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# Role of Pharmacokinetics and Metabolism in Drug Discovery and Development

JIUNN H. LIN[a] AND ANTHONY Y. H. LU

*Department of Drug Metabolism, Merck Research Laboratories, West Point, Pennsylvania*

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[a] Address for correspondence: Jiunn H. Lin, WP26A-2044, Department of Drug Metabolism, Merck Research Laboratories, West Point, PA 19486.

## I. Introduction

Drug research encompasses several diverse disciplines united by a common goal, namely the development of novel therapeutic agents. The search for new drugs can be divided functionally into two stages: discovery and development. The former consists of setting up a working hypothesis of the target enzyme or receptor for a particular disease, establishing suitable models (or surrogate markers) to test biological activities, and screening the new drug molecules for in vitro and/or in vivo biological activities. In the development stage, efforts are focused on evaluation of the toxicity and efficacy of new drug candidates. Recent surveys indicate that the average new chemical entity taken to market in the United States requires 10 to 15 years of research and costs more than $300 million.

Once the target enzyme or receptor is identified, medicinal chemists use a variety of empirical and semiempirical structure-activity relationships to modify the chemical structure of a compound to maximize its in vitro activity. However, good in vitro activity cannot be extrapolated to good in vivo activity unless a drug has good bioavailability and a desirable duration of action. A growing awareness of the key roles that pharmacokinetics and drug metabolism play as determinants of in vivo drug action has led many drug companies to include examination of pharmacokinetics and drug metabolism properties as part of their screening processes in the selection of drug candidates. Consequently, industrial drug metabolism scientists have emerged from their traditional supportive role in drug development to provide valuable support in the drug discovery efforts.

To aid in a discovery program, accurate pharmacokinetic and metabolic data must be available almost as early as the results of the in vitro biological screening. Early pharmacokinetic and metabolic evaluation with rapid information feedback is crucial to obtain optimal pharmacokinetic and pharmacological properties. To be effective, the turnover rate needs to be at least three to five compounds per week for the support of each program. Due to time constraints and the availability of only small quantities of each compound in the discovery stage, studies are often limited to one or two animal species. Therefore, the selection of animal species and the experimental design of studies are important in providing a reliable prediction of drug absorption and elimination in humans. A good compound could be excluded on the basis of results from an inappropriate animal species or poor experimental design.

After a drug candidate is selected for further development, detailed information on the metabolic processes and pharmacokinetics of the new drug is required by regulatory agencies. The rationale for the regulatory requirement is best illustrated by the case of active metabolite formation. Many of the currently available psychotropic drugs form one or more metabolites that have their own biological activity (Baldessarini, 1990). Pharmacokinetically, the active metabolites may differ in distribution and clearance from that of the parent drug. Pharmacologically, the parent drug and its metabolites may act by similar mechanisms, different mechanisms, or even by antagonism. An understanding of the kinetics of active metabolite formation is important not only for predicting therapeutic outcome, but also for explaining the toxicity of specific drugs.

Conventionally, the metabolism of new drugs in humans is studied in vivo using radiotracer techniques as part of clinical absorption and disposition studies. However, this approach often occurs relatively late in the development stage. Ideally, the metabolism of new drugs should be studied in vitro before the initiation of clinical studies. Early information on in vitro metabolic processes in humans, such as the identification of the enzymes responsible for drug metabolism and sources of potential enzyme polymorphism, can be useful in the design of clinical studies, particularly those that examine drug-drug interactions. It is also desirable that the comparison of metabolism between animals and humans be performed in the early stage of the drug development process to provide information for the appropriate selection of animal species for toxicity studies before these toxicity studies begin.

The advance of in vitro enzyme systems used for drug metabolism studies (Wrighton and Stevens, 1992; Guillouzo et al., 1993; Berry et al., 1992; Remmel and Burchell, 1993; Brendel et al., 1990; Chapman et al., 1993), together with the explosion of our knowledge of various drug-metabolizing enzymes including uridine-diphosphate-glucuronosyl-transferases (Cougletrie, 1992), cytochrome P-450s (Henderson and Wolf, 1992; Gonzalez and Nebert, 1990) and carboxylesterases (Wang, 1994; Hosokawa, 1990), allows us to obtain early information on the metabolic processes of new drug candidates well before the initial clinical studies. In addition, the advent of commercial liquid chromatography-mass spectrometry instrumentation and the development of high-field nuclear magnetic resonance as well as liquid chromatography-nuclear magnetic resonance techniques have further strengthened our capability to study the metabolism of new drugs in the early drug discovery stage (Fenselau, 1992; Baillie and Davis, 1993). However, the role of drug metabolism scientists in drug discovery is more than just screening compounds in vitro and in vivo. It really entails a good understanding of the basic mechanisms of the events involved in absorption, distribution, metabolism and excretion; the interaction of chemicals with the drug-metabolizing enzymes, particularly cytochrome P-450; sources of pharmacokinetic and pharmacodynamic interindividual variability; and the consequences of metabolism on potential drug toxicities.

The purpose of this paper is to review the role of pharmacokinetics and drug metabolism in drug discovery and development from an industrial perspective. The intent is to provide a comprehensive, rather than exhaustive, overview of the pertinent literature in the field. Several excellent review articles on individual topics are available and the reader is referred to the most recent articles in the text. It is hoped that with a better understanding of the fate of the drugs, a balanced in vitro/in vivo approach and an intelligent application of sound principles in pharmacokinetics and enzymology, drug metabolism scientists can contribute significantly to the development of safe and more efficacious drugs.

## II. Role of Pharmacokinetics and Metabolism in Drug Design

The history of the pharmaceutical industry shows that many important drugs have been discovered by a combination of fortuity and luck. This serendipity is best exemplified by the discovery of isoniazid. Isoniazid was first synthesized by Meyer and Mally (1912). Its antituberculosis properties were not found until 40 years later, when Robitzek et al. (1952) gave isoniazid to 92 "hopeless" patients with progressive caseous-pneumonic pulmonary tuberculosis that had failed to show improvement after any therapy. Furthermore, both indomethacin and ibuprofen compounds were developed as antirheumatic agents even without any knowledge of their mode of action (Shen, 1972; Adams et al., 1969, 1970). The mode of action was established several years after the drugs were on the market when Vane (1971) showed that these nonsteroidal anti-inflammatory drugs acted by inhibiting the synthesis of prostaglandins.

Another example of serendipity is the discovery of anxiolytics. Diazepam and chlordiazepoxide, the most widely used benzodiazepines, were found to have anxiolytic activity in 1958 and were marketed in 1960. Efforts to determine the mechanism of benzodiazepine action were initiated only after their introduction into the clinic. It was not until 1974 that convincing evidence from behavioral, electrophysiological, and biochemical experiments was accumulated to demonstrate that benzodiazepines act specifically at synapses in which $\gamma$-aminobutyric acid (GABA)[b] functions as a neurotransmitter (Baldessarini, 1990; Haefely et al., 1985; Williams and Olsen, 1989).

Over the past decades, through a better understanding of disease processes, mechanism-based drug design has evolved and produced drugs that interrupt specific biochemical pathways by targeting certain enzymes or receptors. This approach does not require a knowledge of the three-dimensional environment in which drugs act. Recent advances in molecular biology and protein chemistry have provided pure protein in sufficient quantities to allow structural studies to be carried out. Visualization of these structures by sophisticated computer graphics has made structure-based drug design feasible. These rational approaches of drug design have been successful historically in the fields of HIV protease inhibitors (Vacca et al., 1994), hepatic hydroxymethylglutaryl coenzyme A reductase inhibitors (Alberts et al., 1980) and angiotensin-converting enzyme (ACE) inhibitors (Patchett et al., 1980).

Today, the design of new drugs is still received by many medicinal chemists to mean maximization of the desired drug activity within certain structural limits. Sometimes, however, compounds that show very high activity in vitro may prove later to have no in vivo activity, or to be highly toxic in in vivo models. Lack of in vivo activity may be attributed to undesirable pharmacokinetic properties, and the toxicity may result from the formation of reactive metabolites. Therefore, rational drug design should also take both pharmacokinetic and metabolic information into consideration, and the information should be incorporated with molecular biochemical and pharmacological data to provide well-rounded drug design.

### *A. Metabolism and Drug Design*

From toxicological and pharmacological points of view, it is desirable to design a "safer" drug that undergoes predictable metabolic inactivation or even under-

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[b] Abbreviations: 3-MC, 3-methylcholanthrene; 6-TGN, 6-thioguanine nucleotide; ACE, angiotensin-converting enzyme; AFB, alfatoxin B1; Ah, aromatic hydrocarbon; AUC, area under the curve; AZT, zidovudine; BBB, blood-brain barrier; $CCK_B$ cholecystokinin; cL, clearance; $cL_H$ hepatic clearance; $cL_{int}$, intrinsic clearance; CNS, central nervous system; CSF, cerebrospinal fluid; DMBA, 7,12-dimethylbenz[a]anthracene; DMBB, 5-(1,3-dimethylbutyl)-5-ethyl barbituric acid; EM, extensive metabolizer; $f_p$, fraction of unbound drug in plasma; $f_t$, free fraction in tissue; GABA, $\gamma$-aminobutyric acid; GSH, glutathione; $K_i$, dissociation constant of an inhibitor; $K_{inact}$, maximum inactivation rate constant; $K_m$, Michaelis constant; $K_p$, ratio of drug concentration in tissue to that in plasma after drug administration; L-dopa, levodopa; MPH, methylphenidate; NAT, *N*-acetyltransferase; NSAID, nonsteroidal anti-inflammatory agent; PEG, polyethelene glycol; PFDA, perfluorodecanoic acid; PM, poor metabolizers; PPAR, peroxisome proliferator-activated receptors; TMT, thio methyltransferase; TPMT, thiopurine methyltransferase; UDPGT, uridine diphosphoglucose transferase; $V_d$, volume of distribution; $V_i$, velocity of an enzymic reaction in the presence of of inhibitor extensive metabolizers; $V_{max}$, maximum velocity; $V_o$, velocity of an enzymic reaction in the absence of inhibitor.

goes no metabolism. Several approaches have been used for the design of safer drugs.

*1. Hard drugs.* The concept of nonmetabolizable drugs, or so-called hard drugs, was proposed by Ariëns (1972) and Ariëns and Simonis (1982). The hard drug design is quite attractive. Not only does it solve the problem of toxicity due to reactive intermediates or active metabolites, but the pharmacokinetics also are simplified because the drugs are excreted primarily through either the bile or kidney. If a drug is excreted mainly by the kidney, the differences in the elimination between animal species and humans will be dependent primarily on the renal function of the corresponding species giving highly predictable pharmacokinetic profiles using the allometric approach (Lin, 1995; Mordenti, 1985). A few successful examples of such hard drugs include bisphosphonates and certain ACE inhibitors.

Bisphosphonates are a unique class of drugs. As a class, they are characterized pharmacologically by their ability to inhibit bone resorption, whereas pharmacokinetically, they are classified by their similarity in absorption, distribution and elimination. In the clinic, these drugs are used in patients as antiosteolytic agents for the treatment of a broad range of bone disorders characterized by excessive bone resorption. These include hypercalcemia of malignancy, metastatic bone disease, Paget's disease, and osteoporosis.

The discovery of bisphosphonates was based on earlier studies of inorganic pyrophosphate by Fleisch and his coworkers (Fleisch et al., 1966, 1968, 1969; Fleisch and Russell, 1970). They found that pyrophosphate bound very strongly to calcium phosphate and inhibited not only the formation of calcium phosphate crystals, but also the crystal dissolution in vitro. However, pyrophosphate exhibited no effect on bone resorption in vivo. This was later explained by the observation that pyrophosphate is hydrolyzed before it reaches the site of bone resorption. These findings led to a search for analogs that would display the activities similar to pyrophosphate, but would also resist enzymatic hydrolysis. It was found that the bisphosphonates, characterized by a P-C-*P* bond rather than the P-O-*P* bond of pyrophosphate, fulfilled these criteria. As hard drugs, bisphosphonates are not metabolized in animals or humans, and the only route of elimination is renal excretion (Lin et al., 1991c; Lin, 1996a). In general, these compounds are very safe with no significant systemic toxicity (Fleisch, 1993).

Similarly, enalaprilat and lisinopril are considered hard drugs. These two ACE inhibitors undergo very limited metabolism and are exclusively excreted by the kidney (Ulm et al., 1982; Tocco et al., 1982; Lin et al., 1988). Unlike sulfhydryl-containing ACE inhibitors, such as captopril and its analogs, neither enalaprilat nor lisinopril exhibits significant side effects (Kelly and O'Malley, 1990). The most common side effects accompanying the clinical use of captopril are rashes and taste dysfunction (Atkinson and Robertson, 1979; Atkinson et al., 1980). Similar side effects are observed with penicillamine, which is a sulfhydryl-containing heavy metal antagonist used extensively in the treatment of Wilson's disease (Levine, 1975; Suda et al., 1993). It is therefore speculated that captopril interacts with endogenous sulfhydryl-containing proteins to form disulfides that may act as haptens, resulting in immunological reactivity, which may be responsible for these side effects (Patchett et al., 1980). Enalaprilat and lisinopril were designed to avoid these undesirable side effects by removal of the sulfhydryl group (Patchett et al., 1980).

Due to their poor lipophilicity, the bisphosphonates, enalaprilat and lisinopril, are not metabolized in vivo. Ironically, the poor lipophilicity of these compounds results in poor oral absorption. For the bisphosphonate alendronate, the octanol/buffer partition coefficient is 0.0017 (Lin, 1996a). As a result of its poor lipophilicity, alendronate has very poor oral bioavailability in humans (<1%) (Lin, 1996a). To our knowledge, bisphosphonates are the only class of drugs being developed for oral dosage in spite of their poor bioavailability (Lin, 1996a). This is because the systemically available bisphosphonates are largely taken up by the target (bone) tissues, where their elimination is very slow (Lin, 1996a, 1992, 1993b). The half-life of alendronate in bone was estimated to be at least 10 years in humans.

Like bisphosphonates, both enalaprilat and lisinopril have low lipophilicity. The octanol-to-water partition coefficient is approximately 0.003 for both drugs (Ondetti, 1988). Interestingly, enalaprilat, a diacid compound with a net negative charge, is poorly absorbed (<10%), whereas lisinopril, a zwitterionic compound, has acceptable oral absorption (~30%) (Ulm et al., 1982; Tocco et al., 1982). Consequently, enalaprilat was developed as its ethyl ester prodrug (enalapril) to increase its bioavailability, whereas the prodrug approach was not employed for lisinopril.

Bisphosphonates and these two carboxyalkyldipeptide ACE inhibitors were not intentionally designed as hard drugs. The "hardness" came about only as a result of structural improvement. It so happens that the newer ACE inhibitors, such as benazepril, perindopril, and fosinopril, undergo significant metabolism (Kelly and O'Malley, 1990).

Although metabolically inert compounds are highly desirable candidates for drug design, the versatility of the drug-metabolizing enzymes presents quite a challenge to achieve this goal. For example, cytochrome P-450s are known to catalyze numerous oxidative reactions involving carbon, oxygen, nitrogen, and sulfur atoms in thousands of substrates with diverse structures. In addition, cytochrome P-450s are unique in that metabolic switchings can occur when the primary metabolic site of a compound is blocked. Thus, considering the broad substrate specificities and the versatilities of cytochrome P-450s and other drug-metabolizing enzymes, designing drug candidates that are metabolically inert may not always be feasible.

*2. Soft drugs.* In contrast to the concept of hard drugs, Bodor (1984, 1982) and Bodor et al. (1980) have proposed the approach of soft drugs. A soft drug is pharmacologically active as such, and it undergoes a predictable and controllable metabolism to nontoxic and inactive metabolites. The main concept of soft drug design is to avoid oxidative metabolism as much as possible and to use hydrolytic enzymes to achieve predictable and controllable drug metabolism. Most oxidative reactions of drugs are mediated by hepatic cytochrome P-450 enzyme systems that are often affected by age, sex, disease, and environmental factors, resulting in complex biotransformation and pharmacokinetic variability (Hunt et al., 1992; Soons et al., 1992). In addition, P-450 oxidative reactions have the potential to form reactive intermediates and active metabolites that can mediate toxicity (Guengerich and Shimada, 1993). These undesirable effects attributed to oxidative metabolism may be circumvented to some extent by incorporating metabolic structural "softness."

Bodor and his colleagues (Bodor, 1984, 1982; Bodor et al., 1980) have designed soft quaternary-type drugs containing three structural components: an acidic group, an aldehyde, and a tertiary amine. Upon absorption, the soft quaternary drugs are hydrolyzed to three nontoxic components that are rapidly eliminated from the body.

Atracurium, a nondepolarizing muscle relaxant, can be considered a soft drug. This drug contains quaternary *N*-functions and ester groups. Atracurium is metabolized in vivo by two nonoxidative processes: a nonenzymatic metabolism by Hofmann-degradation to form a tertiary amine and an alkene, and hydrolysis of the ester groups by esterases (Mutschler and Derendorf, 1995; Hughes and Chapple, 1981).

Remifentanil, a novel short-acting $\mu$-opioid receptor agonist, may also be considered a soft drug. This drug is a methyl ester and is metabolized extensively by esterases to an inactive acid metabolite, GI-90291, of which over 90% is subsequently recovered in urine. To a much lesser extent, the drug also is metabolized by *N*-dealkylation to a second metabolite, GI-94219 (Feldman et al., 1991; Bürkle et al., 1996; Glass, 1995). The major metabolite GI-90291 is approximately 2000- to 4000-fold less potent compared with remifentanil. Although both hard and soft drug designs are of academic interest, there are only a few successful examples in the drug market.

*3. Active metabolites.* For many years, the process of biotransformation was considered synonymous with the inactivation of pharmacologically active compounds. There is increasing evidence, however, that the metabolites of some drugs are pharmacologically active. Numerous examples of pharmacologically active metabolites being used as a source of new drug candidates exist because these metabolites often are subject to phase II reactions and have better safety profiles.

Perhaps the best known example is acetaminophen, which is an *O*-deethylated metabolite of phenacetin. Acetaminophen shows superior analgesic activity when compared with phenacetin. The main advantage of acetaminophen over phenacetin is that it does not produce methemoglobinemia and hemolytic anemia (Flower et al., 1985). Phenacetin is converted to at least 1 dozen metabolites by *O*-deethylation, *N*-deacetylation, and hydroxylation processes. *N*-hydroxyphenatidine, a metabolite of phenacetin, has been shown to be responsible for the formation of methemoglobin and hemolysis of red blood cells (Jensen and Jollow, 1991). Conversely, acetaminophen primarily undergoes glucuronidation and sulfation exclusively and is quite safe clinically at the recommended dose. Similarly, the analgesic oxyphenbutazone is an active para-hydroxy metabolite of phenylbutazone. Similar to acetaminophen, this active metabolite also shows better analgesic activity than phenylbutazone and causes less gastric irritation (Flower et al., 1985).

Although pharmacologically active metabolites are generally formed by phase I oxidative reactions, phase II conjugation reactions also can produce biologically active metabolites. Morphine 6-glucuronide is more potent as a $\mu$-opioid receptor agonist than morphine itself (Paul et al., 1989; Mulder, 1992). Recent clinical studies in cancer patients given morphine 6-glucuronide indicated that useful analgesic effects are achieved without the side effects of nausea and vomiting that are often associated with morphine (Osborne et al., 1992). These findings have led to the commercial marketing of morphine 6-glucuronide. Sulfation also produces biologically active metabolites. Minoxidil, a potent vasodilator, is a good example. Studies concerning the action of minoxidil revealed that the therapeutic activities were mediated by its sulfate conjugate (Bray and Quast, 1991).

