For most drugs, biotransformation is the major route of elimination, and oxidative metabolism by P450 enzymes is a common metabolic pathway (Rendic, 2002). Therefore, it is important to assess the relative contribution of metabolic pathways to the overall elimination processes and to identify the P450 isoforms responsible for oxidative reactions. In vitro P450 phenotyping has proven to be very successful in predicting the potential of drug interactions and the polymorphic impact on drug disposition (Lin and Lu, 1998; Dahl, 2002). As a result, pharmaceutical companies routinely include in vitro reaction P450 phenotyping in the drug candidate selection process.

Ever since the pioneering work of Smith and coworkers (Mahgoub et al., 1977; Sloan et al., 1978), the 4-hydroxylation of debrisoquine has been recognized as a marker reaction of human CYP2D6. Urinary metabolic ratio defined as debrisoquine to 4-hydroxydebrisoquine measured following an oral dose of debrisoquine is generally considered as an accurate assessment of an individual’s metabolic capacity of CYP2D6. Based on the metabolic ratios, individuals are phenotyped as poor metabolizer (PM), extensive metabolizer (EM), or ultra-rapid metabolizer (UM) phenotypes, each displaying characteristic pharmacokinetic patterns and clinical outcomes (Eichelbaum and Gross, 1990; Dahl et al., 1995; Meyer and Zanger, 1997). The debrisoquine pharmacokinetic profile of an individual with EM phenotype can be changed to a pattern similar to that of an individual with PM phenotype (Brosen et al., 1987) when debrisoquine is coadministered with quinidine, a marker inhibitor of CYP2D6. The interpretation of all these studies and findings is based on the belief that debrisoquine is a highly selective substrate and quinidine is a potent inhibitor of human CYP2D6.

Using a panel of 15 human recombinant cytochrome P450s (1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9*1, 2C9*2, 2C19, 2D6, 2E1, 3A4, 3A5, and 4A11) expressed in microsomes from human B-lymphoblastoid cells, Granvil et al. (2002) recently reported that debrisoquine 4-hydroxylation is catalyzed not only by CYP2D6 but also by CYP1A1. The apparent \( K_m \) and \( V_{max} \) values are 12.1 (\( \mu \)M) and 18.2 (pmol/min/pmol P450) for CYP2D6 and 23.1 (\( \mu \)M) and 15.2 (pmol/min/pmol P450) for CYP1A1. The intrinsic clearance \( (V_{max}/K_m) \) values between these two enzymes are not too far apart, 0.7 \( \mu \)l/min/pmol for CYP1A1 and 1.5 \( \mu \)l/min/pmol for CYP2D6. All other P450 enzymes have insignificant activity toward debrisoquine. Although it is surprising that quinidine inhibits not only CYP2D6-mediated debrisoquine 4-hydroxylation but also CYP1A1-catalyzed hydroxylation, there is a 77-fold difference in \( IC_{50} \) values (1.38 \( \mu \)M for CYP1A1, 0.018 \( \mu \)M for CYP2D6). In their study, various antibodies were also used to study their inhibitory effects on debrisoquine hydroxylation. Anti-CYP1A1 monoclonal antibody abolishes recombinant CYP1A1-mediated hydroxylation whereas anti-CYP2D6 monoclonal antibody completely inhibits recombinant CYP2D6-catalyzed 4-hydroxylation.
alyzed reaction. Although no check of cross-reactivity for these two antibody preparations was conducted in this investigation, the specificity of the respective antibody preparation is expected to be excellent. Thus, this clear demonstration that debrisoquine is not a specific CYP2D6 substrate, and quinidine is not a specific CYP2D6 inhibitor lead these investigators to question the relationship between CYP2D6 genotype and debrisoquine 4-hydroxylase activity. In addition, these findings also raised a key question about the accuracy of data obtained using chemical inhibitors in P450 reaction phenotyping (Guengerich and Shimada, 1991; Madan et al., 2001; Tucker et al., 2001) and underscored the value of using specific antibodies for P450 identification studies.

