

Perspective

THE CONDUCT OF IN VITRO AND IN VIVO DRUG-DRUG INTERACTION STUDIES: A PHARMACEUTICAL RESEARCH AND MANUFACTURERS OF AMERICA (PhRMA) PERSPECTIVE

THORIR D. BJORNSSON, JOHN T. CALLAGHAN, HEIDI J. EINOLF, VOLKER FISCHER, LAWRENCE GAN, SCOTT GRIMM, JOHN KAO, S. PETER KING, GERALD MIWA, LAN NI, GONDI KUMAR, JAMES McLEOD, R. SCOTT OBACH, STANLEY ROBERTS, AMY ROE, ANITA SHAH, FRED SNIKERIS, JOHN T. SULLIVAN, DONALD TWEEDIE, JOSE M. VEGA, JOHN WALSH, AND STEVEN A. WRIGHTON

Abbott Laboratories, Abbott Park, Illinois (S.R.), Amgen Inc., Thousand Oaks, California (G.K., J.T.S.), AstraZeneca Pharmaceuticals LP, Wilmington, Delaware (S.G.), Aventis, Bridgewater, New Jersey (S.P.K.), Bayer Corporation, West Haven, Connecticut (A.S.), Boehringer Ingelheim Corporation, Ridgefield, Connecticut, (D.T.), Eli Lilly and Company, Indianapolis, Indiana (J.T.C., L. N., S.A.W.), GlaxoSmithKline, Research Triangle Park, North Carolina (J.W.), Merck Research Laboratories, West Point, Pennsylvania (J.M.V.), Millennium Pharmaceuticals, Inc., Cambridge, Massachusetts (L.G., G.M.), Novartis Pharmaceuticals Corporation, East Hanover, New Jersey (H.J.E., V.F., J.M.), Pfizer Inc., Groton, Connecticut (R.S.O.), Procter & Gamble, Cincinnati, Ohio (A.R.), Sanofi-Syntelabo Inc., Malvern, Pennsylvania (F.S.), Wyeth, Collegeville, Pennsylvania (T.D.B., J.K.)

(Received January 14, 2003; accepted March 19, 2003)

This article is available online at <http://dmd.aspetjournals.org>

ABSTRACT:

Current regulatory guidances do not address specific study designs for in vitro and in vivo drug-drug interaction studies. There is a common desire by regulatory authorities and by industry sponsors to harmonize approaches, to allow for a better assessment of the significance of findings across different studies and drugs. There is also a growing consensus for the standardization of cytochrome P450 (P450) probe substrates, inhibitors and inducers and for the development of classification systems to improve the communication of risk to health care providers and to patients. While existing guidances cover mainly P450-mediated drug inter-

actions, the importance of other mechanisms, such as transporters, has been recognized more recently, and should also be addressed. This article was prepared by the Pharmaceutical Research and Manufacturers of America (PhRMA) Drug Metabolism and Clinical Pharmacology Technical Working Groups and represents the current industry position. The intent is to define a minimal best practice for in vitro and in vivo pharmacokinetic drug-drug interaction studies targeted to development (not discovery support) and to define a data package that can be expected by regulatory agencies in compound registration dossiers.

Drug-drug interactions can lead to severe side effects and have resulted in early termination of development, refusal of approval, severe prescribing restrictions, and withdrawal of drugs from the market. Regulators, including the U.S. Food and Drug Administration (FDA¹) have therefore issued guidances for in vitro and in vivo drug interaction studies to be conducted during development. These guid-

ances, however, do not address the specific designs of the studies, and there is a desire by regulatory authorities to harmonize approaches and study designs to allow for a better assessment and comparison of different drugs. In addition, the existing guidances cover mainly cytochrome P450 (P450)-mediated drug interactions and the importance of other mechanisms, such as transporters, has been recognized only recently. To address these issues, workshops have been held in Nuremberg (1997), Arlington (1999), and Basel (2000) under the auspices of the European Federation of Pharmaceutical Sciences (EU-FEPS), the FDA, and the American Association of Pharmaceutical Scientists (AAPS) with invited contributions from academia, industry, and regulatory authorities. Furthermore, during a roundtable meeting in May 2001, between the Pharmaceutical Research and Manufacturers of America (PhRMA) Drug Metabolism/Clinical Pharmacology Technical Working Groups and the FDA Center for Drug Evaluation and Research (CDER), PhRMA was invited by the FDA to formulate a White Paper representing their opinion on the conduct of drug-drug interaction studies. Consequently, a PhRMA subcommittee was formed composed of members of the Drug Metabolism, Clinical Pharmacology, and Safety Assessment Technical Working Groups to prepare this consensus opinion.