In addition to the advantages that active metabolites may have in terms of efficacy with fewer unwanted side effects, active metabolites can also be preferred over the parent drugs for kinetic reasons. Many benzodiazepines form active metabolites with similar pharmacological properties. Oxazepam is the common active metabolite of chlordiazepoxide, halazepam, chlorazepate, and diazepam (Caccia and Garattini, 1990). Unlike other benzodiazepines, oxazepam undergoes only glucuronidation and has a shorter half-life than any of its precursors. This kinetic advantage has led to the marketing of oxazepam as a short-acting benzodiazepine in the treatment of sleeping disorders (Baldessarini, 1990).

### *B. Pharmacokinetics and Drug Design*

Many of the failures of drug candidates in development programs are attributed to their undesirable pharmacokinetic properties, such as too long or too short $t_{1/2}$, poor absorption, and extensive first-pass metabolism. In a survey, Prentis et al. (1988) reported that of 319 new drug candidates investigated in humans, 77 (40%) of the 198 candidates were withdrawn due to serious pharmacokinetic problems. This high failure rate illustrates the importance of pharmacokinetics in drug discovery and development.

To ensure the success of a drug's development, it is essential that a drug candidate has good bioavailability and a desirable $t_{1/2}$. Therefore, an accurate estimate of the pharmacokinetic data and a good understanding of the factors that affect the pharmacokinetics will guide drug design. This section includes a discussion of the chemically modifiable factors that influence drug absorption and disposition.

*1. Absorption.* Drug absorption is influenced by many biological and physicochemical factors. The two most important physicochemical factors that affect both the extent and the rate of absorption are lipophilicity and solubility (Leahy et al., 1989). The membrane of the gastrointestinal epithelial cells is composed of tightly packed phospholipids interspersed with proteins. Thus, the transcellular passage of drugs depends on their permeability characteristics to penetrate the lipid bilayer of the epithelial cell membrane, which is in turn dependent on the lipophilicity of the drugs. As in the example of bisphosphonates, drugs with poor lipophilicity will be poorly absorbed after oral administration (Lin, 1996a). The effect of lipophilicity on oral absorption is best exemplified by the classical study of barbiturates conducted by Schanker (1960). In this study, the absorption of these compounds increased with increasing lipophilicity as a result of increased membrane permeability. Similarly, Taylor et al. (1985) have shown that the absorption rates of a series of $\beta$-blockers in rat small intestine correlated well with their lipophilicity. However, it should be noted that although there is a correlation between lipophilicity and increased permeability, lipophilicity, in some cases, is not predictive of permeability because of external factors.

The oral bioavailability of a drug is defined as the fraction of an oral dose of the drug that reaches the systemic circulation. Because the entire blood supply of the upper gastrointestinal tract passes through the liver before reaching the systemic circulation, the drug may be metabolized by the liver and gut wall during the first passage of drug absorption. A drug with high metabolic clearance is always subject to an extensive first-pass effect, resulting in low bioavailability. The lipophilicity of a drug not only affects the membrane permeability, but the metabolic activity as well. In general, the higher the lipophilicity of a drug, the higher its permeability and the greater its metabolic clearance and thereby its first-pass metabolism (Seydel and Schaper, 1986; Toon and Rowland, 1983). The effects of lipophilicity on membrane permeability and first-pass metabolism appear to have opposing effects on the bioavailability. Thus, it is important to balance the effects of lipophilicity on membrane permeability and first-pass metabolism to improve bioavailability. Also, it should be pointed out that there are many factors, in addition to lipophilicity, that can influence first-pass metabolism.

The influence of lipophilicity on the metabolic clearance of drugs is attributed mainly to the increased affinity of drugs for the enzymes. In vitro studies with rat liver microsomes by Martin and Hansch (1971) revealed that variations in maximum velocity ($V_{max}$) values for a series of compounds unrelated in chemical structure were small (only 3- to 5-fold), whereas the Michaelis constant ($K_m$) values varied by approximately 1000-fold. The $K_m$ values were found to correlate significantly with their lipophilicity. The higher lipophilicity resulted in lower $K_m$ values (higher enzyme affinities). Kinetic studies in dogs revealed that there was a positive correlation between metabolic clearance and lipophilicity for dihydropyridine calcium channel blockers in that the metabolic clearance increased with increasing lipophilicity (Humphrey, 1989).

The discovery of fluconazole (Richardson, 1993) is one of the examples of successfully applying the lipophilicity concept in drug design. Pfizer's initial efforts to find a novel antifungal agent resulted in tioconazole, which is clinically effective against fungal infections of the vagina and skin when administered topically. However, tioconazole shows poor efficacy when given intravenously or orally. Pharmacokinetic studies indicated that although this drug was absorbed reasonably well from the gastrointestinal lumen, it was subject to extensive first-pass metabolism, resulting in low oral bioavailability. In addition, the drug also was highly bound to plasma proteins, giving very low circulating levels of the unbound drug. Efforts were made to decrease the lipophilicity of this class of compounds to increase the metabolic stability and to decrease the protein binding. Efforts to decrease the lipophilicity included the replacement of the imidazole function with 12,4-triazole moiety to yield the bistriazole compound, UK-47,265. Although pharmacokinetic evaluation showed excellent absorption and kinetic profiles in several animal species after oral dosing, UK-47,265 exhibited hepatotoxicity in mice and dogs, which could be attributed to the 2,4-dichlorophenyl moiety. This finding led to the synthesis of a 2,4-diflurophenyl analog of UK-47,265, currently known as fluconazole. The introduction of fluorine into a molecule can alter both the metabolism and toxicity of a drug (Park and Kitteringham, 1994). In the case of fluconazole, the fluorine substitution was shown to reduce hepatotoxicity.

Solubility is also an important determinant in drug absorption; a drug must be reasonably soluble in the aqueous environment to be absorbed properly. The discovery of HIV protease inhibitors is an example that illustrates the concept of drug solubility. At Merck Research Laboratories (West Point, PA), starting from an initial peptide renin inhibitor (L-364,505), Vacca and his coworkers (Vacca et al., 1994; Dorsey et al., 1994) successfully developed a novel hydroxyethylene dipeptide isostere series of highly potent and selective HIV protease inhibitors. However, like other HIV protease inhibitors that contain the hydroxyethylene or hydroxyethylamine transition state isosteres, the main drawback of Merck's initial inhibitors was that they were highly lipophilic and poorly soluble, resulting in poor bioavailability. Efforts were made to increase the solubility by incorporating a basic amine into the backbone of this series (table 1; fig. 1). The addition of pyridine to this series lead to the discovery of indinavir (MK-639, L-735,524). As shown in table 1, the aqueous solubility of indinavir is pH-dependent. The solubility of indinavir increased dramatically from 0.07 mg/mL at pH 7.4 to 60 mg/mL at pH 3.5 due to the protonation of the pyridine nitrogen ($PK_a$ = 3.7). For this reason, indinavir sulfate is the clinical formulation, because it maintains the acidity of the gastrointestinal tract and dissolves more rapidly than free base. Indinavir sulfate is well absorbed after oral dosing and was approved recently for the treatment of AIDS.

A different approach to HIV protease inhibitor design was formulated by Abbott Laboratories (North Chicago, IL). The chemists successfully designed a series of $C_2$ symmetric inhibitors to match the $C_2$ symmetric active site of HIV protease. However, once again, the high lipophilicity and poor aqueous solubility limited these inhibitors for oral delivery. A77003, a $C_2$ symmetric inhibitor, was Abbott Laboratories' first HIV protease inhibitor to reach clinical trials for intravenous use (Kempf et al., 1991). Intravenous administration of A77003 was discontinued in phase I clinical trials because the large doses were required as a result of its short $t_{1/2}$ and the accompanying irritation and phlebitis at the injection site. A nonsymmetric analog, A80987, with improved aqueous solubility (pH 4, 122 μg/mL) had greater oral bioavailability and improved $t_{1/2}$ in animals (Kempf et al., 1995). Although A80987 gave reasonably good absorption in phase I clinical trials, the short $t_{1/2}$ limited the ability to maintain plasma levels in excess of the 95% effective concentration for viral replication. Intensive study of a series of A80987 analogs has yielded valuable insight into the relationship of chemical structure to antiviral activity, aqueous solubility, and hepatic metabolism. Application of these insights to drug design resulted in the discovery of ritonavir (Kempf et al., 1995).

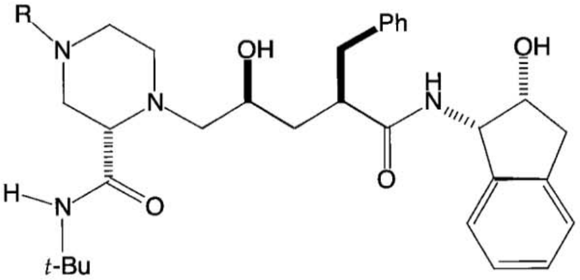

FIG. 1. Chemical structure of some HIV protease inhibitors. See table 1 for the substitutions (R). Reproduced with permission from Dorsey et al. (1994).

In lieu of chemical modification, formulation approaches can be used sometimes to improve oral absorption of poorly soluble drugs. For further information, readers can reference a recent article reviewed by Aungst (1993), which discussed several formulation strategies of improving bioavailability of poorly soluble drugs. L-365,260 [a cholecystokinin ($CCK_B$) receptor antagonist] is a good example in which the formulation modification is applied. This drug has a very poor aqueous solubility of <2 μg/mL. When given orally as a suspension in 0.5% methylcellulose suspension, bioavailability was 14% for the rat and 9% for the dog (Lin et al., 1996c). The low bioavailability of L-365,260 was due mainly to its poor absorption as a result of its poor aqueous solubility, rather than extensive first-pass metabolism. In a separate study, by comparing the drug concentration in the systemic circulation during portal or femoral vein infusion of the drug, the hepatic first-pass metabolism was shown to be low, only 30% for the rat and 14% for the dog.

When L-365,260 was given orally as a solution in PEG 600 to rats and dogs, the bioavailability was increased 3- to 4-fold in rats and 8- to 9-fold in dogs (Lin et al., 1996c). With this information at hand, L-365,260 was dosed in capsules containing PEG 600 in the subsequent clinical studies. This formulation also gave good absorption of L-365,260 in humans. Although the underlying mechanism for the improved absorption is unknown, PEG 600 may have exerted a cosolubilizing effect to maintain a higher drug concentration in solution in the gastrointestinal tract.

TABLE 1

*Effect of solubility of HIV protease inhibitors on drug absorption in dogs after a 5-mg/kg p.o. dose*

| Compound | R | $C_{max}$ (μM)[a] | Solubility (mg/ml) at pH 7.4 (or 3.5) | log *P* |
|---|---|---|---|---|
| L-732,747 | benzyloxycarbonyl | <0.10 | <0.001 | 4.67 |
| L-735,482 | 8-quinolinylsulfonyl | <0.10 | <0.001 | 3.70 |
| L-738,891 | 2,4-difluorophenylmethyl | 0.73 ± 0.15 | 0.0012 | 3.69 |
| L-735,524 | 3-pyridylmethyl | 11.4 ± 2.3 | 0.07 (60) | 2.92 |

[a] $C_{max}$, maximum drug concentration in plasma. See figure 1. Data excerpted from Dorsey et al. (1994).

*2. Prodrugs.* The prodrug concept was first proposed by Albert (1958). Since then, this approach has been widely used in drug design. Although there are many reasons to use prodrugs, improvement of oral absorption is by far the most common. Antibiotic prodrugs comprise the largest group of prodrugs developed to improve oral absorption (Wermuth, 1984). Pivampicillin, talampicillin, and bacampicillin are prodrugs of ampicillin, all resulting from the esterification of the polar carboxylate group to form lipophilic, enzymatically labile esters. The absorption of these prodrugs is nearly complete (98–99%), whereas that of ampicillin is <50% (Loo et al., 1974; Clayton et al., 1974; Bodin et al., 1975).

Enalapril, the most widely prescribed ACE inhibitor, is the ethyl ester prodrug of the active diacid, enalaprilat. Enalaprilat is poorly absorbed from the gastrointestinal tract (<10%), but absorption of the prodrug enalapril is greatly improved (60%). Hepatic metabolic hydrolysis is responsible for its conversion to the active diacid (Ulm et al., 1982; Tocco et al., 1982).

In addition to the simple approaches of ester and amide prodrug formation, more sophisticated manipulation of chemical entities can be used. For example, acyclovir, a potent antiherpes drug, is poorly and erratically absorbed after oral dosing due to its polarity. Although acyclovir possesses a derivatizable hydroxyl group in its structure, esterification of this hydroxy group did not improve the absorption. However, desoxyacyclovir (fig. 2), a prodrug of acyclovir that is activated by xanthine oxidase present in both the gut and liver, gives superior oral delivery of acyclovir over that of the parent drug or its esters (Rees et al., 1986; Krasny and Petty, 1987; Krenitsky et al., 1984). In vivo, phosphorylation of acyclovir is essential for antiviral activity. In normal mammalian cells, phosphorylation of this drug is extremely low, but in cells infected with the herpes simplex virus, there is an induction of a virus-coded thymidine kinase, which effectively catalyzes its phosphorylation (fig. 2). Thus, acyclovir is preferentially activated in virus-infected cells (Krenitsky and Elion, 1982). The example of acyclovir illustrates the point that medicinal chemists can use metabolic and kinetic information to design a better drug.

Sulindac (MK-231), a nonsteroidal anti-inflammatory agent (NSAID) is another interesting example in which medicinal chemists applied metabolic and kinetic principles to design a prodrug. Sulindac is an indene analog of indomethacin. Replacement of the indole nucleus with indene reduces central nervous system (CNS) activity, and the addition of fluoro affords increased analgesic potency. Furthermore, the introduction of a methylsulfinyl (sulfoxide) group not only increases the aqueous solubility but also provides a center for metabolism in vivo (Shen and Winter, 1977). Sulindac, per se, is pharmacologically inactive; it is reversibly reduced to the sulfide metabolite, which is as potent as indomethacin. In vitro study with leukocytes showed a marked difference in the partition and permeation of sulindac and its active sulfide metabolite. The more hydrophilic sulindac tends to remain extracellular, whereas the more lipophilic sulfide accumulates inside the cell with cell/medium ratio of 50:1 (Duggan, 1981). The differential tissue distribution of sulindac and sulfide contributes to its patient tolerance, as well. Although most NSAIDs produce gastrointestinal lesions that are related to local depletion of prostaglandins, the gastrointestinal irritation is reduced by the oral administration of its inactive prodrug (sulindac) and the lack of enterohepatic recirculation of the active sulfide metabolite.

Another promising area for prodrugs is their application to site-specific drug delivery (Stella, 1989; Stella and Himmelstein, 1980). γ-Glutamyl dopa is an example of a site-specific prodrug of levodopa (L-dopa) (Wilk et al., 1978). L-dopa is a precursor of the neurotransmitter dopamine, which plays an important role in the CNS. Aside from its action as a neurotransmitter, dopamine also exerts receptor-mediated vasodilation in the kidney. Intraperitoneal injection of γ-glutamyl dopa into mice led to the selective generation of dopamine in the kidney as a consequence of the sequential actions of γ-glutamyl transpeptidase and L-aromatic amino acid decarboxylase, two enzymes that are highly concentrated in the kidney. The concentration of dopamine in the kidney after γ-glutamyl dopa administration was five times higher than that after administration of an equivalent dose of L-dopa (Wilk et al., 1978). Infusion of 10 nmol·g·30 min of γ-glutamyl dopa to rats produced a 60% increase in renal plasma flow, whereas the same dose of L-dopa had no effect on renal plasma flow. The selective properties of γ-glutamyl dopa suggest that this

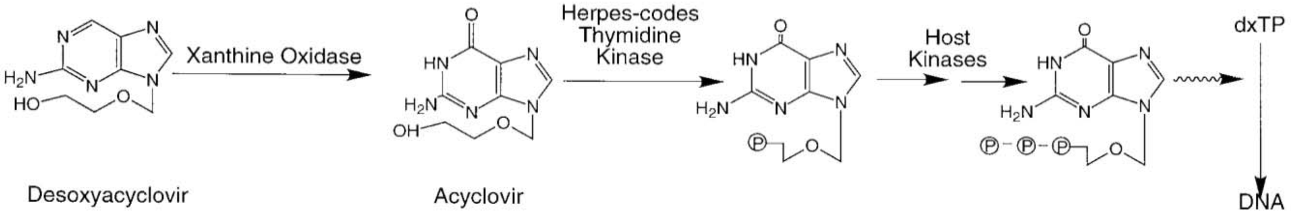

FIG. 2. Desoxyacyclovir, a prodrug of acyclovir that is activated by xanthine oxidase. The ultimate target enzyme of acyclovir is the viral DNA polymerase, which is inhibited by the triphosphate metabolite of acyclovir. Reproduced with permission from Krenitsky and Elion (1982).

prodrug would be beneficial in cases of impaired renal blood flow.

*3. Distribution.* The lipophilicity of a drug not only affects its absorption and metabolism but also its binding and distribution. Generally, the higher the lipophilicity of a drug, the stronger its binding to protein and the greater its distribution (Seydel and Schaper, 1986; Toon and Rowland, 1983). In studies with structure-related sulfonamides, Seydel et al. (1973) have shown that there was a strong positive correlation between plasma protein binding of the drugs and their lipophilicity. Watanabe and Kozaki (1978) found that the volume of distribution increased with increasing lipophilicity when administering 15 basic drugs to dogs.

Studies in the 1950s on the distribution of thiopental and polychlorinated insecticides (e.g., dichlorodiphenyltrichloroethane) have led to the misconception that highly lipophilic drugs tend to accumulate in adipose tissue. Recent studies by Bickel (1994) have shown that although the initial uptake of drugs into adipose tissue is related to their lipophilicity, the degree of adipose tissue storage does not correlate with their lipophilicity. Factors such as drug binding to plasma and tissue proteins also play a significant role in drug storage in adipose tissues.

The brain is different from other organs in several aspects. One of the most important features is that the brain is completely separated from the blood by the blood-brain barrier (BBB). All organs are perfused by capillaries lined with endothelial cells that have small pores to allow for the rapid movement of drugs into the organ interstitial fluid from the circulation. However, the capillary endothelium of the brain lacks these pores and, therefore, drugs must cross the BBB and enter the brain by simple diffusion. To design drugs for CNS activity, it is important to understand the factors that affect drug delivery to the site of action.

Because most drugs cross the BBB by passive diffusion, lipophilicity is an important determinant of brain penetration. Many reports show a correlation between lipophilicity and brain penetration of drugs (Pardridge, 1980; Rapoport, 1976). Ochs et al. (1985) found that the rate of brain uptake of drugs was dependent on their lipophilicity. There was a strong negative correlation between lipophilicity and the time of peak concentration in cerebrospinal fluid (CSF) postdose. The calculated lipophilicities (log D) of salicylic acid, antipyrine, and amitriptyline were −0.9, 0.4, and 3.0, respectively, and the time required to reach the peak CSF concentration after intravenous administration to dogs was 200, 34, and 4 minutes, respectively (Ochs et al., 1985).

Although lipophilicity is an important factor affecting brain penetration, a linear relationship between lipophilicity and brain penetration can only be expected within a certain range. In a recent survey of 257 marketed drugs (fig. 3), the optimum log $P$ value of lipophilicity was between 1 and 2 for the overall beneficial behavior of CNS drugs (Jezequel, 1992). Drugs with extremely high lipophilicity can be as poorly taken up by the brain as those with low lipophilicity. For example, L-365,260, a potent $CCK_B$ receptor antagonist for the treatment of anxiety, is a very lipophilic drug with a log $P$ value of 3.6. However, this drug displays poor BBB penetration (Lin and Lin, 1990).

