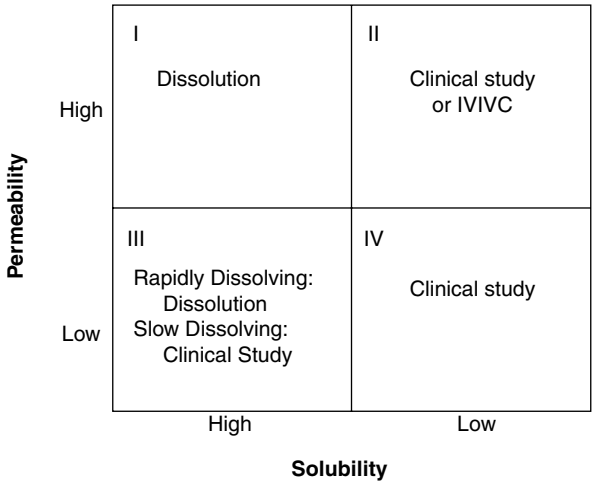

Figure 3 Biopharmaceutics Classification System (BCS): a framework for judging the adequacy of formulation performance for BCS class I–IV compounds. *Source*: From Ref. 24.

example, danazol is a poorly water-soluble drug (10 μg/mL) and shows poor bioavailability (approximately 5%) in humans and dogs. Liversidge and Cundy showed that reducing the particle size of danazol to an average of 85 nm (nanoparticle dispersion) increased bioavailability to 82% in dogs (28).

The solubility and absorption properties of ionizable drugs can depend upon the pH characteristics of the GI tract and may result in significant PK variability among the human population. For example, cinnarazine is a very insoluble drug (15 ng/mL) with two basic groups (pKa values of 1.94 and 7.47, respectively). This drug is very soluble in acidic solutions and its absorption is dependent on the gastric pH (26,29). In individuals with high gastric acid content (i.e., low gastric pH), cinnarazine has good absorption characteristics. Conversely, in those individuals showing low-gastric acid content, AUC and C_{max} were reduced by approximately 75% to 85%.

Carrier-Mediated Transport

As described in Chapter 7, membrane transporters perform a central function in drug disposition and activity. Together with the metabolizing enzymes [e.g., cytochrome P450 (CYP)], membrane transporters form a primary defense mechanism against the potential toxic effects of xenobiotics (30–41). Knowledge of the transporter(s) responsible for the elimination of a compound allows for the elucidation of potential drug interactions (drug–drug, drug–disease) and the identification of possible mechanisms of toxicity. Furthermore, modulation of these transport systems can elicit changes in distribution, clearance, and bioavailability and, consequently, drug activity. Table 2 contains a list of the transporters that play a role in PK processes.

While passive absorption and factors such as solubility and permeability continue to govern the manner in which many new drug candidates are evaluated, new insights regarding the role of the intestine as a selective barrier to drug absorption have emerged. Numerous membrane transport systems are present in the intestine to facilitate the absorption of essential nutrients, systems that may also be responsible for oral absorption of certain classes of medications (Fig. 4). Conversely, transporters in the enterocyte also serve as detoxification mechanisms in the body, which contribute to drug clearance through intestinal exsorption. By understanding the membrane transport mechanisms involved in oral drug absorption, strategies can be developed to enhance drug delivery of poorly bioavailable compounds. Intestinal transport systems that are of important for drug absorption are discussed below.

Peptide Transporter

As reviewed by Walter et al (46) and Wang et al (47), the existence of an oligopeptide transporter (PEPT1) on the apical surface of the intestine provides an efficient route of absorption for poorly lipophilic di- and tripeptides. The transporter has broad substrate specificity. The intestinal oligopeptide transporter (PEPT1) transports β-lactam antibiotics and ACE inhibitors, medications whose bioavailability is greater than predicted on the basis of size and physicochemical characteristics.

In general, peptide transport is electrogenic and is coupled with H^+. Once inside the enterocyte, oligopeptides are subject to proteolytic activity. However, basolateral transport of these poorly lipophilic peptides, albeit a relatively minor mode of transport, is thought to also be carrier-mediated and may involve the same transport system.

The intestinal peptide transport system could be exploited to improve oral bioavailability through a prodrug approach. In theory, a dipeptide prodrug would be absorbed across the apical membrane. Once inside the cell, the active moiety would be released by proteolysis and then be transported (either by passive or active processes) across the basolateral membrane into the blood. One example of this approach is val-acyclovir (48). However, the potential of this strategy for improving oral drug delivery of poorly absorbable compounds has yet to be recognized.

P-glycoprotein (P-gp): Role in Intestinal Efflux and Relationship with Intestinal Metabolism

The expression of the multidrug resistance transporter MDR1, also known as P-gp, on the apical surface of the intestine suggests a role of this transporter in intestinal transport. Indeed, drug efflux by P-gp has been shown to limit oral bioavailability of compounds such as furosemide

Table 2 Summary of Membrane Transporters Involved in Drug Disposition

Transporter	Tissue localization	Substrates
ATP Binding Cassette (ABC) Transporter Family		
P-gp (ABCB1)	Liver, kidney, intestine	Anticancer agents, HIV-protease inhibitors, antifungals, antibiotics, analgesics immunosuppressants
BCRP (ABCG2)	Liver, intestine	Anticancer agents (doxorubicin, mitoxantrone, etoposide), prazosin
MRP1 (ABCC1)	Ubiquitous	Methotrexate, GSH, doxorubicin, vincristine, estradiol-17-β-D-glucuronide
MRP2 (ABCC2)	Liver, kidney, intestine	Methotrexate, vinblastine, etoposide, 2,4-dinitrophenyl-S-glutathione
MRP3 (ABCC3)	Liver, kidney, intestine, bile ducts	Methotrexate, vincristine, estradiol-17-β-D-glucuronide
MRP4 (ABCC4)	Prostate, lung, muscle, pancreas, bladder	Estradiol-17-β-D-glucuronide, cyclic nucleotide (cAMP, cGMP), GSH, PMEA
MRP5 (ABCC5)	Ubiquitous	Cyclic nucleotide analogs, heavy metals (Cd), GSH, 6-mercaptopurine
MRP6 (ABCC6)	Liver, kidney	Endothelial receptor antagonist BQ-123, leukotiene C_4, 6-mercaptopurine
MRP7 (ABCC7)	Colon, skin, testis	Leukotiene C_4, docetaxel, estradiol-17-β-D-glucuronide
MRP8 (ABCC8)	Liver, lung, kidney, fetal tissue	Cyclic nucleotides (cAMP, cGMP), 5-fluorouracil
MRP9 (ABCC9)	Breast, testis, brain, skeletal muscle, ovary	
Solute Carrier (SLC) Transporter Family		
PEPT1 (SLC15A1)	Small intestine	ACE inhibitors, β-lactam antibiotics, anticancer agents
PEPT2 (SLC15A2)	Kidney	
OATP2A1 (SLC21A2)	Ubiquitous	Prostaglandins
OATP1A2 (SLC21A3)	Kidney, liver, brain	BSP, cholate, taurocholate, DHEA-S, E2 17G, PGE2, T_3, T_4, chlorambucil, fexofenadine, ouabain, BQ123, CRC220, ochratoxin A
OATP1A3-v1(OAT-K1, SLC21A4)	Kidney	Taurochorate, E2 17G, ES, DHES, folate, T3, T4, MTX
OATP1A3-v3 (OAT-K2, SLC21A5)	Kidney	Taurochorate, E2 17G, ES, DHES, folate, T3, T4, MTX
OATP1B1 (LST-1, SLC22A6)	Liver	Estrone sulfate
OATP1B3 (LST-2, SLC22A8)	Liver	Estrone sulfate
OATP2B1 (SLC21A9)	Brain, heart, intestine, kidney, liver, intestine	Bromosulphthalein, ES, DHEA-S, benzylpenicillin
OATP3A1 (SLC21A11)	Ubiquitous	ES, PGE2, PC-G
OATP4A1 (SLC21A12)	Ubiquitous	Taurocholate, ES, PGE2, T3, T4, PC-G
OATP1C1 (SLC21A14)	Brain, testis	Digoxin, ouabine
OATP4C1 (SLC21A20)	Kidney	

(*Continued*)

Table 2 Summary of Membrane Transporters Involved in Drug Disposition (*Continued*)

Transporter	Tissue localization	Substrates
OCT1 (SLC22A1)	Liver, kidney, intestine	Amantadine, antivirals (acyclovir, ganciclovir) choline, cisplatin
OCT2 (SLC22A2)	Kidney, intestine	
OCT3 (SLC22A3)	Liver, kidney, intestine	H_2-antagonists (cimetidine, ranitidine), metformin *n*-methylnicotinamide, paraquat, procaine quinine, quinidine tetraethylammonium verapamil
OCTN1 (SLC22A4)	Liver, kidney, intestine	Tetraethylammonium, verapamil, quinidine, pyrilamine
OCTN2 (SLC22A5)	Liver, kidney, intestine	L-carnatine, tetraethylammonium
OAT1 (SLC22A6)	Kidney, brain, skeletal muscle, placenta	PAH, duretics, antivirals, ACE inhibitors, antibiotics, ochratoxin A, NSAIDs, antineoplastics, mycotoxins
OAT2 (SLC22A7)	Kidney, liver	PAH, salicylate, methotrexate, 5-fluorouracil, loop diuretics, carbonic anhydrase inhibitors
OAT3 (SLC22A8)	Kidney, choroid plexus, skeletal muscle	Estrone sulfate, H_2-antagonists, antivirals uremic toxins, methotrexate, β-lactam antibiotics, NSAIDs, pravastatin
OAT4 (SLC22A11)	Kidney, placenta	Estrone sulfate, PAH, ochrotoxin A, tetracycline, zidovudine, bumetanide, ketoprofen
URAT1 (SLC22A12)	Kidney	Urate
OAT5 (SLC22A19)	Kidney	Ochratoxin A
OAT6 (SLC22A20)	Olfactory mucosa	
CNT1 (SLC28A1)	Liver, kidney, intestine, brain	Gemcitabine, cytarabine, lamivudine, AZT
CNT2 (SLC28A2)	Kidney, heart, liver, skeletal muscle, pancreas, placenta, brain, cervix, prostate, small intestine, rectum, colon, lung	ddI, cladribine
CNT3 (SLC28A3)	Mammary gland, pancreas, bone marrow, trachea, intestine, liver, lung, placenta, prostrate, testis, brain, heart	5-fluorouridine, 5-fluoro-2′-deoxyuridine, zebularine, gemcitabine, cladribine, fludarabine, AZT, ddC, ddI
ENT1 (SLC29A1)	Ubiquitous	Cladrabine, gemcitabine, fludarabine, cytarabine, ribavirin
ENT2 (SLC29A2)	Skeletal muscle, heart, pancreas, brain, kidney, small intestine, lung	ddI, ddC, AZT, gemcitabine
ENT3 (SLC29A3)	Kidney, placenta, breast, colon, testis, liver, spleen	
ENT4 (SLC29A4)	Kidney	
MATE1 (SLC47A1)	Kidney, liver, testes, skeletal muscle	Tetraethylammonium, 1-methyl-4-phenylpyridinium, cimetidine, procainamide, metformin, creatinine, cephalexin, cephradine
MATE2-K (SLC47A2)	Kidney	Tetraethylammonium, cimetidine, procainamide 1-methyl-4-phenylpyridinium, metformin, thiamine, *N*-methylnicotinamide, oxaliplatin

Source: From Refs. 34, 37, 39, 42–44.

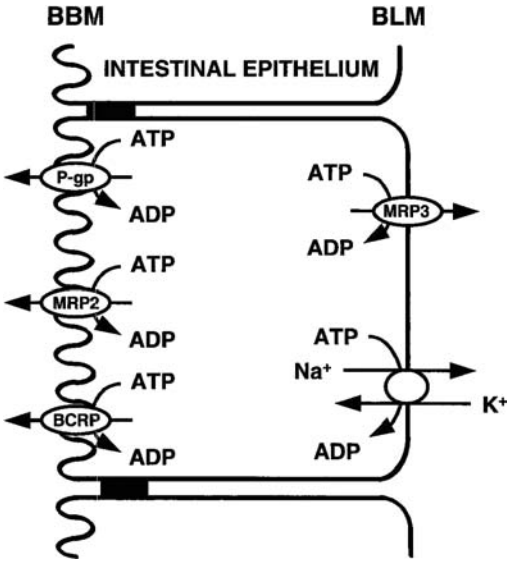

Figure 4 Schematic representation of ATP-dependent efflux transporters in the small intestine. P-glycoprotein (P-gp), MRP2, and BCRP are expressed in the brush border membrane (BBM), whereas MRP3 is located at the basolateral membrane (BLM). *Source*: From Ref. 45.

(49) and etoposide (50). A list of P-gp substrates and inhibitors has been provided in Table 3 (51). Furthermore, there appears to be a cooperative function of this efflux system and intestinal metabolism in limiting drug absorption.

Intestinal phase I metabolism has been established as a contributing factor to limit bioavailability of orally administered medications (52–54). Approximately 70% of intestinal metabolism

Table 3 List of Clinically Relevant Substrates and Inhibitors of P-gp

SUBSTRATES		
Analgesics	*H_2-receptor antagonists*	*Cardioactive medications*
Asimadoline	Cimetidine	Verapamil
[D-Penicillamine2,5]-enkephalin (DPDPE)	Ranitidine	Diltiazem
Anticancer agents	*Antigout agents*	Digoxin
Vincristine	Colchicine	Quinidine
Vinblastine	*Antidiarrheal agents*	*Antihypertensives*
Paclitaxel	Loperamide	Losartan
Doxorubicin	*Antiemetics*	Atovastatin
Daunorubicin	Domperidone	*Immunosuppressants*
Epirubicin	Ondansetron	Cyclosporin A
Bisantrene	*Antifungals*	FK506
Mitoxantrone	Ketoconazole	Tacrolimus
Etoposide	Itraconazole	*Corticosteroids*
Actinomycin D	*Antihistamines*	Dexamethasone
HIV protease inhibitors	Fexofenidine	Hydrocortisone
Saquinavir	Cetirizine	Corticosterone
Ritonavir	*Diagnostic agents*	Triamcinolone
Nelfinavir	Rhodamine 123	*Antibiotics*
Indinavir	Hoechst 33342	Erythromycin
Lopinavir	β-*Blockers*	Gramicidin D
Amprenavir	Talinolol	Valinomycin
INHIBITORS		
First generation	*Second generation*	*Third generation*
Verapamil	Dexverapamil	LY335979 (zosuquidar)
Nicardipine	PSC833 (valspodar)	XR9576 (tariquidar)
Quinacrine	GF120918 (elacridar)	R101933 (laniquidar)
Cyclosporin A	VX-710 (biricodar)	OC 144–093 (ONT-093)

Source: From Ref. 51.

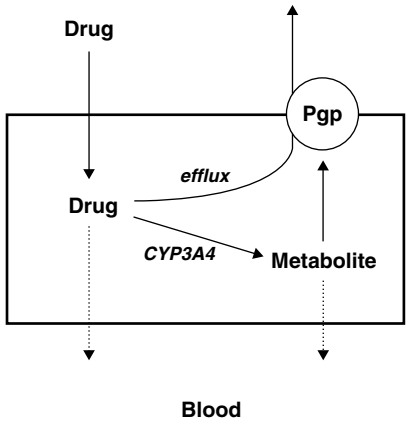

Figure 5 Model that depicts the combined role of P-glycoprotein and CYP3A4 in limiting intestinal drug absorption. Drug entering the intestinal cell via passive transcellular absorption may undergo efflux via P-gp or biotransformation via CYP3A4. Generated metabolite may also be susceptible to cellular efflux. Either metabolite or drug may be subsequently absorbed across the basolateral membrane into the blood. Since intestinal content of CYP3A4 is limited, P-gp recycling may increase exposure of drug to P-gp, thereby increasing presystemic metabolism. *Source*: From Ref. 60.

is mediated by CYP3A4. Interestingly, P-gp and CYP3A4 are induced by many of the same compounds. There exists broad overlap in substrate and inhibitor specificities for these two mechanisms, suggesting that P-gp and CYP3A4 act as a concerted barrier to drug absorption. This so-called drug efflux metabolism alliance is well described in the literature (55). P-gp and CYP3A4, each functions to reduce systemic exposure of substances that undergo passive paracellular transport (Fig. 5). Lipophilic compounds are susceptible to intracellular metabolism or secretion by P-gp. Additionally, P-gp may also act to extrude metabolites generated from the intestinal cell. Based upon these considerations, it appears that oral bioavailability can be enhanced through inhibition of these two detoxification pathways. In addition to MDR modulators listed in Table 3, an example of a P-gp inhibitor is Cremophor EL, a commonly used excipient in oral dosage forms (56–58). Grapefruit juice, a substance widely known to increase oral bioavailability through inhibition of intestinal 3A4 activity, has been shown to activate P-gp-mediated transport (59).

P-gp-mediated secretion also contributes to intestinal drug clearance, an often-neglected route of systemic drug excretion. P-gp-mediated intestinal drug clearance has been demonstrated for fluoroquinolones (61,62) and may be the primary route of excretion of digoxin in patients with severe renal insufficiency (60,63).

Other Intestinal Efflux Transporters

Other membrane transporters may contribute to the intestinal absorption and efflux of medications. Breast cancer resistance protein (BCRP) is an ABC transporter, originally identified by its ability to confer drug resistance that is independent of other ABC transporters including P-gp. Because it contains a single N-terminal ATP binding cassette, BCRP is referred to as a "half-transporter." Analogous to P-gp, these efflux transporters likely limit the oral bioavailability of medications (45,64).

Like P-gp and BCRP, multidrug resistance proteins (MRPs) have also demonstrated MDR to cancer cells. Despite some substrate overlap with P-gp, MRP substrates include conjugates (glutathione, glucuronide) and other organic anions. Consequently, MRP proteins play a crucial role in the export of conjugated drug metabolites out of cells. Both MRP2 and MRP3 are expressed on the apical membrane of the small intestine. In studies comparing normal and mutant (EHBR) rats, a role of these transporters on intestinal exsorption has been demonstrated (65).

Information is beginning to emerge about a class of transporters involved in the uphill cellular transport of nucleosides (66). These sodium-coupled nucleoside transporters may play a critical role in absorption, disposition, and clinical activity of therapeutically active nucleosides (e.g., adenosine) and nucleoside, analogs used in treatment of AIDS (e.g., 3TC and ddI) and cancer (e.g., cytarabine). Of the five major transporter subtypes that have been identified, two are present in the intestine. The relative importance of these transporters to pharmacokinetics is unknown.

Bioavailability Determinations

One of the important first stages in drug development involves assessment of the absolute bioavailability of a new compound (67). Bioavailability is defined as the fraction of administered dose reaching the systemic circulation. Even if a drug is completely absorbed, bioavailability may be low due to presystemic metabolism or degradation in the GI tract.

Typically bioavailability is determined by dosing the drug using two routes. The test product is the intended clinical route of administration (oral, dermal, intraperitoneal, intramuscular, intranasal etc.). Bioavailability is calculated as the ratio of dose-normalized AUC (test vs. reference products). For absolute bioavailability, the reference product is an intravenous dose. If absolute bioavailability is less than 5%, then the drug is considered to be poorly bioavailable. However, this may not be an issue for a highly potent compound, since this bioavailability may be sufficient to produce the desired clinical response. Therefore, one may argue that bioavailability is not a very important factor, particularly when safe plasma concentrations well above the target efficacious concentrations are achievable by the intended clinical route. Nevertheless, a comparison of absolute bioavailabilities in different animal species could provide important information with regard to permeability, absorption, and drug metabolism across species.

Poor bioavailability may sometimes also be the result of extensive hepatic clearance and/or hepatic metabolism rather than poor absorption. In such cases, permeability data generated from cell culture experiments (e.g., Caco-2 cells) and in vitro metabolism studies in liver microsomes/hepatocytes could help guide preclinical programs.

MECHANISMS OF SMALL MOLECULE DISTRIBUTION

Distribution is one of the two critical determinants of drug disposition, the other being clearance. Once a compound reaches the systemic circulation, it is available for distribution throughout the body (Fig. 6). While the therapeutic effect of the drug will depend on its ability to access its site of action or "biophase," drug distribution to other organs and tissues can result in adverse or toxic effects. Additionally, sequestration of drug in an organ or tissue may result in a prolonged residence time in the body (i.e., a long elimination half-life).

The extent of drug distribution in the body is described by V_D. V_D is defined as proportionality constant between the amount of drug in the body and it's concentration in the

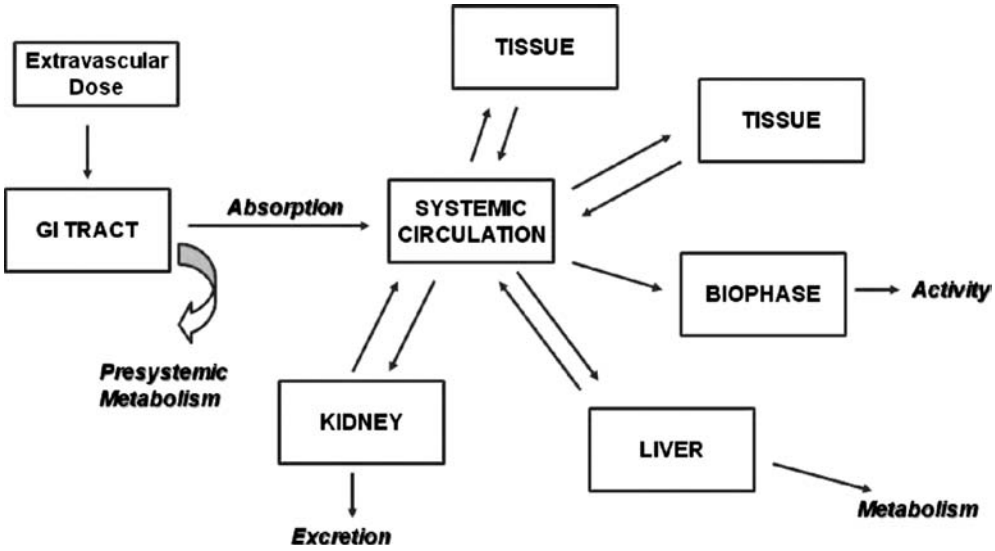

Figure 6 Illustration of drug disposition processes following oral drug administration. Following absorption a across the GI tract, the drug reaches the systemic circulation where it is available for distribution to organs and tissue sin the body. Distribution is generally a reversible process. Included in the figure are the roles of the liver and kidney in drug metabolism and excretion. It should be noted that oral bioavailability (the amount of drug reaching the systemic circulation) depends on various factors including presystemic metabolism by the intestine and liver.

Table 4 Factors Affecting Rate and Extent of Distribution

Rate	Extent
Blood flow to organ/tissues	Protein binding (plasma vs. tissue)
Lipophilicity	Lipophilicity
Ionization	Ionization
Protein binding	

plasma. In preclinical development, it is important to characterize the distribution pattern of a new chemical entity. This is typically done in small animal models as part of a toxicokinetic assessment. As discussed in Chapter 7, tissue distribution studies are conducted in accordance with ICH guidelines. Through these studies, information is obtained regarding the distribution and accumulation of drug and metabolites, particularly in relation to potential sites of action (68).

Since the goal of clinical pharmacokinetics is to establish and target useful therapeutic ranges of drugs, a relationship between plasma concentrations and those at the "biophase" is assumed to exist. The concept that relates these concentrations is distributional equilibrium (DE). DE occurs when the unbound concentration in the plasma is equal to unbound concentration of drug in the tissue. Two important characteristics of distribution are rate and extent (Table 4). In other words, "how quickly does a drug distribute?" and "where is the drug going in the body?" To answer the first question, the rate of distribution depends on two factors: blood flow and the ability of the drug to cross biological membranes. The rate-limiting step to distribution is blood flow to the organ or tissue. Organs such as the liver, kidney, heart, and brain are *highly perfused* and drug reaches these organs rapidly. On the other hand, adipose tissue and skeletal muscle are *poorly perfused* and it takes time for drug to reach them.

In addition to blood flow, the ability of drug to penetrate a biological membrane also affects the rate of distribution. There are two general types of distribution mechanisms: passive uptake and carrier-mediated transport. When drug uptake into organs and tissues is a passive process, it can be described by Fick's Law. Therefore, the rate of absorption depends upon the drug's lipophilicity (partition coefficient), the thickness of the absorbing membrane, ionization state (pH vs. pKa), and plasma protein binding. Ionization is important because of the pH partition hypothesis that assumes that a drug molecule can be absorbed only in its unionized form. Since the driving force for passive diffusion is the concentration gradient across the membrane, if the drug is highly and strongly bound to plasma protein this concentration gradient will be reduced.

Besides the rate of distribution (i.e., time required to reach DE), it is also important to consider the extent of distribution. Here, protein binding is most important, although lipophilicity/polarity and ionization are critical determinants as well. Most drugs can readily exit capillaries. Albumin, the primary binding protein in the plasma, is a large molecule (average molecule weight 69000 Da), which is unable to cross the capillary wall and leave the bloodstream. Therefore, any protein-bound drug cannot leave the plasma (Fig. 7). This is particularly important for weakly acidic compounds (e.g., furosemide, phenytoin). Generally speaking, the volume of distribution of weak acids is relatively small ($<$0.5 L/Kg). This is not only due to plasma protein binding, but also because of poor lipophilicity. For example, aminoglycosides do not bind to plasma albumin and are therefore free to exit the capillary and access the extracellular fluid (ECF). However, these compounds do not cross biological membranes very well and do not distribute further. The reported volume of distribution (V) of these medications is 0.25 L/Kg (69).

In contrast to weak acids, weakly basic drugs are generally lipophilic and not highly plasma protein bound. Because of their physicochemical characteristics, lipophilic compounds readily cross biological membranes and are taken up by tissues. In many cases, the tissue can act as a reservoir for drug and drug is assumed to be "bound-up" by the tissue. An example is the tricyclic antidepressants. The volume of distribution of these compounds can approach 60 L/Kg (70), indicating that they do not stay in the bloodstream, but rather are sequestered somewhere else in the body. Drugs with large volumes usually take time to distribute once they are administered and distribution is extensive.

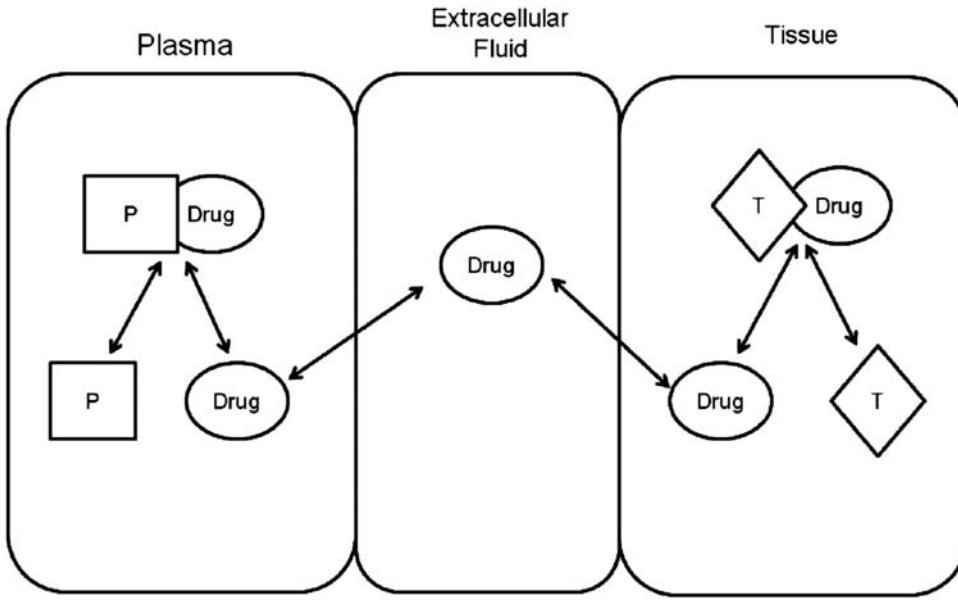

Figure 7 Role of plasma and tissue binding on drug distribution. Only unbound drug (D) is able to distribute between plasma and tissue. At distributional equilibrium, the unbound concentration is equal throughout the body. The extent of drug distribution depends on the relative binding of drug between plasma and tissue.

As demonstrated in Figure 7, the relative binding of drug in the plasma compared to tissue has a significant effect on overall distribution. The apparent volume of distribution (V) can be described by the following equation:

$$V = V_{\mathrm{p}} + \left(\frac{f_{\mathrm{u,p}}}{f_{\mathrm{u,t}}}\right) v_{\mathrm{t}} \tag{2}$$

Where V_{p} and V_{t} represent the true volumes of plasma and tissue. $f_{\mathrm{u,p}}$ and $f_{\mathrm{u,t}}$ are the fraction of drug unbound in the plasma and tissue, respectively (fraction unbound is the ratio of unbound and total concentrations. For medications that are highly plasma bound, this equation predicts limited distribution ($V \approx V_{\mathrm{p}}$). Conversely, when tissue binding is high, extensive distribution is predicted ($V \gg V_{\mathrm{p}}$)

Besides passive uptake, carrier-mediated transport contributes to drug distribution. In addition to the intestine (discussed above), numerous transporters are expressed in other tissues. Of particular importance is the emerging role of transporters on drug distribution to the CNS, as described in Chapter 5. Recent evidence has demonstrated the presence of numerous transport systems that may function in CNS uptake and efflux of xenobiotics (71–75). Understanding the key features of these pathways may allow for improved treatment of diseases of the CNS (e.g., brain tumors, bacterial and viral infections) through enhanced uptake of neuropharmaceuticals. Furthermore, CNS-related side effects of medications could be avoided by blocking these mechanisms.

The results of published investigations have begun to elucidate the role of membrane transporters in the placenta, mammary gland, and testes (74,75). Therefore, transporters provide an important mechanism for the distribution of small molecules.

Based upon the previous discussion, it is evident that plasma protein binding can affect both the rate and extent of drug distribution. The primary binding protein in plasma is albumin, which binds primarily weakly acid molecules (salicylic acid, phenytoin). The large size of this molecule prevents it (and anything bound to it) from exiting the capillary. Therefore, drugs that are highly bound to albumin are generally restricted to the bloodstream. A second

important binding protein is alpha-1 acid glycoprotein (AAG), which binds some weak bases such as propranolol and quinidine. AAG is not the primary binding protein, but the interesting fact about this molecule is that AAG levels can increase or decrease secondary to disease and other factors. Stress, surgery, and malignancy can all increase AAG while pregnancy, malnutrition, and lever disease can decrease AAG. Perturbations in AAG can affect drug binding, distribution and, in certain instances, therapeutic activity (76). Likewise, plasma lipoproteins are potential binding targets for hydrophobic compounds (e.g., cyclosporine A). Changes in the lipoprotein plasma profile of an individual can potentially influence drug disposition and drug activity (77).

Like AAG, changes in albumin plasma concentrations can also affect drug distribution and response. Hypoalbuminemia can occur in elderly patients and those with renal failure. A decrease in the concentration of plasma albumin will increase drug-free fraction (f_u) which increases V. Phenytoin is a useful example (78). Patients with hypoalbuminemia will have an increased f_u of phenytoin, which can potentially result in toxicity (renal failure will further complicate this as uremia increases f_u of phenytoin even more).

A recent review by Benet and Hoener demonstrated that protein-binding changes caused by drug–drug and disease–drug interactions are rarely of clinical importance (79). Except in rare cases (e.g., a compound with a high extraction ratio and narrow therapeutic range), an increase in f_u will result in increased drug clearance. Consequently, clinical exposure of a patient to the drug will be unaffected. Nevertheless, protein-binding measurements are important during drug development for several reasons. First, interspecies differences in f_u will affect allometric predictions of PK parameters (clearance and volume of distribution). Second, knowledge of drug protein binding (f_u) is necessary for establishing a suitable first dose in humans. Third, therapeutic drug monitoring typically involves measuring total drug concentrations. For a highly protein bound compound with a narrow therapeutic range (e.g., phenytoin), this can result in erroneous dosing adjustments for patients with elevated f_u. In the phenytoin example described above, patients with reduced protein binding of phenytoin (e.g., secondary to hypoalbuminemia) tend to have lower total drug concentrations (i.e., below the established therapeutic range), which are often misinterpreted. In this case, attempts should be made to extrapolate observed drug concentrations to "normal binding" conditions in order to avoid unnecessary increases in dose (79).

In addition to plasma proteins, other components of the blood may influence drug disposition. Specifically, the erythrocytes are a potentially important distribution site for medications. The erythrocytes play an important role in the transport and disposition kinetics of medications (e.g., carbonic anhydrase inhibitors) in the blood (80,81). However, the erythrocytes are often regarded as insignificant compartment of drug distribution. For those compounds that accumulate in the erythrocytes to an appreciable extent, characterization of different kinetic events occurring within the erythrocyte can provide significant insight into drug disposition (82).

MECHANISMS OF SMALL MOLECULE METABOLISM

Clearance is the most important determinant of drug disposition, and it is the parameter used to establish suitable doses of medications. Drug metabolism plays a major influential role in interindividual variability in drug clearance in humans. Drug metabolism may be altered in disease states including hepatic and renal failure, resulting in variable drug clearance in humans. Other factors contributing to variability include pharmacogenetics (e.g., polymorphism), gender, age, and drug interactions.

Drug is cleared from the body through two general pathways: metabolism and excretion. As discussed below, excretion implies the elimination of drug from the body intact. While excretion is the predominant pathway for some compounds, many medications undergo some degree of metabolism or biotransformation in vivo. Drug metabolism involves chemical modification of a compound in the body. The resulting metabolites that are formed (either active or inactive) either undergo further metabolism or are excreted by the body. Drug metabolism is an important mechanism for the elimination of lipophilic molecules. These compounds require biotransformation to more polar metabolites that can be readily excreted. Presented here is an overview of drug metabolism. For further information, a number of textbooks are available (83–85)

Liver and intestine are considered the major sites of drug metabolism. There are two general types of metabolic reactions: phase I and phase II. Phase I metabolism includes reactions involved in the biotransformation of molecules that lack a required functional group for conjugation reactions (phase II). Some of the important enzymes that catalyze phase I drug metabolism in these organs are CYP, flavin monooxygenases (FMO), xanthine oxidase, and aldehyde oxidase. Phase II enzymes include glucuronosyl transferases (GT), *n*-acetyltransferase, and sulfotransferases (ST). Recently, a third phase of metabolism has been proposed (phase III), in recognition of the role of membrane transporters on the biliary excretion of drugs and their metabolites, as well as the efflux of these compounds across the hepatocellular membrane (86).

Phase I Metabolizing Enzymes

Cytochrome P450

CYP is the primary enzyme system responsible for the oxidative metabolism of xenobiotics. CYP enzymes are primarily located in the endoplasmic reticulum of cells, when fractionated form vesicles called microsomes. The name P450 stems from absorbance wavelength of the activated enzyme (450 nm), when carbon monoxide was added to reduced microsomes with NADH or dithionite. CYP is a family of enzymes that are classified based upon the structural similarity of their amino acid sequence (83). Enzymes having >40% sequence identity belong to the same family (e.g., CYP1, CYP2). Those enzymes with >55% overlap are grouped into subfamilies (e.g., CYP1A). Eukaryotic enzymes (those identified in eukaryotic systems are designated CYP100 or less. A list of CYP isoforms across various species is provided in Table 5.

The most abundant (>30% total content) CYP enzyme in the human liver is CYP3A4, one of the two primary enzymes for drug metabolism (Fig. 8). The other enzyme, CYP2D6, makes up <2% of total CYP. The predominant isoforms of interest include CYP1A2, CYP2A6, CYP2C8/9/10/19, CYP2D6, CYP2E1, and CYP3A4. Of these CYP3A4, CYP2D6, and CYP2C9 are involved in the metabolism of about 50%, 30%, and 15% of the known drugs, respectively. In addition, many of the CYPs are polymorphic whose expression in human liver is genetically controlled.

A number of reactions are catalyzed by CYP (83,84,87). Examples include oxidative and reductive mechanisms. The oxidative reactions include aromatic and side-chain hydroxylations, N- and O-dealkylations, deamination of primary and secondary amines, N-oxidations, sulfoxidations, desulfurations, and ester cleavage. Reductive reactions catalyzed by CYP include reduction of epoxides, N-oxides, nitroso compounds, hydroxylamines, nitro compounds, azo compounds, nitrosamines and azido compounds, and reductive dehalogenation. The reduction reactions of N-oxides, nitroso compounds, and hydroxylamines are the counterpart of oxidative reactions catalyzed by CYP and should be viewed as bioreversible oxidation–reduction reactions. In contrast, the reduction of nitro, azo azido compounds, and nitrosamines are not mirrored by their oxidative formation and, therefore, are not bioreversible oxidation–reduction reactions. Table 6 provides a list of compounds metabolized by CYP enzymes.

There is a high degree of intersubject variability in drug metabolism. Genetic differences in metabolism are reflected in enzyme polymorphism. All the major human CYP enzymes responsible for drug metabolism exhibit common polymorphisms at genomic level (89). CYP2D6 polymorphisms are of major concern as many of its substrates have narrow therapeutic margin (90). More than 50 alleles of CYP2D6 have been described in literature. There are three categories of individuals (poor metabolizers, extensive metabolizers, and ultrarapid metabolizers) depending on the nature of CYP2D6 polymorphisms. Poor metabolizers have inactivating mutations resulting in inactive or no protein expression. Ultrarapid metabolizers possess several copies of CYP2D6 producing excessive active protein. Approximately 7% of the Caucasian population and <1% of Orientals and African-Americans are poor metabolizers for CYP2D6. This can lead to problems with certain classes of medications (e.g., antidepressants, antiarrhythmics) due to increased plasma levels of drug in these patients. For example, poor CYP2D6 metabolizers respond poorly to codeine therapy because the analgesic activity of codeine depends upon its conversion to morphine in vivo via CYP2D6 (91). The poor metabolizer phenotype of CYP2C19 is present in 20% of Asians, but only 3% in Caucasians (92). An example here is omeprazole. Omeprazole induces CYP1A2, but is a substrate for CYP2C19. In CYP2C19 poor metabolizers,

Table 5 Cytochrome P450 Isoforms: A Species Comparison

CYP family	Human	Mouse	Rat	Rabbit	Dog	Monkey
1A	1A1, 1A2	1a1, 1a2	1A1, 1A2	1A1, 1A2	1A2	1A1
1B	1B1	1b1	1B1	–	–	–
2A	2A6, 2A6v2, 2A7, 2A13, 2A18PC, 2A18PN	2a4, 2a12	2A1, 2A2, 2A3	2A10, 2A11	–	–
2B	2B6, 2B7P	2b9, 2b10, 2b13, 2b19, 2b20, 2b20P1	2B1, 2B2, 2B3, 2B8, 2B12, 2B14P, 2B15, 2B16P, 2B21	2B4, 2B5	2B11	2B17
2C	2C8, 2C9, 2C18, 2C19	2c29, 2c29v2, 2c37, 2c38, 2c39, 2c40, 2c50, 2c51, 2c52P, 2c53P, 2c54, 2c55	2C6, 2C7, 2C11, 2C12, 2C13, 2C22, 2C23, 2C24	2C1, 2C2, 2C3, 2C4, 2C5, 2C14, 2C15, 2C30	2C21, 2C41	2C20, 2C43
2D	2D6, 2D7AP, 8BP	2d10, 2d11, 2d12, 2d13, 2d22, 2d26	2D1, 2D2, 2D3, 2D4, 2D5, 2D18	2D23, 2D24	2D15	2D17, 2D29
2E	2E1	2e1	2E1	2E2	2E1v1, 2E1v2	2E1
2F	2F1P	2f2	2F4	–	–	–
2J	2J2	2j5, 2j6, 2j7, 2j8, 2j9	2J3, 2J3P1, 2J3P2, 2J4	2J1	–	–
2R	2R1	–	–	–	–	
2S	2S1	2s2	–	–	–	–
2T	2T2P, 2T3P	?	2T1	–	–	–
2U	2U1	–	–	–	–	–
2W	2W1	–	–	–	–	–
3A	3A3, 3A4, 3A5, 3A5P1, 3A5P2, 3A7, 3A43	3a11, 3a13, 3a16, 3a25, 3a41, 3a44	3A1, 3A2, 3A9, 3A18, 3A23	3A6	3A12, 3A26	3A8
4A	4A11	4a10, 4a12, 4a14, 4a22	4A1, 4A2, 4A3, 4A8	–	4A4, 4A5, 4A6, 4A7	–
4B	4B1	4b1	4B1	4B1	–	–
4F	4F2, 4f3, 4F3v2, 4F8, 4F9P, 4F10P, 4F11, 4F12, 4F22, 4F23P, 4F24P, 4F25P, 4F26P, 4F27P	4f13, 4f14, 4f15, 4f16, 4f17, 4f18	4F1, 4F4, 4F5, 4F6, 4F9	–	–	–
4V	4V2	4v3	–	–	–	–
4X	4×1	4×1	4×1	–	–	–
4Z	4Z1	–	–	–	–	–
5A	5A1	5a1	5A1	–	–	–
7A	7A1	7a1	7A1	7A1	–	–
7B	7B1	7b1	7B1	–	–	–
8A	8A1	8a1	8A1	–	–	–
8B	8B1	8b1	–	8B1	–	–

(*Continued*)

Table 5 Cytochrome P450 Isoforms: A Species Comparison *(Continued)*

CYP family	Human	Mouse	Rat	Rabbit	Dog	Monkey
11A	11A1	11a1	?	11A1	–	–
11B	11B1, 11B2	11b1, 11b2	11B1, 11B2, 11B3, 11B8P	–	–	–
17	17	?	17	–	–	–
19	19	19	19	–	–	–
20	20	20	–	–	–	–
21	21A1P, 21A2	–	21	–	–	–
24	24	–	24	–	–	–
26A	26A1	26a1	–	–	–	–
26B	26B1	–	–	–	–	–
26C	26C1	26c1	–	–	–	–
27A	27A1	–	27A1	27A1	–	–
27B	27B1	27B1	27B1	–	–	–
27C	27C1	–	–	–	–	–
39	39	39	–	–	–	–
46	46	–	–	–	–	–
51	51, 51P1, 51P2	–	51	–	–	–

omeprazole may be a more potent inducer of CYP1A2 because of elevated plasma levels of drug (93).

The dramatic variability in enzyme expression is a factor contributing to drug-related problems in patient care, a result of administration of an insufficient dose to an ultrametabolizer or a toxic dose to a slow metabolizer. Enzyme phenotyping involves administration of a probe drug for a specific enzyme and comparing the urinary recovery of the probe and its metabolite. A slow metabolizer would be expected to have a low urinary metabolite ratio (metabolite: parent in the urine), where a rapid metabolizer would have a high urinary metabolite ratio. Table 7 lists probe substrates for phenotyping various P450 enzymes. Presently, phenotyping is limited by the time and resources required for testing. As a result, widespread utilization of patient phenotyping is presently impossible. However, cocktail approaches with simultaneous administration of several CYP substrates appear to be a viable alternative approach for CYP phenotyping (94,95). This approach was successfully applied for assessing drug–drug interactions in the clinical setting.

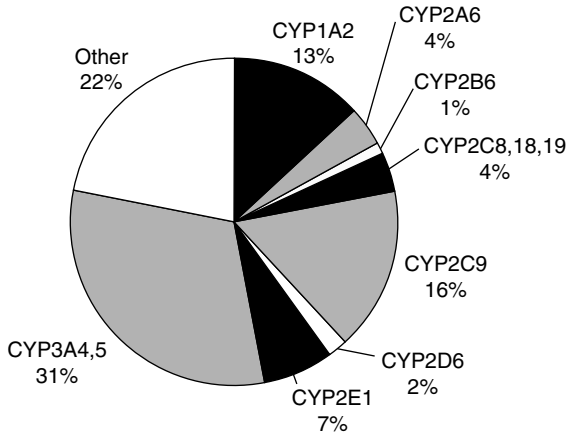

Figure 8 Relative distribution of drug metabolizing CYP isoforms in the human liver.

Table 6 CYP Substrates, Inhibitors, and Inducers

1A2	2B6	2C8	2C19	2C9	2D6	2E1	3A4,5,7
Substrates							
amitriptyline	bupropion	paclitaxel	**Proton pump inhibitors:**	**NSAIDs:**	**β-blockers:**	**Anesthetics**	**Macrolide antibiotics:**
caffeine	cyclophosphamide	torsemide	lansoprazole	diclofenac	carvedilol	enflurane	clarithromycin
clomipramine	efavirenz	amodiaquine	omeprazole	ibuprofen	S-metoprolol	halothane	erythromycin (not 3a5)
clozapine	ifosfamide	cerivastatin	pantoprazole	lornoxicam	timolol	isoflurane	**Not** azithromycin
cyclobenzaprine	methadone	repaglinide	rabeprazole	meloxicam		methoxyflurane	Telithromycin
estradiol			E-3810	S-naproxen	**Antidepressants:**	sevoflurane	
fluvoxamine				piroxicam	amitriptyline		**Anti-arrhythmics:**
haloperidol			**Antiepileptics:**	suprofen	clomipramine	**Others**	quinidine = >3-OH (not 3A5)
imipramine			diazepam = >Nor		desipramine	acetaminophen = >NAPQI	
mexiletine			phenytoin(O)	**Oral hypoglycemic agents:**	imipramine	aniline	**Benzodiazepines:**
naproxen			S-mephenytoin	tolbutamide	paroxetine	benzene	alprazolam
olanzapine			phenobarbitone	glipizide		chlorzoxazone	diazepam = >3OH
ondansetron					**Antipsychotics:**	ethanol	midazolam
propranolol			**Others**	**Angiotensin II blockers:**	haloperidol	*N,N*-dimethyl formamide	triazolam
riluzole			amitriptyline	losartan	perphenazine	theophylline = >8-OH	
ropivacaine			carisoprodol	irbesartan	risperidone = >9OH		**Immune modulators**:
tacrine			citalopram		thioridazine		cyclosporine
theophylline			chloramphenicol	**Sulfonylureas:**	zuclopenthixol		tacrolimus (FK506)
tizanidine			clomipramine	glyburide/ glibenclamide			
verapamil			clopidogrel	glipizide	**Others**		**HIV antivirals:**
(R)warfarin			cyclophosphamide	glimepiride	alprenolol		indinavir
zileuton			hexobarbital	tolbutamide	amphetamine		nelfinavir
zolmitriptan			imipramine N-deme		aripiprazole		ritonavir
			indomethacin	**Others**	atomoxetine		saquinavir
			R-mephobarbital	amitriptyline	bufuralol		
			moclobemide	celecoxib	chlorpheniramine		**Prokinetic:**
			nelfinavir	fluoxetine	chlorpromazine		Cisapride
			nilutamide	fluvastatin	codeine (= >O-desme)		
			primidone	glyburide	debrisoquine		
			progesterone	nateglinide	dexfenfluramine		
			proguanil	phenytoin-4-OH2	dextromethorphan		
			propranolol		duloxetine		
			teniposide		encainide		
			R-warfarin = >8-OH				

rosiglitazone
tamoxifen
torsemide
S-warfarin1

flecainide
fluoxetine
fluvoxamine
lidocaine
metoclopramide
methoxyamphetamine
mexilletine
minaprine
nebivolol
nortriptyline
ondansetron
oxycodone
perhexiline
phenacetin
phenformin
promethazine
propafenone
propranolol
sparteine
tamoxifen
tramadol
venlafaxine

Antihistamines:
astemizole
chlorpheniramine
terfenadine2

Calcium channel blockers:
amlodipine
diltiazem
felodipine
lercanidipine
nifedipine2
nisoldipine
nitrendipine
verapamil

Hmg coa reductase inhibitors:
atorvastatin
cerivastatin
lovastatin
not pravastatin
simvastatin

Steroids
estradiol
hydrocortisone
progesterone
testosterone1

Miscellaneous:
alfentanyl
aprepitant
aripiprazole
buspirone
cafergot

(*Continued*)

Table 6 CYP Substrates, Inhibitors, and Inducers (*Continued*)

1A2	2B6	2C8	2C19	2C9	2D6	2E1	3A4,5,7
							caffeine = >TMU
							cilostazol
							cinacalcet
							cocaine
							codeine-N-demethylation
							dapsone
							dexamethasone
							dextromethorphan2
							docetaxel
							domperidone
							eplerenone
							fentanyl
							finasteride
							gleevec
							haloperidol
							irinotecan
							laam
							lapatinib
							lidocaine
							methadone
							nateglinide
							ondansetron
							pimozide
							propranolol
							quetiapine
							quinine
							risperidone
							not rosuvastatin
							salmeterol
							sildenafil
							sirolimus
							tamoxifen
							Taxol
							terfenadine
							trazodone
							vincristine
							zaleplon
							ziprasidone
							zolpidem

Inhibitors
fluvoxamine[a]
ciprofloxacin[a]
cimetidine
amiodarone
fluoroquinolones
furafylline1
interferon
methoxsalen
mibefradil

thiotepa
ticlopidine

gemfibrozil[a]
trimethoprim
glitazones
montelukast
quercetin

Proton pump inihibtors:
lansoprazole
omeprazole2
pantoprazole
rabeprazole

Others
chloramphenicol
cimetidine
felbamate
fluoxetine
fluvoxamine
indomethacin
ketoconazole
modafinil
oxcarbazepine
probenicid
ticlopidine2
topiramate

fluconazole[a]
amiodarone
fenofibrate
fluvastatin
fluvoxamine
isoniazid
lovastatin
phenylbutazone
probenicid
sertraline
sulfamethoxazole
sulfaphenazole
teniposide
voriconazole
zafirlukast

bupropion[a]
fluoxetine[a]
paroxetine[a]
quinidine[a]

duloxetine
terbinafine

amiodarone
cimetidine
sertraline

celecoxib
chlorpheniramine
chlorpromazine
cinacalcet
citalopram
clemastine
clomipramine
cocaine
diphenhydramine
doxepin
doxorubicin
escitalopram
goldenseal
halofantrine
histamine H_1 receptor antagonists
hydroxyzine
levomepromazine
methadone
metoclopramide
mibefradil
midodrine
moclobemide
perphenazine
ranitidine
red-haloperidol
ritonavir
ticlopidine
tripelennamine

diethyl-dithiocarbamate
disulfiram

HIV antivirals:
indinavir[a]
nelfinavir[a]
ritonavir[a]

clarithromycin[a]
itraconazole[a]
ketoconazole[a]
nefazodone[a]
saquinavir
telithromycin

aprepitant
erythromycin
fluconazole
grapefruit juice
verapamil2
diltiazem

cimetidine

amiodarone
not azithromycin
chloramphenicol
delaviridine

diethyl-dithiocarbamate
fluvoxamine
gestodene
imatinib
mibefradil
mifepristone

(*Continued*)

Table 6 CYP Substrates, Inhibitors, and Inducers (*Continued*)

1A2	2B6	2C8	2C19	2C9	2D6	2E1	3A4,5,7
Inducers							
broccoli	phenobarbital	rifampin	carbamazepine	rifampin	dexamethasone	ethanol	**HIV antivirals:**
brussel sprouts	rifampin		norethindrone	secobarbital	rifampin	isoniazid	efavirenz
char-grilled meat			prednisone				nevirapine
insulin			rifampin				
methylcholanthrene			**NOT** pentobarbital				barbiturates
modafinil							carbamazepine
nafcillin							efavirenz
β-naphthoflavone							glucocorticoids
omeprazole							modafinil
tobacco							nevirapine
							oxcarbazepine
							phenobarbital2
							phenytoin2
							pioglitazone
							rifabutin
							rifampin1
							St. John's wort
							troglitazone1

[a]Denotes a ***strong inhibitor***—A compound that causes more than a fivefold increase in plasma auc or a decrease in clearance greater than 80%.
Source: From Ref. 88.

Table 7 CYP Phenotyping: Examples of CYP Probe Compounds

CYP isoform	Probe compound(s)
CYP1A2	Theophylline, caffeine
CYP2B6	Buproprion
CYP2C9	S-Warfarin, tolbutamide
CYP2C19	S-Mephenytoin, omeprazole
CYP2D6	Desipramine, debrisoquine, dextromethoraphan
CYP2E1	Chlorzoxazone
CYP3A4	Midazolam, busprione, felodipine, simvastatin, lovastatin

Other Phase 1 Enzyme Systems

It should be noted that some metabolic reactions are not exclusively catalyzed by CYP. For example, N-oxidations and S-oxidations are also catalyzed flavin monooxygenases, which are present in the liver and require NADPH to catalyze these reactions. Similarly, N-dealkylations and oxidations of phenols can be catalyzed by other hemeproteins including myeloperoxidase, eosinophil peroxidase, and prostaglandin H synthetase, although the mechanisms of catalysis are different from CYP-catalyzed reactions. In addition, metabolic products of hydrolytic esterase reactions are similar to oxidative ester cleavage although the underlying mechanisms of catalysis are quite different. In addition oxidation of azaheterocycles is catalyzed by molybdenum hydroxylases, aldehyde, and xanthine oxidases.

Phase II Enzymes

Phase II reactions are conjugation reactions. Conjugating agents are the byproduct of protein, carbohydrate, and fatty acid metabolism. A list of phase II pathways is provided in Table 8.

Compared to phase I metabolism, phase II reactions are faster, but they have a limited capacity. In other words, these are more readily saturable. This is the important factor involved in metabolism-based toxicity and carcinogenesis. Generation of toxic species (through phase I metabolism) may overwhelm phase II detoxification pathways, resulting in cell death. Reasons for limited phase II capacity include the following: limited amount of available enzyme (transferase), limited ability to synthesize active intermediate, and limited amount of conjugating agent. An example of the latter is glutathione depletion during acetaminophen overdose (described below). However, phase II conjugation is not necessarily a detoxification pathway. For example, acyl glucuronides can covalently bind to tissue proteins and result in toxicity.

Glucuronidation

Glucuronidation represents the major phase II conjugation pathway in drug metabolism reactions (96–98). Several functional groups are substrates for glucuronidation reactions, which include O-glucuronidation, N-glucuronidation, S-glucuronidation, and C-glucuronidation. O-glucuronidation occurs at several functional groups including alcohols (e.g., hexobarbital), phenols (e.g., estrone), carboxylic acids (e.g., α-ethylhexanoic acid, α-aminobenzoic acid), α− and β-unsaturated ketones (e.g., progesterone), and hydroxylamines (e.g., *N*-acetyl, *N*-phenylhydroxylamine). N-glucuronidation occurs on functional groups containing nitrogen such as carbamates (e.g., meprobamate), arylamines (e.g., 2-naphthylamine), aliphatic tertiary amines

Table 8 Phase II Reactions

Reaction	Conjugating agent	Reactive intermediate	Functional groups
Glucuronidation	Glucuronic acid	UDPGA[a]	−OH, $-NH_2$, −COOH, −SH
Acetylation	Acetyl CoA	Acetyl CoA	OH, $-NH_2$
Sulfate conjugation	Sulfate	PAPS[b]	OH, $-NH_2$
Mercaptopuric acid synthesis	Glutathione	Arene oxides epoxides	Arene oxides epoxides

[a] UDPGA, uridine diphosphate glucuronic acid.
[b] PAPS 3′-phosphoadenosine 5′ phosphosulfate.

(e.g., tripellennamine), and sulfonamides (e.g., sulfadimethoxine). S-glucuronidation occurs with compounds containing free sulfur group such as aryl thiols (e.g., thiophenol), diothiocarbamic acid (e.g., *N*,*N*-diethyldithiocarbamic acid), and finally C-glucuronidation which occurs very rarely and requires 1,3-dicarbonyl functional groups (e.g., phenylbutazone).

The glucuronidation reaction is catalyzed by glucuronosyl transferase isoforms and requires UDP-glucuronic acid as a cofactor. Similar to CYP isoforms, multiple glucuronosyltransferases are present with overlapping and distinct substrate specificities. Table 9 presents the known glucuronosyltransferases that catalyze glucuronidation reactions in rats and humans and their tissue distribution.

The disposition profile of glucuronide conjugates in vivo depends on molecular weight. In rats, compounds having molecular weight <250 appear to be predominantly excreted by kidney, whereas compounds having molecular weight >350 appear to be predominantly excreted via bile. Compounds having molecular weights between 250 and 350 appear to be excreted by either pathway (99). Excretion pathways are described later in this chapter.

Biological Activity and Toxicity of Glucuronides

Glucuronidation is considered to be a detoxication pathway where lipophilic drugs are converted hydrophilic conjugates and are excreted via bile or urine. However, sometimes glucuronides are also bioactive, either contribute to the pharmacological activity or toxicity. For example, morphine-6-O-glucuronide appears to be more potent than morphine itself in its biological activity.

Codeine, structurally related opioid to morphine also appears to produce a glucuronide that is active. In addition, steroids (e.g., testosterone, dihydrotestosterone, estradiol, 17α-ethinylestradiol) containing D-ring glucuronides seems to be cholestatic, whereas the corresponding A-ring glucuronides have the opposite effect (increased bile flow).

Ethinylestradiol, buprenorphine, and lorazepam have been shown to cause jaundice due to their ability to inhibit bilirubin glucuronidation. The glucuronidation pathway of these compounds as well as bilirubin appears to be primarily catalyzed by UGT1A1. Inhibition of bilirubin clearance via glucuronidation by these compounds appears to be responsible for the observed jaundice. Therefore, in toxicology studies, if bilirubin levels in plasma are increased, it should be established whether that drug in question is a substrate and/or inhibitor of UGT1A1.

Reactive acyl glucuronides have received considerable attention over the past decade due to their involvement in toxic adverse reactions. Acyl glucuronides of several drugs (e.g., zomepirac, diclofenac, gemfibrozil) are very reactive and form covalent adducts with proteins that appear to be responsible for the toxicities associated with these compounds. Zomepirac was withdrawn from the market due to high incidence of anaphylaxis following drug administration to humans, which has been related to the covalent binding of its acyl glucuronide (100). Gemfibrozil acyl glucuronide can react with DNA, suggesting that this glucuronide is also genotoxic (101). In addition to acyl glucuronides, N-O-glucuronides of hydroxamic acids such as *N*-hydroxy-2-acetylaminofluorine have also been shown to react with cellular nucleophiles (proteins and DNA), presumably through an arylnitrenium ion (102,103). These reactions have been suggested to play a role in the carcinogenesis of these hydroxamic acids.

Overall, glucuronidation reactions cannot be simply ignored as detoxification pathways and should be regarded as potential mechanisms of bioactivation for biological activity as well as toxicity of drugs. A careful assessment of these reactions should be made in early drug discovery and drug evaluation.

Acetylation

Apart from glucuronidation, acetylation has received attention due to its toxicological relevance. Acetylation is a detoxification pathway for compounds including aromatic amines. However, *N*-acetyl transferases (e.g., NAT1, NAT2) that catalyze this pathway are polymorphic in nature (104). NAT2 is particularly important due to complete deficiency in enzyme activity in a large segment of the population including 50% Caucasians, 10% Asians and 90% North Africans. Medications metabolized by acetylation include dapsone, procainamide, isoniazid, sulfamethazine, and hydralazine. Of these, procainamide, isoniazid, and hydralazine administration to humans produces systemic lupus erythematosus (SLE; an idiosyncratic immunodisease) due to

Table 9 UDP-Glucuronosyl Transferase Isoforms: Species Comparison (Rat vs. Human)

	Rat		Human	
UDPGT Isoform	**Substrate (Rat)**	**Tissue (Rat)**	**Substrate (Human)**	**Tissue (Human)**
UGT1A1	Bilirubin, estradiol, all-transretinoic acid, morphine	Liver	Bilirubin, estradiol, all-transretinoic acid, morphine	Liver, bile duct, stomach, colon
UGT1A2	Bilirubin	Liver	–	
UGT1A2P (pseudogene)	–	–	–	
UGT1A3	Not known	Liver	Low activity, ketoprofen, (R)-ibuprofen, (S)-ibuprofen, fenoprofen, naproxen, ciprofibrate, clofibrate, valproic acid, morphine	Liver, bile duct, stomach, colon, kidney, testes, prostate, small intestine
UGT1A4P (pseudogene)	–	–	–	–
UGT1A4	–	–	–	–
UGT1A5	Not known	Liver	(R)-Naproxen, (S)-Naproxen	Liver, kidney, skin, bile duct, colon
UGT1A6	(R)-Naproxen; (S)-Naproxen	Liver, kidney, duodenum, ovary, testes, epididymis, spleen, lung	(R)-Naproxen, (S)-Naproxen	Liver, kidney, skin, bile duct, colon
UGT1A7	Not known	Liver, duodenum, ovary, kidney, testes, spleen, lung	None known	Stomach, esophagus
UGT1A8	Not known	–	Androgens; morphine, ciprofibrate, clofibrate, valproate, furosemide, diflunisal, 17-EE, and all-transretinoic acid	Esophagus, colon, jejunum, ileum
UGT1A9P	–	–	–	–
UGT1A9	–	–	Fenoprofen, furosemide, ibuprofen, ketoprofen, monoethylhexyltha-late, naproxen, retinoic acid, mefenamic acid, 4-catechol estrogens, estradiol, estrol, 2-catechol estrogens	Liver, kidney, esophagus, colon, stomach, small intestine, testes, ovary, mammary gland, prostate, skin, skeletal muscle
UGT1A10	–	–	–	Bile duct, stomach, esophagus, colon, small intestine, jejunum, ileum

(*Continued*)

Table 9 UDP-Glucuronosyl Transferase Isoforms: Species Comparison (Rat vs. Human) (*Continued*)

	Rat		Human	
UDPGT Isoform	**Substrate (Rat)**	**Tissue (Rat)**	**Substrate (Human)**	**Tissue (Human)**
UGT1A11P (pseudogene)	–	–	–	–
UGT1A12P (pseudogene)	–	–	–	–
UGT2B1	NSAIDS, valproic acid, clofibric acid, bezafibrate, ciprofibrate, morphine, estradiol	Liver (low in kidney, lung, intestine, testes)	–	–
UGT2B2	–	Liver	–	–
UGT2B3	–	Liver (low in kidney, lung, intestine, testes)	–	–
UGT2B4	–	–	–	Liver
UGT2B6	–	Liver	–	–
UGT2B7	–	–	NSAIDS, clofibric acid, valproic acid, estriol, morphine, estradiol, all-trans-retinoic acid	Liver, kidney, esophagus, small intestine, brain
UGT2B8	–	Liver	–	–
UGT2B10	–	–	–	Esophagus, liver
UGT2B11	–	–	–	Liver
UGT2B12	–	–	–	Liver, kidney, testes
UGT2B15	–	–	Dihydrotestosterone	Liver, testes, prostate, esophagus
UGT2B17	–	–	C19 steroids (androsterone, dihydrotestos-terone, testosterone	Liver, kidney, uterus, placenta, mammary gland, adrenal gland, skin, testes, prostate

metabolic activation to reactive intermediates by myeloperoxidase or CYP enzymes. It appears that the susceptibility to SLE is dependent on the acetylator phenotype of human subjects, as slow acetylators rapidly develop the disease than faster acetylators (105).

It should also be noted that this NAT-catalyzed reaction generates more lipophilic metabolites (compared to parent drug). This is in contrast to the general role of metabolism in drug clearance (to increase hydrophilicity). For example, procainamide has a narrow therapeutic range and an active acetylated metabolite *N*-acetylprocainamide (NAPA) has a longer $t_{1/2}$ than procainamide. Therefore, both parent and metabolite concentrations of procainamide must be monitored clinically (106).

Glutathione Conjugation

This pathway is an important detoxification system in the body, responsible for conjugating highly reactive substances (epoxides, quinones, products of phase I). Glutathione (GSH, a tripeptide γ-glutamyl-cysteinyl-glycine) in cells is in millimolar concentrations and therefore can afford protection by scavenging the reactive electrophiles via conjugation (107). If a drug is given at very high doses (e.g., acetaminophen) and all the intracellular GSH is depleted, these

reactive electrophilic intermediates covalently bind to cellular macromolecules causing toxicity, death, and genotoxicity. Conjugation of reactive electrophiles with GSH generally occurs on free sulfhydryl group present on cysteine moiety. GSH conjugates are generally further processed to a *N*-acetyl-L-cysteine conjugate (mercapturic acid) which is excreted in the urine. Therefore, *N*-acetylcysteine, which also contains a free sulfhydryl group, is administered as an antidote in cases of acetaminophen overdose. This provides the cell with a replacement-conjugating agent (to glutathione), to prevent liver toxicity that results from NAPQI, a reactive metabolite of acetaminophen (generated through CYP2E1). Glutathione conjugation of reactive electrophilic intermediates may or may not require glutathione transferases. For example, quinones rapidly react with glutathione directly, whereas epoxides require a transferase to mediate these reactions. Glutathione transferases are also polymorphic enzymes and the biological significance in drug metabolism has not been explored, although they have been to shown to play an important role in environmental toxicology.

Phase III Metabolism: Role of Transporters

While the liver is widely recognized for role in drug metabolism, hepatic elimination involves a sequence of events involving drug uptake from the bloodstream, leading to intracellular metabolism and/or excretion. Hepatobiliary transport processes contribute to the disposition of a number of endogenous substances as well as xenobiotics. Hepatic xenobiotic disposition involves a number of different pathways including uptake into the hepatocyte, intracellular translocation, biotransformation, and egress into blood and/or bile (42,112–115). In terms of drug metabolism, the designation phase III refers to the role of membrane transporters on hepatobiliary disposition.

Figure 9 provides a schematic representation of transport proteins the mediate sinusoidal uptake of drugs into the hepatocyte. These transporters mediate bidirectional drug transport via a facilitative mechanism. The concentration gradient is created by the interplay of intracellular drug metabolism and drug efflux at the sinusoidal and canalicular membrane (42). Hepatic uptake of organic anions is mediated primarily by members of the organic anion transporting polypeptide (OATP) family (OATP1B1, OATP1B3, and OATP2B1). These transporters have a broad substrate specificity. Besides organic anions, type II cations (bulky compounds with one or two charged groups in or near ring structures II) and steroid molecules are taken up by the liver through OATP systems. OAT2 mediates sodium-independent transport of various anionic

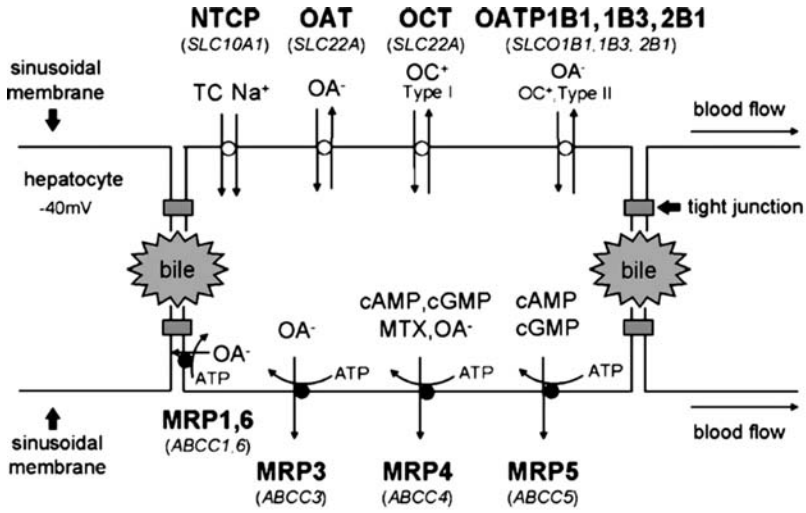

Figure 9 Drug transport across the sinusoidal membrane of the liver. Important basolateral transport proteins (protein name is in bold type with gene symbol listed below) are depicted with arrows denoting the direction of transport. These include OAT, OCT, OATP, and MRP transporters. Typical substrates are listed including organic anions (OA−), organic cations (OC+), methotrexate (MTX) cyclic), adenosine 3′,5′cyclic monophosphate (cAMP), and guanosine 3′,5′-cyclic monophosphate (cGMP) *Source*: From Ref. 42.

compounds including salicylate and methotrexate. OCT1 is involved in the bidirectional transport of small, type I organic cations such as tetraethylammonium and *N*-methylnicotinamide.

Members of the MRP family play prominent roles in hepatic excretion organic anions, including drugs and drug metabolites (43). MRPs are primarily involved in drug efflux from the hepatic cytosol to the bloodstream and include MRP1, MRP3, MRP4, MRP5, and MRP6. It appears that, in addition to drug excretion, hepatic MRPs are important when biliary transport is impaired or blocked. Although expression of MRP1 is normally low in the liver, protein expression is induced during liver regeneration and under conditions of experimentally induced cholestasis (by endotoxin administration or bile duct cannulation). MRP3 expression is induced by drugs such as phenobarbital. Additionally, MRP3 levels are increased in patients with genetic diseases caused by cases of MRP2 deficiency (e.g., Dubin–Johnson syndrome). Under these conditions, upregulation of MRPs by reduces bile acid levels in the hepatocyte by increasing efflux across the sinusoidal membrane into the blood.

Drug Transport Across the Hepatic Canalicular Membrane

Biliary excretion of drug and metabolites involves one of several ATP-dependent transport proteins expressed on the canalicular membrane (42). These proteins are members of the ABC family of transporters and they mediate unidirectional (hepatic cytosol → bile) transport of substrates uphill against a large concentration gradient. As illustrated in Figure 10, five transporters are known to participate in biliary excretion. Among the drug transporters that have been identified, MRP2 (mediates biliary excretion of a diverse number of substrates, including drugs. As noted above, Dubin–Johnson syndrome is a type of hereditary hyperbilirubinemia resulting from absence of canalicular MRP2. To compensate for this deficiency, basolateral MRP3 expression is upregulated.

Besides MRP2, the other important canalicular transporter in terms of biliary drug excretion is P-gp. This widely studied transporter plays a major role in the excretion of numerous endogenous and exogenous compounds by the liver. Drug substrates for P-gp include anticancer agents, antivirals, cardiac medications, and opioid analgesics (Table 3). Another ABC transporter that may play an important role in biliary excretion is BCRP.

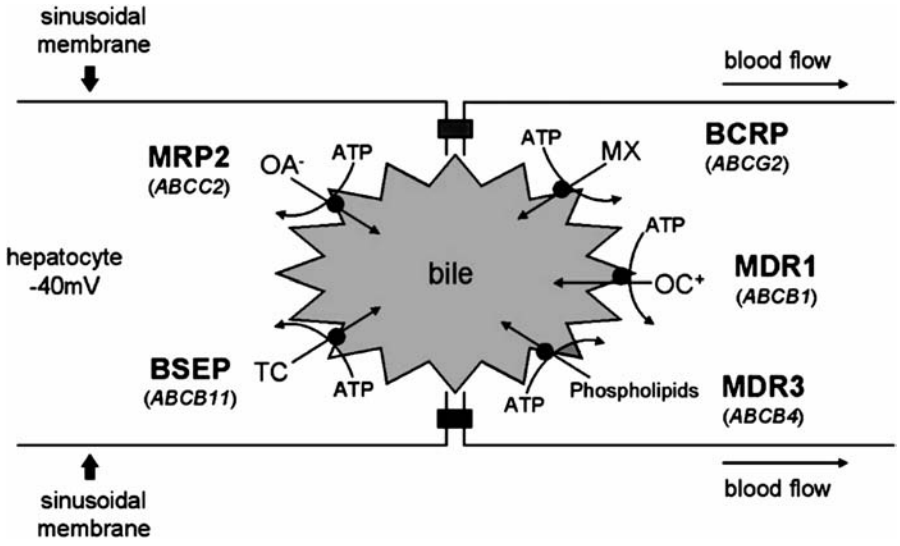

Figure 10 Human hepatic canalicular transport proteins. Important canalicular transport proteins are depicted with arrows denoting the direction of transport and ATP-dependent transporters designated by . Typical substrates are listed. (*Abbreviations*: OA-, organic anions; OC+, organic cations; TC, taurocholate; MX, mitoxantrone). *Source*: From Ref. 42.

Physiologic Factors Affecting Drug Metabolism

Clearance is defined as the volume of blood that is effectively removed of drug by that organ per unit time. Several models of hepatic clearance have been developed to explain and quantitatively predict the influence of several physiologic factors on drug clearance (113–116). These factors are liver blood flow (Q), protein binding, and intrinsic clearance (Cl_{INT}). Perhaps, the most widely adapted model is the venous equilibration model.

The venous equilibration model or "well-stirred" model (115) assumes an eliminating organ is a single well-stirred compartment through which the concentration of unbound drug in the exiting blood is in equilibrium with the unbound drug within the organ. The venous equilibration model describes clearance (Cl) as follows:

$$Cl = \frac{Q \times f_u \times Cl_{INT}}{Q + f_u \times Cl_{INT}} \tag{3}$$

Where f_U is the fraction of drug unbound in the blood. Cl_{INT} is defined as the ability of the liver organ to remove drug in the absence of flow and binding restrictions. In terms of drug metabolism, Cl_{INT} reflects the true metabolizing capability of the liver. In terms of the Michaelis–Menten equation (equation1), $Cl_{INT} = V_{max}/K_M$.

Extraction ratio is defined as the ratio of total drug clearance from an organ to the blood flow supplying that organ. Awareness of the extraction ratio of a drug and its classification as low ($E \leq 0.3$), intermediate ($0.3 < E < 0.7$), or high ($E \geq 0.7$) allows the prediction of the dependence of total organ clearance on the physiologic factors (Q, f_u, Cl_{INT}).

Extraction ratio can be classified as *restrictive* or *nonrestrictive*. This classification is based upon the dependence of drug clearance to binding by proteins in the blood. Generally, the clearance of a high extraction compound is nonrestrictive; that is, the eliminating organ is capable of extracting the entire amount of drug presented to it regardless of the degree of protein binding. In these cases, the clearance approaches a maximum value, the blood flow to the organ ($Cl \approx Q$). Hence, the elimination of a high extraction compound is sometimes referred to as *perfusion rate-limited*. Perfusion rate-limited clearance has been demonstrated for tissue plasminogen activator (*t*-PA) (117).

Conversely, the opposite is observed for a compound with a low extraction ratio. The ability of the eliminating organ to remove drug depends on plasma binding and intrinsic organ clearance ($Cl \approx f_U \times Cl_{INT}$). Such compounds are referred to as restrictively cleared and elimination is dependent upon the free fraction of the drug in the blood. Additionally, alterations in Cl_{INT} directly impact drug clearance for low extraction ratio medications. Alterations in intrinsic clearance are a source of drug–drug interactions, as discussed below.

Metabolic Drug Interactions

A major concern in drug development involves assessment of the potential for drug interactions. In general, many reported drug interactions are not of clinical significance (118). Factors to consider when evaluating the likelihood of a clinically important drug interaction include the therapeutic index of the affected drug, the likelihood for coadministration of the drug and interacting agent in patients, and the effect of the interaction on the clearance of the drug (and therefore plasma levels). While drug interactions have been identified for numerous ADME processes, interactions that result in changes in drug clearance are the most important. In terms of hepatic metabolism, drug interactions are of two general categories: enzyme inhibition and enzyme induction. Enzyme induction and inhibition result in changes in Cl_{INT}.

Enzyme Inhibition

Enzyme inhibition is a fairly rapid process; that is, drug metabolism is affected quickly upon systemic exposure to an interacting drug. Metabolic inhibition can lead to drug toxicity due to elevated plasma levels secondary to reduced clearance. There are several mechanisms of enzyme inhibition: competitive inhibition, noncompetitive inhibition, and irreversible inhibition. Detailed information regarding mechanisms of enzyme inhibition (and methods to study enzyme inhibition) can be found in the literature (118,119)

With regard to CYP metabolism, the list of inactivators for CYP isozymes is fairly well established. Examples of CYP inhibition for therapeutic use include CYP19, an enzyme responsible for estrogen production (120). A possible therapeutic target of estrogen-dependent tumors is irreversible inactivation of this enzyme. Also, the mechanism of action of ketoconazole is inhibition of CYP51, an enzyme involved in lanosterol 14-demethylation and the pathogenesis of fungal infections). For cancer prevention, inhibition of CYP1A1 may be important since the induction of this system may be a risk factor for carcinogenesis (121).

CYP3A4 is the major form of P450 expressed in normal adult human liver and accounts for approximately 30% to 50% of the total P450 content in the human liver microsomes and in intestinal gut wall enterocytes, respectively. This enzyme is also the major isoform involved in drug metabolism, accounting for metabolism of more than 50% of the known drugs on the market. Therefore, CYP3A4-related drug interactions are a major concern during drug development. For example, the calcium channel blocker Mibefradil (Posicor®) was withdrawn from the market because of its potential to inhibit CYP3A4, thus resulting in metabolism based drug interactions (122). Terfenadine (Seldane®), an H_1-receptor antagonist and CYP3A4 substrate, was also withdrawn from market because its metabolism was inhibited by several CYP3A4 inhibitors, resulting in fatal cardiac arrhythmias (118,123).

Overall, successful prediction of clinical drug interactions may be obtained using therapeutically relevant concentrations of the substrate and the inhibitor (119). The use of very high concentrations of drug or inhibitor may produce drug interaction in vitro, which is not observed in vivo.

Enzyme Induction

Induction is an increase in enzyme activity associated with an increased intracellular enzyme concentration. The effect is generally dose-dependent and the duration of exposure to inducing agent can vary from two days up to two weeks (124,125). Also, the inducing effect takes time to dissipate once the inducing agent is removed. Enzyme inducers are not only medications but can also be obtained through diet (e.g., alcohol) and the environment (e.g., smoking). The result of induction is an increased metabolism and therefore increased clearance. It is important to note that induction is only one factor that contributes to intersubject variability in metabolism. One report suggests that intersubject CYP3A4 capability varies 15- to 100-fold (126).

Many CYP isozymes (e.g., CYP1A1/1A2, CYP2B6, CYP2E1, CYP2C9, CYP2C19, CYP3A4) are inducible and as such enzyme induction may contribute to changes in drug clearance and drug toxicity. Interestingly, CYP2D6 is not an inducible enzyme.

CYP enzyme induction may occur by several mechanisms including increased gene transcription, mRNA, and/or protein stabilization (127). CYP1A inducers act by binding to a cytosolic aryl hydrocarbon receptor (AhR). This receptor drug complex then undergoes heterodimerization with Ah nuclear translocator (Arnt) protein in the nucleus. This Arnt-AhR-drug complex binds to the enhancer region in xenobiotic responsive element (XRE) and acts as a transcription factor thus inducing the transcription of CYP1A gene. Similarly CYP3A inducers act by binding to a nuclear pregnane X receptor (PXR), which forms a heterodimer with retinoic acid receptor (RXR). This PXR/RXR complex is activated by CYP3A inducers and binds to the promoter region of CYP3A gene resulting in increased transcription of CYP3A gene. In some cases, CYP enzyme induction may not necessarily be mediated by enhanced gene transcription. For example, CYP2E1 enzyme induction occurs by mRNA or protein stabilization as it occurs during alcohol consumption or during starvation, respectively.

Whether or not induction (or inhibition) is important and clinically depends on a number of factors (discussed previously). Since many medications are metabolism via several pathways that are under control of different enzymes, inhibition of one pathway will result in compensation by other pathways. On the other hand, induction of a potentially toxic pathway is problematic as seen with the effect of alcohol on acetaminophen toxicity (128).

CYP1A2 induction by omeprazole (a proton pump inhibitor) has been associated with severe side effects such as complicated vision disturbances (129,130). Similarly, CYP3A4 induction by troglitazone (an insulin sensitizer) has been associated with hepatic dysfunction and hepatic failure in humans (131,132). Induction of CYP3A4 may also result in drug interactions resulting in loss of efficacy of another drug by inducing drug. For example, rifampin,

an antituberculosis drug is a potent inducer of CYP3A4 in humans and is known to decrease the bioavailability and efficacy of several drugs (reviewed in reference 125). These include analgesics, antidiabetics, antiepileptics, psychotropics, antimicrobials, antifungals, cardiovascular, anticoagulants, hormones, and immunosuppresants. Rifampin coadministration with HIV-protease inhibitors (nelfinavir, indinavir, saquinavir) is contraindicated. Rifampin also decreases the efficacy of oral contraceptives and can result in acute transplant rejection in patients treated with immunosuppressive drugs (e.g., cyclosporin). Similarly, several other CYP3A4 inducing antiepileptic agents increase the CYP3A4 metabolism of oral contraceptives (estrogens and corticosteroids) and decrease their contraceptive potency (133). Therefore, determining the induction potential of the candidate drug early in drug development is crucial for predicting the drug-related toxicities and drug interactions.

MECHANISMS OF SMALL MOLECULE EXCRETION

Renal Excretion

The kidney is the primary organ responsible for the excretion of medications and their biotransformation products from the body. Detailed reviews of renal drug excretion mechanisms are available in the literature (134–136). The major processes involved in the renal elimination of drugs are glomerular filtration, active tubular secretion, and passive reabsorption (Fig. 11). The combined effect of the first two processes is the extraction of drug from the blood into the urine. The last process, reabsorption, involves the movement of drug back into the blood from the primitive urine. Thus, the renal excretion rate of a compound is the net result of these individual mechanisms.

Glomerular Filtration

Urine formation begins with glomerular filtration. The glomerular filtrate normally contains no cells, is essentially protein-free, and contains most inorganic ions and low-molecular weight organic solutes (e.g., glucose and amino acids) in virtually the same concentrations as in the plasma. The quantity of drug that is filtered by the kidney parallels the concentration of unbound drug in the plasma. Overall, the rate of filtration is the product of unbound plasma concentration and glomerular filtration rate (GFR) (138).

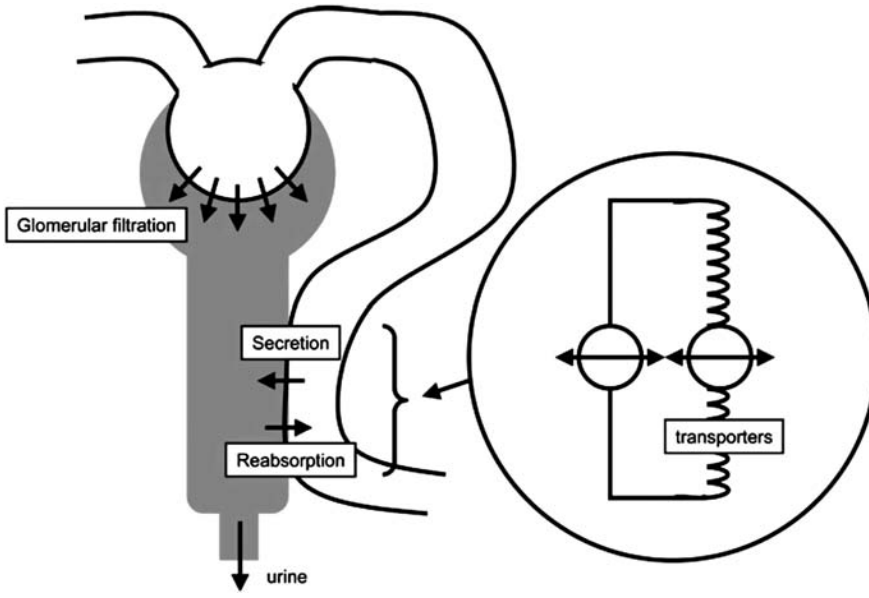

Figure 11 Schematic depiction of a nephron identifying mechanisms of drug excretion. Renal excretion involves glomerular filtration and secretion at the proximal tubules. Drug is returned to the systemic circulation via drug reabsorption. *Source*: From Ref. 137.

Tubular Secretion

Once the plasma is filtered and ultrafiltrate enters the nephron, several forces operate to alter the concentrations of assorted substances in that fluid and ultimately, the mass of each that is excreted. Although most compounds that are renally eliminated undergo glomerular filtration, the extraction of compound via this mechanism is relatively low, particularly if the compound is highly protein bound. A second mechanism by which drug is extracted from the blood into the urine is tubular secretion; that is, the compound is transported from the blood across the kidney tubule cell into the urine.

Tubular secretion is an active, carrier-mediated process that occurs in the proximal tubule. Membrane-bound transporters are responsible for the translocation of xenobiotics across the basolateral and luminal membranes of the kidney cell (38,39,139–144). Consequently, active tubular transport contributes to the cellular accumulation and urinary excretion of medications. Additionally, these transporters are potential sites for significant drug–drug interactions in vivo.

Analogous to metabolic clearance, the venous equilibration model is also applied to renal drug excretion. Cl_{INT} is defined as the clearance of a drug in the absence of flow and binding restrictions (138). For compounds of low renal extraction (renal clearance is small relative to kidney plasma flow), clearance is considered restrictive (protein binding-dependent). Conversely, the renal clearance of high extraction compounds is nonrestrictive (flow-dependent). Such compounds (e.g., para-aminohippuric acid) are excellent substrates for the secretory transport system and are capable of being almost completely extracted from the blood regardless of the degree of protein binding. For this reason, the renal clearance of para-aminohippuric is used as a marker for renal plasma flow.

Proximal tubular secretion is inferred when the rate of excretion of a particular compound exceeds the rate of filtration (138). Since it is carrier-mediated, proximal tubular secretion is a saturable process and accordingly, the kinetics of the secretion can be described by Michaelis–Menten (134).

Tubular Reabsorption

While filtration and secretion systems in the kidney serve to eliminate drug from the blood into the urine, tubular reabsorption serves to counteract excretion from the blood. Active reabsorption occurs for many endogenous compounds (i.e., glucose, electrolytes). The identification of transport systems in the luminal membrane of the kidney cell (e.g., peptide transporters, nucleoside transporters) suggests a role of active transport in drug reabsorption by the kidney. However, the predominant mechanism of drugs reabsorption is passive transport.

The primary driving force for passive reabsorption is the tubular reabsorption of water, which serves to concentrate the drug in urine with respect to plasma (138). It is the establishment of this electrochemical gradient that allows for back diffusion of drug molecules from primitive urine to blood. The degree of reabsorption is dependent upon physicochemical properties of the drug and physiologic variables. The physicochemical properties include polarity, state of ionization, and molecular weight. Small, nonionized, lipophilic molecules tend to be extensively reabsorbed. Physiologic variables that affect reabsorption include urine pH and urine flow rate. Generally, increasing the urine flow rate tends to decrease the concentration gradient, contact time, and subsequently, the extent of reabsorption. Additionally, if the drug is weakly acidic or basic, perturbations in urine pH will influence reabsorption.

When total renal clearance is less than the clearance due to filtrationreabsorption must be occurring. In general, however, it is difficult to accurately quantify the reabsorption of a compound. Equations have been proposed to estimate reabsorptive clearance (135,145,146), but are rarely accurate in predicting observed clearances (147). A particularly useful renal clearance parameter is excretion ratio (XR). XR is simply the renal clearance corrected for filtration clearance:

$$XR = \frac{Cl_{renal}}{f_U \times GFR} \tag{4}$$

Table 10 Substrates for Organic Anion Renal Tubular Transporters[a]

Therapeutic class	Examples
Benzoates	
Carbonic anhydrase inhibitors	Acetazolamide, methazolamide
Cephalosporins	Cephalexin, ceftriaxone
Diuretics	Chlorothiazide, furosemide
HIV inhibitors	Zidovudine, adefovir, cedofovir
NSAIDS	Salicylic acid, indomethacin
Penicillins	flurbiprofen
Prostaglandins	Penicillin G
Sulfonamides	
Miscellaneous	Sulfisoxazole
	Probenecid
	Para-aminohippuric acid
	Methotrexate
	Bile acids
	Saccharin

[a] *Source*: From Refs. 141–143.

Where Cl_{renal} is the overall renal clearance of the medication and f_u represents the fraction of drug unbound in the plasma (determined experimentally). An XR value greater than one is indicative of a net secretory process. Conversely, an XR less than one is reflective of a net reabsorptive process. Therefore, an estimation of XR provides a general indication of the mechanism of elimination for the compound of interest.

Membrane Transporters Involved in Renal Excretion

As noted above, membrane transporters play a fundamental role in renal secretion and reabsorption. The proximal tubule of the kidney contains organic anion transport systems that secrete a wide array of exogenous compounds including many drugs (Table 10). Tubular transport of organic anions proceeds against an electrochemical gradient at the basolateral membrane, with facilitated transport across the luminal membrane into the urine (down an electrochemical gradient). As illustrated in Figure 12, the weak acid transport system (OAT1) is a tertiary active system (139). Organic anion basolateral uptake involves countertransport with α-ketoglutarate (α-KG). Outflow of α-KG occurs along two pathways: a sodium-dicarboxylate transporter and intracellular metabolism.

While OAT1 is the principle anion basolateral transporter in the kidney, other members of the OAT family may also be involved, including OAT2 and OAT3. Additionally, efflux of organic anions across the basolateral membrane has been proposed. This efflux system has been linked to members of the MRP transport family (MRP 3,5,6).

Information is emerging regarding the translocation of organic anions across the luminal membrane into the urine (Fig. 12). Transport proceeds through a facilitated mechanism down an electrochemical gradient. A number of transport pathways have been proposed for the luminal exit of acidic compounds, although the relative contribution of these mechanisms is species-dependent. Both OATP1 and OATP3 are expressed in the brush border membrane of the kidney. Additionally, OAT-K1 and OAT-K2 are kidney-specific transporters, structurally similar to OATP1. These transporters are sodium- and ATP-independent. While it is assumed that these are efflux transport systems (lumen → urine), they may also be involved in luminal reabsorption.

Organic cations are transported by the proximal tubule via a multistep process, as depicted in Figure 13. Consistent with other organ systems, the kidney efficiently secretes a wide range of positively charged medications and their metabolites. Uptake from the blood into the tubular cell proceeds by facilitated diffusion, the driving force being the electrochemical gradient across the basolateral membrane (inside negative potential difference). At least two distinct organic cation transporters have been identified on the basolateral membrane, OCT1, and OCT2 (37,148,149).

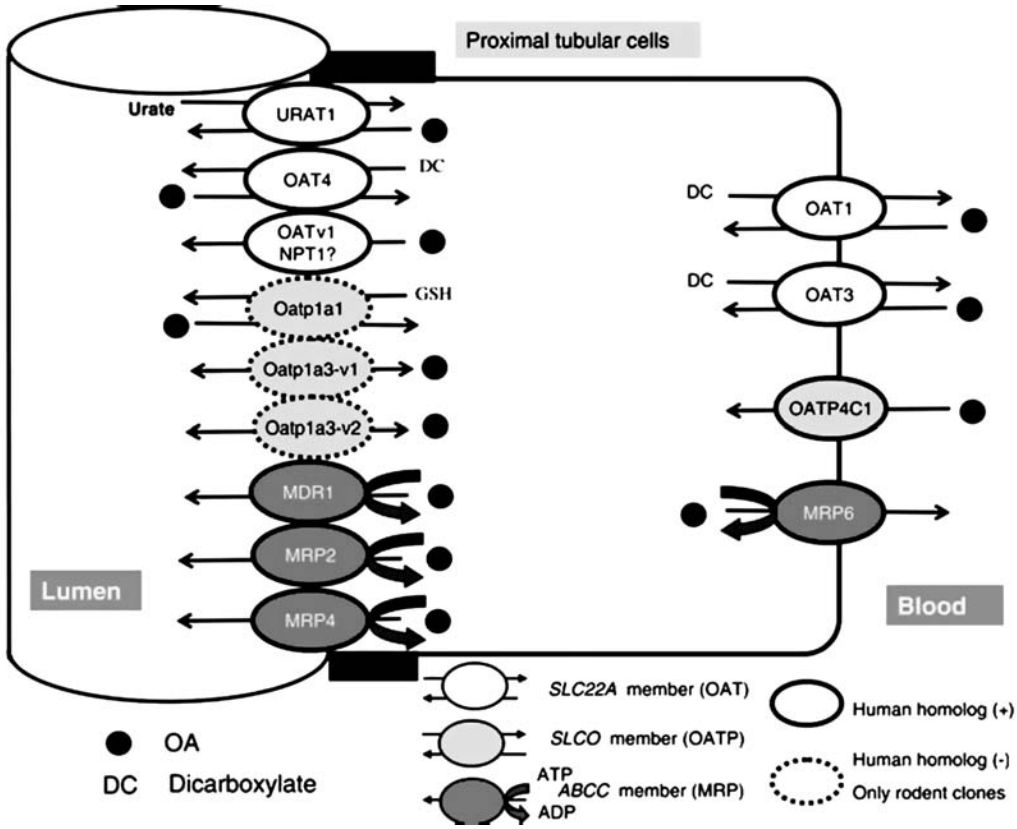

Figure 12 Organic anion (OA) transporters in proximal tubular cells. In the basolateral membrane, OAT1 and OAT3 mediate uptake of a wide range of relatively small and hydrophilic OAs from plasma. OATP4C1 is shown to transport digoxin. In the apical membrane, many OA transporters are identified. The role of URAT1 as an efflux transporter for various OAs into tubular lumen is suggested. In regard to the OATP members, large species differences are noted and their contribution to transepithelial transport of OA is still unclear. Oatp1a3v1 and Oatp1a3v2 could participate in tubular reabsorption and/or secretion of relatively hydrophobic anions such as bile acids, methotrexate, and PGE2. MRP2 and MRP4 extrude type II OAs from the cell into tubular lumen. MRP4 is shown to mediate the transport of PAH. OATv1 and its putative human ortholog NPT1 belong to the distinct transporter family (SLC17A). OATv1 would function as a voltage-driven OA transporter, which mediates efflux of OAs. Transporters whose human ortholog is not identified are depicted by dotted lines. *Source*: From Ref. 44.

Luminal transport of cationic drugs across the brush border membrane into the urine mediated by proton: cation exchange proteins including OCTN1, MATE1, and MATE2-K (150). A Na^+-H^+ exchanger generates the proton gradient (intracellular > extracellular proton concentrations), with intracellular Na^+ levels maintained through Na^+-K^+-ATPase. Another transport system involved in efflux of organic cations is the MDR1/P-gp. Expressed on the brush border membrane the proximal tubule cell, P-gp mediates efflux of a broad spectrum of cationic and hydrophobic drugs via an ATP-dependent mechanism.

The kidney also contains the peptide transporters PEPT2. In contrast to the low affinity, high capacity, PEPT1, PEPT2 is a high affinity, low capacity transporter. Renal disposition of β-lactam antibiotics and ACE inhibitors involves PEPT2-mediated transport (151).