In view of the important findings by Granvil et al. (2002) and the implication of these findings for in vitro and in vivo P450 phenotyping, it is critical to reevaluate the current in vitro approaches used for the identification of P450 responsible for the metabolism of new therapeutic agents. This is a very important issue since P450 reaction phenotyping is a key factor for the evaluation of potential drug-drug interactions. In this commentary, we address many of these issues. No attempt was made to review extensively the literature and to cite all relevant references.

**Current Approaches Used in P450 Identification**

Approaches used currently for the in vitro P450 reaction phenotyping were mostly developed in the late 1980s. At that time, tools available for the P450 identification were less defined and often limited in availability, particularly antibodies against various P450 enzymes (generally polyclonal), recombinant P450 enzymes, and chemical inhibitors. A wise decision was made at the time to use several approaches simultaneously to make unequivocal conclusions regarding the P450 isoform responsible for the metabolism of a drug of interest (Kronbach et al., 1989; Guengerich and Shimada, 1991). Since then, hundreds of papers have been published in this area of research. Typically, most investigators have used multiple approaches, namely, antibody inhibition, chemical inhibition, metabolism of drugs by recombinant P450 enzymes, and correlation analysis. However, since quality of the tools has been greatly improved, it is our belief that not all the approaches are necessary in a P450 reaction phenotyping study, but clearly some approaches are better than others.

**Antibody Inhibition.** Potent, specific and inhibitory antibodies (either polyclonal or monoclonal) against various human P450 isoforms represent the most valuable approach for P450 identification. If the potency and specificity of a particular antibody can be verified with human liver microsomes and recombinant P450 preparations (including the closely related isoforms in the same subfamily, e.g., CYP2C8, 2C9, 2C18, and 2C19), then the antibody can be chosen as the only approach necessary for P450-phenotyping study. In this respect, potent monoclonal antibodies are particularly valuable (Gelboin et al., 1999; Mei et al., 1999). Thanks to the efforts of Gelboin, Gonzalez, Yang, Shou, and coworkers (Gelboin et al., 1999; Mei et al., 1999), specific monoclonal antibodies against most, if not all, of human P450 isoforms relevant to drug metabolism are now commercially available. Although most monoclonal antibodies are specific with respect to P450 isoforms, some are less specific. For example, monoclonal antibodies to CYP2C8, 2C9, and 2C19 are potent and specific (Krausz et al., 2001), while antibodies to CYP3A isoforms are less specific and exhibit cross-reactivity between CYP3A4 and 3A5 isoforms (Mei et al., 1999). In addition, many specific polyclonal antibodies against human P450 enzymes are also commercially available, although some of these antibodies were made against rat enzymes. When used to inhibit the ortholog human P450-catalyzed reactions, the potency of anti-rat P450 antibodies is very often quite low, and only partial inhibition of the reaction is achieved. These results strongly suggest that the usefulness of the anti-rat antibodies in P450 identification study is limited. When polyclonal antibodies are potent and specific (Guengerich and Shimada, 1991; Wang and Lu, 1997), they are as useful as the monoclonal antibodies.

To conduct a good antibody inhibition study, it is highly desirable to examine the effect of a particular antibody in a concentration-dependent manner on the metabolism of the drug of interest. This should include enough low and high antibody concentrations to establish the titration curve. From the inhibition curve, one can assess the potency of the antibody from the slope and the relative contribution of this particular P450 isoform from maximal inhibition (Guengerich, 1988; Wang and Lu, 1997; Yang et al., 1998; Granvil et al., 2002). If there is only one P450 involved in the metabolism, greater than 90% inhibition is expected with pooled and individual human liver microsomes. If more than one P450 enzymes are involved in the metabolism, similar experimental design with various antibodies should be used to examine a large number of human liver samples (ideally 10 to 20 microsomal preparations) so that the relative contributions for each P450 isoform can be clearly demonstrated. The relative contribution of each P450 isoform may vary significantly in microsomes from different donor livers. Regardless the variability in P450 relative contribution, the sum of total inhibition by various antibodies against individual isoform should approach 100% (Gelboin et al., 1999). If the extent of the inhibition increases only in small increments with increasing antibody concentrations and the overall inhibition is not complete, it would imply that either the antibody has poor potency or the antibody is cross-reacting to a closely related P450 isoform. In either case, the usefulness of the antibody employed in the inhibition study is very limited.