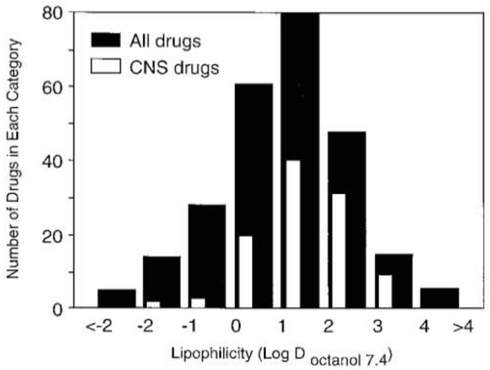

FIG. 3. Distribution of drugs with respect to their lipophilicity. Results of a survey of 257 marketed drugs based on available literature information. Reproduced with permission from Taylor & Francis (Jezequel, 1992).

P-glycoprotein, located on the apical surface of the endothelial cells of the brain capillaries toward the vascular lumen (Tew et al., 1993; Pardridge, 1991), is believed to be responsible for the poor BBB penetration of some highly lipophilic drugs. The poor BBB penetration of L-365,260 may be related to the efflux function of p-glycoprotein. Pretreatment of rats with quinine or verapamil, potent inhibitors of p-glycoprotein, resulted in a substantial increase in BBB penetration of L-365,260 by 3- to 5-fold (Lin et al., unpublished data). These results were consistent with the role of p-glycoprotein in excluding xenobiotics.

Factors other than lipophilicity also may play an important role in the transfer of drugs across the BBB. A strong negative correlation was found between the BBB permeability of steroid hormones and the total number of hydrogen bonds; the greater the hydrogen bond, the lower the permeability (Pardridge, 1980). Similarly, the hydrogen bond potential is a determinant of in vitro and in situ BBB permeability of peptides (Chikhale et al., 1994). It was concluded that a major impediment to BBB penetration of peptides was the energy required to break the water-peptide hydrogen bond.

L-663,581 is an investigational partial agonist of benzodiazepine receptors for potential application in the treatment of anxiety. Studies in rats, dogs, and monkeys have shown that the drug is eliminated mainly by biotransformation. Two metabolites, mono- and bis-hydroxy analogs, were demonstrated to be active in vitro. The potency of benzodiazepine receptor binding ($K_i$) is 3.7 nM for the parent drug, 3.3 nM for the mono-hydroxylated metabolite, and 1.2 nM for the bis-hydroxylated metabolite. Although the metabolites are as potent as, or more potent than, the parent drug in vitro, they are inactive in rats in a conditioned emotional response model (Lin et al., 1994). The lack of in vivo activity of the metabolites cannot be explained by absorption and/or elimination kinetics. Brain uptake studies indicated that permeability of the BBB is high for L-663,581 but very poor for the metabolites (Lin et al., 1994). Because the metabolites have a reasonably good octanol/buffer partition coefficient (log $P$ ranging from 0.7 to 1.2), and because two clinically used benzodiazepines, alprazolam and clobazam, have similar partition coefficients compared to the mono- and bis-hydroxylated metabolites (Arendt et al., 1983; Greenblatt et al., 1983), the poor penetration of the BBB of the metabolites may be due to their hydrogen bonding, rather than lipophilicity. According to Stein's assignment (1967), the mono-hydroxylated metabolite has two more hydrogen bonds, and the bis-hydrogenated analog has four more hydrogen bonds.

*4. Plasma half-life.* Most drugs are administered as a fixed dose given at regular intervals to achieve therapeutic objectives. Generally, the duration of drug action is reflected by its plasma $t_{1/2}$. Thus, the $t_{1/2}$ of drugs in plasma is one of the most important factors that determines the selection of a dosage regimen. Administration of drugs with a short $t_{1/2}$ requires frequent dosing and often results in a significant decrease in patient compliance. Because the $t_{1/2}$ of a drug is determined by its volume of distribution and elimination clearance, the prolongation of $t_{1/2}$ can be achieved by increasing the volume of distribution or decreasing the clearance. It appears to be easier to modify the chemical structure to slow a drug's clearance than to increase its volume of distribution.

Nifedipine, a calcium channel blocker widely used for the treatment of hypertension, has a short plasma $t_{1/2}$ ($\sim$2 h), resulting in a t.i.d. dosage regimen. Nifedipine also undergoes substantial first-pass metabolism and exhibits large interindividual variation in systemic concentrations (Kleinbloesem et al., 1984); these pharmacokinetic properties are not ideal for the chronic treatment of hypertension. Thus, a search was initiated for a backup drug with good oral bioavailability and duration of action that would allow a once-a-day dosage regimen.

The addition of an alkyl amide side-chain linked to the dihydropyridine 2-methyl group yielded amlodipine with a lower clearance, which has an improved oral bioavailability and plasma $t_{1/2}$ without loss of antihypertensive activity (Arrowsmith et al., 1986). Based on pharmacokinetic studies in dogs, amlodipine was chosen as a backup compound to meet the objectives of the program. Clinical studies proved that indeed amlodipine had good oral bioavailability (50–90%) and a prolonged plasma $t_{1/2}$ (30 h) (Humphrey, 1989).

Although chemical modification is preferred due to its ease, prolongation of the $t_{1/2}$ and a decrease in dosage frequency can be achieved by developing sustained-release dosage forms or coadministering inhibitors of drug-metabolizing enzymes. Metoprolol, a $\beta$-blocker, has a relatively short $t_{1/2}$ (<3 h), so a once-a-day sustained-release tablet was developed. This sustained-release dosage form produced a more prolonged and uniform effect on the heart rate and systolic blood pressure than when given as a conventional tablet twice a day (Johnsson et al., 1980).

Sinemet and primaxin are examples of coadministration of enzyme inhibitors to prolong the duration of drug action. Sinemet (Merck Research Laboratories, West Point, PA), a combination product of L-dopa and carbidopa, is widely used for the treatment of Parkinson's disease. When L-dopa is given alone, >90% of the dose is decarboxylated peripherally and only 10% is available for CNS activity. To minimize the decarboxylation of L-dopa outside the CNS, carbidopa, a peripherally active decarboxylase inhibitor that cannot cross the BBB, is coadministered (Marsden, 1976). Primaxin (Merck Research laboratories, West Point, PA), a combination of imipenem and cilastatin, is a widely used $\beta$-lactam antibiotic. Imipenem (MK-787) possesses a broad spectrum of action that comprises most of the gram-positive and gram-negative bacteria. In vivo imipenem is inactivated rapidly by a renal dipeptidase. This inactivation can be slowed by the combination of imipenem with the renal dipeptidase inhibitor, cilastatin (Kropp et al., 1980).

Although it is generally true that the duration of drug action is reflected by its plasma $t_{1/2}$, some drugs are given less frequently than their $t_{1/2}$. Despite its very short plasma $t_{1/2}$ in humans ($\leq$1 h), omeprazole, a proton-pump inhibitor, is given once a day (Regårdh et al., 1985). This drug reduces gastric acid secretion through inhibition of the enzyme $H^+$,$K^+$-ATPase located in the secretory canals of the parietal cells. Omeprazole is a weak base (pka = 4.0) and is rapidly and well absorbed from the alkaline environment of the small intestine. After entry of omeprazole into the parietal cells, the drug is converted to an intermediate (spiro compound) by protonation. The spiro intermediate subsequently forms the active metabolite, cyclic sulfenamide, which binds irreversibly to the enzyme $H^+$,$K^+$-ATPase (Mutschler and Derendorf, 1995). Because formation of the spiro intermediate occurs only in an acidic medium, omeprazole accumulates pH-dependently in the parietal cells and inhibits acid secretion for a long duration.

*5. Stereoselectivity.* Although it has been long known that stereoisomers of a chiral drug often exhibit pronounced differences in their pharmacokinetic and pharmacodynamic properties both in quantitative and qualitative terms, more than 500 drugs are marketed currently as racemic mixtures without relevant pharmacokinetic and pharmacodynamic information for each individual stereoisomer. This neglect of stereochemistry in drug development was widespread until Ariëns' (1984) famous critical review of "sophisticated scientific nonsense" was published. It was Ariëns' review that finally incited drug researchers to consider the importance of stereoselective differences.

MK-927, Merck's first carbonic anhydrase inhibitor to reach clinical trials for the treatment of glaucoma, is a racemic mixture. In 1986, due to the complexity of its stereoselective pharmacokinetic and pharmacodynamic properties (Lin et al., 1991b,d) and in consideration of Ariëns' criticism, the development of MK-927 was terminated and replaced by its more active *S*-isomer, MK-417 (Lin et al., 1990a, 1991a). Subsequently, it was decided to develop dorzolamide (MK-507), which is the *S*-isomer of an MK-927 analog, because of its longer duration of action. Dorzolamide is now on the market for the treatment of ocular hypertension or open-angle glaucoma (Pfeiffer, 1994).

Although in principle it is preferable to synthesize and develop the more active single enantiomer, there are situations in which use of the racemate is justified based on pharmacodynamic or pharmacokinetic information, such as interconversion of stereoisomers or chiral inversion. Recently, Baillie and his coworkers (Zhang et al., 1994; Tang et al., 1994) have demonstrated an acid-catalyzed racemization of stiripentol in rats that takes place at low pH. After oral administration of either the *S*- or *R*-enantiomer, racemization of the drug in the stomach leads to a mixture of the *S*- and *R*-enantiomers before entering the gastrointestinal tract. Because both enantiomers are pharmacologically active (Shen et al., 1992), and because racemization occurs in the stomach, stiripentol is currently being developed as the racemic mixture. Similarly, the clinical use of racemic ibuprofen is justified by evidence of the unidirectional chiral inversion of the inactive *R*(−)-ibuprofen to its active *S*(+)-ibuprofen in vivo (Adams et al., 1976; Lee et al., 1985).

In some cases, enantiomers are purposely combined to optimize their therapeutic profiles. Indacrinone (MK-286) is a 9:1 mixture of the (+)- and (−)-isomers designed to optimize its uricosuric and diuretic activities (Blaine et al., 1982; Tobert et al., 1981). Both isomers are potent uricosuric agents, but the (−)-enantiomer is the more potent diuretic.

Sometimes, the chiral preference of subtypes of certain receptors in specific tissues can provide a basis for novel drug development. Two distinct subtypes of $\beta$-adrenergic receptors have been identified and characterized. In cardiac and pulmonary tissues, the $\beta$-adrenoceptors are predominantly of the $\beta_1$-subtype, whereas in ocular tissues, they are mainly the $\beta_2$-subtype (Weiner and Taylor, 1985). Timolol (MK-950), a nonselective $\beta$-adrenergic antagonist, contains a chiral center in the amino-hydroxypropoxy side-chain. Although both enantiomers inhibit adrenergic activity at $\beta_1$- and $\beta_2$-receptors, the ratio of *R:S* stereoselectivity is substantially greater for the $\beta_1$-receptors than for the $\beta_2$-receptors. The *R:S* ratios of the in vitro $\beta$-blockade by timolol in the guinea pig trachea and atria were approximately 1:80 to 1:90, whereas the *R:S* ratio of the aqueous humor reduction by timolol in the rabbit eye was approximately 1:3 (Share et al., 1984). Thus, timolol was prepared as the optically pure *S*-form for the treatment of hypertension (Keates and Stone, 1984), and the *R*-enantiomer was developed as a topical ocular hypotensive agent in the treatment of glaucoma to circumvent the unwanted cardiac and pulmonary effects (Weiner, 1985). The above examples illustrate that stereoselectivity can be used in novel drug design.

Despite the advances of molecular biology and protein chemistry, drug design is still not a precise science and usually requires an iterative process of reassessing structural changes to obtain optimal pharmacological and pharmacokinetic properties. The examples cited in this section are used to illustrate the principle that both pharmacokinetic and metabolic data can provide important information to guide drug design.

## III. Role of Metabolism in Drug Toxicity

As part of drug development, the safety of a drug candidate has to be evaluated carefully before it can be approved. Due to ethical constraints on performing toxicity studies in humans, relevant safety assessments must be extensively studied in laboratory animals. One of the fundamental challenges drug metabolism scientists face in drug discovery and development is the extrapolation of risk assessment from animals to humans. This extrapolation is far from straightforward. As seen in the marked species differences in metabolism (Lin, 1995; Clark and Smith, 1984), drug-induced toxicity is often species-dependent, both in quantitative and qualitative terms. Some species of experimental animals have such unique mechanisms of developing toxicity that extrapolation of such toxicity assessments to the human situation would be fraudulent (Gregory, 1988; Green, 1991; Boobis, et al., 1990; Park and Kitteringham, 1990). Although there is no single method or model that can extrapolate the toxicity from animals to humans (Boxenbaum et al., 1988), the species differences in toxicity often can be explained by pharmacokinetic or pharmacodynamic effects of drugs. To make an accurate interpretation and a reasonable prediction of potential toxicity in humans, it is important to elucidate the underlying mechanisms responsible for the species differences in metabolism and pharmacokinetics.

### *A. Species Differences in Metabolism*

From an evolutionary standpoint, all mammals are similar because they originate from a common ancestor, yet they have differentiated as a result of their dissimilar environmental adaptation. Biochemistry provides countless examples of similarities and differences between species, of which one of the most instructive is the structure of cytochrome P-450s. Cytochrome P-450s appear to have evolved from a single ancestral gene over a period of 1.36 billion years. To date, at least 14 P-450 gene families have been identified in mammals (Nelson et al., 1996). Although all members of this superfamily possess highly conserved regions of amino acid sequence, there are considerable variations in the primary sequences across species. Table 2 shows the homology of nucleotide and amino acid sequences between humans and animal species (Kamataki, 1995). Even small changes in amino acid sequences can give rise to profound differences in substrate specificity (Lindberg and Negishi, 1989).

Similar to cytochrome P-450s, uridine diphosphoglucose transferases (UDPGTs) and carboxylesterases also show species similarities and differences. At least 10 rat UDPGTs and 8 human UDPGTs have been defined and characterized to date by cDNA cloning (Clarke and Burchell, 1994). Comparison of the amino acid sequences of all UDPGTs indicates that they share a common C-terminal domain, but the *N*-terminal half of these isoforms is quite variable. Examination of each of the UDPGT isoforms has revealed that their substrate specificities are different, although they still have overlapping substrate specificities.

Carboxylesterases, enzymes that are widely distributed in the tissues of mammals, hydrolyze drugs containing ester bonds or amide linkages and play an important role in drug metabolism, particularly for ester prodrugs. Hepatic microsomal carboxylesterases exist as multiple isozymes, and there are significant species differences in the activities of the carboxylesterase (Satoh, 1987; Hosokawa et al., 1987). Hosokawa et al. (1990) have compared the amino acid sequences and substrate specificity of purified carboxylesterase from liver microsomes of mice, hamsters, rats, guinea pig, rabbits, and monkeys. Although high (80–95%) homology in amino acid sequences was shown, all carboxylesterases had a different *N*-terminal amino acid, and their substrate specificities were considerably different.

As a result of the species differences in the amino acid sequences of the isozymes, both the rate of drug metabolism and the metabolite pattern may differ between animal species. Similarly, the response of the enzymes to inducers, inhibitors, and hormones may vary between species due to their enzyme structural differences. This section will discuss the factors with respect to the species differences in drug metabolism.

*1. Oxidation and conjugation.* Indinavir (MK-639, L-735, 524), a potent HIV protease inhibitor, is subject to extensive metabolism in animals and humans. The major metabolic pathways of indinavir in humans are identified as (*a*) glucuronidation at the pyridine nitrogen to yield a quaternized ammonium conjugate, (*b*) pyridine *N*-oxidation, (*c*) para-hydroxylation of the phenylmethyl group, (*d*) 3′-hydroxylation of the indan, and (*e*) *N*-depyridomethylation (Chiba et al., 1996). All oxidative metabolites observed in humans also were formed in rats, dogs, and monkeys, whereas *N*-glucuronide was found only in monkey and human urine (Lin et al., 1996a). An additional metabolite, a cis-2′-3′-dihydroxyindan, was formed in monkeys, but not in other species. The intrinsic clearance ($cL_{int}$) ($V_{max}/K_m$) of the oxidative metabolism of indinavir was in the rank order: rat (157 mL/min/kg) ≈ monkey (162 mL/min/kg) > dog (29 mL/min/kg) > human (17 mL/min/kg) (Lin et al., 1996a). Clearly, indinavir metabolism is qualitatively and quantitatively different among species.

The in vitro metabolism of losartan (MK-954; Dup 753), a potent nonpeptide angiotensin II receptor antagonist, has been studied with liver slices from rats, monkeys, and humans (Stearns et al., 1992). Metabolism of losartan also is qualitatively and quantitatively different among species. In the rat, the primary route of metabolism is oxidative, which leads to either monohydroxylated or oxidized (carboxylic acid) metabolites. In monkeys, glucuronidation of the tetrazole moiety predominates. The metabolism of losartan by human liver slices, however, is not dominated by a single metabolic pathway, as with rats and monkeys but is characterized

TABLE 2

*Cytochrome P-450s: Homology of the nucleotide and deduced amino acid sequences between human and various animal species*

| Isozyme | Human | Crab-eating monkey | Marmoset | Dog | Rabbit | Hamster | Rat | Mouse |
|---|---|---|---|---|---|---|---|---|
| 1A1 | 100 | 95 | — | 84 | 81 | 79 | 81 | 82 |
| | 100 | 94 | — | 82 | 77 | 77 | 79 | 80 |
| 1A2 | 100 | — | 89 | 84 | 83 | 80 | 80 | 79 |
| | 100 | — | 86 | 82 | 78 | 73 | 75 | 73 |
| 2C | 100 | 96 | 89 | 73 | 81 | 79 | 79 | — |
| | 100 | 92 | 79 | 68 | 76 | 80 | 74 | — |
| 2D | 100 | — | 90 | — | — | 76 | 79 | 74 |
| | 100 | — | 90 | — | — | 73 | 78 | 70 |
| 2E1 | 100 | 96 | 93 | — | 81 | 80 | 80 | 79 |
| | 100 | 94 | 89 | — | 79 | 78 | 80 | 77 |
| 3A | 100 | 96 | 92 | 82 | 80 | 74 | 77 | 81 |
| | 100 | 93 | 87 | 77 | 74 | 68 | 71 | 76 |

Upper value, nucleotide sequence (% homology); lower value, deduced amino acid sequence (% homology); —, not known. In the 2C, 2D, and 3A gene subfamilies, the values indicate percentage of pairs that showed the highest identity among the multigene family. Data excerpted from Kamataki (1995).

by an approximately equal formation of both oxidized and glucuronidated metabolites. The investigators of this study suggest that the observed short duration of action of the drug in monkeys may be due to the low formation rate of the pharmacologically active carboxylic acid metabolite in this species. This carboxylic acid metabolite has a much longer $t_{1/2}$ than the parent drug in all species studied.

Stevens et al. (1993) recently compared phase I and phase II hepatic drug metabolism activities using human and monkey liver microsomes. Of the eight P-450–dependent activities measured, only *N*-nitrosodimethylamine *N*-demethylase activity was not significantly different in the two species. Coumarin 7-hydroxylase activity was higher in the humans than in the monkey. In contrast, erythromycin *N*-demethylase, benzphetamine *N*-demethylase, pentoxyresorufin *O*-dealkylase, ethoxycoumarin *O*-deethylase, and ethoxyresorufin *O*-deethylase activities were significantly greater in monkey microsomes than those from humans. Of the seven microsomal and cytosolic phase II activities measured, only 17$\alpha$-ethynyl estradiol glucuronidation was significantly higher in the humans. These results clearly show that the metabolic capacities of the human and Rhesus monkey drug-metabolizing enzymes are quantitatively different.