Nucleosides and nucleoside analogs are used to treat HIV infection as well as certain types of cancers. Many of these drugs are readily excreted by the kidney. Nucleoside transporters are thought to play a key role in the renal disposition of nucleosides. There are two types of nucleoside transport processes: (1) concentrative nucleoside transporters (CNT1–CNT3) and (2) equilibrative nucleoside transporters (ENT1–ENT2). CNTs are primarily localized to the brush border membrane of renal epithelial cells and mediate active reabsorption of substrates into the cell by a sodium electrochemical gradient (Na^+-dependent secondary active transporters). ENTs

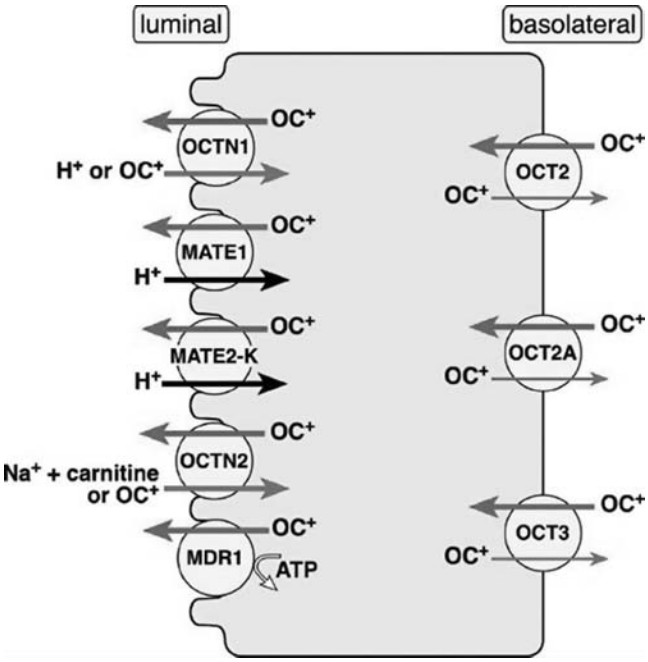

Figure 13 Organic cation transporters in plasma membranes of human renal proximal tubules. For explanation see Figure 2. MATE1 and MATE2-K are secondary active proton–cation antiporters. OCT2A is a splice variant of OCT2. The basolateral localization of OCT3 was observed in unpublished experiments. *Source*: From Ref. 37.

are primarily located on the basolateral membrane, and function bidirectionally by facilitated diffusion (downhill flux of nucleosides), driven by substrate gradients. Accumulating evidence, however, suggests that ENTs may also be expressed at the brush border membrane and are therefore involved in both secretion and reabsorption of nucleosides (39).

Biliary Excretion and Enterohepatic Recycling

Although widely recognized as the major site of drug biotransformation, one of the main functions of the liver is the formation of bile. As described previously, hepatic xenobiotic disposition involves a number of different pathways including uptake into the hepatocyte, intracellular translocation, biotransformation and egress into blood and/or bile.

Biliary excretion contributes to the disposition of a number of endogenous substances as well as medications. Biliary excretion depends on a number of factors including chemical structure and molecular weight. In humans, the molecular weight threshold for biliary excretion is 600 (152). For compounds with molecular weights <600, the primary route of excretion from the body is renal elimination. In rodents, the molecular weight cutoff is 350 for biliary excretion as described earlier.

Enterohepatic recycling involves a sequence of events beginning with drug removal from the circulation by the liver and secretion into bile (in some cases after metabolism). Drug (or metabolite) is transported via the bile into the duodenum, where it is subsequently available for reabsorption back into the circulation. In some cases, reabsorption follows intestinal deconjugation by intestinal bacteria. Accordingly, enterohepatic recycling depends on a number of factors that are associated with each of these processes (absorption, metabolism, excretion). The consequences of enterohepatic recycling include a prolonged residence of drug in the body (i.e., longer $t_{1/2}$) and the possibility of multiple peaks (in the plasma concentration–time profile) following oral administration.

An excellent review of the topic of enterohepatic recycling was recently published (152). Among the medications known to undergo enterohepatic recycling include pain medications (e.g., morphine, NSAIDS), antibiotics, and hormones. Additionally, enterohepatic recycling of a medication is affected by disease, genetics, and coadministration of other compounds.

Excretion into Breast Milk

Although medication use among lactating women generally poses limited risk to a breast-fed infant, excretion of medication into breast milk has potential clinical and toxicological implications (153). There are a number of experimental methods (both in vitro and in vivo) and models to assess drug excretion into breast milk and for determining the potential risk to a nursing infant (154).

Traditionally, drug excretion into breast has been traditionally thought of a passive process, and physicochemical properties of the drug and regional differences in pH (breast milk is more acidic compared to plasma) dictate the concentration of drug in milk. However, recent evidence suggests that membrane transporters in the mammary gland are also involved in drug transfer into breast milk (155). The relative contribution of these carrier-mediated transport systems remains to be elucidated.

SMALL MOLECULE ADME: ISSUES FOR DRUG DEVELOPMENT

Pharmacogenetics

Pharmacogenomics is the study of the relationship between genetic variations and individual differences in drug response (156,157). Today, identification of genetic differences in drug metabolism among different ethnic groups plays a pivotal role in clinical studies to understand the variability in PK variability in humans. The impact of pharmacogenetics on drug metabolism is illustrated by the established polymorphisms of the CYP family and *N*-acetyltransferase. Likewise, polymorphism in membrane transport proteins is expected. While information is building on the pharmacogenomics of drug transporters in general, the effect of polymorphism on P-gp expression and function is the subject of considerable interest.

The pharmacogenetics of MDR1 and its impact on pharmacokinetics and pharmacodynamics has the subject of recent reviews in the literature (158,159). A single nucleotide polymorphism, C3435T, has been associated with a twofold increase in duodenal expression of P-gp, resulting in reduced systemic exposure to orally administered digoxin (160,161). C3435T polymorphism has also been associated with antiretroviral treatment in HIV-infected patients, as some HIV medications (e.g., protease inhibitors) are P-gp substrates. A subgroup of patients with reduced P-gp expression (TT genotype) showed a more favorable response to treatment, presumably due to increase drug penetration into infected cells (162). MDR1 polymorphisms have been reported to be a risk factor for diseases such as inflammatory bowel disease, Parkinson's disease, and renal carcinoma (163).

Information concerning the pharmacogenetics of membrane transporters in fundamental to drug therapy; that is, achieving adequate drug concentrations at the target site with the goal of maximizing therapeutic effectiveness while minimizing toxicity (157). In the not so distant future, drug selection and dosing will be predicated on the genetic profile of the patient. Certainly, understanding the pharmacogenomics of membrane transporters will impact pharmaceutical care in the coming years. In terms of preclinical drug development, extracting the clinically relevant information from available pharmacogenetic data will be a major challenge.

The National Institutes of Health has established a Pharmacogenetics Research Network (PGRN). The PGRN is a collaborative team of multidisciplinary research groups attempting to correlate drug-response phenotypes with genetic variation (164). A major component of the PGRN focuses on specific groups of proteins, membrane transporters, and drug-metabolizing enzymes, which have critical roles in clinical pharmacokinetics. As drug development moves toward personalized medicine, there is a need for solid scientific evidence and that will guide clinicians on how to modify dosages or drug therapies based on the results of pharmacogenetic tests. To the end, the PGRN has established a single, publicly accessible knowledge database, pharmacogenomics and pharmacogenetics knowledge base (PharmGKB).

Species Differences in Drug Disposition

Species Differences in Drug Metabolism

Species variation in drug metabolism is well-established and has led to the identification and characterization of the enzymes involved and their differences in catalytic activities across several animal species (and strains) including man.

With regard to the drug metabolism, species differences have been shown for CYP activity. For example, metabolite profiles of caffeine are quite different in rat, rabbit, monkey, and human, which perhaps reflect species variations in CYP1A2, the primary isoform responsible for caffeine N-demethylation. Coumarin, a CYP2A substrate, is metabolized in rat to 3,4-epoxide that subsequently undergoes ring opening to o-phenylhydroxyacetaldehyde as the reactive toxic species. However, in humans coumarin is primarily metabolized by CYP2A6 to 7-hydroxycoumarin, which is considered to be a detoxification pathway (165). In addition, rat CYP2A1 and CYP2A2 catalyze 7α or 15α hydroxylation of testosterone, but human CYP2A6 does not catalyze these reactions (166). CYP2B isoforms in rat and dog hydroxylate androgens at 16α and 16β positions, whereas guinea pig and monkey CYP2B isoforms catalyze hydroxylation exclusively at 16β position and the rabbit exclusively at 16α position (167,168).

The CYP2C subfamily also shows marked variation in regio- and stereoselectivity of steroid metabolism between different species and also exhibits differences between male and female, which can be ascribed to gender-specific isoforms (169). Species differences in stereoselective 4′-hydroxylation of S- and R-mephenytoin has been demonstrated noted in studies using liver microsomes of mice, rats, dogs, rabbits, monkeys, and humans (170). Humans (CYP2C9) and monkeys preferentially catalyzed S-mephenytoin 4′-hydroxylation (169). Conversely, rats (CYP2C11), rabbits, and dogs catalyzed R-mephenytoin 4′-hydroxylation at rates two- to six-fold higher rates than S-mephenytoin 4′-hydroxylation. However, hydroxylation of both isomers occurred at similar rates in mice. Bogaards et al (170) concluded that none of the CYP activities in the animal species are similar with respect to humans, however, for all the CYP activities but in each species some of the CYP activities can be considered similar to man.

Species differences have also been noted with phase II conjugation pathways. For example, phenol is metabolized by conjugation to glucuronide and/or sulfate, and the relative proportion of these two metabolites depends on the species studied (83). Hepatic metabolism can reduce the bioavailability of drugs even when 100% of the drug is absorbed through GI tract. In such cases, the bioavailability of drugs may be estimated as a total of drug and its metabolites. However, considerable species differences exist in substrate specificities of CYP isoforms. For example, dog liver microsomes catalyzed coumarin 7-hydroxylase and testosterone 6β-hydroxylase activities at rates several fold higher than to human liver microsomes (171). Therefore, such species differences in drug metabolism complicate the prediction of pharmacokinetics in humans from preclinical animal studies. In addition, hepatic drug metabolism by CYP enzymes is very complex as these enzymes exhibit non–Michaelis–Menten kinetic behaviors due to very complicated biochemistry (169) Efforts to predict in vivo hepatic drug clearance and drug–drug interactions from in vitro methods were largely unsuccessful primarily due to these issues.

Species Differences in Membrane Transport

Membrane transporters play a critical role in drug disposition. Mechanisms of ADME are mediated by organ uptake of xenobiotics. Humans and other animal rodent membrane transporters are not orthologous; that is, amino acid sequences of transport proteins are different among between species. Species differences in transporter expression and orthology are an important issue for preclinical drug development. Future research will undoubtedly determine differences in transporter activity (and substrates) among species. Extrapolation of whole animal studies to predict clinical outcomes will depend on the extent of overlap in transporter expression and activity between species (172).

Other Factors Affecting Drug Disposition

Gender

Gender effects on drug disposition are another emerging issue for drug development. The influence of gender on pharmacokinetics and drug activity is well established and has been the subject of recent reviews (173,174). Differences in pharmacokinetics between males and females are the result of biological differences between genders. These differences include body weight, body composition, and hormonal. Additionally, it appears that gender-related differences in drug metabolism and membrane transporters are an underlying cause of these differences (175).

While gender-specific variations in drug response have been demonstrated for several medications, there has been paucity of research in this area. There is an increasing need for clinical studies that emphasize the role of gender on pharmacokinetics and pharmacodynamics. Although there is an emerging body of literature evaluating gender effects on drug disposition, differences in disposition between males and females are not well defined. Elucidation of gender differences in pharmacokinetics is complicated by underlying patient factors including genetics, age, and disease. Consequently, there is a need for better understanding of the role of gender on the disposition and activity of xenobiotics.

Disease

As noted above, the presence of underlying disease can alter the PK profile of a medication (176–178). Of particular importance are diseases that affect kidney and liver function, the two organs responsible for drug clearance. Additionally, alterations in cardiac output and levels of circulating proteins can also affect drug disposition through alterations in organ blood flow and plasma binding, respectively.

The discovery that several endogenous cytokines such as interferons, interleukins decrease CYP enzyme levels and activities suggested that disease state could have profound influence on drug metabolism (179–181). Indeed, bacterial infections have been shown to result in impaired drug clearance in humans (182). Fourteen subjects with acute pneumonia of diverse etiology all had decreased antipyrine clearance during infection. Human volunteers given bacterial LPS showed reduced clearance of antipyrine, hexobarbital, and theophylline that correlated with the initial peak values of tumor necrosis factor and interleukin-6. The involvement of cytokines in altering drug metabolism was later directly demonstrated in studies where humans were given recombinant human IFNα that reduced the clearance of antipyrine, and erythromycin (183,184). These studies indicated that the disease states that alter the endogenous cytokine levels could alter drug metabolism perhaps having an effect on CYP enzymes and their expression.

In terms of renal excretion, changes in kidney function are often assessed through GFR or its clinical marker, serum creatinine. The whole nephron hypothesis assumes that reductions in renal filtration (reflected in GFR) are accompanied by parallel diminutions in secretory and reabsorptive capacity of the nephron (185). Consequently, for medications eliminated primarily via renal mechanisms, dosage adjustments in patients with renal dysfunction are frequently based on GFR (186).

A recent clinical study demonstrated that in patients with renal diseases, expression of organic anion membrane transporters (OATs) correlated with reduced drug secretion (187). It is likely that future studies will further define the impact of disease-induced alterations in transporter expression of drug disposition.

Aging

The biological and physiological consequences of aging can dramatically affect the PK profile of a medication (188). This can have serious implications for pharmacotherapy in the elderly. For example, increased gastric pH, decreased GI motility, and reduced intestinal blood flow can affect the rate and extent of drug absorption following oral administration. The shift in body composition (increased body fat, reduced total body water) with age can affect V_D and $t_{1/2}$ of both lipophilic (↑ distribution) and hydrophilic (↓ distribution) compounds. Likewise, alterations in cardiac output, organ mass, and organ function collectively reduce the ability of aging individuals to clear medications. The pharmacokinetics of a drug is also subject to changes in pediatric subjects, where drug metabolism enzyme expression is considerably different from adult humans (189,190). In recent years, considerable research is devoted in understanding the pharmacogenomics of drug metabolizing enzymes in children also (191). Several other factors related to absorption, distribution, and excretion could also contribute to PK differences in children (51).

The expression of CYP enzymes changes markedly during development. CYP3A7 is the predominant CYP isoform expressed in fetal liver. CYP3A7 peaks shortly after birth, and rapidly declines to undetectable levels, being replaced primarily with CYP3A4. Distinct isoform-specific developmental expression of CYPs has been noted postnatally in the following order of appearance: CYP2E1, CYP2D6, CYP3A4, CYP2C9/19, and CYP1A2 (191). CYP2E1 expression surges

within hours after birth, CYP3A4 and CYP2C appear during the first week of life, and CYP1A2 appears at one to three months of life.

CONCLUSIONS

Pharmacokinetics, the mathematical characterization of drug disposition, is often referred to by the acronym ADME, which signifies the four key aspects of the body's handling of xenobiotics: **A**bsorption, **D**istribution, **M**etabolism, and **E**xcretion. The goal of this chapter was to summarize the major mechanisms involved in drug disposition of small molecules. Since poor PK properties often lead to early development failures, identifying PK properties of a new chemical entity are important factors in the lead optimization process routinely employed by the pharmaceutical industry. The widespread availability of high-throughput screening tools for assessing factors such as enzyme and membrane transporter affinity, intestinal permeability, and protein binding allows scientists to rapidly evaluate their role on drug disposition in vivo. Having this information early on in the development process will positively impact drug candidate selection and allow for development of safer and more efficacious drug therapies.

Looking ahead, advances in molecular biology and genetic engineering will lead to further discoveries of how drugs are metabolized and transported by body organs and tissues such as the kidney and liver. Through advances in protein science, cellular uptake and efflux processes are being characterized at a molecular level. Following in the wake of the our advanced understanding of drug metabolism, the complexity of hepatic drug transport mechanisms has been brought to light over recent years, and our comprehension of these processes continues to evolve. The same can be said for other organ systems such as the kidney, intestinal tract, and CNS, and we have only scratched the surface in terms of our knowledge in this area. This will undoubtedly involve characterization of the substrate-binding site of transport proteins and the mechanisms involved in transporter induction and inhibition. Furthermore, the role of genetic polymorphisms on individual transport systems will become better defined.

In the not too distant future, drug and dosage selection will be individualized based on a patient's genetic profile, which will be readily accessible by a physician at the point of care. This will not only involve selecting the appropriate therapy based on a patient's unique genome related to disease progression, but optimizing to dosing regimen based on the patient's genetic profile with regard to pharmacokinetics. Under this scenario of "personalized medicine," the risks to the patient in terms of ineffective therapy or likelihood for adverse effects will be minimized. In this regard, unraveling the mechanisms of drug disposition is an important endeavor and progress in this area will undoubtedly to play a pivotal role in optimizing therapeutic outcomes in the years to come.

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5 Pharmacokinetics/ADME of Large Molecules

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INTRODUCTION

As discussed in previous chapters, pharmacokinetics (PK) is the study of the processes that are responsible for the time course of the level of an exogenous compound in the body. The processes involved are absorption (A), distribution (D), metabolism (M), and excretion (E). The PK of peptides, proteins, and other biotechnology products are an important factor in their pharmacodynamics (PD), that is, the time course of their pharmacological effect. Therefore, knowledge of PK of a pharmaceutical drug in humans and laboratory animals is required when selecting dose levels and dose regimens. Similarly, the toxicokinetics (PK in toxicology studies, including higher doses than used clinically) are important for the design of toxicology studies (dose levels and dose regimens) as well as in determining safety margins and extrapolating toxicological data to humans.

In this chapter, the PK and ADME characteristics of protein therapeutics will be described. The ADME mechanisms for protein drugs that influence the plasma PK and systemic exposure are usually similar to those that handle endogenous proteins. Receptor-mediated uptake mechanisms that may also be involved in the protein's PD effect play an important role. This is generally different from small molecule drugs that are taken up by cells and distribute into organs, including the biophase, in many cases by simple passive diffusion. In addition, receptor binding of small molecules is important for their PD effect but the fraction bound to the receptors rarely plays a role in their PK. Surprisingly, some large molecule drugs also bind to circulating plasma proteins, which may influence their PK and PD in similar ways as for small molecule drugs. Unlike small synthetic molecules for which different metabolic pathways exist in different species, the clearance mechanism for peptides and proteins is generally conserved across mammalian species. As a result, the PK of many protein drugs can be scaled mathematically between species. In contrast to most small molecule drugs, large molecule therapeutics may be immunogenic and circulating antibodies can influence their PK and PD.

CLEARANCE MECHANISMS OF PROTEIN THERAPEUTICS

It is commonly accepted that peptide and protein drugs are metabolized through identical catabolic pathways as endogenous and dietary proteins. Generally, proteins are broken down into amino acid fragments that can be reutilized in the synthesis of endogenous proteins. Although history has shown that proteins can be powerful and potentially toxic compounds, their end products of metabolism are not considered to be a safety issue. This is in contrast with small organic synthetic drug molecules from which potentially toxic metabolites can be formed. The study of the metabolism of protein drugs is also very complicated because of the great number of fragments that can be produced. The mechanisms for elimination of peptides and proteins are outlined in Table 1.

Proteolysis

Most, if not all, proteins are catabolized by proteolysis. Proteolytic enzymes are not only widespread throughout the body, they are also ubiquitous in nature, and therefore the potential number of catabolism sites on any protein is very large (1–3). It has been shown for interferon-α (IFN-α)) that truncated forms are present in the circulation after dosing of rhesus monkeys with rIFN-α. The rate and extent of production of these metabolites may be dependent on the route of administration. This, and the cross-reactivity of these degraded forms in the ELISA may be responsible for the observation of a bioavailability of more than 100% after subcutaneous (s.c.) administration of rIFN-α (4). Proteolytic activity in tissue may be responsible for the loss of protein after s.c. administration.

Table 1 Clearance Mechanisms for Peptides and Proteins as a Function of Molecular Weight (M_W)[a]

M_W	Site of elimination	Dominating clearance mechanism	Determinant factor
<500	Blood	Extracellular hydrolysis	
	Liver	Passive nonionic diffusion	
500–1000	Liver	Carrier-mediated uptake	Structure
		Passive nonionic diffusion	Lipophilicity
1,000–50,000	Kidney	Glomerular Filtration	M_W
50,000–200,000	Kidney	Receptor-mediated endocytosis	Sugar, charge
		Receptor-mediated endocytosis	
	Liver		
200,000–400,000		Opsonization	α_2-macroglobulin IgG
>400,000		Phagocytosis	Particle aggregation

[a]Other determining factors are size, charge, lipophilicity, functional groups, sugar recognition, vulnerability for proteases, aggregation to particles, formation of complexes with opsonization factors, and so on. The indicated mechanisms overlap, and fluid–phase endocytosis can in principle occur across the entire M_W range.
Source: From Ref. 21.

Renal Excretion and Metabolism

Metabolism studies of peptide and protein drugs were performed to identify the organs responsible for metabolism (and/or excretion) and their relative contribution to the total elimination clearance. The importance of the kidney as an organ of elimination was assessed for rIL-2 (5), M-CSF (6), and rIFN-α (7) in nephrectomized animals. The relative contributions of renal and hepatic clearances to the total plasma clearance of several other proteins are shown in Figure 1.

The different renal processes that are important for the elimination of proteins are depicted in Figure 2. The kidney appears to be the most dominant organ for the catabolism of small

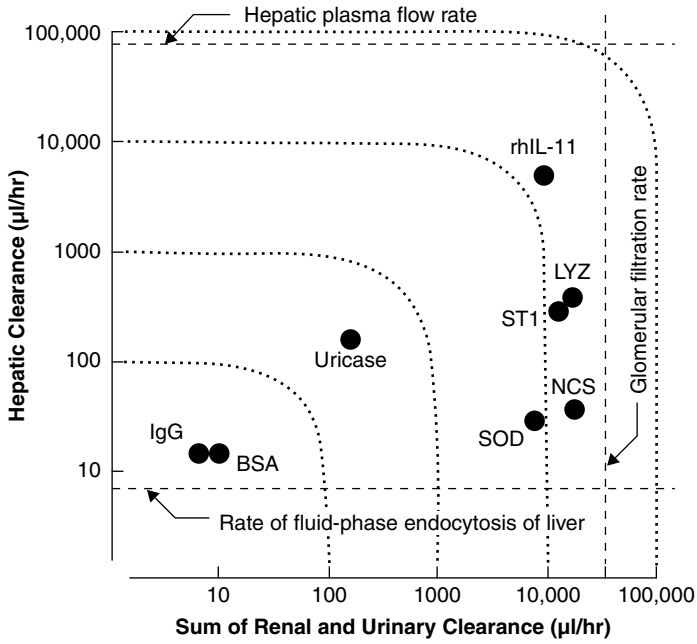

Figure 1 Hepatic and renal clearances of proteins in mice. *Abbreviations*: LYZ, lysozyme; STI, soy bean trypsin inhibitor; NCS, neocarzinostatin; IgG, immunoglobulin G; BSA, bovine serum albumin; rhIL-11, recombinant human interleukin-11. *Source*: From Ref. 96.

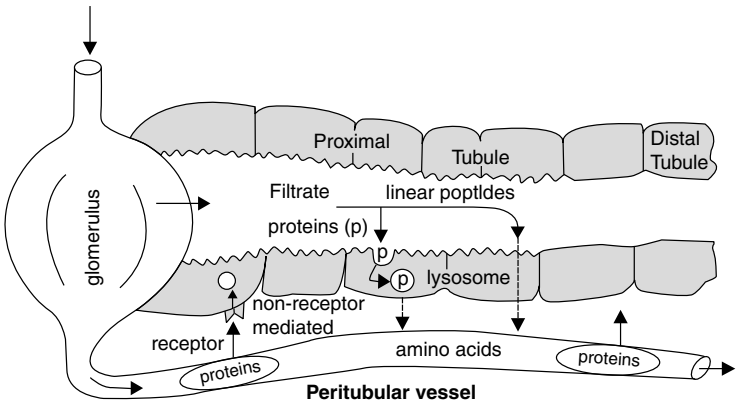

Figure 2 Pathways of renal elimination of proteins, including glomerular filtration, catabolism at the luminal membrane, tubular absorption followed by intracellular degradation, and postglomerular peritubular uptake followed by intracellular degradation. *Source*: From Ref. 11.

proteins (8). Based on the observation that only trace amounts of albumin pass the glomerulus, it is believed that macromolecules must be smaller than 69 kDa to undergo glomerular filtration (9). Glomerular filtration and excretion is most efficient for proteins smaller than 30 kDa (10). Peptides and small proteins (<5 kDa) are filtered very efficiently, and their glomerular filtration clearance approaches the glomerular filtration rate (GFR, approximately 120 mL/min in humans). For molecular weights exceeding 30 kDa, the filtration rate falls off sharply. While there tends to be a reasonable correlation of clearance with molecular weight, the mechanism underlying this correlation is hydrodynamic volume. Indeed, it is the effective molecular radius that determines the degree of sieving by the glomerulus (Fig. 3) (11).

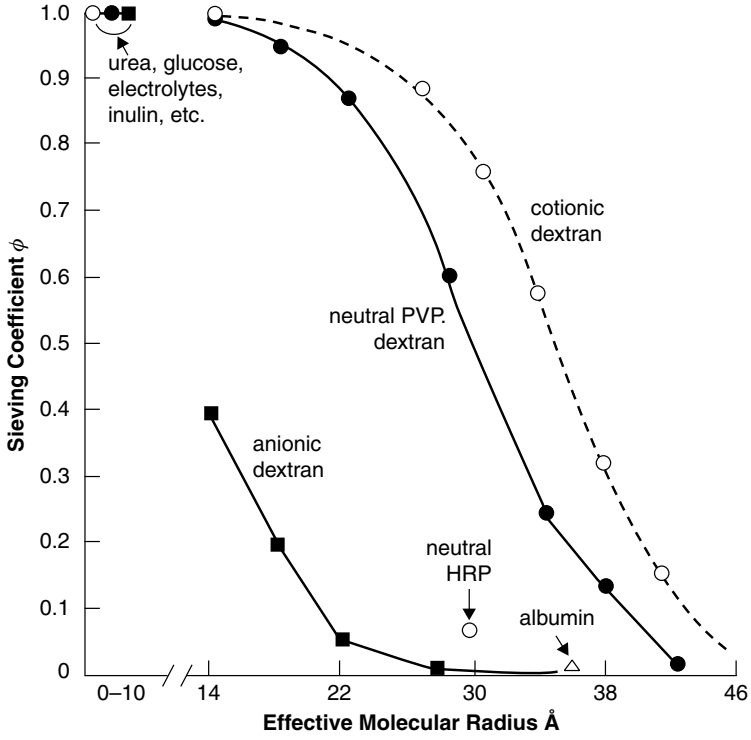

Figure 3 Sieving curves of several macromolecules. The different sieving coefficients reflect the influence of size, charge, and rigidity of molecules. *Source*: From Ref. 97.

The glomerular barrier is also charge selective: the clearance of anionic molecules is impaired relative to that of neutral molecules, and the clearance of cationic macromolecules is enhanced. The influence of charge on glomerular filtration is especially important for molecules with a radius greater than 2 nm (12). The charge selectivity of glomerular filtration is related to the negative charge of the glomerular filter due to the abundance of glycosaminoglycans. Anionic proteins, such as TNF-α and INF-α, are therefore repelled (2).

After glomerular filtration, some peptides (e.g., melanostatin) can be excreted unchanged in the urine. In contrast, more complex polypeptides and proteins are actively reabsorbed by the proximal tubules by luminal endocytosis and then hydrolyzed within the intracellular lysosomes to peptide fragments and amino acids (12,13). The amino acids are returned to the systemic circulation for reprocessing into new protein. Consequently, only small amounts of intact protein are detected in the urine. The kidney appears to be the most dominant organ for the catabolism of small proteins (8). Examples of proteins undergoing tubular reabsorption are calcitonin, glucagon, insulin, growth hormone, oxytocin, vasopressin, and lysozyme (10). Cathepsin D, a major renal protease, is responsible for the hydrolysis of IL-2 in the kidney (14). Important determinants for tubular reabsorption of proteins are their physicochemical characteristics such as net charge and number of free amino groups (8). Cationic proteins are more susceptible to reabsorption than anionic proteins (15). Renal tubular cells also contain an active transporter for di- and tripeptides (16).

Small linear peptides (<10 amino acids) such as angiotensin I and II, bradykinin, and LHRH are subjected to luminal membrane hydrolysis. They are hydrolyzed by enzymes in the luminal surface of the brush border membrane of the proximal tubules, and the small peptide fragments and amino acids are subsequently reabsorbed, further degraded intracellularly, and/or transported through the cells into the systemic circulation (17).

Peritubular extraction of proteins from the postglomerular capillaries and intracellular catabolism is another renal mechanism of elimination (18). This route of elimination was demonstrated for IL-2 (5), insulin (19,20), calcitonin, parathyroid hormone, vasopressin, and angiotensin II (8). It is believed that the peritubular pathway exists mainly for the delivery of certain hormones to their site of action, that is, to the receptors on the contraluminal site of the tubular cells.

Hepatic Metabolism

Besides proteolytic enzymes and renal catabolism, the liver has also been shown to contribute significantly to the metabolism of protein therapeutics. The rate of hepatic catabolism, which determines in part the elimination half-life, is largely dependent on the presence of specific amino acid sequences in the protein (21). Before intracellular hepatic catabolism, proteins and peptides need to be transported from the blood stream to the liver cells. An overview of the different mechanisms of hepatic uptake of proteins is listed in Table 2.

Molecules of relatively small size and with highly hydrophobic characteristics permeate the hepatocyte membrane by simple nonionic passive diffusion. Peptides of this nature include the cyclosporins (cyclic peptides) (22). Other cyclic and linear peptides of small size (<1.4 kDa) and hydrophobic nature (containing aromatic amino acids), such as renin and cholecystokinin-8 (CCK-8; 8 amino acids), are cleared by the hepatocytes by carrier-mediated transport (22). After internalization into the cytosol, these peptides are commonly metabolized by microsomal enzymes (cytochrome P450IIIA for cyclosporin A) or cytosolic peptidases (CCK-8). Substances that enter the liver via carrier-mediated transport are typically excreted into the bile by the multispecific bile acid transporter. These hepatic clearance pathways are identical to those known for most small organic hydrophobic drug molecules.

For larger peptides and proteins, there is a multitude of energy-dependent carrier-mediated transport processes available for cellular uptake. One of the possibilities is receptor-mediated endocytosis (RME), such as for insulin and EGF (23–25). In RME, circulating proteins are recognized by specific hepatic receptor proteins (10). The receptors are usually integral membrane glycoproteins with an exposed binding domain on the extracellular side of the cell membrane. After binding of the circulating protein to the receptor, the complex is already present or moves in coated pit regions, and the membrane invaginates and pinches off to form an endocytotic-coated vehicle that contains the receptor and ligand (internalization). The vesicle

Table 2 Hepatic Uptake Mechanisms for Proteins and Protein Complexes

Cell type/organ	Uptake mechanism	Proteins/peptides transported
Hepatocytes	Anionic passive diffusion Carrier-mediated transport`	Cyclic and linear hydrophobic peptides (<1.4 kDa) (cyclosporins, CCK-8)
	RME: Gal/GalNAc receptor (asialoglycoprotein receptor)	*N*-acetylgalactosamine-terminated glycoproteins Galactose-terminated glycoproteins (e.g., desialylated EPO)
	RME: low-density lipoprotein receptor (LDLR)	LDL, apoE- and apoB-containing lipoproteins
	RME: LDLR-related protein (LRP receptor)	α_2-macroglubulin, apoE-enriched lipoproteins, lipoprotein lipase, (LpL), lactoferrin, t-PA, u-PA, complexes of t-PA and u-PA with plasminogen activator inhibitor type 1 (PAI-1), TFPI, thrombospondin (TSP), TGF-β and IL-1β bound to α_2-macroglubulin
	RME: other receptors	IgA, glycoproteins, lipoproteins, immunoglobulins, intestinal and pancreatic peptides, Metallo- and hemoproteins, transferrin, insulin, glucagon, GH, EGF
	Nonselective pinocytosis (nonreceptor-mediated)	Albumin, antigen-antibody complexes, some pancreatic proteins, some glycoproteins
Kupffer cells	Endocytosis	Particulates with galactose groups
Kupffer and endothelial	RME	IgG-type antibodies *N*-acetylglucosamine-terminated glycoproteins
Cells	RME: mannose receptor	Mannose-terminated glycoproteins (e.g., t-PA, renin)
	RME: fucose receptor	Fucose-terminated glycoproteins
Endothelial cells	RME: scavenger receptor	Negatively charged proteins
	Pinocytosis + binding to the Fc receptor (Brambell or FcRN salvage receptor) and recycling	IgG-type antibodies
	RME: other receptors	VEGF, FGF (?)
Fat-storing cells	RME: mannose-6-phosphate receptor	Mannose-6-phosphate-terminated proteins (e.g., IGF-II)
Liver, spleen	Fixed tissue macrophages	Immune complexes (antigen-antibody complexes)

Abbreviations: RME, receptor-mediated endocytosis.
Source: From Refs. 10, 29, 99, 100.

coat consists of proteins (clathrin, adaptin, and others), which are then removed by an uncoating adenosine triphosphatase (ATPase).The vesicle parts, the receptor, and the ligand dissociate and are targeted to various intracellular locations. Some receptors, such as the LDL, asialoglycoprotein, and transferrin receptors, are known to undergo recycling. Since sometimes several hundred cycles are part of a single receptor's lifetime, the associated RME is of high capacity. Other receptors, such as the IFN receptor, undergo degradation. This leads to a decrease in the

concentration of receptors on the cell surface (receptor downregulation). Others (e.g., insulin and EGF receptors) undergo both recycling and degradation (10).

For glycoproteins, if a critical number of exposed sugar groups (mannose, galactose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, or glucose) are exceeded, RME through sugar-recognizing receptors is an efficient hepatic uptake mechanism (21). Important carbohydrate receptors in the liver are the asialoglycoprotein receptor in hepatocytes and the mannose receptor in Kupffer and liver endothelial cells (26–28). The high-mannose glycans in the first kringle domain of rt-PA have been implicated in its clearance, for example (29).

Low-density lipoprotein receptor-related protein (LRP) is a member of the low-density lipoprotein (LDL) receptor family responsible for endocytosis of several important lipoproteins, proteases, and protease-inhibitor complexes in the liver and other tissues (30). Examples of proteins and protein complexes for which hepatic uptake is mediated by LRP are listed in Table 2. The list includes many endogenous proteins, including some that are marketed or being developed as drugs, such as t-PA, u-PA, and tissue factor pathway inhibitor (TFPI). There are observations indicating that these proteins bound to the cell surface proteoglycans are presented to LRP for endocytosis, thus facilitating the LRP-mediated clearance. It seems likely that proteoglycans serve to concentrate LRP ligands on the cell surface, thereby enhancing their interaction with LRP. Interestingly, none of the LRP ligands compete against each other for the LRP receptor, which is very large (approximately 650 kDa) and contains multiple distinct binding sites (31).

Uptake of proteins by liver cells is followed by transport to an intracellular compartment for metabolism. Proteins internalized into vesicles via an endocytotic mechanism such as RME undergo intracellular transport toward the lysosomal compartment near the center of the cell. There, the endocytotic vehicles fuse with or mature into lysosomes, which are specialized acidic vesicles that contain a wide variety of hydrolases capable of degrading all biological macromolecules. Proteolysis is started by endopeptidases (mainly cathepsin D) that act on the middle part of the proteins. The resulting oligopeptide metabolites are further degraded by exopeptidases. The final metabolic products, amino acids and dipeptides, reenter the metabolic pool of the cell (21). The hepatic metabolism of glycoproteins may occur slower than the naked protein because protecting oligosaccharide chains must be removed prior to hydrolysis of the amino acid backbone. Metabolized proteins and peptides in lysosomes from hepatocytes, hepatic sinusoidal cells, and Kupffer cells may be released into the blood. Degraded proteins in hepatocyte lysosomes can also be delivered to the bile canaliculus and excreted by exocytosis.

A second intracellular clearance pathway for proteins is the direct shuttle or transcytotic pathway (10). The endocytotic vesicle formed at the cell surface traverses the cell to the peribiliary space, where it fuses with the bile canalicular membrane, releasing its contents by exocytosis into bile. This pathway described for polymeric immunoglobulin A, bypasses the lysosomal compartment completely.

Receptor-mediated uptake of protein drugs by hepatocytes, followed by intracellular metabolism, may cause dose-dependent plasma disposition curves due to the saturation of the active uptake mechanism at higher doses. As an example, EGF administered at low doses (50 μg/kg and lower) to rats showed an elimination clearance proportional to hepatic blood flow, since the systemic supply of drug to the liver is the rate-limiting process for elimination. At high doses (>200 μg/kg), the hepatic clearance is saturated, and extrahepatic clearance by other tissues becomes the dominant factor in the total plasma clearance. At intermediate doses of EGF, both hepatic blood flow and EGF receptors responsible for the active uptake affect the total plasma clearance (32).

For some proteins, receptor-mediated uptake by the hepatocytes is so extensive that hepatic blood clearance approaches its maximum value, liver blood flow. As examples, recombinant tissue-type and urokinase-type plasminogen activator (rt-PA and ru-PA, respectively) have been shown to behave as high clearance drugs, and both reductions and increases in liver blood flow affect their clearance in the same direction (33,34). This physiological parameter may have therapeutic implications in patients with myocardial infarction since they can experience variations in liver perfusion caused by diminished cardiac function or concomitant vasoactive drug treatment. Also, liver blood flow decreases during exercise and increases after food intake.

Receptor-Mediated Elimination by Other Cells

For small synthetic drugs, the fraction of the dose bound to receptors at any moment after administration is usually negligible, and receptor binding is reversible, mostly without internalization of the receptor-drug complex. For protein drugs, however, a substantial part of the dose may be bound to the receptor, and receptor-mediated uptake by specialized cells followed by intracellular catabolism may play an important part in the total elimination of the drug from the body. A derivative of granulocyte colony-stimulating factor (G-CSF), nartograstim, and most likely G-CSF itself is taken up by bone marrow through a saturable receptor-mediated process (35). It has been demonstrated for macrophage colony-stimulating factor (M-CSF) that besides the linear renal elimination pathway, there is a saturable nonlinear elimination pathway that follows Michaelis–Menten kinetics (6,36). The importance of the nonlinear elimination pathway was demonstrated by a steeper dip in the plasma concentration profile at lower M-CSF concentrations (Fig. 4). At higher levels, linear renal elimination was dominant, and the nonlinear pathway was saturated. The nonlinear pathway could be blocked by coadministration of carrageenan, a macrophage inhibitor, indicating that receptor-mediated uptake by macrophages was likely responsible for the nonlinear elimination (6). This is especially relevant since M-CSF stimulates the proliferation of macrophages. It is also possible that the receptor-mediated uptake and the effect of M-CSF are closely linked. Indeed, it was observed that after chronic administration of M-CSF, the nonlinear elimination was probably induced by autoinduction since M-CSF increases circulating levels of macrophages. Although autoinduction and consequently accelerated metabolism of most drugs is related to a loss of their pharmacological effect, for M-CSF, it may be an indication of sustained pharmacodynamic activity. Similar kinetics were observed for other hematopoietic stimulating factors such as G-CSF (37) and granulocyte macrophage colony-stimulating factor (GM-CSF) (38). Michaelis–Menten (saturable) elimination was also described for t-PA (39) and for a recombinant amino terminal fragment of bactericidal/permeability-increasing protein ($rBPI_{23}$) (40).

In recent years, several monoclonal antibodies have reached the market, and currently nearly 25% of pharmaceutical biotech products in development are believed to be antibodies or antibody derivatives (41). Their unique structure results in interesting PK. The Fc domain of antibodies and their size is largely responsible for their PK properties with systemic half-lives of several days to weeks. The large size (>150 kDa) prevents excretion through the kidneys. Resistance to proteases is another reason for long half-lives. IgG-type antibodies have an additional mechanism that contributes to their very long half-lives (1–3 weeks). Endothelial cells

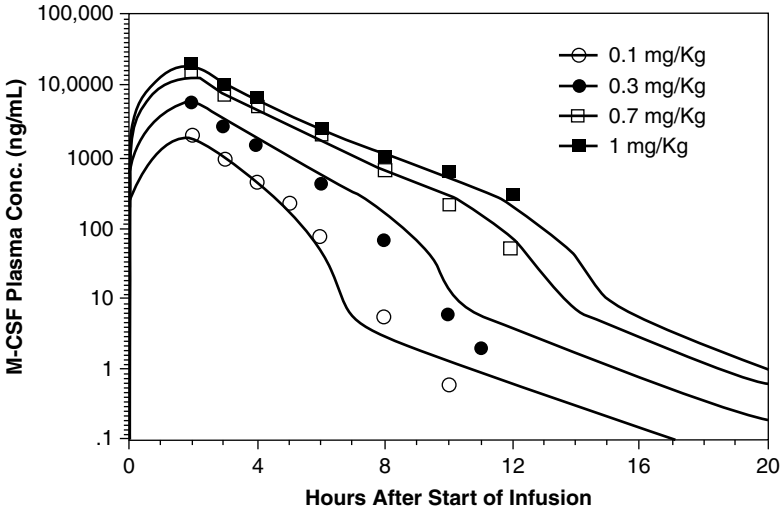

Figure 4 Observed and predicted plasma concentration–time profiles of M-CSF after 2-hour intravenous infusions of 0.1 to 1 mg/kg in cynomolgus monkeys. A two-compartmental pharmacokinetic model with a linear clearance pathway and a parallel Michaelis–Menten elimination pathway was used.

take up most serum proteins by pinocytosis and are consequently degraded in the endothelial cells. IgG antibodies however contain a region in their Fc domain that is recognized by the Fc receptor, called the Brambell receptor, FcRN or salvage receptor. When IgG molecules enter the endothelium, they bind to this receptor in the endosomal compartment, after which the complex moves to the cell surface and the IgG molecule is again liberated into the circulation. This recycling mechanism accounts for the long half-life of IgG-type antibodies (41). Immune complexes (antibodies bound to antigen) are transported to the liver and spleen where they are taken up and degraded by tissue macrophages. This pathway, which is also responsible for the clearance of colloidal particulates (<5 μm) and classical liposomes, is termed the mononuclear phagocyte system (MPS).

DISTRIBUTION OF PROTEIN THERAPEUTICS

Once a molecule reaches the blood stream, it encounters the following processes for intracellular biodistribution: distribution within the vascular space, transport across the microvascular wall, transport through the interstitial space, and transport across cell membranes. The biodistribution of macromolecules is determined by the physicochemical properties of the molecule and by the structural and physicochemical characteristics of the capillaries responsible for transendothelial passage of the molecule from the systemic circulation to the interstitial fluid. In addition, the presence of receptors determines the biodistribution to certain tissues, including extracellular association and/or intracellular uptake. Capillary endothelia are of three types, in increasing order of permeability: continuous (nonfenestrated), fenestrated, and discontinuous (sinusoidal) (10,42). The most likely dominant mode of transport of macromolecules in nonfenestrated capillaries is through interendothelial junctions. Through these junctions, there are two modes of transport (43): the convective transport, often the most important for macromolecules, is dependent on a pressure difference between the vascular and interstitial spaces and the diffusive transport is driven by a concentration gradient.

Capillaries selectively sieve macromolecules based on their effective molecular size, shape, and charge. Because of the large size of proteins, their apparent volume of distribution is usually relatively small. The initial volume of distribution after intravenous (IV) injection is approximately equal to or slightly higher than the total plasma volume. The total volume of distribution is generally up to two times the initial volume of distribution. Although this is sometimes interpreted as a low tissue penetration, it is difficult to generalize. Indeed, adequate concentrations may be reached in a single target organ because of receptor-mediated uptake, but the contribution to the total volume of distribution may be rather small.

In addition to size, it appears that the charge selective nature of continuous capillaries and cell membranes may also be important for the biodistribution of proteins. Information for this is available from studies with different types of Cu,Zn-superoxide dismutase (Cu,Zn-SOD), which are similar in molecular weight (33 kDa), but have different net surface charges, and are isolated from different species (44). Tissue equilibration of the positively charged sheep Cu,Zn-SOD was much faster than for the negatively charged bovine Cu,Zn-SOD. In addition, the positively charged Mn-SOD equilibrated much faster than the negatively charged human Cu,Zn-SOD, although Mn-SOD is much bigger (88 kDa). A trend toward increasing anti-inflammatory activity, for which interstitial concentrations are important, was observed with increasing isoelectric point. It was suggested that the electrostatic attraction between positively charged proteins and negatively charged cell membranes might increase the rate and extend of tissue biodistribution. Most cell surfaces are negatively charged because of the abundance of glycosaminoglycans in the extracellular matrix.

Tissue binding is also important for the biodistribution of the heparin-binding proteins, including the fibroblast growth factor family (such as FGF-1 and FGF-2) (45), vascular endothelial growth factor (VEGF) (46), platelet-derived growth factor (PDGF), tissue factor pathway inhibitor (TFPI) (47), amphiregulin (AR), and epidermal growth factor (EGF). Proteins of this group contain a highly positively charged tail, which electrostatically binds to low-affinity binding sites consisting of heparin sulfate proteoglycans (acidic glycosaminoglycans) (48,49). These binding sites are abundant on the vascular endothelium and liver and are responsible for the majority of cell surface binding of these proteins. The rapid and extensive binding to the vascular endothelium of protein drugs in this class is most likely the explanation for their

rapid distribution phase after IV injection and their relatively large volume of distribution. Binding of growth factors to proteoglycans has been proposed to provide a mechanism for growth factor recruitment at the cell surface, presentation to specific receptors, regulation of their action on target cells at short range, and establishment of a growth factor gradient within a tissue.

A major in vivo pool of some of the heparin-binding proteins appears to be associated with the vascular endothelium and is released into the circulation quickly after injection of heparin. Since heparin is structurally similar to the cell surface glycosaminoglycans, the proteins bind to circulating heparin, depleting the intravascular pool. This was demonstrated, for example, for TFPI (50,51) and basic FGF (FGF-2) (45).

Biodistribution studies with the measurement of the protein drug in tissues are necessary to establish tissue distribution. These studies are usually performed with radiolabeled compounds. Biodistribution studies are imperative for small organic synthetic drugs since long residence times of the radioactive label in certain tissues may be an indication of tissue accumulation of potentially toxic metabolites. Because of the possibility of reutilization of amino acids from protein drugs in endogenous proteins, such a safety issue does not exist. Therefore, biodistribution studies for protein drugs are usually performed to assist drug targeting to specific tissues or to detect the major organs of elimination (usually kidneys and liver).

If the protein contains a suitable amino acid such as tyrosine or lysine, an external label such as ^{125}I can be chemically coupled to the protein (4). Although this is easily accomplished and a high specific activity can be obtained, the protein is chemically altered. Therefore, it may be better to label proteins and other biotechnology compounds by introducing radioactive isotopes during their synthesis by which an internal atom becomes the radioactive marker (internal labeling). For recombinant proteins, this can accomplished by growing the production cell line in the presence of amino acids labeled with ^{3}H, ^{14}C, ^{35}S, and so on. This method is not routinely used because of the prohibition of radioactive contamination of fermentation equipment. Moreover, internally labeled proteins may be less desirable than iodinated proteins because of the potential for reutilization of the radiolabeled amino acid fragments in the synthesis of endogenous proteins and cell structures. Irrespective of the labeling method, the labeled product should demonstrate physicochemical and biological properties identical to the unlabeled molecule (52).

In addition, as for all types of radiolabeled studies, it needs to be established whether the measured radioactivity represents intact labeled protein, or radiolabeled metabolites, or the liberated label. Trichloro-acetic acid-precipitable radioactivity is often used to distinguish intact protein from free label or low-molecular-weight metabolites, which appear in the supernatant after centrifugation. Proteins with reutilized labeled amino acids and large protein metabolites can only be distinguished from the original protein by techniques such as PAGE, HPLC, specific immunoassays, or bioassays. This discussion also implies that the results of biodistribution studies with autoradiography can be very misleading. Although autoradiography is becoming more quantitative, one does not know what is being measured qualitatively without specific assays. It is therefore sometimes better to perform biodistribution studies by collection of the tissues and use specific measurement of the protein drug in the tissue homogenate.

A method was developed to calculate early phase tissue uptake clearances based on plasma and tissue drug measurements during the first five minutes after IV administration (25). The short time interval has the advantage that metabolism and the tissue efflux clearance presumably can be ignored. As an example, with this method, dose-independent (nonsaturable) uptake clearance values were observed for a recombinant derivative of hG-CSF, nartograstim, for kidney and liver (35). In contrast, a dose-dependent reduction in the uptake clearance by bone marrow with increasing doses of nartograstim was observed. These findings suggested that receptor-mediated endocytosis of the G-CSF receptor in bone marrow may participate in the nonlinear properties of nartograstim. Since G-CSF is one of the growth factors that stimulates the proliferation and differentiation of neutropoietic progenitor cells to granulocytes in bone marrow, the distribution aspects of nartograstim into bone marrow are especially relevant for the PD. In addition, since G-CSF and nartograstim are catabolized in the bone marrow cells after receptor-mediated uptake, the biodistribution into bone marrow is also a pathway for elimination of these molecules. Unlike for classical small synthetic drugs, it is not uncommon

for biotechnology-derived drugs that biodistribution, pharmacodynamics, and elimination are closely connected.

Besides receptor-mediated uptake into target organs and tissues, other proteins, or macromolecules in general, distribute into tissues in more nonspecific ways. It was demonstrated in at least one study with tumor-bearing mice that high total systemic exposure of target-nonspecific macromolecules was the most important factor that determines the extent of tissue uptake (9). Consequently, molecules with physicochemical characteristics that minimize hepatic and renal elimination clearances showed the highest tumoral exposure. Compounds with relatively low-molecular-weights (approximately 10 kDa) or positive charges were rapidly eliminated and showed lower tumor radioactivity accumulation; large (>70 kDa) and negatively charged compounds (carboxymethyl dextran, BSA, mouse IgG) showed prolonged retention in the circulation and high tumoral levels. A typical example is the murine urokinase (muPA) EGF-like domain peptide of 48 amino acids, muPA(1–48). This peptide is a urokinase receptor antagonist under consideration as an anticancer drug since urokinase has been implicated in invasive biological processes such as tumor metastasis, trophoblast implantation, and angiogenesis. Scientists at Chiron have fused muPA(1–48) to the human IgG constant region. The fused molecule [IgG-muPA(1–48)] retained its activity of inhibition of the murine UPA receptor, but has a much longer in vivo elimination half-life (79 vs. 0.5 hour, Fig. 5). The half-life increase was due to both a decrease in elimination clearance (4.3 vs. 95 mL/hr/kg) and an increase in the peripheral volume of distribution (434 vs. 43 mL/kg). Although the fused molecule was substantially larger, tissue distribution increased, possibly because of substantial tissue binding. This is in contrast with some polyethylene glycol-modified (PEGylated) molecules such as polyethylene glycol-modified interleukin-2 (PEG IL-2) for which the size increase resulted in a smaller distribution volume compared to the original molecule (see below).

Biodistribution into the lymphatics after s.c. injection deserves special attention since it is a rather unique transport pathway for macromolecules. Following s.c. administration, the drug can be transported to the systemic circulation by absorption into the blood capillaries or by the lymphatics. Since the permeability of macromolecules through the capillary wall is low, they were found to enter blood indirectly through the lymphatic system (53,54). Compounds with a molecular weight larger than 16 kDa are absorbed mainly (>50%) by the lymphatics, while compounds smaller than 1 kDa are hardly absorbed by the lymphatics at all. Lymph recovery after s.c. dosing was apparently linearly related to molecular weight up to 19 kDa (Fig. 6) (54). Negatively charged proteins had increased lymph absorption as compared to positively charged

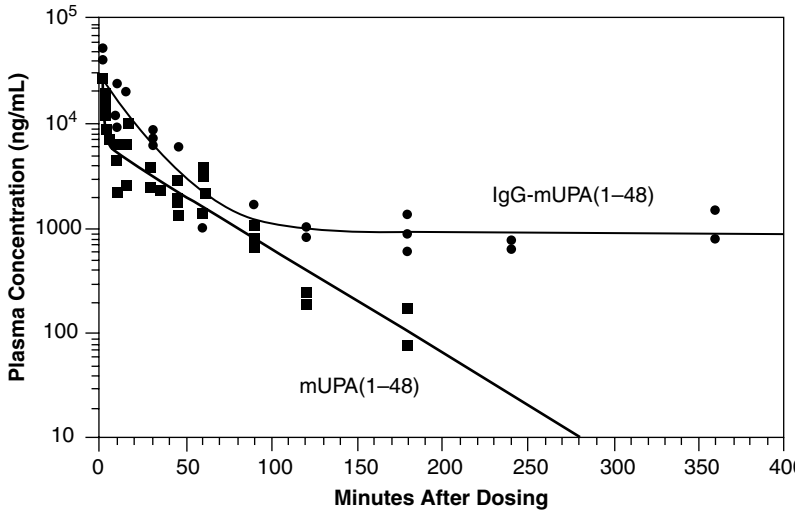

Figure 5 Fusion of the murine urokinase EGF-like peptide of 48 amino acids with human IgG [IgG-mUPA(1-48)] resulted in a much longer half-life than the original peptide [mUPA(1-48)]. The data were modeled according to a linear two-compartmental model.

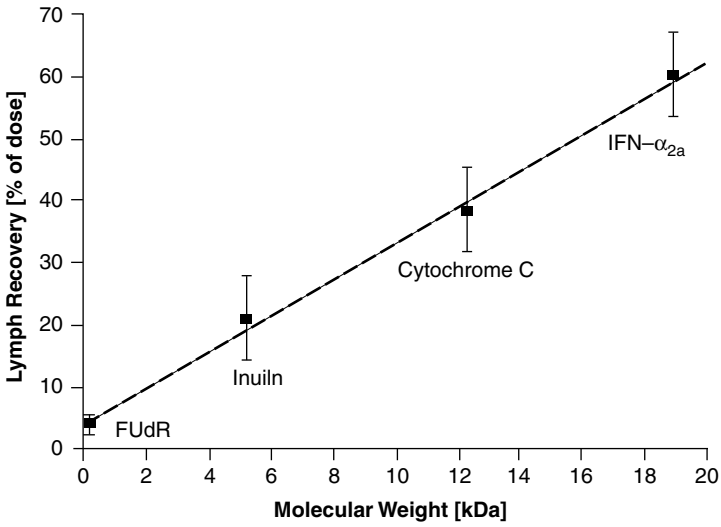

Figure 6 Correlation between the molecular weight and cumulative recovery of rIFN α-2a (M_W 19 kDa), cytochrome *c* (M_W 12.3 kDa), inulin (M_W 5.2 kDa), and FUDR (M_W 256.2 kDa) in the efferent lymph from the right popliteal lymph node following s.c. administration into the lower part of the hind leg of sheep. Each point and bar show the mean and standard deviation of three experiments performed in different sheep. The line drawn is the best fit by linear regression analysis calculated with the four mean values (correlation coefficient r of 0.998, $p < 0.01$). *Source*: From Ref. 54.

proteins with similar molecular weight (55). After lymphatic absorption, compounds circulate within the lymph and are gradually returned to the blood. As a result, lymph concentrations for these proteins may be higher than blood concentrations. Targeting of the lymphatics may be beneficial for proteins that act on the immune system, such as for IL-2. It was shown that s.c. administration of IL-2 in a pig model resulted in higher lymph levels as compared to blood, and at higher doses, absorption was exclusively through lymph (56). The IL-2 receptor-positive T-lymphocytes, that are thought to be primarily associated with efficacy, reside largely in the lymphoid organs. On the other hand, natural killer cells and neutrophils in blood produce cytokines, reactive oxygen intermediates, and proteases, all of which have been shown to be necessary to produce IL-2 toxicities. Therefore, adverse in vivo activity of IL-2 may be related to blood levels, while beneficial activity may be associated to lymph concentrations (56).

Biodistribution into target organs, usually receptor-mediated, is important for the PD of protein drugs. For some proteins, saturable receptor-mediated tissue uptake in target organs is responsible for nonlinear kinetics (57). For example, the uptake clearance of rhEPO by bone marrow and spleen exhibited clear saturation in rats. Also, a single high dose of rhEPO caused a reduction of uptake clearance by bone marrow and spleen, while repeated injections caused an increase of the tissue uptake clearance, especially by the spleen, in a dose-dependent manner (57). Hematopoietic parameters such as hematocrit and hemoglobin concentration changed accordingly, suggesting that changes in the uptake clearance were caused by down- or upregulation of EPO receptors.

PLASMA PHARMACOKINETICS

Although the time course of the compound at the receptor or effector site is the desired knowledge to predict or explain the PD, accurate drug level data at that site are difficult to obtain. In most cases, PK data are limited to plasma concentration data. PK models are widely used to describe and predict the time course of the drug in plasma and tissues. These models include compartmental models and physiological models. A scan of the literature shows that mostly compartmental models are used, in particular one- or two-compartmental models. Terminal-phase elimination half-lives for small- to medium-sized protein drugs in humans range from a couple of hours (e.g., 3.7 hours for rt-PA) to more than 12 hours (e.g., 15 hours for factor VIII).

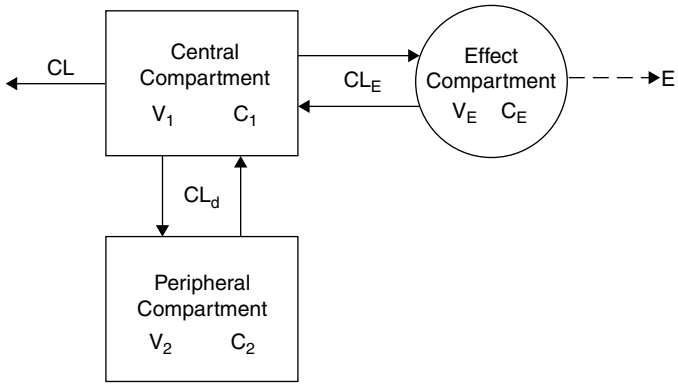

Figure 7 Example of a typical PK/PD link model (98). The PK model is a two-compartmental model with a linear elimination clearance from the central compartment (CL) and a distributional clearance (CL_d). C_1 and C_2 are the concentrations in the central and peripheral compartments, and V_1 and V_2 are their respective apparent volumes of distribution. A hypothetical effect compartment is linked to the central compartment. The concentration in the effect compartment (C_E) drives the intensity of the pharmacodynamic effect (E). CL_E is the linear clearance for distribution of drug to the effect compartment and elimination from the effect compartment. V_E is the apparent volume of distribution in the effect compartment.

Very large protein drugs such as monoclonal antibody-based pharmaceuticals have plasma half-lives ranging from days to several weeks. The IgG1-based recombinant humanized monoclonal antibody trastuzumab (e.g., Herceptin α, 148 kDa)) has a half-life that ranges from 1.7 days to 15 days after IV doses of 10 and 500 mg, respectively (58).

As a modeling example, the PK/PD model used for insulin after a single 10 U s.c. dose in 10 volunteers is depicted in Figure 7 (59,60). The PK model consisted of a classical two-compartment model with first-order elimination from the central compartment. A hypothetical effect compartment is linked to the central compartment to model the PD of insulin, which in this case was the glucose infusion rate to maintain euglycemia. Figure 8 shows the mean serum concentration profile of insulin after a single s.c. injection of 10 U in 10 volunteers, and the corresponding effect measured as the glucose infusion rate to maintain an euglycemic state. The concentration in the effect compartment drives the intensity of the pharmacodynamic effect: The effect compartment of this PK/PD link model cannot be distinguished from the other compartments based on plasma concentration only. Compartmental modeling with plasma

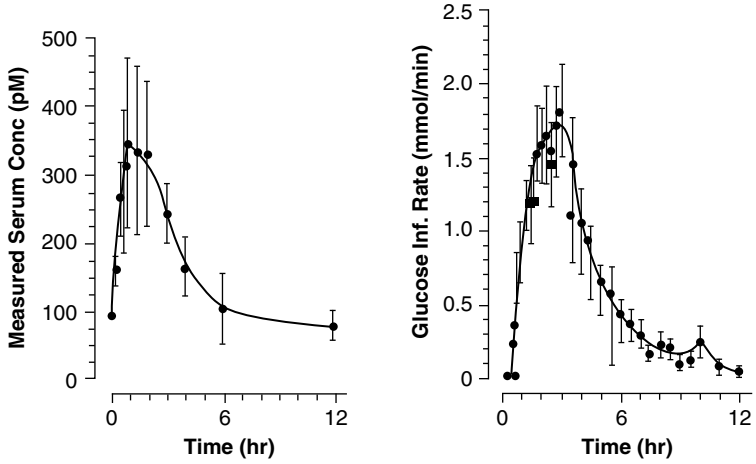

Figure 8 Mean measured serum insulin concentrations after a single 10 U s.c. dose of regular insulin in 10 volunteers (left panel); corresponding glucose infusion rates needed to maintain euglycemia (right panel). *Source*: From Ref. 60.

concentration–time data is in most cases just not sensitive enough to isolate the biophase as a separate compartment without the availability of measured drug concentration data in the biophase. Drug distributes into the effect compartment but since the amount of drug in the effect compartment is rather small, no actual mass transfer is implemented in the PK part of the PK/PD model. This PK/PD link model accounts for the temporal delay of the effect appearance. The delay is typically explained by a distributional delay (61): drug concentrations in a slowly equilibrating tissue compartment with plasma are directly related to the effect intensity. Since the peak level of drug in the biophase is reached later than the time of the peak plasma concentration, the peak effect also occurs later than the plasma peak level. Although this PK/PD model is constructed with tissue distribution as the reason for the delay of the effect, the distribution clearance to the effect compartment can be interpreted differently, including other reasons of delay, such as transduction processes and secondary postreceptor events.

Nonlinear Plasma Pharmacokinetics

As described earlier, many protein drugs are eliminated by receptor-mediated uptake in the liver or by other cells. Sometimes, this uptake is saturated at higher doses, leading to dose-related nonlinearity whereby an increase in dose size does not result a proportional increase in systemic drug exposure. In other instances, the receptors are upregulated after chronic exposure leading to time-related nonlinearity whereby the same dose at a later time after chronic dosing causes a lower drug exposure than after the first dose. As an example, the serum PK of filgrastim (r-methionine-hG-CSF) after s.c. dosing of human volunteers for 10 days and the effect on neutrophilic granulopoiesis after s.c. dosing of human volunteers for 10 days (62) is shown in Figure 9. The PK were modeled with a two-compartment model with elimination from the central compartment. The filgrastim clearance increases because of a time-related increase of the uptake receptors on the neutrophils or because of an increase in the total neutrophil count. This accounts for the observation that the filgrastim peak levels decrease as a function of time Figure 10. However, despite decreasing serum levels of filgrastim with chronic dosing, the

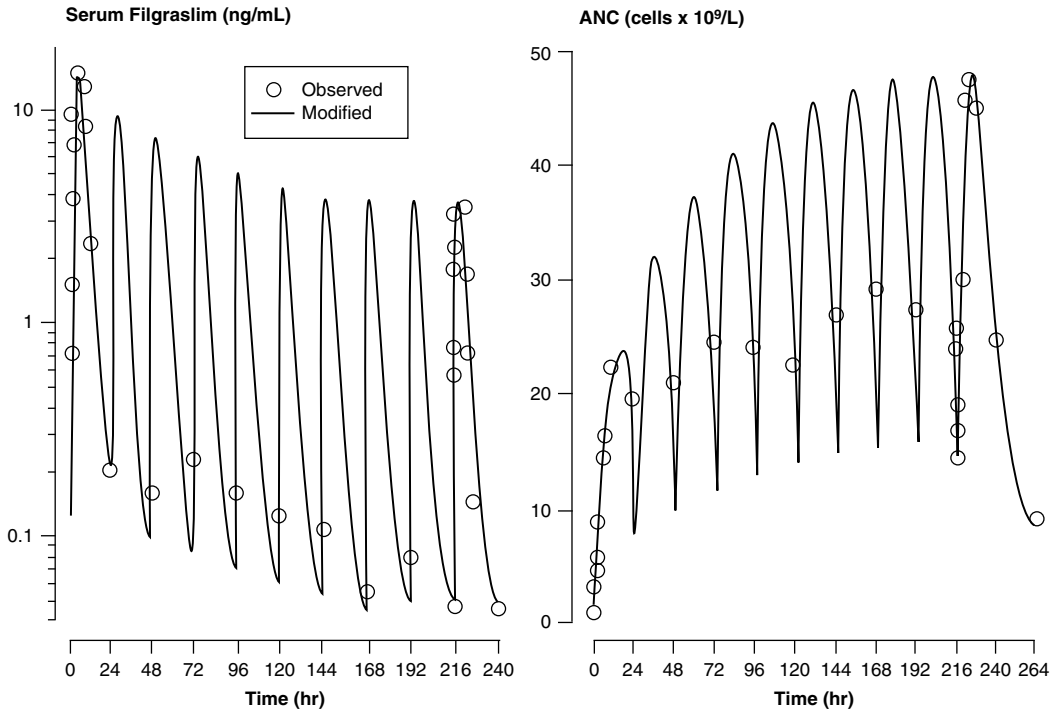

Figure 9 Simultaneous PK/PD modeling of serum filgrastim levels and mean absolute neutrophil counts (ANC) response in normal volunteers receiving s.c. filgrastim (300 μg/day) for 10 days (62).

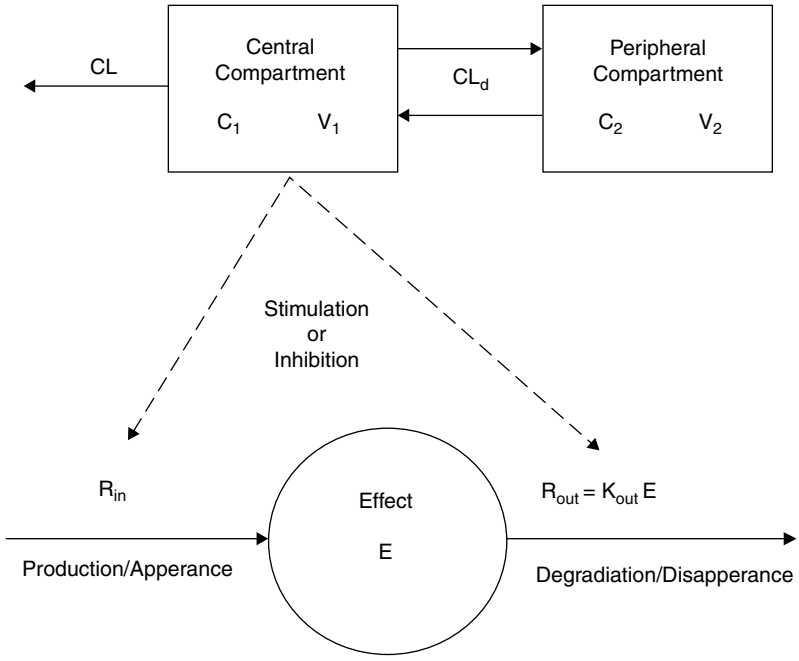

Figure 10 Pharmacodynamic indirect effect model wherein the effect is maintained by equilibrium between a zero-order appearance rate, R_{in}, and a first-order disappearance rate, R_{out}. A drug effect is caused by stimulation or inhibition of R_{in} or R_{out}. The degree of stimulation or inhibition is dependent on the plasma drug concentration. The PD parameters are R_{in}, K_{out} (the first-order rate constant for effect disappearance), EC_{50} (the concentration that produces 50% of maximum inhibition or stimulation), and E_{max} (the maximum inhibition or stimulation). The pharmacokinetic model is identical as in Figure 7. For filgrastim (see Fig. 9), R_{out} is transiently stimulated in the first hour after dosing and R_{in} is stimulated later on causing an increase in neutrophil count after chronic dosing. In addition, the elimination clearance is inhibited by the effect.

pharmacodynamic effect increases and approaches a steady state after approximately six days. The indirect-effect PD model used to model the absolute neutrophil count (ANC) is shown in Figure 9. The transient decrease of blood neutrophils in the first hour after dosing is due to rapid distribution of neutrophils into the marginal blood pool (disappearance process in Fig. 10). The increase in neutrophil count is modeled as a filgrastim concentration-dependent flux into the circulating neutrophil pool (appearance process in Fig. 10). This combined PK/PD model accurately describes the accession of the ANC to steady-state levels (Fig. 9). This example shows how multiple-dose PK/PD data from human trials with nonlinearity in the PK and indirect PD effects can be modeled and predicted.

PROTEIN BINDING OF PROTEIN THERAPEUTICS

The binding of drugs to circulating plasma proteins can influence both the distribution and clearance of drugs, and consequently their PD. Since it is generally accepted for small drug molecules including small proteins that only the unbound drug molecules can pass through membranes, distribution and elimination clearances of total drug are usually smaller than those of free drug. Accordingly, the activity of the drug is more closely related to the unbound drug concentration than to the total plasma concentration. For other protein drugs however, plasma binding proteins may act as facilitators of cellular uptake processes, especially for drugs that pass membranes by active processes. When a binding protein facilitates the interaction of the protein therapeutic with receptors or other cellular sites of action, the amount of bound drug influences the PD directly.

Numerous examples of binding proteins are reported for proteins: IGF-I and IGF-II, t-PA, growth hormone, DNase (63), nerve growth factor, and so on. (64). Some proteins have their own naturally occurring binding proteins that bind the protein specifically. As an example,

six specific binding proteins are identified for IGF-I, denoted as IGFBP-1 to IGFBP-6 (65,66). The IGFBPs are high affinity, soluble carrier proteins that transport IGF-I (and IGF-II) in the circulation (66). In humans, IGFBP-3 appears to be the most important binding protein for IGF-I since it is the most abundant in serum and tissues. At least 95% of the total human serum concentration of IGF-I is bound to IGFBP-3 (67). IGFBP-3 seems to act as a reservoir for IGF-I and as such to protect the organism against acute insulin-like hypoglycemic effects. Indeed, the hypoglycemic effect is related to the free IGF-I plasma concentration. In this case, the binding protein limits the accessibility of IGF-I to receptors since all binding proteins have substantially higher affinities for IGF-I than the IGF receptors (68). In contrast, the delayed, indirect effects of IGF-I, such as its anabolic effects, may be related to the bound IGF-I levels. This is supported by evidence that the IGFBPs may play an active role in the interaction with target cells and may act as facilitators for the delivery of IGF-I to certain receptors (66). One example is the demonstration that the affinity of the binding protein for IGF-I (IGFBP-6) at the cell surface is lower than in solution, which would make it easier for IGF-I to leave its association with the binding protein and to engage in binding with a cell-based receptor. As such, the IGFBPs may act as inhibitors for certain IGF-I effects and as stimulators for other IGF-I effects.

It is demonstrated that the elimination half-life of bound IGF-I is significantly prolonged relative to that of free IGF-I (64,69,70). This suggests that unbound IGF-I only is available for elimination by routes such as glomerular filtration and peritubular extraction. The binding proteins for IGF-I are also responsible for the complicated PK behavior of IGF-I. The IGFBPs can be saturated at high IGF-I plasma concentrations, typically reached after endogenous therapeutic administration of IGF-I. At high doses, the binding proteins saturate and leave a larger proportion of free protein available for elimination. Additionally, the nonlinear PK of IGF-I are complicated by the fact that the concentrations and relative ratios of the IGFBPs change with time during chronic dosing. The binding proteins are also very different between species, which makes interspecies scaling of the IGF-I PK for IGF-I impossible.

Another example is growth hormone (GH), for which a specific high-affinity binding protein homologous with the extracellular domain of the growth hormone receptor is present in human plasma (71.72). At least two GH-binding proteins (GHBP) have been identified in plasma with respectively high and low binding affinities for GH (64). GHBP binds approximately 40% to 50% of circulating GH at low GH concentrations of about 5 ng/mL (73). At higher circulating GH levels, the binding proteins become saturated (Fig. 11). The clearance of bound GH is about 10-fold slower than that of free GH (74). Consequently, the binding proteins prolong the elimination half-life of GH, and as a result, enhance or prolong its activity. On the other hand, plasma binding of GH prevents access of free GH to its receptors, and this could decrease its activity (64).

Other protein therapeutics seem to bind to circulating proteins in a more nonspecific way. As an example, a recombinant derivative of hG-CSF, nartograstim, showed 92% binding in rat plasma, presumably to albumin (35).

INTERSPECIES SCALING

Techniques for the prediction of PK parameters in one species from data derived from other species have been applied for many years (75,76). Such scaling techniques use various allometric equations based on body weight (see chap. 2). The following allometric equation is routinely employed:

$$P = a \cdot W^b$$

where P is the PK parameter being scaled, W is the body weight, a is the allometric coefficient, and b is the allometric exponent. Although a and b are specific constants for any compound and for each PK parameter, the exponent b seems to average around 1 for volume terms such as the volume of distribution and 0.75 for rates such as elimination and distribution clearances. Since the elimination half-life of any drug is proportional to the volume of distribution and inversely proportional to the elimination clearance, b is approximately 0.25 for elimination half-lives. Allometric scaling of PK parameters has been difficult for small synthetic drug molecules, especially for those drugs with a high hepatic clearance and quantitative and/or qualitative

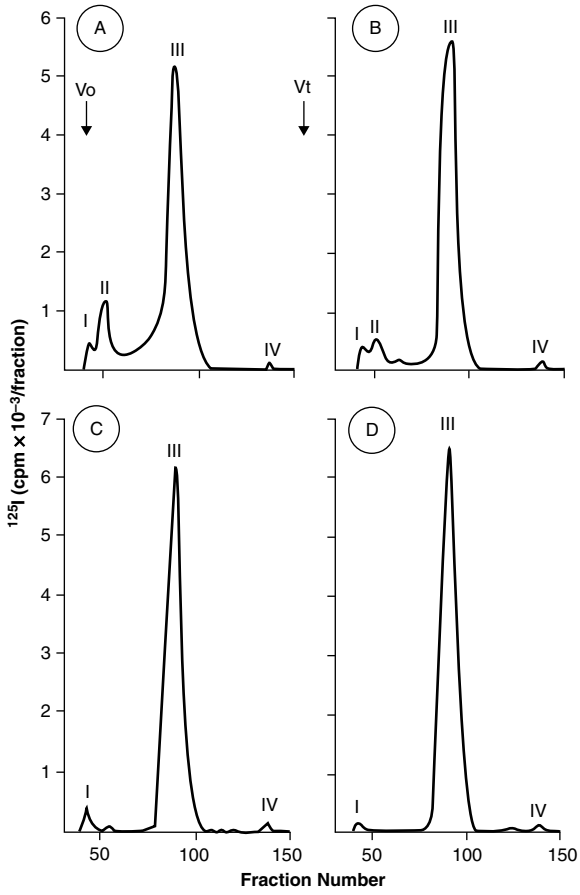

Figure 11 Gel filtration profiles of ^{125}I-hGH in plasma on Sephadex G-100. V_0 and V_t are the void and total volumes, respectively. A. Blank plasma with endogenous level of hGH only; B. 126 ng/mL hGH added; C. 10 μg/mL hGH added; D. tracer only (no plasma). Peak III corresponds to monomeric hGH; peak II and the plateau region between peaks II and III refer to the plasma-bound hGH; peak IV is free iodide. Higher hGH concentrations saturate the binding proteins as peak II becomes smaller relative to peak III (C vs. B vs. A). *Source*: From Ref. 66.

interspecies differences in metabolism. In contrast, the biochemical and physiological processes that are responsible for the PK fate of biologics such as peptides and proteins are better conserved across mammalian species. As such, allometric scaling for those compounds has been more reliable and accurate (77). It is our experience that the systemic exposure in humans of proteins that follow linear PK can be predicted within a factor of two from PK data from three to four animal species. As a typical example, we could scale the PK parameters for IL-2 and PEG IL-2, as demonstrated in Figure 12,for the elimination clearance. Notice that the regression lines for both compounds are parallel, which is expected if PEGylation decreases the clearance to the same degree in all species.

A helpful although potentially less accurate prediction can be made based on PK data from one species to another based on the average allometric exponents for volumes and clearances. Interspecies scaling is helpful in the prediction of doses for pharmacological animal models of disease, toxicology studies, and the first human studies. Indeed, if the efficacious concentration of a protein drug is known from in vitro studies, one might predict the dose needed to reach these levels in an animal efficacy or toxicology model when PK data are know from another species. Similarly, if an estimation of the maximum tolerated exposure can be made, allometric scaling may be helpful to determine the highest dose that should be included in toxicology studies. The dose that results in efficacious concentrations may be taken as the lowest dose that should be included in toxicology studies. Additionally, the efficacious dose in humans can be estimated from the animal PK data. A starting dose in the first human study (usually a dose-escalation study) can be chosen as this estimated efficacious dose, divided by a factor of two or more, based on conservative safety considerations.

It needs to be emphasized that allometric scaling techniques are useful tools to predict a dose that will assist in the planning of dose-ranging studies, but are not a replacement for such

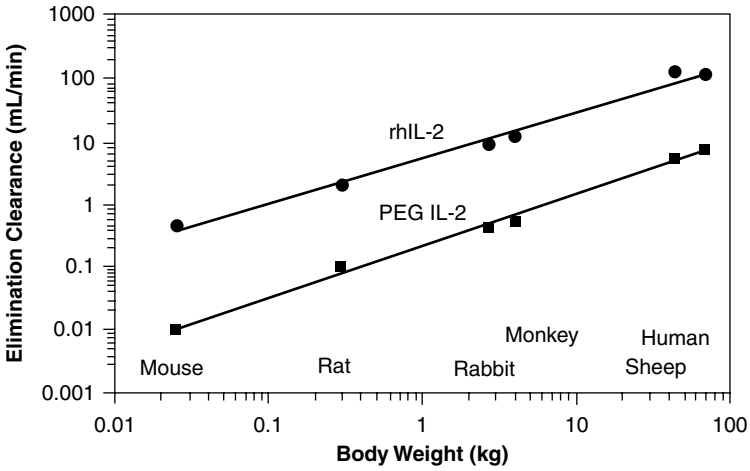

Figure 12 Allometric interspecies scaling of the elimination clearance of IL-2 and PEG IL-2.

studies. The advantage of including such dose prediction in the protocol design of dose-ranging studies is that a smaller number of doses need to be tested before finding the final dose level. Interspecies dose predictions simply narrow the range of doses in the initial pharmacological efficacy studies, the animal toxicology studies, and the human safety and efficacy studies.

HETEROGENEITY OF PROTEIN THERAPEUTICS

The identity, purity, and potency of small synthetic drugs can be demonstrated analytically, and consequently, they are usually completely defined in terms of their chemical structure. Peptides, proteins, and other biotechnologically derived compounds are usually more complex compounds, and it is generally not possible to define them as discrete chemical entities with unique compositions. The physicochemical and biochemical characteristics of proteins are not only dependent on the amino acid sequence (primary structure), but also on the shape and folding (secondary and tertiary structures), and the relationship between the protein molecules themselves, such as the formation as aggregates (quaternary structure). Biotechnologically derived and endogenous proteins may be heterogeneous at each structural level. For natural IFN-α, for example, six naturally occurring C-terminal sequences have been identified (78–80).

In addition posttranslational modifications of proteins, such as the degree of glycosylation of amino acid residues, may be different. The secreted and membrane-associated proteins of almost all eukariotic cells are glycosylated (81,82), and different glycoproteins have also different carbohydrate contents, from approximately 3% for serum IgG to >40% for erythropoietin (EPO). EPO has three N-linked and one O-linked sugar chains. The degree of glycosylation differs according to the cell line used for production. For example, GM-CSF and M-CSF are nonglycosylated in bacterial cell lines such as *Escherichia coli* (*E. coli)*, moderately glycosylated in yeast, and heavily glycosylated in mammalian cell lines. Receptor binding studies with GM-CSF have shown that the receptor affinity decreases with an increase of the level of glycosylation (83).

Another classical example is recombinant human tissue-plasminogen activator (t-PA). Although the active enzyme was first derived from *E. coli* cultures, this cell line lacks several desirable biological activities, such as glycosylation ability and the ability to form the correct three-dimensional t-PA structure. Finally, recombinant t-PA was cloned into a Chinese hamster ovary (CHO) cell line. These mammalian cells carried out the glycosylation, disulfide bond formation, and proper folding similar to human cells (84).

Besides the importance of correct glycosylation for activity, differences in glycosylation may also have an influence on the PK. A typical example is that the removal of terminal sialic acid residues from the sugar chains of EPO (asialo-EPO) causes complete loss of in vivo biological activity, but increases in vitro activity. The loss of in vivo activity of asialo-EPO was explained

by a rapid removal from the systemic circulation, which resulted from hepatic uptake mediated by galactose-recognizing receptors.

CHEMICAL MODIFICATIONS OF PROTEIN THERAPEUTICS

Besides the mostly unwanted heterogeneity of protein drugs introduced by the manufacturing process, other chemical modifications of protein and peptide drugs are intentional to obtain molecules with specified characteristics. Variant proteins can be engineered that differ from natural proteins by exchange, deletion, or insertion of single amino acids, or longer sequences up to entire domains. Small changes in the chemical structure of proteins may cause differences in PK and PD. In addition, mutations may affect glycosylation patterns and conformational changes, which in turn may affect clearance and receptor interactions. A single amino acid mutation in t-PA or the removal of carbohydrate on a single amino acid in t-PA resulted in plasma concentration profiles that were very different from natural t-PA (Fig. 13) (85).

Modification of peptide and protein drugs with the aim of changing the pharmacological activity may at the same time affect the PK behavior of the molecules. In other instances, the increase of duration of response may be exclusively attributed to a change in the PK such as an increase in residence time. Such modifications include amino acid substitution, deletions and additions, cyclization, drug conjugation, glycosylation or deglycosylation, and so on.

The elimination half-life of many peptide and protein drugs is rather small. Consequently, frequent dosing or continuous infusion is necessary to maintain efficacious plasma levels of the drug. Several approaches have been applied to decrease the elimination clearance of biotechnological drugs. One approach is chemical modification such as PEGylation, that is, the attachment of monomethoxy polyethylene glycol polymer (PEG) to the protein. An example is PEG IL-2, which usually consists of a mixture of rhIL-2 molecules (M_W 15 kDa) with 1 to 5 or more PEG polymers attached to each molecule on the α-amino portions of the lysine residues. The production process determines the average number of PEG residues attached, but any process results in a mixture. With each PEG addition, the molecular weight increases with about 7 kDa, but because of the attraction of water molecules, the hydrodynamic size increases even more (95–250 kDa). Increasing the degree of PEGylation decreases the elimination clearance and the volume of distribution (Fig. 14). Since the elimination clearance usually decreases relatively more than the decrease in volume of distribution, the elimination half-life of PEG IL-2 is longer than for IL-2. Based on the relationship between elimination clearance and effective molecular

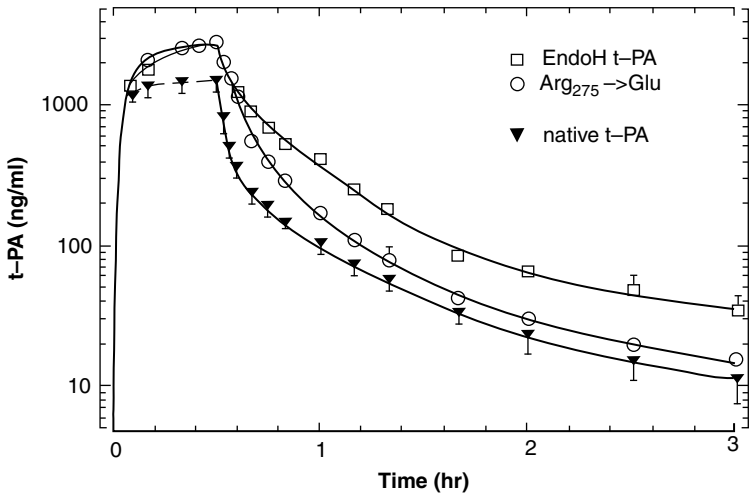

Figure 13 t-PA plasma concentrations after 30 minutes IV infusions of 0.6 mg/kg t-PA in groups of four rabbits. The figure shows the marked effect on clearance of a single amino acid mutation ($Arg_{275} \rightarrow Glu$) or of removal of high mannose carbohydrate at Asn_{114} by the enzyme endoglycosidase H (EndoH t-PA), as compared to native t-PA. *Source*: From Ref. 85.

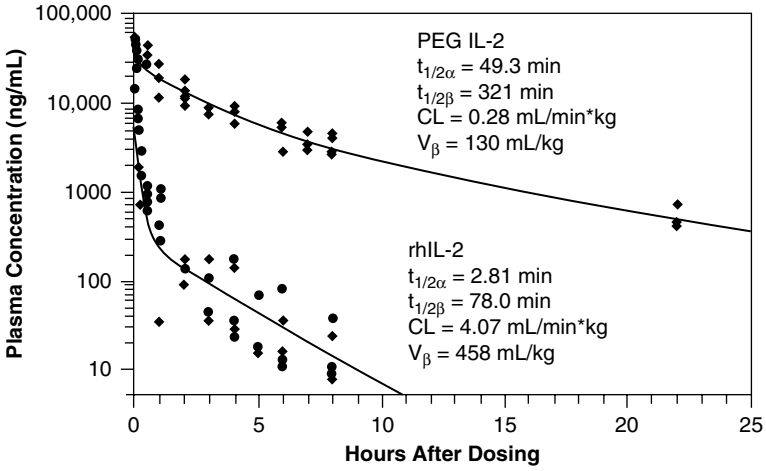

Figure 14 Pharmacokinetics of recombinant human interleukin-2 (rhIL-2) and its PEGylation form (PEG IL-2) in rats after IV bolus administration of 0.25 mg/kg. The data were described by a linear two-compartmental pharmacokinetic model.

weight, it is possible to calculate the optimal degree of PEGylation to obtain the desired systemic exposure (86,87).

The effect of prosthetic sugar groups on elimination and targeting is illustrated by the comparison of the PK of native glucose-oxidase (GO), deglycosylated GO (dGO), and galactosylated GO (gGO) in mice (88). A saturable mechanism was responsible for GO and dGO uptake by mononuclear phagocytes, although there was a substantial difference in elimination half-life (10 minutes for GO; 100 minutes for dGO). In contrast, gGO had a half-life of four minutes and was taken up preferably by hepatocytes, presumably through hepatic galactose receptors. This is an example where RME through sugar-recognizing receptors is an efficient hepatic uptake mechanism for glycoproteins. However, when terminal sialic acid residues on the carbohydrate moieties of glycoproteins shield the receptor-binding sugars, hepatic RME is lower than for the desialylated analogues (21). This has been demonstrated for rEPO and rGM-CSF (29). The protection by sialic residues appears to be a natural mechanism essential for the normal survival of enzymes, acute-phase proteins (such as α_1-acid glycoprotein), and most plasma proteins of the immune system.

IMMUNOGENICITY

Immunogenicity is the ability to induce the formation of antibodies, a prerequisite for antigenicity, which is the ability to react with specific antibodies. Immnogenicity is an important property distinguishing most biologic products from most small drug molecules. An immunogenic response to heterologous (nonhost) proteins is expected, as antibody formation is also often observed after chronic dosing of human proteins in animal studies. However, recombinant human proteins may also stimulate the production of circulating antibodies in chronic human therapy and clinical studies. In this case, immunogenic responses are sometimes associated with the formation of protein aggregates, altered proteins forms or fragments, such as acetylated protein or proteins with broken disulfide bridges (e.g., for IFN). In other cases, impurities from cell substrates or media components are either directly immunogenic or act as adjuvants to stimulate antibody formation against the protein.

Immunogenic responses can cause a wide variety of unwanted effects, with different degrees of severity. Safety issues include the potential for injection site reactions, systemic hypersensitivity reactions, and anaphylactic shock in some cases. As an example, bovine Cu,Zn-superoxide dismutase (Cu,Zn-SOD) (Orgotein) as a treatment for various arthritic diseases was withdrawn from several European countries because of hypersensitivity. Asparaginase from bacterial origin (*E. coli*), indicated in the therapy of acute lymphocytic leukemia, causes a very high level of allergic reactions (3–73% incidence) (89). The manufacturer of asparaginase has

a scheme for skin testing and desensitization should skin tests be positive prior to therapy. Another one of the few nonhuman proteins on the market is the thrombolytic streptokinase, produced in group C β-hemolytic streptococci. Levels of antistreptokinase antibodies can be present in patients as a result of a recent streptococcus infection, and therefore, allergic reactions have been noticed (1–4% incidence), some anaphylactic and anaphylactoid responses (89). The manufacturer cautions against readministration within a period of 5 days to 12 months of either administration of streptokinase or development of a streptococcus infection. Human antibodies have been observed to recombinant human proteins for human IFN, human growth hormone (hGH), human insulin, and human factor VIII. Hypersensitivity reactions are however rather rare. In general, for human recombinant proteins, immunogenicity has not been the primary limitation for their clinical use; poor PK and PD are frequently the major obstacles for efficacy.

Immunogenicity can be a problem in the study (and use) of protein drugs since the presence of antibodies can complicate the interpretation of preclinical and clinical studies by inactivating (neutralizing) the biological activity of the protein drug. Additionally, protein–antibody complex formation may affect the distribution, metabolism, and elimination of the protein drug. Neutralizing antibodies may inactivate the biological activity of the protein by blocking its active site or by a change of the tertiary structure by steric effects. Antibodies are most likely to be induced when the protein is foreign to the host. Examples of such situations are when mouse-derived monoclonal antibodies are administered to humans, or when human recombinant proteins are tested for safety in animals. Extravascular injections (e.g., s.c., i.m.) are also more likely to stimulate antibody production than IV administrations, presumably because of the higher degree of protein precipitation and aggregation at the injection site. This was demonstrated for IL-2 (90) and INF-α (91,92).

Antibodies may directly neutralize the activity of the protein. This has been observed for IFN in the presence of neutralizing IgG, for example. If neutralization occurs, it indicates that at least some fraction of the antibody population binds at or near the active site, which blocks activity (93). Irrespective of the neutralizing capabilities of the antibodies formed, they may also indirectly affect the efficacy of a protein drug by changing its PK profile (Fig. 15). Elimination clearances of protein drugs may be either increased or decreased by antibody formation and binding. An increase of the clearance is observed if the protein–antibody complex is eliminated more rapidly than the unbound protein (94). This may occur when high levels of the protein–antibody complex stimulate its clearance by the reticuloendothelial system (95). In other situations, the serum concentration of a protein can be increased if binding to an antibody slows down its rate of clearance, because the protein–antibody complex is eliminated slower than the unbound protein (93). In this case, the complex may act as a depot for the protein and, if the antibody is not neutralizing, a longer duration of the pharmacological action may occur. For example, the clearance of rIFN α-2a in cancer patients was increased because of an antibody

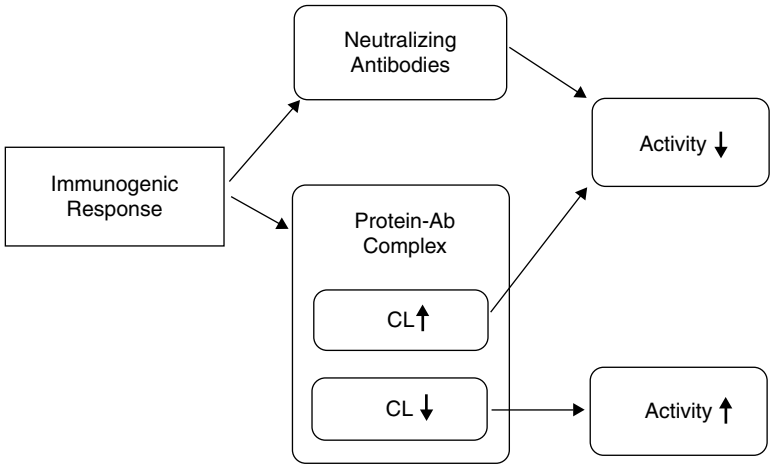

Figure 15 Effect of antibody formation on pharmacokinetics and pharmacodynamics of protein drugs.

response. In contrast, human leukocyte INF-α in rats was decreased 15-fold when circulating antibodies were present. A decrease of clearance in the presence of antibody titers was also detected for t-PA in dogs (93).

Both an increased and decreased clearance is possible for the same protein, dependent on the dose level administered. At low doses, protein–antibody complexes delay clearance because their elimination is slower than the unbound protein. In contrast, at high doses, higher levels of protein–antibody complex result in the formation of aggregates, which are cleared more rapidly than the unbound protein.

The most worrisome situation occurs when neutralizing antibodies are formed during chronic therapy with a protein drug, and when the antibodies cross-react with the endogenous protein or another endogenous factor (89). This is especially a safety concern if the endogenous protein has a unique type of activity, and there is no redundant mechanism to compensate for the activity loss of the neutralized factor. As an example, humans dosed with thrombopoietin (TPO) developed long-term thrombocytopenia, which is believed to be caused by the neutralizing activity of antibodies against endogenous TPO (89). Apparently, TPO is the only factor really important for the formation of platelets. Some patients appeared to have preexisting antibodies to TPO. Preexisting antibodies were also detected for IFN in cancer and HIV patients.

Besides route of administration and product characteristics, other immunogenic determinants are dose and regimen, disease, and concomitant medications. Typically, larger proteins are more immunogenic than smaller ones. The effect of dose size on the antibody response is unpredictable, although cumulative dose may be more important than the daily dose. With IFN, for example, a higher cumulative dose resulted in less neutralizing antibodies. Time, more so than dosing frequency, is important, since any antibody response needs weeks to months to develop fully. In humans, IgM levels appear after five to seven days, while IgG serum concentrations peak three to four weeks after dosing initiation. Patients with infectious diseases, presumably because of a stimulated immune system, showed higher antibody levels than cancer patients, who are typically immunosuppressed. Similarly, autoimmune disease state is a factor that might stimulate immunogenicity responses, while a lower response is possible in patients with kidney and liver disease. Immunosuppressants such as cyclosporin as concomitant medication may diminish the immunogenic response.

