BIOTRANSFORMATION AND METABOLITE ELUCIDATION OF XENOBIOTICS

CHARACTERIZATION AND IDENTIFICATION

EDITED BY
ALA F. NASSAR

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To Sam, Ray (Sam 2), Ruth, and JW

PREFACE

The first seeds for this work were sown in 1994 at the University of Connecticut, when I was a graduate student, trying to analyze metabolites using LC-MS/MS and CE-MS/MS. I could not find a book describing good, modern strategies, techniques, and tools for metabolite identification. After 15 years in the field, working on a variety of drug metabolism studies, I still cannot find a good reference book to guide the drug metabolism scientist. Metabolite identification and characterization have progressed over the years to the point where they now play a major role in drug discovery and development. Clearly, there is a growing need for improving and expanding the education of scientists involved in investigations in these areas. The need for continuing education in the rapidly expanding and dynamic area of metabolite characterization studies is not being met at the university level, and it is often left to lead researchers and managers to teach this critical subject in a piecemeal fashion on the job in the pharmaceutical industries. While this book is written primarily for those scientists involved in designing, developing, and advancing metabolite characterization for drug discovery and development, the information should also be helpful to students, researchers, educators, and professionals in a number of other related fields.

The goal of the work presented herein is to provide a systematic approach for the education of researchers and students at the university level and to improve their knowledge of how and when to perform metabolite identification and characterization. Here we have made an attempt to bring together the most recent FDA guidelines, current tools for metabolite identification, and an outstanding introduction on human biotransformation by authors who not only are great scientists in the field but are also passionate about their work. The material presented

here is intended to guide students, as well as teachers and professionals, in fields such as pharmacy, pharmacology, biochemistry, and analytical chemistry. Another group who would benefit from this book includes physicians, along with scientists and managers in the pharmaceutical industry, researchers in universities and R&D settings, and environmental and agrochemical industries. Any one of these people would benefit from a better understanding of the latest tools, methods, and regulatory requirements for performing metabolite characterization in a safe, effective, and efficient manner.

This textbook consists of eight chapters, starting with an excellent discussion on human biotransformation coauthored by one of the leaders in the field, Dr. Andrew Parkinson. Chapters 2 and 3 present a good background on the tools for metabolite identification, followed by Chapter 4, which discusses some strategies for using these tools to improve drug design, providing important examples of how to manipulate the relationship between metabolic stability, PK properties, and toxicity. Chapters 5 and 6 present recent and relevant case studies illustrating novel rearrangements of the DNA alkylating agent laromustine by collision-induced dissociation, along with identification of the *in vitro* metabolite/decomposition products of laromustine. Chapter 7 provides a well-written discussion of strategies for the detection of reactive intermediates in drug discovery and development. Chapter 8 presents a wonderful overview of the most recent FDA guidelines on human metabolites in safety testing (MIST) and regulatory agency needs, along with their impact on drug metabolism research in the pharmaceutical industry.

With great pleasure and respect, I extend my sincere thanks to my hardworking scientific colleagues for their sacrifices of family time, timely responses, excellent contributions, and consistent cooperation. Also, I would like

to thank the publishers for making this book available at reduced cost to our scientific colleagues in developing countries to enhance their knowledge in the area of metabolite characterization and to help them in furthering their careers in this very important area of research.

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CHAPTER 1

HUMAN BIOTRANSFORMATION

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1.1 INTRODUCTION

Biotransformation is the enzyme-catalyzed process of chemically modifying drugs and other xenobiotics (foreign chemicals) to increase their water solubility in order to facilitate their elimination from the body. For most orally active drugs, biotransformation is the process that alters the physiochemical properties of a drug away from those properties (namely lipophilicity) that favor the initial absorption of the drug from the gastrointestinal tract as well as its reabsorption from the kidney and gut to those properties (namely hydrophilicity) that favor elimination in the urine and feces. Human biotransformation is of interest for several reasons. First, for many drugs, the rate of biotransformation in the small intestine and liver determines the amount of drug that reaches the systemic circulation. Second, the rate of biotransformation in the liver (and occasionally some extrahepatic tissues) determines, in part, the dose of drug required to achieve therapeutically effective drug levels (which is also influenced by the affinity with which the drug binds to its therapeutic target) and the dosing interval (which determines whether the desired