The dihydropyridine calcium channel blockers are eliminated extensively by metabolism. The primary biotransformation route involves oxidation to their pyridine derivatives, a reaction that is known to be catalyzed by cytochrome P-450 (Bäärnhielm et al., 1984). In a recent review article, Smith (1993) compared the $cL_{int}$ (metabolic clearance) of six dihydropyridines (amlodipine, nitrendipine, felodipine, nicardipine, nisoldipine, and nilvadipine) in rats, dogs, and humans. In all cases, the rat showed the highest $cL_{int}$ when compared with dogs and humans. The overall ratio of $cL_{int}$ of these compounds in dogs or rats to those in humans gives values of 1.4 for the dogs and 9 for the rats. For these drugs, the metabolism in humans is quantitatively similar to that in dogs, whereas rats show a much higher capacity for metabolism.

Drugs containing hydroxy groups are subject to both glucuronidation and sulfation reactions. The relative contribution of these two competing pathways depends on the nature of the drugs and animal species being studied. It is generally believed that glucuronidation predominates over sulfation in the rat, whereas in the dog and human, sulfation dominates (Rogers et al., 1987). Consistent with this general belief, xamoterol, a $\beta_1$-adrenoceptor partial agonist, is extensively glucuronidated in the rat, whereas sulfation primarily occurs in the dog (Mulder et al., 1987; Groen et al., 1988). However, this is not the case for acetaminophen, which is predominately sulfated in the rat, but in humans, glucuronidation is quantitatively more important (Lin and Levy, 1986; Slattery and Levy, 1979).

Azidothymidine (AZT), an HIV reverse transcriptase inhibitor, is extensively metabolized in humans, but not in rats. Approximately 75% of an oral dose was recovered in human urine as the 5′-O-glucuronide, and 15% was recovered as unchanged drug (Blum et al., 1988). On the other hand, only 2% of an oral dose was recovered as AZT glucuronide in rat urine, whereas approximately 78% of the dose was excreted as unchanged drug (Good et al., 1986). Consistent with the in vivo data, in vitro studies confirmed that human liver UDPGT catalyzed the glucuronidation of 0.1 mM AZT 10- to 25-fold faster than did rat liver UDPGT (Resetar and Spector, 1989). Similarly, glucuronidation of some drugs, including quaternary amines, has been shown to occur only in human and primate species (Caldwell et al., 1989).

These examples clearly demonstrate that extrapolation of drug metabolism from animals to humans is very difficult, if not impossible, both in the qualitative and quantitative aspects. If drug-induced toxicity is related directly to systemic exposure to the drug and its metabolites, the species differences in the metabolism of the drug are perhaps the most important factors in explaining the observed species differences in toxic responses.

*2. Induction.* In the mid-1950s, Conney et al. (1956) showed that the treatment of animals with 3-methylcholanthrene (3-MC) increased the animals' ability to metabolize methylated aminoazo dyes. Remmer (1958) found that tolerance to barbiturate drugs was the result of the enhancement of their own metabolism by induction of cytochrome P-450. Although the phenomenon of induction has been known for over 4 decades, only in recent years, we began to uncover the mechanism involved in induction.

With the exception of the CYP1A1 isoform (Whitlock et al., 1996), many more studies are needed to explore the molecular mechanisms involved in CYP2B, 2E, 3A, and 4A induction. In the case of CYP1A1, inducing agents bind to the cytosolic polycyclic aromatic hydrocarbon (Ah) receptor and are translocated into the nucleus. The transcriptional process includes a sequence of events: ligand-dependent heterodimerization between the Ah receptor and Ah receptor nuclear translocator interaction of the heterodimer with a xenobiotic-responsive enhancer, transmission of the induction signal from the enhancer to the CYP1A1 promoter, and alterations in chromatin structure. This is followed by the subsequent transcription of the appropriate mRNA and translation of the corresponding proteins.

Although the fundamental mechanisms of CYP1A induction are qualitatively similar in different species, including mice, rats, rabbits, and humans (McDonnell et al., 1992), there are important quantitative differences in the effectiveness of inducer-receptor coupling. For example, the gastric acid-suppressing drug, omeprazole, is a CYP1A2 enzyme inducer in humans but has no such inductive effect in mice or rabbits (McDonnell et al., 1992; Diaz et al., 1990).

Important species differences also exist in the response of other inducible subfamilies of cytochrome P-450s. Phenobarbital induces predominately members of the CYP2B subfamily in rats, whereas in humans, it appears that the major form induced belongs to the CYP3A subfamily (Rice et al., 1992). Furthermore, members of the CYP3A subfamily in rats are inducible by the steroidal agent, pregnenolone-16$\alpha$-carbonitrile, but not by the antibiotic rifampin. The opposite is true in rabbits and humans (Strolin Benedetti and Dostert, 1994; Nebert and Gonzalez, 1990). Thus, drugs that do not induce P-450 enzymes in animals should not be assumed to not have enzyme-inducing capacity in humans, and vice versa. Despite the well-known species differences in the response to P-450 inducers, mice and rats have been routinely used in most pharmaceutical companies to assess the risk of potential drug induction in humans. This type of risk assessment may be of little direct relevance for certain drugs. More recently, however, both in vitro (human hepatocytes) and in vivo (probe drugs for certain human cytochrome P-450s) techniques have become available and have increasingly been used by investigators to evaluate the potential induction of human cytochrome P-450s by a variety of therapeutic agents.

Like a double-edged sword, induction of drug-metabolizing enzymes may lead to a decrease in toxicity through acceleration of detoxification, or to an increase in toxicity due to increased formation of reactive metabolites. Depending on the delicate balance between detoxification and activation, induction can be a beneficial or harmful response. The induction of CYP1A isoforms can reduce the carcinogenicity of certain compounds. For example, intraperitoneal injection of the CYP1A inducer $\beta$-naphthoflavone inhibited tumorogenesis in the lung and mammary glands of rodents treated with 7,12-dimethylbenz[a]anthracene (DMBA), which is a highly carcinogenic compound (Wattenberg and Leong, 1968). In addition, 2,3,7,8-tetrachlorodibenzo-p-dioxin, a potent CYP1A inducer, dramatically reduced the initiation of skin tumors in mice caused by DMBA (Digiovanna et al., 1979). In contrast, CYP1A isoforms can activate some compounds, such as benzo[a]pyrene, to their ultimate carcinogenic forms (Gelboin, 1980), and induction of these isoforms increases the risk of carcinogenicity. Due to the complexity of the factors determining toxicity and carcinogenicity, the issue of whether induction is beneficial or harmful is still highly controversial (Ioannides and Parke, 1993; Beresford, 1993).

In addition to the induction of CYP1A isoforms, binding of an agent to the Ah receptor also leads to the induction of UDPGTs and glutathione (GSH)-*S*-transferases (Bock et al., 1990). The coinduction of phase I and phase II enzymes appears to decrease the risk due to P-450 induction alone. In vitro mutagenicity of benzo[a]pyrene and of benzo[a]pyrene-3,6-quinone was higher in the liver S9 fraction of 3-MC–treated rats than with that of control rats when NADPH was the only added cofactor. The in vitro mutagenicity was decreased substantially by concomitant glucuronidation or GSH conjugation when UDPGA or GSH also was added to the system, and there was no significant difference in the in vitro mutagenicity between 3-MC–treated and control rats. (Bock et al., 1990). Thus, the protective effect appeared to be a result of coinduction of UDPGTs and GSH-*S*-transferases.

*3. Inhibition.* A drug interaction occurs when the disposition of one drug is altered by another. Because metabolism represents a major route of elimination for many drugs, inhibition of drug-metabolizing enzymes is one of the main reasons for drug interactions. Various mechanisms are known to underlie enzyme inhibition, including competition for the catalytic site of the enzyme, noncompetitive (allosteric) interaction with the enzyme, suicide destruction of the enzyme, and competition for cofactors. Among these, competitive inhibition is probably the most common. If enzyme inhibition occurs by the interaction of two substrates competing for the same enzyme, the competitive nature of the inhibition will depend on the $K_m$ value of the substrate and the dissociation constant of an inhibitor ($K_i$) value of the inhibitor as well as their concentrations at the site of enzymes (Segel, 1975). Because there are quantitative differences in the $K_m$ and $K_i$ values between species, it is expected that the degree of enzyme inhibition would be species-dependent

Isoforms of the CYP2D subfamily have been isolated from rats and humans and have been shown to have similar substrate specificities (Guengerich, 1987). Debrisoquine 4-hydroxylation is specifically catalyzed by these isoenzymes (Meyer et al., 1990b). The inhibition kinetics of debrisoquine 4-hydroxylase activity by quinidine and one of its diastereoisomers, quinine, have been compared in human and rat liver microsomes (Kobayashi et al., 1989). Both quinidine and quinine are potent competitive inhibitors of debrisoquine 4-hydroxylation. However, quinidine is a more potent inhibitor of this activity in humans than in rats, whereas the reverse is true for quinine. The $K_i$ values of quinidine for debrisoquine 4-hydroxylation in humans and rats were 0.6 and 50 $\mu$M, whereas with quinine, the values were 13 and 1.7 $\mu$M, respectively. Similarly, furafylline exhibits species-dependent inhibition of phenacetin *O*-deethylase activity of liver microsomes (Sesardic et al. 1990). Furafylline, a mechanism-based inhibitor of CYP1A2, is more potent in inhibiting phenacetin *O*-deethylation in humans than in rats, despite the fact that phenacetin *O*-deethylation is catalyzed exclusively by CYP1A2 in both species.

The in vitro effects of $\alpha$-naphthoflavone on aryl hydrocarbon hydroxylase activity (CYP1A subfamily) were studied in five animal species by Thorgeirsson et al. (1979). The activity of this enzyme was significantly inhibited by $\alpha$-naphthoflavone in mice, rats, and hamsters in a concentration-dependent manner. In rabbits, the aryl hydrocarbon hydroxylase activity was stimulated, rather than inhibited, by this compound. Similarly, species-dependent stimulatory effects of flavonoids were also reported by Huang et al. (1981). Addition of 7,8-benzo[a]pyrene or flavone stimulated the hydroxylation of benzo[a]pyrene in liver microsomes from rabbits, hamsters, and humans by several-fold but had little or no effect on this activity in liver microsomes from rats or guinea pigs. The marked species differences in the inhibitory or stimulatory effects of flavonoids may be due to the involvement of species' different cytochrome P-450 isozymes, or to the structural differences at the active site of P-450 isozymes at which the flavonoids interact.

Recently, Shou et al. (1994) have shown that CYP3A4-catalyzed phenanthrene metabolism was stimulated by 7,8-benzoflavone. Kinetic studies with vaccinia virus coding CYP3A4 revealed that 7,8-benzoflavone increased the $V_{max}$ of phenanthrene metabolism without changing the $K_m$ and that phenanthrene decreased the $V_{max}$ of 78-benzoflavone without increasing the $K_m$. With these data, these investigators speculate that both substrates are simultaneously bound to the enzyme in the active site that has access to the active oxygen and suggest that the increase in the $V_{max}$ of phenanthrene (or the decrease in the $V_{max}$ of 7,8-benzoflavone) indicates that there is competition for the active oxygen between the two substrates. Although the kinetic analyses support their speculation, the underlying mechanism of the stimulatory effect at the molecular level needs to be verified further.

In addition to the reversible competitive inhibitors, compounds can bind irreversibly to the enzyme via a reactive metabolic intermediate. Several drugs have been shown to be irreversible inhibitors or so-called mechanism-based suicide inhibitors (Murray, 1987). L-754,394, a potent HIV protease inhibitor, is a good example. Kinetic studies in rats, dogs, and monkeys have shown that the drug exhibits dose- and time-dependent pharmacokinetics (Lin et al., 1995). The apparent clearance decreased with increasing dose. However, the dose dependency cannot be explained by Michaelis-Menten kinetics. L-754,394 in plasma declined log-linearly with time, but with an apparent $t_{1/2}$ that increased with dose. Furthermore, the apparent clearance of L-754,394 decreased after chronic dosing. Subsequent in vitro microsomal studies revealed that the observed time- and dose-dependent kinetics of L-754,394 may be explained by mechanism-based enzyme inhibition of isozymes of the cytochrome CYP3A subfamily (Lin et al., 1995).

The magnitude of mechanism-based inhibition of cytochrome CYP3A isozymes by L-754,394 appeared to be species-dependent. For liver microsomal testosterone 6$\beta$-hydroxylase, the potency of inhibition by L-754,394 was in the rank order human > monkey > dog > rat. The values of maximum inactivation rate constant ($K_{inact}$) were 2.0, 0.25, 0.20, and 0.04 $min^{-1}$, respectively. Consistent with these results, in vivo kinetic studies indicated that L-754,394 inhibited the metabolism of indinavir (MK-0639), a drug known to be metabolized mainly by the isoforms of the CYP3A subfamily, more significantly in dogs than in rats (Lin et al., unpublished data). Because of this undesirable mechanism-based inhibition, the development of L-354,394 was terminated.

Drug inhibition is usually regarded as potentially dangerous, or at least undesirable. However, there are times when these interactions may be exploited. For example, ketoconazole is used with cyclosporin A to prolong elimination of the latter (Yee and McGuire, 1990; First et al., 1984). Ketoconazole, which is a potent antifungal agent, and cyclosporin A, which is a widely used immunosuppressive agent, are substrates for the same human cytochrome CYP3A4 (Combalbert et al., 1989). The idea is to use the relatively inexpensive ketoconazole to specifically inhibit cyclosporin A metabolism to minimize the cost of long-term therapy with this very expensive drug. Similarly, during World War II, when penicillins were very expensive, probenecid was coadministrated to delay renal excretion of the antibiotics (Weiner and Mudge, 1985). Other successful examples of therapeutic inhibition are carbidopa and cilastatin (Marsden, 1976; Kropp et al., 1980). As mentioned earlier, carbidopa and cilastatin are used as inhibitors to slow the elimination of dopa and imipenem, respectively.

*4. Sexual dimorphism.* Sex-related differences in drug metabolism have been known for more than 60 years, but it was not until recently that the mechanisms for these differences were explored (Shapiro et al., 1995; Skett, 1989). Recent studies have shown that sexual dimorphism in rats, and possibly in other species, results from the differential expression of sex-dependent hepatic cytochrome P-450s. This differential expression, in turn, is largely influenced by steroid and pituitary hormone levels and profiles. Evidence has shown that the sexual dimorphic secretion pattern of growth hormone directly regulates the expression of certain hepatic cytochrome P-450s (Legraverend et al., 1992b; Waxman, 1992; Kato and Yamazoe, 1990).

Such sex-related differences in the levels of cytochrome P-450 expression would be expected to give rise to profound differences in toxicological response because the susceptibility of a tissue to the toxic and/or carcinogenic effects of drugs often is determined by the rate of metabolic inactivation and/or activation by cytochrome P-450. For this reason, regulatory agencies require that equal numbers of males and females of each species be used in toxicity studies of drugs.

Although male rats generally exhibit distinctly higher activities than females, there are instances in which female rats have higher activities than males (Skett, 1989). This results from the fact that cytochrome P-450 can be expressed specifically or preferentially in either males or females. For example, CYP2C11 is expressed only in male rats, whereas CYP2C12 expression is limited to female rats. On the other hand, CYP2A2 and CYP3A2 are male-dominant, but CYP2A1 and CYP2C7 are female-dominant (Kobliakov et al., 1991; Bandiera, 1990; Legraverend et al., 1992a; Waxman et al., 1985, 1990).

The existence of sex-related differences in drug metabolism is not unique to the rat. Such differences have been seen in mice (Macleod et al., 1987), ferrets (Ioannides et al., 1977), dogs (Dogterom and Rothuizen, 1993), and humans (Hunt et al., 1992). However, the magnitude of the sexual differences in these species is invariably far more subtle than that found in rats. Sexual differences in drug metabolism are generally small and not detected easily in humans, due to the large interindividual variability in enzyme activities (Hunt et al., 1992).

Indinavir (MK-639, L-735,524), a potent HIV protease inhibitor, exhibits marked sex-related differences in clearance in rats and dogs, but not in monkeys. The clearance was 89 mL/min/kg for male rats and 41 mL/min/kg for female rats. In contrast to rats, female dogs cleared indinavir more rapidly than male dogs, with a clearance of 26 mL/min/kg for female dogs and 15 mL/min/kg for male dogs (Lin et al., 1996b). Consistent with the in vivo observations, hepatic microsomes from male rats had a substantially higher metabolizing activity toward indinavir than those from females, whereas liver microsomes from female dogs catalyzed the drug at a higher rate than those from male dogs. However, no sexual difference in indinavir metabolism was observed in monkey and human liver microsomes. The functional activity of CYP3A, measured by the formation of testosterone 6$\beta$-hydroxylation, and immunoblot analysis of the level of CYP3A proteins strongly suggest that significant gender differences in the levels of CYP3A isoforms result in the observed sex-related differences in indinavir metabolism in rats and dogs (Lin et al., 1996b). This example demonstrates that the sexual dimorphism in drug metabolism can be species-dependent. The sexual dimorphism in indinavir metabolism is reversed in the rat and dog.

Reverse sexual dimorphism also has been observed in humans. The male has a higher unbound clearance of chlordiazepoxide than the female, whereas the reverse is true for diazepam and desmethyl diazepam (Wilson, 1984). The sex-related differences in drug disposition could be related to the phase of the menstrual cycle, sex hormones, and the use of oral contraceptives.

### *B. Species- and Tissue-Specific Toxicity*

*1. Species-specific toxicity.* Toxic and carcinogenic responses for some drugs are evoked solely by the parent compounds, whereas for other drugs, the responses arise as a result of the formation of reactive toxic metabolites. Sometimes, therefore, the in vivo monitoring of the parent drug alone may have very little relevance. Even if metabolites are monitored, the reactive toxic metabolites are often too labile and too small in quantity to be detected. Consequently, only the chemically stable and nonreactive metabolites are being monitored, yet their presence may be meaningless in predicting toxicity. This complicated situation can be illustrated by species differences observed in the pharmacokinetics and hepatotoxicity of acetaminophen. The elimination $t_{1/2}$ of acetaminophen was longer in rats than in mice by 2- to 3-fold. Acetaminophen caused hepatotoxicity at lower doses in mice (200–300 mg/kg) but evoked only a barely detectable hepatotoxicity in rats at considerably larger doses (>1500 mg/kg). Pretreatment of rats and mice with phenobarbital had little effect on the $t_{1/2}$ of acetaminophen in either of the two species but markedly increased the hepatotoxicity of acetaminophen in both species (Gillette, 1989). These species differences in toxicity depend mainly on the amount of reactive metabolite formed and the amount of GSH present in the liver. Thus, monitoring of acetaminophen and its sulfate and glucuronide conjugates in plasma is of little relevance in predicting hepatotoxicity. Clinically, acetaminophen is well tolerated within the therapeutic dose range; however, hepatotoxicity may occur after ingestion of a single high dose of 10 to 15 g (150–200 mg/kg) of acetaminophen

Dichloromethane is a common industrial chemical that causes lung and liver cancer in mice after chronic inhalation exposure, but not in rats and hamsters under the same conditions (Burek et al., 1984). The compound is metabolized in vivo either by cytochrome P-450 to form carbon monoxide and carbon dioxide or by GSH-*S*-transferase to form GSH conjugates. Although the rate of oxidative metabolism is similar in rats, mice, and hamsters, there are marked species differences in the formation of GSH conjugates. The similar activity of cytochrome P-450 among these species results in very similar levels of carboxyhemoglobin in the blood of rats and mice. On the other hand, mice have substantially higher activity in GSH conjugation compared with rats and hamsters. Biochemical and toxicological studies suggest that the toxicity of dichloromethane is associated with the production of reactive metabolites derived from GSH conjugation via GSH-*S*-transferase (Green, 1990; Reitz et al., 1988, 1989).