Because of the different possible effects of an immunogenicity response on the PK/PD of protein drugs, the study of an antibody response is very important in the drug development process. However, the presence of an immunogenic response in animal studies is rarely a prediction of a similar occurrence in humans. More importantly, the value of certain preclinical toxicology studies may be questioned when large titers of neutralizing antibodies are measured, because a lack of toxicity findings may be caused by the neutralization of the toxicodynamic effect. For the situation in humans, the measurement of antibody, and neutralizing antibody titers, in chronic clinical studies is important.

CONCLUSIONS AND IMPLICATIONS FOR PRECLINICAL DRUG DEVELOPMENT

In summary, the PK/PD of biotechnologically derived molecules is unique and amenable to mechanistic evaluations. These evaluations provide sound fundamental background for extrapolation across species and for prediction of outcomes under various dosing regimens.

Proteins and chemically modified proteins—including glycoproteins—often possess similar absorption, distribution, and elimination mechanisms across species. Through understanding differences in physiology and anatomy of those species, systems analyses can be conducted to extrapolate findings into predicted human outcomes.

Similarly, when the PK/PD of these molecules have been characterized in humans, with the support of the preclinical database, one can predict outcomes when doses, routes of administration, and dose frequencies are modified. It becomes particularly important in human evaluations to understand the mechanism of elimination since it is common for manufacturing changes to occur in the clinical or commercial setting. Here, the preclinical database provides invaluable insight into potential changes in human efficacy or safety.

Antigenicity remains a unique and often troublesome property of these molecules. While antigenicity can result in simple binding complexes, they can also neutralize the pharmacologic activity of the molecule and may cross-react with endogenous or similar molecules. These latter

responses can result in profound and chronic toxicity. Understanding the outcome of induced antibodies on the PK/PD of large molecules in preclinical models provides an understanding of safety that cannot be studied in humans.

While the issues of large molecule drug development are unique from small molecules, those issues can be challenging and complex. Nevertheless, biotechnology has proven itself as a realm of therapeutic intervention that can treat some of our most daunting and destructive diseases. Indeed, our understanding of these diseases, the mechanisms by which we can modulate disease pathways and the technology around development science will continue to fuel the success of biotechnology.

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6 Preclinical Pharmacokinetic–Pharmacodynamic Modeling and Simulation in Drug Development

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INTRODUCTION

Drug development is an evolving, organic process with information being collected from a variety of different sources. Decision makers are forced to merge such diverse data into a form usable for decision making. Even if such data can be merged, decision makers often need to draw extrapolations to conditions not studied in the original experiments. For instance, they may have to make a prediction about how a drug may behave in humans based on data derived from animals. It is well recognized that current drug development processes are inefficient and that the cost of drug development continues to increase while the number of new chemical entities submitted to regulatory authorities continues to decrease.

Regulatory authorities recognize there is a problem and they are taking steps to remedy the problem. In 2004, the Food and Drug Administration (FDA) initiated the Critical Path Initiative, which is designed to stimulate and modernize the drug development process. In 2006, the FDA, in conjunction with external scientists, issued their opportunities report, which identified specific areas that could be improved. One area identified that could improve decision making was the application of "mathematics, statistics, and computational analysis to biological information" (1). Specifically, the FDA stated that model-based drug development (MBDD), which is "the development and application of pharmaco-statistical models of drug efficacy and safety from preclinical and clinical data to improve . . . knowledge management and decision making" (2), "holds vast potential to support more efficient and effective development of drugs and medical devices" (1). Another regulatory authority, the European Medicines Agency concurred with the FDA's findings and stated in a report from a think-tank on innovations in drug development that "further considerations should focus on biomarkers, modeling & simulation, and emerging clinical trials methodology" (3).

Given the imprimatur from these agencies, MBDD is being increasingly applied to aid in decision making. A number of reviews have been written on the subject in recent years (2,4–7) and it would be redundant to write another review article on the subject. Scientists that study how people learn state that incorporating anecdotes and stories into the teaching process facilitates learning. Indeed, the case–study approach is regarded as a highly efficient way to teach and many graduate schools in business and law utilize this approach in their curriculum. This chapter will briefly review what is MBDD and will then focus on some interesting case studies where such models developed preclinically helped guide clinical drug development.

WHAT IS MODEL-BASED DRUG DEVELOPMENT?

A system is a collection of interacting objects that operate in space and time. A car, a computer, or a living organism, all represent different types of systems. A model is any representation of a system that accounts for the properties of the system at some point in space and time. Certainly many classes of models exist. One that comes immediately to mind is a scale model wherein some physical object is recreated and scaled to a size that is more convenient for viewing, for example, an architect's design of a new building. In pharmacokinetic–pharmacodynamic modeling, which is synonymous with exposure–response modeling, the models are mathematical and statistical in nature. A pharmacokinetic model describes the relationship between dose and drug concentration, usually in plasma or serum, while the pharmacodynamic model relates

drug concentration to efficacy, adverse events, or other outcome measures. Given a pharmacokinetic model, predictions can be made regarding changes in dose frequency or total dose administered and their effect on the pharmacodynamic marker.

Translational models extend traditional pharmacokinetic–pharmacodynamic models, for example, a two-compartment pharmacokinetic model with an effect compartment to explain the pharmacodynamics, by treating the compartments as physiologic entities, at least in terms of the pharmacodynamic response. Sometimes physiological-based pharmacokinetic models are linked to more mechanistic models but what is typically seen is an empirical pharmacokinetic model linked to a physiologically relevant pharmacodynamic model (8).

Modeling serves many useful purposes. One is that it characterizes and summarizes a set of data into a cohesive structure. For example, given a set of concentration–time data, a pharmacokinetic model summarizes the data into a few simple parameters, such as clearance and volume of distribution. Second, and most importantly, is that modeling allows predictions to be made, a process that is referred to as simulation. Given a pharmacokinetic–pharmacodynamic model, predictions on outcome or safety can be made regarding changes in dose, dose frequency, or changes in the parameters that describe the system, such as the increase in exposure if renal clearance were decreased in patients with renal failure.

MBDD links pharmacokinetic and pharmacodynamic models from nonclinical, preclinical, and/or clinical data with other models, such as models of disease progression, compliance, and drop-out rate, to gain insight into the factors that determine efficacy and safety (*top* of Fig. 1). Preclinical MBDD uses a similar approach by modeling the pharmacokinetics and/or pharmacodynamics from nonclinical and preclinical studies and then scaling the results to humans (*bottom* of Fig. 1). The power of a MBDD approach is that it allows information from a variety of different platforms to be integrated into a single cohesive framework that can be used to understand the data and answer questions about the data.

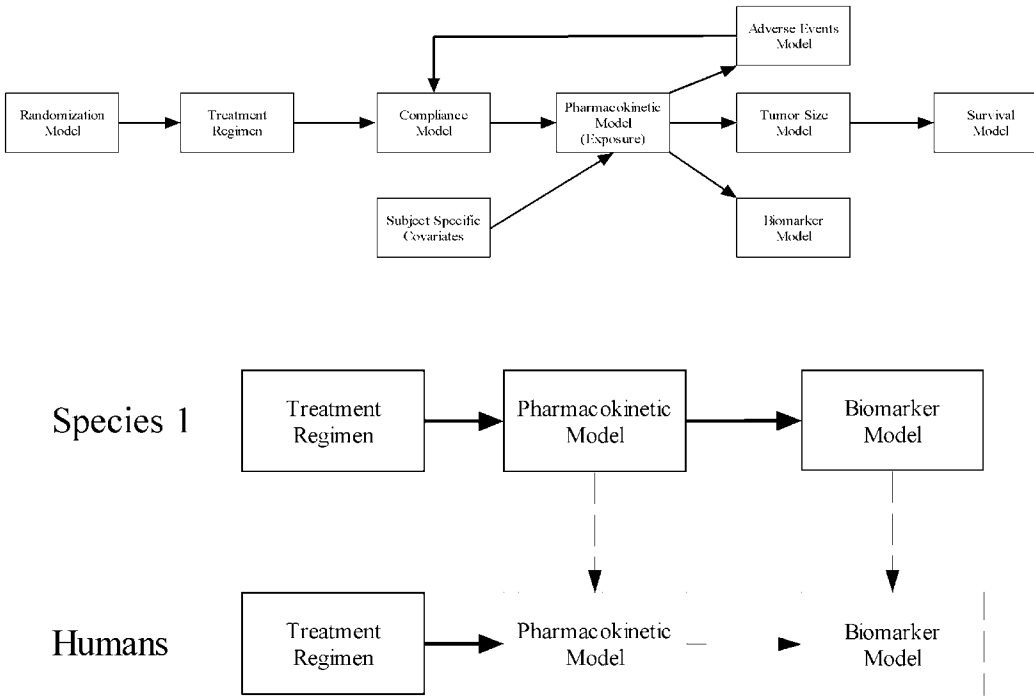


Figure 1 Schematic of an exposure–response model used in clinical model-based drug development (*top*). In this model, patients are randomized to treatment for a new cancer therapy. Each of the component submodels are linked to the model to produce the outcome of interest, survival. In this example, side effects may affect compliance. It is also believed that tumor reduction leads to increased survival. Bottom figure shows a preclinical model where data are available in animals and then are scaled to humans. The dashed lines show models that are scaled based on the preclinical results.

In the first edition of this book, the statement was made to the effect that the application of preclinical models to help guide clinical development was not often done because of the leap of faith required in moving from animals to man. That statement is no longer true. Drug companies are integrating modeling and simulation (M & S) in drug development earlier and earlier. About 10 to 15 years ago, it was recognized that drug attrition was mostly due to pharmacokinetic failures, that is, poor absorption or high metabolism. That has changed. With all the preclinical and nonclinical models available today, such as microsomes, hepatocytes, CACO-2 cells, and so on, pharmacokinetic characteristics related to absorption and metabolism are well understood before entering the clinic. It is now believed that drug attrition is due to poor translation of animal models of efficacy and poor understanding of the factors influencing safety. Successfully implementing model-based decision making into drug development early in the process can impact overall efficiency and success in later stages of development (9).

Before translational pharmacokinetic–pharmacodynamic modeling can occur, a number of conditions should be met before placing any credibility on the extrapolation. First, the biomarker of interest and its relation to the clinical end point needs to be credible, that is, there needs to be some biochemical or physiological rationale for measuring the biomarker. Measurement of drug concentrations and the biomarker(s) of interest, while not needing to be validated to the level indicated by the Guidance to Industry issued by the FDA on Bioanalytical Method Validation (10), should be sufficiently precise, accurate, repeatable, and reliable for the results to have value. Ideally, a good link between pharmacokinetics and pharmacodynamics needs to be established. Greater confidence is placed in models where the relationship between drug concentrations and pharmacodynamic effect is directly related and can be seen with the eye, such as when a linear model or E_{max} model is appropriate. Analysts and project team members more readily accept the outcome. Model credibility is decreased, the greater the degree of mathematical and statistical manipulation that goes into establishing the relationship between drug concentration and pharmacodynamics. Also, the greater the number of assumptions that go into a model, the more likely some of these assumptions are wrong. The impact of these inaccuracies must be assessed before a model will be accepted as credible.

Even if a well-defined relationship between drug concentration and effect is established in animals, there is no guarantee the relationship will hold in humans. We all understand that animals and humans are different, so making the extrapolation from animals to man becomes a leap of faith that animals and man are more similar than dissimilar. The more dissimilar the pharmacology/physiology between the animal species and humans, the more tenuous is the extrapolation. Hence, the physiology of the system under study should be understood and species differences must be identified and corrected for during the extrapolation process. A great help in this regard are lead compounds that have previously had a pharmacokinetic–pharmacodynamic model established preclinically and then tested and validated in humans. Then there is some experience in the validity of the model, and extrapolation should the lead compound fail and back-up compounds having the same mechanism of action are created.

CASE STUDIES

The remainder of this chapter will deal with case studies where pharmacokinetic–pharmacodynamic models established preclinically were used to help guide clinical development or answer some question that could not be addressed in humans.

Case Study 1: Translational Modeling in Oncology

This example will illustrate how preclinical information can be translated to humans and be used to help develop a first-time-in-man (FTIM) study. Cancer is a leading cause of death. Since its creation, the National Cancer Institute's Developmental Therapeutics Program has utilized a variety of different nonclinical and preclinical models to screen potential drug candidates for oncolytic activity. Of these models, mouse tumor xenograft models are the gold standard used to assess anticancer activity. In this model, athymic nude mice are implanted subcutaneously in the hind flank with tumor fragments of human cancers (either direct implantation of patient biopsies or inoculation of continuous human tumor cell lines) that are allowed to grow to measurable dimensions and then are treated with the agent of interest. Tumor size is then followed until death, the tumor is of sufficient size that it is unethical to continue treating the animal in which

case the animal is sacrificed, or the experiment is terminated. Almost all drugs approved for the treatment of solid tumors have been tested in this screen and have shown activity.

The model is not without its controversies, however, with major criticisms being the model is done in a mouse without an immune system, the tumor is growing at an artificial site, and xenograft tumors almost never metastasize (11, 12). Further, activity in the xenograft model does not necessarily correlate with activity in humans. In one study of 39 agents with both xenograft and phase 2 data available, in vivo activity in the xenograft model did not correlate with activity in the particular histology of the tumor in humans, for example, activity in breast cancer cell lines did not correlate with activity in patients with breast cancer. On the other hand, xenograft models have their advantages. In an article by Johnson et al. (13), 45% of drugs that showed activity in at least one-third of the xenograft models tested also showed activity in humans (13). Only lung cancer xenograft models appeared to be predictive of lung cancer activity in humans. Most importantly, however, drugs that are inactive in xenograft models are almost always inactive in humans.

Because of the high false-positive rate, some clinical investigators place little value in preclinical animal models. In these xenograft models, mice are usually dosed at the highest dose that is tolerated without any overt side effects. Inaba and colleagues at the Japanese Foundation for Clinical Research in a series of studies have shown that one reason for the high false-positive rate is that the maximum tolerated dose (MTD) in mice is often four to five times higher than the MTD in humans (14–16). When mice were dosed at doses that produced equivalent concentrations as seen in humans dosed at clinically active doses, the pattern of response was similar between mice and humans (16–18).

Recently, mathematical advances have made it possible to model cancer from a mathematical and statistical point of view (19). A variety of different models have been developed to model tumor growth kinetics. Laird (20) was the first to show that tumor growth could be described by a Gompertz model, which is still considered to be the best mathematical descriptor of tumor growth and is the expectational model for tumor growth based on theoretical considerations (21). The Gompertz equation has the integrated form

$$W = W_0 \exp\left(\frac{A}{\alpha}(1 - \exp(-\alpha t))\right) \tag{1}$$

where W is the tumor size, W_0 is the baseline tumor size, A and α are constants controlling the maximal tumor size and rate of growth, and t is time. Liang and Sha (22) applied this model to xenograft data using a nonlinear mixed effects model. A related model, the logistic model, also called the Verhulst–Pearl or simply the Verhlust equation, was proposed by Swan (23) and takes the form

$$W = \frac{1}{1 + \left(\frac{1}{W_0} - 1\right)\exp(-rt)}, \quad r > 0 \tag{2}$$

where r controls the rate of growth. Both the Gompertz and logistic models are members of the same class of growth curves, the generalized Bertalanffy–logistic model (24), and are generally regarded as being empirical in nature, although it has been argued that the Gompertz has a theoretical basis based on the topology of tumor growth (21,25).

Simeoni et al. (26,27) first reported on a new class of models that were semimechanistic in nature (Fig. 2). In their xenograft studies no plateau phase was observed, which can make fitting a Gompertz or logistic model difficult. To account for this observation, they focused on the exponential and linear phases of tumor growth. They proposed that cell growth in control animals could be explained by the following differential equation

$$\frac{dW(t)}{d(t)} = \frac{\lambda_0 W(t)}{\left[1 + \left(\frac{\lambda_0}{\lambda_1} W(t)\right)^{\Psi}\right]^{1/\Psi}} \tag{3}$$

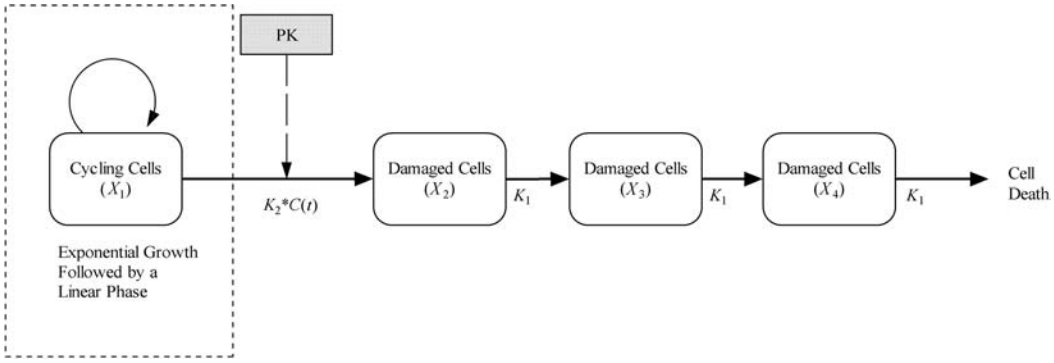


Figure 2 Schematic of the tumor growth model proposed by Simeoni et al. (26). In this model, tumor growth proceeds exponentially at the beginning and then plateaus to a linear phase, that is, from a first-order to zero-order process. Oncolytics cause the cells to start along the path from cycling to damaged cells to cell death. The total weight of the tumor is the sum of weights in each compartment. This model was proposed to account for the lack of a plateau phase in growth kinetics of the tumors in their experimental sets. See text for details.

where λ_0 and λ_1 control the rate of tumor growth and are a measure of the aggressiveness of the tumor and ψ, which is fixed to a value of 20, allows the system to pass from first-order to zero-order kinetics. For mice treated with a cancer drug the complete system of equations is

$$\begin{aligned}
\frac{dX_1(t)}{dt} &= \frac{\lambda_0 X_1(t)}{\left[1 + \left(\frac{\lambda_0}{\lambda_1} X_1(t)\right)^{\Psi}\right]^{1/\Psi}} - K_2 C(t) X_1(t) \\
\frac{dX_2(t)}{dt} &= K_2 C(t) X_1(t) - K_1 X_2(t) \\
\frac{dX_3(t)}{dt} &= K_1 X_2(t) - K_1 X_3(t) \\
\frac{dX_4(t)}{dt} &= K_1 X_3(t) - K_1 X_4(t) \\
W(t) &= X_1(t) + X_2(t) + X_3(t) + X_4(t)
\end{aligned} \tag{4}$$

where $C(t)$ is the concentration of drug at time t. This model was then demonstrated to apply to irinotecan, paclitaxel, 5-fluorouracil, and three undisclosed drugs.

Since their initial publication, this group has continued to explore the use of this model and its properties. Rocchetti et al. (28) showed that the ratio λ_0/K_2 can be used to estimate the threshold concentration such that if animals are exposed to concentrations exceeding the threshold, the model predicts complete tumor eradication. Magni et al. (29) presented a mathematical analysis of the properties of the model, while Simeoni et al. (30) and Poggesi et al. (31) presented the model in the context of a nonlinear mixed effects model.

This model is being increasingly seen in the pharmacokinetics literature and community, most likely due to the recent "advertising" of the model at meetings frequented by other modelers, while the old-standby models like the Gompertz and logistic models are being relegated to the dustbins of history. Gibiansky et al. (32) report on using the model where an effect compartment is used to account for a delay in tumor regression and drug concentrations. Stuyckens et al. (33) reported on how the model can be modified to account for drug resistance through an empirical exponential decline in K_2 over time after some initial lag period. Bueno et al. (34), instead of linking their drug concentration to tumor dynamics, linked a series of biomarkers pSmad and MSPT, to tumor growth kinetics so that the delay in drug effect could be explained mechanistically.

To illustrate the application of the Simeoni model, a potential new oncolytic was tested in the mouse xenograft model. Male nude mice were dosed every other day × 3 which was repeated seven days later. Tumor size was measured for 49 days or until the tumor reached approximately 2500 mm^3 at which time the animals were sacrificed. Tumor size was monitored periodically. In addition, pharmacokinetic data in whole blood were generated in rats after intravenous (IV) administration of 1 mg/kg and in plasma and tumor tissue after IV administration of 2 mg/kg in mice. The data in rats were scaled down to mice assuming all clearance terms had an exponent of 0.7 and volume terms had an exponent of 1.0. So, for example, clearance was modeled as

$$\mathrm{CL}_{\mathrm{mouse}} = \mathrm{CL}_{\mathrm{rat}} \left(\frac{0.02\,\mathrm{kg}}{0.25\,\mathrm{kg}} \right)^{0.7}. \tag{5}$$

Both sets of data in mice and rats were fit to the same model simultaneously. The following assumptions were made:

- whole blood and plasma were in equilibrium;
- drug concentrations in the tumor were dependent on the plasma concentrations of the drug, but plasma concentrations were not dependent on tumor concentrations;
- a delay existed between drug concentration in tumor and tumor size; and
- the effect on the tumor was dependent on the concentration of drug in the delay compartment.

Modeling suggested that a simpler model than the original Simeoni model would support the data (Fig. 3). So, instead of three transit compartments, no transit compartments were used and it was assumed that after the delay in equilibrium cell death would be instantaneous.

The pharmacokinetic model fit the rat data better than the mouse but was a reasonable fit to all sets of data (Fig. 4). The pharmacodynamic model also fit the data well, although

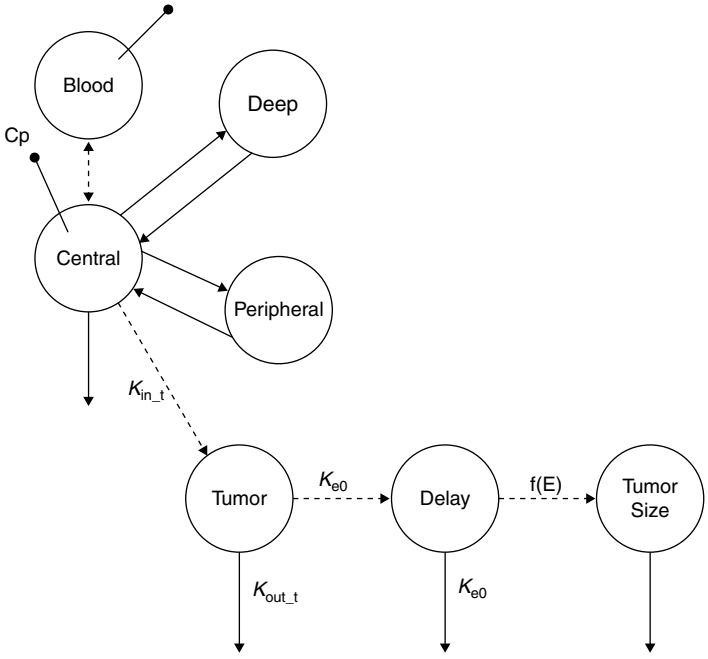
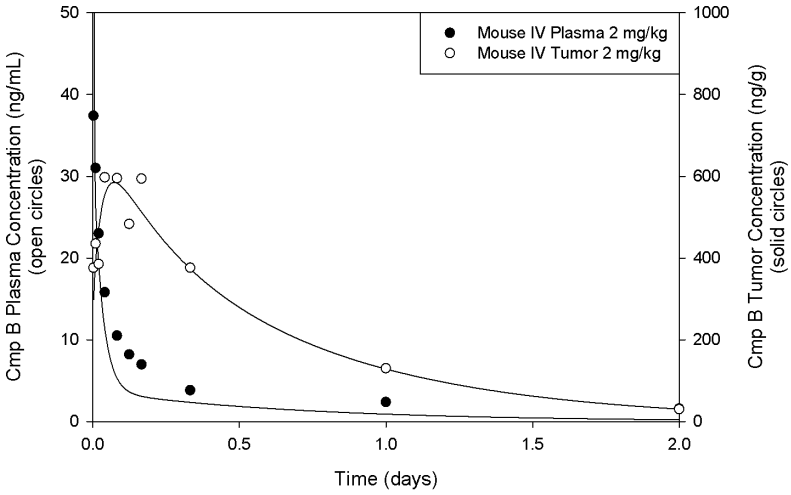
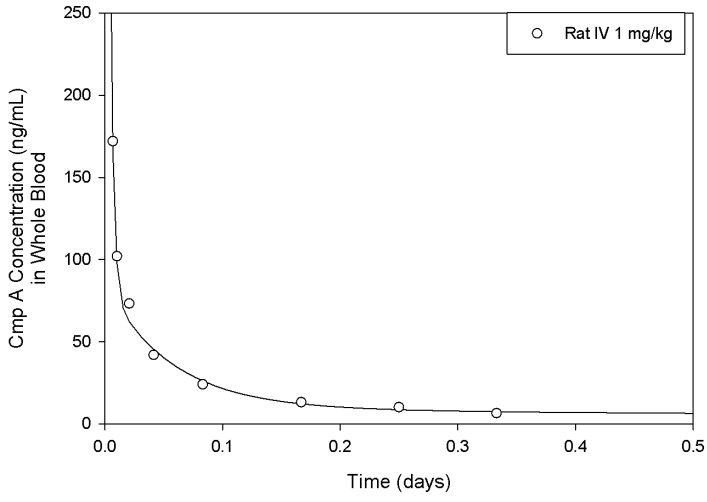


Figure 3 Schematic of the pharmacokinetic and pharmacodynamic model used to model the pharmacokinetics of a new cancer agent. The distribution kinetics of the drug were governed by a three-compartment model, where plasma and whole blood were in equilibrium. Tumor concentrations were driven by concentrations in the plasma but plasma concentrations were not affected by tumor concentrations. A delay between drug concentrations in the tumor and effect on the tumor was created. The size of the tumor was a function of the drug concentration in the delay compartment and was governed by a modification of the model of Simeoni (26).





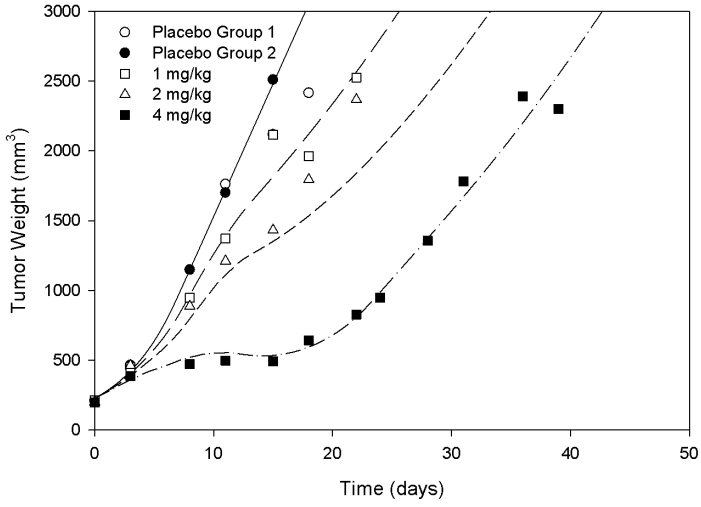


Figure 4 Goodness of fit of the pharmacokinetic data in mice (*top*), pharmacokinetic data in rats (*middle*), and pharmacodynamic (*bottom*) data to the new chemical entity assuming the model in Figure 3.

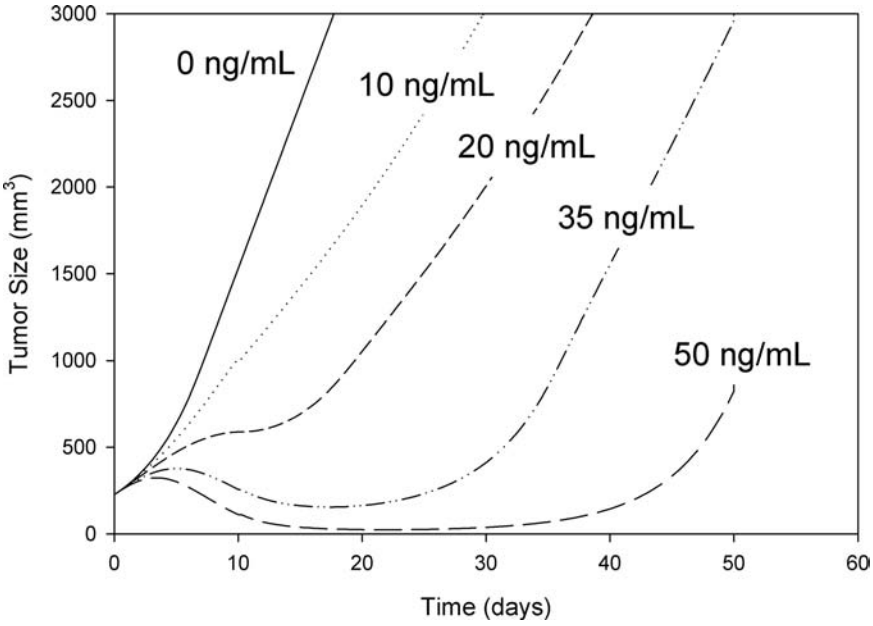


Figure 5 Simulated tumor growth curves for steady-state concentrations of the drug for 10 days. Tumor growth is delayed when steady-state concentrations exceed 35 ng/mL.

it must be stressed that this model has no asymptote and will predict infinite tumor growth if allowed to progress long enough, an effect that is clearly inconsistent with actual tumor growth. In this regard, the Gompertz model is superior. Nevertheless, using the modified Simeoni model, simulating a steady-state plasma concentration of 0 to 50 ng/mL for 10 days produced the simulated tumor growth curves as seen in Figure 5. A steady-state concentration of approximately 35 ng/mL appears to result in a significant delay of tumor growth. In the absence of other information, this concentration then becomes our target concentration in humans.

Given the pharmacokinetic model in mice and rats, the pharmacokinetics in humans can be simulated by extrapolating. So, for example, to extrapolate clearance, Equation (5) is modified to

$$\mathrm{CL}_{\mathrm{human}} = \mathrm{CL}_{\mathrm{rat}} \left(\frac{70\,\mathrm{kg}}{0.25\,\mathrm{kg}} \right)^{0.7}. \tag{5}$$

By applying this extrapolation to all clearance and volume terms in the model and still retaining a three-compartment model, the pharmacokinetics in humans can be simulated. Figure 6 presents two simulations, one being a daily × five-dosing regimen of 1 g/m^2 administered by IV infusion over three hours on each day and a 72 continuous infusion of 100 mg/day for three days. Both regimens produce concentrations in the range of tumor inhibition seen in mice and if these results were consistent with the toxicology studies, some fraction of these doses may then be used as starting doses for the FTIM study.

Case Study 2: Choosing Doses for Phase 1 and 2

Gomeni et al. (35,36) reported on the use of M&S to help select the doses for a FTIM and proof of concept study for a new unspecified agent that affects the CNS. Pharmacokinetic and plasma protein binding were available in rats, cynomolgus monkeys, and dogs as part of the toxicology program. Protein binding was also estimated in human plasma. Rodents and rhesus monkeys were studied in pharmacology efficacy studies. Only the pharmacodynamic response was available in rhesus monkeys, whereas pharmacokinetics and pharmacodynamics were available in the rodent pharmacology study. In vitro receptor binding studies were done in rhesus monkeys and man to compare the binding affinity relative to rodents.

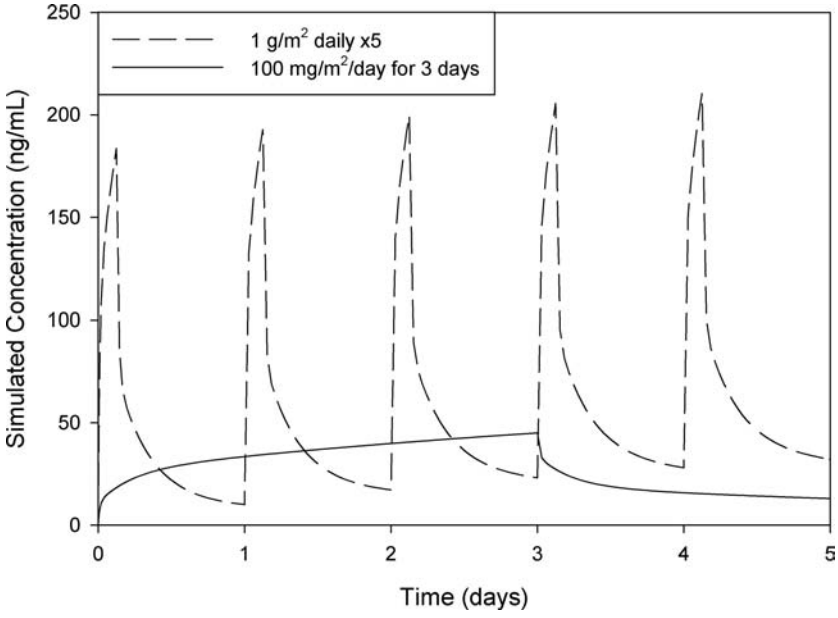


Figure 6 Allometric scaling of drug's pharmacokinetics from mice and rats to a 70 kg (1.83 m^2) human. Simulation of a 3-hour infusion of a 1 g/m^2 dose of the drug once-daily for five days and a continuous infusion of 100 mg/m^2/day for 72 hours.

Certain assumptions were made during the course of the analysis. First, unbound concentrations would be a better predictor of response than total concentrations. This assumption is a common one since only unbound (free) drug tends to cross the blood–brain barrier, unless the drug shows receptor-mediated transport into the brain, which is not that common for small molecules. Second, it was assumed that receptor binding in the brain was directly proportional to the pharmacodynamic effect measured in behavioral tests administered to rats. Hence, measurement of receptor binding could be used as a biomarker for pharmacodynamic activity. Third, the pharmacokinetics of the system were linear and independent of dose. Given these assumptions, the model development approach was as follows:

1. Use allometric scaling to predict the pharmacokinetics in rhesus monkeys based on pharmacokinetic data obtained in rats, cynomolgus monkeys, and dogs.
2. Use the protein binding information in cynomolgus monkeys to estimate the unbound drug concentration in rhesus monkeys.
3. Develop a free drug concentration–behavioral pharmacology (pharmacokinetic–pharmacodynamic) model in rodents. The exact nature of this CNS test is not reported in either manuscript.
4. Predict the pharmacodynamic response in rhesus monkeys using allometrically scaled unbound drug pharmacokinetics (steps 1 and 2) and the pharmacodynamic model in rodents (step 3) adjusting for the differences in receptor binding affinity between rodents and rhesus monkeys.
5. Compare the observed and predicted pharmacodynamic response in rhesus monkeys to validate the underlying pharmacodynamic model.
6. Predict the pharmacodynamic response in humans using
   a. unbound pharmacokinetic parameters estimated from allometric scaling of rat, cynomolgus monkey, and dog pharmacokinetic and protein binding data; and
   b. the pharmacokinetic–pharmacodynamic model obtained in rodents adjusted for the differences in receptor binding affinity between rodents and humans.
7. Optimize the pharmacodynamic response in humans using Monte Carlo simulation by identifying those doses that meet target criteria.

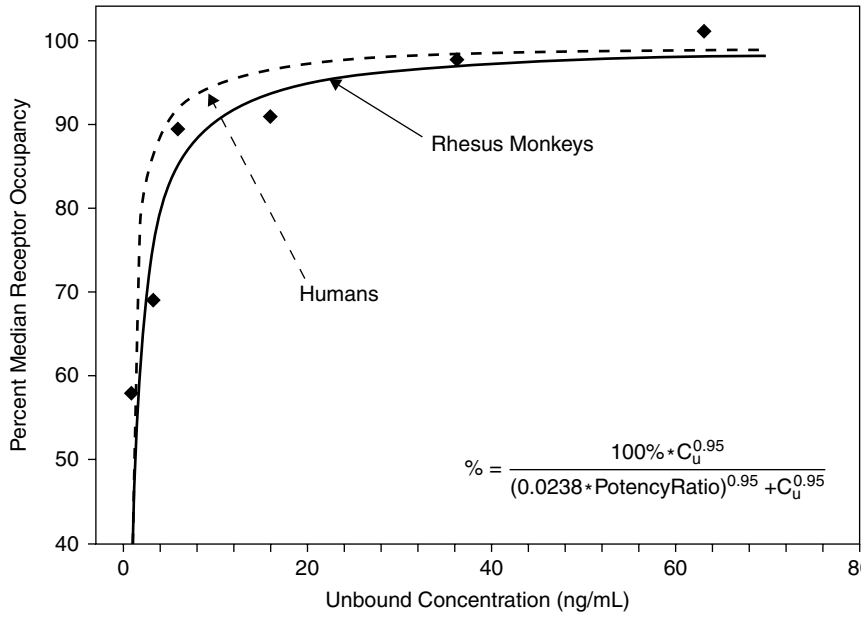


Figure 7 Observed and predicted (solid line) median brain receptor occupancy in two rhesus monkeys as reported by Gomeni et al. (35). Also shown is the predicted occupancy in humans. Predicted occupancy was based on the occupancy model (Sigmoid E_{max}) developed in rodents with EC_{50} adjusted for the differences in affinity between rodents and rhesus monkeys or humans. Maximal binding was assumed to be equal to 100%. Rhesus monkeys have 40-fold lower affinity for the receptor than rodents, whereas humans have 20-fold lower affinity. *Source*: From Ref. 35.

In vitro receptor binding data indicated that humans and rhesus monkeys have 20 times and 40 times less receptor binding affinity than rodents, respectively. The behavioral effect in rodents was best characterized using a Sigmoid E_{max} model with E_{max} fixed to 100% maximal effect, $EC_{50} = 0.0238$ ng/mL, and the shape parameter equal to 0.95. After adjusting the potency from rodent to rhesus monkeys based on in vitro receptor binding, that is, EC_{50} was multiplied by 40, receptor binding in rhesus monkeys was also well characterized (Fig. 7), thus validating the link between pharmacokinetics and receptor occupancy and receptor occupancy and pharmacodynamics.

Having validated the pharmacodynamic model, the authors moved onto finding a dose range for FTIM study. One method to choose the maximal dose in a FTIM study is some fraction of the highest dose that produces no observable toxic effects, called the no observable effect level (NOEL), in the most sensitive animal species. Another method is to choose a dose based on exposure, usually area under the curve at steady state ($AUC_{0-\tau}$), since exposure may better correlate to toxicity than dose. Using the former method, a dose ranging study from 1 to 60 mg was chosen based on one-third of the NOEL from the one-month toxicology study in dogs (5 mg/kg) and one-fifth of the NOEL in rats (3 mg/kg). Monte Carlo simulation was then done to predict the exposure in humans relative to the exposure observed in rats and dogs as part of the toxicokinetic evaluation from those studies. Because interindividual variability was unknown, as was the oral bioavailability and absorption rate constant in humans, several scenarios were evaluated:

1. oral bioavailability was fixed at 40%, 60%, or 80%;
2. interindividual variability on all the pharmacokinetic parameters was assumed to be log-normal in distribution with coefficient of variation 20%, 30%, or 40%; and
3. absorption was first order, fixed at 0.2, 0.5, or 0.8 per hour.

The drug's pharmacokinetics were assumed to follow a two-compartment model with first-order absorption. Monte Carlo simulation was then used to simulate pharmacokinetic profiles after single dose administration. Maximal concentration (C_{max}) and AUC were estimated

for each subject and the population median and 95th percentile determined. Using the most conservative assumptions (80% bioavailability, high interindividual variability of 40%, and rapid absorption of 0.8 per hour) at the highest dose studied (60 mg), the ratio of $AUC_{0-\tau}$ in the rat to the simulated 95th percentile for $AUC_{0-\infty}$ in humans (called a coverage factor) was 1.1. In the dog, the AUC cover factor was 1.6. Hence, based on either exposure or factors of the NOEL dose, both methods indicated that the top dose of 60 mg was an appropriate maximal single dose in humans.

The next set of simulations was aimed at determining how receptor occupancy behaved in a multiple dose setting with doses of 10, 30, and 60 mg once daily. Other drugs from the same family showed previously that receptor occupancy greater than 70% during a 24-hour interval maintained over a period of several weeks is efficacious clinically. Hence, Monte Carlo simulation was used to identify dosing regimens that would achieve 70% receptor occupancy at predose at steady state in the majority of subjects. Predose concentrations at steady state were used as the target variable since this represents the lowest concentration achieved by a drug once steady state is achieved. If at least 70% occupancy is achieved at predose then at least this degree of occupancy will be maintained during the dosing interval immediately after a dose is taken.

The same uncertainties in the single-dose simulations still apply with this set of simulations, but with one additional: how does the uncertainty in the receptor binding potency in humans affect the results? Hence, an additional scenario was examined. Three potency values were compared: (a) equal to results of the in vitro receptor binding study, (b) equal to the potency in rodents, or (c) equal to the average of (a) and (b). Using the most conservative assumptions (low potency equal to the results of the in vitro receptor binding study, low bioavailability of 40%, high interindividual variability of 40%, and slow absorption of 0.2 per hour) at the highest dose studied (60 mg), 95% of subjects had predose receptor binding occupancy very close (66.1%) to the target of 70%. Less pessimistic assumptions (intermediate potency equal to the average of the rodent and the in vitro binding study, intermediate bioavailability of 60%) at the 30 mg dose lead to 95% of subjects attaining predose receptor occupancies of 71%. Hence, based on these results the authors concluded that the proof of concept study should use a dose ranging study from 30 to 60 mg once daily for a week. However, they also recommended that before such a study is conducted, a positron emission tomography study in humans should be done to better characterize the pharmacodynamic model in humans. The authors did not report how well their models actually predicted the results in humans, but the authors, in a personal communication, indicated that their predictions were in full agreement with the observed data, but that due to the nature of the drug and the confidentiality policy surrounding this novel class of compounds are unable to publish their results.

This example illustrates how M&S may be used to help guide early clinical development of the drug. Drug development has historically based many critical decisions on empirical rules of thumb, such as the starting dose for a single-dose FTIM study. Then given the MTD in humans, some fraction of the MTD was used as a starting dose in multiple-dose studies. When tolerability of the multiple-dose study was established, usually in healthy volunteers without the disease of interest, the tolerability in patients having the disease was estimated relative to the efficacious dose used in the preclinical studies. Finally, this dose and maybe one or two others were taken in phase 2.

Preclinical pharmacokinetic–pharmacodynamic modeling aims to change the historical approach by making the decisions less empirical and more rational. Granted, the approach used by Gomeni et al. may seem like a house of cards but a M&S approach is still better than empiricism. The M&S approach requires the analyst to identify what is known and unknown about the drug and then evaluate the impact of those uncertainties on the outcome. Hopefully, in the end, clinical development of the drug will be more scientific and less likely to fail at later, more expensive stages of development.

Case Study 3: Comparison of Pharmacodynamics in Animals and Humans

Ferron, McKeand, and Mayer (37) reported the results of a pharmacokinetic–pharmacodynamic model of pantoprazole, an irreversible proton pump inhibitor for the treatment of reflux esophagitis, peptic ulcers, and other acid-related hypersecretory gastrointestinal disorders. In preclinical studies, a stomach catheter was inserted into female Sprague-Dawley rats and the

acid content of the effluate was measured at 15-minute intervals. Pantoprazole (0.12, 0.23, 0.38, or 1.15 mg/kg) or saline was administered by an IV bolus 1 hour after commencement of a 4.5-hour continuous infusion of 1 μg/min/kg pentagastrin, a drug that maximally stimulates gastric acid secretion. In a separate group of rats the pharmacokinetics of pantoprazole were characterized after IV infusion of 5 mg/kg over one minute.

In the clinical studies used to bridge the preclinical results to the human results, pantoprazole pharmacokinetics and pharmacodynamics were studied in humans in three different studies. In the first pharmacokinetic study, healthy male volunteers were randomized in a four-period cross-over study to receive either 10, 20, 40, or 80 mg pantoprazole as a 15-minute IV infusion, while in a second similar study, healthy male subjects were randomized to receive either 10, 20, 40, or 80 mg enteric-coated tables. In both studies, serial blood samples for drug concentration analysis were collected for 24 hours. In a separate pharmacodynamic study, healthy volunteers who were *Helicobacter pylori* negative were administered pentagastrin 1 μg/kg/hr for 25 hours. Using a crossover design, subjects were randomized to receive either placebo, a single dose of pantoprazole (20, 40, 80, or 120 mg) infused over 15 minutes, or a single oral dose of pantoprazole 40 mg as an enteric-coated tablet. Gastric aspirates were collected by a nasogastric tube every 15 minutes for 2 hours and every 30 minutes thereafter until the end of study. The acid contents of the gastric contents were measured using titration.

The pharmacokinetics of pantoprazole were characterized using a one-compartment model in rats and a two-compartment model in humans. To account for the oral administration of an enteric-coated tablet, a lag compartment was used. Mean concentrations at each dose group were determined and the pharmacokinetic model fit to the data simultaneously for all dose groups. An indirect, irreversible pharmacodynamic response model was used to characterize the pharmacodynamics of pantoprazole in both animals and humans. Since a plot of pH versus time in the placebo group was essentially constant, the rate of acid output in the effluate (R) was described by

$$\frac{dR}{dt} = K_{\text{prod}} - K_{\text{deg}}R \tag{6}$$

where K_{prod} is the zero-order rate of acid production in the absence of drug (with units mass/hour) and K_{deg} is the endogenous degradation rate of acid (with units/hour). At steady state

$$\frac{dR}{dt} = 0 \tag{7}$$

and $K_{\text{prod}} = K_{\text{deg}}R_{\text{ss}}$, where R_{ss} is the basal rate of acid production. In the presence of pantoprazole an irreversible loss to R is added to the model

$$\frac{dR}{dt} = K_{\text{prod}} - K_{\text{deg}}R - kRC_{\text{p}} \tag{8}$$

where k is the rate of apparent reaction constant of pantoprazole with the proton pump and C_{p} is the plasma pantoprazole concentration.

The mean pharmacokinetic parameters were used as inputs to the pharmacodynamic model and the pharmacodynamic model parameters were estimated. The model was able to characterize the pharmacokinetic and pharmacodynamic end-points across all doses studied (Fig. 8) and was able to predict the rate of acid output after oral administration. The apparent reaction rate between pantoprazole and the proton pump was similar between species (0.691 L/mg/hr for rats and 0.751 L/mg/hr for humans) as was the basal rate of acid output (0.44 mmol/hr/kg for rats and 0.33 mm/hr/kg for humans).

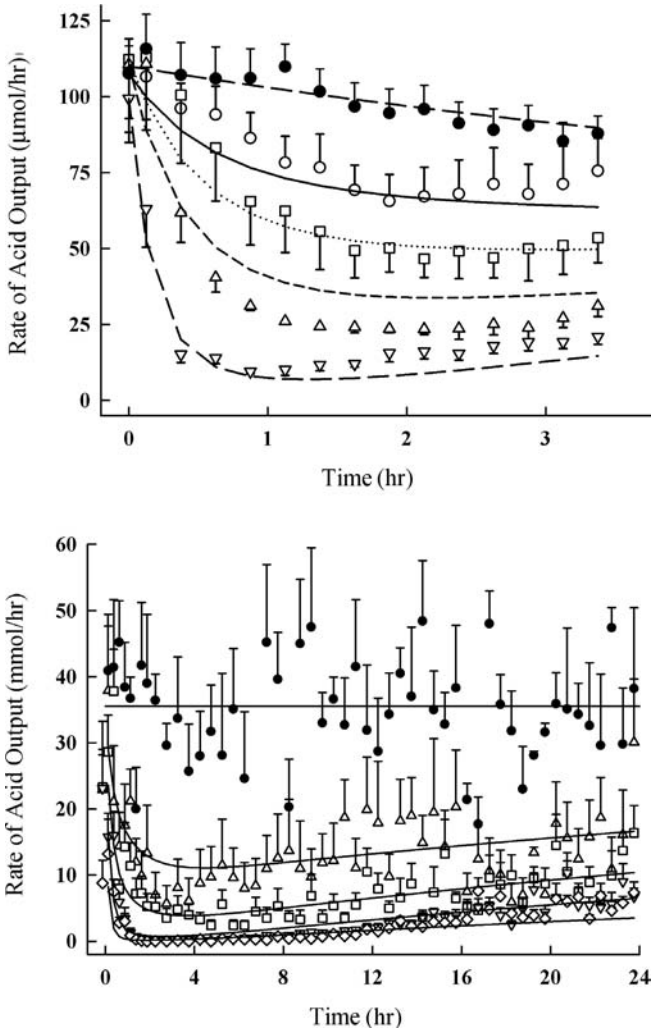


Figure 8 Mean profiles of rate of acid output in rats (*top*) and humans (*bottom*) after IV administration of pantoprazole following pentagastrin acid stimulation as reported by Ferron et al. (37). (*Top*) Solid circle, placebo; open circle, 12 mg/kg; open square, 0.23 mg/kg; open triangle, 0.38 mg/kg; open upside-down triangle, 1.15 mg/kg; open diamond, 5 mg/kg. (*Bottom*) Solid circle, placebo; open circle, 10 mg; open triangle, 20 mg; open square, 40 mg; open upside-down triangle, 80 mg; open diamond, 120 mg. Figure courtesy of Dr. Philip Mayer, Wyeth Laboratories.

Using the estimated pharmacokinetic and pharmacodynamic model, the pharmacokinetic and pharmacodynamic profile after oral administration of the 40 mg enteric-coated tablet was simulated and compared to observed data for validation. The authors then used computer simulation to evaluate the effect of single versus multiple IV and oral doses of pantoprazole 10 to 120 mg and IV infusions of 80 mg with infusion lengths varying from 0.5 to 12 hours. The simulations showed that acid output is related to extent of exposure. Acid inhibition increased and remained inhibited longer as dose was increased.

Using the pharmacokinetic and pharmacodynamic model parameters reported by Ferron, McKeand, and Mayer (37), we simulated plasma concentration and acid output profiles for once-daily dosing of 10, 25, 40, and 55 mg pantoprazole. The results are shown in Figure 9. Despite no accumulation of pantoprazole even at the highest dose, repeated administration suppressed acid output after a few days of dosing, even at the lowest dose. The difference between the doses was largely the time to maximal suppression. Increasing doses resulted in a decrease in the time to maximal suppression, to a point. A difference between 10 and 25 mg

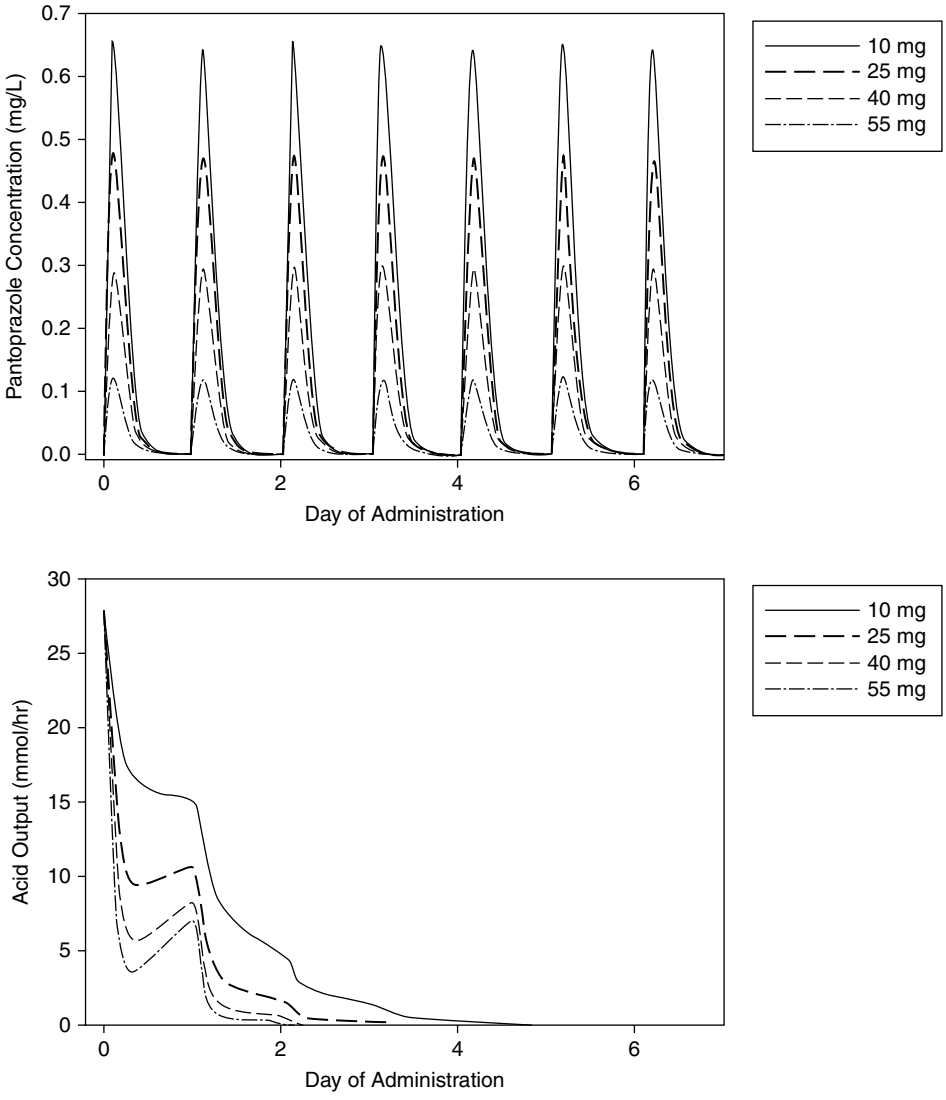


Figure 9 Simulated plasma concentration (*top*) and acid output (*bottom*) profiles in humans dosed once-daily with 10, 25, 40, or 55 mg enteric-coated pantoprazole using the model and parameter values. *Source*: From Ref. 37.

was apparent, as was a difference between 25 and 40 mg, but there was little difference between 40 and 55 mg. Increasing the dose beyond 40 mg appeared to offer little benefit. Interestingly, pantoprazole is marketed as Protonix® as a either a 40 or 20 mg enteric-coated tablet.

Now suppose that pantoprazole did not make it to market (which it did). The authors have now developed a useful pharmacokinetic–pharmacodynamic model that can be used to help develop back-up compounds. If the discovery chemists were able to synthesize a series of compounds thought to inhibit proton pumps, the animal model could be used as a screen to help choose an appropriate back-up compound, as well as aid in dose selection for the FTIM studies. The rat model could also be used to study drug–drug interactions, the effect of food, or any other relevant scientific question deemed of importance and be able to tie these results directly to the pharmacodynamic effects in humans.

Case Study 4: Integrating In Vitro Methodologies into Antimicrobial Drug Development

Traditionally, preclinical implied a study was done in animals. With the advances in cell culturing, molecular biology, and other in vitro methodologies, the traditional use of the word

"preclinical" is too limiting. Today, preclinical needs to be more inclusive and may mean any model system not including humans. With that in mind, Drusano et al. (38) have reported a useful application of pharmacokinetic–pharmacodynamic M & S using antimicrobial sensitivity in isolates from patients infected with a particular pathogen. The first reported use was with evernimicin, the first member of a unique class of oligosaccharide antibiotics active against gram-positive organisms that was later discontinued from clinical development because the drug failed to show a better activity and safety profile compared to already marketed drugs (38). The basic idea is one that has been used often before: use preclinical data to obtain some measure of clinical exposure needed for activity, use PopPK to understand the pharmacokinetic behavior of the drug in humans, then using Monte Carlo simulation vary the dosing regimen until some percentage of patients obtain the target preclinical level.

Antibiotics are classified into two broad classes: bactericidal agents, which kill the organisms by interfering with cell wall synthesis or some other key metabolic function of the microbe, and bacteristatic agents, which inhibit the growth of the organism. The drug concentration that inhibits bacterial growth for 24 hours is called the minimum inhibitory concentration (MIC) and the concentration that inhibits such growth in X% of isolates (aseptically collected specimens from lesions or sputum from patients with the pathogen) is called the MIC_X. For example, the concentration that inhibits 80% growth is called the MIC_{80}. Not all organisms are killed in the same manner by bactericidal drugs. Some drugs kill in a concentration-dependent manner, for example, aminoglycosides, and the higher the blood drug concentration or area under the curve (AUC) relative to the MIC the more effective the drug. For other drugs, it is not the actual drug concentration that is important, but how long drug's concentration remains above the MIC, for example, macrolides and β-lactams. Thus, microbiologists investigate which of three possible parameters relates better to outcome: the ratio of AUC to MIC (AUC/MIC ratio), the ratio of peak antibiotic concentration to MIC ratio (peak/MIC ratio), and the percent of time above the MIC. Which of these three parameters is important for predicting response is drug-dependent.

Drusano et al. (38) used a murine model of infection and studied three pharmacodynamic endpoints: stasis (that value which resulted in no change in the number of bacteria beyond the colony forming unit at the time of inoculation), log killing (calculated from the modeled maximal colony count in the control group), and 90% E_{max} (calculated as the log drop representing 90% of the maximal log drop achievable). The independent variables examined were AUC/MIC ratio, peak/MIC ratio, and percent time above the MIC. Separately, the MICs for each of about 1500 isolates were determined and the MIC_{80} against *pneumococci, staphylococci* , and *enterococci* was estimated. Next the protein binding of evernimicin in mouse and human plasma was estimated. Then the pharmacokinetics of evernimicin in healthy normal volunteers and in patients with hepatic impairment was characterized using PopPK. Lastly, the distribution of MICs and pharmacokinetics of evernimicin were simulated under two-dosing regimens. The percent of subjects meeting the in vivo targets from the murine mouse model was determined. Figure 10 illustrates the process.

The murine model showed that all three independent variables predicted outcome about equally well with AUC/MIC ratio being slightly better than the other two predictors (Fig. 11). Since unbound drug is important for pharmacodynamic activity, any model for antimicrobial pharmacodynamics needs to use unbound concentration as the independent variable. But, in this case, there was no species difference in degree of binding. So to simplify matters, the authors used total drug concentration, instead of free drug concentration, as the independent variable in future simulations. Then using two-dosing regimens, 6 mg/kg and 9 mg/kg evernimicin once daily, the percent of subject attaining the preclinical target was determined.

Table 1 shows the percent of subjects reaching the preclinical targets for each of the pathogens studied. The simulations showed that the lowest dose provided sufficient exposure near the top of the dose–response curve against all three pathogens. Also, evernimicin is such a potent drug that a 50% increase in dose resulted in little change in the number of subjects reaching the preclinical targets.

A second application of this methodology was reported the next year with GW420867X, a nonnucleoside reverse transcriptase inhibitor of human immunodeficiency virus type 1 (HIV-1) (39). In this study, the authors used two preclinical pieces of information: in vitro protein binding of the drug in human plasma and the distribution of concentrations that inhibit 90% of viral

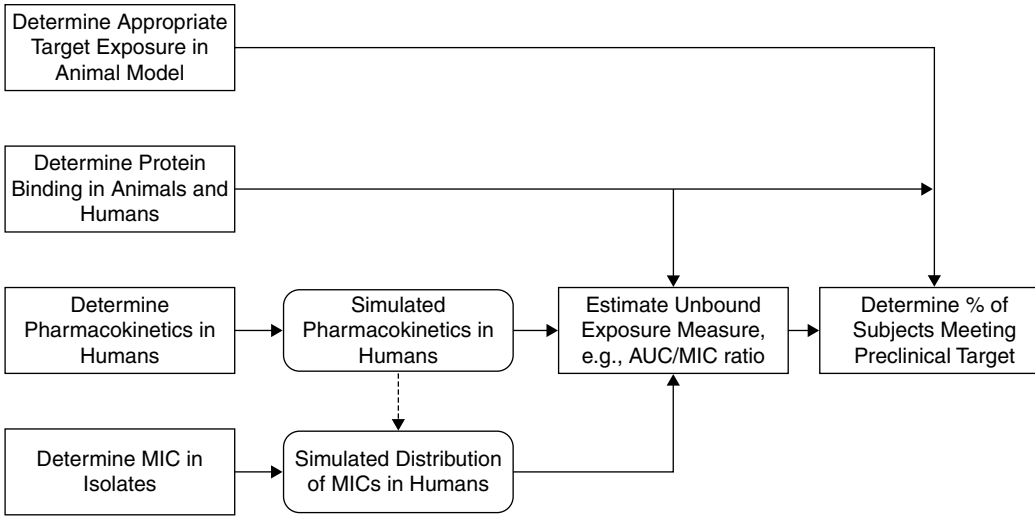


Figure 10 Schematic model used by Drusano et al. (38) in the Monte Carlo simulation of antimicrobial dosing regimens.

growth (EC_{90}) to test HIV isolates. The pharmacokinetics of GW420867X were then characterized using nonparametric population-based methods with data obtained from a multiple-dose study in normal healthy volunteers. Assuming the pharmacokinetics in healthy volunteers will be reflective of the pharmacokinetics in patients with HIV and that time above the EC_{90} will be the important pharmacodynamic target, the clinical information was then combined with the preclinical information to create a joint model predicting unbound drug concentrations in patients at steady state. Using Monte Carlo simulation the authors tested three-dosing regimens (50, 100, and 200 mg once daily) to determine the percent of subjects with simulated unbound trough drug concentrations greater than 10 times the EC_{90} and EC_{50}. Based on the simulation, each of the doses provides >95% target attainment when the EC_{50} was less than 10 nM. At the time of publication, the authors indicated that of the 16 isolates available all had EC_{50}s less than 8 nM. In summary, by combining relevant preclinical targets with clinical information, Drusano et al. were able to either predict a relevant dosing regimen or to make conclusions about differences in already selected dosing regimens.

CLOSING THOUGHTS

Clayton M. Christensen coined the term "disruptive technology" to describe a technology that disrupts the current marketplace and eventually replaces the current technology standard (40).

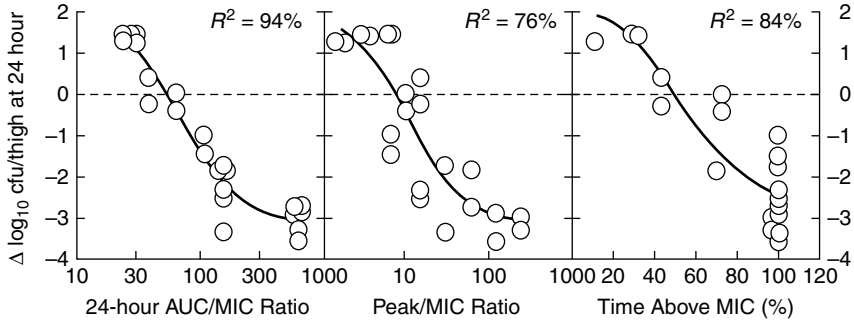


Figure 11 Change in colony forming units (cfu) recovered from mouse thigh model of infection at 24 hours after initiation of therapy with evernimicin as a function of AUC/MIC ratio, peak/MIC ratio, and percent time above the MIC. The AUC/MIC ratio gives slightly better predictability of outcome compared to the other measures. *Source*: From Ref. 38. Courtesy of the American Society for Microbiology.

Table 1 Percent of Subjects Reaching Evernimicin Preclinical Targets

	Staphylococcus pneumonia			*Staphylococcus aureus*			*Enterococci*		
Dose (mg/kg)	**Stasis**	**Log drop**	**90% E_{max}**	**Stasis**	**Log drop**	**90% E_{max}**	**Stasis**	**Log drop**	**90% E_{max}**
6	100	100	96	92	72	34	100	100	58
9	100	100	98	97	85	50	100	100	79

Source: From Ref. 38. Courtesy of the American Society for Microbiology.

A classic example is the personal computer. When PCs were introduced, large mainframe computers were the industry standard and companies like IBM, who at the time was the leader in the computer field, ignored these small machines because they lacked the computing power. However, small companies such as Apple pursued this technology and eventually replaced mainframes to the point of their practical extinction. Is MBDD a disruptive technology? Yes. Can modeling replace current practices? That remains to be seen but seems likely.

The role of modeling in drug development is still developing and growing. Certainly, there are instances where modeling has shown its value and those companies that have recognized this utilize it to a greater degree than those who do not. Still, the field has a long way to go, particularly with regards to incorporating preclinical data into the modeling process. Certainly, the Critical Path Initiate and EMEA think-tank guidance has helped in this regard and proponents of MBDD cite these sources as a way to gain credibility for their cause. Nevertheless, the fact remains that MBDD is but one of many proposed means by regulatory agencies to improve the drug development processes. Modelers need to find opportunities within their organization that whenever possible establish and reestablish the value of modeling as a means to improve decision making in the face of uncertainty, because while it is certainly of value to have regulatory authorities suggest an idea, it is far better for companies to want to implement a technology.

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7 Formulation and Production Strategies for Enhancing Bioavailability of Poorly Absorbed Drugs

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INTRODUCTION

As the pharmaceutical industry continues to develop improved techniques for chemical synthesis, high throughput screening, and computer modeling software, medicinal chemists are able to produce new molecular entities with elevated therapeutic potential quickly and effectively. Often, these drug molecules show great potential for improved therapeutic outcomes; however, these present a variety of challenges to the formulating scientist. Increased lipophilicity and molecular weight and a corresponding decrease in solubility in physiological media are major hurdles that reduce the overall bioavailability of many of these drugs. It is widely accepted that 40% of new drugs developed exhibit poor dissolution and low solubility in aqueous media, leading to reduced therapeutic effect or bioavailability. Bioavailability can be broken down, most simply, into a combination of two characteristics of the therapeutic molecule: solubility and permeability. Indeed, bioavailability has been demonstrated to be a much more complicated concept, as described by Wu and Benet (1), involving potential drug instability, elimination criteria, active transport, and various organ metabolisms. These additional concerns add to a need for production and formulation methods to improve basic principles of drug delivery—solubility and permeability.

Solubilization of a drug molecule occurs when a thermodynamic preference for solute–solvent interactions exceeds the preference for solute–solute interactions due to intermolecular forces (i.e., van der Waals force, hydrogen bonding). Changes in aqueous volume, temperature, and pH as well as the drug molecule's propensity for hydrophilic/lipophilic interactions will determine its inherent solubility. On a macromolecular level, drug particle size and crystalline state have also been proven to influence solubility as modeled by the Ostwald–Freundlich equation. The membrane permeability of a drug molecule is essentially determined by the overall molecular size, polar surface area, and association with active transport mechanisms (2). The polar surface area of a drug molecule directly correlates with the octanol–water partition coefficient or log P value of that molecule. Typically, the more lipophilic a drug is (less polar, higher log P), the more readily the drug will permeate through a physiological membrane. Other general factors affecting permeability (which are more significant to the formulator) that have been shown to influence drug permeability are concentration, temperature, time (viscosity), and surface area. For many drugs, absorption in the stomach has been shown to be minimal in comparison to that of the small intestine. Poor absorption in the stomach has been attributed to factors such as shorter residence time, reduced surface area, and thick epithelial diffusion layer.

The U.S. Food and Drug Administration (FDA) has issued guidelines for a classification system to assist in the categorization of drugs with the biopharmaceutics classification system (BCS) based on research lead by Amidon et al. (3). It should be noted that the BCS is intended for classification of oral bioavailability of drugs; however, because of the common physiological characteristics of fluid and membranes throughout the body, this system may also be loosely applied to assist with formulation in other routes of delivery, particularly those involving permeation across a mucosa. The BCS categorizes all therapeutic molecules into one of the four classes based on drug solubility and permeability: class I (high solubility, high permeability), class II (low solubility, high permeability), class III (high solubility, low permeability), and class IV (low solubility, low permeability). Of the 141 drugs initially classified using this system, only 55 met the criteria necessary for class I status and the rest exhibited characteristics that would

prevent complete drug absorption. The FDA defines a drug as "highly soluble" if the highest dose strength available on market is soluble in 250 mL of aqueous media in pH ranging from 1 to 7.5. "Highly permeable" substances show 90% or more of the administered dose absorbed in humans. Consequently, roughly 60% of all drugs initially categorized by the BCS showed limited bioavailability due to low solubility and/or poor membrane permeability.

When consulting the BCS for formulation purposes, it should be understood that this system was developed to simplify the approval process for generic drug products that have already been formulated and marketed in an innovator product (4). As a result, new therapeutic molecules may not have sufficient human trial data to definitively determine the appropriate human dose or assess permeability across the gastrointestinal (GI) mucosa. Formulation of drugs classified as poorly soluble or poorly permeable serve as a good model for applications of formulation technology and strategy; however, drugs in preclinical stages may lack sufficient data to be classified according to this system. In these cases, pharmacokinetic (PK) modeling becomes necessary for determination of the nature of the new molecular entities. In preclinical formulation, solubility and permeability models affecting overall drug absorption have been developed through the consideration of molecular polar surface area (2,5,6), in vitro membrane permeability studies (7), and animal PK and organ absorption data (8). Alterations of the BCS have been suggested based on the current improvements in permeability models and shortcomings of the methods and limits set forth by the BCS. For example, it has been suggested by Fagerholm that the solubility limits placed by the BCS are too strict and that permeability limits are overly generous, resulting in the under-prediction of the number of molecules with low permeability (9). Some of the suggested classification systems taking into consideration factors influencing drug absorption are the biopharmaceutics drug disposition classification system (BDDCS) (1) and the permeability-based classification system (PCS) (8). In-depth review and analysis of mechanisms for ADME (absorption, distribution, metabolism, and excretion), PK of therapeutic molecules, as well as appropriate modeling techniques have been covered in the previous chapters and will not be discussed in depth here. In this chapter, applications of new pharmaceutical technologies and formulation strategies for the improvement of the overall bioavailability of poorly soluble and/or permeable drugs will be discussed. A general outline of the strategies available to improve the absorption of these drugs is outlined in Figure 1. Of the factors that affect drug absorption, solubility is the first parameter considered because of the relatively simple characterization techniques and the multitude of formulation strategies. The first strategy that is typically considered is ionization of the drug to increase interaction with water molecules. This is normally achieved through salt formation of the drug itself or

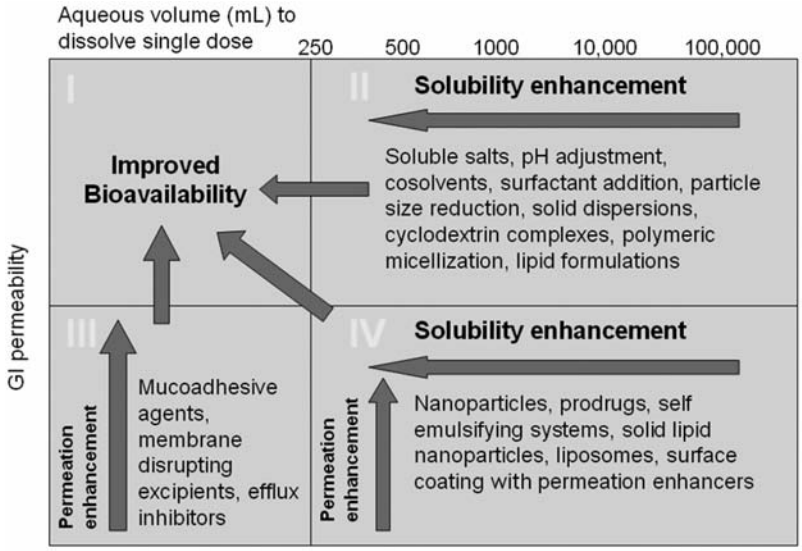

Figure 1 Strategies to enhance bioavailability of BCS class II, III, and IV drugs.

adjustment of the pH of the drug carrier media, since some poorly soluble drugs are more soluble in acidic conditions. Similar to salt formation, chemical attachment of a more soluble molecular species that may be enzymatically removed in vivo is another common strategy. Cosolvents, such as ethanol, propylene glycol, glycerin, and natural oils, are also commonly used for solubilization of lipophilic drugs; however, these often lead to irritation or toxicity at the site of delivery. In addition, dilution of these solvents may also cause precipitation of drugs from solution, leading to complications like nephrotoxicity. To avoid the use of cosolvents, aqueous surfactant solutions have been investigated, although toxicity issues have also been associated with their use.

The purpose of this chapter is to provide the reader with production techniques, examples of appropriate animal models, as well as an overall rationale to employ strategies for improving drug absorption. Methods for enhancing solubility and permeability through various nano-engineering and formulation techniques such as surface stabilized nanoparticles, polymeric micelles, cyclodextrins, solid dispersions, self-emulsifying drug delivery systems (SEDDS), and solid lipid nanoparticles (SLNs) will be discussed in depth. By incorporating these modern technologies for BCS class II, III, and IV drugs as well as similar drugs not included in this classification system, the goal of enhanced drug absorption and expected improvement of therapeutic outcomes can be realized.

SURFACE-STABILIZED DRUG NANOPARTICLES

Particle size reduction as a method of improving dispersion and wettability of pharmaceutical dosage forms has been used for many years in the pharmaceutical industry. To prepare coarse drug particles for incorporation into a wet or dry formulation, micronization has traditionally been used, resulting in a mean particle diameter of 2 to 5 μm (10). This procedure allows for increased product homogeneity and drug dissolution rate. By reducing the mean particle diameter of bulk drug, the available surface area is increased. Surface area is directly proportional to dissolution rate as evident in this modification of the Noyes–Whitney equation:

$$\frac{\mathrm{d}X}{\mathrm{d}t} = \left(A \times \frac{D}{\delta}\right) \times \left(C - \frac{X}{V}\right)$$

where X is the amount of drug in solution, t is time, A is the effective surface area, D is the diffusion coefficient, δ is the effective boundary layer, C is the saturation solubility of the drug, and V is the volume of the dissolution medium (11). By this logic, further increasing the surface area of a pharmaceutical powder will increase dissolution rate and, in turn, the onset of therapeutic effect. Additionally, further size reduction below 1 μm will also increase the saturation solubility of the drug. The Ostwald–Freundlich and Kelvin equations model the effect of particle radius on saturation solubility and demonstrate that increased dissolution pressure due to the high curvature of nanoparticles will increase solubility.

Processing Technology

A multitude of technologies are available for the production of nanoparticles to enhance solubility of BCS class II and IV drugs (Table 1). All of these processes fall under one of the two categories: bottom-up processing or top-down processing. Bottom-up processing, the least used of the two nanoparticle production techniques, involves atomic or molecular assembly that is either naturally occurring due to physicochemical traits (such as crystallization) or manipulated manually. Top-down manufacturing describes processes where bulk material is broken down into smaller particles by machining, milling, and other high-shear techniques. As drug particle size is reduced, overall surface area of that quantity of drug is increased, leading to an increase in free energy of the system. Free energy is directly proportional to surface area as made evident by the following equation:

$$\Delta G = \gamma_{s/l} \times \Delta A$$

where $\gamma_{s/l}$ represents the interfacial tension of a substance and ΔA represents the change in surface area. In order to reduce the free energy and become a stable system, particles within

Table 1 Patents for Production of Surface-Stabilized Drug Nanoparticles by Milling and Homogenization

Technology	Company	Patent application
Hydrosol	Novartis	GB 22 69 536
NanoMorph	SOLIQS/Abbott Laboratories	D 19637517
NanoCrystal	Élan Nanosystems	US 5,145,684
DissoCubes	SkyePharma PLC	US 5,858,410
Nanopure	PharmaSol GmbH	PCT/EPO.0635
NANOEDGE	Baxter Healthcare Corporation	US 6,884,436
IDD-P	SkyePharma PLC	US 5,091,187

Source: From Ref. 136.

a nanodispersion will aggregate to formlarger particles with reduced surface area. A key formulation component used in nearly all nanoparticle production techniques to prevent particles from self-associating is the addition of a surface active agent (surfactant) or a polymer to the production process. Adsorption of these agents to the surface of particles will stabilize a nanodisperse system by electrostatic interaction or by steric hindrance of aggregation. Ionic surfactants will reduce the interfacial tension by associating a hydrophilic polar head with water, while the lipophilic end associates with the surface of the particle. This polar head group functions to repel like charges coated onto other particles. Steric stabilizers form a mechanical shield preventing surface-to-surface particle interaction. Both of these strategies incorporated in one formulation may be used to provide a further enhanced stability where tighter packing of ionic surfactants is allowed due to the inclusion of a neutral polymer.

Top-Down Production

Various technologies have been developed and patented for production of drug nanoparticles, the first of which was patented by Liversidge in 1992 (12). These top-down processes produce surface-stabilized nanoparticles though particle size reduction techniques such as wet milling and high-shear/pressure homogenization processes. Wet milling was the first widely accepted form of drug nanoparticle production due to its avoidance of the use of organic solvents, ability to process small and large batch sizes, and cost effectiveness. NanoCrystal® technology applies wet milling techniques to drug nanonization and is now licensed by Élan Drug Delivery, Inc. (King of Prussia, PA). Beginning with the approval and marketing of Rapamune® in 2000 as an alternative to oral sirolimus solutions, NanoCrystal technology has produced three subsequent FDA approved products exhibiting enhanced oral bioavailability: Emend®, an oral capsule of aprepitant (Merck & Co., Inc.); TriCor®, an oral tablet of fenofibrate (Abbott Laboratories/Groupe Fournier SA); and Megace® ES, an oral suspension of megestrol acetate (Par Pharmaceutical Companies, Inc.). As with all processes for production of crystalline nanosuspensions, a stabilizer is required in addition to the drug and milling media. Stabilizers that are commonly used impart steric or ionic hindrance of particle aggregation and include generally recognized as safe (GRAS) excipients such as povidones, Pluronics, polysorbates, and cellulose derivatives. Laboratory-scale testing should be conducted for process optimization and to determine which stabilizer and what quantities result in the most stable formulation. This demonstrates the importance of a scalable process that may be tested in the laboratory before full-scale production begins. Excessive or insufficient concentrations of stabilizer may result in Ostwald ripening or aggregation, respectively; although, most wet milled nanosuspensions use drug-to-stabilizer ratios ranging from 20:1 to 2:1 (13). The milling process itself incorporates a form of media agitation (either internal or external) as well as the addition of milling beads that produce impacts resulting in fragmentation of drug particles. These beads must be made of very hard substances such as stabilized zirconium dioxide, stainless steel, glass, or highly cross-linked polystyrene resin in order to effectively fragment particles while not self-fragmenting. Contamination due to fragmentation of milling materials has been one of the few concerns regarding this particle size reduction technology (14).

High-pressure homogenization systems are commonly used in the pharmaceutical industry to reduce particle size via mechanical fragmentation. In this process, piston-gap

homogenizers use cavitation energy to fracture crystalline particles suspended in aqueous media. Patented nanoparticle production techniques using piston-gap homogenizers include DissoCubes® (SkyePharma PLC), Nanopure® (PharmaSol GmbH), and NANOEDGE™ (Baxter Healthcare Corporation). Cavitation of aqueous media occurs due to Bernoulli's law, where static pressure of fluid is reduced when it flows through a constricted vessel at high velocities. This same concept is also a fundamental principal behind air-jet nebulizers (15). High flow rates occur in piston-gap homogenizers when the diameter is reduced from 3000 to 25 μm, causing the static pressure decrease as predicted by Bernoulli. After a sudden drop in static pressure to below the vapor pressure, the liquid (typically water) begins to boil, followed quickly by an elevation in static pressure as fluid enters a much wider portion of the homogenizer, causing the gaseous bubbles to collapse or cavitate. By varying the high pressure (power density), number of cycles, or temperature, the size of the nanoparticles produced can be manipulated. Hardness of the drug particle and the crystal packing/structure will also play a role in particle size reduction. It should be noted, however, that as particle size is reduced to submicron levels, more pressure is required to further reduce the particle size. With most drug substances, reduction of particle diameter below 200 nm is difficult, requiring increasingly high-energy input and milling times. As mentioned previously, surfactant selection is paramount for ensuring final product stability and reproducible bioavailability. Further development of the piston-gap technology has led to a variation called Nanopure that allows for the processing of water-soluble or water-sensitive materials by using nonaqueous media. Various homogenization medias, such as pharmaceutical oils, glycerol, liquid or hot-melted polyethylene glycol (PEG), have been used in these processes even though no cavitation is thought to occur. Nanoparticles may still be produced without cavitation by high-velocity impacts and shearing that occurs within the system. Another strategy for producing nanosized drug particles using high-pressure homogenization incorporates the collision of high-pressure jet streams. This technology, called microfluidization or IDD®-P (insoluble drug delivery platform) technology (SkyePharma PLC), uses the high fluid shear and particle collision forces to reduce drug particle size over a number of cycles through a Microfluidizer® processor (Microfluidics). Within the Microfluidizer, the fluid undergoes a torturous path through a nonerodable diamond-coated channels, is split into channels, and then impinges at high velocities back together. As in piston-gap homogenization, these fluidizers also require stabilizers to prevent particle growth and aggregation upon storage; however, phospholipid stabilizers are specifically mentioned for use in this process. When incorporating phospholipid stabilizers, these systems have an added benefit of being immunogenic, avoiding potential toxicity due to high surfactant levels, much like liposomal formulations. The structure, however, of a nanoparticle produced by IDD-P and liposomal formulation are different as discussed by Mishra et al. in their review of IDD technology (16). Liposomal structures used for drug incorporation consist of drug encapsulated or embedded in a phospholipid bilayer membrane, while the homogenized nanoparticles have a more complex phospholipid region made of multiple domains surrounding and stabilizing a solid hydrophobic core.

Bottom-Up Production

Solvent evaporation and drug precipitation methods are the two most common techniques used to construct nanoparticles using bottom-up techniques. Two patented technologies that involve antisolvent precipitation for production of stabilized drug nanoparticles are Hydrosol® (Novartis) and NanoMorph® (SOLIQS). Both of these processing techniques involve drug precipitation, also referred to as nucleation, which occurs after the addition of a drug-containing organic solvent to an aqueous polymeric solution antisolvent. A key caveat to this method is that the drug is soluble in a water miscible solvent, limiting solvent selection due to insolubility of many class II and IV drugs in polar solvents. As in milling and homogenization techniques, precipitation methods also require the use of stabilizing excipients. Stabilizer incorporation is significantly more important in this processing method due to the nature of particle generation and the propensity of these small particles for Ostwald ripening. NanoMorph technology specifically claims the production of stabilized amorphous nanoparticles through the incorporation of stabilizing polymers followed by spray drying of the dispersion. With a combination of high nucleation rates and an effective nonionic amphiphilic polymer (poloxamer 407), it is possible to obtain nanoparticles by precipitation techniques below 300 nm in diameter (17). In combining

solvent evaporation and precipitation methods, evaporative precipitation into aqueous solution (EPAS) also allows for the production of soluble formulations of poorly water-soluble drugs. In this technique, heated and pressurized solvent containing dissolved drug is sprayed into a heated aqueous/stabilizer solution. The solvent evaporates immediately resulting in polymer migration to the hydrophobic particle surface and drug nanoparticles coated with ionic or nonionic surface-stabilizing agents.

In a combination of both top-down and bottom-up processing, NANOEDGE (Baxter Healthcare Corporation) and Nanopure XP (PharmSol GmbH) have been demonstrated as effective methods of nanoparticle production. NANOEDGE uses an antisolvent precipitation technique to crystallize dissolved drug, which then may be homogenized to further reduce the particle size. Specifically, a hydrophobic drug dissolved in aqueous miscible organic solvent is added to aqueous surfactant solution to begin precipitation. In many cases, drug will precipitate out to form crystalline structures or unstable amorphous particles. The final homogenization step, also called the annealing step, will break apart existing crystalline structures and crystallize amorphous particles, allowing for enhanced stability. Nanopure XP also combines precipitation and homogenization to allow for reduced homogenization intensity and nanosizing of drugs with stronger crystal lattice structures. A solvent evaporation step before homogenization reduces crystal strength and may also involve excipients coprecipitated to disrupt the normal drug crystal structure, leading to easier particle fragmenting (18). With this technology, particles in the 100 nm range can be produced while processing times, number of homogenization cycles, and homogenized wear and tear are reduced.

Application Examples

Oral Delivery

Particle size reduction technology has played a significant role in improving drug absorption in drugs that may be limited by dissolution rate and solubility (Table 2). By incorporation of Elan's NanoCrystal technology, a more bioavailable formulation of megestrol acetate was produced while also reducing variability in GI absorption (19). This progesterone agonist used to treat anorexia has been shown in the original formulation to have a bioavailability that is highly influenced by fed or fasted state. When compared to the original formulation in clinical trials, a Megace ES dose of 625 mg dispersed in 5 mL showed a maximum plasma concentration of 1517 ng/mL, proving to be more extensively absorbed than the 800 mg original formulation that showed a C_{max} of 1364 ng/mL. What is most significant is that variability between fed and fasted states was reduced. The fed/fasted ratio (C_{max} fed/C_{max} fasted) in the original megestrol formulation was 7.3, showing a drastic difference between drug absorption in the two states, but was reduced to 1.5 in the Megace ES formulation (19). Subsequent efficacy studies reflected the improvement in bioavailability, showing a 10% improved therapeutic outcome when compared to patients taking the original suspension.

Pulmonary Delivery

Drugs that are poorly absorbed because of low solubility in the GI tract typically will encounter the same problems when delivered via the pulmonary route. While delivery to the lungs has many advantages, such as the avoidance of first-pass metabolism, it can prove difficult because of the small number of approved excipients and the requirement of proper particle aerodynamics for navigation of the pulmonary tree. Elan's NanoCrystal technology has been applied to improve the delivery of budesonide, a poorly soluble asthma medication. When dosed to healthy human volunteers, the nanocrystaline budesonide formulation proved to be safe and homogenously dispersed within aerosolized droplets. The PK of the nanoformulation, when compared to the marketed Pulmicort® Respules® formulation, showed peak blood levels in nearly half the time with double the C_{max}; although, AUC in both formulations proved to be comparable (20). These findings are significant for the indication, though, because of the sudden and potentially lethal nature of asthma. Fluticasone and budesonide were recently involved in a similar study, which compared intravenous solution, nebulized solution, and nebulized nanosuspensions in

Table 2 A Summary of In Vivo Studies Conducted to Determine PK, Efficacy, or Safety of Surface-Stabilized Nanoparticle Formulations

Technology	Drug	Subject	Study	Dose (route) (mg)	Blood AUC (ng h/mL)	Blood C_{max} (ng/mL)	Findings	Reference
Controlled precipitation	Cyclosporine	ICR mice	PK	0.1 (pulmonary)	9665	372	Sustained diffusion from lung to blood	Tam, 2008 (22)
NanoCrystal	Megestrol acetate	Human	PK	625 (oral)	—	1517	C_{max} doubled, T_{max} half compared to control	Adis International Limited, 2007 (19)
NanoCrystal	Budesonide	Human	PK/safety	0.5–1.0 (pulmonary)	—	—	C_{max} doubled compared to control	Kraft, 2004 (20)
Wet milling	Fluticasone	SD rats	PK	0.6 (pulmonary)	—	300[a]	Delayed diffusion, lung retention of 4 hr	Yang, 2008 (21)
Wet milling	Budesonide	SD rats	PK	0.35 (pulmonary)	—	100[a]	Delayed diffusion, lung retention of 1 hr	Yang, 2008 (21)
NanoCrystal	Cilostazol	Beagle dogs	PK	100 (oral)	31,589	4872	Particle size decrease caused increased bioavailability, decreased fed/fasted variability	Jinno, 2006 (23)
DissoCubes	Amphotericin B	Balb/c mice	Efficacy	0.15 (oral)	—	—	More effective than AmBisome, Fungizone® in liver antiparasitic activity	Kayser, 2003 (24)
EPAS	Danozol	IRC mice	PK	0.375 (oral)	1534	430.1	C_{max} doubled compared to marketed drug	Vaughn, 2006 (87)
NanoCrystal	Danozol	Beagle dogs	PK	200 (oral)	16,500	3010	Absolute bioavailability 16 times that of conventional suspension	Liversidge, 1995 (25)

(Continued)

Table 2 A Summary of In Vivo Studies Conducted to Determine PK, Efficacy, or Safety of Surface-Stabilized Nanoparticle Formulations (*Continued*)

Technology	Drug	Subject	Study	Dose (route) (mg)	Blood AUC (ng h/mL)	Blood C_{max} (ng/mL)	Findings	Reference
NanoCrystal	Paclitaxel	CH3 mice	Efficacy	2.64 (IV)	—	—	Better tumor suppression compared to conventional formulation	Merisko-Liversidge, 1996 (26)
NanoCrystal	Camptothecin	CH3 mice	Efficacy	1.2 (IV)	—	—	Little tumor suppression compared to conventional formulation	Merisko-Liversidge ,1996 (26)
NanoCrystal	Etoposide	CH3 mice	Efficacy	3.75 (IV)	—	—	Better tumor suppression compared to conventional formulation	Merisko-Liversidge, 1996 (26)
NanoCrystal	Fenofibrate	Human	PK	145 (oral)	123,800	7900	No bioavailability difference in fed or fasted state	Keating, 2007 (27)

[a]Estimated from plot.
Abbreviations: PK, pharmacokinetic; EPAS, evaporative precipitation into aqueous solution; SD, Sprague Dawley.

Sprague Dawley rats. Results showed that nanosuspension dosing gave a slightly delayed systemic absorption when compared to inhaled solution and injection. This was attributed to the requirement of drug to dissolve before absorption could take place. Also, because of reduced solubility in simulated lung fluid, fluticasone demonstrated longer lung retention as compared to budesonide; although, both showed more prolonged release when compared to solution aerosols (21). Using an antisolvent precipitation technique, Tween 80 stabilized cyclosporine nanoparticles were also studied for their ability to enhance drug bioavailability. Using a permeation model for pulmonary drugs, it was found that using an amorphous formulation with a 10-fold particle diameter reduction could decrease the absorption half-life from 500 minutes to less than 1 minute (22). After aerosol dosing to mice, it was found that amorphous cyclosporine nanoparticles show potential for high drug permeation without the use of potentially irritating solvents (23–27).

POLYMERIC MICELLES

Amphiphilic polymers have many applications in pharmaceutical delivery due to their ability to interact with both hydrophilic and lipophilic moieties. Most commonly, these polymers and surfactants are used to enhance the solubility of a compound by interacting with the surface of the particle or completely surrounding it in a micelle. Drug delivery using polymeric micelles can be very effective in the solubilization of lipophilic drugs in biological fluids and are commonly used in the controlled release of highly potent, poorly soluble drugs. Micelle formation occurs when the polymeric concentration in an aqueous environment reaches a point where self-association of lipophilic polymer chains begins to occur, ultimately forming an encapsulated sphere or micelle. This concentration where micelles form, called the critical micelle concentration (CMC), depends on factors such as polymer molecular weight and proportion of lipophilic and hydrophilic groups. Once the CMC is reached, micelle associated and free polymer chains maintain equilibrium in aqueous fluid. Consequently, concentrations well above the CMC will fortify existing micelles, adding more stability. Because micelles are typically intended for delivery into large aqueous volumes (i.e., GI fluid or blood volume), it is important that a polymer has a low CMC, stabilizing the micelles during processing and preventing their disintegration after dilution. Explanation into electrostatic and chemical properties affecting micelle formation can be very complicated and will not be reviewed in this chapter; however, for an excellent chapter on micellar chemistry, see Ref. (28). Additional benefits of polymeric micelles for drug delivery include the typically small micelle diameter of 10 to 100 nm, ability to protect degradable drugs, and, in the case of highly potent drugs, the potential for sustained-release formulations. Many polymeric micelles have also demonstrated long systemic half-lives and the ability to avoid reticuloendothelial system (RES) uptake due to reduced immunogenicity and shielding provided by long hydrophilic polymer chains (often PEG). PEG, because of its aqueous solubility and biocompatibility, is used as the hydrophilic block in many synthesized copolymers intended for micellar encapsulation. The hydrophobic block of a polymer intended for micelle formation may vary depending on the drug to be solubilized within the micellar core, the desired micelle diameter, and intended release rate. A few examples of molecules for hydrophobic blocks are polymers of propylene oxide, L-lysine, aspartic acid, β-benzoyl-L-aspartate, γ-benzyl:L-gltuamate, caprolactone, D,L-lactic acid, and spermine (29). Small particle diameter and long residence in circulation can be important for these formulations, because it enables the enhanced drug permeation through highly vascularized tissues. The effect of enhanced permeability and retention (EPR) has been extensively investigated for tumor targeting of anticancer drugs and can be attributed to the highly vascular, leaky nature of tumor tissue (30). Emerging methods for further tailoring of targeted release form polymeric micelles include the use of pH-sensitive and temperature-sensitive polymers (31).

Processing Technology

Production of drug-loaded polymeric micelles is quite simple when compared to other pharmaceutical processes, since micelles are self-forming in aqueous media. Most novel studies involving improved drug delivery with polymeric micelles do not focus on the production technique but rather the polymer molecule itself. Research teams are continually investigating

new chemical combinations of hydrophilic and lipophilic molecules to form a new polymer with a low CMC, improved encapsulation efficiency, and high biocompatibility. Typically, the aqueous solubility of the amphiphilic polymer will determine the method of drug-loaded micelle production. If the polymer is somewhat soluble in water then the dissolution method of micelle formation should be used. If the polymer is poorly soluble in water (such as high-molecular-weightor low-HLB polymers) then the dialysis production method is typically chosen (32). The dissolution method involves an emulsification and subsequent solvent evaporation (much like in microencapsulation techniques) or the preparation of a drug–polymer film. Preparation by emulsion formation requires the addition of drug dissolved in organic solvent to an aqueous solution of polymer, followed by stirring and/or heat application. In other cases, the drug precipitates are formed because of the aqueous miscibility of the organic solvent, causing small drug nucleates to form and become encircled by polymer. Yet another method of drug loading of a polymeric micelle by dissolution technique requires the drug and polymer to be dissolved in a common organic solvent and a film to be cast (33). This film can then be shaken in aqueous media to initiate micelle formation.

In cases of poor aqueous solubility, better encapsulation efficiency and micellar size control may be obtained using the dialysis production method. Preparation of drug-loaded micelles by the dialysis method involves the diffusion of organic solvent across a dialysis membrane, causing precipitation of drug and micellar polymer formation. Water at sink conditions passes over the dialysis membrane allowing for solvent diffusion from the dialysis bag, leaving a dispersion of drug-loaded micelles in equilibrium with an aqueous polymeric solution. Processing conditions will not have a profound effect on micelle size, but may greatly influence the drug loading levels, product yield, and encapsulation efficiency (34).