If potent, specific, and inhibitory antibodies (particularly the monoclonal) are so valuable, then why is the use of monoclonal antibodies still not widespread in P450 identification studies? We believe that the major reason for the limited use of monoclonal antibodies (and to a certain extent the polyclonal antibodies too) is due to the high cost and availability of the commercial products. Because of the demand of resources and expertise, not every pharmaceutical company can produce their own monoclonal antibodies. With the exception of a limited number of publications (Guengerich, 1988; Wang and Lu, 1997; Yang et al., 1998; Granvil et al., 2002), most of the antibody inhibition studies used only a single antibody concentration to demonstrate inhibition of the reaction of interest, perhaps due to limited supply of antibodies. If the inhibition at this selected antibody concentration is greater than 90%, one can conclude that the particular P450 isoform is the major enzyme responsible for the metabolism. However, if the extent of inhibition is low, then it would be difficult to determine whether partial inhibition is due to the involvement of other P450 isoform in the metabolism of the drug or simply because not high enough antibody concentrations are used to achieve maximal inhibition.

**Chemical Inhibitor.** Based on their findings, Granvil et al. (2002) questioned the usefulness of chemical inhibitors. In their paper, they stated “this practice (i.e., use of chemical inhibitors) is unsound and should be abandoned in favor of the use of a panel of recombinant P450 and the use of inhibitory monoclonal antibodies that, unlike chemical inhibitors, show much less cross-reactivity with other P450 isoforms.” If so, why should we use them at all in P450 in vitro reaction phenotyping? While acknowledging the value of inhibitory antibodies in P450 phenotyping studies, it is our opinion that chemical inhibitors still have practical value and should not be abandoned. We should consider the following reasons: 1) the pricing and supply of commercially available antibodies (both monoclonal and polyclonal)
will probably not be greatly improved in the near future; 2) one of the valuable attributes of chemical inhibitors is their availability through commercial sources or custom synthesis; and 3) finally, safe and highly selective P450 inhibitors can be used in clinical settings to assess the role of a specific P450 isoform in drug therapy. Antibodies are not suitable for the use in clinical or other in vivo studies.

Since P450 enzymes are very versatile enzymes, they can bind and metabolize a wide range of substrates and inhibitors with diverse chemical structures. Thus, it is unlikely that “absolutely specific inhibitors” can be found. However, well characterized “highly selective inhibitors” with respect to individual human P450 isoforms can be successfully used for P450 identification in carefully designed experiments. To conduct a chemical inhibition study, it is also highly desirable to evaluate the effect of an inhibitor with a wide range of concentrations on human liver microsomal metabolism of the drug of interest. Similar to the situation of antibody inhibition study, the potency of chemical inhibitor, and the relative contribution of P450 enzyme can be assessed from a complete inhibition curve (Newton et al., 1995). In addition, the inhibition curve also allows estimating the IC50 values of inhibitor. Based on the IC50 values for different P450 enzymes, one can then assess the selectivity of an inhibitor. As shown in the study of Granvil et al. (2002), although quinidine inhibits both CYP1A1 and CYP2D6-dependent debrisoquine 4-hydroxylation, the two inhibition curves shown in Fig. 6 of their paper can easily be distinguished because of the 77-fold differences in IC50 values. Therefore, even though not specific, highly selective inhibitors for various P450 enzymes can still provide reliable and meaningful results in P450 identification study when the experiment is properly conducted.

**Recombinant Human P450 Enzymes.** Microsomal preparations containing individual recombinant human P450 isoform provide a valuable means to evaluate the intrinsic ability of each individual isoform to metabolize the compound of interest (Crespi and Miller, 1999). When the metabolism of a drug is catalyzed only by a single recombinant enzyme, interpretation of the result is straightforward. If the drug is metabolized by more than two recombinant enzymes, measurements of enzymatic activity alone do not provide sufficient information to estimate the relative contributions of each P450 isoform to total metabolism of the drug. Further studies with either antibodies or highly selective chemical inhibitors should be conducted in human hepatic microsomes to address the relative contribution. It should be noted that sometimes a recombinant P450, which is shown to be active in metabolism in the absence of other P450 enzymes, may play little or no role in microsomal metabolism in the presence of other P450 isoforms due to the competitive nature of the P450 enzymes. The other approach is to determine the Km and Vm values of the drug with each active recombinant P450 enzyme so that the intrinsic clearance (Vm/Km) values for each reaction can be calculated. Based on the intrinsic clearance and the relative abundance of each P450 isoform in human liver microsomes, the relative importance of different P450 enzymes in the metabolism of the compound of interest in human liver microsomes can be evaluated (Crespi and Miller, 1999; Rodrigues, 1999; Venkatarkishnam et al., 2001).