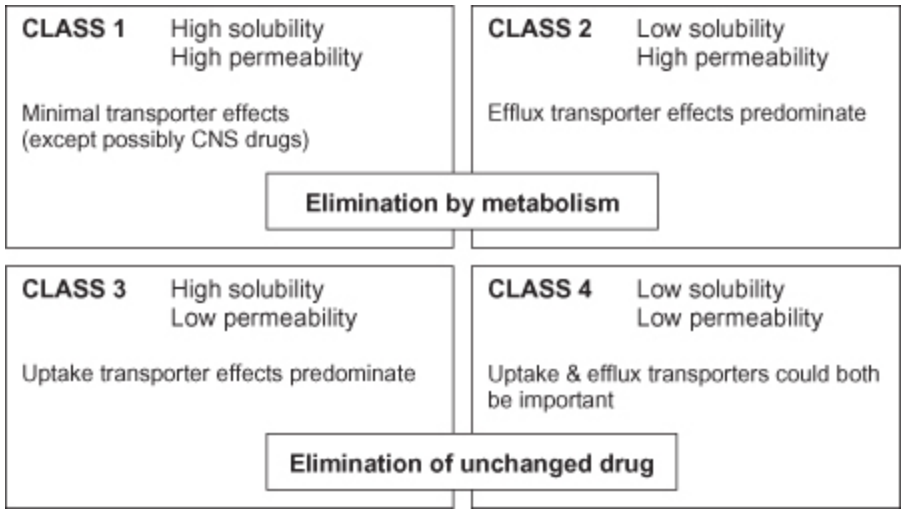
therapeutic goal can be achieved with once-a-day dosing, which for most drugs is highly desirable). Third, the metabolites formed by drug biotransformation must be considered in terms of their therapeutic properties and adverse effects. Fourth, the particular drug-metabolizing enzyme or enzymes involved in the metabolism of a drug can determine the drug's victim potential (i.e., its susceptibility to pharmacogenetic variation in enzyme expression and its interaction with other agents that alter the pharmacokinetics of the drug by inhibiting or inducing that particular drug-metabolizing enzyme or enzymes). The disposition of many drugs is also determined by (or influenced by) their transmembrane transport by various uptake or efflux transporters. Like drug-metabolizing enzymes, drug transporters can influence the uptake, distribution, and elimination of drugs, but they do not chemically modify drugs by converting them to metabolites. This chapter focuses mainly on human drug biotransformation with a final section on drug transport.

1.2 BIOTRANSFORMATION VERSUS TRANSPORT

According to the Biopharmaceutical Classification System (BCS), drugs can be categorized into four classes based on their aqueous solubility and permeability (based on the extent of oral drug absorption; Wu and Benet, 2005). As shown in Fig. 1.1, highly permeable drugs (drugs with high oral absorption) belong to Classes 1 and 2, which are distinguished on the basis of their aqueous solubility (which is high for Class 1 drugs and low for Class 2). Class 3 and 4 drugs both have low permeability (poor oral absorption) but with different aqueous solubility (high for Class 3, low for Class 4). As a general rule, biotransformation represents the predominant route of elimination of Class 1 and 2 drugs

(i.e., the highly permeable drugs that show high oral absorption). Drugs in Classes 3 and 4 tend to be eliminated unchanged. Whereas metabolism plays an important role in the disposition of Class 1 and 2 drugs, transporters (especially uptake transporters) play an important role in the disposition of Class 3 and 4 drugs. Efflux transporters tend to play an important role in the disposition of drugs with low aqueous solubility (Classes 2 and 4).

Figure 1.1 The general role of metabolism and transport in the disposition of drugs according to their Biopharmaceutical Classification (BCS).