Application Examples

Oral Delivery

Cyclosporine, a lipophilic peptide used for immunosuppression, has been studied extensively for methods to improve overall drug absorption and reduce variability in oral bioavailability. The currently marketed oral formulation, Neoral®, is a second-generation formulation of this drug that has been demonstrated to improve oral absorption and reduce variability of blood levels commonly seen with the previous formulation. In the original formulation, bile salts were needed to allow for emulsion-assisted solubilization of the lipophilic drug, and because these salts may vary in concentration on intersubject and intrasubject basis, the overall bioavailability of this product proved to be variable. By preparing cyclosporine in a self-emulsifying microemulsion, Neoral showed a twofold increase in bioavailablity in clinical trials (35). In an effort to further improve cyclosporine bioavailability, Francis and coworkers have investigated a novel polymeric micelle delivery system to enhance oral absorption and reduce P-glycoprotein (P-gp) efflux pump activity. Nontoxic polymeric micelles were formed by hydrophobically modifying the polysaccharides dextran or hydroxypropylcellulose (HPC) with polyoxyethylene cetyl ether. A dialysis method was used to load the polymeric micelles with cyclosporine, producing a polymeric particle for drug delivery that was 14 or 55 nm in diameter for dextran or HPC, respectively. In vitro testing was performed to determine permeability across the GI epithelium using human Caco2-cell monolayer (36). Superior transport across the cell layer was observed in the HPC prepared micelles when compared to dextran micelles or free cyclosporine because of the mucoadhesive properties of the HPC polymer. It was concluded that the use of these micelles for oral lipophilic drug delivery offers high encapsulation efficiencies, reduction in particle size, and less GI toxicity.

Extensive investigation into the effects of Pluronic (poly(ethylene oxide) /poly(propylene oxide) block copolymer) micelles for drug delivery has been conducted due to their biocompatibility and frequent use in pharmaceutical products (37). While much of the research focuses on the micelles themselves, the role of the free polymeric molecules in solution, often called unimers, has also been shown to have some biological significance. The membrane destabilizing properties of Pluronic unimers have been shown to enhance drug penetration into multidrug resistant cancer cells, assisting with delivery of various chemotherapeutic agents. Interestingly Pluronics P85 and L61 have been shown to preferentially affect both the microviscosity and

permeability of cancerous cell membranes while decreasing the permeability of blood cells. Additionally, inhibition of efflux transporters have enhanced drug permeation across the blood–brain barrier (BBB) (38) as well as in Caco-2 cell lines (36–38).

Intravenous Delivery

New polymeric molecules are often designed for polymeric micelle drug delivery to improve the bioavailability of a drug while increasing processing efficiencies and reducing potential for systemic toxicity. Indomethacin, a nonsteroidal anti-inflammatory drug (NSAID) characterized as having low aqueous solubility, has been thoroughly investigated in encapsulation, film coating, and polymeric matrix dispersions to increase the solubility while limiting the adverse side effects. An amphiphilic molecule for solubilization of indomethacin was developed and tested by Uhrich and coworkers and was shown to be nontoxic, biodegradable, and elicit only a mild immune response. These molecules, termed amphiphilic scorpion-like macromolecules (AScMs), can be engineered with a specific HLB by altering the length and number of PEG and acyl chains. When processed by an emulsion technique where volatile solvents are removed under vigorous stirring, the resulting drug encapsulated micelle measures less than 20 nm in diameter and is more thermodynamically stable than other polymer micelles studied (39). It is generally understood that micellular delivery systems are more stable when the CMC is low, preventing micelle dispersion when it is added to large aqueous volumes (such as the human blood volume). These polymeric micelles showed high encapsulation efficiencies (72%) at drug loading levels of 1:10 (drug-to-polymer ratio), proving to be much higher than encapsulation in similar polymers (40). To determine tolerability of this micellular formulation, cytotoxicity assays were performed using human umbilical endothelial cells and compared with PEG and Pluronic P85. Owing to the biocompatible high-molecular-weight PEG shield provided by the AScMs micelle ($M_{12}P_5$), the indomethacin-loaded micelles proved to cause toxicity comparable to that of pure PEG. Since PEG is well-known as a nontoxic, nonimmunogenic polymer, the AScMs micelle ($M_{12}P_5$) was determined as safe for drug delivery.

Polymeric micelles have been investigated for delivery to the brain because of their enhanced membrane permeability, long residence time, and polymer compositions with limited immunogenicity. However, the BBB still presents a permeation obstacle with tight intracellular junctions and P-gp efflux pumps located on the luminal side of blood capillaries. An investigation was conducted for inhibition of the P-gp efflux through application of unimeric Pluronic P85 (38). Batrakova et al. studied changes in permeation of a highly bound P-gp substrate digoxin when Pluronic P85 was incorporated in both in vitro cell layers and in vivo models with and without P-gp gene expression. Using side-by-side diffusion cells, bovine brain microvessel endothelial cells and porcine kidney epithelial cells were investigated for their P-gp efflux activity after Pluronic P85 was applied either apically or bilaterally. Both models showed P-gp efflux inhibition when Pluronic P85 was applied apically, not bilaterally, since receptors are known to be present only on the apical side of the membrane. Further testing of these findings were conducted in vivo using female FVB *mdr1a/b* and wild-type mice by IV injection via the tail vein. After injection of radiolabeled digoxin in phosphate buffered saline (PBS) or 1% Pluronic P85 solution, digoxin concentration in the brain was shown to steadily increase over 10 hours in the Pluronic P85 group. At the 10th hour, digoxin concentration in the blood and brain were essentially the same in the Pluronic P85 dosed group, while the phosphate buffered saline group showed that drug had been eliminated from both compartments (38). These studies showed substantial evidence that the membrane permeability enhancing capabilities of Pluronic P85 may be beneficial for increasing drug bioavailability.

An interesting hybrid between polymeric and lipid-based systems has been developed by Torchilin and coworkers to produce a highly stable biocompatible micelle for loading of hydrophobic drugs, such as anticancer agents. Drugs used in cancer therapies such as tamoxifen, delqualinium, paclitaxel, and chlorine e6 trimethyl ester have been investigated for micelle loading and have shown no significant influence of the micelle size in comparison to empty micelles, thus not affecting permeation characteristics (41). PEG molecules of various chain lengths have been conjugated to phosphatidyl ethanolamine (PE) in a novel study and characterized for micellar drug loading and size as well as investigated for cancer targeting capability in vivo. Female C57B1/6J mice were injected subcutaneously with Lewis lung

carcinoma cells, providing an adequate tumor model within two weeks. Determination of the capability of radiolabeled PEG–PE micelles to penetrate and target tumor tissue were evaluated after tail vein instillation. Enhanced tumor absorption as compared to muscle tissue absorption was noted to be evident after six hours, particularly in high-molecular-weight PEG. Further targeting capability was achieved by attachment of 2C5 antibody to the surface of the micelle, creating what is commonly referred to as an immunomicelle. By allowing long residence time in systemic circulation and small particle diameter, these micelles were able to permeate tumor tissue and preferentially accumulate for drug targeting.

CYCLODEXTRINS

A commonly used means for enhancing the apparent solubility of a lipophilic drug is by molecular complexation via cyclodextrins. Cyclodextrins are cyclic derivations of starch in a chair conformation that have been partially digested by *Bacillus macerans*. For simplicity, cyclodextrins can be thought of as a hollow cone where external hydroxyl groups give the molecule high aqueous solubility. When a poorly soluble or poorly permeable drug is complexed with a cyclodextrin, it is incorporated into the empty cavity of the molecule that essentially takes on the more favorable characteristics of that cyclodextrin. These molecules can be exploited for drug delivery purposes due to their ability to incorporate poorly water-soluble drug molecules within a lipophilic core, increasing the solubility of drug molecules on an individual basis. Because of the direct relationship between number of cyclodextrin molecules and number of solubilized drug molecules, this method of solubilization is often preferred by formulators over an organic solvent approach. Upon dilution (in GI fluid or blood volume), organic solvents will lose their solvent power exponentially, as described by the Hildenbran equation (41), while solvation power of cyclodextrins is reduced linearly. Cyclodextrins are classified by the number of glucose units in the cyclical ring, which typically numbers six (α-cyclodextrin), seven (β-cyclodextrin), or eight (γ-cyclodextrin); however, many new cyclodextrins being introduced are chemically modified versions. Modification of natural cyclodextrins is necessary to avoid aggregation and precipitation of natural cyclodextrins. By replacing one or more of the hydroxyl groups with a moiety that will not promote formation of a crystal lattice, even a lipophilic chain, the cyclodextrin (as well as any complexed drug) will become more soluble. For example, hydroxypropyl-β-cyclodextrin has shown aqueous solubilities upward of 500 mg/mL, while naturally occurring β-cyclodextrin possesses solubility of only 18.5 mg/mL (42). Additionally, by preventing drug and/or cyclodextrin precipitation, many concerns of systemic toxicity are reduced. For a more detailed discussion of solubility parameters and complexation kinetics, refer to an excellent review by Brewster and Loftsson (42).

Further modifications to cyclodextrins have been made to increase their lipophilicity and ability to permeate biological membranes. The addition of one or multiple hydrocarbon chains to a hydrophilic cyclodextrin creates an amphiphilic molecule, conceptually much like copolymers used for micelle encapsulation. Because these amphiphilic cyclodextrins self-associate in many cases, nanoparticulate formulations are also possible. Whether or not one of these amphiphilc molecules self-associates for nanoparticle formation is typically decided by the alkyl chain(s).

Processing Technology

Inclusion of lipophilic drug molecules in cyclodextrins is a process that is self-associating and occurs on the molecular level; therefore, there is not a multitude of manufacturing techniques needed to produce this drug delivery system. When considering formulation with cyclodextrins, it is important to consider a variety of factors including drug/cyclodextrin compatibility, potential mucosal irritation, and quantity of cyclodextrin in the formulation. Cavity size in relation to the lipophilic portion of the drug needs to be considered as well as the ionization of the cyclodextrin and drug in solution. As would be expected, complexation of a drug and cyclodextrin with the same charge will lead to a lower efficiency then when they possess opposite charges. Typically, nonionic combinations of drug and complexation are used to avoid weak complex formation. An increased processing temperature is thought to reduce the interaction forces (such as van der Waals, hydrophobic forces) of drug and cyclodextrin, thus decreasing complexation efficiency (43). Normally, cyclodextrins are included in a formulation between

a 1:1 or 1:4 molar drug-to-cyclodextrin ratio. Adding excess cyclodextrin to a formulation has been shown to have both positive and negative effects on drug permeation. In some cases, when free cyclodextrin is too concentrated, cyclodextrin will compete with the phospholipid membrane for association with the free lipophilic molecule, reducing the quantity of free drug that is able to permeate the membrane. Other studies have shown that cyclosporine will bind with cholesterols in the biological membrane itself, temporarily fluidizing it and enhancing permeability (44). Cyclodextrin association with cell membranes is thought to be not as disruptive as that caused by common surfactants, although completely reversible associations have not been observed in all cases. Solvent evaporation techniques seem to be the most effective in preparation of complexed drug and cyclodextrin. Film casting followed by aqueous redispersion and spray drying are two common pharmaceutical manufacturing processes that have been proven to be effective in complete drug complexation (45).

Application Examples

Oral Delivery

Spray-dried preparations of spironolactone were prepared with one of the four cyclodextrins: β-cyclodextrin, γ-cyclodextrin, hydroxypropylated β-cyclodextrin (HPβCD), or hydroxypropylated γ-cyclodextrin. Although less stable, hydroxypropylated cyclodextrins proved to be better solubilizers of this drug. As mentioned previously, this is mostly due to the improved solubility over the parent cyclodextrin through lack of self-assembly and crystallization. When bulk spironolactone was compared with that prepared with HPβCD via spray drying, oral dosing in beagle dogs showed a 3.5-fold enhancement in bioavailability (46). Many oral cyclodextrin formulations have been investigated and used in marketed products such as Nimedex® (nimesulide), Omeeta® (omeprazole), Sporanox® (itraconazole), Vfend® (voriconazole) and Surgamyl® (tiaprofenic acid). An extensive review of the improvement of oral drug delivery through incorporation of cyclodextrins has been written by Loftsson, Brewster, and Másson (47).

Intravenous Delivery

Sulfobutyl ether β-cyclodextrin (SBEβCD), marketed as Captisol® (CyDex Pharmaceuticals, Inc.), has been used in FDA approved injectable formulations Geodon® (ziprasidone) and Vfend (voriconazole) for enhancing drug solubility. The intrinsic solubility of a poorly soluble lipophilic compound 5-phenyl-1,2-dithiole-3-thione (5PDTT) was shown to improve 480-fold after addition to 10% Captisol aqueous solution. After injection, the highly lipophilic nature of the drug was hypothesized to lead to high erythrocyte binding and a resulting competitive displacement by plasma components (48). Other studies have focused on the PK behavior of voriconazole complexed with SBEβCD in animal and human models (49). Amphiphilic cyclodextrins are an interesting application of cyclodextrins currently receiving attention due to the capability of solubilizing poorly soluble drugs and permeating phospholipid membranes while tailoring release profiles during systemic circulation. These cyclodextrins have also shown the propensity for self-association, leading to the formation of nanoparticles. Encapsulation in formed nanoparticles gives this technology another method of drug loading, and consequently, drug solubilization in addition to cyclodextrin complexation. This further enhancement of drug solubility through incorporation in amphiphilic cyclodextrin nanoparticles was demonstrated with a 33% increase in cyclosporine concentration in cholesterol-associated HPβCD as compared to HPβCD solution alone (50). Distribution of amphiphilic β-cyclodextrin nanospheres was investigated in a mouse model by a radiolabeling technique (51). Results showed that nanoparticles were quickly eliminated from the blood by mononuclear phagocytic uptake and accumulated in the liver. After 1 hour, nearly 70% of the dose administered could be found in the spleen or liver, showing the potential of this system for hepatic targeting of poorly soluble drugs.

Nasal Delivery

As with many drugs exhibiting low aqueous solubility, benzodiazepines have shown to have increased solubility when the pH or the aqueous environment is reduced. At a low pH, drugs such as alprazolam, midazolam, and triazolam undergo reversible ring-opening, where the

primary amine is ionized. By increasing the intrinsic solubility of the drug through ring-opening, cyclodextrin complexation efficiency was shown to increase, further enhancing the drug solubility. SBEβCD was shown to have the greatest influence on midazolam solubility according to Loftsson et al., since complexation was assisted by the ionic attraction between the negatively charged cyclodextrin and the diprotonized drug (52). However, as mentioned previously, charged complexation may lead to a more unstable drug/cyclodextrin complex and result in a reduced efficiency. By addition of a stabilizing polymer [0.1% w/v hydroxymethylpropylcellulose (HPMC)], the drug/cyclodextrin complex was further stabilized, increasing cyclodextrin association and, as a result, the overall drug apparent solubility. To test the bioavailability of this cyclodextrin-solubilized nasal formulation, six healthy human volunteers were dosed with 200 to 300 μL SBEβCD-complexed midazolam and then seven days later with the marketed midazolam IV formulation. Through intranasal instillation, similar serum distribution (two compartment) was obtained in comparison to IV, demonstrating maximum blood concentrations after 15 minutes and 73% absolute bioavailability.

Often, drugs intended for nasal delivery are intended only for local effects on the nasal mucosa. In the case of WIN 51711, a new, poorly soluble anti-rhinovirus drug, mucosal activity is needed; however, poor solubility and susceptibility to hydrolytic degradation limits this drug's therapeutic effect. Additionally, at high level in systemic circulation, this drug was shown to cause asymptomatic crystalluria, which often is a sign of nephrotoxicity. The incorporation of 2,6-di-*O*-methyl-β-cyclodextrin (DMβCD) into the formulation increased solubility substantially (over 3500-fold) while also protecting the drug from hydrolytic degradation. As expected with many cyclodextrins, permeation was enhanced across a bovine nasal membrane mounted on a Franz-type diffusion cell. While drug permeation in this instance was undesirable, it was limited to 20% of the total drug only after two hours by inclusion in the complexed form (53).

SYNTHETIC AND NATURAL CARRIER DISPERSIONS

Complete drug dissolution is needed in all forms of delivery so that the active ingredient may be absorbed by the body and exert the intended therapeutic effect. Formulation techniques involving the dispersion of the active ingredient in a solid matrix carrier have been used to enhance overall bioavailability by preventing nanoparticulate aggregation, stabilizing the active ingredient in a more soluble morphology, and providing excipients that assist in sustaining heightened solubility or increased drug permeation in physiological conditions. The most appropriate drugs for delivery by this strategy are those that are dissolution rate-limited and permeable to biological membranes, or BCS class II. These formulations can include excipients to enhance permeation (i.e., chitosan, fatty acids, phospholipids); however, most of these technologies focus on the release of the drug into solution, not the absorption of an engineered particle. Almost all solid dispersion formulations incorporate the strategy of stabilized nanoparticulate drug in order to enhance solubility. In many cases, nanoparticles are engineered prior to their incorporation into solid dispersions (54), as discussed previously; although, the dispersion processes described here often produce solid dosage forms without any prior active processing. Various production methods such as melt dispersion, solvent evaporation, cryogenic processing, and supercritical fluid (SCF) processing are used to produce formulations with improved bioavailability (Table 3).

Processing Technology

Production of pharmaceutical dispersions by hot melt methods has been used for some time, beginning with the incorporation of drug in a eutectic mixture by Sekiguch and Obi in the 1960s. Further experimentation was conducted to try and elevate the degree of drug saturation within the molten carrier by snap cooling (55). More recently, hot melt extrusion (HME) has gained interest and been adapted for pharmaceutical applications. Melt processing using melt extrusion can result in an increase in drug solubility when the drug is fully or partially miscible in the molten excipients or when shearing levels allow for a substantial reduction in particle size. Briefly, this high-shear process involves feeding, melting, and metering of molten material down a heated barrel. A single or twin screw is responsible for the movement of the material in this process and can be designed for increase or decrease of the shearing forces in the process. When choosing a carrier to enhance the solubility of a drug substance, it is important

Table 3 A Summary of In Vivo Studies Conducted to Determine PK, Efficacy, or Safety of Solid Dispersion Formulations

Technology/ carrier	Drug	Subject	Study	Dose (route) (mg)	Blood AUC (ng h/mL)	Blood C_{max} (ng/mL)	Findings	Reference
URF/poloxamer 407	Tacrolimus	SD rat	PK	1.5 (oral)	450.6	138.5	Enhanced solubility led to more bioavailability compared to Prograf	Overhoff, 2008 (95)
Spray drying/ Pluronic F127	BMS-347070	Beagle dogs	PK	50 (oral)	28,961	1399	Improved bioavailability compared to micronized dispersions, equal to NanoCrystal	Yin, 2005 (54)
HME/HPMC	Itraconazole	SD rat	PK	9 (oral)	2258	291	Bioavailability was improved 2.5-fold over crystalline control	Miller, 2007 (77)
HME/Carbopol 974P, Eudragit L 100-55	Itraconazole	SD rat	PK	9 (oral)	11,107	1198	Carbopol with enteric polymer stabilized supersaturation state in duodenum	Miller, 2008 (78)
Solvent evaporation/ HPMC	Tacrolimus	Beagle dogs	PK	1 (oral)	11	4	Bioavailability was improved nearly 10-fold over crystalline control	Yamashita, 2002 (69)
Solvent evaporation/ HPMC	Tacrolimus	Cynomolgus monkey	BE	5 (oral)	578	38	New preparation method was bioequivalent to old method	Yamashita, 2003 (69)
Solvent evaporation/ HPMC E5	—	Beagle dogs	PK	Unknown (oral)	11,778	449	Drug bioavailability enhanced 30-fold over bulk drug control through stabilization of supersaturated conditions	Vandecruys, 2007 (79)
Emulsification/ solvent diffusion/ chitosan	Cyclosporine	Beagle dogs	PK	100 (oral)	32,801	2762	Cationic/permeation enhancing nanoparticles improved bioavailability 1.7-fold	El-Shabouri, 2002 (96)
Emulsification/ solvent diffusion/ gelatin	Cyclosporine	Beagle dogs	PK	100 (oral)	22,811	2035	Cationic nanoparticles improved bioavailability 1.2-fold	El-Shabouri, 2002 (96)

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(Continued)

Table 3 A Summary of In Vivo Studies Conducted to Determine PK, Efficacy, or Safety of Solid Dispersion Formulations *(Continued)*

Technology/ carrier	Drug	Subject	Study	Dose (route) (mg)	Blood AUC (ng h/mL)	Blood C_{max} (ng/mL)	Findings	Reference
SFL/polysorbate 80, poloxamer 407	Itraconazole	IRC mice	PK	80[b] (pulmonary)	1690	120	Pulmonary ITZ achieved 10 fold high lung levels compared to Sporanox	Vaughn 2006 (97)
URF/lactose	Tacrolimus	IRC mice	PK	30[b] (pulmonary)	1236	402	Stabilized amorphous nanoparticles give higher C_{max} and shorter T_{max} than crystalline	Sinswat, 2008 (88)
Spray drying/ chitosan	BSA	Balb/c mice	Efficacy	5 μg (intranasal)	—	—	Systemic immune response was 40 times higher than chitosan BSA solution	Alpar, 2005 (92)
Spray drying/ polysorbate 80, PVP	Paclitaxel	SD rat	PK	9 (IV)	12,434	8162	Drug partitioning from blood to tissue occurred more rapidly than that with Taxol	Straub, 2005 (94)
Spray drying/ polysorbate 80, PVP	Paclitaxel	NCr-Nu mice	Efficacy	0.45–1.2 (IV)	—	—	Tumor growth reduction comparable to Taxol; does not use Cremophor	Straub, 2005 (94)
Spray dried/ poloxamer 407	Griseofulvin	Wistar rats	PK	12.5 (oral)	13,230	2180	Improved wetting and dissolution lead to improve bioavailability over bulk drug	Wong, 2006 (98)
SCF–aerosol solvent extraction system/HPMC	Itraconazole	SD rat	PK	6 (oral)	2301	173.5	Bioavailability comparable to the marketed product, Sporanox	Lee, 2005 (99)

[a] Estimated from plot.
[b] Drug aerosol preparation.
Abbreviations: BE, bioequivalency; PK, pharmacokinetics; SD, Sprague Dawley.

to determine the miscibility of drug and carrier. By conducting laboratory-scale fusion experiments followed by analysis by dynamic scanning calorimetry (DSC) and the application of the Gordon–Taylor equation, potential carriers for HME can be screened as to expedite the formulation process (56). Thermal treatment of small samples in dynamic scanning calorimetry will assist in the determination of carrier/drug compatibility. Common examples of carriers used in HME are polyethylene glycol, PEO, methacrylate polymers, ethyl cellulose, and hydroxypropyl cellulose. Many carriers due to high glass transition temperatures and melt viscosities require the incorporation of a plasticizer, such as PEG or triacetin, to improve processing conditions. A detailed review of this manufacturing process is provided by Crowley et al. (57).

Formation of solid dispersions can also be produced through solvent evaporation techniques. This method, while simple in concept, can result in the enhancement of drug solubility by creating a fine dispersion in a pharmaceutical carrier. This method was first used in the 1960s by Tachibani and Nakumara (58) when they successfully coevaporated β-carotene in polyvinylpyrrolidone (PVP). By dissolving both drug and carrier in a common solvent and subsequently removing the solvent, dispersed drug-loaded powder can be obtained. The rate of evaporation, solubility of the carrier and drug in the solvent, and miscibility of the drug in the carrier will play a large role in the extent of solubility enhancement. Spray drying is one of the most common methods of solvent removal in the pharmaceutical industry. This technique involves the atomization of a volatile solvent into a temperature-controlled environment so that the solvent is quickly evaporated. Depending on the excipients included, speed of volatilization, and crystalline stability of the drug, amorphous drug particles/domains can be created by this technique. A limitation to this method of solvent evaporation is the inability to manufacture discrete nanoparticles, although spray drying can be used to stabilize premade nanodispersions (59). An excellent review of spray drying in the pharmaceutical industry is provided by Vehring (60). A different method of solvent removal is demonstrated in rapid freezing processes described below. These processes result in the production of highly soluble amorphous materials through the reduction of molecular mobility during the solvent removal process.

Rapid freezing processes using liquid cryogen have been used to make drug dispersions when a solid solution or amorphous homogenous dispersion is desired. Two processes for creating highly porous nanostructured aggregates of hydrophilic carrier and poorly soluble drug use this technique. Spray freezing into liquid (SFL) subjects a feed solution containing drug and excipients(s) to high-pressure atomization beneath the surface of liquid nitrogen. These atomized droplets freeze instantly, holding all dissolved contents in their "solubilized" molecular configuration (61). The solvent is then removed by lyophilization through sublimation in order to ensure no molecular mobility that would be allowed by a liquid state. A similar process, ultra–rapid freezing (URF), incorporates a cryogen-cooled substrate to rapidly freeze a drug/excipients solution. This freezing process may be run continuously, unlike SFL, and may allow for more rapidly frozen product, reducing the chance of phase separation or crystallization (62). Both processes create highly porous, nanostructured powder where drug and excipients are stabilized in the amorphous state. These powders can be created at high potencies (as high as 70% for some drugs) and have been shown to enhance the solubility of poorly soluble drugs. Other cryogenic processes include spray freeze drying (63) and spray freezing into halocarbon refrigerant; although, these processes are subject to problems of agglomeration and particle settling on the surface of the cryogen (64).

SCF processing presents a relatively new method of enhancing the absorption of poorly water-soluble drugs. Two of the main advantages provided by this technology include the limiting of organic solvents and requirement of mild processing temperatures and reducing concerns of potential dangerous solvent residues and degradants. Like other processes for forming solid dispersions, SCF processing has the ability to create stabilized amorphous or polymorphic drug compositions with the ability to exceed normal drug solubility. Additionally, for incorporation of the drugs, a hydrophilic, porous matrix allows for exceptional wetting ability. A review of the SCF technology for production of dispersions with improved solubility was provided by Yasuji et al. (65). Briefly, SCF processing involves the use of CO_2 at an increased temperature and pressure (31°C and 73.8 bar) where it processes both gaseous and liquid qualities. This supercritical CO_2 is nontoxic and may be used to solubilize drugs and excipients, precipitate drugs through antisolvent characteristics, remove organic solvents, or act as a medium for other

processes. Hot melt extrusion and SCF processing have been combined in some studies due to the ability of supercritical CO_2 to effectively plasticize the pharmaceutical carrier, thus reducing the processing temperature and improved processing conditions (66). Variations of this process include gas antisolvent (GAS), supercritical antisolvent (SAS), aerosol solvent extraction system (ASES), and solution-enhanced dispersion with supercritical fluid (SEDS) techniques. A key interest when considering SCF processing is the solubility (or lack thereof) of the pharmaceutical preparation in the SCF. For SCF solvent processes, supercritical CO_2 dissolving power of drug and excipient(s) will determine the resulting powder characteristics, such as particle size and density.

A relatively new technique for forming solid dispersions of drug and polymer has been investigated for the production of drug-loaded nanofibers, which can then be woven into fabrics for topical drug delivery. Electrospinning involves the production of a fluid stream of polymer and drug in a solvent/cosolvent system through a conductive capillary. The polymer stream is subjected to a strong electrostatic field at the end of the capillary, resulting in the formation of a Taylor cone from which small streams of the solution are ejected and solvents are volatilized. The result is the formation of a thin polymeric fiber with a diameter ranging from 100 nm to several microns, depending on the solvent, equipment, and environmental parameters (67,68). Application of these nanofibers have been studied for transdermal drug delivery and wound healing and have shown potential for solubility enhancement of poorly water-soluble drugs such as ketanserin and itraconazole.

Application Examples

Oral Delivery

Improvement of dissolution and solubility for oral formulations is a problem that faces approximately 40% newly developed drugs. Dispersion of a poorly water-soluble drug in a synthetic or natural polymer can often improve the wettability and solubility of the substance by incorporating processes that are readily scalable, high yielding, and cost-effective. These are a few reasons why solid dispersion technology is such an attractive method for improving the oral bioavailability of drugs. Tacrolimus, the leading immunosuppressive drug for the prevention of allograft rejection, is formulated as a solid dispersion in the currently marketed Prograf® (Astellas Pharma, Inc.; Tokyo, Japan). Yamashita et al. described the improvement of the aqueous solubility of tacrolimus (bulk solubility is 1–2 μg/mL) by using a solvent evaporation method. By swelling HPMC in an ethanol solution containing tacrolimus and the subsequent removal of the solvent under elevated temperature and reduced pressure, a 25-fold elevation of in vitro solubility was seen (69). This is attributed to the thermodynamic and kinetic instability of the amorphous tacrolimus created during the solvent evaporation process. More stable crystalline forms do not dissociate as easily in fluid because of their tightly packed, molecularly attracted arrangement, thus limiting the solubility. This study also showed a blood concentration AUC of 10.9 n gh/mL with the solid dispersion formulation as compared to 1.1 n gh/mL with the crystalline formulation after oral dosing to beagle dogs. In a separate study, tacrolimus with various stabilizing polymers was produced by URF (62). As described above, this cryogenic process enabled the production of highly porous, amorphous drug particles stabilized in poloxamer 407, poly(vinyl alcohol) and poloxamer 407, or sodium dodecyl sulfate. When investigated in dissolution and oral rat model testing, it was found that superior wetting and initial concentrations of the URF powders were superior to that of the marketed formulation, Prograf. Tacrolimus and poloxamer 407 formulated in a 1:1 ratio and produced by URF exhibited the highest bioavailability, even exceeding that of Prograf, in a rat model and the reason being its enhanced solubility and wettability, allowing for periods of elevated solubility in the GI medium. Another cryogenic process, SFL, has been used to increase the oral solubility in other poorly absorbed drugs such as danazol and carbamazepine. In vitro testing has demonstrated that dissolution rate was increased when compared to formulations prepared by physical mixture and traditional lyophilization, because the high surface area and high-porosity amorphous properties were afforded by the SFL technique (64). Traditional freeze drying was found to produce semicrystalline powders due to the slow freezing process, allowing for molecular mobility and crystal growth, which resulted in lower porosity, lower surface area, and slower dissolution.

While, many solid dispersion formulations focus on improving the solubility of a given compound, specific polymers may be included in order to enhance membrane permeability through promotion of paracellular transport or increasing formulation residence time. Natural and synthetic mucoadhesive polymers have been incorporated into a variety of solid formulations. These hydrophilic polymers may present some processing challenges during solid dispersion production due to high viscosity and their inability to dissolve in many solvents; however, they can effectively increase the residence time of a formulation for mucosal delivery (particularly important for GI delivery). The most common mucoadhesives are variations of carbomers and chitosans, although other natural polymers like sodium alginate and cellulose derivatives also claim to have mucoadhesive qualities and are discussed in more detail in a review by Grabovac et al. (70). Many mucoadhesive polymers have been chemically altered to increase solubility, such as *N*-trimethylated chitosan (71), which may in turn simplify formulation manufacture. Although not for GI delivery, buccal mucoadhesive films produced by HME have been studied by Prodduturi et al. Solid solution films containing clotrimazole, an antifungal with limited solubility, were produced by extrusion of drug, hydroxypropyl cellulose, and PEO and intended for improved systemic levels while avoiding first-pass metabolism (72). When evaluated for mucoadhesive properties, it was found in this study that increasing levels of PEO increased the adhesion of the film because of increased segmental mobility and increased chain entanglements (72,73). Enhancement of paracellular transport is another method by which low bioavailability drugs may induce a more substantial therapeutic effect. Paracellular transport of hydrophilic drugs is achieved by the chemical opening of the tight junction through disruption of a cell's phospholipid membrane or facilitating the removal of proteins and lipids from the membrane (74). As would be assumed, a high risk of toxicity is associated with many permeation enhancers as cellular membranes are often not able to reform and prevent epithelial cell lysing after disruption. A list of commonly studied tight junction opening agents was studied by Whitehead et al. for safety and efficacy. After evaluation of over 50 permeation enhancers, it was found that phenyl piperazine is the most safe and effective permeation enhancer, as it enhanced the permeability of dextran by 11-fold and allowed for repair of all tight junctions (as measured through transepithelial electrical resistance) within 24 hours (75).

A nanocrystalline formulation of a new COX-2 inhibitor, BMS-347070, was produced by spray drying to increase the solubility and dissolution rate (54). While amorphous formulations have been shown to be advantageous in supersaturating dissolution media, some drugs are highly unstable in their amorphous form and lead to concerns regarding the stability of the formulation lead. In a study conducted by Yin et al., spray drying of the drug and Pluronic F127 in methylene chloride produced nanocrystalline drug dispersed in a polymer matrix. It was found that dissolution rates were greatly improved over physical mixture or separately spray-dried formulations. In oral dosing of beagle dogs, a 1:1 ratio of BMS-347070 and Pluronic F127 formulation was shown to achieve comparable bioavailability when compared to a NanoCrystal preparation (relative bioavailability of 77% and 78%, respectively). The formation of drug nanocrystals in this formulation is due to the ability of amorphous poly(propylene oxide) chains of Pluronic F127 to sequester small area of drug within crystallized PEO domains, leading to a quickly wetting and easily de-aggregated nanocrystalline dosage form. Amorphous drug dispersions produced by spray drying are also used to increase drug solubility and is described by Broadhead et al. (76).

Improvement of the solubility of itraconazole, a poorly water-soluble antifungal, is described by Miller et al. by using a combination of controlled-release polymers and drug blended by HME. Itraconazole, rendered amorphous because of its miscibility in the enteric polymer EUDRAGIT® L 100-55, is able to achieve levels of solubility well above that of bulk powders. Further investigation into sustained release in the upper small intestine was investigated with the hypothesis that slower drug release through a swollen matrix would prevent the extent of drug precipitation from GI fluids. Carbopol® 974P was coextruded with enteric polymer and drug blends to allow for a more viscous release matrix in the upper small intestine, while still providing sufficient gastric protection (77). Application of this theory in oral dosing of Sprague Dawley rats showed that elevated and less variable bioavailability was possible when compared to extrudates formulated without Carbopol (78). Stabilization of supersaturated drug is an interesting concept for enabling an increased duration of high drug concentration

for enhanced drug absorption through the small intestine. Some other commonly used pharmaceutical polymers, HPMC and PVP, have been studied for their ability to prevent drug precipitation from supersaturated solutions and are theorized to prevent crystal growth by hydrogen bonding and diffusion resistance (79). In another study investigating the production of itraconazole solid dispersions, HME technology is combined with the solvent capabilities of SCF technology. Verreck and associates have investigated the use of supercritical CO_2 to reversibly plasticize and foam polymeric carriers during HME. This novel combination of two production strategies allows for the production of solid amorphous dispersions at low processing temperatures and without the stability problems sometimes associated with high plasticizer concentrations. When incorporated in an ethylcellulose 20 cps matrix at 10% drug loading, itraconazole remained completely amorphous and had enhanced wetting and dissolution properties (80). Furthermore, the production of a foamy extrudate by this technique facilitated more efficient milling for powder production. HME was also investigated for the incorporation of nimodipine, a calcium channel blocker, in various extrudable excipients for improvement of the dissolution properties. Dissolution rate was shown to improve when this drug was incorporated in HPMC, Eudragit EPO, and polyvinylpyrrolidone/vinyl acetate (PVP/VA); in addition, nimodipine demonstrated the ability to plasticize Eudragit EPO and PVP/VA, reducing the overall processing temperatures (81).

Polymeric loading of another poorly soluble drug ketoprofen has been conducted by a SCF impregnation process. In a study by Manna et al., amorphous ketoprofen was loaded into PVP at a level up to 58%. High drug loading was enabled by the affinity of ketoprofen for PVP rather than the supercritical CO_2 solvent, mostly due to hydrogen bonding (as determined by FTIR analysis) between the two molecules (82). In other cases, when drug does not passively diffuse into the carrier polymer, entrapment of drug within the polymeric carrier after the removal of the supercritical solvent is the predominant method of drug loading. However, high drug loading (ketoprofen levels of 25% or higher) did not lead to rapid release in dissolution testing because of the high binding affinity between the two molecules. Accelerated dissolution and elevated solubility of indomethacin incorporated in PVP carrier was also demonstrated with a similar supercritical process. In this batch process, Gong and coworkers found that amorphous solid solutions of indomethacin and PVP precipitated out of supercritical CO_2 can be made at levels of up to 20% drug loading without the use of any organic solvent. As indomethacin fractions increased, the preparation increased proportionally in crystallinity (83). Carbamazepine, another poorly water-soluble drug, was prepared as a solid dispersion in PVP K30 by either rotary evaporation or SCF technique. Intrinsic solubility was shown to increase 4-fold when prepared by the SCF method, while it increased only 2.6-fold when prepared by the rotary evaporation method. Interestingly, when the same supercritical preparation was made incorporating the amphiphilic solubilizers Gelucire 44/14 or Vitamin E TPGS, the intrinsic solubilities were actually reduced (84). By not requiring the use of solubility enhancing excipients, this method provides the added advantages of ease of manufacture and less concern for long-term stability. Two other drug molecules with low solubility, Griseofulvin and β-sitosterol, were subjected to rapid expansion of supercritical solutions (RESS) in an effort to reduce the particle size. Without the use of potentially toxic solvents or denaturing thermal processing, these two drugs were dissolved in supercritical CO_2 and precipitated out when pressure was rapidly reduced to normal. This effectively removed the solvent (SCF) leaving pure drug particles in the 200 nm range. Interestingly, when β-sitosterol particles were sprayed into aqueous solution of sodium dodecyl sulfate, particle agglomeration was avoided and a bimodal particle size distribution was measured by dynamic light scattering (DLS). The low range showed particles between 5 and 50 nm in diameter, while the high range showed particles between 120 and 200 nm in diameter. The experimental findings agreed with theoretical modeling of rapid expansion of supercritical solutions produced particles that predicted drug particles as small as 2 to 8 nm (85).

Pulmonary Delivery

Administration of solid dispersions to the lungs has also been studied by multiple groups. Because the delivery to the lungs provides unique formulation challenges such as the requirement of particles of respirable aerodynamic diameter and use of nontoxic biodegradable carriers, formulation technologies for the enhancement of poorly soluble drugs are limited. As

pulmonary delivery of macromolecules and drugs intended for systemic therapy become more popular, techniques to overcome these formulation challenges will become paramount. One such drug that can be intended for both local and therapeutic effects after pulmonary administration is itraconazole. A solid dispersion of itraconazole, polysorbate 80, and poloxamer 407 was prepared by SFL and was shown to be substantially amorphous and have improved wetting and aqueous solubility (86). In an animal study carried out in a murine model, a pulmonary and oral formulation of drug were made by SFL and compared to the marketed formulation, Sporanox. Male ICR mice were dosed with either 0.96 mg SFL itraconazole orally twice daily (b.i.d.), 0.96 mg commercial formulation b.i.d, or pulmonarily with SFL itraconazole and sampled for blood and lung concentrations. Local delivery with SFL-prepared itraconazole formulation produced lung levels 10-fold higher than those of either formulations (87). While the marketed Sporanox produced the highest serum levels, toxic side effects were seen in mice, causing death in 2 of the 12 mice. This was proposed to be due to the cyclodextrins present in the marketed formulation, which has been shown to cause toxicity in humans at elevated concentrations. While blood levels were low in the group dosed with pulmonary itraconazole, the enhanced solubility and permeation of the formulation allowed for sustained trough blood levels above 0.1 μg/mL, which is above the minimum lethal concentration (MLC) for *Aspergillus funigatus* (70 ng/mL). Similar processing techniques for the production of a solid dispersion of amorphous tacrolimus and lactose were produced by URF as described above. Powder X-ray diffraction showed URF production of tacrolimus powders without lactose semicrystalline, proving that lactose is necessary to facilitate the stabilization of the amorphous drug. In dissolution testing using simulated lung fluid with 0.02% dipalmitoylphosphatidylcholine (DPPC) media, this amorphous URF formulation was found to increase the solubility of tacrolimus over 10-fold when compared with bulk crystalline powder. A saccharide dispersion of nanostructured tacrolimus and lactose (1:1) was dosed to mice via a nose-only inhalation chamber for PK evaluation of resulting blood and lung concentrations (88). High blood and lung concentrations were achieved after single dose of the URF tacrolimus formulation due to its ability to supersaturate alveolar fluid, increasing the overall drug bioavailability. In vitro efficacy was shown by lymphocyte suppression in mixed leukocyte culture and mitogen stimulation assays (MSA) and was demonstrated to be more effective than the currently marketed dispersion of tacrolimus dispersed in HPMC (89).

In the lungs, heightened absorption across the pulmonary mucosa can be achieved through prolonged residence time, much like in the GI tract. Pulmonary drugs, however, are removed differently from the pulmonary mucosal surface, either by migration toward the larynx via the mucociliary escalator or by phagocytosis from pulmonary macrophages. Solid dispersion technology has also been used to circumvent these mechanisms resulting in the enhanced drug permeation. Large porous particles, possessing a respirable aerodynamic diameter but a large geometric diameter, enabled the increased bioavailability and elevated systemic levels of insulin and testosterone (90). These particles were produced by an emulsion evaporation technique resulting in poly(lactic acid-co-glycolic acid) (PLGA) particles as large as 20 μm in diameter for drug loading. These particles showed limited macrophage uptake, only 8% immediately after inhalation and 12.5% uptake 48 hours after inhalation as compared to greater than three times as much as when nonporous small particles were delivered. In another study, gelatin and polybutyl cyanoacrylate nanoparticles were loaded into lactose carrier particles by spray drying. By optimizing the spray drying process, fine particle fractions (FPF) and mass median aerodynamic diameters (MMAD) of 40% and 3.0 μm, respectively, were achieved after delivery via dry powder inhalation (91). This technology may allow for better bioavailability of some drugs by solid nanoparticulate delivery to the lungs.

Nasal Delivery

Lymphoid tissue in the upper respiratory tract has been targeted as a potential site for the local delivery of antibody-producing antigens for more patient compliant immunization. Because of its potential for delivery of immunizing macromolecules, nasal associated lymphoid tissue (NALT) has been recognized as a site where high drug absorption may be desired. Typically, macromolecules present a challenge to formulation scientists in that they are poorly permeated due to their large molecular size and sometimes hydrophilic characteristics. Nasal permeability was enhanced for a macromolecular agent through the intranasal instillation of chitosan and

chitosan HCl microparticles in BALC/c mice. Bovine serum albumin (BSA) was added to a solution of chitosan and spray dried to produce a solid dispersion of particles with an average diameter of approximately 3.2 μm loaded with 2% BSA. The immune response elicited by chitosan/BSA microparticles proved to be substantially increased (approximately 40 times) when compared to the response from administration of BSA solution (92). This increase in immune response can be attributed to an increased residence time due to mucoadhesion as well as the potential for chitosan to disrupt mucosal membranes by opening tight junctions.

Intravenous Delivery

Poorly soluble drugs intended for intravenous administration are typically incorporated in a solubility enhancing agent and/or organic solvent in order to provide a fully solubilized formulation. It is important to note that in addition to being fully solubilized after reconstitution prior to administration, these intravenous formulations also have to remain in solution when diluted in the patient's blood volume. Solubility enhancing agents such as Cremophor® EL and polyethoxylated castor oil have been used in many marketed formulations (Taxol®, Sandimmune®) to solubilize poorly water-soluble drugs; however, adverse side effects such as nephrotoxicity, neurotoxicity, and anaphylactic shock have been attributed to this oil and have lead to the use of alternative formulations. Additional studies have shown that Cremophor EL also causes leaching from polyvinylchloride (PVC) tubing, delivering diethylhexyl phthalate (a potential carcinogen) to the patient during intravenous administration (93). To provide enhanced solubility and improved drug absorption of anticancer drug paclitaxel, Straub and coworkers produced high porosity paclitaxel microparticles containing polysorbate 80 and PVP by spray drying. Dynamic scanning calorimetry and dissolution testing revealed that the spray-dried powders were amorphous and rapidly dissolved (95% in 5 minutes) in phosphate buffer solution. Particle size analysis prior to reconstitution gave a mean particle diameter of 1.53 ± 0.07 μm, which is acceptable for intravenous delivery. A PK study in Sprague Dawley rats as well as an efficacy study in human mammary tumor implanted in NCr-Nu mice was performed for intravenous formulation comparison with the marketed, Cremophor containing, paclitaxel formulation. Tissue distribution assayed by LC-MS/MS showed that clearance and steady state volume distribution of the spray dried formulation was fourfold and sevenfold greater than that of an equivalent bolus dose of the marketed formulation, implying that spray-dried paclitaxel is absorbed from the blood to the tissue more rapidly (94). In the efficacy study, the spray-dried formulation was shown to perform comparably to the marketed formulation, both reducing and slowing tumor growth considerably. However, because of the removal of Cremphor from the formulation, maximum tolerated dose for spray-dried paclitaxel was increased, providing the possibility for better therapeutic outcomes through a better tolerated higher dose (95–99).

SELF-EMULSIFYING DRUG DELIVERY SYSTEMS

For class II and IV drugs, a lipid carrier can prove very beneficial in improvement of bioavailability by maintaining the drug in a solubilized state, as it is transported to the mucosa for permeation. However, many lipid-based agents for solubilizing a drug will be diluted in GI media or the blood volume upon administration, causing a decrease in solvent power. Many times this will result in the precipitation of the drug in vivo and a lower and/or erratic bioavailability. SEDDS have been investigated extensively as a solution to these problems and have also seen marketed success in an oral formulation of cyclosporine, Neoral. These self-forming emulsions are defined as isotropic solutions of oils, drug, and surfactant and, in some cases, incorporate water-miscible cosolvents and cosurfactants. The inclusion of high levels of surfactant and its subsequent addition to relatively large aqueous volumes, such as GI fluid, allow for the spontaneous creation of stable and sometimes submicron lipid droplets. Much like explained previously for stabilization of hydrophobic particles in an aqueous dispersion, the thermodynamic stability of these systems can be explained in terms of free energy. However, in this case, change in entropy due to dispersion of oil phase in water phase must be considered so that

$$\Delta G = \gamma_{o/w} \times \Delta A - T \times \Delta S$$

where ΔG is the free energy of formation; $\gamma_{o/w}$ is the surface tension of the oil–water interface; ΔA is the change in interfacial area on microemulsification; ΔS is the change in entropy of the system, which is effectively the dispersion entropy; and T is the temperature (100). When a surfactant enables the significant lowering of the surface tension of the emulsion and the dispersion entropy is relatively high, a negative free energy will be present resulting in spontaneous formation of a stable microemulsion. According to Garrigue et al. (101), typical SEDDS systems result in droplet diameters between 100 and 300 nm, while self-microemulsifying drug delivery systems (SMEDDS) produce droplets below 50 nm in diameter. Although SEDDS are normally intended to form oil/water emulsions in situ, some studies have also investigated the use of water/oil SEDDS for oral dosing of hydrophilic excipients.

Processing Technology

Unlike many of the previous formulation techniques discussed in this chapter, development of a SEDDS formulation does not involve expensive manufacturing equipment or complicated drug loading procedures. The focus on creating a self-emulsifying system is the proper selection of oil phase and stabilizers, consideration of cosolvent/stabilizers, and optimization of all excipients' concentration. The optimization process typically requires the development of one or more pseudoternary phase diagram to model the transitions and properties of the emulsion. As a general rule, self-emulsifying formulations require large amounts of hydrophilic surfactant in order to form small droplets when added to an aqueous phase. Typically, between 30% and 60% w/w of the formulation is composed of a surfactant, which is most commonly a high HLB, nonionic surfactant. In many cases, a cosurfactant/cosolvent (commonly ethanol, PEG, or PG) can be added to the formulation to reduce the amount of surfactant required. Nonionic surfactants, such as polyoxyethylene oleate and ethoxylated polyglycolyzed glycerides, are used because of their lower incidence of GI irritation in comparison to anionic, cationic, or zwitterionic surfactants (101). SEDDS have been studied with a multitude of lipid bases and can be made any of the pharmaceutically accepted fatty acids, fatty alcohols, natural oils and oil esters, phospholipids, or waxes; however, most SEDDS use oils from the medium chain triglyceride or modified vegetable oil categories. Although, many of these oils have already been proven effective when incorporated into a self-emulsifying system, it is important to ensure that the oil has a high loading capacity for the solubilized drug and that an optimized drug/oil/surfactant concentration is reached.

Application Examples

Oral Delivery

An improved oral formulation resulted when reformulation of an oral cyclosporine formulation (Sandimmune) produced a SEDDS that results in a highly bioavailable emulsion when it comes in contact with an external aqueous phase. By using an emulsion stabilizing surfactant that result for the lipolysis of triglyceride, Neoral (Norvartis) is able to achieve therapeutic immunosuppressant levels with less variability. In a multicenter, double-blind clinical study, efficacy in prevention of episodes of heart transplant rejection was shown to be superior in microemulsified cyclosporine when compared to the older formulation. As would be expected, reduced variability in blood PK profiles with the use of Neoral was seen in the first year of treatment (102). Additionally, therapeutic blood targets were met with lower dosing of the microemulsion formulation, proving improved bioavailability. These results agreed with earlier findings reported by Tan et al. in a single dose study in patients awaiting lung transplant suffering from cystic fibrosis. Overall bioavailability of microemulsified cyclosporine was shown to be 1.84 to 2.09 times higher (at 200-mg and 800-mg dose, respectively) than that of the conventional formulation in these patients (35).

SOLID LIPID NANOPARTICLES

As an alternative to polymeric drug delivery systems, lipid-based formulations have also shown distinct advantages over traditional formulations while generally incorporating safe and tolerable pharmaceutical excipients. While drug nanosizing is an excellent formulation strategy for improving the bioavailability of class II drugs (poor solubility, high permeability), there

has not been much evidence that it can also enhance mucosal permeability of hydrophilic drug molecules. Many polymeric excipients and surfactants have demonstrated enhanced permeability; however, some of these polymers may have damaging effects on epithelial tissues that are not readily reversible (103). Lipid-based delivery systems are theorized to enhance membrane permeation by fluidization (or temporary disruption) of mucosal membranes, tight junction opening, and inhibition of efflux mechanisms (104).

Several issues associated with liquid lipid delivery systems, such as broad particle size distribution and instability during production, can be avoided through the use of SLNs. Similar to most lipid-based preparations, SLN formulations incorporate three main components: drug-loaded lipid, emulsifier/stabilizer, and water. Many emulsified lipid carriers allow for drug partitioning between oil and aqueous phases due to the fluidity of the formulation. Additionally, many emulsified systems are quite large in droplet diameter and have a broad size distribution. Formulation techniques for production of SLNs allow for submicron particle sizes and a narrow particle size distribution. The benefits provided by polymeric delivery strategies such as particle stability and controlled release are combined with benefits of biocompatible lipid systems in this formulation strategy (Table 4). Because of their nontoxic nature, SLNs have also been investigated for non-oral routes of administration such as intravenous and pulmonary. It should also be noted that a similar formulation strategy, nanostructured lipid carriers (NLCs), has been shown to improve loading capacity and stability of SLNs by incorporating a blend of solid and liquid lipids that are solid at body temperature (105); however, NLCs are a relatively new technique and have seen less development as pharmaceutical products.

Liposomal formulations share many of the same benefits of SLNs such as small, monodisperse particle sizes and biocompatibility. While liposomal formulations have seen some success on market (AmBisome®, DaunoXome®), many difficulties have been encountered in process scale-up and stability during sterilization. The physical and chemical stability as well as simplification of processing steps make SLNs more attractive in many cases. Unlike liposomes, where a bilayer phospholipid membrane must be produced, SLNs physically encapsulate the therapeutic moiety in a lipid layer/matrix, much like in polymer encapsulation. Similar to liposomes, tailoring for targeted delivery is possible with SLNs since the solid lipid surface allows for attachment of targeting ligands or other surface modifying agents. Choice of processing method will depend on many factors including drug stability to processing conditions, desired drug loading, particle size, and production costs.

Processing Technology

Methods used to produce drug-loaded solid lipid particles in the nanoparticulate range normally involve homogenization processing or particle precipitation. In the early 1990s, two different methods of production were patented by Muller (106) and Gasco (107), independently. Muller produced lipid nanoparticles by a high-pressure homogenization technique of either a suspension (cold homogenization) or an emulsion (hot homogenization). In cold homogenization, drug dispersed in supercooled lipid is milled and then subjected to homogenization while temperatures are maintained below 25°C, minimizing thermal degradation. When formulating a hydrophilic drug for SLN delivery, cold homogenization may be a better suited process due to the lower likelihood of drug partitioning from the lipid particle to the aqueous phase. Additionally, there is less emulsifier needed during this process due to the stability provided by the supercooled temperatures; consequently, only low concentrations of surfactant are added to avoid particle aggregation during milling (108,109). Common emulsifying agents used in the hot homogenization process include lecithins, poloxamers, and sodium glycocholate. These emulsifying agents are necessary, particularly in hot homogenization, to prevent gelation and crystallization of unstable lipid droplets that often require coemulsifiers for complete stability (110). Advantages of hot homogenization include smaller, more monodisperse lipid particles (ideal for intravenous formulations) formed from high shearing of an emulsion; however, because of elevated temperatures and liquid interfaces, drug degradation and loading efficiencies may be less than desired. A more detailed description of the processing steps in both of these techniques is given in Figure 2.

Another preparation method for making SLN incorporates the dilution of a stabilized microemulsion in cold water. Gasco and colleagues developed this process based on the theory of droplet size reduction upon the dilution of a warm emulsion described by Moulik

Table 4 A Summary of In Vivo Studies Conducted to Determine PK, Efficacy, or Safety of Solid Lipid Nanoparticle Formulations

Technology/ lipid	Drug	Subject	Study	Dose (route)	Blood AUC (ng[a]h/mL)	Blood C_{max} (ng/mL)	Findings	Reference
Double emulsion/chitosan-coated tripalmitin	Salmon calcitonin	SD rats	Efficacy	150 IU[b] (oral)	—	—	Lowered serum calcium levels compared to control solution because of permeation enhancement of chitosan	Garcia-Fuentes, 2005 (126)
Warm emulsion/stearic acid	Tobramycin	Wistar rats	PK	1.5 mg (intraduodenal)	709,450	28,000[a]	Lymphatic uptake and slower elimination lead to 100-fold bioavailability increase over IV solution	Cavalli, 2000 (112)
Warm emulsion/stearic acid	Tobramycin	Wistar rats	PK	1.5 mg (IV)	28,500	–	Bioavailability increased 5-fold over IV solution due to longer residence time	Cavalli, 2000 (112)
Warm emulsion/stearic acid	Tobramycin	Wistar rats	PK	1.5 mg (intraduodenal)	1,248,000	31,500	Slower drug clearance allowed by higher number of low potency SLN	Cavalli, 2003 (113)
Hot homogenization/ tristearin	Clozapine	Wistar rats	PK	6 mg (intraduodenal)	11,730	1890	Lymphatic uptake leads to 4.5-fold bioavailability increase over suspension	Manjunath, 2005 (123)
Hot homogenization/ tristearin	Clozapine	Wistar rats	PK	3 mg (IV)	10,240	—	Decreased clearance lead to 2.9-fold bioavailability increase over suspension	Manjunath, 2005 (123)

(Continued)

Table 4 A Summary of In Vivo Studies Conducted to Determine PK, Efficacy, or Safety of Solid Lipid Nanoparticle Formulations *(Continued)*

Technology/ lipid	Drug	Subject	Study	Dose (route)	Blood AUC ($ng^a h/mL$)	Blood C_{max} (ng/mL)	Findings	Reference
Hot homogenization/ vitamin E	Fenofibrate	Wistar rats	PK	30 mg (oral)	2,170,300	200,700	Formulation showed equivalent bioavailability to DissoCubes nanosuspension	Hanafy, 2007 (125)
Spray drying/cetyl alcohol, tripalmitin	5-fluorouracil	Hamsters	PK/tissue distribution	0.188 mg (pulmonary)	—	15	Lipid association causes drug retention in the conduction airways	Hitzman, 2006 (128)
Melted homogenization/ glyceryl behenate	None radiolabeled	Wistar rats	Distribution	200 K/cpm (pulmonary)	—	—	Lipid nanoparticles cause significant lymphatic uptake in lung	Videira, 2002 (129)
Warm emulsion/triolein, DSPC, cholesteryl oleate	Paclitaxel	Tumor implanted Balb/c mice	Safety/efficacy	1.1 mg (intraperitoneal)	—	—	Targeted lipid nanoparticles resulted in significant tumor growth reduction	Stevens, 2004 (130)

[a] Estimated from plot.
[b] Dose given in international units.
Abbreviations: PK, pharmacokinetics; SD, Sprague Dawley.

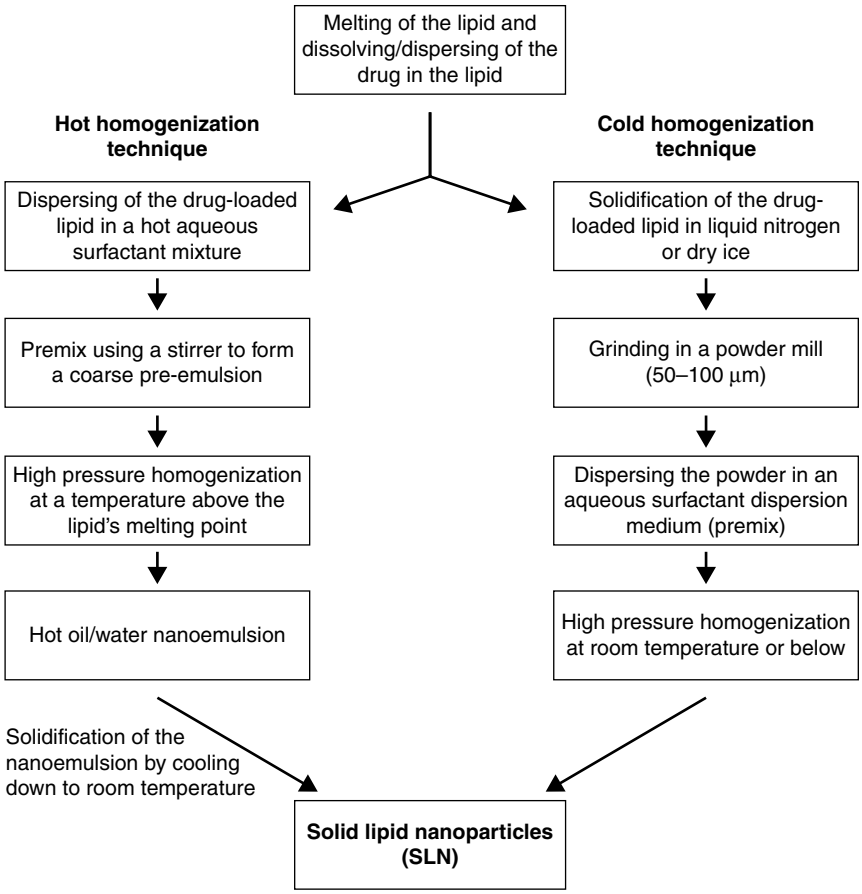

Figure 2 Processing steps for production of SLNs by homogenization. *Source*: From Ref. 109.

and coworkers (111). When the microemulsion is added to cold water for dilution, the water, acting as a heat sink, quickly cools the molten oil droplets and precipitates out the lipid/drug nanoparticle. The lipid phase in this emulsion typically consists of stearic acid stabilized by a surfactant (polysorbate, phosphatidylcholine) and cosurfactant (butanol). These surfactants are removed after particle formation by a rinsing process in order to avoid particle instability and potential human toxicity. It is important that a drug possesses lipophilic characteristics in order to be successfully incorporated into the oil phase. In some cases, such as the incorporation of tobramycin (112,113) and doxorubicin, hydrophilic molecules must be combined in an ion-pair complex by coprecipitation to increase the overall lipophilicity. Tobramycin is often coprecipitated with hexadecyl phosporic acid to produce a lipophilic entity that can readily diffuse into the oil phase of an emulsion. This step is critical to obtain high loading capacities and reduction in particle size. Additional considerations to be noted in using the warm emulsion technique are potential particle aggregation upon storage and loss of surface deposited drug during rinsing procedures. To address these concerns, an alternative warm emulsion technique was proposed where a warm emulsifying wax or Brij 72 (a polyoxyethylene alkyl ether) based emulsion were cooled to room temperature without aqueous dilution, allowing for more potent SLN dispersions and reduced need for lyophilization (114). The warm emulsion process has been shown to be easily scalable because of the limited energy required for particle formation (115) and may be a reasonable choice for incorporation of large molecules since no high shearing is needed.

Other methods of SLN production have been studied; however, these are less widely studied. Very small SLNs, less than 30 nm, have been created by Sjöström et al., using a solvent evaporation technique of a cyclohexane and water emulsion (116). Particles created by evaporation

methods may be superior for targeted delivery; however, low particle yield and residual solvent concern make this method less applicable to large-scale production. A better approach to the use of organic solvents to produce lipid particles may be to use a partially water-miscible organic solvent for solubilization of the lipid phase. By this method, the organic solvent may be removed by dilution with large quantities of aqueous media causing the eventual precipitation of the lipid nanoparticle. One study produced lecithin particles from 150 to 350 nm by continuous dilution of benzyl alcohol (117). Other advanced techniques have been used such as SCF processing for the preparation of insulin containing SLNs and are referenced in a review by Almeida et al. (118). The use of SCF has also been used in the extraction of organic solvents from fine emulsions for the production of lipid nanoparticles for lung delivery (119). An added advantage is given by this extraction method since both drug and lipid are plasticized by supercritical CO_2, creating a homogenous drug–lipid matrix. If processing equipment and capabilities are available, SCF processing can provide advantages for peptide and large molecule delivery due to the mild processing conditions and elimination of toxic solvents.

Application Examples

Oral Delivery

Oral dosing using solid lipid nanoparticle technology provides a variety of advantages for poorly absorbed drugs such as improved dissolution rate, enhanced particle permeability, potential targeting of GI lymphatics, and opportunity for surface modification. Duodenal uptake of SLNs has been investigated thoroughly by Gasco and colleagues in both drug-free (120) and drug-containing (113,121,122) lipid nanoparticles made by the warm emulsion process. In tracking radiolabeled steric acid nanoparticles after duodenal administration in rats, it was observed that up to 20% of the dosed SLNs were detected in the lymph, while only 0.16% were detected in the blood. This apparent targeting of the lymphatic system may be due to the targeting of M cells in the rat GI tract. Further studies by this group have focused on the production and oral delivery of tobramycin containing SLNs. Tobramycin, a poorly soluble and permeable drug, was hypothesized to benefit from incorporation in a lipid nanoparticle to enhance dissolution and solubilization as well as permeation through a physiological membrane. When compared to tobramycin aqueous solution administered IV and duodenally in rats, SLNs of tobramycin (tobra-SLNs) administered by the same routes showed substantial improvement in overall bioavailability (112). Intravenous tobra-SLNs improved bioavailability by fivefold, while duodenally administered tobra-SLNs exceeded 100 times the IV solution bioavailability (duodenal solution was not detectable). The longer residence time and larger elimination half-life due to lymphatic uptake allowed for the permeation and controlled release of tobramycin when administered in lipid nanoparticles. In subsequent studies, tobramycin loading level has been seen to play a role in vivo in release behavior and PK (113). The hot homogenization production technique was used to enhance the bioavailability of clozapine, a lipophilic drug that is highly metabolized by hepatic enzymes CYP1A2 and CYP3A4 (123,124). By applying the strategy of targeting lymphatic tissue using SLNs, first-pass metabolism was substantially reduced and bioavailability was improved up to 4.5-fold. It was also noted in this study that SLN delivery increased the amount of drug delivered to reticuloendothelial tissues and the brain. Fenofibrate, a poorly water-soluble drug, was investigated for formulation in SLNs, a crystalline nanosuspension, and micronized dispersions. After oral dosing to rats, both the nanoparticle preparations achieved nearly double the bioavailability of the micronized formulation; however, no significant difference was seen between SLNs and the nanosuspension (125). The conclusion was drawn that in this lipophilic drug ($\log P = 4.6$), drug absorption was limited more by solubility than permeability. Oral delivery of surface modified SLNs has also been investigated for delivery of peptides such as salmon calcitonin. Garcia-Fuentes and coworkers have studied the use of chitosan and PEG as agents to modify the surface of tripalmitin nanoparticles, assisting with stabilizing the peptide-containing particle in the harsh environment of the GI tract. As hydrophilic polymers, chitosan and PEG essentially create an aqueous boundary layer between the GI peptidases and the drug-loaded particle. Chitosan has shown to also promote a beneficial association with epithelial cells through its mucoadhesive properties; however, in this study it was noted that the positively charged

chitosan reduced the quantity of surface-associated calcitonin, reducing the typical burst effect seen in uncoated and PEG coated particles (126). Chitosan-coated lipid nanoparticles were also shown to disrupt Caco-2 cell monolayers as evidenced by the lowered transepithelial electric resistance (127).

Pulmonary Delivery

Pulmonary applications for SLNs have also been investigated for the aerosolization of drugs with poor absorption, generally due to low solubility in alveolar fluid. Lipid-matrix nanoparticles of poorly water-soluble drug can be dispersed in aqueous media for nebulization and are readily absorbed across the pulmonary epithelial tissue because of the small particle size and enhanced membrane permeability. Lipid nanoparticles with a mean diameter of 30 nm have been shown to have emitted doses comparable to that of aerosolized solutions when dosed with the AERx® Single Dose Platform (Aradigm Corporation, Hayward, CA) (119). Delivery to the lung presents unique challenges to nanoparticle delivery since many polymeric surfactants and stabilizers have been shown to elicit a lung immune response, or are relatively unknown for pulmonary applications. Tolerability of SLNs in intravenous formulations and liposomes in pulmonary formulations (AmBsome) have been improved through the use of biocompatible materials.

An animal model for determination of clearance of lipid nanoparticles in hamster lungs was studied by Hitzman for the determination of clearance rates of the chemotherapeutic agent 5-fluorouracil (128). It was proposed that half-life in the lung (approximately five hours) was longer because of long-term particle retention in the conduction airways. An eight-compartment PK model was also used to determine the amount of free drug not associated with the lipid carrier. In a previous study, lung lymphatic uptake of radiolabelled SLNs was determined by labeling glyceryl behenate with ^{99m}Tc and a lipophilic chelator. The clearance mechanism was proposed to be predominately macrophage uptake, leading to particle concentrations of 7.4%, 6.4%, and 3.2% of the total dose in the periaortic, auxiliary, and inguinal lymph nodes, respectively, 4 hours after administration (129). Pulmonary lymphatic uptake is important when considering targeting lung cancer metastasis and pulmonary immunological diseases, such as asthma.

Intravenous Delivery

SLNs in intravenous formulations are useful in improving solubility and enabling drug diffusion and penetration into tissues that are normally difficult to target. These formulations have been used to enhance aqueous solubility of poorly water-soluble drugs such as paclitaxel (121,130,131), while avoiding systemic toxicity associated with solubility enhancers such as Cremophor EL. An interesting application of the enhanced membrane permeability capabilities of SLNs is their potential to transfect the BBB. The BBB has proven to be very difficult to permeate due to the extent of tight junction bound endothelial tissue, lack of pinocytosis, and active efflux mechanisms. A key concern when designing a delivery system targeted for the brain is the reduction of residual solvents, toxic degradants, and particle aggregates that may lead to stability and toxicity problems. Some characteristics that make SLN a good candidate for drug delivery to the brain are minimal toxicity, formulation stability, minimal membrane disruption in comparison to polymers, potential to attach targeting surfactants and ligands, and controlled-release capabilities (132). Wax nanoparticles were prepared using anionic (sodium lauryl sulfate), cationic (*N*-octadecyl choline), or nonionic (Brij 78) surfactants to determine the effect of surface charge on permeation and toxicity of the BBB. By studying changes in vascular volume resulting from membrane disruption, Lockman and coworkers determined that low concentrations of neutral and anionic wax nanoparticles have little toxic effect, while cationic nanoparticles showed significant disruption and toxicity. Surprisingly, low doses of anionic nanoparticles showed greatly improved BBB penetration, even though the BBB has a negative luminal charge. It was suggested in the study that anionic nanoparticles facilitate transport by binding to low-density lipoprotein receptors on the endothelium (133). The attachment of polysorbate 80 to the surface of SLNs was studied by Göppert et al. for improvement of brain targeting after intravenous injection. Drug targeting, having a direct correlation with the ability of the particle to permeate the BBB, was enabled by the absorption of the plasma proteins apolipoprotein E, apolipoprotein C-II, and albumin and immunoglobulin G to the

surface of polysorbate 80 coated SLNs (134). Furthermore, the extent of apolipoprotein E binding to nanoparticles proved to be proportional to the presence of lipophilic binding sites, meaning more lipophilic surfactants (polysorbate 60 and polysorbate 80) promote better protein absorption, and consequently better BBB permeation. Camptothecin, an antitumor agent most active in its lactone form, exhibits poor solubility and has seen limited use due to instabilities in biological media. As compared to IV injection of camptothecin solution, it was found that camptothecin SLN saw a 10-fold increase in drug delivered to the brain in mice (135). It is hypothesized that transport of intact particles by endocytosis and subsequent drug diffusion was the mechanism of drug delivery.

CONCLUSION

As current trends suggest, the importance of not only enhancing drug solubility in vitro, but the improvement of drug bioavailability in animal and human models is becoming more of the focus of preclinical drug development. There is no shortage of technologies to produce improved formulations; however, many have yet to prove efficacy, safety, and reproducibility in test subjects. It is apparent that better in vitro/in vivo correlation is certainly needed as well as improved understanding of animal/human study relationships.

Strategies for improvement of drug absorption such as particle size reduction, micelle encapsulation, complexation, dispersion, and lipid-based formulation have been studied extensively and shown to improve bioavailability in animal and human models. Through the incorporation of nonimmunogenic carriers, permeation enhancing excipients, and tissue-targeting particle engineering technology continued improvements in drug delivery of poorly absorbed compounds and overall therapeutic outcomes can be realized.

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8 Transporters Involved in Drug Disposition, Toxicity, and Efficacy

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INTRODUCTION

Transporters are proteins that translocate endogenous compounds (such as bile acids, lipids, sugars, amino acids, steroids, hormones, and electrolytes) and xenobiotics (such as drugs and toxins) across biological membranes to maintain the cellular and physiological concentrations of these substances, maintain fluid balance, and provide a means for eliminating potentially harmful foreign substances from cells. Transporter proteins are divided into the adenosine triphosphate (ATP)-binding cassette (ABC) transporter superfamily and the solute carrier (SLC) family of proteins.

SLC transporters act by facilitating the uptake of their substrates into the cells. This family of transporters contains 46 subfamilies and 360 transporters including sodium-bile acid cotransporters (NTCP, SLC10 family), proton oligopeptide cotransporters (PEPT, SLC15 family), organic anion transporting polypeptides (OATP, SLC21 family), organic cation, anion, and zwitterion transporters (OCT/OAT, SLC22 family), and nucleoside transporters (NT, SLC29 family). SLC transporters are divided into facilitative transporter and active transporter classes. Facilitative transporters are not coupled to any energy source and passively facilitate the diffusion of molecules across the membrane down their concentration gradients allowing a rapid equilibrium across the membrane. The active SLC transporters use an energy source that is (*i*) provided by an ion-exchanger, which causes pH alteration in the microenvironment of the cell surface or (*ii*) indirectly coupled to Na^+/K^+ ATPase, which can create a negative intracellular membrane potential due to the imbalance in charge movement.

Recently, the multidrug and toxic compound extrusion (MATE) family has been demonstrated to have an important role in drug disposition. The MATE family was first identified as secondary multidrug transporters in bacteria and confers resistance in antibiotics and antifungal drug therapy (1). Currently, 861 related sequences have been found in a reference protein database by means of a PSI-blast search. These sequences, which include representatives from all three kingdoms of living organisms (i.e., Eukarya, Archaea, and Eubacteria), have been assigned to the MATE family, suggesting that these transporter proteins are common constituents of living organisms and phylogenetic analysis of known sequences has led to division of the MATE family into 3 large subfamilies comprising 14 smaller subgroups. Family 1 comprises bacterial MATE transporters and includes *Vibrio parahaemolyticus* NorM, a prototypic MATE transporter. Family 2 consists of eukaryotic MATE transporters and is divided into four subfamilies: 2A, comprising yeast and fungi MATEs; 2B, comprising plant MATEs; 2C, comprising animal and human MATEs; and 2D, comprising protozoan MATEs. Family 3 consists of bacterial and archaebacterial MATEs (1).

The driving force for MATE is H^+ or Na^+ exchange. Otsuka et al. first cloned the mammalian MATE (family 2C) from human and mouse tissues (2). In humans, the two genes encoding MATE1 (encoded by *SLC47A1*) and MATE2 (encoded by *SLC47A2*) are closely located on chromosome 17 (2). MATE1 is expressed ubiquitously throughout the body, but predominantly in the liver and kidneys, where it is localized on the bile canaliculi and brush border membranes, respectively. In contrast, MATE2 is expressed specifically in the kidneys and is localized on the brush border membranes. When expressed in HEK293 cells, MATE1 is localized on the plasma membrane and mediates H^+-coupled electroneutral exchange of tetraethylammonium (TEA)

and 1-methyl-4-phenylpyridinium (MPP). Studies of *cis*-inhibition suggest that MATE1 recognizes organic cations with highly diverse chemical structures as transport substrates (2). MATE2 also transports various organic cations including TEA, MPP, cimetidine, *N*-methylnicotinamide (NMN), and metformin through H^+ exchange (3). Mouse MATE1 is also predominantly present in renal brush border membranes and bile canaliculi and is involved in the excretion of various organic cations including TEA and MPP (3).

In mammals, the export of organic electrolytes with extremely diverse chemical structures into the urine and bile occurs via transepithelial transport across the basolateral and luminal membranes of renal tubular cells and through the sinusoidal membranes and bile canaliculi of hepatocytes. The biochemical and pharmacological profiles of human and mouse MATE-type transporters, in addition to their subcellular localization, match the proposed properties of the long-sought renal and hepatic organic cation exporter that is principally responsible for the final step of organic cation excretion (2–4). Although MATE1 has been characterized as an organic cation/H^+ antiporter, it has recently been shown that human MATE1 can also transport some organic anions such as estrone sulfate, acyclovir, and ganciclovir, and amphoteric compounds such as cephalexin and cephradine (5). The zwitterionic cephalexin and cephradine were revealed to be specific substrates of *hMATE1*, but not of *hMATE2*. Levofloxacin and ciprofloxacin were not transported by MATEs, but were demonstrated to be potent inhibitors of these transporters (5).

ABC efflux membrane transporters consist of transmembrane domains (TMDs) and nucleotide binding domains (NBDs). They are directly coupled to ATPase activity and hydrolyze ATP to derive energy for pumping substrates across the cell membrane. The full efflux transporters, such as P-glycoprotein (P-gp) and multidrug resistance protein (MRP), possess two NBDs in one polypeptide chain. The half transporters, such as breast cancer resistance protein (BCRP), only contain one NBD (6). The half transporters function as a dimer or tetramer bridged by specific linkages. Among 49 human genes in seven subfamilies of ABC transporters, P-gp [also known as multidrug resistance 1 (MDR1) protein] in ABCB family, MRP1 and MRP2 in *ABCC* family, and BCRP [also known as mitoxantrone resistance protein (MXR), ABCG2, ABCP] in ABCG family are the major ABC transporters to confer resistance in the tumor cells and to efflux xenobiotics (such as drugs or toxins) out of normal tissues.

Uptake (SLC family) and efflux (ABC family) transporters interact dynamically to mediate the accumulation and translocation of drugs or endogenous substrates into a cell. The gene nomenclature, protein name, tissue distribution, driving force, substrate properties and the substrates, inhibitors, and inducers of the common drug-related transporters are listed inTable1 (7–9). Generally, the human gene is designated by all capital letters and the animal gene is denoted by all lower case letters or a capital letter followed by lower case letters.

This chapter reviews our current understanding of transporter types and their functions in drug disposition, toxicity, and efficacy. The clinical impact of transporter polymorphisms is also discussed in this chapter. The methods of evaluating transporters in vitro and in vivo can be found in the first edition of this book and some extensive reviews. This chapter should not be considered as a comprehensive review but rather an update of the current knowledge. References are provided for some of the past reviews in this area for extended reading (6,9–12).

ROLE OF TRANSPORTERS IN DRUG DISPOSITION

Transporter proteins affect drug absorption in the small intestine and drug elimination in the liver and/or kidney by governing drug substance in and out of the intestinal enterocytes, hepatocytes, or renal tubular cells. Transporters can also limit or facilitate the penetration of drugs into the brain, placenta, tumor, T-cells, and so on. The inhibition or lack of transporter function can alter the exposure of drugs to tissues and potentially result in either lack of efficacy or increased toxicity. A classic example of toxicity caused by the reduction in transporter activity is provided by studies on antiparasitic agent avermectin. Avermectin caused neurotoxicity in CF-1 mice and collie dogs deficient in P-gp (13,14).

The role of transporters in drug disposition has been evaluated by using transporter knockout or deficient animals or by using transporter inhibitors in both animals and humans. In humans, the role of transporters in drug absorption has been indirectly shown by

Table 1 Tissue Distribution and Substrate Properties of Major ABC and SLC Transporters

Gene	Protein Name	Tissue Distribution	Substrate Properties	Selected Inhibitor and (Inducer)	Driving Force
ABC Transporters					
ABCB1	MDR1, *P*-glycoprotein	Intestine, liver, kidney, brain, placenta, adrenal, testes, cancer cells	Lipophilic, amphiphilic with weak organic cation, containing hydrogen bond donor and acceptor, such as digoxin, talinolol, vinblastine, paclitaxel, fexofenadine, quinidine, loperamide, topotecan, gleevec, colchicines, daunorubicin, Calceine-AM, rhodamine123	Ritonavir, ketoconazole, cyclosporine, verapamil, erythromycin, quinidine, PSC833, GF918120, LY335979 (rifampin, St John's wort)	Intrinsic ATPase activity and ATP hydrolysis
ABCB11	BSEP, SPGP	Liver	Bile salts and paclitaxel	All major bile salts, CsA, bosentan	Intrinsic ATPase activity and ATP hydrolysis
ABCC1	MRP1	Ubiquitous (mainly in lung, kidney, brain, colon, testis, peripheral blood mononuclear cells), cancer cells	Glutathione, glucuronide, and sulfate conjugates. Hydrophilic with organic anion. Substrates overlap between MRP1, MRP2, and MRP3, such as Calcein, leukotriene C4 (LTC4), methotrexate, and vinblastine	Probenecid, indomethacin, MK571, cyclosporine A (chlorambucil, epirubicin) probenecid, indomethacin, MK571, cyclosporine (dexamethasone, St. John's wort)	Intrinsic ATPase activity and ATP hydrolysis
ABCC2	MRP2, CMOAT	Intestine, liver, kidney, brain, placenta, cancer cells	Cisplatin is a substrate for MRP2 but not for MRP1. Folic acid and monovalent bile salts such as cholate, taurocholate, and glycocholate are substrates for MRP3 but not for MRP1 and MRP2	Benzbromarone (phenobarbital)	Intrinsic ATPase activity and ATP hydrolysis
ABCC3	MRP3	Intestine, pancreas, placenta, adrenal cortex, liver, kidney, prostate, cancer cells			Intrinsic ATPase activity and ATP hydrolysis
ABCC4	MRP4	Prostate, lung, adrenals, ovary, testis, pancreas, small intestine, cancer cells	Nucleoside analogues and cyclic nucleotides (cGMP and cAMP) Unlike MRP4, MRP5 transport GS conjugates but not E17βG.	Probenecid, sildenafil	Intrinsic ATPase activity and ATP hydrolysis
ABCC5	MRP5	Ubiquitous (mainly in skeletal muscle, heart and brain), cancer cells	MRP4: adefovir, zidovudine, monophosphate MRP5: adefovir, mercaptopurine	Probenecid, sildenafil	Intrinsic ATPase activity and ATP hydrolysis

ABCG2	BCRP, MXR, ABCP	Placenta, intestine, liver, breast, brain, cancer cells	Broad substrate specificity, partly overlapping between P-gp and MRP substrates Substrates of BCRP can be either hydrophobic or hydrophilic, negatively or positively charged, xenobiotics or endobiotics, and unconjugated or conjugated, such as Estrone Sulfate, LysoTracker, methotrexate, sulfasalazine, topotecan, and imatinib	Ko143, fumitremorgin, HIV inhibitors, novobiocin, imatinib (Gleevac), gefitinib (Iressa)	Intrinsic ATPase activity and ATP hydrolysis
Solute Carrier Transporters					
SLC10A1	NTCP	Liver	Bile salts, such as taurocholate	All major bile salts, BQ-123, CsA	Na^+ dependent
SLC10A2	ASBT	Intestine, kidney	Bile salts, such as taurocholate	All major bile salts, BQ-123, CsA	
SLC15A1	PEPT1	Intestine, Kidney	Dipeptides, tripeptides, and peptidomimetic	Glycylsarcosine, protonophore, such as carbonyl cyanide 4-trifluoromethoxyphen ylhydrazone (FCCP) and inhibitors of the Na^+/H^+ exchanger, such as amiloride	H^+ dependent
SLC15A2	PEPT2	Kidney	drugs such as glycylsarcosine, valacyclovir, and β-lactam antibiotics (cephalexin, ceftibuten)		
SLC21A8/S LCO1B3	OATP-8, OATP1B3	Liver	Digoxin, bile acids, BQ123, E17βG, DHEAS, estrone sulfate	Digoxin	
SLC21A3/S LCO1A2	OATP-A, OATP, OATP1A2	Brain, liver, testis, prostate	Relative bulky and hydrophobic organic anions (including bile	CsA, verapamil, rifampin, ketoconazole, HIV inhibitors, quinidine,	ND
SLC21A6/S LCO1B1	OATP-C, LST-1, OATP2, OATP1B1	Liver	acid, bilirubin, prostaglandin E_2, tetraiodothyronine,	indocyanine green	
SLC21A9/S LCO2B1	OATP-B, OATP-RP2, OATP2B1	Liver, intestine, pancreas, lung, ovary, testes, spleen	and triiodothyronine), neutral compound ouabain, and organic cations		

(*Continued*)

Table 1 Tissue Distribution and Substrate Properties of Major ABC and SLC Transporters *(Continued)*

Gene	Protein Name	Tissue Distribution	Substrate Properties	Selected Inhibitor and (Inducer)	Driving Force
SLC21A11/ SLCO3A1	OATP-D, OATP3A1	Ubiquitous (strong expression in leukocytes spleen), cancer cells	such as *N*-methyl quinidine and rocuronium Estrone sulfate, E17βG, DHEAS, bromsulfophthalein (BSP),		
SLC21A12/ SLCO4A1	OATP-E, OATP-RP1, OATP4A1	Ubiquitous (mainly in skeletal muscles), cancer cells	fexofenadine, DNP-s-glutathione, LTC_4, pravastatin, rifampicin		
SLC22A1	OCT1	Liver	Small organic cations	Tetraethyl-methyl ammonium, cimetidine, and HIV inhibitors	OCT2 is driven by membrane potential
SLC22A2	OCT2	Kidney, brain	Tetraethyl-methyla mmonium (TEA), MPP^+, metformin, azidothymine (AZT), choline		
SLC22A3	OCT3, EMT	Skeletal muscle, liver, placenta, kidney, heart	Small organic cations TEA, dopamine, guanidine		
SLC22A4	OCTN1	Kidney, skeletal muscle, prostate, placenta, heart	Small organic cations TEA, carnitine, quinidine, verapamil	TEA, verapamil, imipramine, nicotine, procainamide	OCTN1 is driven by H^+
SLC22A5	OCTN2, CT1	Kidney, skeletal muscle, prostate, lung, heart, pancreas, small intestine, liver	Small organic cations Na^+-dependent: carnitine, phaloridine Na^+-independent: verapamil, quinidine, TEA	TEA, cimetidine, acetylcholine, choline, serotonine, quinidine, verapamil	

SLC22A6	OAT1	Kidney, brain	Organic anions. Although cimetidine is an organic cation, it is a substrate that is recognized by both organic cation and anion transporters. PAH, methotrexate, estrone sulfate, prostaglandin, DHEAS		
SLC22A7	OAT2	Liver, kidney			
SLC22A8	OAT3	Kidney, brain			
SLC29A1	ENT1	Ubiquitous, cancer cells	Nucleosides and nucleoside analogues	Nitrobenzyl-thioinosine and dipyridamole for ENT1	Facilitated transport
SLC29A2	ENT2	Ubiquitous, cancer cells	Adenosine, uridine, inosine		
SLC28A1	CNT1	Liver, kidney, intestine, brain	Purine nucleosides, uridine	Na^+ free buffer	Na^+ dependent
SLC28A2	CNT2	Kidney, heart, liver, skeletal muscle, pancreas, placenta, brain, cervix, prostate, small intestine, rectum, colon, lung	Pyrimidine nucleosides, adenosine	Na^+ free buffer	
SLC28A3	CNT3	Mammary gland, pancreas, bone marrow, trachea, intestine, liver, lung, placenta, prostate, testis, brain, heart	Nucleosides and nucleoside analogues	Na^+ free buffer	
SLC47A1	MATE1	Ubiquitously throughout the body, but predominantly in the liver and kidneys, where it is localized to bile canaliculi and brush border membranes, respectively	Organic cations such as TEA, MPP, cimetidine, *N*-methylnicotinamide (NMN) and metformin; Organic anions such as estrone sulfate, acyclovir, and ganciclovir and amphoteric compounds such as cephalexin and cephradine	Levofloxacin and ciprofloxacin	H^+ exchange
SLC47A2	MATE2	Specifically in the kidneys and is localized to brush border membranes	Organic cations such as TEA, MPP, cimetidine, *N*-methylnicotinamide (NMN), and metformin	Levofloxacin and ciprofloxacin	H^+ exchange

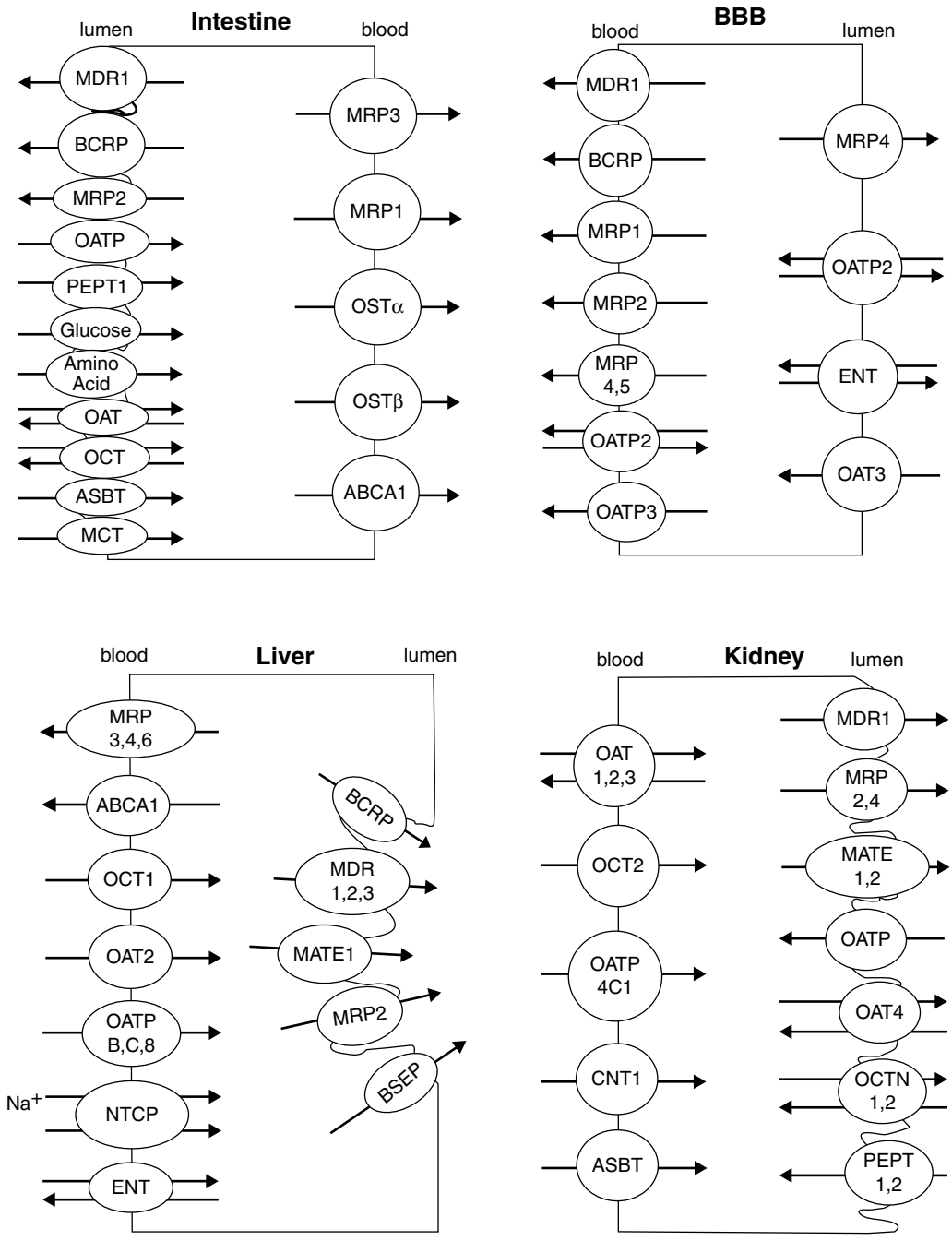

Figure 1 Localization of transporters in intestine, blood–brain barrier, liver, and kidney.

inhibition or induction studies. Transporter-related drug–drug interactions (DDI) can occur during gastrointestinal absorption, hepatic excretion, renal excretion, blood–brain barrier (BBB) penetration, and so on, because of the wide tissue distribution of transporters. Food and formulation effects on P-gp–mediated drug absorption process are also well studied. The localizations of major transporters in human intestine, liver, kidney, and brain are illustrated in Fig.1.

Transporters in Drug Absorption

Uptake transporters have been demonstrated to improve drug absorption through the GI tract. The most successful example is an antiviral prodrug valacyclovir, which shows oral bioavailability three to five times greater than its parent drug acyclovir in human (15). The increased oral bioavailability is attributed to PEPT1-mediated transport, which was demonstrated by an in situ rat perfusion model, Caco-2 cells, and PEPT1 transfected CHO cells (16).

Plasma membrane monoamine transporter (PMAT), which shows low sequence homology to equilibrative nucleoside transporters (ENT) (*SLC29*), has been recently identified and cloned from human intestine. It is expressed in human small intestine and concentrated on the tips of the mucosal epithelial layer and may use luminal protons to drive the absorption of certain organic cation drugs (17). The PMAT transfected cell line showed that PMAT transports metformin. This may cause saturable intestinal transport of metformin and the observed decrease in bioavailability at higher doses in the clinic (17). OATP1A2 (SLCO1A2) is present in human intestine (and Oatp2b1 in mouse intestine) and may be responsible for intestinal uptake of diverse chemicals, such as digoxin and fexofenadine (18,19).

Drug efflux transporters of the ABC family can restrict drug absorption by pumping drugs out of intestinal epithelial cells. Of the known ABC drug efflux transporters, P-gp is localized on the mucosal membrane of intestines and is well documented for its involvement in reducing oral drug absorption. Immunohistological studies showed high P-gp protein levels on the apical surface of columnar epithelial cells but not in crypt cells in human jejunum and colon (20). The mRNA expression of P-gp increased longitudinally along the gastrointestinal (GI) tract in humans (stomach < duodenum < jejunum/ileum < colon) (21,22).

Similar to P-gp, strong staining of BCRP was observed on the luminal surface of the intestine (23). However, BCRP mRNA expression was maximal in human duodenum and decreased continuously down to the rectum (terminal ileum 93.7%, ascending colon 75.8%, transverse colon 66.6%, descending colon 62.8%, and sigmoid colon 50.1%, as compared to the level in duodenum) (24). The role of P-gp or BCRP in reducing the absorption of xenobiotics can be directly examined in *Mdr1a/1b* (−/−) or *Bcrp1*(−/−) mice (25,26). By comparing the oral drug exposure in wild-type (wt) mice and knockout mice, P-gp and Bcrp1 have been shown to play major roles in the reduction of absorption. For example, P-gp and Bcrp1 are important in the absorption of several HIV protease inhibitors, topotecan, etoposide, tacrolimus, paclitaxel, ivermectin, loperamide, and UK-224671 [P-gp substrates (25)], as well as topotecan, nitrofurantoin, ME-3229, GV-196771, and sulfasalazine (BCRP substrates) (9,27).

In addition, Sparreboom et al. have used *Mdr1a*(−/−) mice to demonstrate the effect of gut P-gp on the pharmacokinetics of paclitaxel (28). The area under the plasma concentration time curves (AUC) was two- and sixfold higher in *Mdr1a*(−/−) mice than in wt mice after i.v. and oral drug administration, respectively. Consequently, the oral bioavailability in mice receiving 10 mg paclitaxel per kilogram body weight increased from only 11% in wt mice to 35% in *Mdr1a*(−/−) mice. The cumulative fecal excretion (0–96 hours) was markedly reduced from 40% (after i.v. administration) and 87% (after oral administration) of the administered dose in wt mice to below 3% in *Mdr1a*(−/−) mice. Biliary excretion was not markedly different in wt and *Mdr1a*(−/−) mice. After i.v. administration of paclitaxel (10 mg/kg) to mice with a cannulated gall bladder, 11% of the dose was recovered within 90 minutes in the intestinal contents of wt mice while less than 3% was recovered in *Mdr1a*(−/−) mice. All the data clearly suggest that P-gp limits the oral uptake of paclitaxel and mediates direct excretion of the drug from the systemic circulation into the intestinal lumen.