**Correlation Analysis.** This approach relies on statistical analysis to establish a correlation between the metabolic rates of the drug of interest and marker substrates for each individual P450 enzyme obtained from incubations of individual human liver microsomal preparations. Ideally large numbers of microsomal preparations obtained from different donors should be used to increase the statistical power, but most of the studies reported in the literature use 10 or less preparations. Although results from correlation studies are usually consistent with P450 identification from inhibition and recombinant enzymes studies, inconsistency has been reported for some P450 enzymes (Karanam et al., 1994; Weaver et al., 1995; Heyn et al., 1996; Rodrigues et al., 1997; Stevens et al., 1997). For example, Weaver et al. (1995) reported that the hydroxylation of compound 58C80 is catalyzed significantly by CYP2C9, yet there is no correlation between the 58C80 hydroxylation and CYP2C9 marker substrate activity (r = 0.023). Heyn et al. (1996) observed high correlations between S-mephenytoin N-demethylation and CYP2B6 (r = 0.91), CYP2A6 (r = 0.88), and CYP3A4 (r = 0.74), but other studies established that CYP2B6 is the major enzyme responsible for S-mephenytoin N-demethylation whereas CYP2A6 and CYP3A4 play little or no role in this reaction. Broad overlap of substrate specificity among P450 enzymes and their relative abundance may significantly contribute to the false positive and false negative results. Additionally, inappropriate experimental design also can contribute to the problem. For instance, the correlation analyses were carried out with a small number of microsomal preparations, or the initial rates for metabolic reactions were not properly measured. In some cases, the metabolic rates of marker substrates used for correlation study were provided by supplier for individual microsomes, while the metabolic rates of the drug of interest with these microsomal preparations were determined in the investigators’ laboratory under different assay conditions. For these reasons, correlation analysis approach is a less reliable method for the identification of P450 in drug metabolism (Tucker et al., 2001).

Thus, results from correlation studies in P450 in vitro reaction phenotyping can only be used to confirm the results from inhibition and recombinant enzyme studies.

**Searching for Better Marker Substrates and Chemical Inhibitors**

An ideal marker substrate should be only metabolized by a single human P450, and an ideal chemical inhibitor should inhibit only a P450 isoform. However, in view of the versatile and nonspecific nature of P450 enzymes, it is highly unlikely that one can find truly specific marker substrates and inhibitors for individual P450 enzymes. Indeed, for virtually all of the marker substrates and chemical inhibitors used today, it is not uncommon to see cross-reactivity with other P450 isoforms in varying degrees.

With the available tools at the present time, it is still possible that highly selective marker substrates and chemical inhibitors can be found from the large number of existing chemicals and drugs. As demonstrated in the studies of Granvil et al. (2002) and Stresser et al. (2002), the use of a panel of 15 or more recombinant human P450s provides a valuable tool to screen for highly selective marker substrates and chemical inhibitors. If a compound is metabolized exclusively by a single recombinant P450 isoform, the predominant role of this P450 isoform in microsomal metabolism should be confirmed by careful inhibition titration with antibody against this isoform with a large number of human liver microsomes. Ideally, the inhibition study should include at least 10 microsomal preparations with high and low content of the P450 isoform of interest. Greater than 90% maximal inhibition of the metabolism in all microsomal preparations would indicate that this compound is an excellent marker substrate of this individual cytochrome P450. When two recombinant P450 isoforms are involved in the metabolism of the compound, inhibition study should be carried out in human liver microsomes and the relative contribution of the two active enzymes in the metabolism should be established. If one of the two P450 enzymes contributes greater than 80% of the metabolism in all liver microsomal preparations, this compound can still be considered as a good marker substrate for this particular P450 enzyme. When more than two recombinant P450 isoforms are shown to catalyze the metabolism of a compound at significant rates, it becomes less desirable to serve as marker substrate.
because variable contributions of individual P450 enzyme to microsomal metabolism may become an issue with microsomes obtained from different subjects.