Species-dependent toxicity also is observed with perfluorodecanoic acid (PFDA), a potent peroxisome proliferator. Treatment with PFDA resulted in pronounced hepatomegaly in the rat, but not in the guinea pig (Chinje et al., 1994). In a separate study, PFDA treatment caused a marked induction of lauric acid 12-hydroxylase activity in the rat, but not in the guinea pig, suggesting that hepatomegaly observed in rats may be associated with the induction of isozymes of the CYP4A subfamily mediated by peroxisome proliferator-activated receptors (PPAR) (Johnson et al., 1996). Another peroxisome proliferator, methylclofenapate, showed similar species differences in toxic response. This proliferator caused hepatomegaly in mice and rats, but not in guinea pigs (Bell et al., 1993). These data indicate that peroxisome proliferation is a species-dependent phenomenon most likely reflecting the differences in concentration of PPAR and the affinity of peroxisome proliferators to PPAR from different species.

D-limonene, a major component of orange oil, is an anticarcinogenic terpene. Studies in animals have shown that D-limonene reduces mammary tumorigenesis, although the underlying mechanism of this reduction is still unclear (Elson et al., 1988). Toxicity studies have shown that exposure to D-limonene causes nephrotoxicity only in male rats, but not in mice, guinea pigs, dogs, or monkeys (Webb et al., 1989; Hard and Whysner, 1994). Also, D-limonene causes no nephrotoxicity in female rats. This species- and sex-specific nephrotoxicity is characterized by an exacerbation of hyaline droplet accumulation. The mechanism underlying this accumulation of protein is due to the strong but reversible binding of D-limonene to $\alpha_{2u}$-globulin, a specific protein only produced in male rats (Kanerva et al., 1987; Stonard et al., 1986). Because $\alpha_{2u}$-globulin is not present in humans, it is concluded that D-limonene does not pose any nephrotoxic risk to humans.

Drug-induced thyroid enlargement and tumors are seen primarily in rodents. For example, the sulfonamide, sulfamethoxazole, produced thyroid nodules in rats at a dose of 50 mg·kg·day for 1 year. In Rhesus monkeys, the drug caused no increase in thyroid weight and no morphological alterations even at 300 mg·kg·day administered for the same length of time (Swarm et al., 1973). This species-specific toxicity may be due to the fact that rodents lack thyroxin-binding globulin. As a consequence, the biological plasma $t_{1/2}$ of thyroxin in rats is approximately ten-fold shorter than in humans, i.e., 12 to 24 h versus 5 to 9 days (Döhler et al., 1979). Sulfonamides cause an increase in thyroxine elimination, which leads to a more rapid depletion of the hormone in the rat than in other species and produces a need for prompt regulatory responses by the hypophysis. This leads to the elevation of thyroid-stimulating hormone, which results in a chronic hyperplastic response in thyroid tissue.

Drugs and their metabolites are usually eliminated from the body via urine or bile, or both. The relative contribution of biliary and urinary excretion to the overall elimination of drugs depends on the physicochemical properties of the drug and the animal species. The biliary excretion of drugs varies widely among species. In general, the mouse, rat, and dog are good biliary excreters, whereas the rabbit, guinea pig, monkey, and human are relatively poor (Smith, 1971). Sometimes, biliary excretion of drugs may lead to unwanted adverse effects. Indomethacin, an anti-inflammatory agent, is widely used and highly effective in the treatment of rheumatoid arthritis. However, at high doses, this drug may cause ulcerative lesions in the upper gastrointestinal tract. The biliary excretion of indomethacin appears to be an important factor in the development of intestinal lesions. The tendency for different species to develop intestinal lesions in response to indomethacin appears to correlate well with their respective biliary excretion of this compound (Duggan et al., 1975). Good biliary excreters, such as rats and dogs, appeared to be the most susceptible to indomethacin-induced intestinal lesions.

*2. Site-specific toxicity.* 4-Ipomeanol, a pulmonary toxin, is a naturally occurring fungal catabolite of a furanoterpenoid precursor produced by the moldy sweet potato Ipomoea batatus. It was discovered in the 1970s that 4-ipomeanol was the causative agent responsible for the outbreaks of lethal interstitial pneumonia in cattle. 4-Ipomeanol undergoes metabolic activation to a highly reactive metabolite that binds to nucleophilic tissue macromolecules (Boyd and Burka, 1978). In vivo and in vitro studies in several animal species revealed that the covalent binding occurs primarily in the lung, specifically in bronchiolar Clara cells (Boyd, 1977; Devereux et al., 1982). Because of its lung-specific toxicity, 4-ipomeanol was at one time considered to be a potential agent for the treatment of lung cancer. Although some human lung cancer cell lines, as well as a variety of human lung tumor biopsy specimens, are shown to be capable of activating 4-ipomeanol to a cytotoxic intermediate (Christian et al., 1989), the considerable toxicity of this compound hinders its clinical use in lung cancer therapy.

Although it is generally believed that most reactive intermediates and toxic metabolites of drugs are generated by oxidative reactions, an increasing number of examples suggest that phase II metabolism such as glucuronide, sulfate, and GSH conjugates may be related to drug-induced toxicity (Bock and Lilienblum, 1994; Miller and Surh, 1994; Monks and Lau, 1994). Glucuronides are capable of serving as transport vehicles for carcinogens that are responsible for site-specific toxicities in the urinary bladder or colon epithelium. Aromatic amines such as 2-naphthylamine and 4-aminobiphenyl are found in cigarette smoke and are considered to be a major factor in the incidence of urinary bladder cancer in humans (Mommsen and Aagaard, 1983). The *N*-hydroxy metabolite of 2-naphthylamine has been shown to be carcinogenic. Glucuronidation leads to the formation of *N*-hydroxy-*N*-glucuronide, which is more stable than the *N*-hydroxy metabolite and is excreted into the urinary bladder. In the urinary bladder, the *N*-hydroxy-*N*-glucuronide decomposes under the slightly acidic pH of urine to its protonated nitrenium ion, which readily reacts with DNA, thereby initiating bladder cancer (Kadlubar et al., 1981, 1977). A scheme of underlying mechanism for bladder-specific toxicity caused by 2-naphthylamine is illustrated in figure 4.

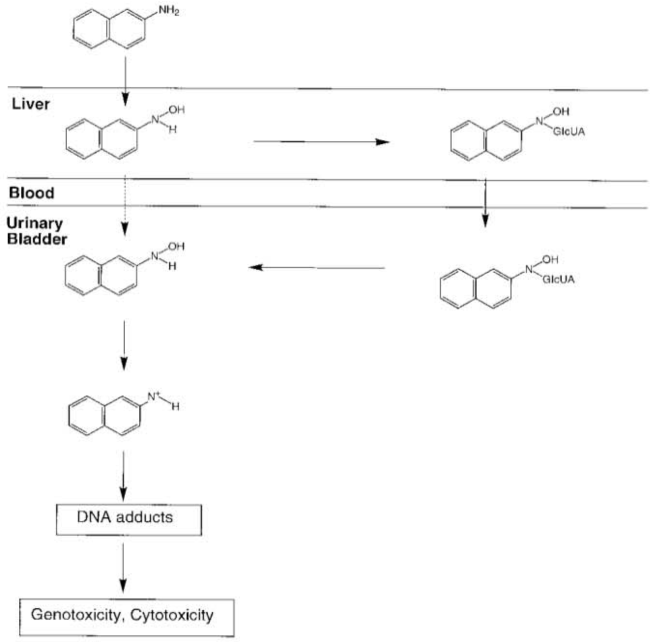

FIG. 4. Hypothesis for 2-naphthylamine-induced bladder cancer. Reprinted from Chem. Biol. Interact., Vol. 33, Kadlubar et al., Alteration of urinary levels of the carcinogen, *N*-hydroxy-2-naphthylamine, and its *N*-glucuronide in the rat by control of urinary pH, inhibition of metabolic sulfation, and changes in biliary excretion, pp. 129–147, 1981, with kind permission from Elsevier Science Ireland Ltd., Bay 15K, Shannon Industrial State, Co. Clare, Ireland.

Similar to the transportable glucuronides of arylamines involved in bladder carcinogenesis, glucuronides also play a major role in the colon cancer caused by heterocyclic arylamines. 2-Amino-1-methyl-6-phenylimidazo[4,5,b]pyridine is converted in the liver to the *N*-hydroxy-*N*-glucuronide metabolite, which is then excreted via the bile into the intestine where the corresponding carcinogenic hydroxylamine is liberated in the colon by bacterial $\beta$-glucuronidases (Alexander et al., 1991).

Although GSH conjugation is probably the most important detoxification function in all mammals, increasing evidence reveals that conjugation with GSH can result in toxicity (Monks and Lau, 1994, 1992). Kidneys possess very high levels of $\gamma$-glutamyl transpeptidase. The high renal activity of the enzyme plays a major role in the kidney-specific toxicity of some compounds. Some GSH conjugate-mediated toxicities involve the intermediary cleavage of the GSH moiety specifically by $\gamma$-glutamyl transpeptidase. The GSH conjugate of 2-bromohydroquinone is a good example of the GSH conjugate transporting form of a reactive metabolite. The nephrotoxicity of bromobenzene in rats probably occurs via its metabolism to 2-bromo-(di-glutathion-*S*-yl)hydroquinone. Although 2-bromo-(di-glutathion-*S*-yl)hydroquinone is formed in the liver, it travels in the blood stream to the kidney, where 2-bromo-(di-cystein-*S*-yl) hydroquinone, a nephrotoxic metabolite, is formed by renal $\gamma$-glutamyl transpeptidase (Monks and Lau, 1994).

Recently, a new mechanism of GSH conjugate-mediated toxicity has been proposed for toxic compounds, such as isothiocyanates and isocyanates (Baillie and Kassahun, 1994; Baillie and Slatter, 1991). Both of these classes are very electrophilic and react readily with GSH. The GSH conjugate is formed in one organ and can be transported to other organs, whereby the reactive moiety can be regenerated upon spontaneous decomposition of the GSH conjugate to GSH and the original reactive moiety. The reversible GSH conjugation may serve to extend the biological $t_{1/2}$ of reactive isocyanates and isothiocynates and influence their tissue distribution. Unlike renal $\gamma$-glutamyl transpeptidase-mediated GSH conjugate toxicity, the toxicity caused by the reversible GSH conjugation is expected to be less site-specific.

### *C. Stereoselectivity and Toxicity*

*1. Stereoselective metabolism.* As stated earlier, enantiomers must be considered as essentially different chemical compounds, because they usually differ greatly in pharmacokinetic and pharmacodynamic properties as a consequence of stereoselective interaction with biological macromolecules (Testa, 1988, 1989b; Ariëns, 1990; Williams and Lee, 1985). During the last decade, there has been growing awareness of the stereoselectivity in pharmacokinetics and pharmacodynamics, and it has become a key issue in new drug development (Campbell, 1990; Testa and Trager, 1990).

As with the examples of stiripentol and timolol described previously (Zhang et al., 1994; Tang et al., 1994; Shen et al., 1992; Adams et al., 1976; Lee et al., 1985; Blaine et al., 1982; Tobert et al., 1981; Weiner and Taylor, 1985; Share et al., 1984; Keates and Stone, 1984), early evaluation of the stereoselective pharmacokinetics and pharmacodynamics of the enantiomers is essential to decide whether to develop a racemate or an individual enantiomer. However, at the early stage, such stereoselectivity studies can only be conducted in animals. Thus, species-related differences in stereoselectivity should be evaluated carefully before the data are extrapolated to humans.

Absorption from the gastrointestinal tract, distribution into tissues, and renal excretion are passive processes for most drugs in which the extent and rate are mainly governed by the physicochemical properties of the drug. Because the physicochemical properties of stereoisomers are similar, stereoselectivity is not expected for these processes unless an active transport system is involved. There are only a few examples of stereoselective drug absorption, tissue distribution and renal excretion, whereas the stereoselective plasma protein binding and metabolism of chiral drugs are well documented (Lee and Williams, 1990; Jamali et al., 1989).

Stereoselective plasma protein binding of drugs differs considerably among species. Plasma binding of MK-571, a potent leukotriene $D_4$ antagonist, has been studied in 12 mammalian species (Lin et al., 1990b). The binding of MK-571 enantiomers to plasma protein was extensive, stereoselective, and species-dependent. In some species, the *S*-enantiomer bound to a greater extent than the *R*-enantiomer. In others, the *R*-enantiomer bound more extensively, and in still other species, there was no stereoselectivity. For both enantiomers, the unbound fraction in plasma differed by a factor of 8 among the species studied. Consistent with these observations, the *R*-enantiomer of MK-571 bound to rat plasma to a greater extent than the *S*-enantiomer, whereas in dog and monkey plasma, the reverse was true (Tocco et al., 1990). The elimination clearance of the enantiomers was related to the stereoselective plasma protein binding, with the greater unbound fraction being cleared more rapidly.

Such stereoselectivity among species also has been seen in metabolism. The stereoselective metabolism of mephenytoin was studied in vitro using livers from different animal species and humans (Yasumori et al., 1993a). The rates of microsomal 4′-hydroxylation were 2 to 6 times higher with the *R*-mephenytoin than *S*-enantiomer in rabbits, dogs, and rats, whereas the rates of microsomal 4′-hydroxylation were 5 to 15 times higher with the *S*-mephenytoin than *R*-enantiomer in monkeys and humans. Reconstituted enzyme systems and immunoinhibition experiments revealed that stereoselective involvement of CYP2C and CYP3A isoforms is the major factor in the species differences in the stereoselective metabolism of mephenytoin (Yasumori et al., 1993a). Propranolol also shows species-dependent stereoselective metabolism. The *S*-propranolol had a higher $cL_{int}$ than the *R*-enantiomer in dogs, whereas the *R*-propranolol had a higher $cL_{int}$ than the *S*-enantiomer in humans (Silber et al., 1982).

Species differences in stereoselectivity have been seen in phase II metabolism reactions, such as glucuronidation. Using immobilized microsomal protein from rabbit, monkey, and human liver, El Mouelhi et al. (1987) have shown that the glucuronidation of three racemic 2-arylproprionic acids, naproxen, ibuprofen, and benoxaprofen, was stereoselective and species-dependent. Similarly, species-dependent stereoselective glucuronidation of oxazepam has been shown among rabbits, dogs, monkeys, miniature pigs, and humans (Sisenwine et al., 1982). The ratios of *S*/*R* oxazepam glucuronides in the urinary excretion was 2.0 for rabbits, 3.0 for dogs, 0.55 for monkeys, 1.2 for pigs, and 3.4 for humans. In addition, species differences in stereoselective hydrolysis have been reported. Methylphenidate (MPH), a methyl ester, is used as a racemic mixture in the treatment of children with attention deficit disorder, and its D-MPH is pharmacologically more active. The plasma esterases of rats, cattle, and rabbits appeared to hydrolyze *L*-MPH faster than the *d*-enantiomer, whereas the plasma of humans, dogs, and horses hydrolyzed *d*-MPH faster than *L*-MPH (Srinivas et al., 1991).

In addition to the quantitative species differences in the magnitude of stereoselective metabolism, qualitative species differences in stereoselective metabolism also occur. The metabolism of disopyramide, a quinidine-like antiarrhythmic agent containing a chiral center, is an example. Cook et al. (1982) have shown that arylhydroxylation is the major metabolic pathway of racemic disopyramide in rats, whereas *N*-dealkylation is the only pathway in dogs and that the *S*-disopyramide is cleared more rapidly than the *R*-enantiomer in both rats and dogs. These results indicate that different metabolic pathways, presumably by different isoforms of cytochrome P-450, are responsible for the stereoselective metabolism between animals. Similarly, the involvement of different enzyme systems in stereoselective metabolism also has been reported for other drugs. Sulindac, an NSAID, contains a chiral sulfoxide moiety and is dosed as a racemate. Sulindac is reversibly reduced to the achiral, pharmacologically active sulfide metabolite. The oxidation of sulfide back to sulindac is stereoselective, forming two enantiomers, sulindac A and sulindac B. In vitro studies with human liver microsomes indicated that sulindac A is formed by flavin-containing monooxygenases, and the formation of sulindac B is catalyzed by cytochrome P-450 enzymes (Hamman et al., 1994).

Considerable interspecies variability exists with respect to the process of chiral inversion. Flurbiprofen, a 2-arylpropionic acid NSAID, is a racemate and is marketed as such. Like other 2-arylpropionic acid NSAIDs, the anti-inflammatory activity of flurbiprofen is believed to reside in the *S*-enantiomer only. A unique characteristic of the metabolism of this class of drugs is the unidirectional inversion of the *R*- to the *S*-enantiomer (Wechter et al., 1974; Hutt and Caldwell, 1983), a process that is species-dependent. The extent of chiral inversion of flurbiprofen is complete in the guinea pig (100%), incomplete in the dog (40%), and very low in the rat and gerbil ($\leq$ 5%) (Manzel-Soglowek et al., 1992). Similar to the rodent species, the inversion of flurbiprofen is also insignificant in humans (Jamali et al., 1988). Species-dependent chiral inversion was also observed for another 2-arylpropionic acid NSAID, ketoprofen. The extent of inversion was high in the rat (Foster and Jamali, 1988), low in the rabbit (Abas and Meffin, 1987), and very small in humans (Jamali et al., 1989).

*2. Stereoselective toxicity.* Drugs exert wanted and unwanted pharmacological effects that are determined, on the one hand, pharmacodynamically by their interaction with the particular enzyme or receptor, and on the other hand, pharmacokinetically by their access to their site of action. Because there are frequently large pharmacodynamic and pharmacokinetic differences between enantiomers, it is not surprising that enantiomers may result in stereoselective toxicity.

Most anticancer drugs are cytotoxic due to their chemical reactivity. For this class of drugs, toxicity is simply an extension of the therapeutic action. Not surprisingly, the major problem with the currently used anticancer drugs is their toxicity toward noncancerous cells. To reduce this undesirable toxicity of antineoplastics, various approaches have been taken to improve their therapeutic indices. One approach has been to exploit the stereoselective toxicity of some chiral antitumor agents. Cyclophosphamide, for example, contains a chiral center at the phosphorus atom and is used clinically as its racemic form. Cox et al. (Cox et al., 1976a,b) reported that the (−)-enantiomer of cyclophosphamide had twice the therapeutic index ($LD_{50}/ID_{90}$) of the (+)-enantiomer against the ADJ/PC6 cell turnover in mice. In clinical applications, however, there was no significant therapeutic advantage gained by using the single enantiomer.