Besides P-gp, other efflux pumps such as BCRP can affect drug absorption. Pretreatment of *Bcrp1*(−/−) and *Mdr1(a/b)*(−/−) mice with gefitinib (Iressa), an oral epidermal growth factor receptor tyrosine kinase inhibitor, increased oral absorption and decreased systemic clearance of topotecan. Gefitinib inhibited the efflux of BCRP and MDR1 substrates and restored vincristine sensitivity in MDR1-expressing cells. Although gefitinib inhibited BCRP more potently than MDR1 (10-fold), the inhibition of both transporters occurred at clinically relevant concentrations (e. g., 1–5 mM) (29).

In general, the influence of efflux transporters on intestinal drug absorption is significant for substrates with either low solubility (30) or low permeability and high affinity to efflux transporters (31). For a substrate with low permeability and high affinity, efflux transporters

may contribute more to membrane clearance (V_{max}/K_m) than passive diffusion and, thus, the changes in the efflux activity may significantly alter their intestinal absorption rate. After oral administration to mice of [^{3}H]vinblastine, a P-gp substrate with low permeability and high affinity, the maximum concentration (C_{max}), and the AUC (0–24 hour) in *Mdr1a/1b* (−/−) mice were approximately 1.5 times greater than those in wt mice, whereas these parameters were not significantly different between the two strains in the case of [^{3}H]verapamil, a P-gp substrate with high permeability and low affinity (31). A low solubility drug, whether it is highly or poorly permeable, tends to have low concentrations coming into enterocytes and, consequently, a lower chance to saturate the efflux transporters

Transporter-Mediated DDIs in Drug Absorption

DDIs due to P-gp–mediated absorption are generally limited to some Biopharmaceutics Classification System (BCS) class II and IV drugs [class II drugs: high permeability and low solubility; class IV drugs: low permeability and low solubility (32)], whereas there is minimal effect on class I drugs with high solubility and high permeability due to the saturation potential of P-gp at high therapeutic doses. Therefore, the importance of P-gp in oral drug bioavailability, drug disposition in the liver, drug efflux in the BBB, and DDI should be considered for BCS class II and IV drugs. This is especially important for drugs with narrow therapeutic windows.

The classic example of the digoxin–quinidine interaction was observed in the early 1980s. Coadministration of quinidine increased the absorption rate constant of digoxin by 30%, the C_{max} by 81%, and the AUC by 77% in patients with cardiac disease (33). Only recently, however, has the underlining mechanism of the digoxin–quinidine interaction been elucidated and attributed to inhibition of intestinal P-gp (and also liver and renal P-gp) by quinidine. Moreover, in healthy volunteers, oral coadministration of 100 mg talinolol increased the AUC (0–6 hour) and the AUC (0–72 hour) of digoxin (0.5 mg orally) significantly by 18% and 23%, respectively, while infusion of talinolol (30 mg) concomitant with an oral dose of digoxin had no significant effects on digoxin pharmacokinetics, indicating that the DDI between talinolol and digoxin is due to the inhibition by tanilolol of P-gp–mediated efflux in intestinal but not in liver and kidney. Digoxin did not affect the disposition of talinolol after both oral and intravenous administration since digoxin is a weak P-gp inhibitor and does not inhibit P-gp at this dose (34). Another study showed that the talinolol (50 mg) AUC (0–24 hour) and C_{max} were significantly increased after administration of oral erythromycin (a P-gp and CYP3A4 inhibitor) at 2 g compared to placebo, while the renal clearance of talinolol was unchanged in healthy volunteers. This suggests that the increase in oral bioavailability of talinolol after concomitant erythromycin administration is caused by a net increase in intestinal absorption of talinolol due to the inhibition of P-gp by erythromycin (35).

P-gp expression levels in humans directly affect oral digoxin and talinolol absorption. Rifampin treatment (600 mg/day for 10 days) increased intestinal P-gp content by 3.5-fold, which correlated with the decreased AUC after oral digoxin (1 mg) but not after intravenous digoxin (1 mg). Renal clearance and half-life of digoxin were not altered by rifampin (36). Similarly, rifampin resulted in increased expression of duodenal P-gp content by 4.2-fold and decreased AUC of intravenous and oral talinolol (21% and 35%) in healthy volunteers, suggesting that rifampin induces P-gp–mediated excretion of talinolol predominantly in the gut wall (37). This implied that individual intestinal P-gp expression differences can contribute to the variation in pharmacokinetics of digoxin and tanilolol.

For drugs with wide therapeutic indices, P-gp–mediated DDIs may not be clinically significant. In healthy volunteers, the overall exposure of dexamethasone, which is widely included in oncology antiemetic regimens, was significantly increased by 24% by valspodar (400 mg), a P-gp modulator used as a chemotherapy adjunct. However, this AUC increase is unlikely to be considered to be clinically significant given dexamethasone's wide therapeutic index and the short duration of coadministration (38).

Food Effects on Transporter-Mediated Drug Absorption

Drug–food interactions can often be caused by food or by health supplements and herbs. For example, potential clinically significant drug interactions were observed with St. John's

wort (16 out of 24 studies), garlic (2 out of 5 studies), and American ginseng (1 study) (39). In other studies, St. John's wort, an herbal medicine used for the treatment of depression, caused remarkable decreases in plasma exposure/concentration of certain drugs such as CsA (40) and digoxin (41) when they are coadministered. The pharmacokinetics of digoxin was investigated in a single-blind, placebo-controlled parallel study. After achieving steady state for digoxin on day 5, healthy volunteers received digoxin (0.25 mg/day) either with placebo ($n = 12$) or with 900 mg/day *Hypericum* extract from St. John's wort ($n = 13$) for another 10 days. Digoxin concentration profiles on day 5 were compared with day 6 (single-dose interaction) and day 15 (tenth day of comedication). No statistically significant exposure change of digoxin was observed after the first dose of *Hypericum* extract at day 6 for placebo and *Hypericum* group, respectively. However, 10 days of treatment with *Hypericum* extract resulted in 25% decrease in digoxin AUC (0–24 hour) ($P = 0.0035$). Furthermore, comparison with the parallel placebo group after multiple dosing showed that the *Hypericum* group had a reduction in trough concentrations and C_{max} of 33% ($P = 0.0023$) and 26% ($P = 0.0095$), respectively. The effect became increasingly pronounced until the tenth day of comedication.

This interaction of St John's wort extract with digoxin kinetics was time-dependent and could be due to the induction of the P-gp (41). The *Hypericum* extract also reduced the mean concentration of cyclosporin from 0.84 ng/(mL × mg) to 0.48 ng/(mL × mg) when coadministered to a kidney transplantation patient (40). This was also attributed to the inducing effect of St John's wort on CYPs and P-gp activities. Since low cyclosporin levels are associated with an increased risk of rejection after organ transplantation, the potential clinical consequence of this pharmacokinetic herb–drug interaction needs to be noted.

Grapefruit, orange, and apple juices at high volume (1200 mL) reduced the oral systemic availability of fexofenadine, a drug transported by OATP1A2 and P-gp, to a mean of 33%, 28%, and 23%, respectively, when compared to water (42,43). Moreover, grapefruit juice at a more commonly consumed quantity (300 mL) decreased AUC and C_{max} of fexofenadine (120 mg) to 58% and 53%, respectively, compared with the consumption of the corresponding volume of water in the healthy volunteers. A larger volume of grapefruit juice (1200 mL) reduced these parameters further to 36% and 33%, respectively. Grapefruit juice, 300 mL and 1200 mL, also reduced the coefficient of variation (CV) of the AUC of fexofenadine by twofold compared to that with a matching volume of water. The mechanism of this decreased oral bioavailability was thought to be due to specific ingredients in the juices selectively reducing intestinal OATP1A2 activity (44).

The Effect of Formulations on Transporters-Mediated Drug Absorption

Formulation strategies to overcome multidrug resistance have been evaluated for their enhancement of membrane permeability of a drug and inhibition of P-gp. Surfactants used in pharmaceutical formulations include nonionic detergents, polyoxyethylene(20)-sorbitanemonooleate (Tween 80), polyoxyethylene-polyoxypropylene block copolymers (e. g., Pluronic P85), and polyoxyethyleneglycoltriticinoleate (Cremophor EL). These agents can modulate drug absorption by multiple mechanisms including inhibition of intestinal P-gp. Water-soluble vitamin E [D-alpha-tocopheryl poly(ethylene glycol)] 1000 succinate (TPGS 1000), which is comprised of a hydrophilic polar head and a lipophilic alkyl tail, has been used as a solubilizer, an emulsifier, and an effective oral absorption enhancer.

In murine monocytic leukemia cells overexpressing P-gp, P-gp–mediated rhodamine123 transport was inhibited by five nonionic surfactants in a concentration-dependent manner in the following rank order: TPGS > Pluronic PE8100 > Cremophor EL > Pluronic PE6100 ≈ Tween 80. In contrast, none of those surfactants showed a significant inhibition of MRP2-mediated efflux in MDCK-MRP2 cells (45). Pluronic P85 was reported to cause a higher degree of inhibition of P-gp than MRP2 and MRP1(46).

In nine healthy volunteers, talinolol solution, containing either talinolol alone (50 mg), talinolol and TPGS (0.04%), or talinolol and poloxamer 188 (0.8%) was administered via nasogastrointestinal tube dosing. TPGS increased AUC of talinolol by 39% and C_{max} by 100%, whereas poloxamer 188 did not significantly alter AUC or C_{max} of talinolol. This in vivo

observation can be explained by Caco-2 data showing abolishment of P-gp–mediated talinolol efflux with TPGS (0.01%), but not poloxamer 188 (47).

TPGS PEG chain length was demonstrated to influence on rhodamine123 transport in Caco-2 monolayers by using TPGS analogs containing different PEG chain length (TPGS 200/238/400/600/1000/2000/3400/3500/4000/6000) (48). Cremophor EL (Cremophor) is a nonionic solubilizer and emulsifier that is used form solutions of some hydrophobic drugs and fat-soluble vitamins. In a study with 12 health volunteers, Cremophor EL increased digoxin (oral dose: 0.5 mg) C_{max} by 22% and AUC by 22% (49).

Although most investigations have focus on the effect of formulations on P-gp activity, formulations may also affect other transporters in the gut and other organs such as the liver and kidney. This remains to be defined, as more knowledge of other transporters becomes better understood.

Transporters in Drug Distribution

Membrane transporters contribute to the drug distribution in certain tissues. Most statins are taken up into the hepatocytes by OATP, excreted into the bile by efflux transporters, and reabsorbed in the intestine, thereby effectively undergoing enterohepatic recirculation which maintains high concentrations in the liver (50). Metformin, a biguanide antidiabetic drug, is distributed into the liver via Oct1 and into the kidney via Oct2 (51).

One of the important tissue barriers for xenobiotics is the BBB. Currently, several ABC transporters, P-gp, BCRP, MRP1, and SLC transporters, amino acid, glucose, organic cation, and organic anion transporters have been identified in the BBB (Fig. 1) (52). The role of the transporters in the BBB has been extensively investigated with transporter knockout mice and inhibitors.

P-gp is highly expressed in the luminal membrane of brain endothelial cells and plays a critical role in restricting the passage of lipophilic compounds into the brain (52). Antihistamines have found their greatest therapeutic value in the treatment and management of various allergic disorders, including seasonal and perennial rhinitis, urticaria, and dermatological conditions (53). However, the most problematic aspect of their use is sedation, which can severely compromise the safe performance of cognitive and psychomotor tasks of everyday life. The third generation antihistamine drug fexofenadine, which is a P-gp substrate (54) and does not cross the BBB, does not cause sedation even at doses two to three times those normally used for seasonal allergic rhinitis (53).

Wolff et al. reported that imatinib (Gleevec, STI-571), a potent and selective tyrosine kinase inhibitor, effectively controlled the systemic proliferation of transduced bone marrow cells in mice but, after two to four months of treatment, many of the mice unexpectedly developed progressive neurological deficits due to leukemia cell infiltration into the brain and leptomeninges. However, imatinib has been shown to effectively inhibit glioblastoma cell growth in preclinical in vitro and in vivo studies (55). These findings suggest that there was inadequate imatinib penetration of the drug into the central nervous system (CNS) (56). Imatinib concentrations in mouse cerebrospinal fluid (CSF) were less than 1% than that in plasma (56). A limited penetration of imatinib into the brain was also observed both preclinically and clinically (1,2,57,58). Both *Mdr1*(−/−) and *Bcrp1*(−/−) knockout mice demonstrated that P-gp and Bcrp can limit the uptake of imatinib into the brain (59). Thus, inhibitors of BCRP and P-gp may improve delivery of imatinib to malignant gliomas.

Lapatinib is a tyrosine kinase inhibitor approved for use in combination with capecitabine to treat advanced or metastatic breast cancers overexpressing human epidermal receptor 2 (ErbB2). In vitro experiments demonstrated that lapatinib is a substrate for both P-gp and BCRP. An in vivo pharmacokinetic study showed that after a 24-hour intravenous infusion of lapatinib to a targeted steady-state plasma concentration of 700 ng/mL (0.3 mg/kg/hr) or 7000 ng/mL (3 mg/kg/hr), lapatinib brain-to-plasma ratios were approximately three- to four-fold higher in *Mdr1a/b*(−/−) double knockout mice (ratio range from 0.09 to 0.16) compared with wt mice (ratio range from 0.03 to 0.04). There was no difference in the brain-to-plasma ratio in *Bcrp1*(−/−) knockout mice (ratio range from 0.03 to 0.04) compared with wt mice. Even more remarkably, *Mdr1a/b*(−/−)/*Bcrp1*(−/−) triple knockout mice had a 40-fold higher brain-to-plasma ratio (ratio range from 1.2 to 1.7), suggesting that P-gp and Bcrp work in concert to

limit the brain-to-plasma ratio of lapatinib in mice. This finding has important potential consequences for the treatment of brain tumors in breast cancer patients treated with tyrosine kinase inhibitors as well as the basic understanding of ATP-binding cassette transporters expressed in the BBB on the CNS disposition of drugs.

The results from the study with lapatinib, along with that for topotecan, highlight the need to understand further the role of BCRP/*bcrp1* and other transporters in the BBB, and how these transporters interact with P-gp, which seems to be the dominant efflux transporter in this barrier. The triple knockout model will be a useful tool to elucidate these mechanisms.

Species differences occur in the brain concentrations of drugs, but the reasons for these differences are not yet apparent. Some radioligands, shown to be P-gp substrates in rodents, are, nonetheless, taken up by the brain in primates (60,61) indicating that there may be species differences in P-gp function. Recently, Syvänen et al. compared brain uptake of three radiolabeled P-gp substrates across species using positron emission tomography (PET) (62). The degree of P-gp involvement was determined by administering cyclosporin A (CsA) to inhibit P-gp. Brain concentrations and brain-to-plasma ratios were compared; [^{11}C]verapamil in rats, guinea pigs, and monkeys; [^{11}C](*S*)-(2-methoxy-5-(5-trifluoromethyltetrazol-1-yl)-phenylmethylamino)-2(*S*)-phenylpiperidine (GR205171) in rats, guinea pigs, monkeys, and humans; and [^{18}F]altanserin in rats, minipigs, and humans. The fraction of the unbound radioligand in plasma was studied along with its metabolism. Pronounced species differences were found in the brain and brain-to-plasma concentrations of [^{11}C]verapamil, [^{11}C]GR205171, and [^{18}F]altanserin with higher brain distribution in humans, monkeys, and minipigs than in rats and guinea pigs. The species differences were still present after P-gp inhibition, although the increase in brain concentrations after P-gp inhibition was somewhat greater in rats than in the other species. The inhibition results suggested that the differences in P-gp transport capacity alone could not explain the observed species differences in brain distribution. In addition, differences in plasma protein binding and metabolism did not explain the species-related differences. These findings are important for interpretation of brain drug delivery when extrapolating preclinical data to humans. Compounds found to be P-gp substrates in rodents are likely to also be substrates in higher species, but sufficient BBB permeability may be retained in humans to allow the compound to act at intracerebral targets (62).

P-gp, BCRP, and MRPs are present in placenta and contribute to the flux of drugs across the maternal–fetal barrier. Their role in restricting placenta penetration of drugs can be demonstrated by using efflux transporter inhibitors and transporter genetic knockouts in pregnant mice. With the pretreatment of GF120918, an inhibitor of both P-gp and BCRP, two hours before intravenous administration of topotecan to pregnant *Mdr1a/1b*(−/−) mice, the plasma levels of topotecan were 3.2- and 1.6-fold higher in fetuses and dams, respectively. These data suggest that Bcrp1 plays an important role in protecting fetuses from potentially toxic xenobiotics (23).

Clinical Evidence for Transporter-Mediated DDIs in Brain Penetration

The high expressions of P-gp and BCRP at the luminal membrane of brain endothelia cells imply that they may play a role during the passage of drugs across the BBB. The function of P-gp in human brain penetration has been demonstrated by DDI studies. In contrast, the impact of other transporters in limiting or facilitating drug passage into the human brain is still not clear.

Loperamide, a potent opiate, is used alone as an antidiarrheal drug without CNS effect due to P-gp restricted entry into the brain. When loperamide (16 mg) was given with quinidine at a dose of 600 mg in healthy volunteers, it elicited central opioid effects exhibited by respiratory depression. This can be explained by P-gp inhibition in BBB and gut, resulting in increased brain penetration of loperamide and increased oral drug absorption (63).

In twelve human healthy volunteers, PET imaging studies have demonstrated that after i.v. infusion, the brain concentration of the P-gp substrate [^{11}C]verapamil was significantly increased upon coadministration of the potent P-gp inhibitor CsA (64), suggesting that P-gp restricts passage across the BBB in man of P-gp substrates such as verapamil. This PET imaging study appears to be the first report to directly demonstrate the function of P-gp in the human BBB.

Ivermectin, a neurotoxic compound in animals with low P-gp expressions, has been safely used in Africa for the prevention and treatment of river blindness. The lack of neurotoxicity in Africans might be due to the high P-gp expression in African population (65). Gene analysis has shown that a higher frequency of the C allele in the African population than in British Caucasian, Portuguese, South-west Asian, Chinese, Filipino, and Saudi populations, which suggests overexpression of P-gp in the African population.

Transporters in Drug Metabolism

It has been recognized that there is a large overlap in the substrate specificity and effects on tissue distribution for substrates and modulators of P-gp and cytochrome P450 (CYP) 3A. Most of the CYP3A4 inhibitors also inhibit P-gp (66). Inducers of CYP3A4, such as St. John's wort, reserpine, rifampicin, phenobarbital, and triacetyloleandomycin, can also induce P-gp expression in cells and in man (66). The spatial relationship of P-gp traversing the plasma membrane and CYP location on the endoplasmic reticulum inside the cells suggests that P-gp may cooperatively influence metabolism by controlling the access of a substrate to the CYP enzymes. In addition, drugs are absorbed into enterocytes, hepatocytes, and renal tubular cells where they can be metabolized by CYP3A and then be exported out of the cells by P-gp. This process creates a unidirectional flux of a substrate to CYP3A, resulting in low bioavailability of CYP3A substrates, less accumulation of parent compound inside the cells, and greater formation of metabolites (66). For example, when LY335979 (zosuquidar trihydrochloride) (0.5 mM) was used to inhibit P-gp in CYP3A4-expressing Caco-2 cell monolayers, it increased the intracellular concentrations of saquinavir and the formation rate of its metabolite, M7, but decreased the intestinal first-pass extraction (Ei) by approximately 50% (67).

Coadministration of rifampin, a cytochrome P450 3A4 inducer and a drug substrate for both OATP and CYP3A4/2C9, usually results in a time-dependent drug interaction in the clinic. The initial increase in trough concentrations of the test drug is the most likely explained by the inhibition of OATP-mediated hepatic uptake of test drug by rifampin, whereas the decrease in exposure to the test drug upon continued dosing is caused by the CYP-inductive properties of rifampin (68–70).

Atrasentan is a highly potent and selective endothelin-A receptor antagonist, which is being developed for the treatment of hormone refractory prostate cancer and other malignancies. In humans, atrasentan is extensively metabolized by glucuronidation and oxidation. In vitro studies suggest that the predominant CYP enzymes involved in the oxidative metabolism of atrasentan are those of the CYP3A family. The UGTs involved in the glucuronidation of atrasentan have not been identified. The FDA considers rifampin as the inducing agent of choice for determining the maximum inductive effect on a CYP3A4-metabolized drug (70). In addition to CYP3A4, rifampin is also known to induce some UGT isoforms, including UGT1A1 and UGT1A6. The effect of rifampin on the pharmacokinetics of atrasentan was assessed in 12 healthy male subjects in an open-label study. Single doses of atrasentan 10 mg were administered orally on days 1 and 12. Rifampin 600 mg was given once daily from days 4 through 14. On day 12, atrasentan and rifampin were administered simultaneously. Blood samples were collected before and during 72 hours after each atrasentan dose. It was expected that coadministration of rifampin would significantly increase the elimination clearance of atrasentan, resulting in decreases in atrasentan C_{max}, AUC_{∞}, and terminal half-life. Consistent with this expectation, coadministration of rifampin decreased the atrasentan half-life by 77%. However, unexpectedly, rifampin significantly increased atrasentan C_{max} and had no significant effect on atrasentan AUC_{∞}. In addition, visual examination of atrasentan concentration–time profiles suggested that the distributive phase of atrasentan was substantially prolonged with coadministration of rifampin.

The expected and unexpected effects of rifampin on atrasentan pharmacokinetics suggest that other mechanisms in addition to enzyme induction were involved in the interaction between rifampin and atrasentan. Rifampin may affect atrasentan pharmacokinetics by acting as both an inhibitor of OATP-mediated hepatic uptake of atrasentan and an inducer of atrasentan metabolism. The effect of rifampin on atrasentan pharmacokinetics may depend on the time of rifampin administration relative to that of atrasentan (70).

Efflux pumps also help to eliminate the metabolites of drugs from systemic circulation. For example, most drug glucuronide conjugates are MRP2 and/or BCRP substrates (71) while most sulfate conjugates are BCRP substrates (71,72).

Transporters in Drug Excretion

Living organisms must deal with environmental toxins, metabolic waste products, and drugs with extremely diverse structures to remain viable. In mammals, these toxic organic compounds are mainly excreted through the kidney and liver. Transporters are involved in biliary and renal excretion, which are the two common routes of drug elimination. In the liver, a drug is first taken up into hepatocytes, then either secreted back to the systemic circulation or excreted into the bile in an intact form or as metabolites via phase I and/or phase II enzymes. Given the involvement of transporters in both uptake at the sinusoidal membrane and efflux at the sinusoidal and canalicular membranes (Fig. 1), the hepatic clearance of a drug can be estimated by a well-stirred model using the following equation (73,74):

$$CL_{H,int} = \frac{PS_{influx}(CL_{H,met} + CL_{Biliary})}{PS_{efflux} + (CL_{H,met} + CL_{Biliary})} \quad (1)$$

where $CL_{H,int}$, $CL_{H,met}$, $CL_{Biliary}$ represent the hepatic intrinsic clearance, hepatic metabolism clearance, biliary excretion, respectively. PS_{influx} and PS_{efflux} are the membrane transport clearances across the sinusoidal membrane. When PS_{efflux} is much smaller than CL_{int} and $CL_{Biliary}$ ($PS_{efflux} << (CL_{int} + CL_{Biliary})$, the overall hepatic clearance will equal to PS_{influx}.

At steady state, the hepatic intrinsic clearance of pravastatin, a substrate for OATP2 and MRP2 (75,76), was regulated by the uptake process, followed by rapid metabolism and/or biliary excretion with minimal efflux to the systemic circulation in rats after infusion. The total hepatic elimination rate at steady state exhibited Michaelis–Menton saturation with the drug concentration and the K_m and V_{max} obtained in rats with different mathematical models (i.e., well stirred, parallel-tube, and dispersion models) were comparable with the initial uptake velocity measured from in vitro hepatocytes (75).

Uptake transporters, such as OCT1, OAT2, OATPB, OATP2, OATP8, and NTCP in the sinusoidal membrane of hepatocytes affect PS_{influx}. Efflux transporters affect PS_{efflux} in the sinusoidal membrane (e. g., MRP3) and $CL_{Biliary}$ in the canalicular membranes (e. g., P-gp, BCRP, and MRP2). After i.v. administration of nitrofurantoin (a BCRP substrate), AUC in *Bcrp1*(−/−) mice was almost twofold higher than in wt mice (139.2 vs. 73.9 min μg/mL). Hepatobiliary excretion of nitrofurantoin was almost abolished in *Bcrp1*(−/−) mice (9.6% vs. 0.2% in wt and BCRP1 knockout mice, respectively) (77).

The impact of P-gp on biliary excretion of its substrate was first indicated by canalicular membrane vesicle studies and isolated perfused rat liver. *Mdr1a*(−/−) or *Mdr1a/1b*(−/−) mice provide more direct evidence to demonstrate the involvement of P-gp in biliary elimination of its substrates, such as digoxin, doxorubicin, and vinblastine. Mrp2 appears to have a less profound impact on the intestinal absorption of its substrates than on their biliary excretion. In order to assess Mrp2-mediated biliary excretion and oral absorption respectively, wt Sprague-Dawley (SD) rats and Eisai hyperbilirubinemic SD rats (EHBR) received an i.v. infusion or oral dose of furosemide, probenecid, or methotrexate (MTX) (78). The biliary clearance of probenecid and MTX was reduced approximately 40-fold in EHBR rats as compared to control rats. Biliary clearance of furosemide was similar in EHBR and control rats. In all cases, no significant difference in absorption was observed between EHBR and control rats. This study demonstrated that MRP2 mediates the biliary excretion of probenecid and MTX but not furosemide.

Species differences in transport mediated by MRP2 were examined using temocaprilat (an angiotensin-converting enzyme inhibitor) in the animal studies and 2,4-dinitrophenyl-S-glutathione (DNP-SG) in the membrane based assay (79). Temocaprilat was infused to examine the biliary excretion rate at steady state. The biliary excretion rates of temocaprilat were 9.8, 39.2, 9.2, 1.1, and 0.8 mL/min/kg for male ddY mouse, SD rats, Hartley guinea pigs, Japanese white rabbits, and beagle dogs, respectively. The in vitro transport clearance (V_{max}/K_m) of

DNP-SG across hepatocyte canalicular membrane vesicles (CMVs) were 25.5, 64.2, 9.4, 8.4, and 7.7 mL/min/kg for mouse, rat, guinea pig, rabbit, and dog, respectively. These values are similar to the transport clearance across the bile canalicular membrane observed among animal species. The clearance rate by MRP2 is the highest in SD rat and lowest in beagle dog (79).

Recently, Li et al. determined the absolute differences of Mrp2/MRP2, Bcrp/BCRP and Bsep/BSEP expression in livers and isolated hepatocytes across species by capillary liquid chromatography (LC) nanoelectrospray ionization quadruple time-of-flight (nano-ESI-Q-TOF) mass spectrometry (80,81). BCRP/Bcrp protein expression showed the following rank order across species: dog > rat > monkey ≈ human, indicating that monkey is the closest preclinical animal to human in the aspect of BCRP/Bcrp transporter. The results for BSEP/Bsep protein expression was as follows: rat ≈ monkey > dog ≈ human. The absolute amount of Bsep protein in rat and monkey was approximately twofold higher than in human and dog. The rank order of MRP2/mrp2 in liver was rat>> monkey>dog ≈ human. The absolute amount of Mrp2 protein in rat was 10-fold higher in liver tissue than in human. A greater variation of MRP2 expression was observed in human liver donors (sixfold ranged from 0.2 to 1.2 fmol/μg protein) and the nonnaive animals (monkey and dog, approximately fourfold, ranged from 0.6 to 2.7 and 0.5 to 1.7 fmol/μg protein, respectively), compared to less than twofold in rat (ranged from 4.6 to 6.1 fmol/μg protein).

Similar to hepatocytes, a drug needs to cross the basolateral membrane of renal epithelial cells before excreting into urine. Metabolism may also occur in the kidney. Efflux transporters on the luminal brush border membrane can pump an intact drug or its metabolites into the urine [Fig. 1(D)]. Renal excretion of drugs can be described by three processes: (*i*) glomerular filtration, (i*i*) renal tubular secretion in which basolateral uptake transporters and apical efflux transporters are involved, and (*iii*) reabsorption from the renal tubular lumen in which apical uptake transporters are involved. Generally, renal clearance can be expressed by the following equation:

$$CL_R = (1 - FR)(f_u GFR + CL_{sec}) \quad (2)$$

where FR, f_u, GFR, and CL_{sec} are the reabsorbed fraction, protein unbound fraction in the blood, glomerular filtration rate, and secretion rate, respectively. GFR is a passive process by which only unbound drugs can be filtered, whereas reabsorption and secretion often involve active transporters. Technically, it is difficult to quantify each process of renal excretion. However, the excretion ratio (ER, which is the $CL_R/(f_u\ GFR)$ ratio) reflects the overall net contribution of each process to renal excretion. If the ER of the drug is greater than unity, the tubular secretion is more dominant. In contrast, when the ER is less than unity, tubular reabsorption is more significant.

Uptake transporter knockout mice, such as *Oat*(−/−), *Oct*(−/−), and *Pept2*(−/−) mice, are available to evaluate the role of these transporters in renal clearance of selected substrates. In the kidney, basolaterally localized OAT1–3 and OCT1–2 (Fig. 1) are important for renal tubular secretion. OAT4 and PEPT1–2 localized in the brush border membrane are mainly responsible for renal reabsorption.

After i.v. administration of [^{14}C]glycylsarcosine (GlySar) (0.05 μmol/g of body weight) to wt and *Pept2*(−/−) mice, both total and renal clearance of GlySar increased twofold in *Pept2*(−/−) mice, resulting in concomitantly lower systemic concentrations compared to wt mice (82). In addition, the ER of GlySar was 0.54 in wild type versus 0.94 in *Pept2*(−/−) mice, suggesting that in *Pept2*(−/−) mice the renal reabsorption of GlySar was almost abolished and GlySar was mainly eliminated by glomerular filtration. Combination of wt mice and *Pept2* (−/−) mice enables to assess the relative contribution of Pept1 and Pept2 on the kidney reabsorption of GlySar via equation 2. Of the 46% of GlySar that was reabsorbed in wt mice, Pept2 accounted for 86% and Pept1 accounted for 14% of the reabsorption.

P-gp is localized on the apical brush border membrane of the proximal renal tubule in the kidney, which implies that it has a role in renal secretion. Hori et al (83) demonstrated that digoxin was actively secreted in the isolated perfused rat kidney with an ER of 2.5. P-gp

inhibitors, quinidine, and verapamil, decreased the ER of digoxin to unity, suggesting that digoxin is actively secreted into urine by P-gp.

The function of Bcrp1, which is expressed on the brush border membrane of proximal tubular cells of the kidneys, in renal secretion was first demonstrated by Mizuno et al. The renal clearance of E3040S(6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)benzothiazole sulfate) was 2.4-fold lower in *Bcrp1*(−/−) mice compared to that in wt mice (84). However, BCRP expression is negligible in the human kidney (83). Given the species differences in the renal localization of BCRP, one must take these differences into consideration when predicting human PK and the route of elimination from preclinical PK data.

BCRP plays an important role in transporting drugs into human milk. BCRP expression is strongly induced during lactation but not in virgin nor in the nonlactating mammary gland epithelia of mice, cows, and humans (85). Nitrofurantoin, a commonly used antibiotic for urinary tract infections, was reported to be secreted into the milk of lactating women (77). The underlying mechanism was demonstrated in mice where milk-to-plasma ratio of nitrofurantoin in wild type was 80 times higher compared to that in Bcrp1 knockout mice. Elimination of nitrofurantoin via milk, however, was comparable to its hepatobiliary elimination (7.5% to approximately 15% vs. 9% of the dose one hour after i.v. injection) in lactating mice. Other BCRP substrates such as PhIP, topotecan, acyclovir, cimetidine, doxorubicin, and doxorubicinol also concentrate in the milk and have high milk/plasma ratios (85). However, some BCRP substrates do not concentrate in milk such as folic acid, DHEAS, porphyrin vitamin B_{12} (85). BCRP is also the first active riboflavin (vitamin B_{12}) efflux transporter identified in mammals, and the first transporter shown to concentrate a vitamin into milk (86). The identification of the role of BCRP in the excretion of drug substrates into milk provides insight to the proper use of these drugs and vitamins in lactating women.

For many years, the transporters responsible for the flux of organic cations from the tubule cell to the tubule lumen were unknown. Recently, Otsuka et al. (2) used database searches to identify two human genes for organic cation transporters designated as *hMATE1* and *hMATE2*. The gene *hMATE1* was found to encode for an organic cation/H^+ exchanger mainly expressed in the apical membrane of renal tubule cells and in the canalicular membrane of hepatocytes. The transporter interacts with structurally diverse small molecular weight cations such as paraquat, TEA, MPP^+, and metformin and is thought to play a potential role in the renal and hepatic elimination of drugs, endogenous compounds, and environmental toxins. A kidney-specific gene, *hMATE2* (*hMATE2-K*), was also cloned and shown to transport various organic cations such as TEA, MPP^+, cimetidine, metformin, and procainamide (3). Paraquat, a widely used herbicide, was demonstrated to be eliminated from the kidney by *hMATE1* but not *hMATE2* (42). These studies suggest that the transporters encoded by both the *hMATE2-K* and *hMATE1* genes play a role in the tubular transport across the brush border membrane of cationic drugs and other small cationic compounds (3).

Transporter-Mediated DDI in Renal Excretion and Hepatic Clearance

As mentioned previously in this chapter, some dosing vehicles can inhibit intestinal efflux transporters and increase oral absorption of the substrates. These vehicles can also inhibit liver and kidney efflux transporters, thereby decreasing clearance and increasing the exposure of the substrates after i.v. administration. For example, when cremophor was administered as a 6-hour infusion every three weeks along with the bolus administration of doxorubicin (50 mg/m^2), the AUC of doxorubicin increased from 1448 (CV of 24%) to 1786 (CV of 15%) h ng/mL in the presence of cremophor. Additionally, the AUC of its metabolite, doxorubicinol, increased from 252 (CV of 42%) to 486 (CV of 22%) h ng/mL. Such interactions can be due to decreased clearance of doxorubicin 612 (CV of 29%) to 477 (CV of 15%) mL/min by inhibiting P-gp in the liver and kidney and inhibiting metabolizing enzymes (49).

Renal excretion is a major elimination route for many antibiotics and antivirals and is partially mediated by the uptake of OATs into proximal tubular cells. Coadministration of probenecid, a nonspecific anion transporter inhibitor, increased the peak concentration, half life, and exposure of many cephalosporins, including cephazedone, cefazolin, cefradine, cefoxitin, cefadroxil, and so on, due to inhibition of renal excretion. This has benefited drug therapy by prolonging the effective exposure of these antibiotics (87). Nonsteroidal anti-inflammatory

drugs (NSAIDs) including diflunisal, ketoprofen, flurbiprofen, indomethacin, naproxen, and ibuprofen inhibit the hOAT1-mediated transport of adefovir at clinically relevant concentrations (IC_{50s} of 0.85–8 μM). Consequently, this NSAIDs-adefovir interaction may reduce or delay the emergence of adefovir nephrotoxicity (88).

Severe DDIs are known to occur between methotrexate and NSAIDs, probenecid, and penicillin G partially due to the inhibition of the renal OAT-mediated secretion of methotrexate. By using mouse proximal tubule cells, which stably express human transporters, methotrexate has been demonstrated to be taken up by hOAT3 and hOAT1 at the basolateral side of the proximal tubule and effluxed or taken up at the apical side by hOAT4. Drug interactions may occur between methotrexate and NSAIDs, probenecid, and penicillin G at both basolateral and apical sides of the proximal tubule (89).

Tsuruoka et al. recently reported the first case of severe arrhythmia caused by the interaction of cetirizine and pilsicainide. These drugs compete for renal excretion via P-gp and OCT. A patient with renal insufficiency who was taking oral pilsicainide was found to have a wide QRS wave with bradycardia three days after taking oral cetirizine. The plasma concentrations of both drugs were significantly increased during the coadministration. A follow-up pharmacokinetic study in healthy volunteers showed that the renal clearance of cetirizine (20 mg) or pilsicainide (50 mg) was decreased by approximately twofold following coadministration of the two drugs. In vitro studies using Xenopus oocytes expressing OCT2 and renal cells transfected with P-gp revealed that these transporters were inhibited by either cetirizine or pilsicainide. The DDIs at either human P-gp or OCT2 in the renal tubule explain the elevated concentrations of these drugs in this patient (90).

A DDI (via transporters such as P-gp, OATP, and CYPs in the liver) explains the increase in the systemic exposure of all statins (lovastatin, simvastatin, pravastatin, cerivastatin, and rosuvastatin) when coadministered with cyclosporine. For example, rosuvastatin has been shown to be a substrate for the human liver transporters OATP2 and BCRP but not P-gp. Its metabolic clearance is low and mainly mediated by CYP2C9. CsA treatment in transplant recipients increased AUC (0–24 hours) and C_{max} of rosuvastatin (10 mg) by 7.1-fold and 10.6-fold, respectively, compared with control values. This pronounced DDI is due to the inhibition of OATP2-mediated rosuvastatin hepatic uptake by CsA (91).

TRANSPORTER-RELATED PHYSIOLOGICAL FUNCTIONS AND ROLES IN TOXICITY

Genetic deficiencies of certain transporters can cause hereditary diseases, such as Dubin–Johnson syndrome (mutation in *ABCC2* (MRP2, cMOAT) (92), X-linked adrenoleukodystrophy (X-ALD, mutation in ABCD1) (93), congenital hyperinsulinism (mutation in *ABCC8*) (94), cystic fibrosis (mutation in *ABCC7*) (95), progressive familial intrahepatic cholestasis (PFIC) (mutations in P-type ATPase, BSEP, and MDR3) (96–98), surfactant deficiency (mutation in *ABCA3*) (99), Stargardt's disease (mutation in *ABCA4*) (100), and Tangier disease (mutation in *ABCA1*) (101), and Wilson disease (mutation in ATP7B) (102).

The physiological functions of transporters have been investigated using genetic knockout mice. Mdr1, Mdr2, Mrp1, Mrp3, Mrp4, Oct1, Oat3, and Pept2 knockout mice appear to be viable, healthy, and fertile (103–108).

As mentioned earlier, P-gp–deficient mice and collie dogs are extremely sensitive to the neurotoxicity induced by ivermectin (15,110). Cyclosporin A or trifluoperazine increased the brain concentration and the acute toxicity of ivermectin in mice after intraperitoneal administration (110).

Bcrp1(−/−) mice are fertile and their life spans and body weights are not different from wild type. Hematological and plasma chemical analysis revealed that there were no abnormalities except for an increase in unconjugated bilirubin in the *Bcrp1*(−/−) mice (111). *Bcrp1*(−/−) mice are extremely sensitive to light after oral administration of pheophorbide A. Pheophorbide A is a phototoxic porphyrin catabolite of a dietary chlorophyll-breakdown product. It is transported by Bcrp1, which limits its intestinal uptake from ingested food and, possibly, facilitates its biliary or renal excretion. In humans, genetic defects in the heme biosynthetic pathway result in the accumulation of photosensitizing porphyrins in the skin which also cause phototoxicity. These porphyrins are analogues of pheophorbide A and are also substrates for Bcrp1. Bcrp1 expressed in erythrocytes and their precursors can protect these cells from the excessive

accumulation of protoporphyrin IX (PPIX), possibly by extrusion of this compound. *Bcrp1*(−/−) mice develop a unique protoporphyria with 10-fold higher erythrocyte levels of PPIX Transplantation with wild-type bone marrow cured the protoporphyria and reduced the sensitivity of *Bcrp1*(−/−) mice to the phototoxin. These results indicate that the lack of BCRP activity in human or animals may increase the risk of protoporphyria and diet-dependent phototoxicities (111).

ABCG5 and ABCG8 are half transporters, forming heterodimers with each other to efflux phytosterols (plant sterols) and cholesterol out of intestine and liver. In patients with sitosterolemia (a rare inherited plant sterol storage disease), mutations in either Abcg5 or Abcg8 lead to increased blood concentration of phytosterols. Abcg5 and Abcg8 knockout mice have elevated blood levels of phytosterols and accumulate phytosterols in brain (112). Some dietary plant sterols disturb cholesterol homeostasis by affecting two critical regulatory pathways of lipid metabolism. Thus, Abcg5/ABCG5 and Abcg8/ABCG8 play a key role in protecting against the disruption of cholesterol homeostasis by dietary plant sterols

The calcification of elastic fibers observed as a phenotype in *Abcc6*(−/−) mice is shared with pseudoxanthoma elasticum (PXE) pathology in humans making *Abcc6*(−/−) mice a useful model to further investigate the etiology of PXE (113).

Generation of bile is regulated by ATP-dependent process and depends on the coordinated action of a number of transporter proteins in the sinusoidal and canalicular domains of the hepatocyte. Dysfunction or inhibition of any of these proteins can lead to retention of substrates or their metabolic products resulting in hyperbilirubinemia or cholestasis. Bilirubin, the end product of heme catabolism, is taken up from blood into hepatocytes by passive diffusion and the sinusoidal membrane transporter, OATP2, conjugated by UGT in the hepatocyte and then excreted into the bile through the bile canalicular membrane transporter, MRP2, mainly as bilirubin glucuronides (113a–116). Mrp2-deficient rats, GY/TR$^-$ (transporter-deficient Wistar rats) and EHBR, suffer hyperbilirubinemia and are good models for Dubin–Johnson syndrome, a human disease, which is characterized by hyperbilirubinemia. This syndrome occurs in the human with a hereditary deficiency in MRP2 (92). Since bilirubin glucuronides are endogenous substrates of MRP2 and excreted from the liver into bile by MRP2 (117), inhibition of MRP2-regulated transport of bilirubin glucuronides into the bile can potentially also cause hyperbilirubinemia. Genetical analysis (119,120) and OATP-transfected cell studies (120) also indicated that lack of OATP or inhibition of OATP can cause hyperbilirubinemia by the increased retention of bilirubin.

Cholestasis, or impaired bile flow, is an important but poorly understood manifestation of liver disease. There are two clinically distinct forms of inherited cholestasis, benign recurrent intrahepatic cholestasis (BRIC) and progressive familial intrahepatic cholestasis (PFIC). PFIC refers to a group of familial cholestatic conditions caused by defects in biliary epithelial transporters. The clinical presentation usually occurs first in childhood with progressive cholestasis. This usually leads to failure to thrive, hepatic failure, and the need for liver transplantation.

PFIC-1 is caused by a variety of mutations in *ATP8B1*, a gene coding for a P-type ATPase-protein, FIC-1, that is responsible for phospholipid translocation (96). It was previously identified as clinical entities known as Byler's disease and Greenland-Eskimo familial cholestasis. Patients with PFIC-1 may also have watery diarrhea , in addition to the clinical features below, due to the expression of FIC-1 in the intestine . How *ATP8B1* mutation leads to cholestasis is not yet well understood. PFIC-2 is caused by a variety of mutations in *ABCB11* , the gene that codes for the bile salt export pump (BSEP). Retention of bile salts within hepatocytes, which are the only cell type to express BSEP, causes hepatocellular damage and cholestasis. PFIC-3 is caused by a variety of mutations in *ABCB4* , the gene encoding multidrug resistance protein 3 (MDR3) (97), which codes for a flippase responsible for phosphatidylcholine translocation. The defective phosphatidylcholine translocation leads to the lack of phosphatidylcholine in bile. Phosphatidylcholine normally chaperones bile acids, preventing damage to the biliary epithelium. The free or "unchaperoned" bile acids in bile of patients with MDR3 deficiency cause a cholangitis . Biochemically, this is of note, as PFIC-3 is associated with a markedly elevated gamma glutamyltranspeptidase (GGT). Biochemical markers include a normal GGT for PFIC-1 and -2, with a markedly elevated GGT for PFIC-3. Serum bile acid levels are grossly elevated. Serum cholesterol levels are typically not elevated, as is seen usually in cholestasis, as the

pathology is due to a transporter as opposed to an anatomical problem with biliary cells (98). The disease is typically progressive, leading to fulminant liver failure and death in childhood, in the absence of liver transplantation. Hepatocellular carcinoma may develop in PFIC-2 at a very early age with even toddlers being affected.

Drug-induced cholestasis, without hepatitis, is observed most frequently with the use of contraceptives and 17α-alkylated androgenic steroids. The most probable mechanism involves interference with hepatocyte canalicular efflux systems for bile salts, organic anions, and phospholipids. The rate-limiting step in bile formation is considered to be BSEP-mediated translocation of bile salts across the canalicular hepatocyte membrane. Inhibition of BSEP function by metabolites of nefazodone, cyclosporine A, troglitazone, bosentan, rifampicin, and sex steroids is an important cause of drug-induced cholestasis (10,122).

Species differences in drug-induced cholestasis have been observed in the rat and human after administration of bosentan. Bosentan, the first orally active endothelin receptor antagonist, was developed for hypertension and heart failure. It caused dose-dependent and reversible liver injury in 2% to 18% of patients and caused a significant increase of serum bile salt, alanine aminotransferase (ALT), and bilirubin levels ($P < 0.01$). In vitro studies demonstrated that bosentan and its major metabolites inhibited BSEP and Bsep-mediated taurochlate transport. These results indicate that bosentan-induced liver injury is mediated, at least in part, by inhibition of Bsep/BSEP–causing intracellular accumulation of cytotoxic bile salts and bile salt-induced liver cell damage. The data further emphasize the pathophysiologic importance of drug-Bsep interactions in acquired forms of cholestatic liver injury. However, in contrast to the human studies, no increases in serum ALT levels were observed in rats, probably reflecting the more hydrophilic and less cytotoxic bile salt pool in rats compared with humans. Recently, it has been demonstrated that bosentan is a more potent inhibitor of rat Ntcp than human NTCP, and this should result in less intrahepatocyte accumulation of bile acids in rats during bosentan treatment.

Pulmonary surfactant forms a lipid-rich monolayer that coats the airways of the lung and is essential for proper inflation and function of the lung. The surfactant is produced by alveolar type II cells, stored intracellularly in organelles known as lamellar bodies, and secreted by exocytosis. The gene for ATP-binding cassette transporter A3 (*ABCA3*) is expressed in alveolar type II cells, and the protein is localized to lamellar bodies, suggesting that it has an important role in surfactant metabolism. Mutation of the *ABCA3* gene causes a fatal surfactant deficiency in newborns (99). *ABCA3* is critical for the proper formation of lamellar bodies and surfactant function and may also be important for lung function in other pulmonary diseases. Since it is closely related to *ABCA1* and *ABCA4*, proteins, which transport phospholipids in macrophages and photoreceptor cells, it may have a role in surfactant phospholipid metabolism (99).

OATP1C1 (also known as OATP14) has been characterized as a specific thyroid hormone transporter. Based on its expression in capillaries in different brain regions, OATP1C1 is thought to play a key role in transporting thyroid hormones, T_4 and T_4 sulfate (T_4S), across the BBB (122). The monocarboxylate transporter 8 (MCT8, SLC16A2) is a high-affinity transporter for the active hormone T_3. Men with mutations in MCT8 have severe, X-linked, psychomotor retardation and high serum T_3 levels (123). A similar phenotype is replicated in Mct8-null mice, which have lower T_3 in brain but higher T_3 in liver, resulting in a decrease in serum cholesterol and an increase in alkaline phosphatase (124,125). Thus, chemicals affecting the expression or function of Oatp1c1 and Mct8 may alter thyroid hormone homeostasis and mental development.

OCTN2 is a Na^+-dependent transporter for carnitine, which is essential for fatty acid metabolism, and its functional defect leads to fatal systemic carnitine deficiency (SCD). OCTN2 is present in various tissues, including kidney, skeletal muscle, heart, placenta, and others. In 1988, homozygous mutant mice, named juvenile visceral steatosis (*jvs*) mice, were found to exhibit cardiac hypertrophy, lipid accumulation in the liver, hyperammonemia with several histological changes (125), and alteration of carnitine disposition. The significance of OCTN2 as the carnitine transporter was clearly demonstrated from *jvs* mice, which exhibited the phenotype of systemic carnitine deficiency (SCD) caused by mutation of the OCTN2 gene (*Leu352Arg*) (126). This suggests an important role for OCTN1 in intracellular carnitine homeostasis.

Many antiviral drugs (e. g., fialuridine; FIAU) produce clinically significant mitochondrial toxicity that limits their dosing or prevents their use in the clinic. Human ENT1 (hENT1) is

expressed on the mitochondrial membrane and this expression may enhance the mitochondrial toxicity of nucleoside drugs such as FIAU (127). However, the lethal mitochondrial toxicity of fialuridine observed in the clinic was not predicted from preclinical toxicity studies in rodents (rats and mice), even at doses that were 1000-fold greater than that used in the human study. In fact, hENT1 but not mouse Ent1 was expressed in the mitochondrial membrane, indicating that fialuridine can get into human but not mouse mitochondria via ENT1. This observation has been confirmed by hepatocyte studies. The mitochondrial uptake of fialuridine was higher in human hepatocytes than that in mouse hepatocytes, and this uptake could be reduced by an ENT inhibitor in human hepatocytes but not in mouse hepatocytes (128). Species differences in transporters may influence the relevance of preclinical toxicity findings to humans.

Adefovir and cidofovir are clinically important antiviral agents and have been shown to cause drug-associated nephrotoxicity in some patients. In vitro studies demonstrated that adefovir and cidofovir were approximately 500-fold and 400-fold more cytotoxic, respectively, in OAT1 transfected CHO cells compared to the vector control transfected CHO cells, suggesting that the drug associated nephrotoxicity could be caused by OAT1-mediated accumulation of adefovir and cidofovir in renal proximal tubules (129).

TRANSPORTERS IN DRUG EFFICACY

Transporters in Drug Resistance

To date, three major mechanisms of drug resistance in cells have been identified by using cellular and molecular biology techniques: (*i*) decreases in the uptake of polar, water-soluble drugs, such as folate antagonists, nucleoside, analogues, and cisplatin, which require transporters to enter cells; (*ii*) changes in cells that affect the capacity of cytotoxic drugs to kill cells, including alterations in cell cycle, increased repair of DNA damage, reduced apoptosis, and altered metabolism of drugs; and (*iii*) increases in the energy-dependent efflux of hydrophobic drugs that can easily enter the cells by diffusion through the plasma membrane (130). Among these mechanisms, both uptake (*i*) and efflux (*iii*) processes involve transporters.

Drug resistance in the oncology field has yet to be fully overcome by inhibiting MDR1/P-gp. Overexpression of P-gp in tumors can confer two orders of magnitude of resistance for drugs that are P-gp substrates (131,132). Combination therapy with transporter inhibitors, such as verapamil, PSC 833, GF120918, and cyclosporine, has offered some help against some refractory tumors (131,132). Such inhibitors in in vitro cell lines have markedly sensitized them to chemoagents. However, the clinical benefit from P-gp modulation is still a question (130). CsA, PSC833, and quinine showed some overall survival benefit in several anticancer drug treatments in P-gp positive patients with poor risk acute myelogenous leukemia (AML), untreated AML, and high myelodysplastic syndrome (MDS), respectively. However, these agents have no effect on the same anticancer drugs on this cancer type in a different clinical trial. These inconsistent results could be partially due to the limitations of MDR inhibitors (such as low potency, low specificity, dose-limited toxicity, and nonoptimal PK profile) and inadequate clinical trial designs (130).

Other efflux transporters such as BCRP and MRP1/2 (133,134) must also be taken into account in the development of drug resistance and new inhibitors are needed for these. For example, lapatinib, an inhibitor for both P-gp and BCRP, increased topotecan accumulation in BCRP- or P-gp–expressing cells in vitro, and the combination showed enhanced efficacy in $HER2^+$ BT474 xenografts. In a phase I study, nausea, vomiting, diarrhea, and fatigue were dose limiting. Pharmacokinetic analyses showed that combined drug administration resulted in decreased topotecan clearance consistent with transporter-mediated interactions. Seventeen (46%) patients had disease stabilization (134).

In addition to the oncology field, efflux pumps have also been shown to confer resistance to drugs that target epilepsy and HIV infection in the CNS, T-cells (inflammation diseases) (135).

P-gp–mediated pharmacokinetics of [^{11}C]verapamil in solid tumors and in the BBB have been studied using PET (136). Both a *MDR1* gene-transfected, P-gp–overexpressing human small cell lung carcinoma GLC_4/P-gp and its P-gp negative small cell lung carcinoma counterpart GLC_4 have been used as tumor models in the rat. Since each rat had xenographs from both P-gp negative and P-gp positive tumors, identical drug systemic exposure to the tumors could be assured.

For validation of the cell lines, in vitro studies were performed. Cellular accumulation of [^{11}C]daunorubicin and [^{11}C]verapamil were 3.3-fold ($P<0.001$) and 2.3-fold ($P<0.001$) higher in GLC4 compared with GLC4/P-gp, respectively. Preincubation with cyclosporin A increased [^{11}C]daunorubicin and [^{11}C]verapamil levels in GLC4/P-gp but not in GLC4. Furthermore, in the cytotoxicity assay, the GLC4/P-gp line was found to be 12-fold more cross-resistant to daunorubicin compared with GLC4. These results demonstrated that GLC4 and GLC4/P-gp cell lines were an elegant model to study P-gp functionality in the tumor-bearing rat model.

Biodistribution studies showed 159% and 185% higher levels of [^{11}C]daunorubicin and [^{11}C]verapamil in GLC4 than in GLC4/P-gp tumor xenographs, respectively. After cyclosporin A, [^{11}C]daunorubicin and [^{11}C]verapamil content in the GLC4/P-gp tumor was raised to the level of GLC4 tumors. PET measurements demonstrated that P-gp limited accumulation of its substrate in the tumors while P-gp inhibition increased the P-gp substrate concentration in tumors that overexpressed P-gp.

Several other strategies for reversing the MDR are also being considered. Some of these involve coadministration of antisense oligonucleotides, hammerhead ribozymes, and short-interfering RNA (iRNA) to suppress P-gp expression (138,139), the antagonism of xenobiotic nuclear receptor SXR involved in the induction of P-gp and CYP3A4 (140,141), the bolstering of the expression of P-gp in bone marrow stem cells, which are more sensitive than other cells to the toxicity of anticancer agents, to limit their exposure to these agents, and the transfection of stem cells with MDR1 cDNA to make them more resistant to chemoagents in order to allow higher doses of the drugs to be used for longer periods of time (141). Although the transcriptional repression of MDR is a promising and attractive strategy, it is still a challenging task to successfully deliver the gene regulators to the cancer cells in vivo (131,138,139). In contrast to normal stem cells, the high expression of drug transporters can also lead to drug resistance in tumorigenic stem cells. During chemotherapy normal cells are killed but the tumor stem cells survive and proliferate, leading to a recurring tumor composed of tumor stem cells and cells of variable, committed lineage (142). Mutations in the tumor stem cells and their descendents can further confer a drug resistance phenotype and result in tumor growth.

For some hydrophilic drug molecules, the rates of passive diffusion through the cell membrane are low and transporter-mediated uptake is a major mechanism for drug entry into target cells. In vitro cell-based assays have demonstrated that inefficient cellular uptake is a potential mechanism for resistance to anticancer drugs such as the nucleosides (144,145) including cytarabine (146,147), fludarabine (147), cladribine (148,149), 5-fluoro-2′-deoxyuridine (FdUrd) (150,151), 5-fluorouracil (151), gemcitabine (152), 6-mercaptopurine (153), and 6-thiolguanine (153).

The NT-deficient murine T-cell lymphoma cells AE1 (154) exhibit greatly reduced uptake of physiological nucleosides and high-level resistance to cytotoxic nucleosides (154). In FdUrd-resistant human HCT-8 colon cancer cells, there was no measurable uptake of FdUrd and no detectable ENT functions resulting in 700-fold resistance to the cytotoxicity of FdUrd compared to naive HCT-8 cell (152). However, the role of uptake transporter deficiencies in clinical drug resistance is less clear partially due to the difficulties of performing transport studies on the malignant cells derived from clinical specimens and quantifying the abundance of transporters in a malignant clone mixed with normal cells.

The efficiency cytarabine uptake by leukemic blast cells has been correlated to clinical outcomes in AML and ALL patients who received a standard dose of cytarabine (100–200 mg/m^2/day). The sensitivity to cytarabine therapy was highly correlated to nucleoside transporter content and a deficiency in NT may impart resistance to cytarabine (146,149).

Transporters as Drug Targets

The cholesterol transporter Niemann-Pick C1 Like 1 protein (Npc1L1) is enriched in the brush border membrane of enterocytes in the small intestine of humans and rodents. In humans, rare variants in NPC1L1 are associated with reduced sterol absorption and plasma low-density lipoprotein (LDL) levels (155). Consistently, Npc1L1-kockout mice have a marked decrease in intestinal absorption of cholesterol and phytosterols (157,158) and are completely resistant to diet-induced hypercholesterolemia (157). Thus, Npc1L1 is essential for intestinal uptake of both cholesterol and phytosterols and is important in cholesterol homeostasis. Ezetimibe, a drug

used to treat hypercholesterolemia in patients, is an Npc1L1 transporter inhibitor. In addition to inhibiting cholesterol absorption, ezetimibe reduces plasma phytosterol levels in patients with hypercholesterolemia, although a molecular mechanism for intestinal phytosterol uptake and absorption has yet to be established.

Ezetimibe also effectively reduces phytosterol levels in patients with sitosterolemia, which is caused by a mutation in the ATP-binding cassette (ABC) cotransporters, either ABCG5 or ABCG8 (157). ABCG5 and ABCG8 are expressed in the mucosa cells and the canalicular membrane, and they resecrete sterols back into the intestinal lumen and from the liver into bile. Defects of either of these transporters lead to the rare inherited disease of phytosterolemia, which is clinically defined by hyperabsorption and diminished biliary excretion of plant sterols.

Bile acids play key roles in the maintenance of bile flow, the absorption of lipids, and the disposition of lipophilic endobiotics and xenobiotics. Over 95% of bile acids secreted into bile are reabsorbed through highly regulated transport systems in liver and gastrointestinal tract. In humans, missense mutations of ASBT (encoded by SLC10A2) at conserved amino acid positions, L243P and T262M, are associated with primary bile acid malabsorption (PBAM) (158). In patients with PBAM, mutations in the ASBT lead to congenital diarrhea, steatorrhea, and reduced plasma cholesterol levels. Studies in Asbt-null mice demonstrate that Asbt is essential for efficient intestinal absorption of bile acids. In the *Asbt*(−/−) mice, fecal bile acid excretion was elevated 10- to 20-fold and was not further increased by feeding a bile acid-binding resin. Despite increased bile acid synthesis, the bile acid pool size was decreased by 80% and selectively enriched in cholic acid in the *Asbt2*(−/−) mice. On a low fat diet, the *Asbt2*(−/−) mice did not have steatorrhea. Fecal neutral sterol excretion was increased only threefold, and intestinal cholesterol absorption was reduced only 20%, indicating that the smaller cholic acid-enriched bile acid pool was sufficient to facilitate intestinal lipid absorption. Liver cholesteryl ester content was reduced by 50% in *Slc10a2*(−/−) mice and, unexpectedly, plasma high-density lipoprotein cholesterol (HDL) levels were slightly elevated (159). Thus, inhibition of Asbt is a potential target for cholesterol-lowering drugs.

Elevated serum urate levels are associated with important common disorders such as gout, metabolic syndrome, diabetes, hypertension, and cardiovascular morbidity and mortality. Uric acid is principally derived from the breakdown of dietary and cellular purines. In humans, the kidney plays a pivotal role in urate management with secretory mechanisms balanced against efficient reabsorption resulting in only 10% of the filtered load actually being excreted in the urine (160). The established urate transporter systems in the proximal nephron include (*i*) the urate anion transporter (URAT1), which is a target of uricosuric drugs; (*ii*) multiple organic anion transporters (OATs 1–4); (*iii*) the urate transporter (UAT); and (*iv*) a voltage-dependent organic anion transporter (OATv1) (160). Recently, using an approach called genome-wide association scanning, Caulfield et al. found that some genetic variants of the human gene SLC2A9 are more common in people with high serum urate levels than in people with normal levels (161). SLC2A9 encodes a glucose transporter (a protein that helps to move the sugar glucose through cell membranes) and is highly expressed in the main urate handling site of the kidney. An in vitro study indicates that SLCA9 is a high capacity urate transporter and plays an important part in controlling serum urate levels. Thus, these findings could eventually lead to new treatments for gout and possibly for other diseases that are associated with increased serum urate levels (161).

Transporters as Disease Prognostic Factors

Many studies have confirmed that the expression of P-gp is an adverse prognostic factor for complete remission and survival in adult AML. However, for prognostications, it should also be taken into account that this association is age-dependent. In two large studies, Leith et al. could show that the prognostic impact of P-gp is more pronounced in elderly patients with AML than in young adults with AML (162). In pediatric patients, it does not have prognostic value at all. There may be two reasons for this age dependence. First, the expression of P-gp is higher in older individuals. Second, pediatric treatment protocols tend to be more aggressive. Thus, the expression of P-gp in younger patients might not be high enough to cause clinically relevant drug resistance and/or drug resistance is overcome by more intensive treatment. In acute lymphoblastic leukemia (ALL), the picture is similar to AML. Most of the studies that

Table 2 Prognostic Impact of ABC Transporters in Acute Leukemia

	Pediatric ALL	Adult ALL	Pediatric AML	Adult AML
P-gp (*ABCB1*)	No prognostic impact in most studies	Indicates poor prognosis	No prognostic impact	Indicates poor prognosis
BCRP (*ABCG2*)	No prognostic impact[a]	Indicates poor prognosis[a]	Indicates poor prognosis[a]	Indicates poor prognosis
MRP1 (*ABCC1*)	No prognostic impact	No prognostic impact	No prognostic impact[a]	Controversial results
MRP3 (*ABCC3*)	Indicates poor prognosis	Indicates poor prognosis[a]	Indicates poor prognosis	Indicates poor prognosis

[a]Based on only one study that was either small or produced only marginally significant results.
Source: From Ref. 164.

analyzed pediatric ALL found no significant association between P-gp and disease prognosis. Only in a few studies did P-gp correlate with poor response to therapy. This discrepancy might be due to different treatment strategies. The expression of P-gp in adult patients is higher than in children and in this age group a prognostic impact was consistently reported (163).

BCRP correlates with poor prognosis for AML in both adults and children. Little is known about the role of BCRP in ALL (163). However, the presence of BCRP-positive cells in esophageal squamous cell carcinoma (ESCC), regardless of the amount, was associated with reduced survival ($p = 0.0088$). Moreover, it was shown to be an independent prognostic factor, along with the extent of the primary tumor and positive lymph node metastasis, in multivariate analysis using the Cox's regression model. The absolute presence of BCRP-positive cells in ESCC as a single independent prognostic factor, suggests that BCRP may play additional underlying roles, other than drug resistance, in the progression of ESCC (164).

Many studies have reported a correlation between MRP1 and poor prognosis in AML patients. Expression of MRP3 has a strong prognostic correlation with AML and ALL, independent of age group. However, MRP3 has not been demonstrated to cause much drug resistance in vitro. Therefore, it remains to be elucidated whether this correlation is due to MRP3 as a cause for poor response or as an epiphenomenon. If MRP3 is important for drug resistance, it would be the ABC transporter with the widest therapeutic spectrum in acute leukemia. *ABCA3* may be an additional cause of drug resistance in AML. It seems to be associated with in vitro drug resistance and poor response to therapy but both aspects need further studies for verification (163). The prognostic impact of ABC transporters in acute leukemia is summarized in Table2.

The human liver-specific organic anion transporter-2 (LST-2/OATP8/SLCO1B3) has been demonstrated to be expressed in various gastrointestinal carcinomas. Recent studies suggest that the liver specific OATP8 is an important prognostic factor for human breast carcinoma. In an immunohistochemical study of 102 cases of breast carcinoma, OATP8 immunoreactivity was detected in 51 cases (50.0%). Of these 51 positive cases, the expression of OATP8 inversely correlated with tumor size and decreased the risk of recurrence and improved prognosis by both univariate and multivariate analyses. In the estrogen receptor positive groups, the LST-2 positive patients correlated with good prognoses. Considering that LST-2 transports estrone-3-sulfate, these results suggest that OATP8 overexpression is associated with a hormone-dependent growth mechanism of the breast cancer. These results support that OATP8 immunoreactivity is a potent prognostic factor in human breast cancer (165).

POLYMORPHISM OF TRANSPORTERS AND INTERINDIVIDUAL VARIATION

It has been estimated that genetics can account for 20% to95% of variability in disposition and the pharmacological effects of drugs although many other factors such as age, organ function, concomitant therapy, DDIs, and the nature of disease also influence drug response (166). Polymorphic genetic variations have been reported in human MDR1 (P-gp), MRP1, MRP2, BCRP, OATP (OATP1B1, OATP1B3, and OATP2B1), OAT1, OCT1 OCT2, and MATE (132,167–170). Generally, implicating alterations in transport activity, such as those caused single nucleotide

polymorphisms (SNPs), to change drug disposition is accomplished in vitro by measuring the efflux or uptake activities of specific substrates in the cells or membranes expressing recombinant protein and in vivo by measuring the expression of mRNA or protein in tissue samples, by assessing the intracellular accumulation of substrates, by evaluating the pharmacokinetic alterations of drug substrates, or by associating changes in clinical outcome from drug substrates (166).

To date, genetic polymorphisms of human MDR1 has been extensively investigated. There are 29 SNPs and 13 of major haplotypes of MDR1 that have been identified. A lot of attention has been focused on silent mutations, not associated with any amino acid change, such as C3435T in exon 26 and nonsynonymous variants, G2677T (Ala893Ser) and G2677A (Ala893Thr). The SNPs of MDR1 were associated with changes in P-gp expression and function and subsequent alteration in drug disposition. However, much of the clinical data has been contradictory or inconclusive (169). Interindividual expression of P-gp is manifold (two- to eightfold) and seems to be related to MDR1 genotype (170). The interindividual variation of P-gp in intestinal expression resulted in a sevenfold range in the oral bioavailability of P-gp substrate digoxin (171) and 30% variability of cyclosporine A plasma concentration (172). The variability in P-gp expression caused greater intersubject variability than intrasubject variability. For example, the intra- and intersubject coefficients of variation for $AUC_{0\text{-inf}}$ of orally administered P-gp substrate tanilolol were 14.0% and 20.4% to 29.5%, respectively (173).

Hoffmeyer et al. (174) were the first group to report that C3435T in exon 26 was associated with altered P-gp expression in human duodenum and thereby intestinal absorption of digoxin. Individuals with the homozygous T allele (TT genotype) had a twofold decreased P-gp protein level ($P = 0.056$) in duodenum biopsies as compared to those with the wild-type alleles (CC genotype). Consequently, the exposure of digoxin was significantly higher in T allele after oral administration. Later, various investigators reported that the T allele is associated with increased P-gp protein expression or has no clearly distinguishable effect (11,167,170,176).

Influence of the *ABCB1* polymorphisms 2677G>T/A and 3435C>T on placental P-gp expression and function was evaluated in perfused human placental with the well-established P-gp substrate saquinavir. The results indicate that the variant allele 3435T was associated with significantly higher placental P-gp expression than the wild-type alleles. Although the *ABCB1* polymorphism 3435C>T altered the expression levels of P-gp in the human placenta, this did not have any consequences on P-gp–mediated placental transfer of saquinavir (176). P-gp function at the BBB, evaluated by integration plot analysis of the first 3-minute data using ^{11}C-verapamil as a probe was not significantly different between the haplotypes of MDR1 genes (*1236TT, 2677TT, 3435TT* vs. *1236CC, 2677GG, 3435CC*).

Because of conflicting results of the functional significance of MDR1 exon 26 C3435T SNP on the disposition of digoxin in different ethnic groups, Chowbay et al. performed a meta-analysis of the published data investigating the influence of C3435T SNP on the pharmacokinetics of digoxin as well as MDR1 expression in Caucasian and Japanese populations. The following outcomes were included exposures to digoxin measured by AUC and C_{max}, the mean expression levels of intestinal MDR1 mRNA and P-gp in the absence of digoxin administration. The results of the meta-analysis indicated that the synonymous MDR1 C3435T SNP does not affect the pharmacokinetics of digoxin and the expression of MDR1 mRNA. Future studies should focus on the impact of MDR1 haplotypes on the pharmacokinetics of MDR1 substrates rather than the C3435T SNP alone (177).

Although it is common to see controversial conclusions from published clinical observations about P-gp polymorphism in drug disposition, even when the same probe drug and ethnic group were studied, there are several possible reasons for these inconsistent data, including (*i*) different experimental conditions (e. g., probe drug used, dose level, single dose vs. repeat dose), (*ii*) small sample sizes, (*iii*) sample selection, or (*iv*) heterogeneity in the diversity of the ethnic population studied. In addition, many probe substrates for transporters are also substrates for drug metabolizing enzymes or other transporters. For example, transports studies with cyclosporine and tacrolimus may be complicated by the involvement of CYP3A metabolism. Digoxin and fexofenadine are also substrates of OATPs. Consequently, it is also possible that the metabolism and transport by other competing proteins, apart from P-gp, contribute to the observed variability in drug disposition.

ABCC genes have been screened in Japanese, Chinese, and Caucasian subjects (178–182). Although a high number of rare mutations, which lead to amino acid changes, have been reported in the literature and the database (Pharmacogenetics and Pharmacogenomics Knowledge Base, www.pharmgkb.org), there are few common nonsynonymous SNPs that are striking. The contribution of common nonsynonymous SNPs on genetic variability of expression and function of MRP transporters is limited. Little success has been made to characterize the functional effects of MRP1, MRP2, and MRP3 variants by in vitro assays and clinical trials.

BCRP has been systemically screened for single nucleotide polymorphism (SNP) in 90 different ethnic populations. More than 40 nonsynonymous and synonymous SNPs have been revealed in the promoter as well as in both the exon and intron sequences (184,185). The two most frequent naturally occurring SNPs G34A and C421 A have been identified in humans (185). G34A variant in exon 2 resulting in a Val12Met amino acid change has been associated with low BCRP protein expression and an altered efflux function in cancer cells (185–187). All Mexican-Indians screened possessed at least one variant allele, while the frequency in Caucasians was only 4.7%. Recent studies suggested that nasopharyngeal carcinoma patients who were wild type for the G34A showed a trend toward lower systemic exposures of irinotecan compared with patients with one or two variant alleles (Table 2) (188).

SNPs in drug uptake transporters such as OATP1B1 (OATP2) have been shown to influence the exposure of HMG-CoA inhibitors and fexofenadine in healthy volunteers (189–191). To date, 44 polymorphism of *SLCO1B1* (encoding OATP1B1) have been identified in coding regions, including 17 nonsynonymous (change of an amino acid), 4 conservative (no change of amino acid), and 20 in the intron and 3 in the promoter regions. Of the nonsynonymous variants, seven were common. Two of these: A388G (Asnl30Asp in OATP1B1*1b), and T521C (Va1174Ala in OATP1B1*5) occurred in African-Americans (74% and 1%), Asians (63% and 16%), and Caucasians (40% and 14%) while five of these: A452G (Asnl51Ser, OATP1B1*16) was detected specifically in Asians (3.8%), and C463A (Prol55Thr, OATP1B1*4) (8%) and A1929C (Leu643Phe, OATP1B1*19) (9%) were specific for Caucasians and G1463C (Gly488Ala, OAP1B1*9) (9%), and A2000G (Glu667Gly, OATP1B1*11) (34%) was found only in African-Americans (191–194).

Nishizato et al. (195) provided the first evidence in humans that the *SLCO1B1* variants were associated with altered pharmacokinetics of pravastatin. Subjects with the *OATP1B1*15* allele (130Asp174Ala) had reduced total and nonrenal clearance, with concomitant increased plasma concentrations, of pravastatin compared with those with the *SLCO1B1* allele (130Asp). These findings suggest much of the functional loss in hepatic uptake of pravastatin associated with the *SLCO1B1* haplotype is related to the Val174Ala mutation. Subsequently, several groups demonstrated that *SLCO1B1* variant haplotypes had significant effects on the disposition, efficacy, and toxicity of other HMG-CoA reductase inhibitors. The genetic polymorphism of *SLCO1B1*, T521C (Val174Ala), considerably increases the plasma concentration of simvastatin acid and moderately increases those of pravastatin but seems to have no significant effect on fluvastatin.

Recently, the ongoing Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH) collaboration group has carried out a genome-wide association study using approximately 300,000 markers and additional fine-mapping in 85 subjects with definite or incipient myopathy in patients taking 80 mg of simvastatin daily (196). This study identified a single strong correlation of myopathy with the rs4363657 SNP located within OATP1B1 on chromosome 12. The noncoding *rs4363657 SNP* was in nearly complete linkage disequilibrium with the nonsynonymous *SNP,rs4149056*, ($r^2 = 0.97$) which has been linked to statin disposition. The prevalence of the *rs4149056C* allele in the population was 15%. Among participants taking 80 mg of simvastatin daily, *rs4149056 CC* homozygotes had an 18% cumulative risk of myopathy, with occurrence primarily during the first year, whereas the CT genotype was associated with a cumulative risk of approximately 3%. In contrast, the cumulative risk of myopathy was only 0.6% among TT homozygotes. Overall, more than 60% of these cases of myopathy could be attributed to the *rs4149056C* variant in *SLCO1B1*. No SNPs in any other region were clearly associated with myopathy. This genome-wide study from the SERACH group has identified common genetic variants in *SLCO1B1* that are associated with substantial increases in the risk of simvastatin-induced myopathy. These findings are likely to apply

to other statins because myopathy is a class effect, and *OATP1B1* polymorphisms are known to affect the blood levels of several statins (196). Moreover, these variants may be relevant to the effects of other classes of drugs transported by OATP1B1. Consequently, the genotyping of *SLCO1B1* polymorphisms may be useful in the future for tailoring both the dose of statins and in identifying risk factors in order to optimize the benefits of statin therapy with greater safety.

Studies with rifampicin, a potent inducer of CYP3A4 and a substrate of OATP2, have demonstrated, in vitro, that OATP variants also affect the uptake of rifampicin. Tirona et al. (197) demonstrated that various SNPs in *SLCO1B1* markedly reduced the hepatocellular uptake of rifampin, suggesting that patients with these functionally deficient OATP-C variants may exhibit reduced capacity for rifampin-mediated induction of hepatic drug metabolizing enzymes and transporters. Later, Niemi et al. demonstrated that *SLCO1B1* polymorphisms did not affect the extent of induction of hepatic CYP3A4 by rifampicin in humans, probably because other uptake transporters, such as OATP1B3, can compensate for reduced uptake of rifampicin by OATP1B1 (198). These findings suggest that transporters, drug metabolizing enzymes, and regulatory factors should be viewed and evaluated as an integrated system when analyzing the potential of genetic changes on drug response.

Genotyping studies have identified over 200 SNPs in the *OCT1* genes and most of the variants have been functionally characterized with in vitro studies. Clinical and *Oct1*(−/−) knockout mice studies demonstrated that *OCT1* expression may correlate with metfomin efficacy (199). OCT1 mRNA levels tend to be lower in Met408Val (A1222G) variant although this is not statistically significant. In healthy volunteers, metformin treatment resulted in significantly elevated plasma glucose concentrations and prolonged plasma insulin levels in subjects expressing M408V (V1222G) although the basal plasma glucose levels were similar in the OCT variant and reference subjects. However, in Japanese patients the polymorphism of OCT1 (including Met408Val) and OCT2 had little affect on the clinical efficacy of metformin (200).

To date, polymorphisms in OATP, BCRP, and OCT1 have been shown to have clinic impact on the disposition, efficacy, and toxicity of its substrates. The clinical impact of MDR1 (P-gp) polymorphism is more uncertain. The contributions of genetic variants of other transporters to the interindividual variability in drug disposition and efficacy remains controversial as contradictory results have been reported. Most published studies have been limited by the small sample sizes, in relation to the allele and genotype frequency, of the studied variant and from potentially confounding factors of the probe drug that affect their interpretation (169). This will require further clinical investigation.

Numerous environmental factors which also affect the phenotypic expression of drug transporter activities must also be considered. For example, food constituents, herbal preparations, and/or the therapeutic drugs used may induce or inhibit the function or expression of the protein. Thus, these nongenetic factors might mask the potential genetic effects on transporter function.

The route of drug administration and drug-specific differences in metabolism and excretion may also contribute to discrepancies observed for various substrates of drug transporters. For instance CsA is a substrate for P-gp and CYP3A4, but digoxin is only a substrate for P-gp. Rifampicin is not only a substrate for OATP2 but also for OATP8, which may compensate for reduced uptake of rifampicin by OATP2 variants (198). Finally, distribution of other unidentified variation(s) in the same gene and/or other genes relevant to drug disposition might be different among the different human populations studied.

CONCLUSIONS AND PERSPECTIVES

Although the transport mechanisms of most therapeutic drugs remain unknown, some clinical DDIs and toxicities in humans have been linked to transporters. With the large number of transporters cloned, their functions characterized in in vitro assays and in animals, and the recent increase in clinical studies and retrospective gene analysis, the roles of transporters in the absorption, distribution, metabolism, and elimination of drugs and, consequently, the efficacy and toxicity of drugs in humans is being recognized. Therefore, regulatory agencies and the pharmaceutical industry have acknowledged the need to evaluate new drug candidates for

their potential as substrates or inhibitors of drug transporters in causing potential drug–drug, drug—endobiotic, or drug–food interactions in humans.

The effort needs to be continually made to better predict transporter-mediated drug interactions by knowing the complexity of localization and function of transporters and their interplay with phase I and phase II enzymes. At this point, it is difficult to extrapolate the results from in vitro or animal studies to human. The challenge of understanding the quantitative significance of transporters in drug development remains. Research focusing on the development of methodology to delineate the contribution of major transporters to drug disposition, the establishment of in vitro–in vivo correlations, and the further understanding of the clinical impact of transporter polymorphisms will help us better use our knowledge of drug transporters in drug discovery and development.

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9 Toxicity Evaluations, ICH Guidelines, and Current Practice

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CHAPTER OVERVIEW

This chapter is provided to address the conduct of nonclinical[a] toxicity studies in the drug development process and specifically the role of the International Conference on Harmonization (ICH) of the Technical Requirements for the Registration of Pharmaceuticals for Human Use Guidances in current toxicology practices. This chapter should be useful to the beginning toxicologist as well as persons in the pharmaceutical industry with no formal training in toxicology but whose roles may require them to be involved in the design and conduct of the studies. The seasoned toxicologist also might find this chapter to be a good review of current practices. My hope is that this chapter will be a springboard into knowledge and as such, I have provided numerous Website addresses to find additional information.

The chapter is divided into four major sections. In the first section, an introduction to the ICH process is provided. It is important as one moves into the actual ICH topics to have a basic understanding of the parties to ICH and the process by which ICH operates to propose and approve guidances for the pharmaceutical industry.

The second section is a presentation of the nonclinical toxicity guidances in the overall safety designation of ICH topics. Among the topics included in this particular section are ICH guidance for single- and repeated-dose toxicity studies, reproductive toxicity studies, genotoxicity and carcinogenicity studies, and the safety assessment of biotechnology-derived pharmaceuticals.

The third section provides a short presentation of the nonclinical safety topics that fall outside the traditional toxicology realm. These nonclinical ICH safety guidances are in the area of pharmacokinetics/toxicokinetics and evaluation of systemic exposures in nonclinical studies and safety pharmacology, an area of intense recent interest.

The fourth and final section is a presentation of case studies involving a series of nonclinical toxicology packages of studies that were submitted to support the marketing approval of specific drugs. These case studies of Celebrex™, Herceptin®, Rituxan®, and Remicade® are each illustrative of the unique nature of the different drug development programs. It is my hope that the case studies, together with the ICH guidances, provide a constructive starting point for the design of the package of nonclinical toxicity studies that may be required for a new drug.

INTRODUCTION

The ICH is a regulatory and scientific undertaking initiated with the goal of improving the drug development process in the three regions, Europe, Japan, and United States (US), through harmonization of the regulatory guidances among these regions. Established in 1990, ICH is an ongoing joint project between governmental regulatory authorities and pharmaceutical industry experts from each of the three major regions. These six parties to ICH meet with the goal of reaching scientific consensus on various regulatory issues, thereby standardizing the regulatory guidances globally. This standardization of regulatory guidances has to date significantly reduced the duplication of development activities that previously occurred when a company desired to obtain worldwide marketing approval. The positive influence of the ICH

[a] As these toxicity studies are conducted prior to the initiation of clinical trials, the word "preclinical" is often used. However, as toxicity studies are also conducted during the course of clinical trials, it is perhaps more correct to refer to these studies as "nonclinical."

Table 1 Four Categories of ICH Topics

Letter designation	Category	Topic
Q	Quality	Guidances in the Area of Chemistry, Manufacturing, and Control (CMC)
S	Safety	Guidances in the Area of Nonclinical Toxicology, Pharmacology, and Pharmaco/Toxicokinetics
E	Efficacy	Guidances in the Area of Clinical Studies of Safety and/or Efficacy
M	Multidisciplinary	Guidances That Cross-Categorical Lines

process is readily apparent in the improved relationship among government regulators and the pharmaceutical industry. The industry is now more able to implement strategies for drug development that allow registration in multiple regions. Much of the credit for the success of ICH has been the end result of the commitment of the regulatory authorities to implement the tripartite harmonized recommendations and guidelines in each of three regions.

ICH Topics

ICH has divided the topics into four major categories designated Q, S, E, and M. These categories correspond to the overall areas for drug development and regulatory approval as illustrated in Table 1.

In this chapter, the focus of the presentation will be on safety topics addressed by ICH. For guidances in other areas, the reader is referred to the ICH Website (see www.ich.org). As will be seen, toxicology study guidelines have been the primary area addressed in the realm of the safety topics. Before proceeding to the discussion of the safety topics, however, it may be beneficial to first review the ICH process and parties involved to understand the basis and context of the current regulations.

Parties to ICH

ICH is composed of six representative parties from three regions: regulatory representatives from Europe, Japan, and US; and pharmaceutical industry representatives from these same three regions. Each of these parties is described briefly below.

European Union (EU)

The European Union (EU) formerly the European Commission, represents the member countries of the EU. The EU is working, through harmonization of technical requirements and procedures, to achieve a single market in pharmaceuticals that would allow free movement of products throughout the EU. The European Agency for the Evaluation of Medicinal Products (EMEA) was established by the EU in 1993 and headquartered in London. The EMEA is responsible for providing advice and guidance on research and development programs and evaluating pharmaceutical products for human use, and the EU subsequently authorizes the marketing of products on the basis of the EMEA's opinion. Technical and scientific support for ICH activities is provided by the EU via the Committee for Proprietary Medicinal Products (CPMPs) of the EMEA.

European Federation of Pharmaceutical Industries and Associations

European Federation of Pharmaceutical Industries and Associations (EFPIA) is situated in Brussels and is made up of member associations in sixteen countries in Western Europe. These member associations ensure that the EFPIA's views of proposed ICH guidelines are representative of the pharmaceutical industry in the EU. Companies in membership of EFPIA are manufacturers of prescription medicines and include all of Europe's major research-based pharmaceutical companies.

Japan—Ministry of Health, Labor and Welfare

The Ministry of Health, Labor and Welfare (MHLW) was formed in 2001 from the Japanese Ministry of Health and Welfare and the Japanese Ministry of Labor. The MHLW has, as one of its responsibilities, the protection and promotion of the health and welfare of the Japanese people. The MHLW is responsible for a wide range of administrative activities encompassing the approval of drugs as safe and effective.

Japan Pharmaceutical Manufacturers Association

Japan Pharmaceutical Manufacturers Association (JPMA) represents ninety member companies. Membership includes all the major research-based pharmaceutical manufacturers in Japan. Among the objectives of JPMA is the development of a competitive pharmaceutical industry with a greater public awareness and understanding of issues in the development of new pharmaceuticals. JPMA promotes and encourages the adoption of international standards by its member companies.

United States—Food and Drug Administration

The FDA consists of administrative, scientific, and regulatory staff organized under the Office of the Commissioner and has several centers with responsibility for the various products that are regulated. Technical advice and experts for ICH work are drawn from the Center for Drug Evaluation and Research (CDER) and the Center for Biologics Evaluation and Research (CBER).

Pharmaceutical Research and Manufacturers of America

Pharmaceutical Research and Manufacturers of America **(**PhRMA) represents the research-based industry in the US. The association represents the country's leading pharmaceutical research and biotechnology companies, which are involved in the discovery, development, and manufacture of prescription medicines. There is currently, as of 2009, one international affiliate and Associate affiliates in the areas of: Researchers, Contract Research Organizations (CROs), Advertising and Communication Services, and Consultants and Drug Discovery Software Firms. The list of member companies can be found at www.phrma.org. PhRMA, which was previously known as the U.S. Pharmaceutical Manufacturers Association (PMA), coordinates its technical input to ICH through its Scientific and Regulatory Section. Special committees of experts from PhRMA companies have been created to deal with ICH topics.

Additional Participants

The observers are the World Health Organization (WHO), the European Free Trade Area (EFTA), and Health Canada. Each of the observer parties has a seat on the ICH Steering Committee.

In addition, the International Federation of Pharmaceutical Manufacturers Association (IFPMA) is a non-profit, non-governmental federation of member associations representing the research-based pharmaceutical industry and other manufacturers of prescription medicines in 56 countries throughout the world. IFPMA has been closely associated with ICH since its inception to ensure contact with the research-based industry outside the ICH regions. IFPMA has two seats on the ICH Steering Committee and runs the ICH secretariat.

Steps in the Process of Harmonization in the ICH process

The approval process of ICH guidelines is a five-step process. The status of each proposed or implemented guidance is provided on the ICH Website.

Step 1: Consensus building

An initial draft of a guideline (or recommendation) is prepared by the ICH rapporteur and the ICH Expert Working Group (EWG). This guideline is prepared in consultation with experts designated to the EWG. When consensus on the guideline is reached, the final revised consensus guideline is submitted by the EWG to the ICH Steering Committee for adoption, and the guidance moves to the next step in the process.

Step 2: Start of regulatory action

When the Steering Committee agrees that the proposed guideline contains sufficient scientific consensus on the technical issues laid out in the guideline, the draft guideline is deemed ready to go to regulatory consultation stage. The six parties to the ICH confirm, with signatures, that the ICH scientific committee agrees with the ICH EWG proposed guideline and the guidance moves to the next step.

Step 3: Regulatory consultation

At this stage, the guideline is circulated by the three regions for regulatory consultation: in the EU, it is published as a draft CPMP guideline; in Japan, it is issued by the MHLW; and in the US, it is published as a draft guidance in the Federal Register. With this circulation comes the opportunity for regulatory authorities and industry persons from non-ICH

regions to comment on the Draft Document. The three regulatory parties review these comments with the goal of reaching a single, harmonized wording of the guideline. The final revised guideline is approved by the regulatory parties of the three regions.

Step 4: Adoption of a tripartite harmonized text

Since the guideline may have been revised from that proposed by the ICH Steering Group, at Step 4 the guideline is returned to ICH and reviewed by both industry and regulatory experts to ensure that the proposals in the guideline remain acceptable subsequent to the consultation edits. If both regulatory and industry delegates are in agreement with the guideline, the text of the guideline is adopted and the guideline signed by the three regulatory parties to ICH; at this point, the guideline is recommended for adoption by the regulatory bodies in the three regions.

Step 5: Implementation

The tripartite harmonized guideline is implemented by the regulatory bodies. In the EU, the guideline is published by the EU in volume III of the *Rules Governing Medicinal Products in the European Union*. In Japan, the Pharmaceutical and Medical Safety Bureau (PMSB) is responsible for the promulgation of the guideline. In the US, the guideline is published by FDA.

ICH PRECLINICAL TOXICITY GUIDELINES

The preclinical toxicity testing of a new drug (including biotechnological products) is a fairly well-defined process in terms of the five general areas of testing, although the actual studies and protocol elements of the studies within each of these general areas may vary depending on the class of drug and intended clinical program. In this chapter, ICH activity is discussed and additional comments are provided regarding study design in each of the following areas of toxicity testing:

Single-dose toxicity
Repeat-dose toxicity
Reproductive toxicity
Genetic toxicity
Carcinogenicity
Immunotoxicity

A tabular listing of the ICH guidances in the "Safety" and "Multidisciplinary" categories that are specifically toxicity testing guidances is provided in Table 2. All of these guidances have reached the Step 4 adoption of the guidance text and have been implemented by the regulatory bodies from each of the three regions (Step 5).

Single-Dose Toxicity Guidance (ICH Topic S4)

One of the first guidances implemented by ICH was the guidance regarding single-dose toxicity. The main intent of this guidance was to remove the classical acute lethality dose determination (LD_{50}) from acute toxicity testing protocols, thereby altering the objective of these studies from one of determining the dose that leads to death (and perhaps an inordinate amount of suffering to the animal) to one of determining the maximum tolerated dose (MTD). This guidance was agreed upon prior to the first ICH meeting in 1991 and was published in the proceedings of the First International Conference on Harmonization. In the US, the guidance was published in the Federal Register with FDA revision (1). This FDA revision modified the ICH guidance to include wording that would allow the use of single-dose toxicity studies to support single-dose clinical trials in humans, for example, "in the screening of multiple analogues to aid in the selection of a lead compound for clinical development." This modification is consistent with the ICH position on acute toxicity testing but should be noted to be an FDA specific modification of the ICH guidance.

The ICH guidance provided some specific protocol elements to be addressed in the design of single-dose toxicity studies. Agreement was reached that determination of the lethal dose killing 50% of animals (the LD50) approach would be abandoned. Instead, the range of doses should include those doses that cause no adverse effect to those that cause life-threatening (but not death as an endpoint) toxicities. The drug should be administered by two routes, the

Table 2 ICH Preclinical Toxicity Testing Guidelines

Topic designation	Title
S1	**Carcinogenicity Studies**
S1A	Guideline on the Need for Carcinogenicity Studies of Pharmaceuticals
S1B	Testing for Carcinogenicity in Pharmaceuticals
S1C	Dose Selection for Carcinogenicity Studies of Pharmaceuticals
S2(R1)	**Genotoxicity Studies**
S2(R1)	Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use (previously coded as S2A and S2B)
S4	**Toxicity Testing**
S4	Single-Dose Toxicity Tests
S4A	Duration of Chronic Toxicity Testing in Animals (Rodent and Nonrodent)
S5(R2)	**Reproductive Toxicology**
S5(R1)	Detection of Reproduction for Medicinal Products & Toxicity to Male Fertility (previously coded as S5A and S5B)
S6	**Biotechnological Products**
S6	Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals
S8/S9	**Immunotoxicology Studies**
S8	Immunotoxicity Studies for Human Pharmaceuticals
S9	Nonclinical Evaluation for Anticancer Pharmaceuticals
M	**Multidisciplinary**
M3(R2)	Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals

intended clinical route and the intravenous (IV) route, and should be administered to at least two mammalian species, including a nonrodent species. In contrast to previous studies that required large numbers of animals to calculate lethality parameters, the ICH guidance calls for a limited number of animals, for instance three to five rodents per sex per group and even smaller numbers of nonrodents (not actually specified as to number). In the investigations in nonrodents, acute dose range-finding (RF) studies may be acceptable to provide the requisite acute toxicity data. The animals should be monitored for 14 days subsequent to dosing at which time a gross necropsy should be conducted.

The FDA guidance adds additional study elements for those special instances in which the data will be used to provide support for single-dose clinical trials. The guidance calls for the study to include pharmacokinetics (PK) and assessment of dose–response relationships and for more detailed toxicity assessments to include clinical pathology and histopathology evaluations at an early time (at which toxicity might be expected to be greatest) and later at termination (14 days) to evaluate recovery.

Repeat-Dose Toxicity Guidances

Repeat-dose toxicity studies in laboratory animals form the crux of the studies characterizing the potential toxicities of the compound. The toxicologist needs to identify the target organs for toxicity in the animal and potential surrogate markers in man, define the dose–response relationships for any observed toxicities including the threshold dose for observing toxicities, determine systemic exposures resulting in toxicities for extrapolation to man, and determine the potential for reversibility of observed toxicities.

Two questions come immediately to mind when considering repeat-dose toxicity studies in the overall development plan: "How long should each particular toxicity study be?" and "When do the studies need to be conducted to support clinical trials?" Both of these questions have been addressed in the ICH guidances.

Duration of Chronic Toxicity Testing in Animals (Rodent and Non Rodent Toxicity Testing) (ICH Topic S4)

Prior to the ICH guidance regarding the length of a chronic toxicity study, there was general consensus across the three regions that a 6-month duration was sufficient for a chronic toxicity study in rodents and, hence, the ICH S4A guidance regarding the study length in rodents was

Table 3 Duration of Repeated-Dose Toxicity Studies to Support Phase I and II Trials in EU and Phase I, II, and III Trials in the US and Japan[a]

Duration of clinical trials	Minimum duration of repeated-dose toxicity studies	
	Rodents	Nonrodents
Single dose	2 wk[b]	2 wk
Up to 2 wk	2 wk	2 wk
Up to 1 mo	1 mo	1 mo
Up to 3 mo	3 mo	3 mo
Up to 6 mo	6 mo	6 mo[c]
> 6 mo	6 mo	Chronic (9 mo)[c]

[a]In Japan, if there are no phase II clinical trials of equivalent duration to the planned phase III trials, conduct of longer duration toxicity studies is recommended as given in Table 4.
[b]In the US, as an alternative to 2-week studies, single-dose toxicity studies with extended examinations can support single-dose human trials.
[c]Data from six months of administration in nonrodents should be available before the initiation of clinical trials longer than three months. Alternatively, if applicable, data from a 9-month nonrodent study should be available before the treatment duration exceeds that which is supported by the available toxicity studies.

readily harmonized at six months (2). However, the length of the chronic repeated-dose toxicity study in nonrodents was different in the three regions. In the US, the FDA generally held that the repeated-dose toxicity study in nonrodents be 12 months in duration to be considered chronic, whereas in Japan and Europe, a repeat-dose toxicity of 6 months was considered sufficient. Consequently, both 6- and 12-month studies were often being performed in nonrodents, an action that might be considered to be a redundant use of animals. Following a review of 6- and 12-month data by regulatory reviewers from all three regions, ICH proposed and reached agreement that a study duration of 9 months in nonrodents would be considered an acceptable duration for a chronic toxicity study. This harmonization is an excellent example of the ICH process meeting its goal to eliminate duplication of testing.