¹ Abbreviations used are: FDA, U.S. Food and Drug Administration; AAPS, American Association of Pharmaceutical Scientists; ADME, absorption, distribution, metabolism, and excretion; AUC, area under the curve; AZT, azidodeoxythymidine; CDER, Center for Drug Evaluation and Research; CNS, central nervous system; CsA, cyclosporin A; P450, cytochrome P450; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; EUFEPS, European Federation of Pharmaceutical Sciences; EM, extensive metabolizer; FMO, flavin-containing monooxygenase; GLP, good laboratory practice; GST, glutathione S-transferase; HPLC, high performance liquid chromatography; hPXR, human pregnane X receptor; MAO, monoamine oxidases; MD, multiple dose; Mo-CO, molybdenum-containing oxidases; NAT2, N-acetyltransferase; NME, new molecular entity; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; PhRMA, Pharmaceutical Research and Manufacturers of America; Pgp, P-glycoprotein; PK, pharmacokinetics; PM, poor metabolizer; ST, sulfotransferase; T4, thyroxine; TAO, troleandomycin; TEER, transepithelial electrical resistance; UDPGA, uridine diphosphate glucuronic acid; UGT, UDP-glucuronosyltransferase; ZDV, zidovudine.

Address correspondence to: Volker Fischer, Novartis Pharmaceuticals Corporation, One Health Plaza, East Hanover, NJ 07836-1080. E-mail: volker.fischer@pharma.novartis.com

package that can be expected by regulatory agencies in compound registration dossiers. The goal was also to achieve consistency in quality and predictability for drug-drug interaction studies but not to limit innovation and experimental approaches. The outcomes of the previous workshops (Tucker et al., 2001) served as a basis for the current article. This document does not attempt to define the stage in the drug development process when certain experiments should be performed nor address pharmacodynamic interactions. In addition, it was beyond the scope of this report to address the emerging issue of interactions between drugs and foods or herbal products.

In Vitro Drug-Drug Interaction Studies

In vitro drug-drug interaction studies must be performed with high quality and consistency, particularly when the studies ultimately influence the design of clinical trials. The experimental procedures and documentation of data for in vitro work should be rigorous, reproducible, with specific analytical methods and documentation of assay procedures and results.

Data obtained in support of drug-drug interaction studies should be collected and documented with the same level of quality applied to other non-GLP (good laboratory practice) preclinical metabolism and pharmacokinetic data (i.e., signed and witnessed legal notebooks, good electronic data audit trails). Importantly, sources of human-derived reagents and their characterization should be well documented so that preparation procedures can be tracked and verified. Standard within-laboratory procedures and practices for biological reagent generation should be developed and documented. Chemical reagents should possess test notes for percentage of purity, including authentic standards of metabolites used in construction of calibration curves whenever possible. For some metabolites, chemical synthesis of authentic standards in large enough quantities for comprehensive analytical testing is not possible. In such cases, it must be understood that conservation of these limited valuable materials is necessary and that, therefore, all analytical criteria typically required for bioanalytical standard materials will not be able to be fulfilled. Such limitations should be documented.

Although scientific soundness is important for all experiments, it is most critical for negative in vitro findings. Positive in vitro findings are likely to be followed up with a clinical investigation, in which the clinical results will supersede the in vitro data with regard to clinical relevance of the involvement of metabolizing enzymes or transporters in the pharmacokinetics of the new molecular entity (NME) or that of coadministered compounds. However, negative in vitro findings may comprise the only information in support of a claim regarding the lack of involvement of a particular enzyme in the metabolism of the NME or inhibition of the enzyme(s). Therefore, it is essential that data in support of these negative findings be expertly obtained and well documented. In vitro inhibition data should be described in detailed study reports that underwrite summary documents submitted to regulatory authorities and should be suitable for inclusion in new drug application submissions.