Because they are well absorbed from the gastrointestinal tract, BCS Class 1 and 2 drugs contain a large number of orally active drugs. Their high permeability is largely due to their lipophilicity. This same property prevents their elimination in urine and feces because, even if eliminated, the unchanged drugs can readily be reabsorbed from the kidney and intestine. For this reason, biotransformation is the predominant route of elimination of lipophilic, high permeable drugs (those in BCS Classes 1 and 2) because their conversion to water-soluble metabolites permits their excretion in urine and feces. As a general rule, biotransformation to water-soluble metabolites is required to eliminate drugs with a $\log D_{7.4} \geq 1$. For highly lipophilic

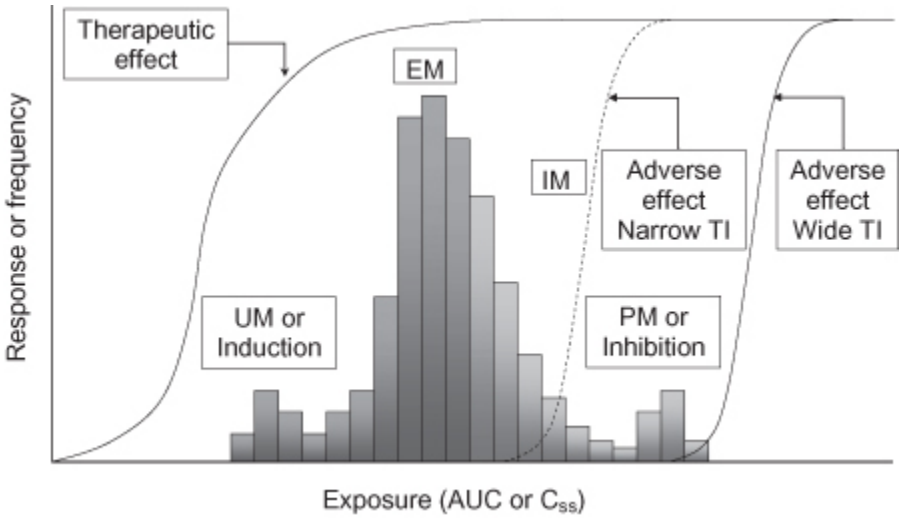
drugs, two or more biotransformation reactions may be required to achieve urinary or biliary excretion.

1.3 PHARMACOKINETIC VARIATION AND THERAPEUTIC INDEX

In a clinical setting, there is always some interindividual variation in systemic exposure to an oral drug, which is usually measured as plasma C_{max} (the maximum concentration of drug in plasma) or AUC (the area under the plasma concentration vs. time curve). This pharmacokinetic variability has three major causes: variation in the extent of absorption (perhaps due to food effects or differences in the extent of first-pass metabolism in the intestine and liver); variation in the rate of systemic clearance (due largely to differences in rates of hepatic uptake and metabolism), and variation in rates of excretion (due to impaired renal or biliary elimination as a result of impaired kidney and liver function, respectively). The variation in systemic exposure due to variation in rates of drug metabolism can arise for both genetic and environmental reasons. Genetically determined differences in the expression of a drug-metabolizing enzyme, such as CYP2D6, give rise to four phenotypes designated as UM, EM, IM, and PM, which respectively stand for ultra-rapid metabolizers (individuals who express multiple copies of the gene encoding the drug-metabolizing enzyme), extensive metabolizers (individuals who express at least one fully functional allele), intermediate metabolizers (individuals who express one or two partially functional alleles), and poor metabolizers (individuals who express no functional alleles and are completely deficient in the affected drug-metabolizing enzyme) (Zineh et al., 2004). Even for enzymes that show

little pharmacogenetic variation (such as CYP3A4), considerable variation in enzyme expression can be caused by environmental factors including medication. In the case of drug-metabolizing enzymes like CYP3A4, UMs can be created pharmacologically by enzyme-inducing drugs whereas PMs can be created pharmacologically by inhibitory drugs. This topic is discussed later in the section on Drugs as Victims and Perpetrators. Whatever its cause, the impact of interindividual differences in systemic exposure to an oral drug depends on the magnitude of the pharmacokinetic differences relative to the drug's therapeutic index, as illustrated in Fig. 1.2. When a drug has a large therapeutic index, it is possible that no dosage adjustment is required either to achieve therapeutic efficacy in UMs or to prevent adverse effects in PMs, assuming that the therapeutic and adverse effects are both mediated by the parent drug (which is often but not always the case, as discussed below in the section on Parent Drug versus Metabolite). When a drug has a narrow therapeutic index, the standard dosage may need to be increased in UMs (to achieve a therapeutic effect) or decreased in PMs (to prevent an adverse effect). The U.S. Food and Drug Administration (FDA) defines a narrow therapeutic range as either "less than a 2-fold difference in median lethal dose (LD_{50}) and median effective dose (ED_{50}) values" or "less than a 2-fold difference in the minimum toxic concentrations and minimum effective concentrations in the blood, and safe and effective use of the drug products require careful titration and patient monitoring." An ideal drug is not necessarily a drug that shows no interindividual differences in systemic exposure but rather one with a therapeutic index that encompasses interindividual differences in systemic exposure such that everyone derives therapeutic benefit from the same dose and no dosage adjustment is required for UMs or PMs.