Timing of Conducting Preclinical Toxicity Studies (ICH Topic M3(R2))

The timing of when to conduct the repeat-dose toxicity studies to support clinical trials is of utmost importance. The timeline for initiation of each phase of clinical trial (phase I, II, and III) is the critical path in the later-stage development of the compound. To support each of these stages of clinical trials, a combination of animal toxicity data and previous clinical trial data is used to progress to the subsequent clinical trial. ICH Guidance M3 is a multidisciplinary guidance that defines at a particular stage of clinical development the realm of preclinical safety studies generally required to proceed with a particular clinical trial (3).

The durations of the repeat-dose toxicity studies in laboratory animals need to be evaluated in light of the duration, therapeutic indication, and scale of the proposed clinical trials. In principle, the duration should be equal to, or exceed, the duration of the clinical trial up to the maximum recommended duration of the repeat-dose toxicity studies; the studies should be conducted in two species, including a nonrodent species. The proposed durations of repeated-dose toxicity studies are presented in Table 3 and Table 4.

There are a several points in these tables regarding the duration of repeated-dose toxicity testing that bear mentioning. As discussed in the section on "Single-Dose Toxicity Guidance", the US will allow single-dose toxicity studies to support single-dose phase I trials provided that these single-dose studies include clinical pathology determinations and histopathological assessment at early and later time points. However, this is not universally accepted. For clinical trials of longer duration, the guidance also provides for the possible conduct of a 6-month repeat-dose toxicity study in nonrodents (Table 3), a study that, as discussed previously is not required under the ICH guidance regarding length of chronic preclinical toxicity studies. This type of 6-month repeat-dose study would only be required if the data were needed to support a clinical trial of longer than 3 months, and if 9-month repeat-dose data in a nonrodent species were not available. The likeliest scenario if time were absolutely crucial to the initiation of a clinical 6-month trial

Table 4 Duration of Repeated-Dose Toxicity Studies to Support Phase III Trials in the EU and Marketing in All Regions[a]

Duration of clinical trials	Minimum duration of repeated-dose toxicity studies	
	Rodents	Nonrodents
Up to 2 wk	1 mo	1 mo
Up to 1 mo	3 mo	3 mo
Up to 3 mo	6 mo	3 mo
> 3 mo	6 mo	9 mo

[a]This table reflects the marketing recommendations in the three regions except that a chronic nonrodent study is recommended for clinical use > 1 month.

would be to conduct a preclinical 9-month study with an interim necropsy at 6 months. An important regional difference is illustrated in Table 4, where the length of repeated-dose toxicity studies to support phase III clinical trials in Europe is longer than in Japan and the US.

The enrollment of men and women in clinical trials and their reproductive capability, or impairment of such function, are of considerable importance and are addressed in this ICH Guidance. The ICH M3(R2) guidance draws special attention to the enrollment of women of childbearing potential and maintains some regional differences in the timing of reproductive toxicity studies to support clinical trials involving this population. The assessment of embryo–fetal development should be completed prior to the enrollment of women of childbearing potential in both Europe and Japan. Japan goes further by suggesting that female fertility studies as well be conducted prior to enrollment of women. In Europe, female fertility studies should be completed prior to phase III trials.

In the US, the inclusion of women of childbearing potential has been an issue for some time. Historically, women were excluded from early clinical trials in the US because of concern over birth defects in children of treated mothers. The 1977 FDA guideline *General Considerations for the Clinical Evaluation of Drugs* (www.fda.gov/cder/guidance/old034fn.pdf) states that "In most cases, women of childbearing ... should be excluded from phase I." However, more recently, the FDA has initiated regulatory reforms to address the perceived barrier to the enrollment of women in clinical trials in 1993 by emphasizing the critical importance of including women in all phases of clinical trials (http://www.fda.gov/cder/guidance/women.pdf), and in 1998 by amending its regulations to require effectiveness and safety data across demographic subgroups including women (4), and imposing the possibility of a penalty of a clinical hold on studies under an investigational new drug (IND) if women with reproductive potential are excluded from participation in a study only because of the risk or potential risk of reproductive or developmental toxicity from use of the drug (5). Under the ICH M3 guidance, women of childbearing potential in the US may be included in early, carefully monitored trials without reproduction toxicity studies in animals. Nonetheless, because of US guidances stressing inclusion of women and the requirement for teratogenicity studies in animals prior to phase I in Japan and Europe, it is becoming common for pharmaceutical companies to include reproductive studies, notably embryo–fetal development studies, at an early stage of the drug development process in the US. Under ICH M3 guidance, the recommendation in the US is that the assessment of embryo–fetal development and female fertility should be completed prior to phase III.

The ICH guidance is more straightforward with regard to the timing of reproduction toxicity studies and the enrollment of men in clinical studies. The consensus across the three regions is that men may be included in phase I and II trials, since the male reproductive organs are part of the gross anatomic and histopathological tissues for examination in repeat-dose toxicity studies. The male fertility study should be completed prior to initiation of phase III trials enrolling men. Likewise, with regard to the timing of the pre- and postnatal development study in animals, there is consensus across the three regions that the study should be submitted for marketing approval, and hence, can be conducted late in the drug development program and earlier if there is a cause for concern.

The ICH M3 guidance provides recommendations for the timing for conducting local tolerance studies, genotoxicity studies, and carcinogenicity studies. Local tolerance should be

evaluated using the relevant clinical route. Since repeat-dose toxicity studies generally involve the clinical route of administration, these studies can evaluate local tolerance as well by including, for example, histopathological evaluation of administration site. The ICH guidance suggests that the genotoxicity package of studies be at least partially completed by the time of first human exposure, specifically, in vitro assays for mutagenicity and chromosomal damage. The entire battery of testing recommended in ICH guidance S2(R1) should be completed prior to phase II clinical studies. Regarding carcinogenicity testing, these studies are generally not required to support clinical trials; these studies, if required, may be conducted prior to marketing approval or as a postmarketing commitment.

In terms of clinical trials in pediatric populations, the ICH M3(R2) guidance states that human adult clinical trial data will be most relevant to the conduct of pediatric trials. Juvenile animal repeat-dose toxicity studies should be considered when previous animal and human data might be considered insufficient. Lastly, the guidance calls for all reproduction toxicity testing, all genotoxicity testing, and the appropriate repeated-dose toxicity studies be completed prior to initiation of pediatric clinical trials.

The ICH M3(R2) guidance also mentions the timing of safety pharmacology and toxicokinetics/pharmacokinetic studies; these specific ICH guidances are addressed on Page 249 (see "Additional Safety Topics and Guidelines"). Safety pharmacology studies should be evaluated prior to any human exposures. These evaluations may be conducted as stand-alone studies and/or in combination with a standard toxicity study. PK data in animals should be available by the time the first clinical trial in humans is conducted. In order to compare the absorption, distribution, metabolism, excretion (ADME) characteristics of the drug in humans, animal studies should be completed by the time that phase I clinical trials are completed.

Additional Guidance Information and Protocol Elements in Repeated-Dose Toxicity Studies

All three regions (USA, Europe, and Japan) provide additional guidance with respect to protocol elements to be included in repeated-dose toxicity studies (6–8). In the US, recommendations are provided in the Redbook, a Center for Food Safety and Applied Nutrition set of guidances for the safety assessment of food additives. Although not directly written to address drugs and biologics, the guidances are often referred to by default. The regional protocol elements are provided in Table 5 and Table 6, with some further discussion and recommendations for the reader. The actual listing of clinical pathology parameters and of the tissues to be collected for histopathological examination are not provided in these tables but can be found in the guidances as well as in the literature (9).

The elements of repeat-dose subchronic toxicity testing are listed in Table 5. The recommended species for these tests are generally rats and dogs. Repeat-dose studies in mice are generally conducted with the primary objective (notably in the 13-week study) of establishing dose levels for a carcinogenicity bioassay and not with the goal of defining no adverse effect levels (NOAEL) or target organs for toxicity. An important consideration for subchronic repeat-dose toxicity studies in rodents is the emphasis in Europe on including an immunotoxicity component in at least one 28-day repeated-dose study in rodents; bone marrow cellularity, lymphocyte subsets, and natural killer cell activity or the primary antibody response to a T cell-dependent antigen are recommended (7). The FDA is also moving rapidly to address the importance of immunotoxicological evaluation of new drugs (10). Recovery groups of animals are usually included in subchronic toxicity studies, especially those studies of four weeks in duration; the recovery period is usually two to four weeks. The guidances provide general recommendations for clinical pathology evaluations in rodent and nonrodent studies. In a 13-week study, clinical pathology determinations might also be recommended at a 1-month interim if the treatment levels are different from those used in a 4-week study.

The protocol elements for chronic toxicity testing are listed in Table 6. Note that the protocol elements of the chronic toxicity studies are similar to those listed for the subchronic toxicity studies. The FDA recommends an increase in rodent group size to 20/sex/group. However, a group size of 15/sex/group should be sufficient to address target organ toxicity. With regard to a recovery period, recovery groups should not be included in chronic studies since reversibility has usually been demonstrated at this point in the toxicity evaluation; including recovery

Table 5 Protocol Elements of Subchronic Toxicity Studies (2–13 Weeks Duration)

Protocol elements	US	Europe	Japan
Species	1 rodent and 1 nonrodent species; rats and dogs preferred	1 rodent and 1 nonrodent; 1 species may be acceptable if it is the only relevant species	1 rodent and 1 nonrodent (rabbit cannot be considered as nonrodent species)
Age at which study starts	Rats, 6 wk Dogs, 4–6 mo	Not defined	Not defined
Number and size of groups	At least 3 treated and 1 control; for rodents, 10/sex/group, individually housed; for nonrodents, at least 4/sex/group (2/sex/group for a pilot study)	3 treated, 1 control; group sizes not specified, depends on design including interim and recovery sacrifices	At least 3 treated plus vehicle control, untreated control group may be necessary; for rodents, 10/sex/group, individually housed; for nonrodents, 3/sex/group
Recovery period and TK evaluation	Recovery groups recommended, systemic exposure of oral dose should be ensured	Recovery groups recommended, TK are essential to include	Recovery groups and TK are recommended
In-life observations	Observations twice daily[a], body weight and food consumption weekly, water consumption only if administered in drinking water; ophthalmology at baseline and termination; ECG not addressed	Observations daily, body weight and food consumption weekly, ophthalmology in rodents and nonrodents required but frequency not defined; ECG not addressed	Observations daily, body weight and food consumption weekly, ophthalmology in all animals at least once during study, ECG in nonrodents as appropriate
Clinical pathology	Hematology, clinical chemistry, urinalysis in 10 rodents/sex/group and in all nonrodents at predose and termination	Hematology, clinical chemistry, urinalysis required; important to include immunotoxicity evaluation in at least one repeated-dose rodent study	Hematology and clinical chemistry in all animals at necropsy, urinalysis once during study; rather than allowing death, euthanize moribund animals to collect clinical pathology data
Histopathology	For rats, full histopathologic evaluation on all high dose and control and moribund/dead animals, target tissues in mid- and low-dose groups and recovery groups; full histopathology on all nonrodent species of all dose groups	For rats, full histopathologic evaluation on all high dose and control and moribund/dead animals, target tissues in mid- and low-dose groups and recovery groups; full histopathology on all nonrodent species of all dose groups	For rats, full histopathologic evaluation on all high dose and control animals; full histopathology on all nonrodent species of all dose groups

[a]This likely refers to mortality checks and not detailed and recorded clinical signs.

Table 6 Protocol Elements of Chronic Toxicity Studies (Six Months in Rodents and Nine Months in Nonrodents)

Protocol elements	US	Europe	Japan
Species	Same as subchronic	Same as subchronic	Same as subchronic
Age at which study starts	Same as subchronic	Same as subchronic	Same as subchronic
Number and size of groups	Same as subchronic except for rodents, 20/sex/group, increase size by 10/sex/group for each interim necropsy	Same as subchronic	Same as subchronic
Recovery period and TK	Recovery groups not routinely used, TK useful to include	Same as subchronic	Same as subchronic
In-life observations	Same as subchronic; body weight and food consumption measured weekly to 13 weeks and monthly thereafter	Same as subchronic	Same as subchronic; body weight and food consumption measured weekly to 3 months and every 4 weeks thereafter
Clinical pathology	Hematology, clinical chemistry, urinalysis in 10 rodents/sex/group at predose, days 30 and 60, and termination and in all nonrodents at predose and at 3-month intervals thereafter	Hematology, clinical chemistry, urinalysis required	Same as subchronic
Histopathology	Same as subchronic	Same as subchronic	Full histopathologic evaluation on all animals, rodents and nonrodents

groups prolongs the study duration at a time when these chronic toxicity studies may lie on the critical path for the development of the drug. Full toxicokinetic (TK) profiles do not generally need to be determined for a chronic study unless the study uses dose levels for which no data exist. To substantiate systemic exposures and provide comparisons to subchronic toxicity studies, a limited blood sample collection for drug concentration analysis of two to three time points at 3-month intervals is recommended. Clinical pathology evaluation in rodents should be conducted at the same 3-month intervals as in nonrodents. Rodents should be randomly allocated into subgroups of 10/sex/group (in other words, not all of the rodents in each group need to be evaluated) from which clinical pathology parameters are collected. Ophthalmologic evaluation in rodents and nonrodents and ECG collection in nonrodents should be collected at 3-month intervals; in rodents, again it is feasible to use subgroups of 10/sex/group rather than the full 15/sex/group.

Reproductive Toxicity Guidelines

One of the first topics addressed by ICH was the area of reproductive toxicity testing. The complexity of the studies and the questions being addressed resulted in numerous and diverse protocols of testing strategies employed across the different countries. The ICH has provided considerable guidance and harmonization of reproductive toxicity testing in the current ICH Topic S5(R2) (11,12).

In addition to drug products, reproductive toxicity is a component of testing in the chemical realm as well, for example, pesticides, environmental chemicals, and workplace chemicals. Interestingly, the testing for these chemicals often encompasses multigeneration reproductive

Table 7 Reproductive Toxicity Testing—3-Study Design

Number designation	Study title	Recommended species
4.1.1	Fertility and Early Embryonic Development	Rats
4.1.2	Pre- and Postnatal Development	Rats and rabbits
4.1.3	Embryo–Fetal Development	Rats

toxicity testing, the rationale being that chemical exposures in the workplace and in the environment may occur unexpectedly over ill-defined periods of times. The question may be raised as to why multigenerational studies are not part of the reproductive toxicity testing of drug products. ICH comments on this and points out that reproductive toxicity testing with medicinal products is much more defined. Hence, the reproductive toxicity testing at specific stages of reproduction is more reflective of humans taking drug products at specified periods, and therefore, is a better assessment of actual human risk.

As an aside, the FDA has published a draft guidance regarding the integration and interpretation of study results obtained in the reproductive toxicity studies and this is an additional useful resource for the reader (13).

Detection of Toxicity to Reproduction for Medicinal Products (ICH Topic S5(R2))

ICH S5(R2) provides a three-study combination that should be sufficient for testing of most drug products (Table 7). Rats are the primary rodent species and rabbits are the primary nonrodent second species for embryo–fetal effects. The rationale for both is similar and is based on large litter size, predictable gestation period, ease of handling and housing, and, most importantly, the large historical background information available.

The study protocol elements for each of these studies are fully described in the ICH Guidance Document. A comprehensive summary of the guidance is beyond the scope of this chapter. For the purposes of this chapter only the major harmonization highlights will be presented.

ICH study 4.1.1 looks for effects in males and females from before mating to implantation. Importantly, the duration of treatment for males has been shortened significantly from 9 to 10 weeks to 4 weeks (and subsequently shortened to 2 weeks in ICH S5(R2). A 1:1 mating ratio is suggested in this guidance, with the sacrifice of males delayed until the outcome of mating is known to allow for remating with untreated females if necessary. Females are terminated between gestation days 13 and 15, in contrast to gestation days 20 and 21; this time is considered adequate to assess fertility and reproductive indices.

ICH study 4.1.2 is an evaluation of the pre- and postnatal effects of the drug. Dosing is initiated in the first generation dams (F0) at the implantation stage and continues through to weaning of the first generation (F1), while observations are continued through to sexual maturity of the F1 generation to allow for the appearance of any delayed effects. ICH S5(R2) recommends that one male and one female from the F1 generation be used for both behavioral/functional testing and testing of reproductive function since such dual use will allow for correlations among the assessments. However, ICH S5(R2) does accept that some laboratories use separate sets of animals and this is accepted in the guidance as being valid as well. One important note in this ICH guidance is the use of culling of the F1 population. This is recognized as a controversial issue among the three regulatory regions, and the issue is still under discussion. Finally, since the study design does not cover exposures of the F1 generation from weaning to maturation, if the intended clinical population is of infants and/or juveniles, the potential toxicity of drug products on these age groups should be considered with separate studies unique to the age group in question.

ICH study 4.1.3, an evaluation of embryo–fetal effects of the drug, is the only guidance in the reproductive toxicity studies that requires two species unless there is strong rationale to conduct such an assessment in a single species. If this embryo–fetal study is conducted in a single study or two-study combination of fertility and/or pre- and postnatal development in rodents, an embryo–fetal study must still be performed in the second nonrodent species. The

litter size in ICH S5A is standardized at 16 to 20 litters. The guidance accepts that examination of offspring in the low- and mid-dose groups for visceral and/or skeletal abnormalities may not be necessary if the high dose and control groups show no significant treatment-related differences.

Lastly, the selection of dose levels is imperative as in any testing of toxicity. As such, RF studies are often conducted prior to the definitive studies. While the ICH guidance does not state a requirement for an initial dose RF study, such RF studies are usually included in the conduct of the embryo–fetal development studies (ICH study 4.1.3); RF studies are not needed for ICH studies 4.1.1 and 4.1.2 in rats, since repeat-dose toxicity data usually exist for this species at the time these studies are to conducted. The RF embryo–fetal development studies may follow one of three designs: (*i*) evaluation of maternal toxicity only with animals terminated after final exposure, (*ii*) evaluation of maternal and fetal toxicities where the fetuses are delivered and examined externally (no detailed evaluation of skeletal and visceral abnormalities), and (*iii*) study conducted as if it were the definitive study with full fetal skeletal and visceral examinations. In rats, it is usually sufficient to conduct the second design as the only RF study. In rabbits, it may be preferable to conduct the first design initially to avoid excessive maternal toxicity and effects on the fetus that may result from an overly excessive maternal toxicity. Alternatively, one may conduct a study along the lines of the second design with 4 to 6 treatment dose levels as opposed to the usual three treatment groups. In any instance, even though these studies are RF, they should still be conducted under adherence to GLP guidelines. The norm is also to include collection of blood samples from satellite animals in these RF studies for TK purposes. If TK samples are not collected in the RF studies, they should be collected in the definitive studies to provide an assessment of systemic exposures.

Maintenance of the ICH Guideline on Toxicity to Male Fertility (formerly ICH Topic S5B)

This former ICH guidance has been incorporated into S5(R2). The duration of exposure of males in study 4.1.1 (the assessment of drug effect on fertility) has been shortened from four to two weeks, provided that there is no indication of male reproductive toxicity in repeat-dose studies of at least two weeks duration.

Genotoxicity Guidelines

The ICH guidance provides a harmonized battery of tests to be used to investigate the potential genotoxicity of a drug and provides some specific protocol elements and guidance regarding interpretation of test results.

Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use (ICH Topic S2(R1))

The core battery of testing recommended by ICH and specific protocol elements are provided in Table 8.

As illustrated in the table, ICH S2(R1) outlines the standard base set of strains to be used in the bacterial mutation assay; an important consideration since each of the three global regions had particular preferences to be included in this assay. ICH S2(R1) recommendations for the in vitro assays include the range of concentrations to be evaluated, for the most part the high-dose level, and the elements required in the selection of drug concentrations in the in vitro testing of drugs that are poorly soluble. An adequate rationale for the selection of the high dose is crucial as this is an area that is sure to be scrutinized by regulatory authorities when reviewing the test data.

ICH S2(R1) also provides a discussion as to the interchangeability of different test systems to evaluate in vivo genotoxicity of a test material. The bone marrow micronucleus test in rats or mice, the analysis of chromosomal aberrations in mouse or rat bone marrow cells, and the peripheral blood micronucleus test in mice were all considered adequate to assess the in vivo clastogenicity of the drug. The selection of the sex (either one or both) should be dependent on the PK and toxicity of the drug. Male rodents are sufficient provided there are no real gender-specific effects.

Perhaps one of the most important points provided in ICH S2(R1) pertains to the interpretation of the test results. Since no assay will provide 100% predictivity, it is especially

Table 8 Standard Battery of Tests for Genotoxicity Assessment Including Specific Test Elements

Core battery testing	ICH Topic S2(R1) guidance	Additional author comments
Bacterial mutagenicity	*Salmonella typhimurium* strains TA 98, 100, 1535, and TA 1537 or TA 98 or TA 97 a; in addition, *S. typhimurium* strain TA 102 or *Escherichia coli* strains WP2uvrA or WP2PKM101 Highest concentration should show significant cytotoxicity For soluble nontoxic compounds, limit concentration at 5 mg/mL for bacteria and higher of 5 mg/mL or 10 mM for mammalian cells For poorly soluble compounds, use lowest precipitating concentration at as the high dose, within the constraints of the limit concentration (above)	Nonaudited range-finding cytotoxicity assay to select dose levels causing decreased viability Two independent assays recommended. In the first, plate incorporation method is suggested. If a negative response is obtained, the liquid preincubation method with metabolic activation is suggested Definite assay should include at least 5 dose levels tested in triplicate Test substance considered positive if revertant colonies number more than twice the negative control and if increase is dose-dependent
In vitro mammalian mutagenicity	Mouse lymphoma TK test (MLA) recommended; appropriate assays also include HPRT test with CHO cells, V79 cells or L5178 cells, GOT-(XPRT) test with AS52 cells, and human lymphoblastoid TK6 test 3–4 hour treatment (MLA); if negative, include a 24 hr treatment in absence of metabolic activation (MLA) $\pm$ S9 fraction (MLA) Highest concentration produce at least 80% toxicity Include solvent control and positive test compound	Mouse lymphoma TK assay on L5178Y cell line using microtiter method recommended Nonaudited preliminary cytotoxicity assay to select concentrations producing 20–100% survival 2 independent mutagenicity assays At least 4 concentrations tested in triplicate
In vitro chromosome aberration	CHO-WBL cell line or human lymphocytes Highest concentration produce $>$50% reduction in cell number for cell lines, or $>$50% inhibition of mitotic activity in lymphocyte cultures $\pm$ S9 fraction 3–6 hour exposure Sampling time of approximately 1.5 normal cell cycles for beginning of treatment	Nonaudited preliminary cytotoxicity assay to select concentration producing $<$ 50% survival 2 independent clastogenicity assays At least 3 concentrations tested in triplicate Two harvest times (18 and 42 hr) 200 metaphases scored/concentration
In vivo mutagenicity	Mice or rats acceptable Chromosome aberration in bone marrow cells or measurement of micronuclei in bone marrow cells, both are acceptable Males sufficient, unless there are clear qualitative differences in metabolism Suggest TK to evidence systemic exposure	Mice preferred by Japan 5 animals/dose 3–4 dose levels; top dose $=$ MTD Single dose—oral, IP, or IV Bone marrow sampling times 24 (all doses) and 48 hr (top dose only) after administration

important to be able to differentiate between a true response and a false result (positive or negative). Criteria to be considered in the interpretation of the response(s) are provided in this ICH guidance.

ICH S2(R1) also provides excellent guidance as to the standard battery of tests to support clinical trials and the marketing application of a drug (Table 8). This standard battery should be followed without substitution of alternative tests unless there is valid scientific rationale for the substitution. There are three important points that ICH S2(R1) addresses relating to the core battery of testing. First, the ICH panel of experts involved in the review of the different assays found a high level of congruence between the in vitro chromosome aberration testing and the in vitro mammalian cell mutagenicity testing of different drugs. For that reason, the guidance provides a recommendation that it is not necessary to conduct both testing schemes if the compound is negative in the other assays. Hence, if the compound is negative in the bacterial mutagenicity testing, in vitro chromosome aberration testing, and in vivo mammalian genotoxicity testing, then in vitro mammalian mutagenicity testing is not required. Second, in accordance with the standard core battery of testing, the experts also recognized that there may be instances where bacterial mutagenicity may not be appropriate or may not provide sufficient information. In this instance, the in vitro mammalian mutagenicity testing should be conducted as part of the core battery of genotoxicity testing. Third, where conflicting test results are obtained in the core battery testing, the ICH guidance provides some recommendations for additional genotoxicity tests that can be added to the standard battery.

Lastly, ICH S2(R1) provides specific procedural elements that can be followed in the conduct of the tests, for example, the use of RF tests as sufficient replications of complete tests and the timing/durations of the exposure to the test drug. As this chapter is not intended to be a description of all methodology, the reader is referred to the guidance for protocol elements beyond those presented.

Carcinogenicity Study Guidelines

The historical norm for carcinogenicity studies has been to conduct such studies in both rats and mice for two years in duration. As more of these studies have been conducted, both in the support of pharmaceuticals and in the evaluation of potential environmental carcinogens, the investigation has focused on the relevance of animals studies to human cancer risk assessment, especially given the long duration and large numbers of animals involved. The ICH has commented on the provided guidance in the area of carcinogenicity testing in three separate documents (16–18). These documents are summarized below.

Guideline on the Need for Carcinogenicity Studies of Pharmaceuticals (ICH Topic S1A)

Carcinogenicity studies are generally required only in cases where the drug is to be administered over a chronic period of time to human subjects. In Europe and Japan, carcinogenicity studies were typically conducted when intended drug therapy was six months or longer. In the US, drug administration for three months or longer generally required carcinogenicity studies. This ICH guidance defines the conditions under which carcinogenicity studies should be submitted to support the approval of a drug.

Generally, drugs that are administered for three months continue to be administered for six months and beyond. The guidance has been harmonized to state that carcinogenicity studies should be performed for those drugs that are administered for at least six months. In those instances where the exposure may not be continuous for six months, but does occur intermittently over the lifetime of an individual, carcinogenicity testing should also be conducted. Carcinogenicity studies should also be considered for drugs given for less than six months, when there exists risk factors suggestive of potential cancer risk in humans.

There are instances where, despite chronic administration, carcinogenicity studies are not required. Drugs that are known to be genotoxic are hence presumed to be transspecies carcinogens. In these cases, no additional benefit would be gained by conducting long-term carcinogenicity studies, and these compounds are not generally tested in traditional 2-year bioassays. In addition, when the life expectancy of the target population is short, long-term carcinogenicity studies may not be needed. Anticancer drugs fall into this category. In instances

where the drug is not systemically bioavailable (i.e., topical drug administration), there may not be a need to conduct the studies unless there is a cause for photocarcinogenic potential. Lastly, endogenous peptides or proteins do not generally require carcinogenicity testing.

With regard to timing, carcinogenicity studies are usually not required prior to initiation of any clinical trials. Rather, the studies are usually completed in time to support the application for marketing approval. For certain disease indications, the speed of approval is such that the carcinogenicity studies might be conducted and/or submitted as a postmarketing approval commitment.

Testing for Carcinogenicity of Pharmaceuticals (ICH Topic S1B)

Of particular concern to the ICH is the large numbers of rats and mice used for carcinogenicity studies. This particular guidance poses the question as to whether one carcinogenicity bioassay would provide sufficient data to evaluate the cancer risk in humans. In conjunction with a single long-term bioassay, could alternative shorter-term studies be conducted that may better evaluate the carcinogenicity of a drug, the mechanisms involved, and determine whether the drug poses a cancer risk in humans, all with the added benefit of using fewer numbers of animals and a shorter duration of testing? The answer to this question is Yes. The data from a chronic rodent bioassay in one species combined with other appropriate studies will enhance the assessment of carcinogenic risk in man. ICH S1B accordingly provides guidance as to the selection of the rodent species (the rat in the absence of data favoring one species over another) for use in the standard carcinogenicity bioassay, additional short to medium-length studies that will support the determination of cancer risk, and mechanistic studies that allow interpretation of a tumorigenic response and relevance of the response to man.

ICH S1B does provide a listing of potential carcinogenicity models that could be used as short to medium studies investigating potential carcinogenicity. These include initiation–promotion models of specific organ systems, transgenic mouse models including *p53*(+/−), Tg.AC, and TgHras models, and the neonatal rodent tumorigenicity model. A number of these studies are currently being evaluated for validity using pharmaceutical compounds of known carcinogenic potential, or lack thereof.

At this point, it is not clear as to which alternative shorter-term study(s) is favored by regulatory authorities. The available Summary Basis of Approval (SBA) Documents discussed as case studies in the second half of this chapter generally followed the traditional long-term bioassays in rats and mice. However, these documents were part of development plans that existed prior to Step 5 approval of this ICH guidance by FDA in 1998. Nonetheless, the trepidation on the part of the toxicologist to conduct a shorter-term study in place of the chronic bioassay in the second rodent species is understandable if the results of such short-term studies consistently led regulators to ask for more insight from the sponsor by conducting the traditional rodent bioassay in the second species. Such a regulatory response would certainly be detrimental to the career of the toxicologist at their company as the timelines to drug approval could be drastically altered. The ICH S1B guidance does include the statement that "a long-term carcinogenicity study in a second rodent species is still considered acceptable." Hence, some toxicologists may continue to recommend rodent chronic bioassays in two species to avoid the potential for equivocal/irrelevant data in a short- to medium-term study and subsequent delays in the critical path timelines. The pharmaceutical industry standards and practices will only become clearer as more SBA Documents are released and the trends in the pharmaceutical industry and the regulatory environment become more evident.

Dose Selection for Carcinogenicity Studies of Pharmaceuticals (ICH Topic S1C(R2))

The appropriate dose levels for carcinogenicity assays are crucial. Too high, and the dose level will need to be lowered midstudy or, worse still, the mortality is so great that entire treatment groups are terminated early (see Case Study #1: Celebrex, Page 253). Too low, and one runs the risk of the criticism that the doses did not adequately address the carcinogenic potential of the drug. ICH S1C(R2) addresses these concerns by providing specific guidance on the proper selection of the doses for the chronic carcinogenicity assays, including the subchronic toxicity studies used to assist in the selection of dose levels.

Dosage selection, particularly the high dose, for cancer bioassays has been based on the MTD determined from subchronic toxicity studies, usually of three months in duration. In the US, high-dose selection has been traditionally based solely on the MTD. In Europe and Japan, there has been the recognition that the MTD may far exceed the expected human exposure and not be relevant to assessment of human risk. Consequently, in addition to the MTD, an acceptable alternative to the MTD has been a high multiple of the maximum recommended human dose (> 100-fold on a mg/kg basis). In keeping with one of the overall objectives to reach harmonization of study requirements, ICH S1C(R2) represents the culmination of mutually acceptable, rational, and scientifically based criteria for selection of the high dose for carcinogenicity studies.

General guidances for the dose-range finding studies to select the high dose for the carcinogenicity studies are provided in ICH S1C(R2). Importantly, the ICH Expert Working Group agreed that a consensus on the use of toxicity endpoints other than the MTD would be difficult, and accepted the continued use of the MTD, determined in both males and females in a subchronic toxicity study, as an endpoint for high-dose selection. The definition of the MTD in each of the three regions is provided in the "Notes" section of the guidance.

The role of the use of PK in the selection of the bioassay high-dose level is a primary feature of the ICH S1C(R2) guidance. Systemic exposure may be especially important as an appropriate endpoint for nongenotoxic carcinogens that might be expected to have a threshold effect. In a retrospective analysis of the data from carcinogenicity studies for which there were sufficient rodent and human PK data, a review of systemic exposures, expressed in terms of the area under the concentration–time curve (AUC), was conducted. These systemic ratios were analyzed with respect to exposure and/or dose ratios for known or probable human carcinogenic pharmaceuticals (IARC class I and 2A pharmaceuticals with positive rat findings). ICH S1C(R2) concludes from these evaluations that a relative systemic exposure ratio of at least 25 (man–rodent ratio) is an acceptable PK endpoint to use for high-dose selection. Further, in order to establish the actual dose on a milligram basis, comparisons between man and rodents of systemic exposures as a function of dose found that systemic exposures were better estimated by mg/m^2 rather than mg/kg. Accordingly, the guidance concludes that the high dose in the rodent carcinogenicity study should be at least 25-fold higher than the anticipated human clinical dose on a mg/m^2 basis.

Since systemic exposure is of critical importance in determining the potential high-dose level, ICH S1C(R2) provides some specific guidances as to the conduct of specific studies (which may be the 3-month toxicity study with suitable evaluation of TK) that will be used to determine the systemic exposure ratio (note that this also assumes that there is adequate human exposure data at anticipated dose regimen), including the use of doses across the anticipated carcinogenicity dose range and for durations of time sufficient to allow for any time- and repeated-dose–dependent changes in PK parameters.

Once the selection of the high-dose level for the carcinogenicity study is complete, the selection of the mid- and low-dose levels is somewhat more straightforward. Selection of the mid and low doses should take into account saturation of metabolism leading to a plateau of blood concentrations as well as potential saturation of absorption and elimination. Dose selection should also take into account alterations in rodent physiological parameters (e.g., the drug is anticipated to exert hormonal effects), as well as mechanistic information and the potential for threshold effects and human exposure and therapeutic dose.

An addendum to the original ICH S1C regarding the addition of a limit dose was added to the original guidance as ICH S1C(R1). This addendum states that the limit dose for carcinogenicity testing should be 1500 mg/kg/day. This limit dose may be exceeded if the systemic exposure in animals resulting from such a dose does not exceed the human systemic exposure by at least one order of magnitude.

Additional Guidance Information and Protocol Elements in Carcinogenicity Studies

All three regions (USA, Europe, Japan) provide guidance with respect to protocol elements to be included in carcinogenicity studies (6,8,19). These recommendations are provided in Table 9.

Each of the regions requires a group size of at least 50/sex/group. If the study involves daily oral intubation and the staff is inexperienced in conducting a study of this length, it may be useful to increase the group size (e.g., 65/sex/group) to compensate for technical error.

Table 9 Protocol Elements of Long-Term Carcinogenicity Studies

Protocol Elements	US	Europe	Japan
Species	2 recommended, rats and mice	Rats recommended in absence of evidence for a more appropriate species	2 recommended, rats, mice, or hamsters
Duration	104 wk	24 mo (rats) and 18 mo minimum for mice and hamsters	24 mo (rats) and 18–24 mo for mice and hamsters; survival should not be less than 50% in low and control groups at termination
Age at which study starts	6 wk	As soon as possible after weaning and acclimation	6 wk
Dose Frequency	Daily	Daily	Daily in feed, but oral gavage 5 day/wk is acceptable
Number and size of groups	3 treated and 1 vehicle control; minimum of 50/sex/group	3 treated and 1 vehicle control; minimum of 50/sex/group	3 treated, 1 vehicle control, 1 nontreated control; minimum of 50/sex/group
TK	No	No	No
In-life observations	Observations twice daily, body weight and food consumption weekly to 13 weeks and monthly thereafter	Observations daily, body weight, food consumption required but frequency not defined	Observations daily, body weight and food consumption weekly to 3 months and every 4 weeks thereafter
Clinical pathology	Hematology, clinical chemistry, urinalysis at predose and months 3, 6, 12, 18, and termination; number of animals not defined	Hematology, clinical chemistry, urinalysis requested but frequency and animal numbers not defined	Only hematology evaluation is requested, animal number and frequency not defined
Histopathology	Histopathologic evaluation on all high dose and control and moribund/dead animals, target tissues in mid- and low-dose groups	Histopathologic evaluation on all high dose and control and moribund/dead animals, target tissues in mid- and low-dose groups	Histopathologic evaluation on all high dose and control and moribund/dead animals, target tissues in mid- and low-dose groups

Some laboratories use 65/sex/group as a standard to ensure sufficient group size at the study termination, especially in mice, for long-term studies (two years in duration).

A carcinogenicity study is not a chronic toxicity study, but rather a study only to assess the potential carcinogenicity of the compound. Thus, some elements that are part of repeat-dose toxicity studies are absent here (e.g., ophthalmology and ECG assessment). There is also some debate over the utility of clinical pathology determinations in the carcinogenicity bioassay. One preference is to include determinations as outlined in the FDA guidance in randomly allocated subgroups of 10 rats/sex/group. For mice, it is necessary to further separate animals into those being bled for clinical chemistry determinations and those being bled for hematology evaluation as the blood sample volume at a nonterminal time point is usually not of a sufficient volume to evaluate both clinical chemistry and hematology; this may be why the Japanese guidance asks only for a hematology evaluation. Given the inherent sensitivity of mice to carbon dioxide anesthesia and potential for death, combined with the desire to have sufficient group size at study termination, some investigators prefer not to collect blood samples for clinical pathology in a mouse carcinogenicity bioassay.

Finally, systemic blood exposures and PK are essential in the selection (and justification to regulatory authorities) of the dosage levels in a carcinogenicity bioassay. Hence, these data should be well-defined prior to the conduct of the carcinogenicity study and there is no added value to include TK in these bioassays.

Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals (ICH Topic S6)

ICH Topic S6 expressly addresses the preclinical safety evaluation of biotechnology drugs (20), but the recommendations are consistent with those provided for single-dose toxicity (ICH S4A), repeat-dose toxicity (ICH S4B), and timing and duration in support of clinical trials (M3). Development of the biologic product should follow these guidances. However, there are some potential differences in study design that are worthy of further discussion.

One likely difference lies in the selection of the animal species to conduct these single- and repeat-dose toxicity studies. Because of the nature of the species specificity of many biotechnology products, the selection of the relevant species may sometimes rule out commonly used laboratory animals (e.g., rodents and dogs), in favor of a species in which the biotechnology product has pharmacological activity, often primates. While the use of two animal species as discussed in the repeated-dose toxicity studies is similarly recommended for biotechnology products, the identification of a single relevant species and toxicity studies in this single species may be justified. In fact, ICH S6 goes so far as to discourage the use of a nonrelevant species in toxicity testing of the biotechnology product. In instances where no relevant species exists to test the human product, a homologous animal biotechnology product should be considered. The testing of the human product in a transgenic animal model in which the animal expresses the human target protein of interest is also an alternative, although a potential lack of the full spectrum of interacting human proteins in transgenic animals might make this model less than ideal.

Another potential difference is in the subchronic to chronic duration of the repeat-dose toxicity studies of biotechnology drugs. Many of the biotechnology products, by nature of their intended use, will be immunogenic when administered to animals on a repeated basis. Hence, the detection and characterization of an antibody response to the biologic are crucial. An antibody response may change the PK of the drug and may alter the pharmacological and/or toxicological profile of the biotechnology drug. If the antibody response is truly an immune response that neutralizes any pharmacological and/or toxicological activity of the biologic, then this may serve as a criterion for the early termination of a repeat-dose toxicity study and a justification for not conducting repeat-dose toxicity studies of longer length.

While the immunogenic response of the animal to the biotechnology-derived product is important to characterize, ICH S6 does state that the routine–tiered-testing approach for immunotoxicology evaluation is not recommended for biotechnology products; the immunotoxicology tier approach has not been addressed by ICH but guidance can be found in the FDA *Guidance for Industry* entitled *Immunotoxicology Evaluation of Investigational New Drugs* (10). For information on that tier-testing approach, see that document.

In contrast to classic small-molecule drugs, genotoxicity testing is not routinely required for biotechnology drugs. Nonetheless, as illustrated in the case studies presented later in this chapter, it is not uncommon to see the ICH recommended battery of genotoxicity studies conducted with biotechnology products. Carcinogenicity studies are also not generally performed on these biotechnology products, although the guidance does indicate circumstances where animal bioassays may be relevant. Similarly, reproductive toxicity testing is dependent on the biotechnology product and the intended clinical population. Again, the submission of such studies, or lack thereof, is illustrated in the case studies presented later in this chapter.

ADDITIONAL SAFETY TOPICS AND GUIDELINES

The basic preclinical toxicity testing of a drug was outlined in the previous section. The guidance on Immunotoxicity Assessment was not discussed in detail; the reader is referred to the S8 Guidance document. The S9 Guidance document detailing the nonclinical toxicity testing of cancer therapeutics is currently at the Step 2 consultation step (as of Nov 2008) and is not discussed in this chapter. Toxicity is one of the three major areas evaluated during preclinical development; the other two being pharmacology and PK (administration, adsorption, metabolism, and elimination, hence the acronym ADME). This section will briefly discuss the ICH guidelines to date

Table 10 Additional Preclinical Safety Topics

Topic designation	Title
S3	**Pharmacokinetics and Toxicokinetics**
S3A	Toxicokinetics: Guidance on the Assessment of Systemic Exposure in Toxicity Studies
S3B	Pharmacokinetics: Guidance for Repeated-Dose Tissue Distribution studies
S7	**Pharmacology Studies**
S7A	Safety Pharmacology Studies for Human Pharmaceuticals
S7B	Safety Pharmacology Studies for Assessing the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals

in the realms of pharmacology and PK. The preclinical safety topics in these areas are provided in Table 10.

Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies (ICH Topic S3A)

The demonstration of systemic exposures to the drug product in toxicity studies is extremely important, perhaps best illustrated by the fact that this topic was addressed early on in the ICH proceedings (21). The incorporation of blood sample collection in animals, either the actual study animals or separate satellite groups, provides an evaluation and correlation of systemic exposure with toxicity endpoints and can validate the relevant selection of the animal species in the toxicity study. TK data collected in animals can provide vital comparisons with the clinical data, allowing for assessment of potential risk and possible margin of safety of the drug product in humans. This ICH guidance provides strategies for incorporation of PK data collection in toxicity studies, termed toxicokinetics. Excellent real-life examples of how these data have been collected in the conduct of toxicity studies and how the data are used, especially to design subsequent toxicity studies, are provided in the Case Studies in this chapter.

ICH S3A provides some guidance for the collection of TK data as a part of the toxicity testing discussed above, that is, single- and repeat-dose toxicity studies, reproductive toxicity testing, genotoxicity testing, and carcinogenicity assessment. In single-dose toxicity studies, drug assays often are not developed. Consequently, TK is not a routine requirement in these studies. However, the ICH guidance does suggest that plasma samples be collected and stored for possible future analysis.

In the realm of repeat-dose toxicity testing, ICH S3A calls for profiling (collection of samples at more than four time points to allow for determination of area under the concentration–time curve, the AUC) or monitoring (defined as collection of samples at one to three time points to estimate peak systemic concentration and time to peak) to be incorporated appropriately into the repeat-dose toxicity studies. Minimally, full profile TK data should be collected at the start and toward the end of the treatment period of the first repeated-dose toxicity study; the study must be at least 14 days in duration. In practice, the standard toxicity package generally includes a 28-day toxicity study as a pivotal study and a full TK profile is routinely obtained in this study after the first and last dose of the drug. The ICH guidance states that further collection of TK data in toxicity studies of different duration is not necessarily required if the dose levels and drug formulation are unchanged and that collection of TK data past six months of exposure is not essential. In practical terms, dose levels often change as the length of the repeat-dose toxicity study is increased. During repeat-dose toxicity studies of three, six, and/or nine months, one should obtain full TK profiles after days 1 and 28 at any dose levels for which the data do not exist. If the TK profile data have been collected in a previous study, it is appropriate to monitor (1–3 samples) on days 1 and 28 as opposed to collecting a full AUC profile. In these 3-, 6-, and 9-month studies, monitoring is also recommended prior to dosing and at the estimated C_{max} at each 3-month time point. Examples of other approaches can be found in the Case studies in this chapter.

For in vivo genetic toxicity testing, it may be useful to include monitoring of systemic blood concentrations to establish that the animals were exposed to the drug.

Carcinogenicity bioassays, as discussed previously in this chapter, are based on 13-week dose–setting studies to determine MTD, and these should include determination of systemic

exposures for comparison to human exposures. In the definitive carcinogenicity bioassay, the ICH guidance suggests monitoring at several occasions before six months. One suggestion is to monitor at predose and at estimated C_{max} on day 1, and at the end of three and six months.

The collection of TK data in reproduction studies will be based on the extent of data collected to that point. For example, if exposures have been documented in repeat-dose rat toxicity studies at similar dose levels, it is not essential to include TK data collection in the fertility and the peri-/postnatal studies if conducted in rats. However, for the embryo–fetal development studies in rats and rabbits, it may be prudent to include TK data collection since there is the possibility that the PK profile may be different in pregnant animals. These TK data are usually collected in satellite pregnant animals to avoid any influence of the blood collection procedure on the data in the main study animals. Since systemic exposure data collected in the RF studies are used in the selection of doses, it is not a requirement that these data again are generated in the definitive study if the conditions and dosing regimen in the study are not different from those of the RF study.

Pharmacokinetics: Guidance for Repeated-Dose Tissue Distribution Studies (ICH Topic S3B)

This ICH guidance is a short list of circumstances under which repeat-dose tissue distribution studies should be considered in the preclinical development of a drug (22). Repeat-dose tissue distribution studies should be considered in cases where (*i*) the tissue half-life is much greater than the plasma half-life, (*ii*) steady-state levels with repeated-dose studies are significantly higher than that predicted from single-dose studies, (*iii*) histopathological changes are observed for which tissue distribution studies may clarify the interpretation of the findings, and (*iv*) the drug is targeted to a specific tissue in the body. The dosing duration specified in the guidance is from one to three weeks. The guidance reiterates that for most drug development programs, single-dose tissue exposure tissue distribution studies are sufficient for regulatory authorities.

Safety Pharmacology Studies for Human Pharmaceuticals (ICH Topic S7A)

This guidance by the ICH is intended to provide a specific harmonized guidance for the scope of safety pharmacology studies to be conducted for marketing approval (23). Safety pharmacology studies can be thought of as studies that evaluate the unintentional potential pharmacological effects of the drug on organ systems, that is, the pharmacological effects beyond those, which the drug was designed to possess. In addition to this ICH guidance, the Japanese authorities have a well-written chapter that provides guidance for safety pharmacology testing (the chapter is entitled *Guidelines for General Pharmacology Studies* with the definition of general pharmacology in the JMHW guidelines being roughly equivalent to the definition of safety pharmacology in the ICH guidance) (8). For a comprehensive understanding of the study designs and protocol elements, the reader is encouraged to consult these Japanese guidelines along with the ICH Guidance Document.

The package of safety pharmacology studies will encompass evaluation of the effects of the drug product on the major organ systems in the body: the cardiovascular system, the central and autonomic nervous systems, the respiratory system, the gastrointestinal system, and the renal system. ICH identifies a core battery of testing to encompass the effects of the drug on the central nervous system (CNS), the cardiovascular, and the respiratory systems. ICH Topic S6 also recommends that this core battery of testing be utilized in the development of biotechnology-derived products. Depending on the class of compound and pharmacological mode of action, safety pharmacology testing can also include, or exclude with sufficient rationale, effects on the gastrointestinal, peripheral nervous system, and renal system. Examples of observations and endpoints that can be used to evaluate potential drug effects on each organ system are provided in Table 11. The safety pharmacology testing in the core battery should be conducted in compliance with GLP. Supplemental testing should be conducted in the spirit of GLP regulations to the extent feasible since absolute compliance to the GLP may be difficult.

As can be seen from the listing in Table 11, there are a number of safety pharmacology endpoints that can be incorporated as components of the toxicity testing studies with potential cost savings both in terms of money as well as in total numbers of animals used. Motor activity, behavioral changes, coordination, and body temperature are all endpoints that are easily

Table 11 Safety Pharmacology Studies—Battery of Testing

Target organ system	Potential observations/endpoints
Primary organ systems (core battery)	
Central nervous system	Spontaneous locomotor activity, behavioral changes, coordination, sensory/motor reflexes, general anesthetic effect (i.e., pentobarbital sleeping times), effect on chemically induced analgesia and convulsion Functional observation battery (FOB) or Irwin's test are commonly conducted
Cardiovascular system	Blood pressure, heart rate, electrocardiogram (ECG), cardiac output
Respiratory system	Respiratory rate, tidal volume, airway resistance, blood gases and blood pH
Secondary organ systems	
Gastrointestinal system	GI transit time, drug effect on isolated ileum
Renal system	Urine volume, urine chemistry, glomerular filtration rate
Autonomic nervous system	Stimulation of autonomic nerves and measurement of cardiovascular responses, baroreflex testing

captured in the conduct of single- and repeat-dose toxicity testing. Similarly, urine volume and urine chemistry should also be components of repeat-dose toxicity testing.

The following case example illustrates the potential value of including safety pharmacology endpoints in toxicity testing. A study director was assigned a drug that was about to enter phase III trials. At the end of the phase II meeting with FDA regulatory authorities, concerns were raised over the potential cardiovascular effects of the drug, an intravenous anti-infective molecule, even though there was no apparent reason why any such effect might be observed. This FDA concern would have easily been addressed had the repeat-dose toxicity study in dogs included collection of ECG data in all dogs at all dose levels at various time points during the study. However, the previous study director believed that such testing would not add any value (and was being instructed from management to keep the cost down) since this particular class of drug was not known to have any cardiovascular effects. Therefore, full ECG testing was not performed. Instead, ECG testing was done in just two control and two high-dose dogs at one time point. Subsequently, the FDA requested a full cardiovascular safety pharmacology study, with the collection of ECG parameters over a 24-hour period before and after dose administration, in the end costing money, time, and the use of extra animals.

ICH S7A provides some guidance as to the timing and applicability of safety pharmacology testing in the drug development process. Safety pharmacology testing is not required for locally applied agents and/or instances where systemic exposures are anticipated to be minimal. Studies with cytotoxic drugs in cancer are usually excluded from any safety pharmacology testing. Biotechnology products with highly specific targets and mechanisms of action may also be exempt from safety pharmacology testing, although biotechnology products with less specific targeting or unknown mechanisms of action should have the core battery assessment of testing. With regard to timing, safety pharmacology testing in the core battery of systems should be completed prior to administration of the drug in the clinical setting.

Safety Pharmacology Studies for Assessing the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals (ICH Topic S7B)

The high profile for the cardiovascular system in the assessment of safety pharmacology is emphasized with this ICH guidance extending the guidance discussed in ICH Topic S7B to specifically identify and assess the risk of potential cardiovascular effects, specifically effects on the QT interval (24).

ICH S7B provides general recommendations for the testing strategy to evaluate risk of a drug product to cause a prolongation of the QT interval in man. The guidance calls for evaluation in four particular areas. First is the evaluation of the pharmacological class to which the drug product belongs and whether this class is known to possess cardiovascular effects. Second is an

evaluation of the drug effects in an ionic current assay in vitro (e.g., isolated animal or human myocytes, cultured cardiac cell lines). Third is an evaluation of action potential parameters in isolated cardiac preparations or, alternatively, the measurement of specific electrophysiological parameters indicative of action potential duration in animals. Fourth is an in vivo QT assessment. This assessment should be a component of the core battery cardiovascular study conducted as part of the safety pharmacology evaluation described in ICH S7A. An investigator can expand the scope of the in vivo QT assessment to include regional information relating to ventricular repolarization, and thereby satisfy testing in the third area. Each of these evaluations should be complete prior to initiation of clinical trials.

ICH Topic S7B also provides extensive guidance and protocol elements of investigational test systems to address drug effects in each of the areas, second, third, and fourth, described above. A discussion of each of the test systems in the ICH guidance is beyond the scope of this chapter and the reader is referred to the ICH S7B Guidance Document.

NONCLINICAL DEVELOPMENT PROGRAMS

This section presents some case studies of drug development programs that have been reviewed by the regulatory authorities in the US and/or in Europe and approved to support the marketing approval of the product. Under the Freedom of Information Act (FOIA), and subsequent amendments, federal agencies including the FDA place FOIA materials (including drugs and biologics approvals) in publicly accessible electronic reading rooms. The Internet address for FDA's SBA Documents in CDER is www.fda.gov/cder/approval/index.htm and the address in CBER is www.fda.gov/cber/products.htm. All of the information discussed in each of the following drug development case studies is part of the public record and can be found in the Approval Documents at one of these sites. The SBA Documents are invaluable in providing insight into current regulatory practices and development programs and the reader is encouraged to review the Regulatory Review Documents for other drugs and biologics. In addition, analogous review and Approval Documents are available for drug products approved for use in Europe; these can be found on the EMEA Website at www.emea.eu.int/index/indexh1.htm. A note here to the reader – the SBA will be pulled from the FDA website of approved drugs if the drug is withdrawn. Hence, one should consider saving the documents of interest as a pdf file.

In reviewing different nonclinical drug development programs, it becomes apparent that the regulatory guidances are not to be used as a cookbook to design in ubiquity one drug development program after another. Each drug development program is likely to be unique and full of particular challenges such that, on some days, one may yearn for just one "simple" drug in development. The old adage "don't miss the forest for the trees" is quite apt for nonclinical drug development if one considers the forest to be the nonclinical plan of studies to support the drug and the trees to be each study. It is important to keep in mind that the purpose of the nonclinical studies is not to provide a checklist of studies that have been conducted with the resulting toxicities in the animals. Rather, the purpose of the nonclinical studies is to support the safe dosing of humans with the new drug in clinical trials.

It is when one realizes that nonclinical toxicity studies are conducted with the goal of being supportive to the assessment of the safety in man that one can fully utilize the guidelines. The most appropriate package of nonclinical toxicity studies will be the one that provides the best extrapolations of toxicities in animals to potential toxicities in humans. This mindset of thinking makes clear that the best nonclinical toxicity program is one that uses the most appropriate animal species, doses, dosing regimen, duration of dosing, and study endpoints to predict effects in humans.

When assessing treatment-related effects, the following factors should be used to evaluate the significance of differences between treated and control groups:

- dose-related trends,
- reproducibility,
- related findings,
- the magnitude and types of differences, and
- occurrence in both sexes.

Finally, before proceeding to the presentation of case studies, one last item bears mentioning. As most persons in the pharmaceutical industry will point out, when you buy a medicine

you are not so much paying for the cost of the ingredients used to prepare the drug as you are paying for the package insert that comes with the medicine. Consequently, all drug development should be geared toward the contents on the label. It is important that the package of toxicity studies address any labeling concerns for marketing the drug to physicians and the general public.

As an example, a package insert contains a use-in-pregnancy rating system to classify the risk to the fetus. These risks of fetal harm are divided into categories A, B, C, D, and X. Category A is defined as "controlled studies show no risk" in which adequate, well-controlled studies in pregnant women do not demonstrate a risk to the fetus. Category B is defined as "no evidence of risk in humans" in which animal studies have been conducted and show no fetal risk but there are no controlled studies in pregnant women. Category C is defined as "risk cannot be ruled out"; in this category, animal studies and human controlled studies are lacking or animal studies have shown a risk to the fetus and there are no human controlled studies. In this category, drugs should only be given if the potential benefit outweighs the potential risk to the fetus. Category D is defined as "positive evidence of risk" where there is evidence of human fetal risk but the benefits for use in pregnant mothers may be acceptable despite the risk. Category X is defined as "contraindicated in pregnancy" where studies have demonstrated fetal abnormalities and the risk of drug use outweighs any potential benefit. In some instances, reproductive toxicity testing may not be required for marketing approval. However, a competitor product may have a category C label because no reproductive toxicity studies were conducted. It may be desirable, then, to conduct the reproductive toxicity testing in order to use a category B label (assuming no adverse effects were detected). Similarly, additional nonclinical studies that may be important for inclusion in the product label insert should be considered in the context of the development program.

Case Study #1: Celebrex (NDA 20–998, Celecoxib, SC-58635)

Celebrex is a well-known drug approved for the treatment of acute and chronic signs and symptoms of rheumatoid arthritis and osteoarthritis and management of acute and chronic pain. The drug is a new chemical entity (NCE), a nonsteroidal anti-inflammatory inhibitor of the cyclooxygenase 2 (Cox-2) enzyme, and as such was reviewed by CDER. Celebrex is provided in capsules at strengths of 100 and 200 mg and is to be administered orally. The sponsor, G.D. Searle & Co., submitted the new drug application (NDA) for Celebrex for review on June 29, 1998, and the Pharmacology review was completed within six months on November 24, 1998. The SBA can be found under the Freedom of Information Act at www.fda.gov.cder/approval/index.htm. Given the widespread usage of the drug, it is interesting to examine the studies used to support the marketing application in the US. Indeed, for the novice toxicologist, the list of studies evaluated in the SBA provides an invaluable blueprint of those studies that would likely be required for support of a standard new chemical entity.

The list of toxicity studies in support of the new drug application (NDA) for Celebrex is given in Table 12. The studies listed were standard to support chronic administration of a drug to a potentially large population of patients. Rats were the primary rodent species examined, while dogs were the primary nonrodent species. It is interesting to note the different forms of the drug administered in these toxicity studies. In rats, the drug was administered by oral gavage of a methycellulose/Tween 80 suspension, whereas in dogs, capsules were administered. In mice, the drug was administered in the diet.

For mice, the studies described are classic studies that one would conduct in series with the overall end goal being the evaluation of carcinogenicity of the drug. The first study investigated whether administration of the drug in the diet could provide adequate systemic exposures and, perhaps, a consistent estimation of a target daily dose. The advantage of diet administration is obvious in not having to oral gavage dose several hundred mice daily for two years, a task requiring highly skilled animal technicians to avoid accidental dosing deaths. The target doses chosen in the 2-week study were 100, 300, 1000, and 3000 mg/kg/day, with a group size of 10/sex. Males were more sensitive than females to the toxic effects of the drug, correlating with greater systemic exposure in this gender; the NOAEL was 100 and 300 mg/kg/day for males and females, respectively. The dose levels in the 13-week study (to define the doses for the long-term carcinogenicity study) were consequently set at 0, 75, 150, and 300 in males and 0, 150, 300, and 1000 mg/kg in females with a group size of 20/sex. Both 2-week and 13-week studies

Table 12 Toxicity Studies[a] Submitted to Support Marketing Approval of Celebrex in the US

Mice	Rats	Dogs	Other studies
2-wk diet admix toxicity study; included TK	Acute oral toxicity studies; some included TK	Single dose oral toxicity study	Acute limit study oral monkeys
13-wk diet admix toxicity study; included TK	4-wk oral toxicity study with 4-wk recovery; included TK	4-wk oral toxicity study with 4-wk recovery; included TK	Teratology, embryo–fetal (2 RF and 1 definitive) studies—oral rabbits
104-wk diet admix carcinogenicity study	13-wk oral toxicity study with 4-wk recovery; included TK	13-wk oral toxicity study with 4-wk recovery; included TK	AMES assay
	26-wk oral toxicity study; included TK	52-wk oral toxicity study with 4-wk recovery; included TK	In vitro mutagenicity in CHO
	104-wk oral carcinogenicity study	7-day exploratory IV toxicity study; included TK	In vitro chromosome aberration in CHO
	Fertility, early embryonic development (three separate studies)—oral		In vivo rat bone marrow micronucleus assay
	Embryo–fetal development (2 separate studies)—oral		Antigenicity
	Perinatal/postnatal development study—oral		Guinea pig maximization
			Primary irritation—dermal and ocular in rabbits

[a]Not listed in this table are studies conducted with chemical intermediates in the production process; these were an acute oral toxicity study in rats, primary dermal and ocular irritation studies in rabbits, a guinea pig maximization test, and an AMES assay.

included a large number of satellite mice for TK purposes; the TK samples were collected on days 1, 45, and 87 in the 13-week study. The outcome of the 13-week study was a NOAEL of 150 mg/kg/day in females and <75 mg/kg/day (the low dose) in males; systemic exposures were twofold higher in males at equivalent target dietary doses. The major target organ in the study was the gastrointestinal tract. One can comment here on dose setting. It would have been beneficial to identify a NOAEL in males to have a greater degree of confidence in the doses for the carcinogenicity bioassay.

The carcinogenicity study in mice used target doses of 0, 25, 50, and 75 mg/kg/day (diet) in males and 0, 50, 100, and 150 mg/kg/day in females. The number of animals were 90/sex/group, far in excess of the 50/sex/group suggested in the ICH guidance, but the increased numbers allowed for a scheduled interim necropsy at one year (such an interim necropsy is not required and is somewhat unusual). There were changes in dose levels throughout the study that were reflective of the close monitoring of mice during the study by the Study Director and, although not ideal, reflected the need for flexibility in changing the dose levels in a study as necessitated by the animals rather than risk the entire study by stubbornly adhering to an original design. All dose levels for both males and females were halved at week 19 due to excessive toxicity. Oddly enough, the high dose (and only the high dose) for females was returned to the original 150 mg/kg/day at week 23. The interim necropsy at week 52 revealed no marked treatment-related findings at any dose level. Nonetheless, at week 80, all high-dose animals of both sexes were terminated due to poor survival with gastrointestinal toxicity being the main finding. To maintain study validity and provide control animals at this unscheduled necropsy and at scheduled termination, the Study Director ingeniously terminated the control satellite PK mice to provide control-matched tissues. The remaining mid- and low-dose group mice survived up to 2 years, with gastrointestinal changes observed histopathologically in only a minority of animals and no evidence of carcinogenicity. Overall, these studies conducted in mice are a good illustration of the difficulties in dose setting with a compound showing a steep

dose-response curve and of the real-time adjustments in study design necessary to gain the most complete picture of toxicity that is possible with such a compound.

The standard toxicity studies were conducted in rats for Celebrex, with some additional unusual design elements. The 4-week toxicity study consisted of 6 groups of 10 to 15/sex allocated to dose groups of 0, 20, 40, 80, 400, and 600 mg/kg/day; the extra 5/sex were animals assigned to a 4-week recovery period. Interestingly, the study included TK evaluations in satellite animals as would normally be recommended in a 4-week study, but only in the mid and high doses and only on day 31 as opposed to the first and last day of the study. There was very little toxicity in this study. The NOAEL of 80 mg/kg in males and 400 mg/kg in females was not driven by marked toxicities across the animals in these groups, but by the death of one male at 400 mg/kg and one female at 600 mg/kg; these two rats had marked gastrointestinal lesions.

The 13-week rat study was a standard toxicity study with groups of 25/sex allocated to dose groups of 0, 20, 80, and 400 mg/kg/day; 10/sex/group of which were assigned to a 4-week recovery period. This 4-week recovery period is not a standard design element in a study of this length, but most likely reflects the fact that there were no toxicities observed in the 4-week study for which reversibility needed to be assessed. The study, in terms of TK evaluation, was unusual in that a radiolabeled version of the drug was administered on the TK profiling days 1, 37, and 86. Hence, the TK component of the study included evaluation of urine and feces along with measurement of drug metabolites; such PK studies with radiolabeled drug are usually conducted separately from the toxicity studies and for much shorter-study durations. In contrast to the greater systemic exposure in male versus female mice, in rats females were found to have the higher exposures relative to similarly dosed males. The NOAEL in this 13-week rat study for both sexes was the highest dose of 400 mg/kg/day. One could argue that the high-dose level could have been increased in this study, but the highest dose administered in the 13-week study was based on death-related MTD of 400 and 600 mg/kg in the previous 4-week study.

The 26-week rat study utilized the same dose regimen and group size of 25/sex/group as the 13-week study. Again, this study included a 4-week recovery period with likely a similar rationale for the inclusion of the design element. Like the 13-week study, this 26-week study also included satellite groups that received radiolabeled compound on days 1 and 177; full TK profiles were collected following dosing on these days along with evaluation of urine and feces. The lack of TK data collection at interim time points in this 26-week study demonstrates that one does not need to collect redundant data, that is, there is no need to collect TK data at the same time points as in a previous shorter-duration study when the dose levels are the same. As with the previous studies, females were found to have higher systemic exposures to the drug. These higher exposures correlated with the gastrointestinal injury and death of females at the 80 and 400 mg/kg/day dose levels; the NOAEL in males was again the high dose of 400 mg/kg/day.

There is one additional observation in the repeat-dose oral toxicity studies in rats that bears mentioning for toxicologists designing study protocols. In these rat studies, several deaths were attributed to technician errors in the dosing procedure. These errors can only be confirmed when the protocol states that satellite (TK) animals will be necropsied in the event of an unscheduled death. These deaths are not uncommon and without necropsy results it may not be possible to rule out a drug-related toxicity. It is in the best interest of the study sponsor to ensure that all unscheduled deaths will be investigated by a protocol mandate.

The oral rat carcinogenicity bioassay was a 2-year study with 80 rats/sex/group allocated to dose groups of 0, 20, 80, and 400 mg/kg. A cohort of each group (10/sex/group) was terminated at an interim necropsy at week 53. The rationale for the increased group size (> 50/sex/group recommended by ICH) could not be ascertained from the SBA Document, but was perhaps reflective of the uncertainty in dose selection and the interim necropsy. It should be noted that the treatment initiation date for this carcinogenicity bioassay was the same month as the 26-week repeated-dose toxicity study; this timing itself is not unusual in that these studies are often run in parallel rather than in sequence for the sake of time. Consequently, the dose levels in the carcinogenicity bioassay in rats were determined by the data obtained in the 4-week and 13-week repeat-dose studies as in the mice; the toxicities in females at 26 weeks were not a factor in dose selection in the carcinogenicity study. For reference, the systemic exposures in

males in the high dose of 400 mg/kg/day, based on TK data from the 13-week study, were 5-fold and 10-fold greater than systemic exposures expected in humans receiving the expected clinical doses of 400 and 200 mg, respectively. The experimental design elements of an interim necropsy and the inclusion of satellite animals to provide full TK profiles at weeks 1, 26, 52, and 78 are unusual and not normally seen in a carcinogenicity bioassay. The results indicated the lack of carcinogenic potential for the drug. Before proceeding, there is one last note regarding the test article. There is no absolute requirement that the study utilizes the same lot of drug across all studies. In the rat study, different lots of test compound were used during the course of the bioassay.

The oral toxicity studies in dogs were based on complicated, and not entirely conventional, study designs. In the 4-week toxicity study, groups of 4/sex were allocated to receive 0, 25, or 50 mg/kg daily for four weeks. Additional groups of 8/sex received 0, 100, or 250 mg/kg daily for two weeks followed by two weeks of recovery. There was an interim necropsy of 4/sex/group at day 17 with the remaining animals terminated at day 29. This is an atypical study design and may reflect a desire to really push the highest doses to produce toxicity and evaluate the reversibility of such toxicities. As would be standard protocol for a 4-week toxicity study in dogs, blood samples were collected for TK at numerous time points following dosing on days 1 and 28 to provide full drug profiles. An additional unusual study design element was the inclusion of satellite TK groups of 2/sex administered 25 or 100 mg/kg/day for 28 days, with radiolabeled drug given on days 1 and 28 to allow identification of metabolites and quantitation of elimination in urine and feces; something seen previously in the rat studies. Gastrointestinal findings were the major toxicity in this 4-week study, again with a steep dose response curve; the NOAEL was 25 mg/kg/day while doses of 50 mg/kg/day exceeded the MTD with moribundity and death observed in dogs at these higher doses.

There were additional pathological findings of interest to the FDA reviewer in the 4-week dog study. Interdigital pyoderma and focal areas of subcutaneous inflammation (cellulitis) with necrosis and abscess formation in the caudal-ventral neck were seen in several treated dogs. The sponsor pointed out that interdigital pyoderma is a common bacterial infection of the pedal skin of short-haired breeds of dogs and these observations were not noted to be dose-dependent. Therefore, the sponsor concluded that these findings were not associated with administration of the test article. However, the FDA reviewer did not agree since these findings are not commonly observed in the laboratory setting. Moreover, the FDA reviewer also had knowledge that similar cutaneous lesions were observed in dogs administered other Cox-2 inhibitors. Also observed in the study was a perivascular/periventricular lymphocytic inflammation in the brains of several dogs that was seen with a slightly higher incidence in the drug treatment groups. The FDA reviewer noted that a relationship to treatment could not be ruled out without additional study to determine whether there was a relationship to drug treatment or whether the changes were due to an underlying viral inflammation or another causes. It is worth noting that these findings did not adversely affect the drug development or drug review process by requiring an additional mechanistic study since these findings were observed only at doses in excess of the MTD.

The 13-week dog study also used an elegant, nonstandard study design to incorporate multiple endpoints in the single study. Given the steep dose-response curve observed in the 4-week study, this study design included a dose group receiving single daily doses of the NOAEL of 25 mg/kg and dose groups receiving twice-daily administration at 0, 7.5, 12.5, and 17.5 mg/kg ($n = 4$/sex/group). Note that the twice-daily administration of 12.5 mg/kg/dose allowed direct comparison of 25 mg/kg/day given all at once or in two divided doses. The higher 17.5 mg/kg dose allowed one to ascertain whether the NOAEL could be increased by twice-daily dosing, keeping in mind the mortality observed when one approached 50 mg/kg/day. The control and high-dose groups also included an additional 2/sex/group for a 28-day recovery period. The inclusion of recovery groups in a study of 13 weeks is likely reflective of the change in dosing regimen from the previous 4-week study. Similar to the 4-week oral toxicity study, there were two satellite groups of 3/sex/group that were administered radiolabeled drug on days 1, 39, and 88. However, in contrast to the 4-week study, TK evaluations were only conducted on these satellite dogs and not on main study animals. To maximize the information in this study, the sponsor also included in vitro metabolic activity in the livers of control and treated main study

dogs. These additional PK add-ons were quite valuable with the sponsor demonstrating distinct populations of fast and slow metabolizers of the drug. No remarkable toxicities were observed in this study indicating a NOAEL in excess of 17.5 mg/kg twice daily and 25 mg/kg once daily.

The 52-week dog oral toxicity study had the same treatment groups as in the 13-week study. The group size was 8/sex/group with an additional 4/sex/group assigned to a 4-week recovery for the control and high-dose groups. Again, the inclusion of recovery groups is unusual but likely to allow for recovery of effects that might have been seen with this more chronic drug administration. Of the 8/sex/group, half were terminated at an interim necropsy at 26 weeks. An interim necropsy on a chronic dog study is atypical but likely reflects a desire to initiate a clinical trial of up to 26 weeks and the need for this supporting nonclinical data prior to completion of 52 weeks of chronic animal dosing. An additional 4/sex/group were allocated to satellite TK groups to receive 7.5 and 12.5 mg/kg twice daily, with radiolabeled drug administered on days 1, 176, and 358. As in the 13-week repeat-dose toxicity study, there were no remarkable drug-related toxicities at the highest-dose levels of 25 mg/kg once daily or 17.5 mg/kg twice daily. This lack of toxicity at any dose level would be a potential criticism in both studies, in that the major objective of the experimental design is to allow elucidation of dose levels at which toxicity occurs along with a dose-response relationship and identification of a NOAEL dose. However, in this instance the MTD was established as 50 mg/kg/day in the 4-week oral dog toxicity study, and consequently, to have administered that dose level in studies of longer duration would not have been prudent as such a dose level would have been overly toxic. Hence, the Sponsor was prudent in the selection of dose levels for the 13- and 52-week studies.

In summary, the oral toxicity studies in dogs illustrate several important points. First, it is possible to expand normal toxicity studies to encompass a variety of endpoints that one might normally associate with separate studies by independent groups of researchers. Second, the results of chronic toxicity studies are valid even in the absence of observed toxicities and provided that the MTD has been established in a repeated-dose study of shorter duration. Third, one can report findings (in this case histopathological findings in the 4-week dog study) that do not require a full explanation of definitive causality to drug treatment and/or mechanistic interpretation and extensive (often fruitless) investigation down blind trails.

The sponsor is obligated, under certain circumstances, to more fully characterize a toxicity in animals and the potential toxicity risk in humans. Such was likely the case in the 7-day repeat-dose IV study in dogs conducted in 3 dogs/group (across sex randomly) at doses of 0, 15, and 40 mg/kg/day. This study was carried out to determine the relationship between the gastrointestinal activity of the drug and the systemic exposure. If the gastrointestinal activity were merely a local effect, one would likely not see such toxicity with IV administration. This mechanistic study evaluated only gastrointestinal histopathological and biochemical changes with the end result being a greater understanding of the gastrointestinal effects of the drug. To this end, the sponsor demonstrated gastrointestinal effects consistent with the known properties of the drug.

The dog was the primary nonrodent species used to characterize the toxicity of the drug. Interestingly, a single-dose study was conducted in 3 monkeys/group administered 25 or 250 mg/kg. This is a peculiar study because the objective was only to determine the limit of lethality over a 14-day period following a single dose; there was no clinical pathology evaluation, no necropsy, and the PK sampling was limited to two time points on day 1. Hence, this study would have been of limited value in the overall marketing application and the purpose is not known. The timing of the study indicated that it preceded the definitive single-dose studies in rats and dogs and it may have been simply an exploratory screening study. The study is reported here only to remind the reader that all studies performed with a compound are required to be submitted in the marketing application, even if they are not conducted with expressed intent to support clinical trials.

The standard ICH package of studies was conducted for Celebrex although there were some redundancies with repeated studies. The design elements of the studies are briefly covered here to illustrate concordance with the ICH guidance and rationale for repeated studies. Of particular note is that of the studies listed in the ICH guidance, the embryo–fetal development (ICH 4.1.3) and the male and female fertility (ICH 4.1.1) studies were initiated at roughly the

same time period, while the peri-/postnatal development (ICH 4.1.2) was initiated roughly a year later. This timing is fairly typical in that the embryo–fetal development studies are frequently completed early in the nonclinical development often prior to initiation of human clinical trials, whereas the peri-/postnatal development study is often completed later in the nonclinical development of a drug.

There were three fertility/early embryonic studies (ICH study 4.1.1) conducted with Celebrex. All three studies used a group size of 25 rats/group. The first two were conducted after the 4-week repeat-dose rat study was completed and hence, likely relied on that study for dose levels. In the first study, dose levels were 0, 60, 300, and 600 mg/kg/day and males were exposed for four weeks prior to mating; note that the study was conducted prior to the ICH S5B(M) guidance. Females were exposed at these same dose levels from two weeks prior to mating to gestation day 7. There were no effects on male fertility, but there were decreases in live fetuses and implantation sites and increased postimplantation losses in females at all dose levels. Subsequently, a second study was conducted only in females at dose levels of 0, 15, 30, 50, and 300 mg/kg/day, again for two weeks prior to mating to gestation day 7. In this second study, the Study Director included an extra fourth dose group, probably in an attempt to bracket a range of exposures that provided the highest possible NOAEL, that is, an investigator could choose a very low dose (say 1 mg/kg/day) to definitely obtain a NOAEL but such a lower NOAEL would provide less of a margin of safety in humans. Similar results were observed in this study at higher doses, but the NOAEL was established at 30 mg/kg/day. Finally a third study was conducted in females to assess the reversibility of the effects. In this study, females were administered 0, 60, or 300 mg/kg for 14 days followed by a 14-day recovery period prior to mating. No adverse treatment-related effects were observed in this study. Hence, the 3 rat studies established NOAELs of 600 mg/kg/day in males and 30 mg/kg/day in females and demonstrated that effects in females were reversible even up to a high dose of 300 mg/kg/day.

The peri- and postnatal development study was conducted at dose levels of 0, 10, 30, or 100 mg/kg administered from gestation day 6 to days 21–23 postpartum to groups of 25 rats/group. It is worth mentioning some design elements although there were no effects on the F1 and F2 generations. At day 4 postpartum, the litters were culled to eight pups, four males and four females (recall that litter culling is still under scrutiny by ICH in 2003). Physical development in the F1 pups was assessed as pinna unfolding, tooth eruption, and eye opening and reflexological development was assessed with geotaxis testing and the startle response testing. For the adult F1 generation, one male and one female were selected from each litter on day 21 postpartum. Consequently, the behavioral testing and reproductive testing of the F1 generation were conducted in the same animals as suggested in the ICH guidance. Physical development of the selected animals was evaluated as day of vaginal opening and day of preputial separation and visual function as papillary closure and visual placing on day 21 postpartum. Behavior performance was evaluated by motor activity in "Figure 8" mazes on days 35 and 60 postpartum, auditory startle habituation on day 55 postpartum, and performance in "E" water maze on days 60 and 70 postpartum. Mating of the F1 generation was initiated on day 85, the females were allowed to deliver the F2 generation, and the F2 generation was terminated on day 5 postpartum.

Two embryo–fetal development studies (ICH Study 4.1.3) were conducted in rats. Both studies used doses of 0, 10, 30, and 100 mg/kg/day on gestation days 6 to 17. The first study used 20 rats/group with additional satellite pregnant rats for TK profiles on gestation days 6 and 16. This study found a slight decrease in live fetuses at 100 mg/kg/day and increased incidences of wavy ribs at 30 and 100 mg/kg/day. Hence, the NOAEL for fetal development was 10 mg/kg/day. The second study was conducted approximately two years later and used a larger group size of 30/group with additional satellite pregnant rats for TK profiles on gestation days 6 and 17. This group size of 30/group was significantly larger than the group size of 16 to 20 recommended in the ICH guidance, but might reflect a desire to confirm with a larger group size whether or not the skeletal effects observed in the first study were definitively related to drug treatment. Unlike the first study, there was no increased incidence of wavy ribs or a decrease in number of live fetuses, but there was a dose-related increased incidence of diaphragmatic hernia at 30 and 100 mg/kg/day. Consequently, the NOEAL for fetal development was again 10 mg/kg/day. With these results, the drug is labeled category C in the package label insert.

The increased incidence of skeletal abnormalities is detailed and the increased incidence of diaphragmatic hernia noted in the package insert.

The embryo–fetal development studies in rabbits (ICH 4.1.3) consisted of a dose RF study, a pilot study, and the definitive study. The RF study consisted of 6 animals per group allocated to receive 0, 6, 30, 60, 300, or 600 mg/kg/day on gestation days 7 to 18. In this RF study, blood samples for TK analysis were collected from the study animals on gestation days 7 and 18. A necropsy was conducted on gestation day 29 and fetuses were examined externally. The study found significant maternal and embryo–fetal toxicity at 300 and 600 mg/kg/day. The pilot study was conducted to further define the high dose for the definitive study. Rabbits ($n = 2$/group) were dosed on gestation days 19 to 23 or 21 to 25 at doses of 200, 400, or 600 mg/kg/day. On the basis of this study, the drug was considered toxic at 600 mg/kg/day. In the definitive study, 20 rabbits/group (litter size consistent with ICH Study 4.1.2 recommendations) were allocated to receive 0, 60, 150, or 300 mg/kg/day from gestation days 7 to 18. Again, blood samples for PK evaluation were collected on gestation days 7 and 19. The collection of blood samples from main study animals is not necessarily the norm as some investigators fear that collection of the blood samples from main study animals might affect the study outcome. Therefore, it is not surprising to see separate satellite TK groups of pregnant animals that are terminated after the final day of dosing with no evaluation of fetal parameters, with the possible exception of drug concentration determinations in fetal tissues. A slight dose-dependent increase in skeletal abnormalities was observed at 150 and 300 mg/kg/day.

The ICH recommended battery of genetic toxicity tests were conducted with Celebrex and all assays were conducted as outlined in the ICH S2A and S2B guidances. The AMES^{TM} bacterial mutagenicity assay included 6 concentrations over 2 logs of concentrations. The highest concentrations were precipitating, and colony counts were not determined. The highest nonprecipitating concentration was toxic, fulfilling the guidance recommendation that significant cytotoxicity be observed. The sponsor conducted both in vitro mammalian genotoxicity assays, that is, the in vitro assay for chromosome aberration and the in vitro assay for mammalian cell mutagenicity. Chromosome aberrations were evaluated in Chinese hamster ovary (CHO) cells. The initial RF assay used a 4-log concentration range and found significant toxicity at the highest concentrations with no viable cells. The definitive assay used three concentrations based on the RF assay. The exposure times were 4 and 24 hours in the absence of metabolic activation and 4 hours in the presence of metabolic activation. An increased cell endoreduplication was observed in cells treated at the highest two drug concentrations in the presence of metabolic activation. The biological significance of the finding was not known. This finding likely led the sponsor to conduct the in vitro mammalian mutagenicity assay, the evaluation of hypoxanthine-guanine phosphoribosyltransferase (HGRT) mutations in CHO cells. The assay used concentrations over 3 logs incubated with drug for 20 to 24 hours in the absence of metabolic activation and 4 hours in the presence of metabolic activation. Apparently the dose RF assay was unable to reach a maximum concentration causing at least 80% cytotoxicity. The definitive study did reach cytotoxic concentrations in the presence, but not the absence, of metabolic activation. The conclusion was that Celebrex was not mutagenic in this assay at the highest assayed concentrations. The in vivo micronucleus assay was conducted using male and female Sprague Dawley rats. Five rats/sex were allocated to receive vehicle control, cyclophosphamide (positive control), or drug at 150, 300, or 450 mg/kg for three days. This study was conducted approximately two years after the 4-week repeated-dose toxicity studies. Rats were terminated 24 hours after the final dose and bone marrow was extracted from the tibia. Slides were evaluated for micronuclei. Celebrex was not clastogenic in this assay. Hence, the sum of the studies did not reveal a mutagenic potential for Celebrex.

The above studies make up the package of toxicity tests generally required for marketing approval. In the case of Celebrex, there was some additional toxicity testing conducted. These special toxicology tests are listed in Table 12. The objective of the special testing of the drug was to evaluate antigenicity, skin sensitization, and the potential to cause local irritation to the skin or eyes. The rationale for carrying out these studies is not clear, given that the drug is an oral product. However, the studies were conducted early in the development of the drug at about the same time that the 13-week repeat-dose toxicity studies were being conducted in rats; hence, they were not suggested by regulatory authorities late in the development of

the drug. One explanation could be that the studies were conducted with the manufacturing personnel in mind, for the preparation of a Material Safety Data Sheet (MSDS), rather than the clinical population. That possibility is further evidenced by the conduct of skin sensitization and dermal and ocular irritation studies with starting chemical material for the synthesis of Celebrex. Acute toxicity testing in rats and an AMES assay were also conducted with the starting chemical material. Interestingly, the starting material was an extremely potent dermal sensitizer in guinea pigs and a local irritant, in contrast to the finished drug that was negative in all of the special toxicity studies.

Specific safety pharmacology studies were also conducted to support the marketing application. These studies are listed here but are not discussed in detail; see the SBA Document. In mice, CNS effects were evaluated in single-oral dose studies to monitor general activity and behavior, spontaneous locomotor activity, hexobarbital sleeping time, induced convulsions, and analgesia. In addition, a gastrointestinal transit study of the effect of a charcoal meal on the drug was conducted in mice. In rats, body temperature and renal effects were evaluated in animals given single oral doses. In dogs, drug effects (following administration of single oral dose) on respiratory and cardiovascular physiology were evaluated. Finally, in guinea pigs, the effect of drug concentrations on isolated ileum was evaluated to assess effect on autonomic nervous system and smooth muscle. These safety pharmacology studies encompassed the spectrum of examination of the major physiological systems as outlined in the Japanese guidelines for safety pharmacology evaluation (8).

In summary, the drug safety evaluation of Celebrex is a classic example of the package of studies required to support marketing approval of a drug with anticipated wide and potential long-term usage. The rat and the dog were the primary species in which repeat-dose toxicity testing was conducted. Mice were used in so far as the carcinogenicity of the drug in the second species is required. A single-dose monkey study was conducted, which is highly unusual given that the dog was an appropriate nonrodent species. The design of the studies and the comments by the FDA reviewer also reveal some interesting study components for the toxicologist to consider when planning a development program for their drug product.

Case Study #2: Herceptin (CPMP/1774/00, Trastuzumab)

Trastuzumab is a recombinant humanized monoclonal antibody (MAb) that binds to the extracellular domain of the human epidermal growth factor receptor 2 protein otherwise known as HER2. The MAb is an IgG1 kappa antibody that contains the human framework regions with the mouse complementary determining regions that bind to HER2. In the case study of Herceptin, the European Review Document was used as the source of the studies conducted to support marketing approval in Europe. Roche is a marketing authorization holder in Europe. The Scientific Discussion Document for Herceptin can be found at the EMEA Website at www.eudra.org/humandocs/humans/EPAR/Herceptin/Herceptin.htm. The Application for Marketing Authorization was submitted to EMEA on February 11, 1999, and the biologic was granted Marketing Authorization on May 25, 2000. The delays in approval were primarily related to manufacturing issues.

Trastuzumab, the active substance, is produced in a cell-based system, a CHO suspension culture. A significant sidebar of note is that an early development cell line was used to support nonclinical toxicity testing and phase I and II clinical trials, while a later cell line was developed for production of the intended marketed product. The finished product, Herceptin, is administered as an initial loading dose of 4 mg/kg MAb over a 90-minute infusion period, followed by weekly maintenance dose of 2 mg/kg given over a 30-minute period. In the US, the biotechnology-derived product is lyophilized and reconstituted with bacteriostatic water containing 1.1% benzyl alcohol as a preservative. The reconstituted solution is diluted in normal saline for infusion into the patient. In Europe, the use of a preservative is contrary to the Ph. Eur. requirements and the drug product is reconstituted with sterile water without preservative.

Herceptin is an interesting case study since it provides an overview of the extent of toxicity testing one might expect to conduct to support administration of a human biotechnology-derived protein. The list of toxicity studies conducted in support of the marketing application for Herceptin is given in Table 13. The ICH guidance regarding the studies to support registration of a biotechnology drug product should be read in conjunction with this case study (ICH Topic

Table 13 Toxicity Studies Submitted to Support Marketing Authorization of Herceptin in Europe

Primates	Other studies
Single dose IV toxicity study—rhesus monkeys	Single dose IV toxicity study—mice
4-wk IV toxicity study-–rhesus monkeys	Coadministration (cancer chemotherapeutics) PK/toxicity in rhesus monkeys
13-wk IV toxicity study-–cynomolgus monkeys	Local tolerance study—rabbits
26-wk IV toxicity study-–cynomolgus monkeys	Tissue cross-reactivity study—adult human and cynomolgus monkey tissues
Fertility and reproductive toxicity study-–cynomolgus monkeys	In vitro mutagenicity in CHO
	In vitro chromosome aberration in CHO
	In vivo mouse bone marrow micronucleus assay

S6. As stated in the guidance, the toxicity studies should be conducted in the relevant species and, in some instances, this will result in the testing of the drug in only one species, as was the case for Herceptin. The selection of nonhuman primates for the toxicity studies was not, however, consistent with the action of the MAb in humans. In nonhuman primates, unlike humans, there is no overexpression of the p185HER2 protein and no production of shed antigen. In addition, nonhuman primates possess an uncharacterized protein on epithelial cells to which the humanized MAb binds. Nevertheless, monkeys were chosen as the most relevant species and the toxicity studies were mostly limited to monkeys.

With that , although limited to a single species, the standard durations of repeated-dose toxicity studies were conducted. Hence, the package of studies (in terms of length of repeated-dosing toxicity studies) parallels those studies submitted to support approval of the drug Celebrex. Thus, a single-dose toxicity study of trastuzumab was followed by studies of 4, 13 and 26 weeks. In Europe, prior to ICH Topic S4A, the standard length of a chronic repeated-dose toxicity study in nonrodents was six months (26 weeks) as opposed to the current ICH S4A guidance of nine months. One interesting fact regarding the toxicity testing in monkeys was that the program initially appeared to utilize the rhesus monkey while later studies utilized the cynomolgus monkey.

The single-dose toxicity of trastuzumab was evaluated in rhesus monkeys and in mice. The biologic was administered at 0, 4.7, 23.5, and 47 mg/kg by IV bolus to monkeys and at 0, 9.4, 47 and 94 mg/kg by IV bolus to mice. The Review Document notes that there were several different formulations evaluated. However, the details of these acute studies and the other toxicity studies are not provided to the level reported in the SBA Document for Celebrex. Endpoints included clinical chemistry, determination of antibody formation, and gross and histopathological evaluations. The no –observable effect level (NOEL) was found to be the highest dose studied in either species: 47 and 94 mg/kg in rhesus monkeys and mice, respectively.

The repeated-dose toxicity studies are discussed as a single package of studies in the submission, and the study designs are not provided. Rhesus monkeys were used for the 4-week study, while cynomolgus monkeys were used for the 13- and 26-week studies. The reason for the switch to the cynomolgus monkeys is not known, but the reason may be that purpose-bred cynomolgus monkeys are more easily obtained than rhesus monkeys. No dose levels are provided in the review. Blood samples were collected for TK analyses, but no details of the sampling intervals were provided. There was minimal toxicity observed, with the single observation being injection site effects in the 4-week study. A rabbit local tolerance test, where trastuzumab was administered as a single bolus injection into the ear vein, revealed no local irritation. Neutralizing antibodies were detected in only one monkey, a single-dose female in the 26-week study. The overall incidence was 1 out of 84 monkeys treated with trastuzumab in repeated-dose studies.

There were no adverse toxicities in rhesus monkeys (in-life observations, clinical pathology only) when trastuzumab was coadministered with Taxol, Adriamycin, or a Cytoxan/Adriamycin combination. However, coadministration with Taxol did result in a twofold reduction in clearance of trastuzumab; this was not seen with the other cytotoxic drugs.

Reproductive toxicity studies were conducted in cynomolgus monkeys with trastuzumab. This is somewhat unusual for a biologic that is not active in rats or rabbits, but likely reflects

the intent to treat population, patients (women) with metastatic breast cancer whose tumors overexpress HER2. At dose levels of up to 25 times the weekly maintenance dose in women, there were no effects on reproductive function in females, no maternal toxicity or embryotoxicity, and no adverse peri- or postnatal toxicities. These studies allowed for labeling of the biologic as a pregnancy category B. Placental transfer of the biologic was observed during the gestational period and this is noted on the labeling insert.

Trastuzumab was investigated for mutagenic potential in the ICH recommended battery of genetic toxicity tests. Again, for this class of biologic, genotoxicity is not an absolute requirement, but a review of recent approvals of biologics by CBER shows an increasing trend to conduct these studies. An AMES bacterial mutagenicity assay, an in vitro evaluation of chromosome aberrations in human peripheral lymphocytes, and an in vivo mouse micronucleus assay (single IV dose of 29.5, 59, and 118 mg/kg) gave negative mutagenicity results.

Tissue cross-reactivity studies were conducted with human and monkey tissues as requested in the FDA guidance for the testing of MAb (FDA, CBER, 1977).

Finally, Herceptin does contain a boxed warning in the package label insert regarding the potential for cardiotoxicity manifested as development of ventricular dysfunction and congestive heart failure. The single-dose toxicity study with trastuzumab and Adriamycin, discussed previously, did not reveal any evidence of cardiac effects. Using a surrogate rat c-erb-B2 antibody, there was no enhancement of Adriamycin-induced toxicity for trastuzumab. Further investigations in dogs were not conducted due to the lack of pharmacological activity in this species. Possible anthracycline models in monkeys were considered unsuitable based on lack of well-defined endpoints.

To sum, the toxicity studies conducted with Herceptin are illustrative of the toxicity testing of a biologic that is active only in man and in nonhuman primates. Reproductive toxicity and genotoxicity testing represent trends in study conduct to support marketing, although these are not absolute requirements. The use of a 26-week repeat-dose toxicity study to support chronic dosing is characteristic of the European guidance regarding chronic study duration prior to the ICH harmonization of the study duration at nine months.

Case Study #3: Rituxan (BLA 97–0260, Rituximab, IDEC-2BC8)

Rituximab is a chimeric (murine/human) IgG1K MAb directed against the CD20 antigen present on the surface of malignant, and normal, B lymphocytes. CD20 is present on the surface of greater than 90% of all B cell non-Hodgkin lymphomas (NHL) (25). The IgG1 structure of the MAb is designed to possess effector function through binding of the Fc portion of the constant region of the MAb to the Fcγ receptors expressed on immune effector cells. Hence, the MAb binds specifically to CD20 on the malignant B cell and linkage to the effector cell results in destruction of the tumor cell. The biologic is produced in CHO suspension culture and is provided as concentrated liquid. The sponsor, originally Idec and now know as Biogen Idec, submitted the Biologics License Application (BLA) for Rituxan, while Genentech submitted the additional manufacturing BLA 97–0244. The SBA can be found under the Freedom of Information Act at www.fda.gov/cber/products/ritugen112697.htm. The biologic was approved in November 1997 for the treatment of relapsed or refractory CD20+ B cell non-Hodgkins lymphoma (NHL).

Rituxan is a good case study to include here, as an example of the limited scope of nonclinical toxicity testing that may be required for a drug (biologic) that is to be given for a short period of time and for the treatment of a life-threatening disease. The clinical course of treatment is 375 mg/m^2 given by IV infusion once weekly for four weeks. The list of toxicity studies conducted in support of the BLA for Rituxan is provided in Table 14.

Tissue cross-reactivity studies were conducted with human tissues as requested in the FDA guidance for the development/testing of monoclonal antibodies (26). It is not clear if studies were also conducted in cynomolgus monkey tissues, but there is no reference to monkey tissues in the SBA Review Document. It would be unusual if the sponsor did not conduct such a study in monkey tissues, since the goal of tissue cross-reactivity studies, in addition to evaluating whether or not there is an unexpected binding in human tissues, is to determine the comparability of binding between human and monkey tissues. Similar patterns of tissue cross-reactivity between humans and monkeys demonstrate that the monkey is a relevant laboratory animal species to perform toxicity testing.

Table 14 Toxicity Studies Submitted to Support Marketing Approval of Rituxan in the US

Primates	Other studies
Single dose, dose escalation IV toxicity study—cynomolgus monkeys; included TK	Tissue cross-reactivity study—adult human tissues
4-wk and 8-wk repeated dose IV toxicity study-–cynomolgus monkeys; included TK	Single dose IV PK study—rats
Embryo–fetal development study—cynomolgus monkeys; including TK[a]	
Peri- and postnatal development study – cynomolgus monkeys[a]	

[a]Reproductive toxicity studies are being conducted postapproval for NHL as the sponsor is looking to expand the clinical indication into non–life-threatening diseases. The embryo–fetal study has been completed and the peri- and postnatal development study is being planned as of March 2003.

The single-dose study in cynomolgus monkeys utilized a dose escalation study design. One monkey per sex per group was administered the drug intravenously at 10, 30, or 100 mg/kg and followed for 14 days of observation and blood sample collection (for TK and hematology purposes). As with a human phase I clinical trial, the monkeys were administered the low dose and followed for the observation period prior to initiating the subsequent higher dose to the next set of two monkeys. Blood sampling time points for TK were not provided in the review document, but the number of samples was sufficient to allow the sponsor to define one- and two-compartment kinetic models. Transient decrements in the numbers of lymphocytes were observed in all dose groups.