The search for better chemical inhibitors follows a similar strategy, i.e., initial screening for compounds that show inhibition selectivity with a panel of recombinant P450 enzymes, followed by a careful inhibition titration of this compound on microsomal marker substrate metabolism with a panel of microsomal preparations. If the chemical shows good inhibition selectivity, the results must be confirmed by inhibition studies with antibodies against the P450 isoforms of interest. Once confirmed, the chemical inhibitors against individual P450 isoform can be used with greater confidence. In all of these studies, it is critical that the antibody used (either monoclonal or polyclonal) be specific and potent and that wide range of antibody concentrations be used to construct good inhibition curves so that contributions of specific P450 in microsomal metabolism can be accurately established.

Recently, azamulin, an azole derivative of the pleuromutilin class of anti-infectives, has been identified as a highly selective CYP3A4/5 inhibitor. The IC50 value for CYP3A4 was at least two orders of magnitude greater than all other non-CYP3A enzymes (D. M. Stresser and C. L. Crespi, personal communication).

Is Debrisoquine 4-Hydroxylation a Valid in Vitro and in Vivo Probe for CYP2D6?

The results reported by Granvil et al. (2002) cast doubt on the validity of using debrisoquine for CYP2D6 phenotyping. Before this issue can be definitively resolved, two questions must be addressed. The first question is whether debrisoquine is still a good marker substrate for in vitro CYP2D6 phenotyping. To address the question, it is important to assess the relative contribution of CYP1A1 to total 4-hydroxylation of debrisoquine in human liver microsomes. It is well known that CYP1A1 is predominantly expressed in extrahepatic tissues. Although the expression of CYP1A1 in human liver has been reported, the hepatic expression of CYP1A1 is much lower than CYP2D6 (Murray et al., 1993; Schweikl et al., 1993; Pastrakulic et al., 1997). The relative contribution of CYP1A1 to the overall 4-hydroxylation of debrisoquine in human liver microsomes can be established by the extent of maximal inhibition of debrisoquine metabolism by an antibody against CYP1A1. Given the fact of low hepatic CYP1A1 expression and its lower intrinsic clearance, the contribution of CYP1A1 is likely to be very limited, if any. It is highly likely that debrisoquine is predominantly metabolized by CYP2D6. Since the contribution of CYP1A1 in hepatic microsomal debrisoquine 4-hydroxylation is limited, quinidine should still be a good selective inhibitor for CYP2D6 isoform.

If debrisoquine can be established as a good in vitro probe, then can the same be said of debrisoquine as a good in vivo probe? Similarly the answer relies on the relative contribution of CYP1A1 (both hepatic and extrahepatic) to the overall in vivo debrisoquine 4-hydroxylation in humans. Most of the in vivo phenotyping studies use the metabolic ratio of debrisoquine to 4-hydroxydebrisoquine in urine collected over a time interval to identify PM and EM of CYP2D6 enzyme. Owing to incomplete urinary recovery, quantitative assessment of CYP1A1 contribution from this type of data are difficult. A more desirable way is to measure the plasma AUCs of debrisoquine and its metabolite 4-hydroxydebrisoquine, and total amounts of the parent and metabolite in urine in both genotypes EM and PM. Since the CYP2D6 PM individuals have little or no CYP2D6 enzyme activity, any product formation of 4-hydroxydebrisoquine in the CYP2D6 deficient individuals can be assumed due to the contribution of CYP1A1.