Figure 1.2 The impact of genetic polymorphisms (such as the UM, EM, IM, and PM phenotypes of CYP2D6) and the corresponding impact of enzyme induction or inhibition of a drug-metabolizing enzyme like CYP3A4 on systemic exposure to a drug whose rate of clearance is largely determined by its rate of biotransformation.

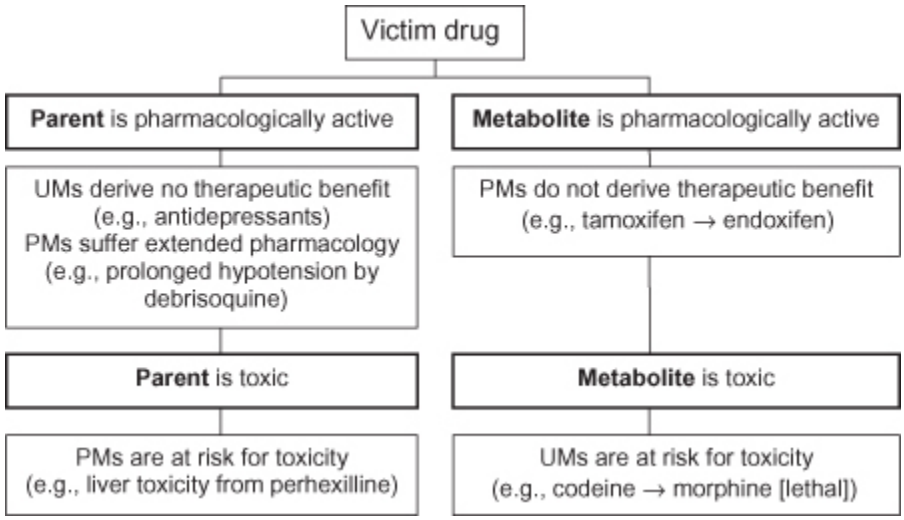


1.4 PARENT DRUG VERSUS METABOLITE

The metabolism of a drug will terminate its pharmacological action and lessen its toxicity when the therapeutic and adverse effects are due to the parent drug. Conversely, the metabolism of a drug will increase its therapeutic and adverse effects when one or more metabolites mediate the pharmacological and toxicological effects. This principle is illustrated in Fig. 1.3 for drugs that are metabolized by CYP2D6, a genetically polymorphic drug-metabolizing enzyme. When the parent drug is pharmacologically active (as in the case of the antihypertensive drug debrisoquine and various antidepressants), UMs are at risk of deriving no therapeutic benefit whereas PMs are at risk of exaggerated or prolonged pharmacological effects. The converse occurs when the pharmacological effects of a drug are mediated by

a metabolite; in this case, PMs are at risk from deriving little or no therapeutic benefit, which is why, for example, CYP2D6 PMs are at increased risk for breast cancer recurrence following tamoxifen adjuvant therapy because these individuals cannot convert tamoxifen to endoxifen, which is 30- to 100-fold more potent than tamoxifen in suppressing estrogen-dependent cell proliferation (Goetz et al., 2005).

Figure 1.3 The therapeutic and safety consequences of ultra-rapid metabolism (UM) and poor metabolism (PM) of a drug as a function of whether the pharmacological or adverse effects of the drug are mediated by the parent drug or its metabolite(s). The examples shown are drugs metabolized by the genetically polymorphic enzyme CYP2D6.



When an adverse effect is due to the parent drug (such as the hepatotoxic effects of the antihypertensive drug perhexilline), PMs are at increased risk. Conversely, when an adverse effect is due to a metabolite, UMs are at increased risk. For example, in the case of codeine, which is converted by CYPD6 to morphine (an effective analgesic that causes respiratory depression), CYP2D6 UMs are at increased risk of morphine toxicity. The death of an elderly man administered a recommended dose of codeine and the death of a baby

who was breast-fed by a woman on codeine have been attributed to the CYP2D6 UM genotype that causes rapid conversion of codeine to morphine (which causes respiratory depression) (Gasche et al., 2004; Koren et al., 2006).