Analytical Considerations for in Vitro Studies

In vitro drug-drug interaction studies can be performed using both radiolabeled and nonradiolabeled substrates/test compounds. If non-radiolabeled compounds are used in the experiments, quantitation of metabolite or substrate should be done using standard bioanalytical methods, e.g., liquid chromatography/mass spectrometry. Standard assay procedures should include a defined standard curve range, a blank standard to ensure no assay interference by endogenous components of the assay matrix (blanks from multiple individuals should

not be necessary) and quality control standards to verify the accuracy and precision of the assay. Special assurance should be provided that the substrate or inhibitor (or its metabolites) do not interfere in the analysis of the substrate metabolite. Long-term storage stability, a hallmark of method validation for analysis of blood or urine samples, is not as necessary for analytical methods designed to measure in vitro samples, since it is not common to store such samples for extended periods. However, stability of the analyte through the length of time and under conditions that in vitro samples are stored and maintained needs to be ascertained.

If authentic standards of metabolites are not available, but a radio-labeled investigational compound is, a quantitative HPLC-radiometric approach can be used in analysis of in vitro reactions. In this approach, recovery of the radiolabel from the incubation should be assessed. Standard curves for metabolite quantitation are not used, but rather, quantitation is done based on percentages that each metabolite peak comprises the total radioactivity in the chromatogram. This is the same way by which metabolites are quantitatively assessed in radio-labeled excretion studies. The radiochemicals used must be of high purity and should not interfere with quantification of the metabolites. The lower limit of quantitation will be defined by a lower limit signal-to-noise ratio rather than a lower limit defined by a standard curve. Upper limits of quantitation will not be reached if the scientist uses appropriate "as low as reasonably achievable" (ALARA) radiochemical practices.

Cytochrome P450 (P450) Inhibition

In Vitro Test Systems. A majority of drugs are cleared via P450-mediated metabolism, therefore the inhibition of P450 enzymes can lead to serious clinical drug interactions. The potential for such interactions is highest when concomitant drugs are metabolized by the same P450 enzyme. In addition, many compounds can also be strong inhibitors of P450 enzymes, which are not directly involved in the clearance of the drug, and could greatly affect the metabolism of coadministered drugs. The information from enzyme inhibition studies is extremely valuable because it could allow extrapolation of the data to other compounds and of drug interactions in organs other than liver (e.g., the intestine) depending upon the degree of the metabolism by the specific organ. The availability of human liver tissue, cDNA-expressed P450 enzymes, and specific probe substrates have been valuable tools in the assessment of a drug's potential to inhibit different P450 enzymes in vitro. Inhibition of P450 activity by drugs is most frequently examined in human liver microsomal preparations. For inhibition of enzyme-selective activities, it is sufficient to use tissue from individual donors as long as the activity for the reaction is sufficiently present. Alternately, recombinant P450s can be used when a specific enzyme is to be investigated. Microsomes and recombinant P450 enzymes are the preferred test system as they are more readily available than human hepatocytes, and P450 kinetic measurements are not confounded with other metabolic processes or cellular uptake. A disadvantage of microsomes or recombinant enzymes is that they do not represent the true physiological environment (e.g., not all Phase II enzymes are present) if that is of interest to study. Phase II enzymes are discussed later in this article.

In Vitro P450 Probe Substrates and Inhibitors. The selection of P450 enzyme-specific substrates and inhibitors, as listed in the consensus paper following the conference held in Basel, November 2000 (Tucker et al., 2001), seems to be a good compromise between selectivity, sensitivity, and availability of the probe compounds (Table 1).

For the purpose of high throughput drug-drug interaction screening, some P450 substrates have been developed specifically to form a

TABLE 1

Examples of recommended in vitro probe substrates and inhibitors for P450s (adapted from Tucker et al., 2001)