In the past, barbiturates were used extensively as hypnotics. These compounds are rarely used today because of the numerous adverse reactions that have been associated with their use. One of the untoward effects of barbiturates is their excitatory aftereffects. The excitation phenomena range from mild tremors to conclusive seizures. 5-(1,3-dimethylbutyl)-5-ethyl barbituric acid (DMBB) has been used extensively in the investigation of the mechanism of the excitatory effects associated with barbiturate administration. The *S*-(+)-DMBB isomer induced extensive seizures, whereas the *R*-(−)-isomer induced preanesthetic excitation without seizures (Downes et al., 1970; Downes and Williams, 1969). The $LD_{50}$ of the *S*-(+)-DMBB in mice was 3 mg/kg i.v., whereas that of the *R*-(−)-isomer was 72 mg/kg i.v., indicating stereoselective toxicity in that species.

The hypnotic drug thalidomide was taken off the market in Europe after it was tragically found to cause a rare birth defect known as phocomelia. The tragedy led to the passage of the Harris-Kefauver Amendment to the Federal Pure Food and Drug Act in the United States in 1962 to ensure that approved drugs have proof of safety and efficacy (Blaschke et al., 1985). Thalidomide contains a chiral center, and both enantiomers are equally sedating; thus, during its use it was supplied as the racemate. In studies with mice and rats, Blaschke and his coworkers (Blaschke et al., 1979; Blaschke, 1980) found that the *S*-enantiomer of thalidomide was teratogenic, whereas the *R*-isomer was not teratogenic. After intraperitoneal administration of the *S*-enantiomer (200 mg·kg·day) to pregnant animals, the percentage of fetuses born deformed was approximately 30% in mice and 50% in rats. However, no deformed fetuses were found when the *R*-isomer was given intraperitoneally at the same dose to a similar population of animals. By contrast, both enantiomers of thalidomide appeared to be equally teratogenic when administered to rabbits, and the racemate appeared to be even more teratogenic (Fabro et al., 1967; Simonyi, 1984). The percentage of deformed fetuses born from rabbits which were given these treatments was approximately 40% for the racemate, but only 16 to 17% for either *S*- or *R*-enantiomer when given at an equal daily dose (150 mg·kg·day) to pregnant rabbits. Clearly, the stereoselective toxicity of thalidomide is species-dependent. A sad thought is that if the underlying mechanism of the species-dependent stereoselective toxicity was carefully explored, then the thalidomide tragedy could have been avoided.

Due to the limits in available technology in the 1960s and 1970s, studies carried out with thalidomide as a racemate and each of its enantiomers could not be clearly elucidated regarding the mechanisms that resulted in the stereoselective toxicities. With advances in knowledge of molecular biology and stereochemistry, the underlying mechanisms of many stereoselective toxicities are beginning to be understood. For instance, it is now known that stereoselective bioactivation plays a very important role in the carcinogenesis of environmental pollutants (Testa, 1989a; Trager and Testa, 1985; Trager, 1989).

One of the best-documented examples illustrating stereoselective bioactivation is the biotransformation of benzo[a]pyrene by CYP1A1 (Thakker et al., 1988; Vermeulen, 1989; Jerina et al., 1979). Initial oxidation of benzo[a]pyrene by the CYP1A1 results in the selective formation of the 7R,8S-arene oxide, which, upon hydrolysis by epoxide hydrolase, is converted to 7R,8S-dihydrodiol-benzo[a]pyrene. This compound is then converted in a highly stereoselective reaction by the same cytochrome P-450 isozyme to the diastereomeric (+)benzo[a]pyrene 7R,8S-diol-9S,10R-epoxide-2 (>80%) (fig. 5). For the diastereomeric pairs of bay-region diol epoxides

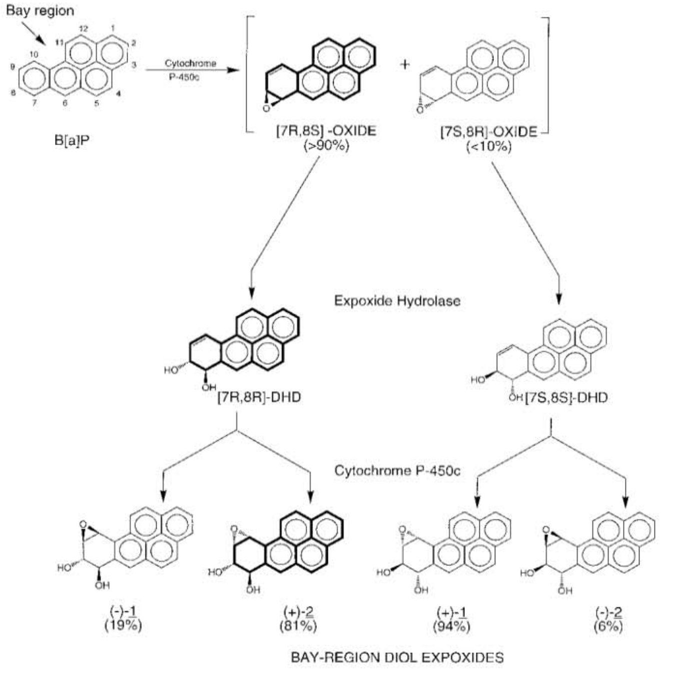

FIG. 5. Regio- and stereoselectivity of CYP1A and epoxide hydrolase in the formation of bay-region diol epoxides of benzo[a]pyrene. Reprinted from Thakker et al. (1988).

of benzol[a]pyrene, only diol epoxide-2 diastereomers show substantial carcinogenic activity, indicating that stereochemical factors play an important role in the carcinogenicity of benzo[a]pyrene. It has been proposed that the hydroxy groups in the bay region are predominately axial in diol epoxide-1 and equatorial in diol epoxide-2 and that their absolute configuration is directly related to their carcinogenicity (Thakker et al., 1988).

Similar to benzo[a]pyrene, alfatoxin B1 (AFB) is a potent carcinogen that undergoes stereoselective bioactivation by cytochrome P-450 isozymes to form AFB exo-epoxide plus small amounts of the AFB endo-epoxide (Baertschi et al., 1989; Raney et al., 1992). The exo-epoxide reacts readily with DNA to give high adduct yields, but the endo-epoxide is nonreactive (Baertschi et al., 1988; Iyer et al., 1994).

## IV. Role of Pharmacokinetics and Metabolism in Drug Development

In recent years, there has been a large expansion in both the range and use of in vitro systems to study absorption and metabolism. Due to the simplicity of in vitro systems, they are very useful in studying the factors influencing drug absorption and metabolism. A trickier task is to use these in vitro systems to predict quantitatively in vivo drug absorption and metabolism. The difficulty in extrapolating in vitro to in vivo lies in the complexity of the whole body with its greater number of interdependent events. Therefore, it is important to carefully set up the in vitro experimental conditions that simulate the in vivo situations. In addition, a good understanding of pharmacokinetic principles will help the in vitro/in vivo extrapolation.

### *A. In Vitro Studies of Drug Metabolism*

*1. Determination of metabolic pathways.* In drug development, early information on human metabolism of a new drug is critical in predicting potential clinical drug-drug interactions and in selecting the appropriate animal species for toxicity studies. For human risk assessment, it is required by regulatory agencies to demonstrate that the systemic exposure of an unchanged drug and its major metabolites in animal species used in the toxicity study exceed that expected in humans to ensure a safety margin. It is important, therefore, to select animal species that have metabolite profiles similar to humans. However, the in vivo human drug metabolism normally is not carried out until the later stages of drug development, which is often too late for animal selection. Fortunately, the increased availability of human tissues and the advances in bioanalytical and biochemical technologies have provided opportunities for in vitro studies of human metabolism at the early stage of drug development before the toxicity studies (Wrighton et al., 1993; Chiu, 1993; Rodrigues, 1994; Powis, 1989).

The metabolite profile of a drug obtained in vitro generally reflects the in vivo metabolite pattern, although limited to qualitative aspects. From physiological and biochemical points of view, precision-cut liver slices are especially useful to obtain the complete in vitro metabolite profile of a drug, because this system retains the physiological conditions of enzymes and cofactors of both phase I and phase II reactions and, therefore, better simulates the in vivo situation (Dogterom, 1993).

As mentioned earlier, the major metabolic pathways of indinavir (MK-639, L-735,524) in humans have been identified as: (*a*) glucuronidation at the pyridine nitrogen to yield a quaternary ammonium conjugate, (*b*) pyridine *N*-oxidation, (*c*) *para*-hydroxylation of the phenylmethyl group, (*d*) 3′-hydroxylation of the indan and (*e*) *N*-depyridomethylation. The metabolite profile of indinavir obtained from human liver slices accurately reflected the in vivo human metabolite pattern (Balani et al., 1995). Athough all of the oxidative metabolites of indinavir also were formed by human liver microsomes, the *N*-glucuronide was not detected when indinavir was incubated with native or Triton X-100–treated (Sigma, St. Louis, MO) human liver microsomes in the presence of 10 mM UDPGA (Lin et al., 1996a). The reason for the inability of human liver microsomes to form the *N*-glucuronide is not clear. Nevertheless, these results suggest that the liver slice is a better in vitro model to study the metabolic pathways of drugs.

Although liver slices are valuable in identifying metabolic pathways, their use in obtaining kinetic parameters may be limited. Houston and his coworkers (Worboys et al., 1996) have shown that the values of $cL_{int}$ ($V_{max}/K_m$) of a series of drugs in slices are consistently less than those in hepatocytes by a factor ranging from 2 to 20. These results strongly suggest that a distribution equilibrium is not achieved between all the cells within the slice and the incubation media, due to the slice thickness (~260 μm).

Isolated and cultured hepatocytes also are used often as in vitro models for identifying metabolic pathways of drugs. In vitro metabolism of ketotifen, an antiasthmatic drug, by cultured rat, rabbit, and human hepatocytes was consistent with the in vivo metabolic pathways, namely oxidation in rat hepatocytes, oxidation, glucuronidation, and sulfation in rabbit hepatocytes, and reduction and glucuronidation in human hepatocytes (Le Bigot et al., 1987). However, the results obtained from hepatocytes should also be interpreted with caution when quantitative comparison is the objective, because many enzyme activities decline spontaneously during hepatocyte isolation or culture. The metabolism of biphenyl has been compared by isolated hepatocytes and liver slices from rats, dogs, and humans (Powis et al., 1989). Human and dog, but not rat, isolated hepatocytes had decreased drug-metabolizing activities of oxidation and conjugation reactions of biphenyl as compared with liver slices. Furthermore, it has been reported that substantial loss of cytochrome P-450 content was observed during the first 24 h of culture (Padgham and Paine, 1993; Padgham et al., 1992).

Another important consideration is the choice of drug concentrations for in vitro studies. The major metabolic pathway may be shifted, depending on the drug concentration used. The clinical studies indicated that *N*-demethylation is the major metabolic pathway of diazepam in humans (Bertilsson et al., 1989). However, in vitro studies in human liver microsomes showed that 3-hydroxylation was the major metabolic pathway of diazepam metabolism when a high (100 $\mu$M) drug concentration was incubated (Inaba et al., 1988). This in vitro and in vivo discrepancy could be due to the differences in the substrate concentration used. Indeed, the major metabolic pathway of diazepam is *N*-demethylation in human liver microsomes when an in vivo relevant substrate concentration (2–4 $\mu$M) is used (Yasumori et al., 1993b).

*2. Identification of drug-metabolizing enzymes.* Over the last 10 years, a great deal of information on human cytochrome P-450s and phase II drug-metabolizing enzymes at the molecular level has become available (Gonzalez et al., 1991; Nelson et al., 1993, 1996; Burchell et al., 1991). This information, with the availability of antibodies and probe substrates, has made it possible to determine which isozyme(s) is/are responsible for a specific reaction of a drug in vitro and in vivo.

To identify which cytochrome P-450 isozymes are responsible for metabolizing drugs in humans, several in vitro approaches have been developed, including (*a*) use of selective inhibitors with microsomes, (*b*) demonstration of catalytic activity in cDNA-based vector systems, (*c*) metabolic correlation of an activity with markers for known enzymes, (*d*) immunoinhibition of catalytic activity in microsomes, and (*e*) catalytic activity of purified enzyme isoforms (Tuengerich and Shimada, 1993). Each approach has its advantages and disadvantages, and a combination of approaches is usually required to accurately identify which cytochrome P-450 isozyme is responsible for metabolizing a drug.

Metabolism of drugs is usually very complex, involving several pathways and various enzyme systems. In some cases, all the metabolic reactions of a drug are catalyzed by a single isozyme, whereas, in other cases, a single metabolic reaction may involve multiple isozymes or different enzyme systems. The oxidative metabolic reactions of indinavir (MK-639, L-735,524) are all catalyzed by a single isozyme, CYP3A4, in human liver microsomes (Chiba et al., 1996). Similarly, CYP3A4 catalyzes both the *N*-dealkylation and *C*-hydroxylation of the antihistamine drug terfenadine in humans (Yun et al., 1993). In contrast, two isozymes, CYP1A2 and CYP3A4, are involved in imipramine *N*-demethylation in human liver microsomes (Lemoine et al., 1993). The *S*-oxidation of 10-(N,*N*-dimethylaminoalkyl)phenothiazines in human liver microsomes is catalyzed by several cytochrome P-450s, including CYP2A6, 2C8, and 2D6 (Cashman et al., 1993). The complexity of metabolism results from the multiplicity of enzyme systems.

The stereoselective metabolism of drugs may result from the involvement of different isoforms. Warfarin, an oral anticoagulant, is marketed as a racemic mixture consisting of equal amounts of *R*- and *S*-warfarin, and its metabolism is stereoselective. Humans metabolize *S*-warfarin almost entirely to form *S*-7-hydroxywarfarin and a smaller amount of *S*-6-hydroxywarfarin. On the other hand, *R*-warfarin is converted mainly to *R*-6-hydroxywarfarin and some 7-hydroxywarfarin (Lewis et al., 1974). In vitro studies with human liver microsomes indicate that both 6- and 7-hydroxylation of *S*-warfarin are catalyzed exclusively by CYP2C9, whereas 6- and 7-hydroxylation of *R*-warfarin is mediated mainly by CYP1A2 and CYP2C19 (Kunze et al., 1996). Similarly, the 4′-hydroxylation of *R*- and *S*-mephenytoin is stereoselective and catalyzed by different isoforms. The rate of microsomal 4′-hydroxylation was 2 to 3 times higher with *R*-mephenytoin than its *S*-enantiomer in rats. Reconstituted systems and immunoinhibition studies suggest that 4′-hydroxylation of *S*-mephenytoin in rats is catalyzed by CYP2C11, whereas 4′-hydroxylation of *R*-mephenytoin is metabolized by CYP3A1/2 (Yasumori et al., 1993a). The stereoselective metabolism of mephenytoin is species-dependent. In contrast to rats, 4′-hydroxylation of *S*-mephenytoin is catalyzed preferentially in human liver microsomes, and this reaction is mediated exclusively by CYP2C9 (Yasumori et al., 1993a).

Like the cytochrome P-450s, multiple UDPGT isoforms can be involved in the glucuronidation of drugs. In vitro evidence has shown that at least two forms of human liver UDPGTs catalyze morphine 3-glucuronidation (Miners et al., 1988). Studies of the effects of different enzyme inducers, such as phenobarbital, 3-MC, and $\beta$-naphthoflavone, in rats suggest that multiple forms of UDPGTs are involved in the glucuronidation of diflunisal (Lin et al., 1987a).

Recently, Stearns et al. (1995) have demonstrated that losartan (MK-954) is converted to its active carboxylic acid metabolite (L-158,641) via the aldehyde intermediate (L-158,610) in human liver microsomes. In an atmosphere of $^{18}O_2$, losartan and L-158,610 were converted to the active metabolite L-158,641 in a reaction that was both NADPH- and oxygen-dependent. The investigators have also shown that CYP2C9 and 3A4 are the major enzymes responsible for each of the two-step oxidative reactions of the formation of the active metabolite (Stearns et al., 1995). However, published in the same issue of *Drug Metabolism and Disposition*, Yun et al. (1995) concluded that only CYP3A4 is involved in the biotransformation of losartan to its active metabolite. Although the reason for this discrepancy is not clear at the present time, differences in experimental conditions between the studies may have led to different conclusions. Furthermore, because different human liver microsomal preparations were often used by different laboratories, differences in intrinsic properties of the cytochrome P-450 population (such as the presence of allelic variants) also may contribute to different results regarding specific cytochrome P-450 involvement.

Because several cytochrome P-450 isoforms with distinct $K_m$ values can contribute to the metabolism of a single drug, selection of the substrate concentration in enzyme identification is important. For example, a CYP2C antibody shows no inhibition of diazepam *N*-demethylation at a substrate concentration of 200 $\mu$M but inhibits over 80% at a substrate concentration of 20 $\mu$M (Kato and Yamazoe, 1994). This clearly illustrates the importance of using clinically relevant substrate concentrations for in vitro studies, either in determining metabolic profiles or identifying drug-metabolizing enzymes. The selection of concentrations of isozyme-selective inhibitors also is important. A recent study by Newton et al. (1995) has shown that the specificity of isozyme-selective inhibitors of cytochrome P-450 is concentration-dependent. Quinidine, a selective inhibitor of CYP2D6, exhibited maximum inhibitory effect on CYP2D6-catalyzed bufuralol 1′-hydroxylation activity at 5 to 10 $\mu$M. At higher (>20 $\mu$M) concentrations, quinidine also inhibited CYP3A4-mediated testosterone 6$\beta$-hydroxylation activity. The concentration-dependent selectivity of inhibitors also has been reported by other investigators (Guengerich, 1986; Ward and Back, 1993). Judicious selection of inhibitor concentration is of importance when determining the contribution of a specific cytochrome P-450 isoform(s) to a given reaction.

*3. Drug-drug interaction.* Concomitant administration of several drugs is common and, indeed, is often the situation in hospitalized patients. Whenever two or more drugs are administered over similar or overlapping time periods, the possibility for drug interactions exists. Although drug interactions can be explained by pharmacokinetic or pharmacodynamic effects, in many cases, the interactions have a pharmacokinetic, rather than pharmacodynamic, basis.

Interaction by mutual competitive inhibition between drugs is almost inevitable, because metabolism represents a major route of drug elimination and because many drugs can compete for the same enzyme system. The risk of clinical consequences from drug-drug interactions is higher with some drugs than with others. Patients receiving anticoagulant, antidepressant, or cardiovascular drugs are, due to the narrow therapeutic index of these drugs, at much greater risk than patients receiving other kinds of drugs (May et al., 1977). Most of the interactions are predictable and manageable, usually by appropriate dosage adjustment, whereas a few are potentially life-threatening. Coadministration of terfenadine, an antihistamine agent, and ketoconazole led to fatal ventricular arrhythmias in some patients (Monahan et al., 1990). Studies by Honig and his colleagues (1993, 1992) revealed that terfenadine is metabolized extensively by CYP3A4 isozymes, and ketoconazole, a potent CYP3A4 inhibitor, inhibited the metabolism of terfenadine, resulting in elevation of terfenadine, which, in turn, caused the prolongation of the QT interval.

Drug-drug interaction studies have become an important aspect of the development process of new drug candidates because of potential adverse effects. Because studies of all possible interactions are neither practicable nor economic, careful selection of a limited number of drug combinations to be studied is essential. Principally, the selection of drug interaction studies is usually based on two main criteria: the likelihood of coadministration and the therapeutic index (Tucker, 1992). Even with these criteria, clinical studies to assess drug interactions with new drug candidates are still very costly and time-consuming. With the advanced in vitro technologies in drug metabolism available today, an alternative approach is to use in vitro systems. These systems are valuable aids as screening tools to predict drug-drug interactions (Peck et al., 1993; Wrighton and Ring, 1994). Increasing evidence has demonstrated that in vitro interaction studies can accurately reflect the in vivo situation (Wrighton and Ring, 1994; Riesenman, 1995; Shen, 1995).