1.5 DRUGS AS VICTIMS AND PERPETRATORS

From a drug interaction and pharmacogenetic perspective, drugs can be evaluated for their victim and perpetrator potential. *Victims* are those drugs whose clearance is predominantly determined by a single route of elimination, such as metabolism by a single cytochrome P450 (CYP) enzyme. Such drugs have a high victim potential because a diminution or loss of that elimination pathway, either due to a genetic deficiency in the relevant CYP enzyme or due to its inhibition by another, concomitantly administered drug, will result in a large decrease in clearance and a correspondingly large increase in exposure to the victim drug (e.g., plasma AUC). *Perpetrators* are those drugs (or other environmental factors) that inhibit or induce the enzyme that is otherwise responsible for clearing a victim drug. Genetic polymorphisms that result in the partial or complete loss of enzyme activity (i.e., the IM and PM genotypes) can also be viewed as perpetrators because they have the same effect as an enzyme inhibitor: they cause a decrease in the clearance of—and an increase in exposure to—victim drugs. Likewise, genetic polymorphisms that result in the overexpression of enzyme activity (i.e., the UM genotype) can be viewed as perpetrators because they have the same effect as an enzyme inducer: they cause an increase in the clearance of—and a decrease in exposure to—victim drugs. Several drugs whose elimination is largely determined by their metabolism by CYP2C9, CYP2C19, or

CYP2D6, three genetically polymorphic enzymes, are victim drugs because their clearance is diminished in PMs, that is, individuals who are genetically deficient in one of these enzymes. Drugs whose disposition is dependent on uptake or efflux by a transporter or on metabolism by a drug-metabolizing enzyme other than CYP can also be considered from the victim/perpetrator perspective. From a drug interaction perspective, *victim* drugs are also known as *objects*, whereas *perpetrators* are also known as *precipitants*.

Reaction phenotyping is the process of identifying which enzyme or enzymes are largely responsible for metabolizing a drug or drug candidate. When biotransformation is known or suspected to play a significant role in the clearance of a drug candidate (which applies to most drugs in BCS Classes 1 and 2, which are well-absorbed drugs with a $\log D_{7.4} \geq 1.0$), then an *in vitro* reaction phenotyping or enzyme mapping study is required prior to approval by the FDA and other regulatory agencies (Tucker et al., 2001; Bjornsson et al., 2003a,b; Williams et al., 2003; US FDA, 2006; Huang et al., 2008). Reaction phenotyping allows an assessment of the victim potential of a drug candidate. The FDA also requires drug candidates be evaluated for their potential to inhibit the major CYP enzymes involved in drug metabolism which can be evaluated *in vitro* with human liver microsomes or recombinant human CYP enzymes (US FDA, 2006; Huang et al., 2008; Grimm et al., 2009). This allows an assessment of the perpetrator potential of the drug candidate. Drugs can also cause pharmacokinetic drug interactions by inducing CYP and other drug-metabolizing enzymes and/or drug transporters, which can be evaluated *in vitro* with cultured human hepatocytes (US FDA, 2006; Huang et al., 2008; Chu et al., 2009).

Terfenadine, cisapride, astemizole, and cerivastatin are all victim drugs, so much so that they have all been withdrawn

from the market or, in the case of cisapride, made available only with severe restrictions. The first three are all victim drugs because they are extensively metabolized by CYP3A4 (US FDA, 2006; Ogilvie et al., 2008). Inhibition of CYP3A4 by various antimycotic drugs, such as ketoconazole, and antibiotic drugs, such as erythromycin, decrease the clearance of terfenadine, cisapride, and astemizole, and increase their plasma concentrations to levels that, in some individuals, cause ventricular arrhythmias (QT prolongation and torsade de pointes) which can result in fatal outcomes (Backman et al., 2002; Ogilvie et al., 2008). Cerivastatin is extensively metabolized by CYP2C8. Its hepatic uptake by OATP1B1 and CYP2C8-mediated metabolism are both inhibited by gemfibrozil (actually by gemfibrozil glucuronide), and the combination of cerivastatin (Baycol®) and gemfibrozil (Lopid®) was associated with a high incidence of fatal, cerivastatin-induced rhabdomyolysis, which prompted the worldwide withdrawal of cerivastatin (Ozdemir et al., 2000; Shitara et al., 2004, Ogilvie et al., 2006, 2008; http://www.emea.europa.eu/pdfs/%20human/referral/Cerivastatin/081102en.pdf).