| P450 | Substrates | | Inhibitors ^a | |
|---------|--|---|---|---|
| | Preferred | Acceptable | Preferred | Acceptable |
| CYP1A2 | Ethoxyresorufin, phenacetin | Caffeine (low turnover), theophylline (low turnover), acetanilide (mostly applied in hepatocytes), methoxyresorufin | Furafylline | α -Naphthoflavone (but can also activate and inhibit CYP3A4) |
| CYP2A6 | Coumarin | | 8-Methoxypsoralen | Coumarin (but high turnover), Sertraline (but also inhibits CYP2D6) |
| CYP2B6 | S-Mephenytoin (N-desmethyl metabolite) | Bupropion (availability of metabolite standards?) | | |
| CYP2C8 | Paclitaxel (availability of standards?) | | Glitazones (availability of standards?) | |
| CYP2C9 | S-Warfarin, diclofenac | Tolbutamide (low turnover) | Sulphaphenazole | |
| CYP2C19 | S-Mephenytoin (4-hydroxy metabolite), omeprazole | | | Ticlopidine (but also inhibits CYP2D6), nootkatone (but also inhibits CYP2A6) |
| CYP2D6 | Bufuralol dextromethorphan | Metoprolol, debrisoquine, codeine (all with no problems, but less commonly used) | Quinidine | |
| CYP2E1 | Chlorzoxazone | 4-Nitrophenol, lauric acid | Clomethiazole | 4-Methyl pyrazole |
| CYP3A | Midazolam, testosterone (strongly recommended that at least two structurally unrelated substrates be used) | Nifedipine, felodipine, cyclosporin A, terfenadine, erythromycin, simvastatin | Ketoconazole (but recent evidence indicates that it is also a potent inhibitor of CYP2C8), troleandomycin | Cyclosporin A |

^a Specific inhibitory antibodies may also be used.

fluorescent product to easily monitor the inhibition of metabolism. These compounds are mostly not P450 enzyme-specific and can only be used with individually expressed enzymes. These substrates cannot be tested in humans in vivo, and most importantly, metabolites of the "inhibitor", if fluorescent, can interfere with these assays and lead to false negative results. The above-recommended probes (Table 1) can readily be measured by more specific assay procedures such as liquid chromatography/mass spectrometry or HPLC with radiometry. In addition, there appears to be a poor correlation of inhibitory potential using different fluoroprobes (Stresser et al., 2000). Therefore, fluorescent probes for in vitro studies are not recommended for regulatory submission.

P450 Probe Substrate Inhibition Assays. *General study outline and validation requirements.* To determine whether an NME inhibits a particular P450 enzyme activity, changes in the metabolism of a P450-specific substrate (probe substrate) by human liver microsomes (or recombinant P450) with varying concentrations of NME are monitored. Potency of the inhibition and rank order of the inhibition of different P450 enzymes can be assessed by the determination of the K_i or IC_{50} value (NME concentration, which reduced the metabolism of the P450 probe substrate by 50%). The concentration of P450 probe substrate used should be at or below its Michaelis-Menten constant (K_m). Therefore, before performing in vitro P450 inhibition studies with NMEs, the test system (e.g., human liver microsomes) needs to be established, and kinetic parameters of the P450 probe substrate (K_m , V_{max}), as well as inhibition (K_i or IC_{50}) by a typical P450 inhibitor (see Table 1) determined and compared with reference values. Such a determination does not need to be repeated, unless the test system is changed, e.g., from microsomes to recombinant P450.

To determine the kinetic parameters for P450 probe substrate metabolism, or in general for any P450 substrate, the turnover of the substrate by the test system must first be optimized; turnover should be linearly dependent on time and less than 20% of the substrate should be consumed. It is desirable to utilize the lowest amount of protein in the incubation that yields readily quantifiable metabolite concentrations. A concentration of below 0.5 mg of microsomal protein \cdot ml⁻¹ is suggested. The low protein concentration would help maintain minimal protein binding, especially with basic lipophilic compounds. Therefore, adding albumin or human serum to microso-

mal incubations to mimic in vivo binding is not recommended. The data with albumin and CYP2C9 substrates are confusing, and cannot be readily explained (Ludden et al., 1997). In addition, for lipophilic substrates, which are dissolved in organic solvents, using a low percentage of organic solvent in any kinetic incubation, e.g., <0.5% final (v/v) to sustain P450 activity is recommended.

Assay validation for probe substrate. At a minimum, the following experiments are needed to establish accurate kinetic parameters. First, a reaction time course experiment should be performed in which the incubation is conducted at a single concentration of protein near the lowest probe substrate concentration anticipated to be used in subsequent experiments, and isoform-specific metabolite formation measured at several time points. Second, the relationship between enzyme concentration and reaction velocity at an incubation time determined in the former experiment should be established. Thus, all subsequent in vitro incubations are performed using the condition that ensures linearity with time and enzyme concentration, conditions should also be such that less than 20% of the initial substrate is consumed. If studies are performed using a pool of samples from individual donors, e.g., pooled human liver microsomes, it should not be necessary to redefine these conditions for each individual lot of material, provided that substrate consumption is low. However, if the source of enzyme changes, for example from liver microsomes to expressed enzyme, then these experiments will need to be repeated. Once optimal conditions are obtained, e.g., incubation time, microsomal concentration, the substrate concentration dependence on the rate of metabolite formation is examined. The K_m value is determined by nonlinear regression of a plot of enzyme activity versus substrate concentration. Substrate concentrations should span a range of at least $1/3 \cdot K_m$ to $3 \cdot K_m$ with at least six concentrations, to obtain an accurate measurement of K_m value. In some cases, limitations of assay sensitivity or solubility of the substrate may prevent gathering of data over this range of concentrations, and caution should be applied to interpretation of the data.