Many pharmaceutical companies now use in vitro techniques to predict the potential drug interactions of new drug candidates. However, several factors need to be considered when the in vitro approach is employed. It is essential to accurately identify those enzyme systems involved in metabolizing particular drugs and to evaluate the relative contribution of the metabolic pathways being inhibited to overall elimination of the drug. A significant drug-drug interaction occurs only when drugs compete for the same enzyme system and when the metabolic reaction is a major elimination pathway.

Another important factor is the use of clinically relevant concentrations of inhibitor and substrate. For competitive inhibition, the velocity of an enzymic reaction in the absence ($V_o$) and presence ($V_i$) of inhibitor can be expressed as:

$$V_o = \frac{V_{max} \cdot S}{K_m + S} \qquad [1]$$

$$V_i = \frac{V_{max} \cdot S}{K_m\left(1 + \frac{I}{K_i}\right) + S}, \qquad [2]$$

where $V_{max}$ is the maximum velocity, $K_m$ is the Michaelis constant of the substrate, $K_i$ is the inhibition constant of the inhibitor, and S and I are the substrate and inhibitor concentrations, respectively. By rearrangement of equations [1] and [2], the percent of inhibition can be described as:

$$\frac{V_o - V_i}{V_o}(\%) = \frac{\dfrac{I}{K_i}}{1 + \dfrac{I}{K_i} + \dfrac{S}{K_m}} \times 100. \qquad [3]$$

As shown in equation [3], the percent of inhibition is dependent on both the ratio of $[I/K_i]$ and $[S/K_m]$. Thus, an understanding of the relationship between substrate and inhibitor concentrations is critical to the design and interpretation of in vitro inhibition studies. Although the metabolic reactions of most drugs in their clinical dose range follow linear kinetics and the ratio $[S/K_m]$ can be neglected, there are several drugs, such as antiviral and anticancer drugs, for which the $[S/K_m]$ ratio can be very high in relation to the $[I/K_i]$ ratio.

As in the case of the terfenadine-ketoconazole interaction, an understanding of the mechanisms involved in drug interactions also is essential to provide a rational basis for interpreting and preventing adverse effects. Warfarin, an oral anticoagulant, exists in enantiomeric forms, in which the *S*-enantiomer of warfarin is much more potent than the *R*-enantiomer. As noted earlier, the more potent *S*-warfarin in humans is eliminated almost entirely as *S*-7-hydroxywarfarin, whereas *R*-warfarin is metabolized mainly as *R*-6-hydroxywarfarin (Lewis et al., 1974). Furthermore, these two hydroxylation reactions are mediated by different cytochrome P-450 isoforms (Kunze et al., 1996). Coadministration of enoxacin, a quinoline-azaquinoline antibiotic, resulted in a decrease in the clearance of *R*-warfarin but not in the clearance of *S*-warfarin. The decreased clearance of *R*-warfarin was found to be a consequence of inhibition by enoxacin on the (*R*)-6-hydroxywarfarin metabolic pathway. As expected, enoxacin did not affect the hypoprothrombinemic response produced by warfarin, because this antibiotic had no effect on *S*-warfarin elimination (Toon et al., 1987). Similarly, cimetidine inhibited human metabolism of *R*-warfarin while having little effect on that of *S*-warfarin (Somogyi and Gugler, 1982). Further studies in healthy subjects indicated that treatment with cimetidine resulted in a significant decrease in the formation of *R*-6- and *R*-7-hydroxywarfarin but had no effect on the formation of *S*-6- and *S*-7-hydroxywarfarin (Niopas et al., 1991). Because only *R*-warfarin metabolism is inhibited by cimetidine, and because *R*-warfarin is much less active, it is expected that cimetidine has little effect on the anticoagulant activity of warfarin.

The two examples above illustrate the importance of an understanding of the mechanisms for interpreting drug interactions and predicting their clinical consequences. Another good example is omeprazole-diazepam interactions. Omeprazole is a proton pump blocker used to treat peptic ulcers and reflux esophagitis. This drug is metabolized mainly by CYP2C19 (Andersson et al., 1990). Diazepam, an antianxiety agent, also is metabolized predominantly by CYP2C19 (Andersson et al., 1990). The CYP2C19 isoform is known to be polymorphic; approximately 2 to 6% of Caucasians or 14 to 22% of Asians are found to be poor metabolizers (Wilkinson et al., 1992; Kalow and Bertilsson, 1994). Coadministration of omeprazole resulted in a significant decrease in plasma clearance of diazepam in extensive metabolizers (EMs) but had no effect on diazepam clearance in poor metabolizers (PMs) (Andersson et al., 1990). Because both omeprazole and diazepam are metabolized mainly by the same enzyme, CYP2C19, this explains why the two drugs interact in EMs but not in PMs. In PMs, there is no enzyme for which diazepam and omeprazole could compete. Similarly, coadministration of a quinidine CYP2D6 inhibitor has been shown to increase plasma concentrations of encainide (CYP2D6 substrate) in EMs but had little effect on the plasma concentrations in PMs (Turgeon et al., 1990).

Although it is easy to determine in vitro drug-drug interaction, the accurate interpretation and extrapolation of in vitro interaction data also require a good understanding of pharmacokinetic principles. If the elimination of a drug is mainly by the liver, the total clearance is approximately equal to the hepatic clearance ($cL_H$) that can be expressed as (Lin, 1995; Wilkinson, 1987):

$$cL_H = Q_h \cdot E = Q_h \cdot \left(\frac{f_p \cdot cL_{int}}{Q_h + f_p \cdot cL_{int}}\right), \qquad [4]$$

where $Q_h$ is the hepatic blood flow, E is the hepatic extraction, $f_p$ is the free fraction in plasma, and $cL_{int}$, the intrinsic clearance, is a measure of the drug-metabolizing activity ($V_{max}/K_m$).

Kinetically, drugs can be classified by whether their hepatic clearance is "enzyme-limited" or "flow-limited" with an intermediate class (Wilkinson and Shand, 1975). When the $cL_{int}$ of a drug is very small relative to the hepatic blood flow ($Q_h \gg f_p \cdot cL_{int}$), then the hepatic clearance is low, and the $cL_H$ is directly related to $f_p$ and $cL_{int}$ as shown in equation [5]:

$$cL_H \risingdotseq f_p \cdot cL_{int}. \qquad [5]$$

Thus, a decrease in the $cL_{int}$ caused by metabolism-based drug interaction will result in an almost proportional decrease in the clearance of "low-clearance" drugs. On the other hand, if the $cL_{int}$ is so high that $f_p \cdot cL_{int} \gg Q_h$, then the hepatic clearance is limited by the hepatic blood flow as shown in equation [6]:

$$cL_H \risingdotseq Q_h. \qquad [6]$$

Thus, a decrease in the $cL_{int}$ caused by drug interaction has little effect on the hepatic clearance of "high-clearance" drugs.

Because the hepatic first-pass effect reflects the hepatic $cL_{int}$, hepatic bioavailability (F) can be expressed as:

$$F = 1 - E = \frac{Q_h}{Q_h + f_p \cdot cL_{int}}, \qquad [7]$$

and the area under the curve (AUC) after oral dosing can be described as:

$$AUC_{po} = \frac{F \cdot dose}{cL_H} = \frac{dose}{f_p \cdot cL_{int}}. \qquad [8]$$

As shown in equation [8], a decrease in the $cL_{int}$ caused by metabolism-based drug-drug interaction will yield an almost proportional increase in the AUC after oral dosing, regardless of whether it is a low- or high-clearance drug. In contrast, after intravenous administration, a decrease in the $cL_{int}$ only affects the clearance and AUC of low-clearance drugs (fig. 6).

Indinavir (L-375,524, MK-639) is a high-clearance drug that is cleared rapidly with a clearance of 80 to 90 mL/min/kg in rats and 15 to 17 mL/min/kg in AIDS patients. These values are greater than rat hepatic blood flow (60–70 mL/min/kg) or close to human hepatic blood flow (20 mL/min/kg). In vitro studies with rat and human liver microsomes indicate that ketoconazole competitively inhibited the metabolism of indinavir with a $K_i$ value of approximately 2.5 $\mu$M. Pretreatment of rats with ketoconazole (25 mg/kg p.o.) had little inhibitory effect on the clearance of indinavir and its AUC after intravenous administration of indinavir. The clearance decreased from 87 mL/min/kg in control rats to 83 mL/min/kg in ketoconazole-pretreated rats. However, ketoconazole significantly increased the bioavailability of indinavir and its AUC after oral dosing. The bioavailability increased from approximately 20% in control rats to 89% in ketoconazole-pretreated rats (Lin, 1996b). Similarly, coadministration of ketoconazole (6 mg/kg p.o.) increased the AUC of indinavir in AIDS patients by approximately 62% after oral administration (McCrea et al., 1996).

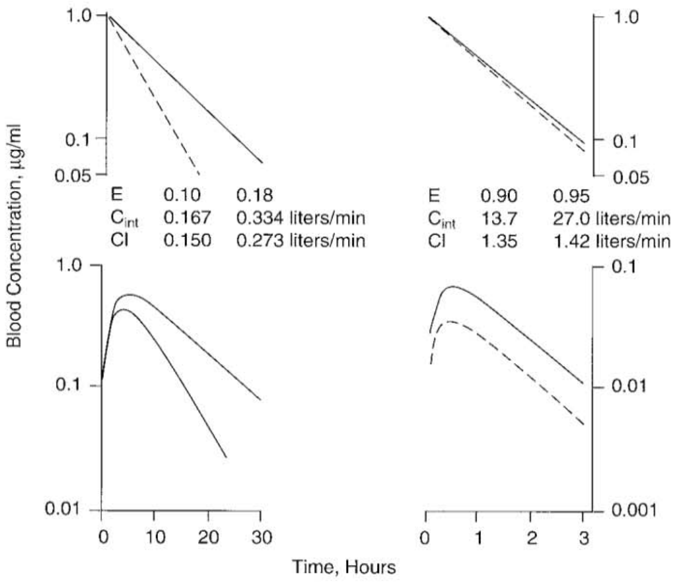

FIG. 6. Effect of $cL_{int}$ on the disposition of low-clearance drugs (left panels) and high-clearance drugs (right panels) after intravenous (upper panels) and oral (lower panels) administration of equal doses of two totally metabolized drugs. Dotted line indicates normal situation; solid line indicates decreased $cL_{int}$. Reproduced with permission from Wilkinson (1987). Each reprint must be printed or stamped on the first page with authorship, title or article, name of journal, volume, Copyright date, The American Society for Pharmacology and Experimental Therapeutics. Reproduced by permission. Further reproduction is prohibited.

On the other hand, ketoconazole is a low-clearance drug with a plasma clearance of 6 to 7 mL/min/kg in rats. In vitro studies with rat liver microsomes revealed that indinavir also competitively inhibited the metabolism of ketoconazole with a $K_i$ value of 4.5 $\mu$M. As expected, pretreatment of rats with indinavir (20 mg/kg p.o.) significantly increased the AUCs of ketoconazole by two-fold after both intravenous and oral administration of ketoconazole (Lin, 1996b).

*4. Prediction of in vivo metabolic clearance.* One of the main objectives of in vitro metabolism studies is the quantitative prediction of in vivo drug metabolism from in vitro data. The prediction of metabolic clearance from in vitro systems is difficult and highly controversial. Some scientists believe that in vitro/in vivo extrapolation is possible, whereas others are less optimistic and believe that it is extremely difficult, if not impossible, to predict in vivo metabolism from in vitro metabolism data, especially in quantitative terms. Each group can cite examples from literature in support of their views (Sugiyama et al., 1989; Pang and Chiba, 1994; Houston, 1994; Gillette, 1984). Despite the difficulty of extrapolating in vitro data, we believe that quantitative in vitro metabolic data can be extrapolated reasonably well to in vivo situations with the application of appropriate pharmacokinetic principles.

There are many examples of good quantitative correlation between in vitro and in vivo drug metabolism. Ethoxybenzamide, an antipyretic agent, is exclusively metabolized to salicylamide by rat liver microsomes. The in vitro $V_{max}$ and $K_m$ values (3.46 $\mu$mol/min/kg and 0.378 mM) are in good agreement with those obtained in vivo by application of a two-compartment model (3.77 $\mu$mol/min/kg and 0.192 mM) (Lin et al., 1978). Indinavir (MK-639, L-735,524), a potent HIV protease inhibitor, exhibited marked species differences in hepatic clearance. This drug was metabolized mainly by isoforms of the CYP3A subfamily to form oxidative metabolites in all species examined (Lin et al., 1996a). The in vitro hepatic clearance of indinavir estimated from in vitro $V_{max}/K_m$ values using liver microsomes from rats, dogs, and monkeys was in good agreement with the corresponding in vivo hepatic clearance values. The in vitro hepatic clearance of indinavir was 31, 25, and

7.8 mL/min/kg for rats, monkeys, and dogs, respectively, and the corresponding in vivo hepatic clearance was 43, 36, and 11 mL/min/kg (Lin et al., 1996a). Chiba et al. (1990) have successfully predicted the steady-state concentration of imipramine and its active metabolite, desipramine, in rats using the $V_{max}$ and $K_m$ values obtained from in vitro microsomal studies. Felodipine, a calcium channel blocker, is primarily metabolized to its pyridine analog in rats, dogs, and humans. The hepatic clearance of this drug obtained from in vitro studies with hepatic microsomes was 16 L/h for rat, 39 L/h for dog, and 259 L/h for humans and agreed reasonably well with those observed in vivo; the corresponding values were 6.2 L/h, 88 L/h, and 321 L/h (Bäärnhielm et al., 1986). Similarly, a good in vitro and in vivo correlation of the clearance of cytarabine hydrochloride has been reported by Dedrick et al. (1972). Furthermore, Iwatsubo et al. (1996) successfully predicted the in vivo clearance and bioavailability of YM796, a CNS drug for the treatment of Alzheimer's disease, using a recombinant system of human CYP3A4 together with the information of the content of this isoform in human liver microsomes (Shimada et al., 1994). Thus, these examples clearly show that in vitro to in vivo extrapolation is indeed possible if appropriate pharmacokinetic principles are employed.

However, the literature review revealed that in some cases, in vitro metabolic data filed to predict in vivo clearance. Sources of inaccuracy in predicting the in vivo metabolic clearance may include the nature and design of in vitro experiments, presence of extrahepatic metabolism, and active transport in liver. Unfortunately, the reason for the lack of in vitro/in vivo correlation has rarely been examined.

### *B. In Vitro Studies of Drug Absorption*

Good absorption is one of the most important criteria in selecting new drug candidates for development. In the discovery stage, drug absorption studies can be performed only in laboratory animals and/or in vitro systems in an effort to characterize the absorptive process both qualitatively and quantitatively. Therefore, one must ask whether the in vitro models are useful in predicting drug absorption in humans or whether animal absorption data can be extrapolated to humans.

*1. Extrapolation of in vitro absorption data.* Numerous in vitro techniques have been developed for the study of drug absorption. These techniques include the use of everted intestinal sacs, everted intestinal rings, isolated brush border and basolateral membrane, and Ussing diffusion cells (Osiecka et al., 1985; Weiser, 1973; Windmueller and Spaeth, 1975; Grass and Sweetana, 1988). The limitations associated with these techniques often restrict their usefulness in the study of drug absorption. In 1989, Hidalgo and Borchardt introduced the Caco-2 cell monolayer model into the research field of drug absorption. During the last few years, the use of Caco-2 cells in the study of drug absorption has increased dramatically. The Caco-2 cell line is derived from a human colorectal carcinoma. It spontaneously differentiates into monolayers of polarized enterocytes under conventional cell culture conditions. After 2 to 3 weeks in cell culture, the monolayers have well-developed junctional complexes. Recently, a new cell line 2/4/A1, isolated from rat fetal intestinal epithelial cells, was used in studying drug absorption (Milovic et al., 1996).

Drugs pass through the intestinal lumen into the blood stream via two routes: (*a*) transcellularly, in which the drugs are transported actively or passively into and through epithelial cells into the blood circulation; and (*b*) paracellularly, in which drugs reach the blood circulation via the tight junctions between the epithelial cells. Because the surface area of the epithelial cell membrane is >1000-fold larger than the paracellular surface area (Pappenheimer, 1987), it is reasonably assumed that absorption of drugs via transcellular transport is always much better than that via paracellular transport. To date, most studies with Caco-2 cells are used to characterize whether a drug is actively or passively transported across the intestinal epithelium and to provide new insight into the regulation of drug transport. Bisphosphonates are poorly absorbed from the gastrointestinal lumen, and the bioavailability was approximately 0.7% for alendronate, 0.3% for pamidronate, and 1 to 2% for clodronate (Lin, 1996a). The poor absorption of bisphosphonates is speculated to be attributed to their very poor lipophilicity, preventing transcellular transport across the epithelial membrane, and therefore, the drugs must be absorbed via the paracellular route. Recently, in vitro studies with the Caco-2 cells have proven that two bisphosphonates (pamidronate and tiludronate) indeed are transported paracellularly (Boulenc et al., 1993; Twiss et al., 1994). Although it is believed that the paracellular permeability of hydrophilic compounds is inversely related to their molecular size (Chadwick et al., 1977), in vitro studies with the Caco-2 cell model show that, in addition to molecular size, flexibility of the drug's geometric structure is also an important factor in determining the permeation through the paracellular pathway (Artursson et al., 1993). Furthermore, the Caco-2 monolayer model was used to illustrate the influence of lipophilicity on the epithelial permeability of a series of $\beta$-blockers with similar $PK_a$s and molecular weights but different lipophilicities (Artursson, 1990). The Caco-2 cell model also has been employed by Conradi et al. (1991, 1992) to show that the permeability of peptides through the intestinal epithelial membrane is goverened by hydrogen bond potential rather than lipophilicity. These examples demonstrate the usefulness of Caco-2 cells for determining the factors that influence drug absorption.

Although most studies of the Caco-2 cells are of a mechanistic nature, attempts have been made to predict quantitatively drug absorption in humans. In their classic study, Artursson and Karlsson (1991) have correlated the epithelial permeability of 20 structurally unrelated drugs in Caco-2 monolayers with the extent of drug absorption in humans after oral administration. They concluded that drugs with complete (100%) absorption were found to have high permeability coefficients ($P_{app} \geq 1 \times 10^{-6}$ cm/s) in the Caco-2 cells, whereas poorly absorbed drugs had low permeability coefficients ($P_{app} < 1 \times 10^{-7}$ cm/s). However, in a similar correlation study, Rubas et al. (1993) reported that compounds with complete absorption in humans had Caco-2 permeability coefficients $>7 \times 10^{-5}$ cm/s, whereas compounds with poor absorption had permeability coefficients $<1 \times 10^{-5}$ cm/s. These values were approximately 70 to 100 times greater than those obtained by Artursson and Karlsson (1991). In a more recent study, Stewart et al. (1995) claimed that compounds completely absorbed in humans had Caco-2 permeability coefficients $>3 \times 10^{-5}$ cm/s. The reasons for this discrepancy in the reported permeability values between laboratories are not clear. Such results indicate that although the Caco-2 cell line is a useful model in ranking the permeabilities of drugs, it cannot be used quantitatively in predicting human absorption in vivo. Gan et al. (1993) reported that the Caco-2 permeability coefficient of ranitidine was $1.0 \times 10^{-7}$ cm/s. If solely based on this in vitro value, one might predict poor absorption of ranitidine and throw away a billion-dollar drug. Actually, ranitidine has a good (50–70%) bioavailability in humans (Lin, 1991). Similarly, a poor Caco-2 permeability coefficient ($<1.0 \times 10^{-7}$ cm/s) was obtained for cimetidine, which is absorbed well in humans (personal communication with J. Hochman).