Posicor® (mibefradil) is the only drug withdrawn from the U.S. market largely because of its perpetrator potential (Huang et al., 2008). This calcium channel blocker not only caused *extensive* inhibition of CYP3A4, but it also caused *prolonged* inhibition of the enzyme by virtue of being a metabolism-dependent inhibitor of CYP3A4. By inactivating CYP3A4 in an irreversible manner, such that restoration of normal CYP3A4 activity required the synthesis of new enzyme, mibefradil inhibited CYP3A4 long after treatment with the drug was discontinued.

Victim potential can be quantified on the basis of fractional metabolism according to the following equation:

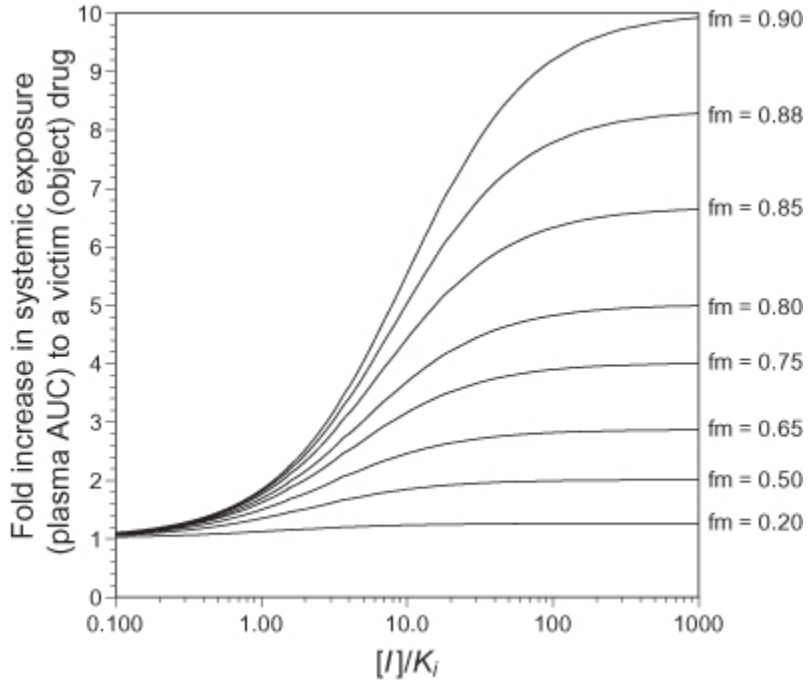
$$\text{Fold increase in exposure} = \frac{\text{AUC}_{\text{i}} \text{ or } \text{AUC}_{\text{PM}}}{\text{AUC}_{\text{ui}} \text{ or } \text{AUC}_{\text{EM}}} = \frac{1}{1 - f_m}$$

(Eq. 1.1)

where AUC_i (AUC inhibited) and AUC_{ui} (AUC uninhibited) are the plasma AUC values of the victim drug in the presence and absence of inhibitor, respectively, and where AUC_{PM} and AUC_{EM} are plasma AUC values in genetically determined PMs and EMs, respectively. The relationship between fractional metabolism (f_m) by a single enzyme and the fold increase in drug exposure that results from the loss of that enzyme is shown in Fig. 1.4, which shows that the relationship is not a linear one. (The same principle applies to a drug whose elimination is determined by a transporter, although in this case the term f_e is used instead of f_m). Loss of an enzyme that accounts for 50% of a drug's clearance (f_m = 0.5) results in a twofold increase in AUC, whereas it results in a 10-fold increase in AUC when the affected enzyme accounts for 90% of a drug's clearance (f_m = 0.9). In the case of oral drugs that undergo significant pre-systemic clearance (i.e., first-pass metabolism in the intestine and/or liver), the impact of enzyme inhibition (or the PM genotype) can be twofold: it can increase AUC of the victim drug by (1) decreasing pre-systemic clearance (which increases oral bioavailability) and (2) decreasing systemic clearance. In the case of drugs administered intravenously, enzyme inhibition increases AUC only by decreasing systemic clearance. Consequently, the magnitude of the increase in AUC for certain drugs depends on their route of administration, as illustrated by the interaction between ketoconazole (perpetrator) and midazolam (victim): ketoconazole, a CYP3A4 inhibitor, increases the AUC of midazolam 3- to 5-fold when midazolam is administered intravenously, but it causes a 10- to 16-fold increase when midazolam is administered orally (Tsunoda et al., 1999). The same is true when the perpetrator is an enzyme inducer. For