Once the K_m of the probe substrate is established for the test system, an IC_{50} value of a known specific P450 inhibitor (Table 1) can be determined by using the probe substrate concentration at or below the K_m . The determination of an IC_{50} or K_i value can be used to verify the inhibition experiments by comparing the experimentally obtained

IC_{50} or K_i value with known literature values. The rate of the probe substrate turnover is assayed in the presence of various inhibitor concentrations, and the percentage of activity remaining (percentage of the original rate) with respect to inhibitor concentrations are plotted to derive an apparent IC_{50} value. To estimate the relationship between the IC_{50} and the K_i value, the following equations may be used. When the probe substrate concentration is equal to the K_m value, the concentration of inhibitor at which the activity of the enzyme reaction(s) is decreased by one-half (IC_{50}) will be the same as the K_i value if the type of inhibition is noncompetitive or approximately twice the K_i value for a competitive inhibitor ($K_i = IC_{50}/[1 + (S)/K_m]$) or an uncompetitive inhibitor ($K_i = IC_{50}/[1 + K_m/(S)]$). Actual calculation of a K_i value can be determined as described below.

Inhibition by NME: IC_{50} determination. The IC_{50} value for the inhibition of the P450 probe substrate can then be determined for NMEs, as described above for the specific P450 inhibitor. The concentration range of NME is based upon solubility of the compound and concentrations, which would cover, at least, the anticipated plasma concentration. Activities from the blank samples (assays with the substrate but without the inhibitor) should be compared with the historical data (data obtained previously for the same reaction conditions) for quality control purposes. In the case of major circulating metabolites, P450 inhibition studies may also be of importance. The term "major circulating metabolite" refers to 25% of the total drug-related material in human circulation, as defined previously (Baillie et al., 2002).

Inhibition by NME: K_i determination. To examine the type of P450 inhibition, a K_i value (i.e., dissociation constant for the enzyme-inhibitor complex) may be determined for inhibitors where a clinical interaction is likely or possible (see *Clinical implications*). These K_i values are determined from incubations of the NME with human liver microsomes and a P450-selective substrate or with recombinant enzyme and a substrate, at several substrate and inhibitor (the NME) concentrations. The previous section, which described the establishment of reaction conditions to produce accurate kinetic parameters, should be used to guide the determination of the substrate concentrations used in the estimation of K_i values. Furthermore, a preliminary IC_{50} determination guides the inhibitor concentrations used in these studies (inhibitor concentrations should encompass the IC_{50}). The rate of formation of the metabolite of interest in the presence and absence of inhibitor is determined. Using nonlinear regression analysis, the data obtained are used to determine whether the inhibition observed best fits various models of inhibition often including competitive, noncompetitive, mixed competitive/noncompetitive, and uncompetitive types of inhibition. The model that best fits the data, determined by a number of statistical criteria, indicates the type of inhibition observed and the K_i value for the NME.

Time-dependent inhibition. Time-dependent inhibition of P450 enzymes by the drug candidate may complicate the prediction of the extent of drug-drug interactions in vivo. Drug-drug interactions predicted from in vitro data, based upon competitive models (for instance), may underestimate the true interaction that could occur in vivo in the presence of a time-dependent inhibitor. Investigation of this type of P450 inhibition by NMEs can occur when deemed appropriate. Some indicators of time-dependent P450 inhibition may be loss of metabolizing activity of the NME with time, if the inhibited enzyme is involved in the metabolism of the NME. Structural elements of the compound may also indicate a possible inactivator of P450 enzymes, such as side chains with unsaturated carbon-carbon bonds and furan ring systems, alkylamino and methylenedioxy functional groups (Murray, 1997). To examine time-dependent inhibition of P450 enzymes by the NME, it is common to incubate microsomal