Attempts also have been made to compare in vitro and in vivo drug permeability. The permeabilities of a series of drugs were investigated in Caco-2 cells and in the human jejunum in situ using a double balloon technique (Lennernäs et al., 1996). Although the rank order of the permeability of these drugs was similar between the Caco-2 monolayers and the human jejunum, the permeability values of all drugs were much greater in the human jejunum than in the Caco-2 monolayers. The permeabilities of the drugs with complete absorption differed 2- to 4-fold between in vitro and in situ models, whereas the permeabilities of drugs with poor absorption differed as much as 30- to 80-fold. Thus, the permeability measured by Caco-2 cells can only be used for qualitative comparison, but not for quantitative purposes. Nevertheless, because drug transport studies in Caco-2 monolayers are easy to perform and require only small quantities of drugs, the Caco-2 cell monolayers can be used for screening of drug absorption (by ranking the permeability) at the early stages of drug discovery. Recently, Caco-2 monolayers were used to screen the permeability of a synthetic peptide library containing 375,000 compounds (Stevenson et al., 1995). Because the properties of Caco-2 monolayers can be varied with time in culture (Wilson et al., 1990), the passage number (Walter and Kissel, 1995), and the cell culture medium (Jumarie and Malo, 1991), it is therefore important to include a reference drug for comparison purposes when screening the permeability of drugs.

*2. Extrapolation of animal absorption data.* In addition to the drug's permeability, many other factors, such as gastric and intestinal transit time and hepatic and intestinal metabolism, can influence the rate and extent of absorption. Because the in vitro models cannot provide quantitative prediction of drug absorption in humans, alternatively one can use animal absorption to predict human drug absorption. A rough estimate of human drug absorption from animal data is possible if species differences in the magnitude of first-pass metabolism can be assessed accurately. This is based on the assumption that the membrane permeability of drugs is similar across species. Membrane permeability is characterized as the relative magnitude of the interaction of the drug with the aqueous environment and lipophilic interior of the membrane, and is a function of the lipophilicity, molecular size, and $PK_a$ of drugs (Ho et al., 1983). Because the nature of the biomembrane of the intestinal epithelial cells is similar across species, and because the main absorptive process (simple diffusion) is basically an interaction between the drug and the biomembrane (Wilson et al., 1989; Jackson, 1987), the permeability of a drug across the wall of the gastrointestinal tract is expected to be similar among species. There are numerous examples that support species similarity in the epithelial permeability. Amidon et al. (1988) successfully predicted the fraction of dose absorbed from the gastrointestinal tract in humans, using rat intestinal membrane permeability for a series of structurally unrelated compounds. Similarly, a good correlation between drug absorption rate constants in the human Caco-2 model and in a rat intestinal in situ model was obtained for a series of $\beta$-blocking agents (Artursson, 1990). In addition, the permeabilities of drugs that are transported by paracellular transport, due to their inability to cross the epithelial membranes, have been demonstrated to be similar among species. The paracellular permeability of a series of hydrophilic compounds obtained from human Caco-2 cells were quantitatively in good agreement with those from rat colon (Artursson et al., 1993).

Another key factor controlling drug absorption is first-pass metabolism. The oral bioavailability of a drug is defined as the fraction of an oral dose of the drug that reaches the systemic circulation. Because the entire blood supply of the upper gastrointestinal tract passes through the liver before reaching the systemic circulation, the drug may be metabolized by the liver and gut wall. Kinetically, the oral bioavailability (F) of a drug can be described as:

$$F = f_{abs} \cdot (1 - f_g) \cdot (1 - f_h), \qquad [9]$$

where $f_{abs}$ is the fraction of drug absorbed from the gastrointestinal lumen, and $f_g$ and $f_h$ are the fractions of drug metabolized by the gut wall and liver during the first passage of drug absorption (Lin, 1995). The $f_{abs}$ of a drug is expected to be similar among species because it is determined mainly by its permeability. On the other hand, the $f_g$ and $f_h$ of a drug could be substantially different from one species to another.

Marked interspecies differences in the bioavailability of indinavir (MK-639, L-735,524) were observed when the drug was given orally as a solution in 0.05 M citric acid. The bioavailability varied from 72% in the dog to 19% in the monkey and 24% in the rat (Lin et al., 1996a). The low bioavailability observed in rats and monkeys was due to extensive hepatic first-pass metabolism. By comparing the drug concentration in the systemic circulation during portal or femoral vein infusion, hepatic first-pass extraction was estimated to be approximately 68% in rats. On the other hand, in situ studies with rat isolated intestinal loop preparation showed that intestinal first-pass metabolism was minimal (<8%). Consistent with in vivo and in situ studies, in vitro intestinal and hepatic first-pass extraction ($f_g$ and $f_h$) were estimated to be 5 and 55%, respectively, for the rat using the intestinal and liver microsomal $V_{max}/K_m$ data. Although in vivo hepatic first-pass extraction was not determined for the dog and monkey, the in vitro values were estimated to be 17% and 65%, respectively, using dog and monkey liver microsomes (Lin et al., 1996a). Taking the hepatic first-pass metabolism into account, the extent ($f_{abs}$) of indinavir absorbed from the gastrointestinal lumen was quite similar among species (~55 to 80%). Thus, observed species differences in the bioavailability of indinavir were due mainly to the differences in magnitude of hepatic first-pass metabolism. Using human intestinal and hepatic microsomes, the intestinal and hepatic first-pass metabolism of indinavir in humans were estimated to be 5 and 26%, respectively (Chiba et al., 1997). With the extent of absorption (55–80%) obtained from animal studies, we predicted the bioavailability of indinavir in patients would be 40 to 60%. As predicted, when clinical data became available, the bioavailability of indinavir was found to be approximately 60% (Yeh et al., unpublished data).

Another example that shows species similarity in drug absorption is L-365,260, a potent $CCK_B$ receptor antagonist for the treatment of anxiety. The bioavailability of L-365,260 was 14% for the rat and 9% for the dog when given orally as a suspension in 0.5% methylcellulose (Lin et al., 1996c). The limited bioavailability was attributed mainly to poor absorption as a result of its low aqueous solubility (<2 μg/mL), because the hepatic first-pass metabolism was low and estimated to be 30% for the rat and 14% for the dog (Lin et al., 1996c).

When L-365,260 was given as a solution in PEG 600, the bioavailability increased to 50% in the rat and 70% in the dog. Taking hepatic first-pass metabolism into consideration, the extent ($f_{abs}$) of L-365,260 absorbed from the gastrointestinal lumen was similar between rats and dogs (~70–80%). With this information at hand, L-365,260 was dosed in capsules containing PEG 600 in the subsequent clinical studies. As expected, the formulation gave good absorption of L-365,260 in healthy volunteers. The $C_{max}$ and AUC were, respectively, 2.3 μg/mL and 450 μg·min/mL for the dogs and 0.5 μg/mL and 148 μg·min/mL for normal human subjects when the same dose (50 mg) of L-365,260 in polyethelene glycol (PEG) capsules was given orally to dogs (12 kg) and normal volunteers (70 kg) (Lin et al., unpublished data). The $C_{max}$ and AUC values were comparable in dogs and humans when compared on a weight-normalized dose basis.

The examples of indinavir and L-365,260 suggest that drug absorption in humans can be extrapolated reasonably well from animal data when information on first-pass metabolism is also available. Indeed, Clark and Smith (1984) have reported in a survey that the fractions ($f_{abs}$) of dose absorbed from the gastrointestinal lumen for a large variety of drugs are remarkably consistent between animal species and humans. The bioavailability, however, differs substantially among species, presumably as a result of species differences in the magnitude of first-pass metabolism.

### C. In Vitro Studies of Protein Binding

A basic tenet of biochemical pharmacology is that the intensity and duration of drug action is mediated via the time course of unbound drug concentrations at the site of action. Although direct measurement of unbound drug concentrations at the site of action is seldom possible, the unbound drug concentrations in plasma often bear a proportional relation, such that unbound drug concentrations in plasma can be used in lieu of site unbound concentrations. This assumption implies that drugs bind reversely to plasma and tissue protein and that equilibrium of unbound drug occurs readily between plasma and tissues. Several reports are available to support this tenet that the unbound drug concentration correlates with pharmacological response and toxicity better than the total drug concentration (Yacobi and Levy, 1975; Yacobi et al., 1976; Mungall et al., 1984; Booker and Darcey, 1973; Rimmer et al., 1984; Huang and Øie, 1982; Øie and Chiang, 1991).

*1. In vitro/in vivo protein binding.* There are numerous in vitro methods for the determination of protein binding, including equilibrium dialysis, dynamic dialysis, ultrafiltration, ultracentrifugation, exclusion chromatography, and circular dichroism. The advantages and disadvantages of each method have been discussed, and the reliability of these methods was compared (Kurz et al., 1977; Kurz, 1986). It is concluded that equilibrium dialysis and ultrafiltration are most likely to provide both an accurate and precise assessment of plasma protein binding. Koike et al. (1985) compared an ultrafiltration technique with an equilibrium dialysis method for measuring the unbound phenytoin fraction in plasma in 36 patients with normal renal function and 6 uremic patients. The unbound concentrations of phenytoin determined by the ultrafiltration and equilibrium dialysis were esentially identical in both normal and uremic plasma obtained from patients under treatment.

Because the binding of drugs to plasma proteins is an important factor in determining their pharmacokinetics and pharmacological effects, plasma protein binding is routinely determined in vitro for drugs in discovery and development. The question is whether the in vitro binding data accurately reflects the in vivo binding.

The ratio of CSF drug concentration to plasma drug concentration has been used to determine in vivo drug binding. CSF is a very low-protein fluid, and therefore, drug in CSF is considered to be almost unbound. Chou and Levy (1981) demonstrated that the in vitro free fraction of phenytoin serum (0.155) obtained by equilibrium dialysis was essentially identical with the in vivo CSF:serum drug ratio (0.183). Similarly, Bertilsson et al. (1979) showed that the CSF:plasma ratio of demethylchlorimpiramine (0.026) was similar to the in vitro free fraction of the drug determined by ultrafiltration (0.035). These results suggest that in vitro plasma protein binding may accurately reflect in vivo binding. However, the CSF:plasma concentration ratio can only be viewed as an in vivo free fraction if there is no active transport involved in brain penetration. Enprofylline and theophylline have virtually identical in vitro free fractions in plasma (0.53 and 0.51, respectively) (Tegner et al., 1983). However, in a clinical study, the CSF: plasma ratios averaged 0.095 with enprofylline and 0.36 with theophylline (Laursen et al., 1989). The lower CSF levels of enprofylline than theophylline may be explained by the active transport of enprofylline, but not of theophylline, from CSF to blood.

Recently, microdialysis has been developed for measuring the unbound drug concentration in biological fluid. The use of microdialysis to determine the plasma protein binding of drugs was evaluated by comparing with ultrafiltration and equilibrium dialysis. Values of the free fraction of several drugs determined in vitro by microdialysis agreed very well with those by ultrafiltration and equilibrium dialysis (Herrera et al., 1990; Ekblom et al., 1992). The development of microdialysis technique provides the potential use of direct measurement of in vivo plasma protein binding. Recently, we used microdialysis to assess the in vivo plasma protein binding of warfarin, salicylate, and acetaminophen under steady-state conditions in conscious rats. Microdialysis probes were implanted in a jugular vein and continuously perfused with saline. The in vivo free fraction measured by microdialysis was 0.041 for warfarin, 0.185 for salicylate, and 0.76 for acetaminephen. These values correlated very well with the corresponding in vitro values determined by ultrafiltration (0.048, 0.192, and 0.62) (Wong and Lin, unpublished data). Similarly, microdialysis was performed in vivo to determine the plasma protein of the nonindolic melatonin analog S 20098 in rats under steady-state conditions, yielding similar free fraction values (0.26) to those obtained in vitro (0.24) (Quellec et al., 1994).

In view of the evidence presented above, it appears that the in vitro binding data determined by ultrafiltration and equilibrium dialysis accurately reflect the in vivo binding situations. However, care still must be exercised in determination of in vitro binding when the goal is to represent the in vivo situation. For example, in some cases, the metabolite of a drug may also bind to the plasma proteins and thus may be in competition with the parent drug for binding sites. Therefore, an ex vivo experiment in which plasma is taken from a species that has already received the drug may better reflect the in vivo binding situations. Dorzolamide (MK-507) is a good example. This drug is a potent carbonic anhydrase inhibitor used for the treatment of glaucoma. Carbonic anhydrase predominantly localized in red blood cells, accounting for >90% of the enzyme in the body. After administration of dorzolamide to the rat, a substantial fraction of the drug was converted to the *N*-demethylated metabolite, which is also a potent carbonic anhyrase inhibitor. Both dorzolamide and its *N*-demethylated metabolite bind extensively to erythrocytes. The rat erythrocyte:plasma concentration ratio of dorzolamide was approximately 200 when the drug was added to blood in vitro to yield a concentration of 30 $\mu$M. However, the ratio of dorzolamide was <10 at the same drug concentration when the ratio was determined ex vivo with the blood obtained from the rats that received a 25-mg/kg i.v. dose (Wong et al., 1996). The discrepency between in vitro and ex vivo erythrocyte:plasma ratio is attributed mainly to the competitive binding interaction betwenn dorzolamide and its *N*-demethylated metabolite. Similarly, competition in plasma protein binding between parent compound and its metabolite has been reported for sulfamethazine and its *N*-acetyl metabolite (du Souich and Babini, 1986).

*2. Plasma and tissue protein binding.* It is generally believed that only the unbound drug can diffuse across membranes that restrict distribution of a drug from the vascular compartment to the tissues and vice versa. Therefore, drug protein binding in plasma and tissues can affect the distribution of drugs in the body. Kinetically, the simplest quantitative expression relating the volume of distribution ($V_d$) to plasma and tissue binding (Lin, 1995) is given as:

$$V_d = V_p + \sum V_t \frac{f_p}{f_t}, \qquad [10]$$

where $V_p$ is the plasma volume, $V_t$ is the tissue volume, and $f_p$ and $f_t$ are the fraction of unbound drug in plasma and tissue, respectively. From this relationship, it is seen that the $V_d$ increases when $f_p$ is increased and decreases when $f_t$ is increased.

Rearrangement of equation [10] yields:

$$V_f = \frac{V_d}{f_p} = \frac{V_p}{f_p} + \sum \frac{V_t}{f_t}, \qquad [11]$$

where $V_f$ is defined as the volume of distribution of unbound drugs. From this equation, it is clear that a change in $f_t$ has a greater effect than $f_p$ on $V_f$, because $\Sigma V_t$ is much greater than $V_p$.

Although it is easy to determine the plasma protein binding of drugs, the study of tissue binding is hampered by methodological problems. Several methods have been developed for the study of tissue binding. These include perfused intact organs, tissue slices, or tissue homogenates. In principle, these methods allow the direct determination of tissue binding but require removal of tissues from the body, which limits their applicability. Furthermore, the necessary handling of tissues, such as of tissue slices and homegenization, may alter binding properties. The technical difficulties associated with determinations of drug binding to tissues are reflected by the very limited amount of published information on that subject (Fichtl et al., 1991).

Despite the technical difficulties, attempts have been made to extrapolate the in vitro tissue binding to that of in vivo. Assuming that the unbound drug concentration in tissues and plasma is equal at distribution equilibrium, the ratio ($K_p$) of drug concentration in tissue to that in plasma after drug administration is equivalent to the ratio of free fraction in plasma ($f_p$) to the free fraction in tissue ($f_t$). By applying this principle, Lin et al. (1982) showed a good agreement between the in vitro $K_p$ values of ethoxybenzamide obtained from tissue homogenate binding and those from in vivo study of ethoxybenzamide in nine tissues of rats. Schuhmann et al. (1987) determined the $K_p$ values for 11 drugs in muscle, liver, lungs, and kidneys of rabbits after constant rate infusion. For muscle tissue, a good agreement between the in vivo- and in vitro-calculated $K_p$ values of the 11 drugs was observed, whereas in the other tissues (liver and lung), the in vivo and in vitro $K_p$ values of some drugs were not in agreement. For example, the in vivo $K_p$ values of quinidine, imipramine, and buphenine were 10- to 20-fold greater than the corresponding in vitro $K_p$ values calculated from in vitro binding data. Similarly, major discrepancies between in vitro and in vivo $K_p$ values for other drugs also were reported by other investigators (Igari et al., 1982; Harashima et al., 1984). These results suggest that in vivo binding of drugs to tissues may not be predicted readily by simple in vitro methods, because distribution of drugs in tissues may involve active uptake and secretion or metabolism processes.

As shown in equation [12], a drug's $t_{1/2}$ is directly related to its $V_d$. Therefore, it is very useful if one can predict the $V_d$ in humans before its initial clinical studies. Unfortunately, it is difficult to predict the $V_d$ on the basis of in vitro binding data, because the $V_d$ is determined by both its plasma and tissue binding as indicated in equation [10] and because it is difficult to assess tissue binding. Alternatively, it is hoped that $V_d$ of drugs in humans can be extrapolated from data of animals.

Fichtl et al. (1991) reported that there were striking species differences in plasma protein binding and $V_d$ of propranol. The values for the $V_d$ varied by >20-fold, being lowest in monkeys and highest in rabbits. However, when the $V_d$ was corrected for the $f_p$, the volume of distribution of unbound propranolol, $V_f$, was virtually the same for all species. Consistent with this, Sawada et al. (1984a) reported that the $V_f$s of 10 basic drugs were quite similar among species including humans. Based on these results, Fichtl et al. (1991) proposed that the $V_f$ of drugs should be similar in humans and other species. Therefore, with knowledge of the $V_f$ from laboratory animals and of $f_p$ from human plasma protein determined in vitro, one can predict the $V_d$ ($V_f \times f_p$) in humans before the initial clinical studies. Unfortunately, this approach is not valid for all drugs. Boxenbaum (1982) compared the pharmacokinetic parameters for 12 benodiazapines in dogs and humans. Eight of the 12 benzodiazapines had quite different $V_f$ values between the dog and human, the differences being as much as seven-fold for lorazepam. The large species differences in the $V_f$ values also were reported for β-lactam antibiotics (Sawada et al., 1984b). Thus, the species similarity in the $V_f$ of propranolol observed by Fichtl et al. (1991) could be fortuitous. In conclusion, these results suggest that the $V_d$ of drugs in humans cannot be extrapolated from animal data.

*3. Protein binding displacement interactions.* Like metabolism-based competitive interactions, binding displacement interaction occurs when drugs compete for a common binding site of plasma proteins. Substantial drug displacement occurs when the displacing agent occupies a significant portion of the binding. Human plasma contains over 60 proteins. Albumin is the major component of plasma proteins responsible for the binding of most drugs in plasma. The concentration of albumin in normal subjects is approximately 650 μM (Lin et al., 1987b). The concentration of $\alpha_1$-acid glycoprotein can vary considerably in several physiological and pathological conditions. In healthy subjects, the concentrations of $\alpha_1$-acid glycoprotein ranged from 10 to 30 μM (Kremer et al., 1988). Although it is generally believed that basic drugs bind mainly to $\alpha_1$-acid glycoprotein and acid drugs bind to albumin, it has been shown that acid drugs bind to $\alpha_1$-acid glycoprotein, and basic drugs bind to albumin as well (Urien et al., 1986; Israili and El-Attar, 1983). Because of the high albumin concentration, a relatively high concentration of inhibitors (displacers) would be