The repeat-dose toxicity study consisted of four groups of cynomolgus monkeys. Groups 1 and 2 were administered vehicle ($n = 1$/sex/group) while groups 3 and 4 were given 20 mg/kg Rituximab ($n = 3$/sex/group). Groups 1 and 3 were dosed weekly for four weeks and groups 2 and 4 were dosed weekly for eight weeks. Monkeys in all groups were terminated two weeks after the last dose. Clinical pathology assessments were made on all animals at time points during the study, and a necropsy was conducted on all animals at termination. There were decrements in the numbers of B cells in the MAb-treatment groups and histopathological evidence for depletion of B cells in spleens and lymph nodes. Somewhat unusual in this study was the fact that animals were not terminated until two weeks after final dosing, allowing some recovery from compound effects. It is more common in the design of a repeat-dose study to include subgroups, one terminated following final dosing and the other allowed a recovery period.

The repeat-dose toxicity study included an assessment of TK, although time points were not provided. Given the long half-life relative to the dosing interval (that is, the MAb may not have been eliminated significantly from the body before the next subsequent dose of MAb was administered), there was likely not a collection of full profiles but rather an assessment of peaks and troughs following dosing. While repeat-dose studies often do not collect full profiles during the first doses of the compound (since the dose interval is too frequent to allow adequate determination of PK parameters such as half-life), a study will often include sample collection at numerous time points following the final dose to determine PK parameters. In this repeat-dose toxicity study, such data collection and interpretation would have been hindered by the fact that anti-MAb antibodies were observed in all monkeys after repeated doses of Rituximab.

The PK of different lots and methods of manufacturing of the MAb were also studied in Sprague Dawley rats and cynomolgus monkeys to demonstrate bioequivalence; however, details regarding study design were not provided in the SBA Review Document.

Rituximab was not evaluated in genotoxicity or carcinogenicity testing. Such nonclinical testing is not an absolute requirement for this class of drug (biologic) intended for a life-threatening clinical indication. The initial approval of Rituxan also did not include any reproductive toxicity testing, and the current approved labeling is pregnancy category C, noting that animal reproductive toxicity studies have not been conducted.

While the initial filing and approval of Rituxan for the treatment of CD20+ B cell NHL was based on the limited toxicity testing outlined in Table 14, this limited package of

toxicity studies would likely not support approval of the MAb for non–life-threatening disease indications. In looking to expand the clinical indication for Rituxan into immune disorders (for example, rheumatoid arthritis), the sponsor has completed an embryo–fetal development study in cynomolgus monkeys and is presently defining a peri- and postnatal development study in cynomolgus monkeys (27).

In the embryo–fetal development study, 48 female cynomolgus monkeys were allocated 12/group to receive vehicle control or rituximab at 20, 50, or 100 mg/kg weekly from gestational day 20 to gestational day 50; loading doses of 0, 15, 37.5, and 75 mg/kg, respectively, were administered on gestational days 20, 21, and 22 to provide consistent exposure. The study parameters included clinical pathology determinations and collection of blood samples for MAb and anti-MAb antibodies. Monkeys underwent cesarean section on gestational day 100. All fetuses were examined externally and visceral and skeletal examinations were conducted. The spleen and lymph nodes were also examined histopathologically and immunohistochemically. Maternal and fetal systemic exposures to rituximab were confirmed at C-section. There were no adverse treatment-related effects on embryo–fetal development.

The abbreviated nonclinical toxicity package of studies to support marketing approval of Rituxan is an example of what might be considered the minimal testing platform that one will likely encounter. The limited scope is based on the clinical indication for a life-threatening disease and the limited duration of treatment. The case study also illustrates how additional toxicity studies may be required as the clinical indication is expanded.

Case Study #4: Remicade (BLA 98–0012, Infliximab)

Infliximab is a chimeric (murine/human) MAb containing the murine variable region amino acid sequence. Approximately 30% of sequence is murine, with the remaining 70% of the antibody corresponding to human IgG1K. The MAb binds specifically to human tumor necrosis factor alpha (TNFα). TNFα is a proinflammatory cytokine and elevated levels of the cytokine are thought to play a role in a number of immune disorders. The MAb binds to both the soluble and transmembrane forms of TNFα, thereby inhibiting the binding of TNFα to its receptor. Binding to the soluble form results in an amelioration of the induction of proinflammatory cytokines and migration of inflammatory cells to areas of inflammation. Like Rituxan, infliximab is an IgG1 isotype intended to have effector function, and binding of the MAb to transmembrane TNFα leads to cell lysis by effector cells or complement.

The first approved indication for Remicade was for Crohn's disease and this case study is primarily based upon the SBA Document for this indication found at www.fda.gov/cber/products/inflcen082498.htm. The sponsor of this BLA was Centocor Ortho Biotech, Inc. The BLA was received at CBER on December 30, 1997, and the pharmacology review was completed on May 12, 1998. The intended treatment regimen for Crohn's disease is 3 IV administrations of 5 mg/kg (an initial dose and at two and six weeks). One should be cognizant of this short duration of treatment when reviewing the studies submitted in the SBA Document.

The initial indication for Crohn's disease was subsequently expanded to include rheumatoid arthritis, in which 3 mg/kg Remicade is administered (IV infusion) initially, at two and six weeks, and then every eight weeks thereafter (28). The labeling also provides for dosing of up to 10 mg/kg administered every four weeks. The SBA Document (US) for this indication is not available, however, additional data can be found in the European Scientific Discussion Document for Marketing Authorization in Europe for Remicade (CPMP/1901/99); the Website is www.eudra.org/humandocs/Humans/EPAR/Remicade/Remicade.htm. In addition, the Physician's Desk Reference (PDR) is especially useful as the animal toxicity labeling has changed several times during the span of 2000 to 2003 (28–30). The use of both FDA and EMEA Documents in this case study provides a sense of the toxicity information required when moving from the limited treatment of Crohn's disease to the more prolonged treatment of rheumatoid arthritis.

Two tissue cross-reactivity studies were conducted with human tissues as requested in the FDA guidance for the development/testing of monoclonal antibodies (26). Cross-reactivity was observed in cells and tissues known to express TNFα; no unexpected cross-reactivity was observed. The ability of infliximab to neutralize TNFα activity in vitro was the assay used to investigate the species cross-reactivity of the biologic. No inhibition of activity was observed using TNFα from baboon, rhesus and cynomolgus monkeys, pig-tailed macaque, marmoset,

Table 15 Toxicity Studies Submitted to Support Marketing Approval of Remicade for Crohn's Disease

Chimpanzees	Mice (with murine analogue cV1q MAb)	Other studies
Single dose IV toxicity study Single- and repeated-dose study (up to 5 doses) IV toxicity study 3-day IV toxicity study	Range-finding embryo–fetal development study—cV1q MAb Embryo–fetal development study—cV1q MAb 6-mo toxicity/tumorigenicity study —cV1q MAb[a] Tumorigenicity studies in mice deficient in TNFα[a]	In vitro inhibition of TNFα activity in multiple animal species-–rhesus and cynomolgus monkeys, marmoset, tamarin, pig, rabbit, rat, mouse tissues Tissue cross-reactivity study-–adult human tissues Single-dose IV toxicity study-–rats (2 studies) 7-day IV toxicity studies—rats (3 studies) Single-dose IV and IM studies—rabbits AMES bacterial mutagenicity assay In vitro chromosome aberration assay in human lymphocytes In vivo mouse micronucleus assay

[a]These studies were not submitted in the initial BLA, but are found in the later EMEA scientific discussion document to support marketing authorization.

tamarin, pig, rabbit, rat, or mouse. The potency in the dog was five orders of magnitude less than that observed with human and chimpanzee TNFα. The cross-reactivity with chimpanzee TNFα was expected since the protein sequence of the chimpanzee cDNA was identical to that of humans.

The lack of a relevant species for infliximab and the fact that the treatment regimen (initially solely for Crohns' disease) consisted of three total infusions provides the rationale for a very atypical nonclinical testing program to support marketing authorization of Remicade (Table 15). Single- and repeat-dose toxicity studies were conducted in chimpanzees and reproductive toxicity studies were conducted in mice with a murine analogue of infliximab. Two single-dose pilot studies were conducted in dogs since there was some reactivity with dog TNFα in vitro, albeit orders of magnitude less than seen in humans. However, an immediate hypersensitivity reaction was observed in association with increased plasma histamine levels and, consequently, the dog was not considered a practical species to evaluate the toxicity of the biologic. Toxicity studies were also conducted in rats, but the FDA reviewer noted that these studies have little relevance to the safety evaluation and served only to evaluate nonspecific toxicities.

Before discussing these nonclinical toxicity studies, it is of interest to note that infliximab is not unique as a MAb that is cross-reactive only in humans and chimpanzee. Keliximab is a Primatized™ anti-CD4 MAb that was in development for the treatment of autoimmune disorders. Like infliximab, this MAb recognized only human and chimpanzee CD and not CD4 from other species including rhesus and cynomolgus monkeys. Consequently, like infliximab, nonclinical studies with keliximab were conducted in chimpanzees (31). However, rather than utilize a murine analogue of the MAb, nonclinical studies with keliximab used a different approach, the administration of the human MAb to transgenic mice expressing human CD4 (32). The reader is referred to these papers to note the similarity in the nonclinical development programs.

In the single-dose study, infliximab was administered to one male and one female chimpanzee (IV dose 30 mg/kg). This study was conducted to support the use of the biologic in combination with Centoxin® (an MAb to an endotoxin protein from gram-negative bacteria). Both chimpanzees were monitored for 14 days, with collection of ECGs, ophthalmic examinations, clinical pathology, and blood samples (to determine MAb concentrations) and anti-infliximab determinations. Infliximab was well-tolerated in this study.

In the single-dose/multiple-dose study, one male/group was administered a single dose of vehicle or 30 mg/kg, one male and one female received 30 mg/kg for three consecutive days, and two males and one female received 15 mg/kg for either four or five consecutive

days. Two additional females were administered vehicle for either three or five consecutive days. All chimpanzees were monitored for two weeks with the same collection of parameters as described for the single-dose study. In this study (as in all of the chimpanzee studies), animals were anesthetized for infusion of the test article. However, in this particular case, cumulative doses of ketamine were included since sedation and respiratory depression were observed in one animal treated with vehicle and 15 and 30 mg/kg dose groups. The anesthesia protocol was altered and an additional two animals were administered 30 mg/kg for three consecutive days. Other than the effects related to anesthesia, there were no adverse effects associated with infliximab infusion.

In the final chimpanzee toxicity study, 1 sex/group received vehicle or 30 mg/kg infliximab by IV infusion (1–2 hours) for three consecutive days. In this study, animals were monitored out to six weeks. Observations included ECGs prior to, during and immediately after the first and last dose, on day 4, and at termination. Ophthalmologic examination was performed prior to each dose and at termination. Blood samples for clinical pathology, serum–infliximab concentrations, and anti-MAb antibodies were collected prior to the first two doses, on day 4, and weekly thereafter. As with the other studies, there were no adverse effects of infliximab in this study.

In summary, infliximab was well tolerated at doses of 30 mg/kg for three consecutive days and 15 mg/kg for five consecutive days with no treatment-related toxicities. Systemic exposures were demonstrated in all treatment groups. Anti-infliximab antibody determinations were problematic. Because of the long elimination half-life of the compound, infliximab was still present in the blood (the presence of the MAb would interfere with the ELISA assay by binding any anti-MAb antibodies that might be present in the blood).

Local tolerance testing was carried out in rabbits to evaluate nonspecific irritant effects. An acute IV study was conducted in which groups of 16 male New Zealand white rabbits were allocated to receive 3-hour infusions of infliximab, human serum albumin, or saline for injection. Additional groups received similar treatment subcutaneously to evaluate potential extravasation during infusion. IV infusions were well-tolerated, although subcutaneous administration of infliximab did cause slightly greater irritation relative to the other treatments. In the acute intramuscular study, seven New Zealand white male rabbits were assigned to receive infliximab, human serum albumin, or cefoxitin sodium (Mefoxin®) at separate sites (the same animal received all treatments at separate sites, thereby serving as its own control). Injection sites were scored daily for three days. Infliximab produced less irritation than Mefoxin, a drug approved for intramuscular injection.

As discussed previously, a relevant animal model for an evaluation of the reproductive toxicity of infliximab was not available because infliximab was not reactive with TNFα from rats or rabbits. Nonetheless, it was important for the sponsor to obtain a pregnancy category B labeling for Remicade, and the sponsor pursued this by conducting embryo–fetal studies of a murine analogue, cV1q, in mice. This murine MAb cV1q was validated for specificity for murine TNFα in vitro, and the pharmacological activity was validated in vivo in a transgenic murine colitis model.

An exploratory embryo–fetal study was first conducted in which groups of 16 mated female mice were allocated to receive an IV bolus of 0, 10, 20, or 40 mg/kg cV1q on gestation day 6; the 40 mg/kg dose was determined to be the therapeutic dose in mice in a murine model of colitis. Blood samples were collected at 24 hours and 4, 7, and 12 days after dosing on gestational day 6 from 4/group at each time point. The blood collection was terminal. A C-section and gross necropsy were performed and fetal endpoints (e.g., numbers of corpora lutea, implantation sites, viable/nonviable fetuses, fetus weight) were evaluated.

The definitive embryo–fetal study was conducted with groups of 33 mated female mice allocated to receive an IV bolus of 0, 10, or 40 mg/kg cV1q. Since blood levels of cV1q in the exploratory study were not maintained to gestational day 18, mice were treated on gestational day 6 and again on gestational day 12. Because of a shortage of cV1q antibody, only 22 and 23 mice in the 10 and 40 mg/kg groups, respectively, were dosed on gestational day 12. On gestational day 14, 8 mice/group underwent cesarean section and blood samples were collected for maternal and fetal MAb concentrations and a gross necropsy was conducted for determination of fetal parameters. Remaining mice were terminated on gestational day 17 with collection

of maternal blood samples. Fetuses were evaluated for gross external alterations and one-half of the fetuses were examined for visceral alterations, and the other half for skeletal alterations. Administration of cV1q was not associated with any maternal or developmental toxicity. Maternal and fetal blood concentrations of cV1q were observed, indicating fetal exposure throughout organogenesis. The murine analogue cV1q had the same specificity, pharmacological activity, and PK profile as infliximab. Consequently, the reproductive toxicity studies in mice with the murine analogue, with the lack of any adverse reproductive toxicity, were determined by the FDA reviewers to be relevant for the pregnancy class labeling and inclusion in the Remicade package insert.

A 6-month study of the murine analogue cV1q was submitted to the EMEA in 2001 (see also the later text pertaining to carcinogenicity of Remicade). A likely presumption is that this study was required to support the extended duration of treatment in rheumatoid arthritis. This is revealing in that one might expect the sponsor to argue that the plethora of human safety data generated from the approved use of the product in Crohn's disease would be of more relevance to the assessment of safety for the extended treatment for rheumatoid arthritis than would chronic animal data. Either the regulatory authorities asked for this study or the sponsor acted proactively in conducting the study and providing the data. Mice received 25 weekly doses of 0, 10, or 40 mg/kg by IV infusion (study protocol elements are lacking). The study did not find any treatment-related mortality, clinical signs, or histopathological findings, but there was a dose-dependent increased incidence of bilateral crystalline deposits in the lens capsules of male mice. The relevance of this finding to humans is not known.

Genotoxicity assays are not required for monoclonal antibodies, but infliximab is another example of the studies nevertheless being conducted. Infliximab was evaluated in the AMES assay, an in vitro chromosome aberration assay with human lymphocytes, and an in vivo mouse micronucleus test. Infliximab was nongenotoxic in all of these assays.

Carcinogenicity bioassays also are not typically required for monoclonal antibodies, yet this has been an area of interest for Remicade and the progression of the package labeling in the PDR is notable. The Remicade package insert in the 2000 PDR for Crohn's disease states that long-term animal studies to evaluate carcinogenicity have not been conducted (29). However, the labeling does make reference in the precautions and adverse reactions to the patient population being at risk for the development of lymphoma and the unknown impact of Remicade on the development of malignancy. In the Remicade package labeling in the 2002 PDR for the treatment of both Crohn's disease and rheumatoid arthritis, the precautions includes the following statement "Tumorigenicity studies in mice deficient in TNFα demonstrated no increase in tumors when challenged with known tumor initiators and/or promoters" (30). Interestingly enough, this labeling statement may have come from the submission of literature data and not specific studies conducted by the sponsor. This conclusion is based on the EMEA Scientific Discussion, which mentions that information from studies with TNFα knock-out mice and mice treated with murine anti-TNFα does not provide evidence of an increased risk of tumor development. There is no reference to a specific study with the murine cV1q MAb. Regardless, it is interesting that the sponsor was able to obtain on the package labeling this specific reference to lack of carcinogenicity in laboratory animals deficient in TNFα. The Remicade labeling pertaining to carcinogenicity was again revised in the 2003 PDR (28). This labeling would indicate that the 6-month chronic study of cV1q in mice discussed previously was actually a tumorigenicity study; although tumorigenicity is not mentioned in the EMEA Scientific Discussion Document comments on this 6-month study. As the dose levels and duration are the same, this is likely one and the same study. The Remicade labeling in the 2003 PDR has dropped the reference to the lack of tumorigenicity in TNFα-deficient mice provided in the 2002 PDR and stated that a 6-month repeated-dose study with cV1q anti-mouse TNFα found no indication of tumorigenicity in mice.

In summary, the lack of cross-reactivity and a relevant animal species, the expanded clinical population, and the extended clinical dosing regimen provide for a fascinating case study of Remicade. Remicade was first approved for use in patients with Crohn's disease in which a total of three doses of the biologic were to be administered. As infliximab was found to be cross-reactive only with TNFα in chimpanzee, an endangered species, the regulatory authorities did not have serious issues with the lack of repeated-dose administration nonclinical

toxicity data to support the short duration of clinical exposures. Nevertheless, the sponsor was concerned with the pregnancy category labeling and developed a murine surrogate for use in reproductive toxicity testing in mice. The negative data generated in these studies allowed the sponsor to use a pregnancy category B in the labeling package insert. It is interesting to note that in moving Remicade forward in the treatment of rheumatoid arthritis, the availability of safety data for the biologic in thousands of Crohn's disease patients did not preclude the sponsor from conducting a chronic toxicity/tumorigenicity study of the murine analogue. The submission of novel carcinogenicity studies not involving administration of the biologic in question to support a labeling statement is also a remarkable component of this case study, as is the progression in the PDR (consecutive years) of the studies included in the carcinogenicity section of the package labeling.

CONCLUSIONS

Drug development is a dynamic, ever-changing process that is not easily compartmentalized into a checklist of nonclinical toxicity studies that should be conducted as one moves toward marketing approval. Despite the challenges in study design, the questions to be addressed in these studies generally remain the same. Characterization of target organs, dose–response relationships and correlative systemic exposures, and reversibility of any observed effects are all major issues that must be addressed in laboratory animals prior to administration of the drug in the first phase I clinical trials. The safety of the study drug continues to be investigated by the toxicologist during the conduct of phase II and phase III clinical trials. Nonclinical toxicity studies of greater duration and specialized toxicity studies investigating the potential of the drug to affect, for example, fertility and carcinogenicity, are conducted to provide additional support for the safety of the drug as it is administered in clinical trials utilizing more prolonged exposures and/or greater numbers of subjects. Subsequent to the completion of these nonclinical toxicity studies, the toxicologist will complete an overall assessment of the safety of the drug, based on the nonclinical toxicity data, and will provide the pertinent toxicity data to be included in the package insert of the product.

The design of the overall nonclinical package of toxicity studies and the conduct of the individual studies is best thought of as an art form rather than a static exercise in standardized studies. The toxicologist must tailor each study to fit the intended clinical population and the properties of the drug or biologic in question. The case studies provided in this chapter provide excellent examples of the differences in the package of studies submitted for approval. In this context, a fascinating range of nonclinical studies and study packages were explored, from Celebrex, a drug with activity in a wide range of animal species intended to treat non–life threatening indications of rheumatoid arthritis and/or acute and chronic pain in a potentially large patient population with intermittent drug exposures over a lifetime, to Rituxan, a biologic with restricted activity in animal species intended to treat patients with life-threatening B cell nonHodgkin lymphoma. The toxicity studies, individually and in the sum package of studies, have posed challenges for the toxicologist, whether it be in study design, conduct of the in-life segment of the study, interpretation of the study results, and/or applicability to the clinical population.

In the past, regulatory authorities from the different regions of the world have added their different views regarding both the overall package of studies required to support clinical trials and the protocol elements of the studies themselves, further complicating the already dynamic nature inherent in the design and conduct of the package of nonclinical studies. To confront these differences in viewpoints, the formation of ICH has been an invaluable endeavor to provide guidances in the safety assessment of a drug as well as in the areas of quality and clinical trials. The application of these guidances has eliminated redundancies across the three major regions comprised of the US, Europe, and Japan. Equally important, ICH has addressed the science behind the toxicity studies and has opened a constructive dialogue among regulatory authorities and the pharmaceutical industry. The work accomplished by ICH has been significant and represents important contributions from both sides. Just as drug development is an ever-changing process, so too will ICH continue to evolve and address areas of concern, all the while with an eye to the goal of improving human health.

APPENDIX

Websites of Interest

ICH: http://www.ich.org/
EC: http://pharmacos.eudra.org/
EMEA: http://www.emea.eu.int/
EFPIA: http://www.efpia.org/
MHLW: http://www.mhlw.go.jp/english/
JPMA: http://www.jpma.or.jp/12english/
FDA: http://www.fda.gov/
FDA CDER Guidance Documents: http://www.fda.gov/cder/guidance/index.htm
FDA CBER Guidance Documents: http://www.fda.gov/cber/guidelines.htm
FDA Redbook 2000: http://www.cfsan.fda.gov/~redbook/red-toca.html
Federal Register Online: http://www.access.gpo.gov/su_docs/aces/aces140.html
PhRMA: http://www.phrma.org/
IFPMA: http://www.ifpma.org/
WHO: http://www.who.int/en/
EFTA: http://secretariat.efta.int/
TPD: http://www.hc-sc.gc.ca/hpb-dgps/therapeut/htmleng/index.html
Medicines Control Agency (MCA) of the United Kingdom Department of Health: http://www.mca.gov.uk/
Therapeutic Goods Administration (TGA) of the Australian Department of Health and Ageing: http://www.health.gov.au/tga/index.htm

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15. ICH Topic S2A Document. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Step 4. July 19, 1995.
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10 Application of Pathology in Safety Assessment

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INTRODUCTION

Positioning of Pathology

Pathology contributes to two main aspects of toxicological studies, namely, results from

- clinical pathology for detection of *biochemical toxicity* and
- postmortem examinations for detection of *structural toxicity*.

Pathological investigations are a cornerstone for the assessment of the general toxicity of a compound, and postmortem examinations are *the* endpoint in lifetime rodent bioassays (carcinogenicity studies) (1). Pathology has also become increasingly important for research support (2–5), and histopathological examinations are performed for the clarification of certain macroscopic findings in reproductive toxicology studies (not covered in this chapter) (6).

Pathology is a holistic investigative tool, which allows conclusions to be drawn as to the overall integrity or sickness of an individual. In-life observations and clinical pathology findings in blood, serum, and urine are important elements of a toxicity study. However, they are rarely the basis for crucial decisions such as discontinuation of development of a compound in the absence of corresponding pathological findings. Exceptions include electrocardiographic findings (in particular QT prolongation) and seizures. Both of these changes are related to *functional toxicity*, where pathological alterations are minimal or not detectable with conventional techniques.

Pathologists often act as study directors of toxicity evaluations and particularly of lifetime bioassays. In cases where the duties are limited to clinical and/or postmortem pathology, close interaction between the study director and the pathologist is a valuable necessity. The pathologist should be involved from the planning to the reporting of the study and must be ready to answer questions posed by regulatory authorities, especially, but not only in the case of carcinogenicity studies (Table 1) (7).

This chapter is written mainly for nonpathologists to provide them with an overview of procedures used in postmortem toxicologic pathology, to increase their understanding of the terminology used by pathologists, and to ease their collaboration with pathologists during the conduct and evaluation of toxicity studies.

Pathology Methods

Clinical Pathology

Clinical pathology investigations are related to blood cells (hematology), clinical chemistry analysis of blood (e.g., pattern and amount of enzymes leaking from organs or tissues) and urine (urinalysis), and investigation of the coagulation of blood (8). A selection of important clinical pathology parameters is provided in Tables 2A–C.

Table 1 Involvement of the Study Pathologist During Different Phases of a Toxicity/Carcinogenicity Study[a]

Phase	Involvement of pathologist
Study planning	Contributes to design of the study including dose selection and parameters to be investigated, particularly in view of previous pathological findings
Study conduct	Works closely with study director. Needs to see important in-life observations
Necropsy (macroscopic inspection, organ weights, sampling)	Supervises necropsy generally conducted by experienced technicians
Histotechnology	Supervises especially trimming generally conducted by experienced technicians
Histopathological evaluation	Conducts evaluation and records findings Correlates pathological findings in different organs and with in-life observations including clinical pathology results
Report writing	Writes pathology section of report and contributes to the overall assessment of the findings
Preparation of registration documents	Reviews the toxicology-related part of the registration documentation, in particular, the final toxicology summary and assessment
Answering of regulatory queries	Contributes to and reviews the answers to regulatory queries related to general toxicology

[a]Shown is the minimal involvement. The pathologist may also act as study director, final report writer, and responsible scientist for writing the toxicology-related registration documents (2, 4, 6, 38).

Table 2A Clinical Pathology—Hematology

Code	Parameter	Method of determination (example)	Unit
Hb	Hemoglobin concentration	Spectrophotometric measurement of cyanmethemoglobin	gr/dL *or* mmol/L
RBC	Red blood cell count	Flow cytometry	10^{12}/L *or* t/L
PCV	Packed cell volume	Calculated (PCV = MCV × RBC × 10) or measured by sedimentation	%
RETA	Reticulocytes	Supravital staining with oxazin 750, assessed by flow cytometry	% of RBC
RET ABS	Absolute reticulocytes	(RBC × RETA) ÷ 100	10^{12}/L *or* t/L
MCV	Mean cell volume	Flow cytometry	fl
MCH	Mean cell hemoglobin	MCH = (Hb ÷ RBC) × 10	pg *or* fmol
MCHC	Mean cell hemoglobin concentration	MCHC = (Hb ÷ PCV) × 100	gr/dL *or* mmol/L
RDW	Red cell distribution width	Flow cytometry	%
PLAT	Platelets	Flow cytometry	10^9/L *or* g/L
MPV	Mean platelet volume	Flow cytometry	fl
PT	Prothrombin time	Fibrin formation using rabbit brain thromboplastin to activate coagulation cascade	sec
APTT	Activated partial thromboplastin time	Fibrin formation using micronized silica to activate coagulation cascade	sec
WBC	White blood cell count	Flow cytometry	10^9/L *or* g/L
N	Neutrophils	Manual:	% of WBC
L	Lymphocytes	Visual appraisal of a blood smear using a Romanowski-type stain	*or* g/L
M	Monocytes		
E	Eosinophils	Automated:	
B	Basophils	Cytochemical staining with a peroxidase stain and detection of light scatter using a photo-optical method; basophils enumerated using differential lysis and laser light scattering	
LUC	Large unstained cells		

Reference values depend in particular on species, strain and age of animals. For reference values see Ref. 311. Some labs use different units e.g. for concentrations (e.g. RBC: 10^6/mm^3)

Table 2B Clinical Pathology—Clinical Blood Chemistry

Code	Parameter	Method of determination (example)	Increased (examples)	Decreased (examples)
AM	Amylase*	Enzyme activity measured photometrically as NAD-NADH concentrations	Pancreatic diseases	Usually not clinically significant
γ-GT	γ-glutamyltransferase*	Quantitative enzyme detection method	Hepatic disease Generally parallels ALK PHOS in hepatobiliary diseases Cholestasis Hepatic fibrosis	Not clinically significant
AST	Aspartate aminotransferase	Optimized UV method using L-aspartate and alpha-oxoglutarate as primary substrates	Hepatitis Hepatic necrosis Skeletal muscle damage Myocardial necrosis Acute pancreatitis Hemolysis (artefact)	Not clinically significant
ALT	Alanine aminotransferase	Optimized UV method using L-alanine and alpha-oxoglutarate as primary substrates	Hepatitis Hepatic necrosis Cholestasis Hepatic neoplasms	Not clinically significant
ALK PHOS	Alkaline phosphatase	Colorimetric method using p-nitrophenyl phosphate as substrate	Steroids Cholestatic disorders Acute pancreatitis Neoplasms of or in bone Diabetes mellitus Certain drugs (e.g. anticonvulsants)	Not clinically significant
CK	Creatinine kinase*	Enzyme activity measured photometrically as NAD-NADH concentrations	Seizures Myocardial necrosis Intramuscular injections Hemolysis (artefact)	Not clinically significant Lack of refrigeration of serum
GLDH	Glutamic dehydrogenase*	Quantitative enzyme detection method based on NADH concentrations	Hepatic necrosis/inflammation Bile duct obstruction	Not clinically significant
Na	Sodium	Ion-selective electrode	Hemolysis (except dogs) Adrenocortical hyperfunction	Hemolysis (in dogs) Excessive loss (e.g. hypoadrenocorticism, vomiting, diarrhea, renal failure)

*Special investigations, as needed

(*Continued*)

Table 2B (*Continued*)

Code	Parameter	Method of determination (example)	Increased (examples)	Decreased (examples)
K	Potassium	Ion-selective electrode	Hypoadrenocorticism (Addison's) disease Decreased excretion (e.g. renal failure) Improper sample collection and handling (hemolysis in some species)	Hyperaldosteronism
Cl	Chloride	Ion-selective electrode	All causes of increased sodium	All causes of decreased sodium
Ca	Calcium	Colorimetric method using o-cresolphthalein complexone in alkaline solution	Primary hyperparathyroidism (e.g. because of parathyroid adenoma or carcinoma)	Malabsorption Pancreatic necrosis Renal secondary hyperparathyroidism Hemolysis (artefact)
IN PHOS	Inorganic phosphorous	Colorimetric method based upon ammonium molybdate in acid conditions	Decreased renal clearance (e.g. renal disease/failure, hypoparathyroidism) Hemolysis or aging RBCs in sample	Malabsorption Hyperparathyroidism (primary or secondary)
Mg	Magnesium	Colorimetric method with Calmagit	Renal failure Adrenocortical insufficiency	Decreased absorption, reduced feeding) Renal loss (e.g. increased diuresis)
UREA	Urea	UV method using a coupled urease procedure	Pre-renal azotemia (e.g. dehydration, hypoadrenocorticism) Renal azotemia (kidney failure) Post-renal azotemia (e.g. obstruction)	Decreased synthesis (e.g. severe hepatic disease)
T BILI	Total bilirubin	A colorimetric method. Indirect bilirubin is liberated by detergent.Total bilirubin is coupled with a diazonium compound to give corresponding azobilirubin	Hemolysis Hepatic disease (e.g. cholestasis hepatocellular injury/failure) Fasting	Exposure of serum to sunlight Decreased RBC production
CREAT	Creatinine	Colorimetric method	Decreased renal perfusion (e.g. dehydration, hypoadrenocorticism, heart failure) Renal azotemia (kidney failure) Post-renal azotemia (e.g. obstruction)	Not clinically significant
T PROT	Total protein *plus* generally electrophoretic investigation of proteins	Colorimetric method based on Biuret reaction	Monoclonal gammopathies (e.g. multiple myeloma) Polyclonal gammopathy (e.g. infections, hepatic disease) Lipemia (artefact) Hemolysis (artefact)	Chronic hepatic disease Malabsorption Nephrosis Glomerulonephritis
ALBUMIN (A)	Albumin	Colorimetric method based on the BCG reaction	Dehydration Lipemia (artefact)	Increased loss (e.g. nephritic syndrome, glomerulonephritis) Vasculitis Decreased production (e.g. chronic hepatic disease)

GLOBULIN (G)	Globulin	globulin = total protein – albumin	Polyclonal gammopathy (e.g. infections or parasites) Monoclonal gammopathy (e.g. myeloma) Hepatopathy	Decreased production (e.g. hepatic disease, immunodeficiency)
A:G RATIO	Albumin/globulin ratio	AG ratio = albumin /(total protein – albumin)	Hyperalbuminemia with normal or low globulins	Hyperglobulinemia with normal or low albumin
TOT CHOL	Total cholesterol	Enzymatic method using cholesterol oxidase/esterase	Hypothyroidism Increased fat mobilization (e.g. diabetes mellitus, hyperadrenocorticism), pancreatitis, cholestatic diseases, nephrotic syndrome Recently fed; high fat diet (usually only mild increase)	Decreased uptake (e.g. low fat diet, malabsorption) Decreased production (e.g. advanced hepatic disease) Increased loss or catabolism (e.g. protein-losing enteropathy, hyperthyroidism)
TG	Triglycerides	Colorimetric method using peroxidase	Hepatic disorders incl. biliary obstruction Hypothyroidism (see also under TOT CHOL)	Estrogens Spironolactone Other reasons see also under TOT CHOL
GLUC	Glucose	UV method using a coupled hexokinase procedure	Transitory (stress) Diabetes mellitus, pancreatitis, hyperadrenocorticism, epinephrine, pituitary neoplasm, pheochromocytoma, hyperthyroidism	Adrenal insufficiency Hyperinsulinism (insulinoma) Hepatic disease
Hormones				
• *Peptide hormones*	Luteinizing hormone (FSH) Follicle stimulating hormone (FSH) Thyroid stimulating hormone (TSH) Adrenocorticotropic hormone (ACTH) Growth hormone (GH) Etc.	Radioimmunoassay (RIA)	Hypertrophy, hyperplasia and neoplasia of organ, where hormone is produced	Primary or secondary atrophy of pituitary or of organ, where hormone is produced
• *Steroid hormones and others*	Estrogens Progesterone Testosterone Triiodothyronine (T3) Thyroxin (T4)	Radioimmunoassay (RIA) Enzyme-linked immunoassay (ELISA)	Hypertrophy, hyperplasia and neoplasia of pituitary Hypertrophy, hyperplasia and neoplasia of organ, where hormone is produced	Primary or secondary atrophy of pituitary or organ, where hormone is produced

Reference values depend in particular on species, strain and age of animals. See also (8, 10, 11, 308, 312–314 in particular 311)

Table 2C Clinical Pathology—Urinalysis

Code	Parameter	Analyser/Reagent/Kit (examples)	Interpretation
VOL	Volume	Measuring cylinder	Low toxicological significance
SP GR	Specific gravity	Observed using a clinical refractometer	Geneally the higher the volume, the lower the specific gravity
pH	pH	Dip sticks	Low toxicological significance
PROT	Protein	Dip sticks	Present only in case of kidney disease (glomerular leakage)
GLUC	Glucose	Dip sticks	Present only in case of hyperglykemia
RED SUB	Reducing substances	Clinitest	Positive e.g., in case of diabetes mellitus, galactosemia, etc.
KET	Ketones	Dip sticks	Generally only present in fasting condition
UROBIL	Urobilinogen	Dip sticks	Increased in case of hepatic diseases associated with decreased bile secretion
BILI	Bilirubin	Dip sticks	Increased in case of hepatic diseases associated with decreased bile secretion
BL	Blood	Dip sticks	Present, e.g., in case of kidney disease (e.g., glomerulopathy)
RBC	Red blood cells	Microscopy	RBC in case of glomerular disease or bleeding into urinary system
WBC	White blood cells		WBC, e.g., in case of infections of the urinary tract
EPITH	Epithelial cells		Increased number of epithelial cells, e.g., in case of urinary tract infection
SPERM	Sperm cells	Microscopy	Without toxicological significance
CASTS	Casts	Microscopy	Protein casts (amorphous) in case of proteinuria Cellular casts (granular) in case of increased cellular components in the urine (see above)
PHOS	Phosphates	Microscopy	Increased occurrence under various conditions
URATE	Urates		
UR AC	Uric acid		
Ca OX	Calcium oxalate		
AM DEB	Amorphous debris		
BACT	Bacteria		

For reference values see in particular (311).

Investigation of clinical pathology parameters are undertaken as follows:

- Nonrodents (all animals and all doses): Pretest, during treatment (see below), at the end of treatment, and, if applicable, at the end of the treatment-free recovery period.
- Rodents: Specialized investigations can be limited to a subset of animals at each dose level and control groups or to top dose and control groups during treatment (see below), at the end of treatment, and, if applicable, at the end of the treatment-free recovery period.

Investigation during treatment means sampling at appropriately spaced intervals (e.g., sample after three months for six-month chronic toxicity studies). For details see specialized textbooks and articles (9–11).

Postmortem Investigations

Postmortem investigations start with a macroscopic inspection of the carcass and visual observation of the internal organs and tissues upon dissection. With exceptions (e.g., acute toxicity studies), macroscopic inspection is followed by microscopic evaluation of samples of organs, tissues, cells, and cellular components in tissue sections or smears by means of a light or electron

microscope. Microscopic inspections need histological processing of organ/tissue sections (for details see "Technical Postmortem Procedures").

For answering specific questions, microscopic investigations can be complemented by special methods:

- *Morphometry* to count numbers (e.g., cells per surface area) or dimensions such as diameters and volumes (12–15). Digital image analysis has greatly simplified morphometric measurements by allowing some automation (16). Nevertheless, morphometrical measurements remain time consuming and cannot be regarded as a routine method. Morphometrical measurements are done on cells in their tissue context and allow estimation of various parameters concomitantly. In particular, digital image analysis also allows assessment of texture characteristics (e.g., nuclei) that are related, among others, to nuclear activity. One of the most important applications of morphometry is determination of the no-effect level, for example, with regard to hypertrophy and/or hyperplasia in critical cases.
- *Flow cytometry* to measure ploidy (number of chromosome sets) and receptor density and to classify immune cells (17–19). Flow cytometric measurements are performed on a large number of cells and various parameters are assessed simultaneously and in a short time. The cells can be sorted (e.g., by receptor type) and used for further analysis. The disadvantage of flow cytometry is that cells have to be isolated under disruption of the tissue architecture and that preparation techniques are not trivial.
- *Microdissection techniques* (20, 21) to obtain microscopically well-defined samples of complex tissues (e.g., of single cells) for special investigations including *molecular analyses* (22).

Molecular techniques are important tools for modern pathologists to investigate special questions mainly related to the presence of certain genes (e.g., viral genes) or the expression of genes (gene activation, gene transcription, protein synthesis) (23–25) and include, among others, the following

- *Polymerase chain reaction (PCR)* for rapid synthesis of large quantities of DNA fragments using primers and DNA or RNA isolated from tissues and fluid of interest. It involves a repeated process of separating the two complementary DNA strands by heat and synthesizing new complementary DNA strands from each single strand. For example, the synthesized copies are used for in situ hybridization, that is, for rendering visible the occurrence and localization of DNA or RNA that contains that particular sequence.
- *Blotting techniques:* Gel electrophoresis for separation and detection of RNA fragments (Northern blot), DNA fragments (Southern blot), and proteins (Western blot) isolated from diseased tissues and compared to standard probes with regard to their electrophoretic characteristics.
- *Toxicogenomics and proteomics* to investigate aspects of gene expression (22,26–31).

TECHNICAL POSTMORTEM PROCEDURES

Introduction

This section provides a general description of routine techniques. The procedures need to be adapted depending on the study type. The main determinants for tissue sampling, processing, and histopathological investigations are as follows:

- *Study duration:* For *acute* studies, histopathological investigations are limited to unclear macroscopic findings because histopathological findings are generally unspecific (e.g., necrosis, edema) and reflect acute intoxication with failure of various organs. For *subacute/subchronic* (one to three months) and *chronic* (six to nine months) toxicity studies, extensive histopathological investigations are carried out according to regulatory requirements, as described in this chapter. This also applies to *lifetime bioassays*, which include additional organs generally not sampled in studies of shorter duration (e.g., lachrymal gland) and additional samples (e.g., additional liver sections).
- *Route of application:* The application site has to be sampled and evaluated carefully. Examples include, for example, skin (dermal application), muscle (intramuscular application), subcutaneous tissue (subcutaneous application), vein (intravenous application) (32), nasal cavity

(inhalation studies by nasal application), or implantation site (implantation, e.g., of chips, etc.) (33).

- *Type of tested compound, device, etc.:* In addition to conventional chemical entities, newer types of test material include skin equivalents (34), humanized monoclonal antibodies (mAb) (35), synthetic oligonucleotides (36), gene therapy (37), and other biotechnology products (38, 39).
- *Species:* Organ/tissue sampling procedures are significantly influenced by the size of the animal and its organs. While cross sections of the whole left and right liver lobe are taken for rodents, samples, which are much smaller than the whole organ, are preserved from different liver lobes of dogs.
- *Purpose of investigation:* While for a standard toxicity study the procedures are regulated mostly according to ICH guidelines (chapter 9), the investigator running a mechanistic study for elucidation of unclear findings in a previous study is free to concentrate on the most important parameters and organs/tissues.

The awareness that immunotoxicity can be an issue has significantly increased over the more recent years and requires careful pathological investigation of the hemo-lymphoreticular system, at times complemented by functional tests (40–42).

Necropsy

All animals in a toxicity study or lifetime bioassay are subjected to a postmortem necropsy. After euthanasia, the animal is generally exsanguinated. Consistent bleeding is important for achieving reproducible organ weights. The animal is then placed on its back and the abdominal and thoracic cavities are widely opened. Experienced technicians generally carry out dissection, but a pathologist must be available for the examination of unclear findings. Macroscopic inspection of the corpse, the organs, and the contents (if any) in cavities is very important, as lesions once missed macroscopically are generally not preserved and therefore unavailable for histological examination.

A selection of important organs is weighed (for details see Table 3) using animals from toxicity studies but usually not from animals of lifetime bioassays. For reliable organ weights, organs have to be cleaned carefully from fat and adhering water and blood (43). Organ weights are easy to record and can be very helpful, for example, liver weights may be more sensitive than qualitative histopathological evaluation; liver cell hypertrophy can be easily missed under the microscope, as a volume increase of, for example, 20% corresponds to only $\sqrt[3]{1.2}$ in linear dimension, which cannot be recognized without morphometric measurements.

Table 3 also lists *standard organ/tissues* to be sampled according to study type and duration. In addition to this standard list, all macroscopic findings must be sampled.

Sampling procedures influence the result of the histopathological procedure significantly. For example, the number and size of pancreatic islets regulating blood sugar vary according to location within the pancreas. The prostate has various distinct regions that react differently to toxic injuries and to age. Particularly in rodent species, lesions tend to be small and are, therefore, often detected only histologically. It is evident that the size of the sample influences the probability of such a lesion being detected. These few examples illustrate that it is of utmost importance that consistent sampling procedures and techniques are used throughout the study and across all dose groups and sexes. Standard sampling procedures are available in the literature (44–46). Special procedures are at times necessary for special investigations, such as investigations of nerves (e.g., teased fiber technique) (47).

In addition to organ/tissue samples, smears (e.g., of bone marrow or of a turbid exudate in body cavities) can be crucial for the correct diagnosis of a condition. Smears need to be taken at necropsy on unfixed fresh material.

Histotechnology

Organs/tissues as specified in Table 3 should be examined histologically as follows:

- Rodents

  Toxicity studies: At least top dose and control group. Examination of organs/tissues from lower dose groups may be limited to gross lesions and to organs/tissues showing possibly treatment-related lesions in the top dose.

Table 3 Organ List

Organ	Remarks	Organ weights
Adrenal glands		+
Aorta		
Blood smears	Not required in lifetime bioassays, but may be very useful to diagnosing proliferative hematological disorders	
Bone with bone marrow and joint with cartilage	Generally femur; in toxicity studies often complete joint preserved and investigated	
Brain	At least three levels to include medulla/pons, cerebellum, and cerebellum	+
Epididymides		(+) sometimes with testes
Esophagus		
Eyes with optic nerves	In rodents the adjacent Harderian gland should be preserved, at least in lifetime bioassays for histological investigation, if needed	
Gallbladder	In mice (with liver), dogs, and monkeys	
Heart		+
Kidneys including urether		+
Lacrimal gland		
Large intestine (cecum, colon)	Rectum should be preserved for lifetime bioassays for histological investigation, if needed Including Peyer's patches if relevant	
Larynx	At least in lifetime bioassays	
Liver		+
Lung with bronchi and bronchioli		+ inhalation studies
Lymph nodes (representative LN at least at two locations)	e.g., mesenteric LN in case of infeed/gavage studies, bronchial LN in case of inhalation studies	
Mammary glands	Both sexes; in males, subcutaneous tissue at the site of mammary glands to be preserved for histological investigation, if needed	
Nasal cavity, nasopharynx, and paranasal sinuses	In inhalation studies; for other application routes at least in lifetime bioassays to be preserved for histological investigation, if needed	
Nerve, peripheral	e.g., sciatic	
Ovaries		+
Pancreas		
Parathyroid glands	In rodents preserved and examined together with thyroid	
Pituitary gland		+
Preputial/clitorial glands	Lifetime bioassays only	
Prostate		(+)
Salivary glands (mandibular, parotid, sublingual)	For lifetime bioassays to be preserved for histological investigation, if needd	(+)
Seminal vesicles with coagulating glands	Rodents only	(+)
Spinal cord	Generally two to three levels	
Skeletal muscle	e.g., biceps femoris	
Skin/subcutaneous tissue		
Small intestine (duodenum, ileum, jejunum)		
Spleen		+
Stomach (forestomach of rodents and glandular stomach)		
Testes		+
Thymus		+
Thyroid gland		+

(*Continued*)

Table 3 Organ List (*Continued*)

Organ	Remarks	Organ weights
Tongue		
Trachea		
Urinary bladder		
Uterus with uterine cervix and oviducts		+
Vagina		
Zymbal's gland with external ear	Should be preserved, at least for lifetime bioassays for histological investigation, if needed	
Gross lesions including masses, target organs, application sites		

[a] The organs listed generally need to be preserved during necropsy and investigated histologically for all types of studies (with the exception of acute studies) and all species, unless otherwise indicated.
Note: Organ weights are generally not recorded for lifetime bioassays.
+ organ weight needed.
(+) organ weight optional.
For details see also Refs. (45, 315–320).

Lifetime bioassays: Usually all animals (terminal sacrifice, intermediate deaths, and termination in extremis) from all control and dose groups.

- Nonrodents: From all animals and all dose groups including control groups.

The following section provides a brief overview of histotechnical procedures, as far as they are important for a basic understanding of the preparation of organs and tissues for microscopic evaluation.

Histotechnology comprises the following steps (48,49):

- Fixation of organs/tissues to prevent autolysis.
- Trimming of the hardened fixed samples to appropriate size (difficult with unfixed "soft" organs/tissues).
- Dehydration of the sample, that is, replacement of the water in the sample with an organic solvent.
- Embedding for light microscopic investigations, most commonly in paraffin wax to increase the consistency of the organ/tissue and thus facilitating the histological sectioning.
- Histological sectioning.
- Removing of the embedding material to allow coloration of the sections.
- Coloration of the section to increase contrast between different cellular components.
- Mounting the section on glass slides (for light microscopy) and placing a coverslip on the section for protection and to avoid uneven surfaces distorting the optical image.

Each of these steps is explained in more detail in the following text.

To prevent autolysis, proteins of organs and tissues have to be denatured (cross-linked) by so-called *fixatives*. The most common fixative is 40% aqueous paraformaldehyde called formalin, often in a buffered neutral solution. Compared to alternatives such as Bouin's solution, formalin offers the advantage that the tissue can be left in the formalin solution without becoming too hard. Generally, organs and tissues are immersed in formalin, which then penetrates into the organ/tissue at the rate of a few millimeters over 24 hours. Therefore, samples must be reasonably thin (less than one centimeter) to allow a rapid and, therefore, good fixation. Other fixatives (e.g., glutaraldehyde) penetrate tissues at a much slower rate, which allows fixing only thin organ slices. The big advantage of the latter fixative is the much better preservation of the cellular microarchitecture, which makes it the preferred fixative for electron microscopy (EM). In contrast, because of unequal shrinkage of cellular components, formalin creates some artifacts (clefts) in tissues. Such artifacts are generally acceptable in most organs. However, particularly for the following organs and for special investigations, alternative fixatives are used such as

various formulas of Zenker's or Davidson's fluid (50) for eyes and bone marrow, Bouin's or modified Davidson's fluid (50,51) fixative for testes or a mixture of formalin and glutaraldehyde for nervous tissue (52). Lungs are often fixed by intratracheal instillation of formalin (53).

Vascular perfusion of the whole animal or parts of it with fixative yields much better results than immersion, as denaturation of proteins occurs almost instantly. Fixation by vascular perfusion is particularly meaningful for special morphological investigations of delicate organs such as the testis (using, e.g., Bouin's fixative) or for electron-microscopic investigations using glutaraldehyde (54). However, the perfusion technique is time consuming and is not a method of choice for routine toxicity studies.

Once an organ or a tissue is fixed, it can be *trimmed* into a smaller piece that contains the region of interest (generally at the most 15 × 20 × 5 mm). Bones have to be decalcified to allow their sectioning. For the reasons mentioned under sampling procedures, it is important that trimming is done consistently. Standard sampling procedures are available in the literature (44–46,55). The water naturally present in organ/tissue samples has to be replaced generally by a liquid, in which the embedding material is soluble (usually an organic solvent). This process is called *dehydration.*

The trimmed samples are then *embedded* with paraffin wax or a resin. Paraffin is the most commonly used embedding material; it is cheap, easy to handle, and absolutely adequate for routine purposes. To embed a tissue/organ, it is immersed in liquid paraffin of approximately 58°C. This temperature results in some distortion of the tissue architecture that can be avoided by using resins, which harden at room temperature. The latter also tend to be harder than paraffin, which allows cutting thinner sections. This is particularly important for cutting ultrathin sections for electron microscopic investigations. However, the stainability of resin sections is much lower than that of paraffin sections, mainly because of the difficulty to remove the embedding resin.

If organs/tissues are frozen and then sectioned, no fixation and embedding are needed. However, the quality of frozen section is generally lower than that of fixed and paraffin-embedded material.

Embedded organs/tissues are *sectioned* at a thickness of around 3 to 5 μm using a microtome and the sections are *mounted* on a glass slide. After removal of the embedding material by solvents, the sections are *stained* to increase contrast and to differentiate between different cellular components. Hematoxylin–eosin (H&E) is the most common stain used for routine paraffin sections. A selection of more specialized stains is listed in Table 4. Enzyme/immunohistochemical stains serve for the investigation of cell kinetics (BrdU, Ki 67, etc.) (56,57). In situ hybridization, a tool of molecular pathology, has gained good acceptance in toxicologic pathology to investigate gene expression.

A summary of the basic methods for histopathological preparations is presentedin Table 5.

Evaluation of Histological Slides

Microscopes

The pathologist usually examines histological sections using a transmission *light microscope.* More specialized procedures involve Nomarski illumination, which increases the contrast particularly of membranes and creates the illusion of three-dimensional images. Confocal microscopy is used for optical sectioning of thicker sections (30+ μm) and thus lends itself to the three-dimensional reconstruction of cells and organelles.

Investigation of *fluorescence* is another specialized technique. Fluorescence means that molecules that are excited by light of one particular wavelength emit light of a different wavelength. Fluorescence can occur with substances "naturally" present in cells such as some pigments, or can be introduced with fluorescent labeling of cellular structures and molecules (58). For dark field investigations, only light that is deviated by structures such as crystals enters the microscope.

EM uses an electron beam and magnetic lenses, instead of light and optical lenses. Because of the much smaller wavelength of an electron beam, EM allows much higher magnifications than light microscopy. To take advantage of this, sections need to be extremely thin (in the order of Ångströms) to decrease superposition and loss of resolution. EMs are classified as

Table 4 Basic Histological Stains (Examples)

Stain	Result	Advantage/use	Remarks
Traditional stains			
• **Hematoxylin–eosin (H&E)**	Nuclei: blue Cytoplasm: red	Rapid, easy, cheap Works well on formalin-fixed tissue Universal stain	Routine stain in toxicologic pathology Allows, e.g., to suspect SER proliferation (cytoplasm turns intensively eosinophilic) or foci of cellular alteration (cytoplasm turns blue because of RER proliferation)
• **Periodic acid Schiff's reagent (PAS)**	In combination with hematoxylin: Glycogen: rose/purple Mucin: blue Basement membrane: pink	Particularly for certain storage products and for rendering visible basement membranes	Can also be combined with alcian blue and methenamine silver, etc.
• **Klüver Barrera**	Myelin: greenish-blue Cells: pink/violet	For nerve fibers	
• **Sudan red**	Neural lipids: red	For demonstration of fat storage	Other Sudan dye varieties: Sudan black, etc. Lipid demonstration requires frozen sections
• **Cresyl echt Violet**	Nissl substance: blue	For staining of nerve cell body	
Histochemistry			
• **For acid phosphatase**	Lysosomes: black		Also works for EM
• **For dehydrogenase**	Mitochondria: dark dense precipitate		Also works for EM
Enzyme/immunohistochemistry			
• **Bromodeoxyuridine (BrdU) incorporation**	Newly synthesized DNA	Demonstration of cell kinetics without use of radioactivity	BrdU incorporated into replicating DNA can be traced, e.g., by fluorochrome labeled antibody
• **Ki 67**	Ki67 nuclear antigen expressing cells (= all proliferating cells)	Demonstration of cell kinetics without pretreatment	Ki67 is demonstrated by specific antibodies, which are then visualized, e.g., by a biotinylated second antibody
In situ hybridization	Known DNA sequences produced by PCR are hybridized with homologous sequences expressed in tissues	Demonstration of specific DNA or RNA sequences	Appearance depends on the tracing system

For details see (48, 49, 57, 204, 208, 321).

Table 5 Basic Methods for Histopathological Preparations

Fixative		Embedding material	Thickness (μ)	Characteristics of sections			Field of application
Application	Type			Size	Quality	Staining	
Immersion	Formalin	Paraffin	5–7	Regular	(+)	Normal stains	Routine
	Bouin's	Paraffin	5–7	Regular	+	Normal stains	Routine
		GMA	2	Regular	++	Normal stains	Special cases
Perfusion	Bouin's[a]	GMA	2	Regular	+++	Normal stains	Research
	Glutaraldehyde	Epon, araldite	< 1	$(2–3\ mm)^2$	+++	Toluidine blue	Research

[a]Or mixture of formalin and glutaraldehyde.
Abbreviation: GMA, glycol methacrylate.
See also (261).

transmission EM (similar to transmission light microscopy) and scanning EM, mainly used to investigate surfaces by aiming an electron beam on to the surface of the sample from the observer's side, who then looks at the reflection of this beam (59).

Depending on the organ under investigation and the purpose of the investigation, additional procedures are available, such as three-dimensional visualization of brain lesions by nuclear magnetic resonance (NMR) (60).

Nomenclature

The task of the pathologist is to recognize organ and tissue alterations and to record these findings in a reproducible way (48,61,62). The pathologist uses a standardized nomenclature, which is not limited to describing single findings (see "General Pathology") but which summarizes a physiological or pathological status of the organ/tissue or system by using diagnostic terms of diseases. For example, an abscess at a subcutaneous injection site may consist of necrotic fat tissue in the center with leukocyte infiltration and a leukocyte wall around this center with, depending on the age of the lesion, lymphocytic components, some edema, and possibly the beginning of a connective tissue capsule.

Over the past 30 to 40 years pathologists have agreed to a significant extent on standardized nomenclatures particularly for the diagnostically difficult field of neoplasia (tumors, including precursor lesions such as hyperplasia) (63,64). Examples of standardized nomenclature systems are WHO/IARC/RITA nomenclature: *The Rat* (65), *The Mouse* (66), STP nomenclature (67), IARC nomenclature for rats (68) and mice (69), *Monographs on the Pathology of Laboratory Animals* (70) as well as further books (71,72).

Control Data

For toxicity studies, the pathologist generally examines the animals on an organ-by-organ basis, while for lifetime bioassays the pathologist looks at all organs/tissues per animal. The first approach allows a good comparison between control groups and dose groups and sexes, while the latter provides a better picture of the disease status of any particular animal, which is important for lifetime bioassays.

A toxicity study or rodent lifetime bioassay is an epidemiological study, where findings in dosed animals are compared to those in the control group(s). Therefore, it is important that incidental or background findings and lesions are recorded (48) with regard to their occurrence, severity and, if multiple time points of investigation are available, their first observation and further development. Many findings are not of pathological significance. For example, lymphocytic foci in or under the intestinal mucosa are normal, but their occurrence and extent might be increased in case of irritant compounds. The *concurrent control* is the best control. Sometimes the concurrent control group may show an unusual incidence of a particular lesion (low or high), or rare findings not present in the concurrent control group may be found in one or the other dose group. This is particularly important for the evaluation of results from lifetime bioassays. Under such circumstances one might want to use *historical control data* from the same strain and facility. Ideally, these data should not be older than five to eight years because of the genetic

drift and evolution of diagnostic nomenclature. If insufficient data are available from the same facility, one might consider using data from a *historical control database*. Good historical control databases share the following characteristics (64,**?**–77):

- Quality control (consistent nomenclature, peer review, data check).
- Data on possible modifying factors, such as strain, breeder, husbandry, age of the animals, sampling, and histotechnical procedures.
- Audit trail for changes of nomenclature over time available.
- Data on stability of tumor pattern over time.
- Visual documentation of characteristic lesions in the database is a special asset.

Statistical Testing

Incidences of histopathological observations in *toxicity studies* are generally only analyzed by biostatistical methods (e.g. t test) for their statistical significance in case of a problem with interpretation.

For *lifetime bioassays*, the following tests are routinely used (75,78–83):

- Fischer test: pairwise comparison; good for small numbers.
- Peto test: survival-adjusted trend test (asymptotic form) stratified according to time (often in four periods); distinguishs fatal and nonfatal tumors; needs larger number of animals/test group (50+) (84).
- Exact Peto: combination of Peto and Fischer test; good for small numbers.
- Cochran–Armitage test: trend test (simpler than Peto test); no need to determine cause of death; often used for nontumorous lesions.

It is important to remember that *statistical significance* flags out only a particular finding but does not tell anything about its *biological significance*. Biological significance can only be derived from sound scientific judgments taking into account all information available about the observation, the experiment as such, and scientific knowledge at large.

Quality

Quality assurance is also highly important in pathology. The general good laboratory practice (GLP) rules have to be observed.

The pathologist can judge the *quality of the test animals* with regard to their health, in particular the occurrence of subclinical infections. As will be explained later, the latter are evident from the incidence and size of inflammatory foci (e.g., in livers and lungs).

Tissue accountability is an important factor to judge the quality and validity of toxicity and in particular of lifetime bioassays (48,85). It is influenced by various factors, such as autolysis, age of the animal (e.g., thymus), size of organ, and amount of tissue available and preserved during necropsy and embedded in paraffin blocks. There are no guidelines, but a recommended minimum standard for lifetime bioassay studies is 90%/sex/group with the following exceptions or specifications (74):

- Parathyroid glands: for at least 70%/sex/group sufficient tissue from at least one gland
- Adrenal glands: cortical and medullary tissue available bilaterally in at least 70%/sex/group and unilaterally in 90%/sex/group
- Thymus: for at least 80%/sex/group
- Mammary gland (rat)
  Males: for at least 90%/group, a section of subcutaneous tissue from the mammary area (caudal portion of the ventral abdomen) with or without male mammary gland
  Females: for at least 90%/group subcutaneous tissue with mammary gland components
- Mammary gland (mouse): similar to rat, but for both males and females at least 70%/group

Blinded slide evaluation means evaluation of slides without knowing the treatment group. It is not recommended for routine examinations (86). However, blinded reading of an organ with an equivocal or subtle finding can be very helpful. If done, it should be recorded in the raw data and the study report, and this will enhance the confidence of the regulatory reviewer on the validity of the data (74).

In addition, *peer reviewing* has become a standard procedure in pathology (87–89). Specifically, a pathologist examines a selection of the histological slides of a study. This can mean that 10% of all animals at random plus all target organs are reexamined by the reviewing pathologist. The procedure has to be recorded; in particular, discrepancies and their resolution should be noted.

GENERAL PATHOLOGY

An organism and its organs/tissues have a limited spectrum of reactions to nonphysiological conditions, including toxicity and carcinogenicity.

This section defines the basic pattern of reactions, gives some insight into the pathogenesis of these basic lesions, and addresses their consequences as far as relevant for toxicology.

Type of Lesions

The basic types of pathological lesions are summarized in Table 6 and selected examples from rat studies are shown in Figures 1 to 10 (exception Fig. 2 shows monkey study).

The following lesions are frequently seen in toxicity studies:

- *Regressive alterations* including, cytotoxicity and cell death, the classical endpoints of acute to subacute toxicity.
- *Progressive alterations*, including repair processes following toxic injury as well as adaptive changes such as hypertrophy (increase in cell size) and hyperplasia (increase in cell number). Progressive changes are important particularly in long-term studies, especially in lifetime rodent bioassays. Irrespective of the underlying pathogenesis, cell proliferation observed in shorter-term studies generally indicates an increased likelihood for finding tumors in lifetime rodent bioassays at comparable doses (90–96). This is related among other factors to an increased risk of proliferating cells to become genetically abnormal (point mutations, chromosomal abnormalities, etc.). For example, various substances bind to α2μ- globulin in renal tubules, thereby leading to kidney toxicity with necrosis of single tubular cells and tumors in rats (97,98). However, cell proliferation is not always followed by neoplastic changes (99).
- Signs of *inflammation*, be it as consequence of small infections (particularly in nonrodent species) or following toxic injury with cell death.
- Alterations associated with *metabolic changes*, which includes storage of various endogenous substances (e.g., fat) and of administered chemicals.
- *Circulatory and respiratory disturbances* are less frequent, but can be observed, as in the case of arteritis and phospholipidosis.
- *Malformations* are the endpoint of reproductive toxicity studies (not addressed here), but can also occur spontaneously.

Some Important Subcellular Alterations

Toxic injury may be first seen in the electron microscope at the subcellular level before histopathological changes are evident under the light microscope, which then later might result in macroscopic lesions (see also below). The following organelles react most frequently to exposure to chemicals:

- The *smooth endoplasmic reticulum (SER)*, particularly of the liver, is the site of metabolizing enzymes; among them also those metabolizing exogenous chemicals. Exposure to chemicals is often accompanied by a proliferation of SER. Chemicals of the barbiturate-type can lead to spectacular SER proliferation (14), which microscopically translates as cellular hyperplasia and manifests itself by eosinophilia and at necropsy by increased organ weights. Lifetime studies with significant SER proliferating agents generally result in liver tumors in rodents that are of no significance for man (100).
- *Lysosomes* take up especially exogenous substances by a process called endocytosis. Lysosomes are particularly abundant in cells specialized in phagocytosis (e.g., leukocytes and macrophages). Lysosomal enzymes in these so-called phagolysosomes can often degrade and thus remove the ingested material. If the endocytosed material is not degradable, the resulting phagosomes can accumulate in the cell or be excreted from the cell to be transported by

Table 6 General Pathology

Lesion	Definition	Examples	Regulation/Pathogenesis	Consequences	Remarks/Interpretation
1. Malformations and aberrations	Malformation = teratogenic effect: often a consequence of incomplete organogenesis (6)	e.g., cleft palate	Essentially by exposing germ cells, embryos and, with lower probability, fetuses to chemicals (see textbooks of reproductive toxicity). The molecular basis of malformations is essentially • Genetic alterations (mutation, chromosome aberration, etc.) • Interference with vital cellular processes by chemicals, radiation, etc.	Malformations can be insignificant (e.g., additional toe) to lethal (e.g., missing closure of the neural crest)	As only healthy animals are selected for toxicity and carcinogenicity studies, significant malformations are not seen
	Aberration: e.g., remnants (322) and ectopic tissue (323, 324)	Cyst with respiratory epithelium in thymus	Often spontaneous	Ectopic tissue is generally without physiological significance	—
2. Regressive alterations					
2.1 Atrophy	Loss of substance: e.g., cell volume, organ size, etc.	Age-related atrophy of the thymus and other organs in adult animals	Physiological involution and/or decreasing protein synthesis with age (e.g., because of tighter binding of histones to DNA)	Reduced functional spare capacity of organ	Lymphatic and hemopoietic tissues are regularly atropic in animals from life-time studies
		Pressure atrophy: e.g., atrophic tissue around growing tumor	Among other effects: pressure leads to diminished vascular perfusion	If extensive, can lead to failure of the organ	Formation of tumor capsule, which, however, offers little protection against tumor spread
		Hunger atrophy	Reduction of vital processes, lack of essential nutrients, etc.	Can lead to death	Can occur in high-dose animals of toxicity studies to various degrees
		Hormonally induced atrophy: e.g., steroid-induced atrophy of the lymphatic tissue	Physiological receptor-mediated effect	E.g., increased susceptibility for infections	Partly reversible upon cessation of exposure to substance

Lesion	Definition	Examples	Pathogenesis	Consequences	Remarks/Interpretation
2.2 Apoptosis	Orderly cell death (cell turn-over)	In many organs, e.g., in the intestine	Spontaneous or induced. Starts, e.g., with increased intracellular Ca and activation of endonuclease	Not accompanied by inflammation	Apoptosis is a process to remove old or damaged cells, including neoplastically transformed cells
2.3 Necrosis	Cell death	Any organ	Can be the ultimate consequence of a series of alterations starting with reversible changes (e.g., cellular swelling or fatty accumulation) At the molecular level, disturbance of DNA expression, inhibition of vital enzymes, disturbance of membrane integrity, etc.	Depending on extent and organ, can result in complete or partial reparation	Depending on extent and organ, death of parenchymal cells is always a significant finding. Particularly easy to see with acute toxicity. However, single cell necrosis also occurs in long-term toxicity studies.
3. Progressive alterations					
3.1 Parenchymal regeneration	New cells are of the same kind as replaced cells	Spontaneous shedding of intestinal epithelia and replacement by cells from the crypts	Physiological process to replace, e.g., aging cells	Maintenance of functional integrity; tissue does not change	All cells with exception of nerve cell bodies can regenerate Can be impaired by chemicals, e.g., antimitotic or mutagenic agents
		Cell division in liver following hepatotoxicity with single cell necrosis	Activation of undifferentiated hepatocytes	Restitutio ad integrum, but increased cell proliferation is generally associated with tumor formation in lifetime rodent bioassays	
3.2 Scar	New cells are different from replaced cells	Wound healing	Activation of fibroblasts to form granulation tissue, which produces connective tissue fibers	Incomplete repair	

(*Continued*)

Table 6 General Pathology (*Continued*)

Lesion	Definition	Examples	Pathogenesis	Consequences	Remarks/Interpretation
3.3 Hypertrophy	Increase of cellular volume because of increased organelle numbers	Hepatocellular hypertrophy	Adaptive change generally to cope with increased workload, e.g., metabolism of exogenous substances	Organ weight increased Full reversible, if underlying condition ceases (e.g., following termination of exposure to chemical)	Hypertrophy per se is not a sign of toxicity, but a physiological response (231) Note: Increased cell volume because of excessive storage, e.g., of fat, is not called hypertrophy.
3.4 Hyperplasia	Increase in cell number in an organ Cells have normal appearance	Hepatocellular hyperplasia	Mitogenic stimuli following increased workload, cytotoxicity or genetic toxicity		Generally reversible; hypertrophy and hyperplasia often occur concomitantly and are difficult to distinguish
3.5 Neoplasia	Autonomous growth of a tissue Cells often structurally abnormal (e.g., larger nuclei with different texture, etc.)	Liver cell adenoma	Autonomous mitogenic stimuli with loss of contact inhibition of growth; therefore, compression of surrounding tissue, resulting in capsule formation	May lead to atrophy or necrosis of significant portions of the organ because of compression	Irreversible (though some tumors, which fulfill the histological criteria of a tumor, were found to regress following termination of exposure to chemicals)
		Liver cell carcinoma	In addition, production of proteases, resulting in infiltration into surrounding tissue and vascular vessels leading to distant metastatic foci of tumor cells Activation of oncogenes	Because of infiltrative and rapid growth, often fatal	Irreversible
4. Alterations associated with metabolic changes	Often degenerative changes, where cellular processes become defective	Vacuolar cell degeneration, e.g., of renal tubular epithelia	Barrier function of the cell membranes disturbed, e.g., because of insufficient production of ATP in mitochondria	Initially reversible Can progress to cell death	Edema formation and vacuolar cell degeneration are seen in acute intoxication and conditions such as intestinal ulceration, chronic inflammation, etc.

Lesion	Definition	Examples	Regulation/Pathogenesis	Consequences	Remarks/Interpretation
		Deposits of endogenous or exogenous material	Endogenous material: deposits of pigments (insoluble proteins increasingly formed with age), fat, excessive formation of immunoglobulins (amyloidosis), etc.	In severe cases, cell function can be reduced, e.g., by reducing metabolic exchange between cells and blood	Age-related pigments (lipofuscin) are regularly seen in older test animals. Occurrence and extent are sometimes increased in toxicity studies without obvious consequences
			Exogenous material deposits, e.g., of test compound or its metabolites		
5. Circulatory disturbances	Insufficient perfusion of an organ/tissue with blood	Arteritis	Narrowing of the vascular lumen leads to insufficient perfusion	Infarction of affected organ Can lead to death because of organ failure or be followed by partial restitution (scars)	Circulatory disturbances are not frequent in toxicity studies
		Persistent tachycardia in dogs treated with vasoactive drugs	Vascular perfusion is normal, but insufficient to cover the increased demand for oxygen	Scar formation	Is not predictive for man, as this degree of tachycardia is tolerated by man
6. Respiratory disturbances	Disturbance of the gas exchange in the lungs between the alveoli and the blood vessels	Spontaneous alteration: pneumonia	Bacterial and/or viral infection	Can heal or be lethal	Respiratory disturbances are not frequent in toxicity studies
		Induced, e.g., by mistake during gavage of liquid test formulation into the trachea	Irritation of bronchial and alveolar epithelium by chemical(s)	As above	
7. Inflammation	Characterized by leuco/lymphocytic infiltrates, hyperemia and edema	Abscess formation in subcutaneous tissue, e.g., following s.c. injection Pneumonia (see above)	Infection (bacteria, virus, fungi) Chemicals (irritation) As a consequence of cell necrosis	Can be accompanied by fever. Generally painful and impairing the function of affected organ Often restitutio ad integrum	Inflammation is frequently seen as a secondary phenomenon in toxicity studies

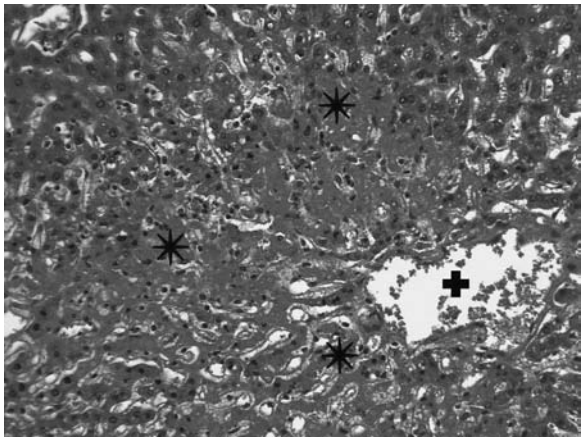

Figure 1 (See color insert) Focal necrosis (liver)—a focus of homogenous eosinophilic (pink) necrotic hepatocytes (∗) adjacent to a lobular central vein (+). Note loss of normal hepatocellular structure and round nuclei, while elongated nuclei of sinusoidal cells lining the blood spaces are still present.

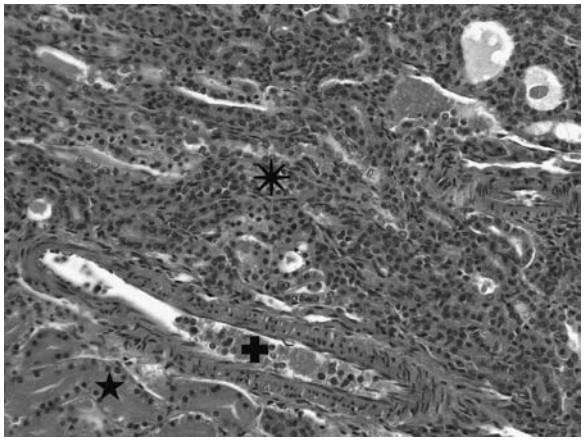

Figure 2 (See color insert) Regenerating proximal renal tubules (kidney)—in contrast to normal eosinophilic tubules (*), regenerating tubules (∗) are basophilic (blue) and cellular (higher cell density). On this microphotograph, normal and regenerating tubules are separated by a renal artery (+).

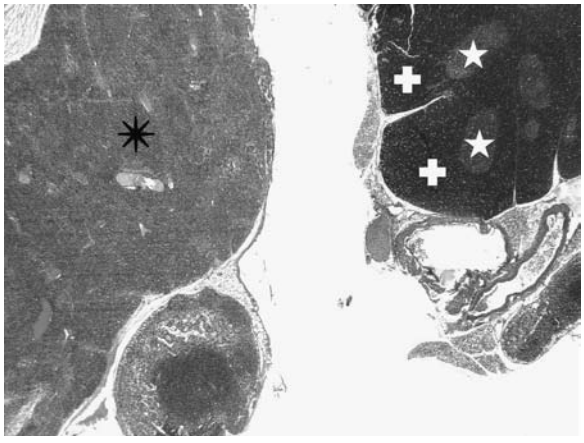

Figure 3 (See color insert) Thymus: atrophy of the lymphatic tissue (*)—note loss of structure in comparison with normal thymus, where cortex (+) and medulla (∗) can be clearly distinguished.

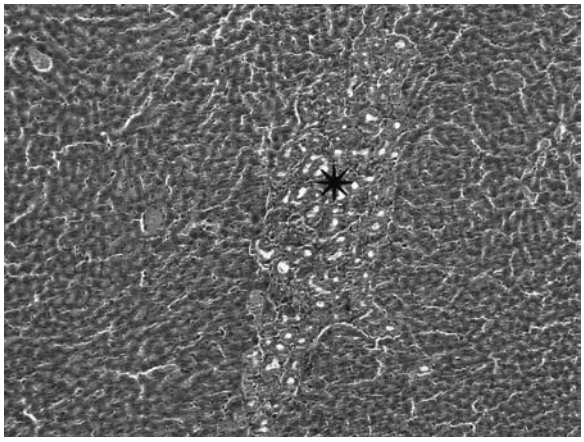

Figure 4 (See color insert) Bile duct hyperplasia (liver)—a focus of proliferating bile ducts (∗) surrounded by normal hepatic parenchyma.

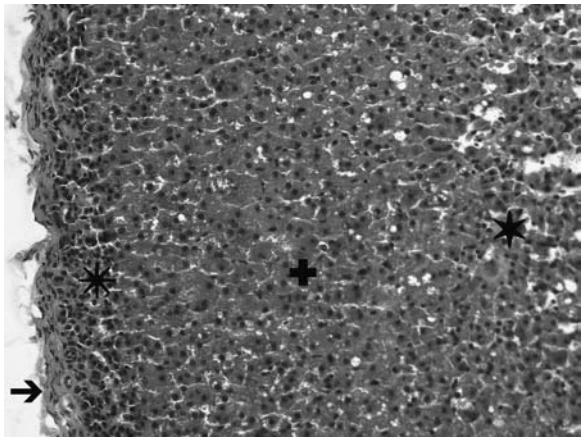

Figure 5 (A) (See color insert) Adrenal cortex, normal—note the appearance of the three cortical zones, namely, the zona glomerulosa (∗), zona fasciculate (+), and zona reticularis (*). Adrenal capsule (→).

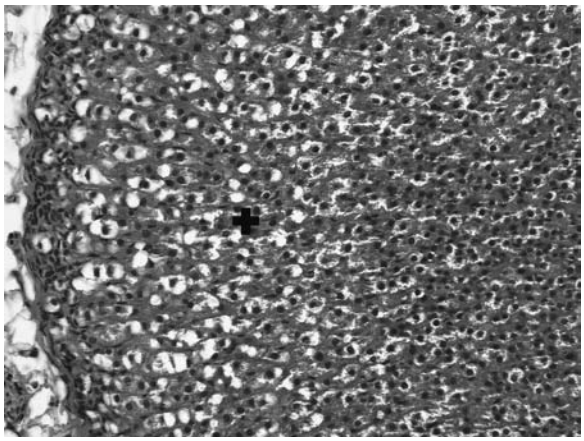

Figure 5 (B) (See color insert) Adrenal cortical hypertrophy—cells of the zona fasciculate (+) are enlarged and vacuolated (optically empty spaces, as the lipid was removed during histological processing).

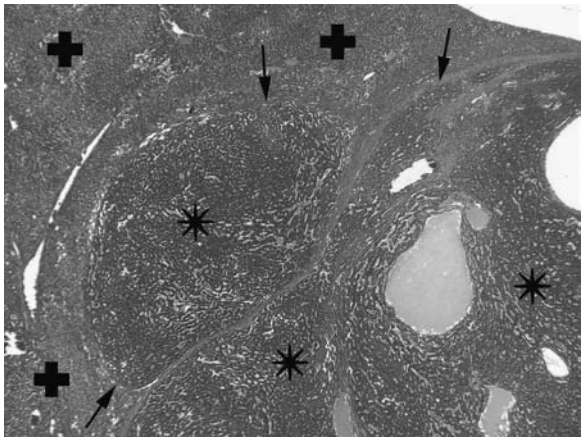

Figure 6 (A) (See color insert) Liver adenoma (low magnification)—large areas of proliferating cells (∗) with some sinusoidal structure (vascular spaces), compression of normal hepatic parenchyma (+), and partial capsule formation (→).

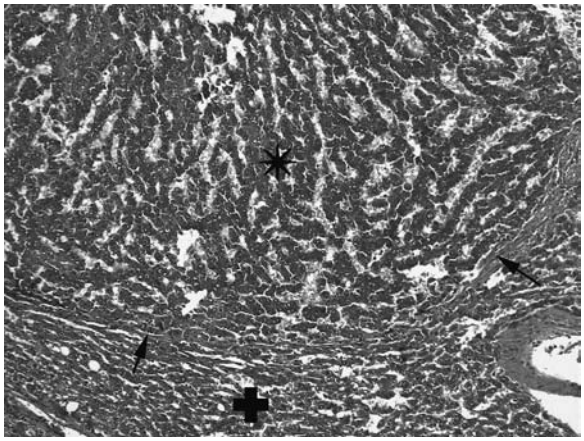

Figure 6 (B) (See color insert) Liver adenoma (higher magnification)—note the clear delineation between adenoma (∗) and normal compressed parenchyma (+). Fibrous strands of early capsule formation (→).

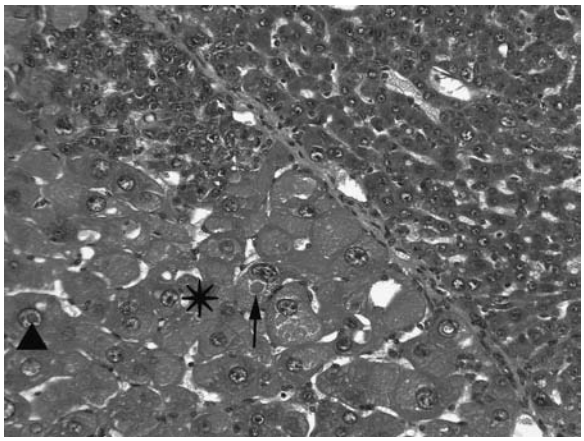

Figure 7 (See color insert) Liver carcinoma–high magnification of a hepatic carcinoma (∗) showing mostly large eosinophilic (pink) tumor cells with nuclei of variable size (nuclear pleomorphism), intranuclear (►), and intracytoplasmic (→) inclusions and lack of clear sinusoidal structure. Mitotic figures are not apparent on this photomicrograph.

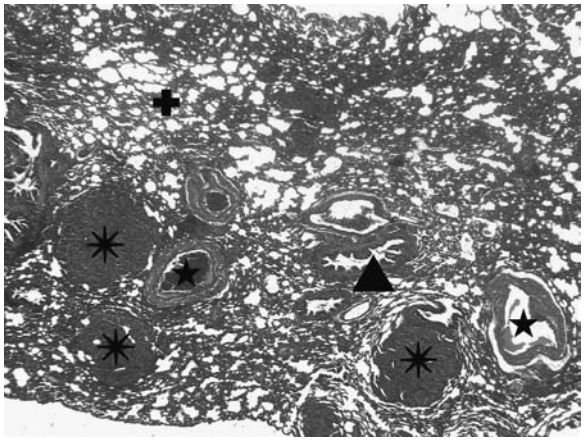

Figure 8 (See color insert) Lung metastases of fibrosarcoma (∗)-–note also normal lung parenchyma (+), large blood vessels (*), and airways (►).

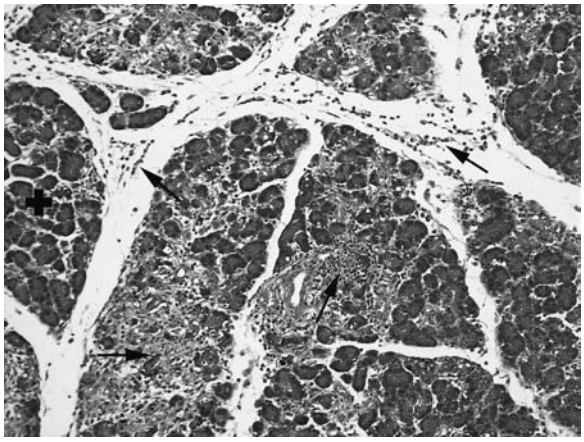

Figure 9 (See color insert) Pancreas inflammation-–inflammatory cells (→) infiltrating into and around pancreatic lobules. Note also some exocrine atrophy in comparison with normal portion of a lobule (+).

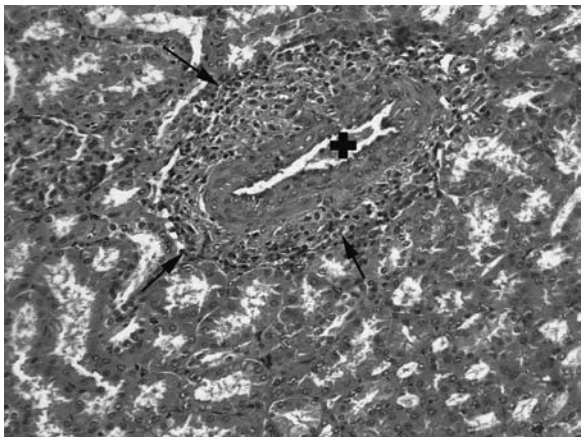

Figure 10 (See color insert) Kidney vasculitis–inflammatory cells (→) mainly around a small blood vessel (+). The surrounding renal tubules are normal.

the lymphatic system and accumulate in lymphatic organs (e.g., spleen or reticuloendothelial system of the liver). Significant accumulation of phagosomes is called a storage-type disease, which can impair the physiological functioning of the cell and even lead to single cell necrosis. Drug-induced storage disorders can also occur in other organs including kidneys (101).

- *Peroxisomes* are small vesicles in the liver, which contain among others peroxide-reducing enzymes. There is a whole class of chemicals, the so-called peroxisome-proliferating chemicals, which makes these peroxisomes proliferate. In lifetime rodent bioassays, this class of compounds generally induces liver tumors, which are considered of little to no significance for man (96,102,103).
- Isolated reports (personal communications and observations) indicate that *other subcellular organelles* can also proliferate under chemical exposure; among them are mitochondria. The toxicological significance is not known.

Time Course of Lesions

A toxic injury generally *starts* with functional impairment (e.g., cellular membrane impairment), which then leads to structural changes such as microvesiculation (accumulation of water, in mitochondria or lysosomes for example), and later becomes visible under the light microscope as vacuoles (generally empty spaces).

If the injury *persists*, cell organelles might be disrupted and the cell might eventually die. Cell necrosis liberates chemical substances, which attract leukocytes and possibly macrophages, that will then phagocytose the cell debris. Lymphocytes generally appear somewhat later in the process, but often stay longer at the scene and are, therefore, indicative of a process that is a few days (or more) older.

However, early toxic lesions may disappear under continuation of exposure to a particular chemical because of *adaptation* of the organism. Thus, partial acute tubular necrosis in kidneys of various species can be seen initially, but already after one or two weeks of exposure the kidney appears again normal, with the exception of tubular basophilia in H&E sections, which characterizes the regenerated tubular cells.

With *progressing severity* of a lesion, the likelihood of complete repair decreases. Thus, early lesions are generally fully reversible, if exposure to the toxin ceases. Cell death is per se an irreversible process. However, some tissues, including the liver and kidneys, have a high capacity for regeneration. Repair can be complete (restitutio ad integrum) or partial. In the latter case, replacement with regular cells is achieved only partially and the remaining defect is filled with unspecific cells of the connective tissue type called fibroblasts, which produce connective fibers and mature to fibrocytes.

In selected cases tumor development in internal organs can also be observed in living animals by suitable techniques (e.g., nuclear magnetic resonance) (104).

Modifying Factors

There are a number of factors that can modify the outcome of toxicity and, in particular, of a lifetime rodent bioassay studies (105–109). The most important factor for the latter is *feeding* (105,107) and standardization of procedures (**?**). Feeding ad libitum significantly increases tumor incidences of the pituitary, the mammary gland, and the lung in rats and of the liver in mice (110–114). However, the incidence of uterine tumors in rats may decrease (114). Feeding ad libitum also significantly increases the incidence and severity of degenerative diseases, including nephropathy, myocarditis, polyarteritis, and prostatitis (115). Nephropathy might significantly impact the excretion of the test compound. Overall, feeding ad libitum shortens the life span of test animals.

By contrast, reduced feeding (116,117) and feeding ad libitum of modified diets rich in fibers (118) significantly decreases incidences of most proliferative lesions, in particular, those of endocrine organs. It also lowers the occurrence and severity of age-related degenerative diseases but may increase the incidence of tumors of testis and uterus in rats (114). Caloric restriction can decrease the metabolism of xenobiotics and herewith modify the outcome of lifetime bioassays (119).

Toxicity is generally associated with decreased *well-being* of the test animals and therefore with decreased feed intake. In addition, poor palatability of the test compound given by feed

admixture reduces feed intake. Not infrequently and despite careful dose selection to produce only minimal signs of toxicity, this may happen in lifetime bioassays (120), thus biasing their outcome for the reasons explained above. Signs of reduced feeding and of unspecific toxicity are also seen in shorter-term studies and consist of the following:

- Retarded growth of young animals.
- Weight loss (in certain cases).
- Decreased organ weights, particularly of the lymphatic system (thymus, etc.).
- Decreased resistance to infections with multiple foci of acute to chronic infection. Such infections are not such infrequent, particularly but not exclusively in nonrodents, and can jeopardize the outcome of toxicity studies (121–123).

TYPICAL NONNEOPLASTIC ALTERATIONS SEEN IN TOXICITY STUDIES

Typical *nonneoplastic alterations* seen in toxicity studies with rodents, dogs, monkeys, and minipigs are summarized in Table 7.For further details, a number of good histopathology atlases (70–72,124) and textbooks (125,126) are available.

Toxicity manifests itself often in single *target organs* but can also affect whole systems (e.g., the reticuloendothelial system). For this reason, storage diseases (e.g., phospholipidosis) are seen throughout the body and are manifested in lung, liver, lymphatic system, and partly also in other organs (127).

The target organ also depends on the physical characteristics of the test compound. This is best illustrated by inhalation studies: Test compounds in powder form with particle sizes >5 μm are deposited in the nose and, therefore, may exert an irritative potential only locally. Particles of 1 to 5 μm deposit in the airways and <1 μm particles reach the alveoli (128,129).

The following reactions are *difficult to elicit* in test animals used in toxicity tests:

- Immunological reactions, including anaphylaxis and eczema.
- Arteriosclerosis: mild atherosclerotic lesions can be induced by excessively high lipid diets.

Furthermore, idiosyncratic drug reactions in humans are likewise generally unpredictable by toxicological investigations. Such reactions are rare and often based on a particular sensitivity of the affected individual, such as in the case of a rare metabolic peculiarity.

Lesions associated with impaired or changed organ function can also affect other organs resulting in "syndromes" such as the hepato-renal syndrome (130), thyroid changes associated with liver hypertrophy (131–135), changes in bones (136) and parathyroids (137) associated with renal disease, or the association of hypoxemic lung pathology with adrenal medullary proliferations related to stress (138). Some of these syndromes will be explained in more detail below (see "Pathogenesis of Tumors").

TYPICAL NEOPLASTIC CHANGES SEEN IN LIFETIME BIOASSAYS

Introduction

The most important lesions in lifetime rodent bioassays are tissue proliferations: hyperplasia, benign tumors, and malignant tumors. The *biological behavior* of these lesions is often not well-understood, as—in contrast to human pathology—no biopsies and follow-ups are available. This also renders the establishment of the cause of death difficult (75,139). Histopathological diagnosis of proliferative lesions in rodents is partly based on assumptions and agreement among pathologists, including size criteria to label a tumor as benign or malignant (12). Therefore, standardization of diagnostic criteria is particularly important and the method should be referenced in the report (63,64,77). Deviation from standardized diagnostic criteria is permissible but must be justified and defined.