example, the CYP3A4 inducer rifampin decreases the AUC of midazolam by a factor of 9.7 when midazolam is administered orally, but it decreases the AUC by a factor of only 2.2 when midazolam is administered intravenously (Gorski et al., 2003). The difference caused by route of administration is more dramatic for the inductive effect of rifampin on the AUC of nifedipine (12-fold for oral vs. 1.4-fold for intravenous; Holtbecker et al., 1996) and even more dramatic for *S*-verapamil (30-fold for oral vs. 1.3-fold for intravenous; Fromm et al., 1996).

Figure 1.4 The effect of fractional metabolism of a drug by cytochrome P450, $f_{m(CYP)}$, on the theoretical increase in systemic exposure to a victim drug with increasing enzyme inhibition by a perpetrator drug (as reflected by increasing $[I]/K_i$ values).



When an enzyme accounts for only 20% of a drug's clearance (f_m = 0.2), complete loss of the enzyme activity causes only a 25% increase in AUC, which is normally considered to be bioequivalent (the so-called bioequivalence goalposts range from 80% to 125%,

meaning that AUC values within this range can be considered equivalent and, therefore, acceptable). Therefore, an “unacceptable” increase in AUC requires an f_m of greater than 0.2. Actually, the FDA urges the characterization of all elimination pathways that account for 25% or more of a drug’s clearance (i.e., $f_m \geq 0.25$).

Fractional metabolism by an enzyme determines the magnitude of the increase in drug exposure in individuals lacking the enzyme, but it does not determine its pharmacological or toxicological consequences. These are a function of therapeutic index, which is a measure of the difference between the levels of drug associated with the desired therapeutic effect and the levels of drug associated with adverse events. For drugs with a large therapeutic index, a high degree of clearance by a polymorphically expressed enzyme is not necessarily an obstacle to regulatory approval. For example, dextromethorphan is extensively metabolized by CYP2D6. Its fractional metabolism is estimated to be 0.93 to 0.98 such that the AUC of dextromethorphan in CYP2D6 PMs or in EMs administered quinidine is about 27–48 times greater than that in EMs (Gorski et al., 2004; Pope et al., 2004). Dextromethorphan has a large therapeutic index; hence, despite this large increase in exposure in CYP2D6 PMs, dextromethorphan is an ingredient in a large number of over-the-counter (OTC) medications. Strattera® (atomoxetine) is another example of a drug whose clearance is largely determined by CYP2D6 ($f_{m(CYP2D6)} \approx 0.9$). Its AUC in CYP2D6 PMs is about 10 times that of EMs, but Strattera has a sufficiently large therapeutic index that it was approved by the FDA in 2002.

Genetic polymorphisms give rise to four basic phenotypes based on the combination of allelic variants that encode a fully functional enzyme (the wild type or $*1$ allele

designated “+”), a partially active enzyme (designated “∗”), or an inactive enzyme (designated “−”). These four basic phenotypes are (1) EMs, individuals who have at least one functional allele (+/+, +/∗, or +/−), (2) PMs, individuals who have no functional alleles (−/−), (3) IMs, individuals who have two partially functional alleles or one partially functional and one nonfunctional allele (∗/∗ or ∗/−), and (4) UMs, individuals who, through gene duplication, have multiple copies of the functional gene ([+/+]n). This traditional classification scheme has been revised recently on the basis of an activity score, which assigns to each allelic variant a functional activity value from 1 (for the wild type or ∗1 allele) to 0 (for any completely nonfunctional allele), as reviewed by (Zineh et al., 2004). The basis of the activity score, as it applies to CYP2D6, is illustrated in Table 1.1.

TABLE 1.1 The Relationship between Genotype and Phenotype for a Polymorphically Expressed Enzyme with Active (wt), Partially active (*x), and Inactive (*y) Alleles