The study of Dalen et al. (1999) represents one of the very few examples that the pharmacokinetics of parent drug and its metabolite 4-hydroxydebrisoquine in plasma were examined in individuals with different CYP2D6 genotypes following oral dosing of debrisoquine. For our discussion, we focus on white individuals with homozygous PM genotypes (five subjects, all CYP2D6*4/*4; metabolic ratio 23 to 132) and homozygous EM genotype (five subjects, all CYP2D6*1/*1; metabolic ratio from 0.20 to 1.08). Plasma 4-hydroxydebrisoquine AUC for EM homozygotes is 702 ± 185 (nmol/h/liter), whereas in PM individuals, plasma concentration of 4-hydroxydebrisoquine was very low, and the AUC of this metabolite was estimated to be <37.5 (nmol/h/liter). Assuming that the formation of 4-hydroxydebrisoquine in CYP2D6 PM homozygotes is completely metabolized by CYP1A1 and that the elimination clearance of debrisoquine in PM individuals is similar to that in EM subjects, the relative contribution of CYP1A1 is estimated to be less than 5%. Consistent with the estimation from the data of plasma AUCs, the relative contribution of CYP1A1 to the overall debrisoquine metabolism in humans is estimated to be 2% based on the urinary recovery data. The recovery of 4-hydroxydebrisoquine in the urine over a 96-h period following drug administration was 0.6 ± 0.3% of total dose for PM subjects and 26.8 ± 3.5% for EM heterozygotes. Similar results have also been reported by other investigators. Smith and colleagues found that urinary recovery of 4-hydroxydebrisoquine in PM individuals was only 5 to 8% of that in EM subjects (Idle and Smith, 1979; Sloan et al., 1983).

From the results of these studies, one can conclude that although hepatic, and most likely extrahepatic, CYP1A1 can contribute to debrisoquine 4-hydroxylation in vivo, its role is very limited. Since CYP1A1 is inducible by cigarette smoking and certain foods, its contribution to debrisoquine 4-hydroxylation in vivo may be increased under these circumstances. The extent of this increase cannot be assessed with certainty until such induction studies are conducted in humans in the future. Nevertheless, it is reasonable to conclude from existing data that CYP2D6 is the predominant enzyme responsible for the 4-hydroxylation of debrisoquine in vivo and that CYP2D6 phenotyping results reported in the literature based on debrisoquine 4-hydroxylase activity are meaningful and reliable. Since debrisoquine is not approved for clinical use in the United States, it is important to search other highly selective CYP2D6 marker substrates for clinical use. Hopefully, future phenotyping studies for CYP2D6 or other P450 enzymes will include determination of product formation in both plasma and urine so that the specificity of marker substrates can be evaluated in PM and EM subjects.

Variable Cytochrome P450 Contribution and the Hidden Factor

When two or more P450 isoforms are involved in the microsomal metabolism of a given compound, results of P450 phenotyping can sometimes be confusing, mostly due to the use of different as well as limited numbers of human liver samples. For example, data on the role of CYP2D6 in the 4-hydroxylation of tamoxifen by human liver microsomes was equivocal until a panel of 10 human livers with a wide range of 4-hydroxylase activity was carefully examined by Crewe et al. (1997). These investigators found that the relative contributions of CYP2C9, CYP2D6, and CYP3A4 to 4-hydroxylation of tamoxifen varied considerably between different microsomes prepared from the 10 livers. The 4-hydroxylation of tamoxifen was catalyzed by CYP2C9 and CYP3A4 in 4 of the 10 liver samples, by CYP2D6 and CYP3A4 in another 2 livers, and by all three P450 enzymes in the remainder of liver samples. Yamazaki et al. (1997) also reported different contributions of CYP2C9 and CYP3A4 to the oxidation of omeprazole in human liver microsomes, depending on the contents of these two P450 enzymes in individual samples. Using
inhibitory monoclonal antibodies, Gelboin et al. (1999) reported very large variability in the contribution of individual P450 enzyme to the metabolism of various drugs. For example, CYP2D6 contributes to 10 to 70% of bufuralol 1’-hydroxylase activity in different liver samples (Gelboin et al., 1997) while CYP2B6 contributes to 8 to 42% of phenanthrene metabolism in all liver microsomal preparations examined (Yang et al., 1998). Thus, when conducting P450 in vitro reaction phenotyping, it is desirable to use pooled human liver microsomes to obtain a composite picture of the P450 enzymes responsible for the metabolism of compound of interest. In addition, it is important to use individual microsomal preparations to evaluate the variable contributions of individual enzymes to the metabolic pathway being studied.