Analysis and interpretation of lifetime rodent bioassays is among the most *demanding* tasks a pathologist must face (140,141). The development of a compound can be jeopardized by adverse pathological findings just before introduction to the market. On one hand it is good to remember that there are many more animal carcinogens than human carcinogens (142,143). On the other hand, toxicologists and toxicologic pathologists have the responsibility to make sure that humans and the environment are not exposed to real carcinogens. The diagnostic quality

(*text continues on page 316*)

Table 7 Typical Nonneoplastic Toxicological Lesions (for General Textbooks and Overviews See 70–72, 124–126, 325–328)

7.1 Liver

Organ Cell type Lesion type	Lesion	Pathogenesis	Consequence	Examples	Remarks
1. Hepatocyte					
1.1 Storage	Fatty change	Can have many reasons, such as organelle injury, metabolic disorders, etc.	Organ weight increased Per se reversible If excessive: liver cell function disturbed	Ethionine overdosage	Frequent, unspecific sign of toxicity
	Glycogen storage	Higher than normal storage of glycogen	Organ weight increased Per se reversible If excessive: liver cell function disturbed	Acetaminophen	Unspecific, frequent finding in toxicity studies
	Other cytoplasmic inclusions	e.g. multilamellar bodies resulting from undegraded membranes	If excessive: liver cell function disturbed	e.g. tricyclic antidepressants (329)	
	Hepatocellular and sinusoidal pigmentation	Storage of Fe, lipofuscin (an age-related natural pigment), hemosiderin (e.g. after hemolytic anemia), test compound	Probably none	Many different compounds, e.g. vincamine alkaloids	Normal with increasing age, but can be increased after exposure to chemicals
1.2 Nucleus	Increase in size and/or number	Hyperploidy of hepatocyte nuclei or fusion of hepatocytes to form multinucleated cells	—	Nitro compounds, pyrrolizidine alkaloids	Unspecific reaction of hepatocytes to hepatocellular injury
1.3 Regressive changes	Hydropic/cystic degeneration	Intracytoplasmic accumulation of fluid Partly probably derived from Ito cells (330)	Can lead to cell death	Radio-opaque agents	Relatively frequent, unspecific sign of toxicity
	Single cell necrosis	Cytotoxicity	Liver enzymes elevated Regeneration by proliferation of hepatocytes can lead to hepatocellular tumors in life-time bioassays	Many chemicals after high doses, e.g. acetaminophene	Can be monitored in man by checking liver enzymes (see also *table 2B*) To be distinguished from apoptosis

1.4 Progressive changes	Hypertrophy	e.g. proliferation of • smooth endoplasmic reticulum (14, 100, 233) • peroxisomes (96, 102, 103, 262, 263, 272)	Increased liver weight Eosinophilic hepatocellular cytoplasm Per se reversible. In rodent long-term studies generally associated with liver tumors	• Barbiturates • Some lipid lowering drugs	Relatively frequent, unspecific adaptation to chemical overload, often with hepatocellular hyperplasia, particularly centrilobular
	Foci of cellular alteration (333–339)	Various types; the basophilic type is characterized by proliferation of the rough endoplasmic reticulum	Highly active cells with increased protein synthesis and generally increased mitotic index May lead to liver tumors in long-term studies	Many animal carcinogens, which are not mutagenic	The basophilic type is considered preneoplastic, but is essentially still reversible Can also occur spontaneously at lower incidence in older rodents
	Nodular hyperplasia	Nodular hyperplasia of hepatocytes, e.g. following longer term liver toxicity	May result in impaired liver function and hepatic blood flow	Many liver toxins	Generally accompanied by fibrosis
2. Bile ducts	Cholestasis	Bile retained in hepatocytes, Kupffer cells and bile canaliculi, most likely because of increased bile viscosity and/or impaired bile excretion	Increased alkaline phosphatase "Jaundice" Depending on severity: any outcome possible from recovery to liver failure	Experimental antidepressive agents, griseofulvin (mice)	Rare in animals, but relatively frequent as side-effect to drugs in man
	Cholangitis	Inflammation of the intra- and sometimes the extrahepatic bile ducts. Acute or chronic	May lead to cholangiofibrosis, associated with significant impairment of liver function	Phytotoxins and mycotoxins	Relatively rare finding in toxicity studies
	Hyperplasia	Proliferation of bile duct epithelia sometimes accompanied by periductular inflammation and fibrosis	Outcome mainly determined by underlying disease	Many compounds	Frequent, particularly in rats, sometimes also in dogs
3. Sinuses	Sinus dilatation	Cardiomyopathy with resulting congestion of the liver	Severe liver congestion results in impairment of the liver	Daunomycin	Also a consequence of hepatocellular atrophy because of unspecific toxicity or as terminal hemostasis
4. General	Foci of chronic inflammation	Spontaneous infections	None, if present in small number		Incidence is a measure for the quality of the test animals
	Cystic degeneration	Possibly arising from altered fat storing perisinusoidal cells	Loss of liver parenchyma	Nitroso compounds	Seen particularly in long-term rodent studies
	Fibrosis	Proliferation of fibroblasts with increased formation of connective fibers, often creating a nodular appearance of livers	May progress to liver cirrhosis with significant impairment of liver function	Many liver toxins	Incomplete regeneration after liver toxicity

(*Continued*)

Table 7 Typical Nonneoplastic Toxicological Lesions (for General Textbooks and Overviews See 70–72, 124–126, 325–328) (*Continued*)

7.2 Alimentary system (338)

Organ system Organ/organ part	Lesion	Pathogenesis	Consequence	Examples	Remarks
1. Mouth	Hyperplasia of gingiva	Not known	Impairs feeding	Cyclosporine Ca channel blockers (339)	Sometimes not fully reversible
	Alterations of teeth	Cytotoxicity of tooth forming cells Hypercalcification	Impairs feeding	Antitumor agents (340) PTHrP (341)	
2. Salivary glands	Acinar hypertrophy	Stimulation of sympathetic innervation	Can lead to acinar necrosis upon prolonged treatment	Isoproterenol	Reversible if not accompanied by acinar necrosis
	Acinar atrophy	Unknown	Feeding and digestion can be impaired	Some antineoplastic agents	Also seen in animals in bad general condition for prolonged time
3. Stomach	Proliferative lesions of the forestomach	Especially hyperplasia of the keratinized stratified squamous epithelium because of irritation following gavage or in-feed administration of test substances	None	Many different chemicals, e.g. antineoplastic agents	Hyperkeratosis also seen in rodents with depressed feeding
	Proliferation of enterochromaffin -like cells (ECL)	Therapeutic achlorhydria (no gastric HCl) results in hypergastrinemia, which exerts a constant stimulus on ECL	Can give rise to carcinoids (ECL tumors) and adenocarcinomas in long-term rodent bioassays	H2 blockers	Regarded as not being significant for man (273–275)
	Ulcers (missing epithelium with necrosis of underlying tissue and inflammation)	Inhibition of prostaglandin formation with decreased formation of mucus, which protects the epithelium against digestion by gastric and intestinal enzymes Other mechanisms (e.g. mucosal hemorrhage) also occur	Impairs the general well-being Can lead to gastrointestinal hemorrhage and perforation Accompanying inflammation can result in intraabdominal adhesions	Non-steroidal anti-inflammatory drugs (342) Steroids (251)	Ulcers are sometimes very small and only detected microscopically Ulcers can also occur in the small intestine
4. Intestine	Villus stunting, particularly in small intestine	Cytotoxicity of mucosal and particularly of crypt cells	If minor: reversible If extensive: lethal	Cytostatics	Proliferating cryptal cells are particularly sensitive
5. Pancreas					
5.1 Exocrine					Toxicologically relevant non-neoplastic changes are rare in the exocrine pancreas
5.2 Endocrine					See endocrine system

7.3 Urinary system (115, 245, 246, 343–351)

1. Kidney					
1.1 Glomerula	Glomerulo-nephrosis	Age-related spontaneous	May alter excretion of compounds		Toxicologically relevant non-neoplastic changes of the kidney glomeruli are rare.
1.2 Tubules	Cytoplasmic vacuolation	Early sign of tubular toxicity: increased membrane permeability leads to 'osmotic nephrosis'	Generally fully reversible	Many compounds: e.g. aminoglycosides (243, 244), cephalosporin (352)	
	Cytoplasmic basophilia	Most likely excessive cell turn-over due to tubulotoxicity	Per se little. May lead to kidney tumors in life-time bioassays	Many compounds (see above)	Relatively frequent in repeat dose studies. May be an early sign of nephrotoxicity
	Cytoplasmic inclusions	Storage of many different materials including proteins, compound, pigments, etc.	Probably little, with exception of α2-microglobulin tubulopathy, which results in tubular tumors in life-time bioassays	Many compounds α2-microglobulin induced by hydrocarbons	Relatively common finding α2-microglobulin associated tumors are of no significance to man (98, 353)
	Degeneration/ necrosis	Cytotoxicity	Prolonged tubular toxicity can lead to tubular tumors in life-time bioassays If extensive: kidney failure	Many compounds, e.g. cephalosporins (352), aminoglycosides (243, 244)	Tubular epithelia have a big potential for regeneration and adaptation: despite continuous exposure, tubulotoxicity can be transitional
	Hypertrophy	Often age-related but occasionally increased after chemical exposure	Increased organ weight	Daunomycin	
	Hyperplasia	Spontaneously or following nephrotoxicity	Preneoplastic lesion, if multilayered (clear or eosinophilic cells)	Induced by many compounds, e.g. cisplatin	
1.3 Tubular lumen	Crystals	Consequence of concentrating properties of kidney and/or pH changes	May lead to tubular damage and occlusion	Sulfonamides	
	Dilatation / cysts	e.g. tubular stasis following nephrotoxicity	Can reduce kidney function in old animals in the context of other renal diseases	Many nephrotoxins	
1.4 Interstitial space	Mineralization	Spontaneous and induced lesion, often associated with increased urinary excretion of Ca	Can reduce kidney function in old animals in the context of other renal diseases	Induced by vitamin D, PTH, Ca gluconate, etc.	In rats, mineralization tends to occur at the corticomedullary junction
1.5 Papilla	Necrosis	Reduced renal papillary blood flow because of reduced prostaglandin formation	Can reduce kidney function in old animals in the context of other renal diseases	e.g. non-steroidal anti-inflammatory drugs (252, 354)	Mainly in rats and humans
2. Urinary bladder	Calculi	Spontaneously and induced	Can lead to cystitis and/or mild hyperplasia and metaplasia of the urothelium Can result in bladder cancer in life-time bioassays (92, 93, 355)	Induced e.g. by sulfonamides, saccharine (160)	Spontaneously frequent in rodents with no consequences

(*Continued*)

Table 7 Typical Nonneoplastic Toxicological Lesions (for General Textbooks and Overviews See 70–72, 124–126, 325–328) (*Continued*)

7.4 Endocrine system (259, 356, 357)

Organ Organ part Part of cell	Lesion	Pathogenesis	Consequence	Examples	Remarks
1. Adrenal-Cortex (358)					
1.1 Regressive changes	Lipidosis (especially Zona fasciculata and reticularis)	e.g. by inhibition of steroidogenesis with build-up of precursors	Could lead to Addison-like symptoms	Aminoglutethimide and other compounds	Functional impairment rarely seen
	Inclusions of hyaline droplets	Resorption of proteins with formation of vacuoles	Probably none	Starvation, methyl-androstenediol	
	Atrophy	e.g. decreased stimulation of aldosterone production in Zona glomerulosa	Decreased organ weight Generally reversible	Timolol maleate, captopril, corticosteroids	
	Necrosis	e.g. by retrograde embolization of adrenal medullary cells into cortical capillaries	If limited: none	Various compounds incl. hexadimethrine	
	Cystic degeneration of zona fasciculata / reticularis	Unknown	Unknown	Estrogens, tamoxifen etc. administered to male rats	
1.2 Proliferative changes	Hypertrophy	e.g. increased stimulation of aldosterone production in Zona glomerulosa	Organ weight increased Can lead to tumors in long-term bioassays	Diuretics, spironolactone	Reversible. Various compounds induce hyperplasia of the adrenal cortex by different mechanisms (359)
	Focal nodular hyperplasia	Regenerative reaction to cortical toxicity	Can lead to tumors in long-term bioassays	Diuretics, spironolactone	
1.3 Sinuses	Vacuolation of sinusoidal cells	e.g. multilamellated bodies resulting from indigestible membranes in phospholipidosis	Unknown	Tricyclic antidepressants	Sinusoidal cells are part of reticulo-endothelial system with phagocytic function
2. Adrenal-Medulla (358)	Proliferation	Spontaneous and induced, possibly associated with hypercalcemia	Unknown	Induced e.g. by reserpine, retinoids, thiouracil	Especially seen in rats

3. Pancreatic Islet cells					Infrequently the target of toxicity
3.1 Regressive changes	Necrosis	β-cell toxins	Diabetes mellitus	Alloxan, streptozotocin	
	Vacuolation	β-cell hypersecretion	Reversible	Cyproheptadiene, cyclizine	
	Atrophy	Exhaustion of β-cells: Consequence of prolonged stimulation e.g. through lowering of circulating insulin levels	Diabetes mellitus	Growth hormone and others (360)	
3.2 Progressive changes	Hypertrophy	Corticosteroids increase blood glucose levels and therefore stimulate β-cells	In severe cases, the β-cells become exhausted and atrophic	Corticosteroids	
	Hyperplasia	Prolonged stimulation of β-cells	Can progress to islet cell adenoma	Any compound causing hyperglycemia	
		Stimulation of insulin secretion in mice (trophic effect on β -cells)	Can progress to islet cell adenoma	Prolactin	
4. Thyroid (361)					
4.1 Follicles	Hypertrophy/ hyperplasia	Increase of TSH as a consequence of decreased T3 and T4 levels because of decreased production or increased metabolism	Increase of organ weight Can progress to follicular adenoma	Thyrostatic agents Compounds, which induce metabolic enzymes (131, 134, 135) or liver mass (362)	The rat is particularly sensitive
	Atrophy	Decrease of TSH levels	Decreased organ weight, hypothyroidism	Thyroxin (T4), triiodothyronine (T3)	
	Pigmentation	Pigment granules (lipofuscin, melanin, derivatives of test compound) in follicular epithelial cells	Probably none	Minocycline, vincamines	
4.2 Parafollicular cells	Hyperplasia	Physiological response to increase of blood Ca levels	May progress to adenoma	Vitamin D	
5. Parathyroid	Hyperplasia	Physiological response to decrease of blood Ca levels, particularly because of severe nephropathy with secondary hyperparathyroidism	Increased organ weight May progress to adenoma	Age	Frequent in lifetime bioassays
6. Pituitary	Infarction	Unknown	May lead to atrophy of endocrine organs	Hexadimetrine	Rare
	Vacuolation	Unknown	Unknown	Anticancer drugs	Rare
	Hypertrophy / hyperplasia	If function e.g. of thyroid is significantly decreased, thyrotrophs become first hypertrophic, then hyperplastic and may finally become exhausted and atrophic	Increased organ weight If last stage (exhaustion, atrophy) is not reached, hyperplasia might transform into a tumor	Thyrostatics, contraceptives and neuroleptics producing hyperprolactinemia Calcitonin (205, 363)	

(*Continued*)

Table 7 Typical Nonneoplastic Toxicological Lesions (for General Textbooks and Overviews See 70–72, 124–126, 325–328) (*Continued*)

7.5 Reproductive system (259)

Organ system Organ Part of cell	Lesion	Pathogenesis	Consequence	Examples	Remarks
1. Ovary (255)					
1.1 Follicules	Oocyte destruction	Cytotoxicity	Sterility if primordial follicles are destroyed (present since birth without regenerative capacity) Can result in atrophy of the whole ovary	Radiation, polycyclic aromatic hydrocarbons, anticancer agents	Primordial (young) follicles are particularly sensitive
	Atretic follicles	Physiological process and increased by hormonal effects and through cytotoxicity	Generally reversible with hormonal compounds May be irreversible with anticancer agents (see above)	Induced by contraceptive steroids, anticancer agents	
	Absence of corpora lutea	Disturbance of the estrogenic cycle, which prevents maturation of follicles to corpora lutea	Generally reversible	Contraceptive steroids, in particular estrogens	
	Persistence of corpora lutea	Delayed luteolysis through disturbance of the estrogenic cycle	Organ weight increased Generally reversible	Butyrophenone	
		Bromocriptine decreases prolactin and prevents normal luteolysis	Organ weight increased Generally reversible	Bromocriptine (186)	
	Cysts	Retention of fluid in follicles	Organ weight increased	Estrogenic compounds, β-stimulants, bromocriptine	
1.2 Interstitium	Tubular hyperplasia	Proliferation of tubular structures with Sertoli cell-like cells		Hypophysectomy, growth hormone	
	Mesovarial smooth muscle hyperplasia	Proliferation of the smooth muscle cells in the tissue between follicles		β-adrenoceptor-stimulant drugs	Mainly in rats
	Mesothelial hyperplasia	Proliferation of mesovarium covering ovary		Estrogenic compounds	Mainly in dogs

2. Uterus (255)					
2.1 Endometrium	Endometritis	Often associated with squamous metaplasia of glands and/or endo/myometrial hyperplasia	Chronic irritation can result in uterine tumors in life-time bioassays	Estrogenic and progestational compounds	
	Squamous metaplasia	Response of endometrial epithelium to chronic irritation	Can result in hydrometra (fluid accumulation) and pyometra (pus)	Estrogenic compounds, vitamin A deficiency	
	Hyperplasia	Response of endometrial glands to prolonged irritation or stimulation	Increased uterine weight	Estrogenic and progestational compounds	
	Decidualization	Unknown	Reversible	Growth hormones	Mainly in rats. Can be mistaken for a uterine neoplasm
	Endometriosis	Endometrium-like tissue (glands and stromal elements) in ectopic (unusual) locations	Probably not highly relevant	Stilbestrol and other estrogenic compounds	Mainly rabbits and mice, where it is also called adenomyosis
	Atrophy	Prolonged stimulation of the progesterone type	Marked reduction in uterine weights Infertility	Progestational compounds Butyrophenones	Mainly in monkeys Mainly in dogs
2.2 Myometrium	Hyperplasia	Physiological response, see also endometrial hyperplasia above	Increased uterine weight	Estrogenic and progestational compounds	
3. Vagina/cervix	Hyperkeratosis / hyperplasia	Increased estrogenic stimulation of the squamous epithelium	Reversible, probably with exception of late stages	Estrogenic compounds	Mainly dogs and monkeys
	Mucification	Increase of mucus producing cells in vagina and cervix	Can lead to marked distention of the cervical canal in monkeys	Progestational compounds	
	Atrophy	Mediated through diminished ovarian activity	Reversible, probably with exception of late stages	Progestational compounds	Mainly dogs and monkeys
4. Mammary glands	Glandular development in immature females or in males / glandular hyperplasia in mature females	Physiological response with proliferation mainly of the ducts and – after prolonged exposure – also of the glands (lobules)	Results, particularly in rats, in glandular secretion with cystic dilatation of the ducts	Estrogenic compounds (various species), dopamine antagonists (rats and dogs), progesterone (dogs)	Mammary gland differentiation and hormonal receptor expression are influenced by the diet (364)
	Hyperplastic nodules	Proliferation of glandular and stromal components to various proportions	Can be associated with regressive changes such as sclerosis, etc.	Progestational compounds	
	Atrophy	Lowering of prolactin levels	Reversible, probably with exception of late stages	Bromocriptine (186)	

(*Continued*)

Table 7 Typical Nonneoplastic Toxicological Lesions (for General Textbooks and Overviews See 70–72, 124–126, 325–328) (*Continued*)

Organ system Organ Part of cell	Lesion	Pathogenesis	Consequence	Examples	Remarks
5. Testis (51, 54, 253, 254, 256–258, 260)	Necrosis	Cytotoxicity to germ cells at various stages of their development (from spermatogonia to spermatids)	Depletion of spermatogonia will result in sterility with complete atrophy of the seminiferous tubules, so called Sertoli cell-only tubules	Anticancer agents	Mainly seen in acute to subacute toxicity studies Generally accompanied by cell debris and multinucleated germ cells in the epididymis
	Atrophy	Cytotoxicity	Organ weight decreased Sertoli-cell only tubules Sterility	Anticancer agents, estrogenic and progestational compounds. Dopaminergic antagonists and agonists	The most common finding in case of prolonged testicular toxicity
		Spontaneous	Organ weight decreased Foci of Sertoli-cell only tubules If not too extensive, testicular function preserved		Foci of atrophic seminiferous tubuli are frequent, particularly in dogs in subcapsular area (365)
	Leydig cell hyperplasia	Inhibition of spermatogenesis increases Leydig cell activity	Organ weight often decreased because of Sertoli-cell only tubules In long-term bioassays often leading to tumors	Estrogenic compounds dopaminergic antagonists and agonists	
6. Epididymis (51, 254)	Spermatic granuloma	Rupture of epididymal ducts	Extravasation of spermatozoa into the interstitium leads to a granulomatous reaction Can be associated with infertility	DL-ethionine, guanethidine and other compounds (366)	Can also occur in the testis
7. Prostate (85, 254)	Atrophy	Physiological and often unspecific reaction	Reduction of organ weight In severe cases sterility	Estrogenic and progestational compounds	Frequent finding particularly in animals in poor condition
	Hypertrophy / hyperplasia	Physiological reaction	Organ weight increased	Androgens	
	Squamous metaplasia	Transformation of acinar epithelium	Impaired function	Estrogenic compounds	Particularly in dogs and mice

7.6 Immune system (41, 42, 232, 247–249, 270, 367–368)

1. Thymus					The thymus is a so called lympho-epithelial organ
1.1 Lymphatic component	Atrophy	Loss mainly of T-cells with concomitant reduction of the thymal stromal component Physiological involution in adolescence	Organ weight decreased If occurring pre/peri- or early postnatally: T-cell deficiency with reduced resistance to infection	Stress Decreased levels of growth hormone Corticosteroids Anticancer agents, e.g. cyclophosphamide	
1.2 Epithelial component	Hyperplasia	Unknown	Can result in cyst formation, etc.	Estrogenic compounds	
2. Lymph nodes (LN), spleen (S)					
2.1 General	Extramedullary hematopoiesis	Physiological phenomenon Sometimes a consequence of systemic diseases (e.g. bone marrow toxicity) Unspecific sign of toxicity	—	Many compounds	Particularly megakaryocytes (produce thrombocytes) and granulopoiesis
	Congestion, hemorrhage	Generally agonal	—		Frequent, generally without toxicological significance
	Necrosis	Possibly because of microinfarctions due to erythrocyte agglutination	Granulomatous partial reparation	Ethyl palmitate, concanavalin A	
		Infarction because of vasoconstriction	If not extensive: none	Vasoconstrictive agents	
2.2 Lymphatic components	Decreased cellularity	Decreased number of T-lymphocytes in LN paracortex / in periarteriolar sheaths of S and/or of B-lymphocytes in follicles (LN/S)	If extensive: resistance against infection is reduced	Cyclophosphamide (B and partly T cells) Indomethacin, aspirin (T cells)	
	Atrophy	Depletion of lymphatic tissue	Spleen weight decreased Resistance against infection may be reduced	Corticosteroids at high doses for prolonged periods of time	
	Increased cellularity	LN: e.g. through mobilization of lymphocytes from the spleen	-	LN: Heparin, polysaccharide sulfates	
	Germinal center hyperplasia	Physiological stimulation	Often accompanied by plasmocytosis (B cells)	Antigens, infection	
2.3 Sinuses	Histiocytosis	Storage of lipids and various kinds of pigments in the LN, into which the application site of xenobiotics (e.g. mesenteric, bronchial, etc.) drains	Can lead to hypersplenism with reduced half-life of erythrocytes and ensuing anemia	Various compounds, including e.g. macromolecular polymers	The lining of the sinuses is part of the reticulo-endothelial system (phagocytosis), to whjch e.g. also the hepatic sinuses belong

(*Continued*)

Table 7 Typical Nonneoplastic Toxicological Lesions (for General Textbooks and Overviews See 70–72, 124–126, 325–328) (*Continued*)

7.7 Hematopoietic (250, 369) and circulatory system (242, 370, 371)

Organ system Organ Part of cell	Lesion	Pathogenesis	Consequence	Examples	Remarks
1. Bone marrow	Atrophy	Cytotoxicity: proliferating cells are particularly sensitive to toxicity.	(Partial) replacement of blood forming bone marrow by fat tissue Complete bone marrow atrophy is fatal	Anticancer drugs, phenylbutazone (dogs)	Partial atrophy is physiological with age
	Myelofibrosis	Possible consequence of cytotoxicity resulting in proliferation of fibroblasts	Depending on extent. If extensive: bone marrow failure	Irradiation, saponin	Rare
	Hyperplasia	Proliferation of megakaryocytes	Unknown	β adrenergic compounds	
		Proliferation of monocytes under immunostimulation	Unknown	Muramyl peptide	Dogs only
		Myeloid hyperplasia as a reaction to inflammatory and/or hemorrhagic conditions	Most likely fully reversible	Secondary to various compounds	
		Formation of germinal centers	Most likely fully reversible	Immunostimulants, infection	
	Foam cells	Accumulation of phospholipids	No major consequences	Amphiphilic drugs, such as tricyclic antidepressants	Easily induced in rats
2. Cardiovascular system - Heart	Necrosis	Cytotoxicity	Multifocal scars, possibly with calcification. If extensive: cardiac insufficiency	Doxorubicin and other anticancer agents (27)	
		Hypoxia by prolonged tachycardia (mainly dogs) or decreased blood pressure	As above	Catecholamines, vaso- and broncho-dilating agents (372)	
	Hypertrophy	Enlargement of myocardial fibers, partly because of increased workload	Increased organ weight	Ca channel blockers, thyroxine	
3. Cardiovascular system-Vessels	Vasculitis	Unclear, possibly vasoconstriction of vasa vasorum and/or insudation of plasma through leaking endothelial cells	Can lead to thrombotic occlusion of the vessel, particularly if evolving to necrotizing vasculitis	Immunostimulators Theobromine, theophylline, dopaminergic vasodilators (238), phosphodiesterase inhibitors (237) and others (375)	Vasculitis can occur spontaneously, particularly in dogs (373, 374)

7.8 Respiratory system (241)

1. Nasal chambers and airways					
1.1 Respiratory epithelium	Degeneration / atrophy	Cytotoxicity	Necrosis of the respiratory epithelium may lead to ulcerations and disturbance of the nasal innervation (376)	Irritant chemicals administered by (nasal) inhalation, e.g. formaldehyde	
	Hypertrophy / hyperplasia / squamous cell metaplasia	Reaction to chronic irritation of respiratory epithelium	Squamous epithelium is more resistant against chemical irritation Probably reversible	Irritant chemicals administered by (nasal) inhalation	
	Goblet cell proliferation	Reaction to subacute irritation of respiratory epithelium	Protective effect of mucus covering the respiratory epithelium	Irritant chemicals administered by (nasal) inhalation	
1.2 Olfactory epithelium	Degeneration / atrophy	Cytotoxicity	The regenerative capacity of the olfactory epithelium is limited and cell loss might be irreversible	Irritant chemicals administered by (nasal) inhalation Acetaminophen, phenacetin	3-methylindole administered systemically can damage the olfactory epithelium in mice

(*Continued*)

Table 7 Typical Nonneoplastic Toxicological Lesions (for General Textbooks and Overviews See 70–72, 124–126, 325–328) (*Continued*)

Organ system Organ Part of cell	Lesion	Pathogenesis	Consequence	Examples	Remarks
2. ***Lung parenchyma***	Congestion, alveolar hemorrhage	Generally an agonal finding, but can accompany severe toxic lung lesions	If toxicity-related: disturbance of gas exchange between alveoli and blood vessels. If agonal: without significance	Bleomycin, paraquat, hyperbaric oxygen, cyclophosphamide	
	Edema, exudation of fibrin, etc.	Often secondary to toxic lung injury or pneumonia	Increased organ weight Can be associated with fibrin deposits on the alveolar wall, hyaline membranes. Can be resolved or lead to intraalveolar and interstitial fibrosis In severe cases necrotizing pneumonitis with respiratory insufficiency	Bleomycin, paraquat, hyperbaric oxygen, cyclophosphamide	With unexpected animal deaths in gavage studies instillation of test solution by mistake into the trachea to be considered
	Diffuse, unspecified alveolar injury	Cytotoxicity mainly of type 1 pneumocytes	May lead to epithelialization (cuboid type 2 pneumocytes, which later transform into type 1 pneumocytes = recovery) Gas exchange likely to be impaired	Bleomycin, paraquat, hyperbaric oxygen, cyclophosphamide	
	Adenomatosis	Adenomatous hyperplasia of type 2 pneumocytes	If not generalized: no significant consequences	Dimethylhydrogen phosphite	
	Intracellular storage of various substances	Essentially in type 2 pneumocytes e.g. phospholipids Dust particles	Even with extensive storage disease generally no clinical signs	Amphiphilic drugs given systemically (phospholipidosis) (329) Inhalation e.g. of quart dust	Storage can also occur in macrophages present in alveolar septa, especially in subpleural, peribronchiolar and perivascular regions
	Chronic inflammatory foci	Accidental infection	None, if the number of inflammatory foci is not excessive	Spontaneous Exacerbation with immunosuppressive agents	The number of infectious foci is a measure of the quality of the test animals
	Perivascular granulomas	Embolization after intravascular injection of crystalline material, hair and skin fragments	Embolization leads to thrombosis, perivascular granulomas and can evolve into focal interstitial pneumonia	Compounds, which are difficult to dissolve Long-term iv. studies (skin and hair fragments introduced into vessels)	

7.9 Nervous system (52, 264, 265, 377–378)

1. General	Vacuolar encephalopathy	Unknown	Unknown	Incidence and severity can be increased by treatment with certain compounds	Vacuolation in the nervous system is often an artifact
2. Nervous tissue					
2.1 Neural cell body	Degeneration and necrosis	Cytotoxicity	Damage to the cell body usually results in degeneration of axons and dendrites, as well as the myelin sheath	Mercury, tri/tetramethyl, lead	Rare in toxicity studies
		Excessive stimulation of neurons with exhaustion	Loss of innervation	Excitatory amines, e.g. glutamate and derivatives	
2.2 Axons	Degeneration (with fragmentation and variation of axonal thickness)	Interference with vital axonal processes. Probably often associated with disturbed transport of vital nutrients and components (e.g. proteins) from the cell body to the peripheral axon	Axonopathy is followed by secondary demyelination and by chromatolysis, but generally not death of the cell body After discontinuation of exposure some recovery is possible	Chloramphenicol, diphenylhydantoin, isoniazid, nitrofurantoin, vincristine, organophosphates	Axonopathy occurs mostly in distal axons
2.3 Myelin sheath	Myelin vacuolation and degradation	Direct action on myelin, on myelin producing Schwann cell or on oligodendrocyte metabolism	After discontinuation of exposure some recovery is possible	Hexachlorophene, isoniazid, triethyl tin, actinomycin D	Long myelinated axons are particularly vulnerable
3. Glia	"Activation": incl. proliferation around lesions	Secondary reaction to toxic injury to neurons	Phagocytosis of neuronal debris, etc.	Hexachlorophene, isoniazid, triethyl tin, actinomycin D	Primary toxic changes to glial cells are uncommon
4. Choroid plexus	Vacuolation	Unknown	Unknown	Disobutamide, chloroquine	

(*Continued*)

Table 7 Typical Nonneoplastic Toxicological Lesions (for General Textbooks and Overviews See 70–72, 124–126, 325–328) (*Continued*)

7.10 Sensory system

Organ system Organ Part of cell	Lesion	Pathogenesis	Consequence	Examples	Remarks
1. Eye (379–381)					
1.1 Cornea	Keratitis / conjunctivitis Ulceration	Generally following topical application of irritant chemicals or accidentally (e.g. fighting among animals)	Chronic irritation can lead to neovascularization of cornea, which is normally devoid of vessels Ulceration might lead to perforation of cornea with loss of eye	Any irritant chemical	
	Corneal vacuolation	Phospholipidosis	Impairment of vision	Amphiphilic drugs	
1.2 Uveal tract	Uveitis, vacuolation, etc.	Unknown	Unknown	Seen occasional with a variety of chemicals	
1.3 Lens	Cataracts (turbidity)	Osmotic stress	Impairment of vision	High amounts of lactose, xylose, glucose, certain drugs (382)	Also known to occur in diabetes mellitus
		Secondary to keratitis, iridocyclitis, etc.	Impairment of vision	Any irritant chemical	
1.4 Retina	Retinal degeneration	Direct effects on photoreceptor and secondary to degeneration of the optic nerve	Debris of the degenerating photoreceptors are phagocytosed by the retinal pigment epithelium	Light, propionic acid derivatives, hexachlorophene	The retina particularly of albino animals is very light sensitive (383)
	Degeneration of the tapetum lucidum	Chelation of Zn in tapetum lucidum	Most likely impairment particularly of night vision	Ethambutol, dithizone	Dogs only
1.5 Optic nerve	Papillary edema	Cytotoxicity	Can result in retinal atrophy	Methanol	
2. Lachrymal glands	Decreased lachrymal secretion, partly with some inflammation and cell necrosis	Partly unknown, partly pharmacological effect (e.g. with anticholinergic drugs)	Leads to keratoconjunctivitis sicca (see also above under keratitis)	Sulfonamides, 5-aminosalicylic acid, anticholinergic drugs	Mainly in dogs
	Atrophy	Unclear, possibly autoimmune disease	Dry eye syndrome with corneal ulceration and possibly perforation	β adrenergic receptor blockers	
3. Harderian gland	Chromodacryorrhea (pigmented tears)	Normal porphyrin excretion in female animals	—	Increased in inhalation studies with irritant chemicals	No Harderian glands in man
4. Ear	Degeneration of parts of the organ of Corti	Cytotoxicity of hair cells	Impairment or loss of hearing	Aminoglycoside antibiotics, quinine	Because of its location in the cranial bones, the investigation of the ear is rather difficult and not done routinely
	Edema of the stria vascularis	Possibly by swelling of the vascular endothelium and ensuing hypoperfusion	Lowers the potassium level in the endolymph and therefore the function of the organ of Corti	Salicylates, furosemide	

7.11 Skin, musculoskeletal system

1. Skin	Inflammation	Cytotoxicity of topically applied chemicals	Can lead to ulceration	Methylsalicylate, benzalkonium	
	Hyperplasia	Chronic irritation by topically applied chemicals	Increase of resistance against irritation	Non-steroidal anti-inflammatory drugs	
	Alopecia	Loss of hair/fur related to endocrinopathy	Can be associated with dermatosis-like skin changes	Estrogens, corticosteroids	
2. Locomotor muscles (384)	Atrophy of muscle fibers	Malnutrition (e.g. because of severe toxicity) Nerve damage (e.g. because of neurotoxicity) Inactivity associated with painful alterations of skeletal system	If extensive: impairment of locomotor activity	Any toxin given at high enough dose See nervous system	Overall, locomotor muscles are rarely affected by toxicity
		Cytotoxicity	See above	Emetine	
		Endocrine regulation, which favors replacement of myocytes with fat cells	See above	Corticosteroids	Monkeys
	Necrosis	Toxicity at the site of intramuscular injection or after systemic application	Followed by regeneration of myocytes or by (partial) scar formation If extensive: impairment of locomotor activity	Any irritant compound given im. Some lipid-lowering drugs given systemically (385)	
		Cytotoxicity	See above	Adriamycin	
3. Skeletal system					Overall, skeletal system infrequently affected by toxicity
3.1 Joints	Arthropathy	Cytotoxicity of chondroblasts	Limits locomotor activity Can be associated with synovial hyperplasia	Alkyl pyridone carboxylic acid and derivatives, quinolone antibiotics Certain immunostimulants	Mainly dogs
	Arthritis	Cytotoxicity of chondroblasts, immunological effects, etc.	As above	6-sulphanil-aminoindazole	Rats
3.2 Bones	Osteoarthritis	Age-related, but may be aggravated by administration of sex hormones	As above	Spontaneously Sex hormone	Mice
	Osteomalacia	Inadequate calcification of bone matrix and wide osteoid seams because of lack of Ca	Can lead to deformation of bones	Vitamin D deficiency, tetracycline, bisphosphonates	
	Osteoporosis	Decreased osteoblastic activity	Can lead to fractures	Estrogens	
		Increased osteoclastic activity	As above	Retinoic acid	
		Decreased absorption of intestinal Ca	As above	Corticosteroids	
		Renal loss of Ca	As above	Kidney failure with secondary hyperparathyroidism	
	Proliferations	Bone marrow injury, followed by local inflammation and proliferation of endostium and myeloid stroma	Unknown	Anticancer agents (386)	

depends on the qualifications and ability of the pathologist, which necessitates many years of postgraduate training in pathology (144). Board certification (US, EU, IATP) or registration for toxicologic pathology (EU, Japan) is another hallmark of qualification in pathology (74,145).

As lifetime bioassays are large studies with 500 and more animals, each with some 60 organs, and as these studies are generally performed toward the very end of development, they are *time-critical*. Nevertheless, it is advised that only one pathologist should read the histological slides. If, for deadline reasons, two pathologists must share the task, then splitting into male and female groups may be considered. Splitting by dose groups should be avoided. All the slides from one animal are generally read consecutively, as the various lesions are interdependent and often have biologically significant effects on the overall condition of the animal (as mentioned above in "Typical Nonneoplastic Alterations Seen in Toxicity Studies").

Sometimes, *transgenic mice* are used for carcinogenicity testing. Examples include the *p53*(+/−), TgHras2 and XPA transgenic mice, or neonatal mice (146–158). The theoretical advantage of these models is the earlier appearance of tumors, which allows shortening of the experimental duration. So far these models have not gained full acceptance, partly because it was recognized that the genetic construct is unstable and can be lost. The technology underlying transgenic models and the role of transgenic animals in preclinical drug development is the focus of chapter 3.

Because of the complexity of tumor diagnosis and since information about diagnostic criteria of various tumor types is readily available in the scientific literature (65–72) and on the internet (65,66), this section is limited to a general description of the pathogenesis of tumors, their histological appearance, as well as their precursor lesions.

Pathogenesis of Tumors

The basic pathways of tumor formation include

- *Genetic alterations* (mutagenicity, chromosomal anomalies, etc.). With today's genotoxicity testing, genotoxic chemicals are rarely tested in lifetime bioassays. However, the relatively large number of spontaneous tumors may well have a genetic component, resulting from inbreeding and/or spontaneous genetic alterations.
- *Epigenetic alterations (*nongenotoxic mechanisms) are now more likely to account for tumor induction in lifetime bioassays (159). As mentioned earlier, essentially all conditions with increased cell proliferation are likely to result in increased tumor incidences in the long-term. Increased cell proliferation is present in so-called tumor precursor lesions, particularly in the following conditions:
  - *Chronic tissue irritation*. Test substances may crystallize and form aggregates with proteins and lipids from body fluids in the excretory system. One such example is saccharine that leads to urinary bladder carcinoma if fed for lifetime to male rats (92,160). Similarly, solid-state carcinogenicity occurs mainly in rodents, for example, at subcutaneous injection sites in rats (161) or at implantation sites of microchips in mice as used for animal identification (162,163). The latter connective tissue tumors are of little relevance in man, whose connective tissue is much less sensitive to chronic irritation.
  - *Increased physiological stimulation of cells* leading to cellular hypertrophy (increase in size, generally with proliferation of cellular organelles) and hyperplasia (increase in cell number). It is well-known that hepatomegaly seen with liver enzyme inducers in mice during subchronic toxicity studies is often an early indicator for liver tumors in the mouse lifetime bioassays (164). Liver enzyme inducers can lead not only to liver tumors because of long-term stimulation of hepatocytes, but also to thyroid tumors because of increased T3 metabolism and the consequent TSH increase with chronic stimulation of the thyroid follicular cells (131,134,135**)**. Both findings are of no relevance to man.

Species Specificity of Tumor Formation

The influence of the species is reflected in the wide variation of incidences of spontaneous pathology findings among different rodent *species*. Mice have a high incidence of spontaneous tumors particularly in lung and liver and also in Harderian and adrenal glands, the hematopoietic

system, and ovaries (165–174). Rats have a high incidence of mammary gland and pituitary tumors (172,175,176). Rats and mice may also differ in their response to lifetime exposure to chemical substances, but in most cases, data from mice and rat lifetime bioassays are in agreement (177).

There are also marked differences in tumor incidences between *different strains* of the same species. Examples include the following:

- Leydig cell tumors: 88% to 96% in F344 rats, below 10% in Sprague Dawley (SD) rats, and 1% to 2% in Long–Evans rats (178).
- Mononuclear cell leukemia: 20% in F344 rats and rare in SD rats (179).
- Mammary gland tumors, the incidence of which varies widely between different strains, possibly due to endocrine differences (180) and viral infections (123).

Significant *intrastrain differences* exist also between animals from different breeders (181). Gene expression can vary in genetically homogenous animal populations, leading to differences within that population (182).

Formation of tumors in endocrine organs resulting from disturbance of *hormonal balance* is particularly frequent in lifetime rodent bioassays. However, their occurrence is of little significance to man, mainly as the physiology of rodents is quite different from man in several respects (183). Particularly, the endocrine regulation of rodents is special and easily disturbed. For example, rats lack high-affinity thyroxin-binding globulin (184,185); the estrogen/progesterone ratio is 1:100–200 in rats but 1:1 in women; reproductive senescence means progesterone dominance in old rats, while in women the sex hormone production just decreases (186); prolactin has a trophic effect on the mammary gland of rats, while it maintains lactation in women (187); rat, but not human, Leydig cells react with tumor formation following long-term stimulation by high LH levels (178).

Rodents have additional *anatomical particularities*. The following organs are not found in man: forestomach, Harderian gland (eye region), Zymbal's gland (ear), and preputial/clitoral gland. Tumors occurring exclusively in these organs are often regarded as not relevant for man (188,189), although tumors in the forestomach might indicate, for example, a risk for esophageal tumors in man and the similarity of Zymbal's and preputial/clitoral glands to human sebaceous glands must be kept in mind.

Further differences in the reaction of rodents and man to toxic injury, particularly also with regard to tumor formation, may be based on *pharmaco/toxicokinetic differences* (133,190,191). Therefore, among other aspects the following need clarification: Which metabolites are formed and could they be genotoxic (e.g., higher amount of genotoxic tamoxifen metabolites in test animals) (192)? Does the compound have a special affinity for a particular organ? How is the compound excreted? (See also chapters 2, 5, and 6.)

Histopathogenesis of Tumors

Tumors, which are induced by chemicals, are generally morphologically not different from spontaneous tumors. The development of tumors is often a continuous process, though not all stages have to occur in a textbook-like manner (Table 8). Because of their continuous *nature*, the various stages often overlap and classification into one or the other stage is arbitrary (e.g., based on the size of the lesion). This holds true in particular for the distinction between precursor lesion (hyperplasia) and benign tumor of the endocrine organs. Leydig cell hyperplasia in the testis of more than a certain diameter is called an adenoma, though this "tumor" could possibly still regress (and, therefore, not fulfill the criterion of a tumor), if the endocrine regulation would revert to normal (normal LH levels). In contrast to human pathology, animal pathology generally does not have the benefit of a follow-up of the individual (biopsy, surgery with histological assessment, after surgery, etc.).

Similarly, and for the same reasons as given above, the distinction between *benign and malignant tumor* is also not always clear-cut. Metastases are not frequently seen in lifetime bioassays. In view of the small size of the animals, metastases may also be missed macroscopically with the consequence that the affected portion of the organ is not sampled for histology.

Table 8 Tumor Formation—Histopathological Sequence

Type of lesion		Definition	Underlying reason	Example
Precursor lesions: alterations are reversible and do not compress or infiltrate surrounding tissue	(Hypertrophy)	Increase in cell size, often associated with and difficult to distinguish from hyperplasia	Proliferation, e.g., of SER	Barbiturates, which induce P450 cytochrome drug metabolizing enzymes
	Metaplasia	Transformation of cells into a type generally not seen at this location	e.g., replacement of respiratory epithelium by squamous cell epithelium	Chronic irritation during inhalation studies, with many compounds at doses high enough to elicit some irritation
	Hyperplasia	Increase in cell number, often associated with hypertrophy	Chronic stimulation of cells	Chronic irritation and inflammation at subcutaneous injection site
Tumor: Not reversible. At least some autonomous growth with compression of surrounding tissue	Benign	Tumor remains delimited by connective tissue capsule Does generally not infiltrate surrounding tissue	Expansion of (spontaneously) initiated cells (mutagenic defect) by mitogenic stimulus	Thyrostatic agents induce adenomas of thyroidal follicular cells
	Malignant	Tumor infiltrates surrounding tissue and often also blood vessels, which results in metastases to distant organs	Expansion of (spontaneously) initiated cells (mutagenic defect) by mitogenic stimulus	Any agent which induces benign tumors Anticancer agents

Table 9 Tumor Types Seen Particularly in Lifetime Rodent Bioassays[a]

Cell of origin	Cell/tissue	Benign tumor	Malignant tumor
Epithelium	Squamous	Squamous cell papilloma	Squamous cell carcinoma
	Transitional	Transitional cell papilloma	Transitional cell carcinoma
	Glandular		
	Liver cell	Hepatocellular adenoma	Hepatocellular carcinoma
	Follicular cell (thyroid)	Follicular adenoma	Follicular adenocarcinoma
Connective tissue	Fibroblasts, fibrocytes	Fibroma	Fibrosarcoma
	Cartilage	Chondroma	Chondrosarcoma
	Bone	Osteoma	Osteosarcoma
	Fat	Lipoma	Liposarcoma
Muscle	Smooth muscle	Leiomyoma	Leiomyosarcoma
	Skeletal muscle	Rhabdomyoma	Rhabdomyosarcoma
	Cardiac muscle	Rhabdomyoma	Rhabdomyosarcoma
Neural tissue	Nerve sheath	Schwannoma	Neurogenic sarcoma
	Glioma	Glioma	Malignant glioma
	Astrocytes	Astrocytoma	Malignant astrocytoma
	Embryonic cells	—	Neuroblastoma
Endothelium	Lymph vessels	Lymphangioma	Lymphangiosarcoma
	Blood vessels	Hemangioma	Hemangiosarcoma
Hematopoietic	Bone marrow	—	Leukemia
Lymphoreticular	Lymph nodes	—	Lymphosarcoma

[a]Examples of a few common tumors encountered in lifetime bioassays.
For histopathological atlases of rodent tumors see e.g. (65–70, 387, 388). For hamsters consult the following publications (389, 390).

Tumor Types

Tumors are classified according to

- Organ.
- Cell type.
- Biological behavior: benign, malignant (see "Histopathogenesis of Tumors").
- Degree of differentiation: the less differentiated, the more aggressive they generally are.
- Growth: with increasing malignancy, generally the velocity of growth also increases.

An overview of common tumor types found in lifetime rodent bioassays is given in Table 9.

Basically, all types of cells and all tissues can develop into tumors, both of the benign and the malignant type. Species, strain, breeder, and dose and route of application of the test substance influence the organ-specific tumor incidence (193). Overall, there are no marked differences in the distribution of tumors induced by mutagens and nonmutagens (194,195), although differences were described for some substances, such as chlormethine (196,197). Epigenetic carcinogens usually increase tumor incidences in organs with a high spontaneous rate of tumors and lead to neoplasms with similar histology as their spontaneous counterparts (105). This is further supported by data published in the *Physician's Desk Reference* (142), which cites the most frequent target organs in rats and mice:

- Rats: thyroid, liver, testis, mammary gland, adrenal, and pituitary.
- Mice: liver, lung, mammary gland, bone marrow, and ovary.

Small, but statistically significant, increases in the above tumor incidences are often regarded as less relevant than the appearance of new tumor types. This is the case with tumors in livers (198), particularly in mice livers (199,200).

Pooling of Incidences of Similar Tumors

To increase statistical power of rare observations, one often has to combine the incidences of similar tumors, for example, tumors with similar histogenesis such as benign and malignant tumors of the same cell type in the same organ/organ system (e.g., liver adenoma and carcinoma, leukemia and lymphoma). At times it is reasonable to combine tumors also with precursor lesions (e.g., liver adenomas and carcinomas with hyperplasia/metaplasia). The rationale for combining proliferative lesions is as follows:

- Distinction among hyperplasia, benign tumors, and malignant tumors is often arbitrary, as explained earlier.
- As generally only one section per lesion is examined, the most malignant portion of a neoplasm might not be available.
- One cell type can give rise to different types of proliferative lesions through differentiation (e.g., epithelial basal cells) and dedifferentiation (generally associated with increased malignancy) and shows various growth patterns (e.g., mammary gland and endocrine neoplasms: cystic, papillary, solid growth patterns). Progression over time can change the histological appearance of proliferative lesions significantly.
- For risk assessment, the distinction between benign and malignant generally is not of great importance, with exception of rare cases, when a substance only increases the aggressiveness of tumors.

The following proliferative lesions can generally be combined in lifetime bioassays (201):

- Neoplasms in the respiratory tract or in the peripheral lung, but generally not from both locations together.
- Squamous cell neoplasia of the upper alimentary tract including forestomach.
- Epithelial neoplasm of the small intestine and the large intestine and, depending on the type of proliferative lesions, also of small and large intestine.
- Smooth muscle neoplasia body-wide with exception of those of the GI tract and reproductive tract, which must be evaluated separately.

The type of proliferative lesions that can be combined have often to be determined on a case-by-case basis (202).

INVESTIGATION OF UNCLEAR PATHOLOGICAL FINDINGS

Additional Investigations of Available Samples

If unclear histopathological findings are detected, additional investigations might be warranted (203). They can sometimes be performed on samples already available from the study in question, or from previously conducted toxicity studies (204). Examples for possible investigations include

- *Blood samples for hormones* (205,206), particularly for findings in endocrine organs. As already mentioned, hormone levels provide important insights into the trophic effect on a particular endocrine regulated organ. Increased or decreased stimulation of an organ is associated with hypertrophy/hyperplasia or, conversely, atrophy of the organ in question. Hyperplasia is often associated with tumor formation in that particular organ in lifetime rodent bioassays.
- *Blood or tissue samples for gene or mRNA expression or for marker proteins* or protein patterns by proteomics (207). Such investigations have become fashionable in the past few years. However, the future will have to show to what extent they are actually useful.
- *Tissue samples for investigations by EM or by immunohistochemical methods*. Applications include identification of the proliferative cell type (16,208), measurement of cell proliferation (by PCNA) (57,209), or apoptosis (210). Even with formalin-fixed tissue, EM investigation may show important subcellular details, which help to confirm or reject a pathogenetic

mechanism. The presence of a phospholipidosis can easily be proven by EM investigations on formalin-fixed tissue. Molecular epitopes are astonishingly stable on fixed tissue and can be demonstrated immunohistochemically even after many years in wet tissue and paraffin-embedded tissue. It is always worthwhile to test such material as long as negative and positive controls are run simultaneously.

- *Morphometry* is important to prove or disprove numerical or volume changes in tissues and particularly to establish a no-effect level (13).

Tailor-Made Mechanistic Studies

Additional studies may be envisaged, particularly for the following purposes:

- *To repeat any of the above investigations on properly sampled and prepared tissue,* for example, after fixation with glutaraldehyde by EM (204) or more detailed cell kinetic studies (proliferation, single cell necrosis, etc.) (211).
- *Investigation of early findings and their development over time* (e.g., by sequential necropsies in a time-course study).
- *Reversibility studies*, with necropsies also after a treatment-free period.
- *Mechanistic studies* to test hypotheses that cannot be tested with enough certainty on material already available.
- *Special* in vitro *studies* with cell or tissue cultures, organ slices, or the perfused target organ itself to investigate metabolism (212), effects on subcellular organelles (see"Some Important Subcellular Alterations") or on gene expression (22,26–31), or dose–effect relationships at subcellular or molecular levels (213). Receptor studies (214,215) can be very helpful, while studies of intercellular communication (216) and initiation-promotion assays (217) may not contribute much.

INTERPRETATION OF PATHOLOGICAL FINDINGS

Interpretation of toxicological findings always has to start with a number of questions, such as

- Is the study technically valid?
- Is the model valid?
- Is there indeed an adverse effect? Could the adverse effect be species-specific?
- Were there other relevant findings?
- What is the pathogenesis of the lesion and what is known about other substances of the same class?

Each of these questions is described next.

Is the Study Technically Valid?

There are a number of reasons why a study can be flawed. Three selected aspects are mentioned below.

- One key factor is *dose selection*, particularly in rodent lifetime bioassays (218–221). The high dose must induce minimal signs of toxicity but should not significantly interfere with the growth of test animals or exert overt toxicity (222–224).
- Was the *test substance* analytically characterized according to current standards (225)? How pure was it and what were the impurities? Could an impurity, such as a by- or degradation-product, contaminant (225), or in case of a racemate, the pharmacologically less effective enantiomere (226), be responsible for the toxic response seen?
- Were the *technical pathology procedures* appropriate? This includes, for example, consistent necropsy, sampling, and histotechnical procedures (44,46). Tissue accountability is an important factor to judge the quality and validity of a lifetime bioassay. The quality of study conduct depends significantly on the personnel involved and particularly on the experience of the study director and study pathologist (74,145).

Is the Model Valid?

In particular, is the *species* tested representative for man with regard to anatomy and physiology, absorption, distribution, metabolism, and excretion of the test compound or to the

pharmacodynamic action of drugs (133,227–229)? However, for long-term bioassays, there may not be valid alternatives. For example, albino rodents are not an appropriate model if toxicity studies in pigmented animals (dogs, minipigs, etc.) have shown a particular affinity of the test compound to the pigmented cells. Is the breeder, who supplied the test animals, reliable (182) and the *quality of animals* acceptable with regard to latent infections (121–123)? Was the husbandry good and the diet nutritionally sufficient (230) and without contamination?

To extrapolate findings in test animals to human beings a number of conditions have to be fulfilled:

- Comparable systemic exposure with regard to
  - test compound and its metabolite and
  - distribution, plasma levels, and elimination half-life in vivo.
    If adverse effects in test animals occur only at plasma levels that are at least 100 times more than those achieved in man, there is a good chance that the effects are not clinically relevant.
- The finding must occur in an organ with similar physiology and anatomy as in man. Rodents have anatomical particularities, as discussed in "Species Specificity of Tumor Formation." Aspects of extrapolation of high-dose animal findings to risk at low-dose exposure in humans are discussed in "Extrapolation of High-Dose Findings in Animals to Low Doses As Typically Applied to Man."

Is There Indeed an Adverse Effect?

Pathologists compare the incidence, severity, and time of onset (as far as possible) of lesions in dosed and control animals. Occasionally, control animals might appear to have an unusually low (or high) incidence of a particular lesion. This can be troublesome, particularly when it comes to the evaluation of long-term rodent bioassays, mainly for the following reason: Overtly genotoxic drugs are recognized early in development and, with the exception of certain classes such as antineoplastic drugs, they are not further developed. Drugs, which tested negatively in short-term mutagenicity and genotoxicity assays, may still have a *weak carcinogenic potential* in lifetime rodent bioassays, usually because of epigenetic mechanisms. The variability of spontaneous rodent tumor incidences (64) and the large number of statistical tests performed on tumor data from lifetime rodent bioassays often result in equivocal results (81). If small increases of tumor incidences achieve statistical significance, then one needs to differentiate between a biologically nonrelevant finding and a true carcinogenic effect. This is particularly difficult in the case of a rare tumor or with a slightly increased incidence of a common tumor in high-dose groups. Historical control data can be invaluable in these circumstances. For a discussion regarding the use of historical data, please refer to "Control Data."

Crucial and not infrequently controversial is the distinction between adverse effects and, for example, exaggerated pharmacological or adaptive effects (231–233). This is addressed in the section below.

Were There Other Relevant Findings?

Pathological findings must be *correlated* with in-life observations, clinical chemistry findings, and any other findings, such as results from pharmacological or mechanistic studies. There must be close interaction among the pathologist, the study director, and any other scientist involved in the study (or anyone having performed or is planning to do further studies with the same compound).

Clinical chemistry findings of biochemical toxicity must not necessarily have a histopathological correlate (structural toxicity), but may help the pathologist to correctly interpret histopathological observations. Since investigations of clinical chemistry are conducted repeatedly during a toxicity study, they also provide information on the time course of a target organ lesion.

Findings of other studies, including mechanistic studies, can be very helpful to interpret pathological findings. If new findings were noticed in longer-term studies, reexamination of that particular organ from shorter-term studies is a must. Furthermore, published data and data obtained from regulatory agency (e.g., under the Freedom of Information Act) are additional

sources of information. Knowledge of the mechanism of action helps considerably, especially in the assessment of tumorigenic findings in lifetime rodent bioassays (133,234–236).

What Is the Pathogenesis of the Lesion and What Is Known About Other Substances of the Same Class?

What is the pharmacological action of the compound? Is the toxicity just a consequence of exaggerated pharmacological action or is it an intrinsic part of pharmacological action? To answer these questions, it is always worthwhile to review the published literature or to obtain information form regulators about similar compounds.

Toxic Lesions

The following is a summary of some important toxic effects generally seen with particular classes of drugs:

- Arteritis with phosphodiesterase inhibitors (237), some dopaminergic compounds (238), some endothelial antagonists (239), and other compounds (240).
- Phospholipidosis with amphiphilic drugs, such as tricyclic antidepressants (241).
- Cardiotoxicity with anticancer drugs of the anthracyclic antibiotic type (27,242).
- Nephrotoxicity with aminoglycosides and other antibiotics (243–246).
- Atrophy of rapidly proliferating tissues, in particular, of bone marrow, lymphoid organs, seminiferous tubules with anticancer agents (247–250).
- Ulcers in the intestinal tract and papillary necrosis in kidneys; the latter particularly in dogs with nonsteroidal anti-inflammatory drugs (NSAID) (245,246,251,252).
- Seminiferous tubule atrophy with estrogenic compounds (54,253,254,256–261).
- Pseudopregnancy with persistence of corpora lutea with progestational and dopaminergic compounds, for example, with neuroleptics (259).
- Thyroid insufficiency with sulfonamides (259).
- Hepatomegaly because of SER proliferation, for example, with phenobarbital (14,100,233) or because of peroxisome proliferation, for example, with certain hypolipemic drugs (96,102,103,262,263).
- Neuronal toxicity with organophosphates (264,265).
- Myocardial infarction with vasoactive substances (242).
- Cardiopathy with positive inotropic agents in dogs (242).

Carcinogenicity

There are two basic types of tumorigenesis, namely, genetic and epigenetic (96,266–268).

There are a number of compounds on the market that are carcinogenic in animals and humans because of their mode of action. Examples include alkylating anticancer drugs (140–142) and immunosuppressive drugs (269–271). For this type of compound, extrapolation from test animals to man is regarded as meaningful.

However, several classes of drugs are known to be animal carcinogens but are regarded as safe for man (142), as discussed previously (i.e., precursor lesions). These compounds belong to the class of epigenetic carcinogens, which are classified below.

Carcinogenic class effects (epigenetic carcinogenicity) are as follows:

- Compounds inducing *drug metabolizing enzymes* (best known representative is phenobarbitone) (100,233).
- *Peroxisome proliferators* such as hypolipemic drugs, including the clofibrates (103,262,272).
- *Hormones* and other compounds with some endocrine (side) effects: hyperplastic and neoplastic changes are often seen following hormonal and other receptor-mediated stimulation (259). Bromocriptine and mesulergine, both dopaminergic compounds that lower prolactin levels, significantly decrease the incidence of endocrine tumors but increase that of uterine tumors [bromocriptine (188)] and of Leydig cell tumors [mesulergine (208)]. It is also known that tumors of endocrine organs are often associated with tumors of the adenohypophysis.
- Compounds that, by their intended pharmacological action, *block the normal function of an endocrine organ* or system often lead to tumors in the organ or system (259). For example,

H2-blockers lead to "carcinoids" in the rat stomach (273–275) and antithyroid agents result in thyroid tumors (134,135). These tumors are without relevance to man.

- Similar findings are reported with other compounds, which *disrupt endocrine function* through an adverse action, for example, sulfonamides interfere with thyroid function and lead to thyroid tumors only in rodents (134,135,276).
- β2*-agonists* (e.g., salbutamol or terbutaline) cause hyperplasia of salivary glands and mesovarian leiomyomas in rats, the pathogenesis of which is not well understood (277). Their occurrence can be suppressed by simultaneous administration of a β-blocker, such as propranolol.

The list of animal and human carcinogens is regularly updated in the IARC monographs (140,141,278).

Extrapolation of High-Dose Findings in Animals to Low Doses As Typically Applied to Man

Extrapolation of high-dose findings in animals to estimate the risk of side effects and toxicity at low dose exposure of man is an old and still controversial subject (281–286; chapters 2, 4, and 12).

- Effects produced by *genotoxic and mutagenic compounds* in animals are generally assumed to be relevant for man. In addition, such effects may not have a threshold below which exposure is considered safe (285).
- General *toxicity and epigenetic tumorigenicity* are considered to have a threshold and therefore, many rodent carcinogens are considered safe for man (90,142,286,287).

For overall safety assessment, often only a *weight-of-evidence* approach is possible (234) and the relevance of toxic or tumor findings has to be evaluated on a case-by-case basis (288,289). One also notes a considerable evolvement over time, particularly regarding the assessment of toxicity and tumor findings, which has become less schematic and more pathogenesis oriented (290,291).

On the other hand, much discussion is ongoing regarding the *shape of dose–response curves*. The possibility of, for example, bell-shaped dose-response curves cannot be discounted a priori (292,293). Compounds have various effects that can at least theoretically counteract each other. For instance, a carcinogen might induce fewer tumors at higher doses because of toxicity; splenic toxicity is often associated with decreased incidences of leukemia in mice (294).

Furthermore, there can be no doubt that lifetime rodent bioassays are not a completely reliable *test system* (295) and their interpretation is at times controversial (296). They are difficult to validate and are sometimes questioned with regard to their usefulness (297,298). Nevertheless, lifetime rodent bioassays are widely accepted (299). Low sensitivity to detect a carcinogenic effect is of greater concern than low specificity [i.e., carcinogenic findings of no relevance to man (300,301)].

Safety Factors

If toxicity or an epigenetic carcinogenic effect of possible relevance to man is found, the single most important parameter to evaluate is the no observed adverse effect level (NOAEL) for toxic or carcinogenic effects in relation to the exposure. Is there a sufficient safety factor defined as exposure at the NOAEL in the most sensitive species (as far as this species is of relevance to man) relative to the maximal human exposure (dose/concentration and duration of exposure) occurring or intended? For the reasons given below, no strict rules are available. Generally, a safety factor of 10 for extrapolation from animal to man and an additional safety factor of 10 for interindividual variation in man (for a total safety factor of 100) is considered acceptable. However, there are many compounds on the market with a considerably lower safety factor.

The *therapeutic indication* of drugs is particularly important. Lower safety factors are acceptable for life-saving indications compared to non–life-saving indications. In general, higher safety factors are needed when children or young adults are the target treatment population compared to medications used for patients toward the end of their natural life span. Are potentially safer alternatives available or has the substance in question a true advantage over the alternatives

that would justify a minimal risk? How did regulatory authorities assess similar findings with other drugs? For chemicals other than drugs, generally none or limited human data are available. Furthermore, exposure to these agents happens accidently or through contamination. Therefore, risk assessment tends to be more conservative for agrochemicals (302), other environmental chemicals (218,282), consumer products (303), residues in food (304), and chemicals at the workplace (305). The general principles are also summarized in the textbooks of general toxicology or in various publications (306).

Especially high safety margins are required for compounds that induce lesions, which are considered irreversible. Lesions in the category include necrosis of neurons and ocular changes (particular retinal degeneration or degeneration of the lenses).

Certain authorities are also very cautious in case of toxicity to the reproductive organs, which could lead to sterility (besides the possibility of teratogenic effects).

REPORTING

Study Report

Good reporting of potentially adverse effects, including a candid discussion and scientifically sound conclusions are very important.

The *method section* must include a good description of the procedures followed during the study and for the evaluation. In particular, the pathology methods must be described with reference to the standard nomenclature used for the histopathological diagnosis of lesions, the recording method for tumor multiplicity, and concomitant hyperplasia and neoplasia in the same organ or system, as well as the peer review procedures. The statistical evaluation procedures for tumors must be described, including pooling of groups and lesions. If additional investigations were performed on the material of the study in question, they have to be reported or a reference has to be given to indicate where the results are or will be reported. In general, additional mechanistic studies are reported separately.

The *result section* must comprise the following: an individual animal pathology report, with the findings for each animal in the study, namely, macro- and microscopic findings including correlation with relevant in-life data, in particular the first occurrence of palpable masses (mainly rodent bioassays) and cause of death for animals that died or were killed in moribund state. In addition, summary pathology tables should be included, where treatment and control groups are identified separately as are decedent and terminal sacrifice animals within each control/treatment group. Summary tables should be available at least for cause of death or moribund state per group, incidences per group for macroscopic lesions, nonneoplastic and neoplastic lesions as well as grading/severity for selected nonneoplastic lesions (as appropriate). For selected organs/tissue systems, one may want to consider including summary tables with combined benign and malignant tumors or hyperplasias as described in "Pooling of Incidences of Similar Tumors." Data regarding the normal variation of tumor incidences in the particular species and strain should be included as far as necessary.

In the *discussion section,* each finding should be addressed including decreased incidences of spontaneous lesions and their possible relationship with exposure or treatment (288,307) as well as pharmacokinetic and toxicokinetic considerations (133,227–229). The interpretation of nonrodent findings can be particularly challenging because of the relatively small number of animals used (308). A highly important aspect of the final discussion is that of possible mechanisms of toxicity (96,235,266,276,309,310).

Technical Documentation for Regulatory Authorities

In the discussion of an overall summary to be submitted to the authorities, a review of all internal (preclinical and clinical) and external (published) data of relevance to the issue should be performed and copies of all cited reports and publications submitted together with a final conclusion and recommendation to the authorities. Among other issues, the following items should be addressed: factors, which may have contributed to the findings; species-related particularities; pharmacology of the substance in relation to the toxicity/carcinogenicity findings; drug metabolism aspects; presence or absence of precursor lesions; and any other findings of importance. Particularly for the risk assessment part, relevant human data must be included

and discussed, if available. In this context, it is particularly important to point out the differences between man and test animals. One should also not be afraid to discuss, if appropriate, the worst-case scenario, that is, risk assessment in the unlikely event that the toxicity or the proliferative effect seen in the test species is relevant for man. A conclusion is not just an opinion, but must be justified so that one can attempt extrapolation from experimental animals to humans (309).

CONCLUSIONS

This overview is intended to introduce the reader to the science of pathology investigations and illustrate the value of pathology in preclinical development. Pathology provides mechanistic insight into pharmacologic and toxic effects. It also permits the preclinical development scientist to predict potential human outcomes to drug exposure by careful extrapolation from nonhuman studies.

Early microscopic assessment of nonhuman tissues may reveal subtle toxicity and subsequently lead to a timely discontinuation of the development project. Pathology assessments can also reveal potentially serious toxicities that might not become apparent, for example, until long-term clinical trials have been conducted.

In all cases, appropriate pathology support will increase the likelihood of safe drugs proceeding into and through clinical development. It is incumbent upon the preclinical development scientist to utilize pathology expertise early and frequently during the development of a drug candidate.

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R.A. Ettlin has managed this collection at its early stage. With the dissolution of the Institute of Toxicology, the collection passed into the custody of the Preclinical Safety Department of Novartis Pharma AG in Switzerland. Teaching slide sets with 24 to 30 duplicates of representative selected cases can be obtained for up to 4 weeks at no costs with the exception of those for mailing and insurance at CaseCollection@eurotoxpath.org.

Microphotographs of representative lesions from this collection are part of a CD-ROM edited by a group of pathologists coordinated by PG Germann. This CD-ROM is available from the European Society of Toxicologic Pathology (ESTP) (391).

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11 Utilizing the Preclinical Database to Support Clinical Drug Development

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One of the important goals of the preclinical phase in drug development is to generate knowledge and information that leads to the rational and informative design and conduct of clinical studies in human subjects. This goal can be achieved through a variety of in vitro or ex vivo technologies such as isolated organs and tissues, cell cultures, cellular fragments, subcellular organelles, receptors, ion channels, transporters and enzymes, and whole animal in vivo investigations (1–3). Traditionally, preclinical studies have focused on early clinical drug development, that is, clinical pharmacology, pharmacokinetic (PK), and pharmacodynamic (PD) studies (4,5).

From the perspective of clinical drug development, the aim of preclinical studies should be evaluations of both safety and efficacy in various experimental settings of animal species that can be integrated into the information database for the safe and effective use of the drug in humans. More specifically, the preclinical safety evaluation should focus on identification of (*i*) an initial safe dose and subsequent dose escalation schemes in humans, (*ii*) potential target organs for toxicity and their reversibility, (*iii*) safety parameters for clinical monitoring, and (*iv*) at-risk populations (6,7). Likewise, the specific aim of efficacy evaluation during the preclinical phase is primarily to elucidate the PD characteristics of the agent and their impact upon the therapeutic activity (8). Therefore, the determination of the PK profile of the drug and/or the metabolites in animals as a guide to these characteristics in humans is indispensable. Additionally, the development and use of biomarkers in animal models is strongly recommended, because these can demonstrate early signals of efficacy and toxicity in humans (9–14).

In this chapter, several real drug development cases will be introduced in which the preclinical database was adequately utilized to provide drug developers with supportive, and sometimes decisive, evidence necessary to design efficient and informative clinical development programs, thereby facilitating final regulatory approvals. These examples will be followed by a brief discussion on the implication of using the preclinical database in a specific drug development setting.

UTILIZING THE PRECLINICAL DATABASE TO SELECT HUMAN DOSE

The selection of dose for the first-time-in-man (FTIM) study is one of the most important and difficult decisions to be made when entering the clinical phase of drug development. The dose for the FTIM study has to be small enough to not to cause harm to the subjects, but, at the same time, a starting dose that is too small must be avoided since it will increase the time needed to reach the maximum tolerated dose (MTD) or the clinically efficacious dose. Many methods to determine the safe human dose have been introduced with mixed results (15–17).

Determination of Starting Dose: A Regulatory Agency's View

The U.S. Food and Drug Administration (FDA) released the *Guidance for industry for estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers* ("*Dose Guidance*" hereafter) in July 2005 (18), outlining a standardized process for deriving the maximum recommended starting dose (MRSD) for adult healthy volunteers for first-in-human clinical trials of new molecular entities. This guidance was the finalized version of the earlier draft guidance in 2002. The *Dose Guidance* defined the MRSD as the highest initial dose recommended in a clinical trial that is predicted to cause no adverse reactions in adult healthy volunteers.

The steps for selecting the MRSD are summarized in Figure 1. The MRSD is selected after the determination of the no observed adverse effect levels (NOAELs), based upon the analysis

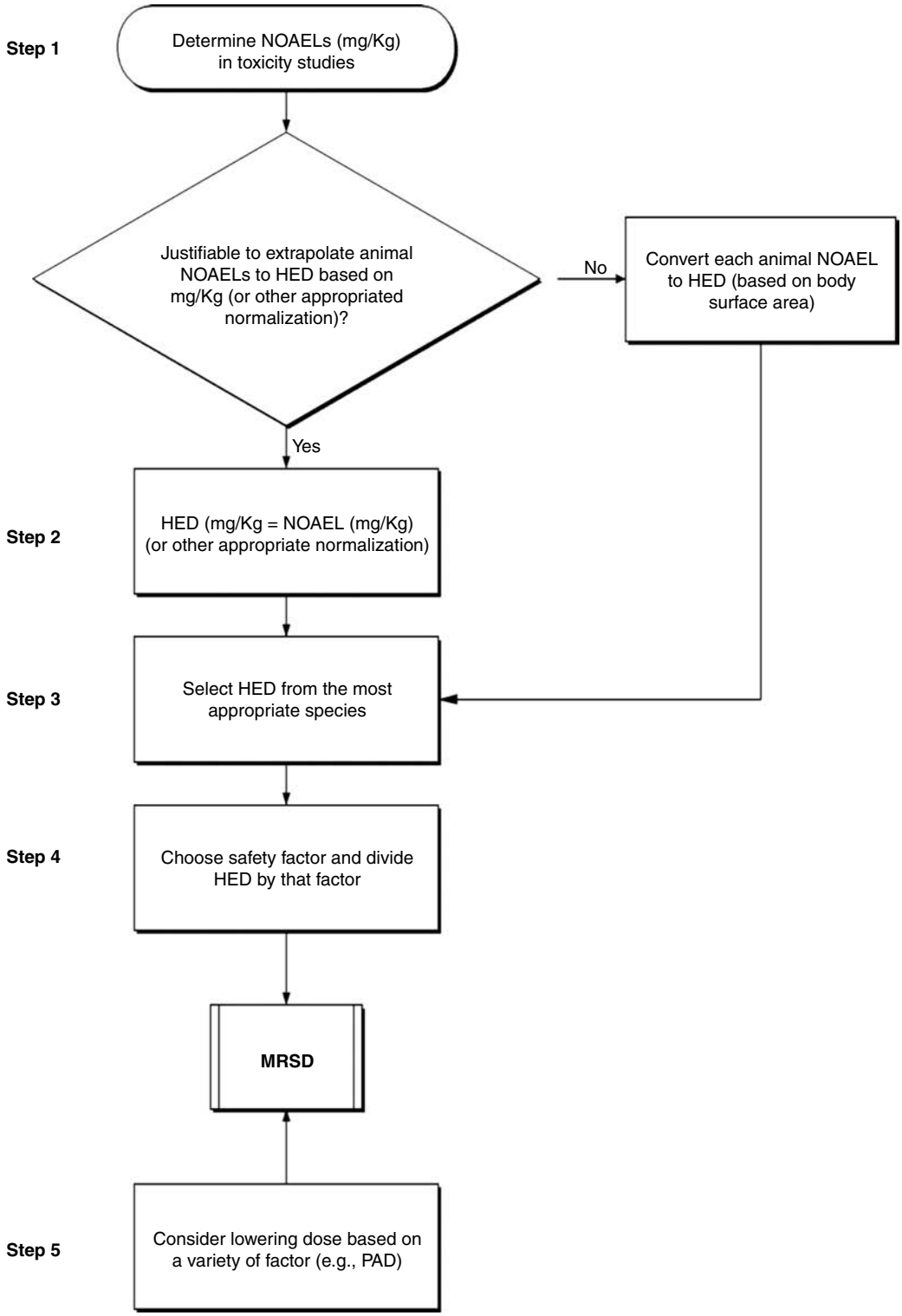

Figure 1 Selection of the maximum recommended starting dose for drugs administered systematically to normal volunteers. *Abbreviations*: NOAELs, no observed adverse effect levels; HED, human equivalent dose; MRSD, maximum recommended starting dose; PAD, pharmacologically active dose. *Source*: From Ref. 18.

of toxicity data from preclinical animal studies. Although only the NOAEL is used in the first step of the algorithm, other data (exposure/toxicity relationships, pharmacologic data, or prior clinical experience with related drugs) will affect the choice of the most appropriate species, scaling, and safety factors in later steps.

The NOAEL for each species tested should be converted to the human equivalent dose (HED) by appropriate scaling factors. The *Dose Guidance* strongly recommends that the conversion be based on the normalization of doses to body surface area for most systemically administered therapeutics. In some cases, however, extrapolating doses based on other parameters (e.g., body weight) may be more appropriate; therefore, this decision should be based on the data available for the individual case. The body surface area normalization and the extrapolation of the animal dose to human dose should be done in one step; the NOAEL for each species is divided by the appropriate body surface area conversion factor, a unitless number that converts mg/kg dose for each animal species to the mg/kg dose in humans. This results in a HED, which is equivalent to the animal's NOAEL on a mg/m^2 basis. The species that generates the lowest HED is called the most sensitive species but may not necessarily be the most appropriate species.

When information indicates that a particular species is most relevant for assessing human risk, the HED for that species should be used in subsequent calculations, regardless of whether this species is the most sensitive one. This case is common for biologic therapies, many of which have high selectivity for binding to human target proteins, and limited reactivity in species commonly used for toxicity testing. In such cases, in vitro binding and activity studies should be done to select appropriate, relevant species before toxicity studies are designed (7). Additionally, a species might be considered an inappropriate toxicity model for a given drug if a dose-limiting toxicity in that species was concluded to be of limited value for human risk assessment (based on historical comparisons of toxicities in species to those in humans across a therapeutic class). In this case, data from that species should not be used to derive the HED. Without any additional information to guide the choice of the most appropriate species for assessing human risk, the most sensitive species is used, because using the lowest HED would generate the most conservative starting dose.

A safety factor should then be applied to the HED to increase assurance that the first dose in humans will not cause adverse effects. The use of the safety factor is based on the possibility that humans may be more sensitive to the toxic effects of a therapeutic agent than predicted by the animal models, that bioavailability may vary across species, and that the models tested do not evaluate all possible human toxicities. In general, a safety factor of 10 is recommended, and the MRSD should be obtained by dividing the HED by the safety factor. Safety concerns or design shortcomings noted in animal studies may increase the safety factor and reduce the MRSD further. Alternatively, if the pharmacologic class is well-characterized (with extensive human clinical and preclinical experience), information about the class may allow reduction of the default safety factor and an increase in the MRSD. The process described here derives the MRSD; a dose lower than the MRSD can be used as the actual starting dose. The MRSD is in units of mg/kg, a common method of dosing used in phase I trials. As previously stated, for purposes of initial clinical trials in adult healthy volunteers, the HED should ordinarily be calculated from the animal NOAEL. If the HED is based on an alternative index of effect, such as the pharmacologically active dose (PAD), this exception should be clearly stated in descriptions of starting dose calculations in the study protocol.

Mechanistic Approaches to Determine Human Dose

Although it is intended to offer a pragmatic and conventional standard method as for establishing the MRSD, the FDA's *Dose Guidance*, as intended, may be incomplete; it does not mention or call sufficient attention to other scientifically valid and more mechanistic approaches (19). For example, the *Dose Guidance* (18) placed little emphasis on the animal PK–PD modeling approach, stating that

> . . . , in the majority of investigational new drug applications (INDs), animal data are not available in sufficient detail to construct a scientifically valid, pharmacokinetic model whose aim is to accurately project an MRSD.

This view, however, appears inconsistent with other recent FDA guidelines that emphasize the importance of PK–PD relationships in establishing the safety as well as the efficacy of a drug in development (20,21). In other words, the *Dose Guidance* has focused only on dose, which does not necessarily guarantee a specific exposure. Therefore, mechanistic approaches that take full advantage of existing preclinical information are needed to estimate the dose for a FTIM study.

Recent developments in advanced PK–PD modeling and simulation technology can be applied to design better-informed clinical studies using existing data (22–26); this topic was discussed in detail in chapter 6. In some cases, PK–PD modeling and simulation can be also used to bridge the gap between preclinical and clinical phases of drug development. For example, PK–PD modeling and simulation was actively used to design the FTIM and the proof-of-concept studies of a new neurological agent using only preclinical data without the support of any human in vivo information; rhesus monkey and human PK were initially estimated using allometric scaling on data collected in dogs, cynomolgus monkeys, and rats (15). A PK–PD model was then derived from a study conducted in rodents and validated by comparing the model-predicted response to that observed in a positron emission tomography experiment conducted in rhesus monkeys.

Preclinical Database to Determine Human Effective Dose

In some cases, preclinical data are the sole source of information by which the human effective dose (i.e., the dose for proof-of-concept study) has to be determined. For example, palivizumab (Synagis®), a humanized monoclonal antibody against the respiratory syncytial virus (RSV), was approved by the FDA in 1998, and the indication was prophylaxis of serious lower respiratory tract disease caused by RSV in pediatric patients at high risk of RSV disease (27). Three in vitro neutralization studies (i.e., microneutralization assay of RSV, plaque reduction neutralization assay, and neutralization of clinical isolates) were conducted during the preclinical phase in which palivizumab was shown to inhibit RSV replication (28). Additionally, in vivo intravenous (IV) treatment, intramuscular (IM) prophylaxis, and IV prophylaxis studies were conducted using the cotton rat animal models loaded with RSV, where the rats were challenged intranasally with 10^5 plaque forming unit (pfu) of RSV, and lung tissue was collected and pulmonary viral titers were determined. These studies showed that palivizumab was effective in both prophylaxis and treatment of the RSV pulmonary infection, and serum concentrations of $\geq$40 μg/mL were shown to reduce the pulmonary RSV replication in the RSV cotton rat models. For example, in the IV prophylaxis study, a reduction in RSV titer $\geq$ 100-folds (i.e., 2 logs) corresponded to a mean serum antibody concentration of 25 to 40 μg/mL at the time of challenge (Table 1).

The human PK studies were conducted in infants and children during the RSV season, and the mean trough concentration after single IM and IV doses of palivizumab, 15 mg/kg, were 49 μg/mL and 60.6 μg/mL, respectively. In addition, after quarterly IV doses of palivizumab, 15 mg/kg, the mean trough concentration was 96.9 μg/mL (29). Consequently, 15 mg/kg was

Table 1 Prophylaxis by Intravenous Pavilizumab Administration in Cotton Rats Loaded with Respiratory Syncytial Virus

Treatment	Number of animals	Dose (mg/kg)	Concentrations of human IgG (μg/mL)	Lung viral titer (pfu[a]/g)
BSA[b]	15	10	0	$1.3 \times 10^5 \pm 1.2$
Pavilizumab	7	0.312	2.67 ± 0.60	$4.6 \times 10^4 \pm 1.5$
Pavilizumab	17	0.625	5.27 ± 0.27	$2.7 \times 10^4 \pm 1.3$
Pavilizumab	18	1.25	10.1 ± 0.29	$3.3 \times 10^3 \pm 1.4$
Pavilizumab	17	2.5	28.6 ± 2.15	$9.6 \times 10^2 \pm 1.5$
Pavilizumab	15	5.0	55.6 ± 3.43	$1.3 \times 10^2 \pm 1.2$
Pavilizumab	18	10.0	117 ± 5.09	$1.0 \times 10^2 \pm 1.0$

[a] Plaque forming unit.
[b] Bovine serum albumin (control).
Source: From Ref. 28.

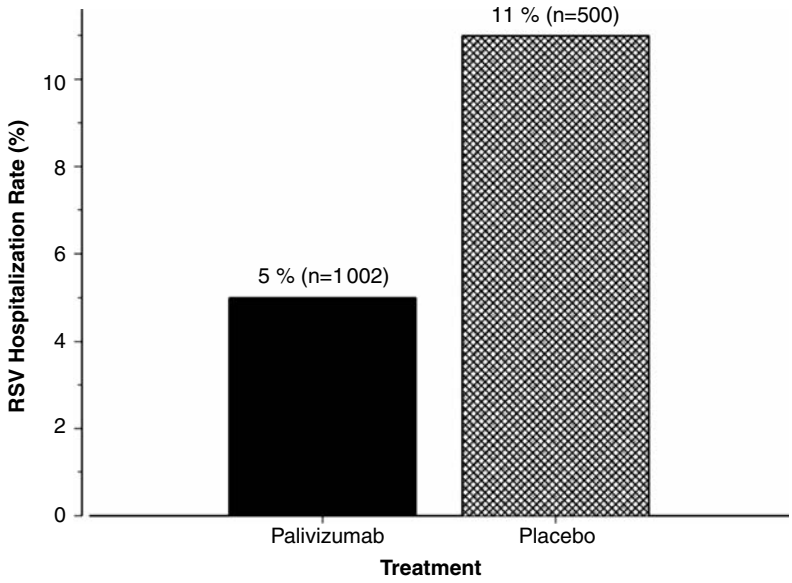

Figure 2 RSV hospitalization rates by palivizumab (15 mg/Kg IM, monthly for 5 months) and placebo, $p < 0.0001$, 2-sided Fisher's exact test. *Source*: From Ref. 29.

selected as the human effective dose for the phase II and III studies with palivizumab largely based on the preclinical RSV lung infection cotton rat model and the PK profiles obtained in the phase I studies.

It is interesting to note that the clinical reviewer of palivizumab found the sponsor's approach for dose selection was *acceptable* even though no dose-response relationship was formally investigated in the target population, that is, pediatric patients at high risk of RSV disease (29). The clinical reviewer commented that since the cotton rat model is a fairly well-accepted one and that, due to its low incidence, it would have been very difficult to conduct a dose-response study using RSV hospitalization rate as the primary endpoint in human patients, the utilization of the preclinical information seemed reasonable.

Using 15 mg/kg as the human effective dose based on the preclinical database, a randomized, double-blind, placebo-controlled, multiple-dose (IM monthly injection for 5 months) phase III study of palivizumab was conducted in infants and children at high risk for severe RSV disease. The RSV hospitalization rate was used as the primary endpoint and was significantly lower in the palivizumab-treated group compared to that in the placebo-treated patients (Fig. 2), confirming that the dose-selection approach was appropriate. This palivizumab case adequately exemplified how informative the preclinical database can be to select the human effective dose when the clinical dose-response studies are difficult or impossible to conduct.

UTILIZING THE PRECLINICAL DATABASE TO SUPPORT THE EFFICACY CLAIM FOR REGULATORY APPROVAL

Preclinical efficacy findings, if adequately coupled with the well-understood pathophysiology of a disease and the mechanism of action of a drug, could serve as the confirmatory evidence to support the conduct of a single phase III clinical trial for regulatory approval (30). This is a huge saving in terms of both time and resource for drug developers given that the current FDA position is to require at least two adequate and well-controlled phase III studies, each convincing on its own, to establish effectiveness for final regulatory approval (31). Preventive vaccines are good examples; one adequate and well-controlled clinical trial may be supported by compelling animal challenge/protection models, if accompanied by human serological data, passive antibody data, or pathogenesis information demonstrating that there is a previously accepted correlation with clinical effectiveness and serological response data (20).

Table 2 Reduction in Colorectal Polyp Counts by Treatments

Treatment	Number of patients	% Reduction	p-value[a]
Placebo	15	4.5	
Celecoxib, 100 mg b.i.d.[b]	33	14.5	0.327
Celecoxib, 400 mg b.i.d.[b]	30	28.0	0.003

[a]Compared to placebo treatment.
[b]Twice a day.
Source: From Ref. 34.

In some cases, efficacy data from animal models have been the most important or the sole confirmatory evidence supporting the final regulatory approval. For example, Celebrex™ (celecoxib) was approved by the FDA in 1999 for the reduction of the number of adenomatous colorectal polyps in patients with familial adenomatous polyposis (FAP) as an adjunct to usual care (e.g., endoscopic surveillance, surgery) (32). This was a supplemental new drug application (sNDA) for celecoxib, which was first approved in 1998 for the relief of the signs and symptoms of osteoarthritis and rheumatoid arthritis (33).

For the FAP indication, the sponsor submitted the results of a single randomized, double-blind, placebo-controlled, dose-response phase III study in which a total of 83 patients received either placebo, celecoxib 100 mg b.i.d. (twice a day), or celecoxib 400 mg b.i.d. for 6 months. Patients on the celecoxib 400 mg b.i.d. showed a statistically significant reduction (mean = 28%) in the number of colorectal polyps from the baseline, which was used as the surrogate endpoint (34). A dose-response relationship was also observed (Table 2).

A significant body of evidence suggested that the cellular expression of cyclooxygenase-2 (COX-2) is prominent in some tumors, for example, colon (35–37), skin (38), prostate (39,40), lung (41,42), and breast (43,44) as well as precancerous lesions including the adenomatous polyp (45,46). On the basis of this understanding on the role of COX-2 in cancer pathophysiology, celecoxib was evaluated in two animal models of colon cancer (34,47). In the adenomatous polyposis coli (APC) mutant Min mouse model, which represents a genetic model of human FAP, celecoxib, which selectively inhibits COX-2, showed effectiveness for prevention (i.e., "early" treatment before development of adenomatous polyps) and regression (i.e., treatment after most adenomatous polyps are established) comparable to that of the positive control of piroxicam. Celecoxib caused dramatic reductions in the multiplicity of tumors in a dose-dependent manner (Table 3) (34,47,48).

Additionally, in the rat colon cancer model induced by azoxymethane, treatment with celecoxib for 11 weeks resulted in a 40% reduction in aberrant crypt foci that was similar to that observed for the positive control, sulindac, given at its maximum tolerated dose (320 ppm) (34,49). When administered for 1 year in the diet, 1500 ppm of celecoxib reduced tumor incidence by 93%, surpassing the results observed in similar studies with various nonsteroidal anti-inflammatory drugs (NSAIDs) (34,50).

Table 3 Effect of Celecoxib Treatment on Tumor Multiplicity in the Adenomatous Polyposis Coli (APC) Min Mouse Model

		Tumors/mouse	
Drug	Dose in diet (ppm)	Early treatment (30–80 days)	Late treatment (55–80 days)
Vehicle	0	22.4 ± 9.0	22.9 ± 6.8
Celecoxib	150	15.8 ± 9.5	18.0 ± 7.6
	500	15.8 ± 4.6	16.3 ± 6.2
	1500	6.5 ± 4.2	11.1 ± 6.8
Piroxicam	50	5.2 ± 4.0	7.9 ± 4.8

Source: From Refs. 34, 47, and 48.

In the review document, the medical reviewer clearly indicated that the approval of celecoxib for the reduction of the number of adenomatous colorectal polyps in FAP patients was supported by (*i*) evidence from animal colon tumor models, demonstrating a reduction in the incidence and multiplicity of tumors with its exposure; and (*ii*) numerous clinical studies, mostly small, uncontrolled series, demonstrating the ability of other NSAIDs, notably sulindac, to reduce colorectal polyps in FAP patients (34). This example adequately illustrates how preclinical animal models, when coupled with the well-understood pathophysiology of a disease, can be successfully used to support the efficacy claim for regulatory approval.

UTILIZING PRECLINICAL DATABASE TO ADDRESS SAFETY CONCERNS

When the FDA reviews a submitted NDA package, any safety concerns raised during the preclinical or nonclinical phase are given special attention if they were not adequately addressed in the clinical development, or if those clinical studies addressing the same safety issues were limited in terms of scope and exposure time. This can become more serious if the same or similar safety issues were seen in a drug of the same class. For example, cardiac hypertrophy was observed in all animal species tested with pioglitazone (51,52), a thiazolidinedione developed for the treatment of type 2 diabetes mellitus; this was also seen in preclinical studies with troglitazone (53) and rosiglitazone (54). Since diabetic patients are obese in general and are likely to have other chronic diseases such as hypertension and congestive heart failure, cardiac hypertrophy was viewed as one of the main safety concerns by the medical reviewer at the FDA (55).

In order to directly address this issue, the sponsor conducted a 26-week, placebo-controlled, randomized, double-blind study to compare changes in the echocardiogram in patients with type 2 diabetes treated with four doses of pioglitazone (7.5, 15, 30, and 45 mg). Patients who had valvular abnormalities, ischemic heart disease leading to left ventricular motion abnormalities, or symptomatic heart failure were excluded, and 80 patients were assigned to each dose group. No significant changes from the baseline echocardiogram were noted, and there were no clinically meaningful differences among the treatment groups. However, the design of this study was criticized by the medical reviewer at the FDA, because poor glycemic control in the placebo-treated patients could produce harmful effects on the heart. Additionally, the analysis was not appropriately adjusted for body weight and lipid profiles that could also have an impact on the heart, and more serious patients, that is, New York Heart Association (NYHA) functional class III or IV patients, were not included in the study.

On the basis of this reasoning, the medical reviewer concluded that the cardiac safety concern needed to be clarified further because, as the drug reached the market, patients with different, possibly more serious, profiles from those studied in the pivotal clinical trials would be exposed and the exposure time increased. Likewise, the pharmacology reviewer, concurring with this view, commented that clinical safety should have been demonstrated for approval. Pioglitazone was finally approved by the FDA in 1999; however, the sponsor was requested to conduct a phase IV, randomized, placebo-controlled six-month clinical study in patients with type 2 diabetes and New York Heart Association class II and early class III patients with congestive heart failure, along with six additional phase IV commitments (56).

The pioglitazone NDA case clearly illustrates how seriously the FDA considers any major safety concerns seen in the preclinical studies in the final NDA review process. Therefore, drug developers should take a serious look at any major safety issues raised during the preclinical phase, and clinical studies should be designed to produce clinically meaningful information to address these issues.

UTILIZING THE PRECLINICAL DATABASE TO DESIGN IN VIVO METABOLIC DRUG–DRUG INTERACTION STUDIES

Hepatic metabolism and renal excretion are the two main elimination routes of a nonprotein drug or its metabolites (57). Many metabolic pathways, including the hepatic cytochrome P450 enzymes (CYP), can be inhibited, activated, or induced by the administration of a concomitant drug. More and more, preclinical in vitro studies using suitable probes and appropriately validated experimental methods, for example, human liver microsomes (58,59) or recombinant cytochrome P450 (58,60), can be used as screening mechanisms to rule out a possible metabolic

pathway and drug–drug interactions that could occur via this pathway. In many cases, negative findings from early in vitro studies, if coupled with similar results from early clinical studies, can eliminate the need for later in vivo clinical investigations (61,62).

On the other hand, when positive findings are seen in in vitro metabolic studies, in vivo clinical studies are recommended. The clinical importance of the potential drug–drug interaction should be quantitatively estimated for safe and efficient use in patient populations that were not necessarily tested in clinical development and for which the interaction may be sufficiently large to necessitate dosage adjustment or therapeutic monitoring of the drug itself or concomitant medications (61). For example, the potent CYP3A4 inhibitor, ketoconazole, inhibited 85% of in vitro pioglitazone metabolism at equimolar concentrations, suggesting that pioglitazone may be a substrate for the CYP3A4 metabolic pathway (63). However, the sponsor did not conduct any in vivo drug–drug interaction studies based on these preclinical results; instead, they conducted in vivo drug–drug interaction studies of pioglitazone with digoxin, glipizide, warfarin, and metformin in which no significant changes in PK parameters were found. The sponsor's approach to address possible drug–drug interactions focused mainly on a specific group of drugs that were likely to be coadministered with pioglitazone (digoxin, hydrochlorothiazide) or for which the clinical consequences of potential interactions were of concern.

Additionally, troglitazone, another thiazolidinedione, was known to potentially induce CYP3A4 (64), but pioglitazone was not tested for this potential, neither in vitro nor in vivo. The clinical pharmacology and biopharmaceutics reviewer at the FDA discussed that the drug interaction potentials of pioglitazone were not investigated using the understanding of the metabolic pathways of related agents; instead, the sponsor's approach made it hard to draw conclusions for general drug–drug interaction profiles (62,63). As a result, the FDA requested the sponsor to conduct two drug–drug interaction studies as the phase IV commitments for regulatory approval of pioglitazone: a two-way crossover drug interaction study of single-dose pioglitazone and single-dose ketoconazole (i.e., pioglitazone as a *substrate* of the CYP3A4 pathway), and a two-way crossover drug interaction study of steady-state pioglitazone and single-dose midazolam (i.e., pioglitazone as a possible *inducer* of the CYP3A4 pathway) (56).

Again, this pioglitazone drug interaction study exemplifies why it is so important for clinical drug development to be guided by information and knowledge obtained from preclinical studies.

LIMITATIONS AND PREDICTIVE VALUE OF THE PRECLINICAL DATABASE

As presented in the previous sections, the preclinical database can play a significant role in clinical drug development by providing supportive, sometimes critical, information on the safety and/or efficacy of the drug in humans for the purposes of regulatory approval. Although new in vitro techniques have reduced the need for animal studies, these conventional studies still have been the primary method to understand the in vivo pharmacology, toxicology, efficacy, and safety of a drug for clinical development.

However, toxicity testing using animals is time consuming and expensive, especially primates [e.g., a monkey costs approximately $3500 to $5000 (65)], which may restrict the breadth of the preclinical database for clinical drug development (66). Additionally, there are some inherent limitations of preclinical studies, which may render a naïve empirical extrapolation from animals to humans less useful or even meaningless for the purposes of an informed decision during clinical drug development (67). For example, the variability between human individuals can be overestimated due to extrapolation across different animal species; diversity even exists within inbred strains of homogenously derived and maintained laboratory animals (68,69). Furthermore, there are few animal models or animal-based in vitro or ex vivo systems that can duplicate the structure or function of humans, and drug developers must validate the animal systems as models for human systems at all levels (70–72).

Therefore, the value of animal models in predicting the efficacy and toxicity of a drug in humans can be realized only in cases where findings are congruent in both animal models and humans (73,74), that is, efficacy/toxicity found (75–77) or not found (78), in both animals and humans (Table 4). When animal models failed to find a drug's efficacy and toxicity, seen later in humans (third row of Table 4), these animal models had no predictive value (79); in practice, the

Table 4 Predictive Value of Animal Models

Findings in		Predictive value	
Animal[a]	**Human[b]**	**Efficacy**	**Toxicity**
Found	Found	Yes	Yes
Not found	Not found	Yes	Yes
Not found	Found	No	No
Found	Not found	No	?

[a]Multiple of human dose.
[b]Human effective dose.
Source: From Ref. 73.

drug could have dropped from further clinical development at preclinical stage. But, the failure to find efficacy in animal models does not necessarily preclude further clinical development. For example, none of animal models of osteoarthritis (OA), including spontaneous OA in mouse and guinea pig, meniscectomy and ligament transection in guinea pigs, meniscectomy in rabbits, and meniscectomy and cruciate transection in dogs, have been used with success to predict the efficacy in humans, because no agents have been shown to provide anything other than symptomatic relief in human OA (80).

Of course, if the efficacy of a drug was seen in animal models but not found in humans, the drug would fail to show proof of concept in humans and is likely to be dropped from further development. The most difficult decision, however, has to be made when toxicity, found in an animal model, is not necessarily seen in humans, after which the predictive value of this animal model may be questioned. In this case, drug developers must carefully plan and design clinical studies to adequately address safety issues raised in animal models, and the preclinical database can give them meaningful insight into potential safety concerns as exemplified in the case of pioglitazone (51,55,56).

Physiologically-based PK (PBPK) modeling, although not widely used in drug development because of its technical complexity, may be useful for internal decision making to assess the predictive value of the preclinical database for clinical drug development (81–83). Because physiologically-based PK approaches help drug developers link toxicity data from animal species to expected clinical observations using the exposure–response (i.e., toxicokinetic safety) relationship, it can serve as a valuable tool for understanding what preclinical PK, safety, and efficacy results ultimately mean in humans (84).

CONCLUSION

Although there are some limitations of preclinical studies, they provide the sole source of data upon which the assessments of drug efficacy and safety are to be made before human data become available (73). Therefore, if utilized adequately, coupled with more physiological models that account for the biological inter- and intraspecies diversity and variability based on a mechanistic understanding of a drug action, the preclinical database is a valuable tool to support an efficient and informative clinical drug development that leads to a successful regulatory approval.

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about the book...

This ***Second Edition*** of ***Preclinical Drug Development*** provides clinical chemists, drug development scientists, biologists, and researchers the tools they need to manage and improve all development and regulatory aspects of preclinical formulation and routes of administration. This breakthrough resource delivers the A to Z's of the latest industry trends and compliance guidelines that every bench scientist needs to succeed.

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