In many of the studies reported in the literature, investigators often used “well characterized” microsomal preparations from commercial sources. Suppliers generally provide data for catalytic activity for each individual P450 enzyme of a given preparation based on the metabolic rates of marker substrates. While this information is useful to evaluate the catalytic activity (high, medium, and low) of individual P450 enzyme in a particular liver sample, one should not be surprised to find that different P450 variants (the hidden factor) could exist in some of the human livers. While a single amino acid change in a P450 enzyme can result in a substantial decrease in the enzymatic activity toward one substrate, it may retain full activity for another (Matsunaga et al., 1990; Crespi et al., 1995; Sullivan-Klose et al., 1996). Therefore, the variant may have normal enzymatic activity for the marker substrate but not for the drug of interest. The presence of these hidden variants in human liver microsomes is hard to detect simply by activity measurement and could be the cause of some conflicting results regarding P450 in vitro reaction phenotyping. For example, the biotransformation of losartan, an angiotensin II receptor antagonist for the treatment of hypertension to its active carboxylate metabolite was reported to involve both CYP2C9 and CYP3A4 by Stearns et al. (1995) but only CYP3A4 by Yun et al. (1995). The reason for the different P450 assignment in losartan metabolism is unclear. However, different P450 involvement from these two studies cannot be attributed to differences in experimental conditions or assay methods, but most likely due to the presence of a CYP2C9 variant with altered properties in the particular microsome used by Yun and coworkers (R. A. Stearns, R. R. Miller, and R. W. Wang, personal communication). Recent in vitro study by Yasar et al. (2001) and in vivo study by McCrea et al. (1999) indicate that CYP2C9 is the major human P450 isozyme responsible for losartan oxidation.

**Mystery Unsolved**

While in vitro studies usually provide a valuable insight into a drug’s metabolic profiles in humans, sometimes there are discrepancies between in vitro results and in vivo findings. Unfortunately we are unaware of any published papers regarding this observation. Therefore, we only present a general scenario here. In the past we have experienced unusual observations that some compounds were so metabolically stable in the human liver microsomal incubation system that no metabolites could be detected. However, oxidative metabolites via typically P450-catalyzed reactions were found in the urine of animals or even in humans. To explore the possibility that extrahepatic tissues or enzymes other than P450 system (such as peroxidases, flavin-containing monoxygenase) might be responsible for the metabolism observed in vivo, these compounds were incubated with human liver mitochondria, cytosol, S9, liver slices, hepatocytes, kidney, and gut and lung homogenates. However, no metabolism could be demonstrated in any one of these enzyme systems.

Since the in vitro system failed to demonstrate significant metabolism, negative drug-drug interaction results might be expected. However, in reality drug interactions have often been observed in clinical studies for these metabolically stable compounds. This situation can create considerable difficulty in the interpretation of in vivo drug-drug interaction studies because in vitro data do not provide any meaningful guidance. This discrepancy between in vitro and in vivo metabolism findings remains a challenge for future study.

**Other Drug-Metabolizing Enzymes**

Even though P450 enzymes play a prominent role in drug metabolism, other drug-metabolizing enzymes such as flavin-containing monoxygenase, monoamine oxidase, epoxide hydrolase, UDP-glucuronosyltransferase, glutathione S-transferase, sulfate transferase, methyltransferase, and N-acetyltransferase can also be important when the compound is primarily metabolized by a non-P450 enzyme. Since many of these enzymes exist in multiple forms, it may become necessary to identify the specific enzyme responsible for the metabolism of a compound, particularly when a coadministered drug is also biotransformed by similar metabolic pathway so that the potential for drug-drug interaction can be evaluated in vitro.

Unlike the P450 system, some of the analytical tools necessary for the in vitro reaction experiments are still not available for many of the non-P450 enzymes. Specific or highly selective probe substrates and chemical inhibitors for individual isozyme are still not available for most of these non-P450 enzymes. Furthermore, antibodies against many of these enzymes are often not inhibitory and selective. Therefore, inhibition study either by antibodies or chemical inhibitors could not be carried out for isoform identification or for the assessment of contribution of specific enzyme to the metabolism of the compound. For an enzyme such as UDP-glucuronosyltransferase, a panel of recombinant enzymes remains to be the only tool for reaction phenotyping (Burchell et al., 1995; Remmel, 2001). Further development of better analytical tools for in vitro reaction phenotyping involving non-P450 enzymes is needed.

**References**