protein, with and without NADPH (reduced nicotinamide adenine dinucleotide phosphate), with the test inhibitor at various concentrations (a range of 1- to 10-fold the clinically relevant plasma concentration). Samples are then aliquoted at various time points (e.g., 0, 15, 30, 45, and 60 min) and diluted, e.g., 10-fold with fresh assay buffer containing a P450 probe substrate to measure residual activity. The dilution needs to be sufficient to enable the probe substrate to occupy the active site of the uninactivated enzyme. Testosterone or midazolam could be used as a probe substrate for CYP3A4 and troleandomycin (TAO) as a positive control inhibitor. Kinetic parameters of the inactivation, K_I and k_{inact} are determined as described by Silverman (1995), for example. Briefly, the initial rate constant for enzyme inactivation (k_{obs}) at each concentration of inhibitor is estimated from a plot of the log percent activity remaining versus incubation time, where the slope of a linear regression line is $-k_{obs}$. The relationship of the initial rate of inactivation (k_{obs}) with K_I and k_{inact} is defined by the equation: $k_{obs} = k_{inact} \cdot I/(K_I + I)$, where I is the initial inhibitor concentration, k_{inact} is the maximum rate constant for inactivation, and K_I is the inactivator concentration at half the maximal rate of enzyme inactivation. The K_I and k_{inact} values for the test inhibitor are determined from linear regression of a double-reciprocal plot of the k_{obs} values versus the inhibitor concentrations where the y-intercept is $1/k_{inact}$ and the x-intercept is $-1/K_I$. The correlation of clinical drug-drug interactions and the degree time-dependent inhibition is still being defined. However, an in vitro model to predict the extent of in vivo CYP3A4 inhibition at a select inhibitor concentration has been described (Mayhew et al., 2000) and may be useful to qualitatively rank order the inhibition by NMEs with known time-dependent inhibitors.

Non-Michaelis-Menten kinetics (cooperativity, activation, and substrate inhibition). In cases of nonhyperbolic velocity with respect to substrate (probe substrate or NME) concentration relationships, e.g., activation kinetics, substrate inhibition, cooperativity, etc., a wider range of substrate concentrations may need to be tested to define the reaction kinetics. In particular with CYP3A4, it is recommended that two substrates be used in inhibition studies with NMEs, such as testosterone and midazolam, which show positive cooperativity (autoactivation) and hyperbolic increases with negative cooperativity (autoinhibition), respectively (reviewed in Houston and Kenworthy, 2000). The in vivo relevance for such kinetics in humans is not known at this time (Houston and Kenworthy, 2000; Tang and Stearns, 2001). There has been only one report where increased diclofenac clearance in the presence of quinidine in monkeys has been attributed to enzyme activation (Tang et al., 1999).

Clinical implications. The clinical implications of P450 enzyme inhibition by NMEs are dependent upon the in vivo concentration of the NME and the role of that P450 in the biotransformation of the coadministered drug. In vitro inhibition data may be used to rank order the inhibition of particular P450 enzymes with the objective to test the clinical relevance for the most likely affected P450 enzyme. However, quantitative comparisons between different substrates are difficult. A tentative guideline for decisions was discussed in a previous report (Tucker et al., 2001). The guideline was based upon the equation $[AUC]/[AUC] = (1 + [I]/K_i)$ for competitive inhibition. As a conservative approach, the inhibitor C_{max} at steady state and at the highest clinical dose expected should be used in the estimation of AUC change. It was estimated that for reversible inhibition an interaction would likely occur if the ratio of inhibitor C_{max}/K_i was greater than 1 (Table 2). Interactions are possible if the ratio is between 1 and 0.1 and remote if below 0.1.

TABLE 2

Prediction of clinical relevance of competitive P450 inhibition

| $[I]/K_i$ | Prediction |
|--------------------------|------------|
| $C_{\max}/K_i > 1$ | Likely |
| $1 > C_{\max}/K_i > 0.1$ | Possible |
| $0.1 > C_{\max}/K_i$ | Remote |

Cytochrome P450 Reaction Phenotyping

Overview of P450 Reaction Phenotyping. P450 reaction phenotyping is defined as a set of experiments that aim to define which human cytochrome P450 enzyme(s) is involved in a given metabolic transformation. Such data are useful in the prediction of pharmacokinetic drug-drug interactions and interpatient variability in drug exposure.

The science underlying P450 reaction phenotyping has developed throughout the 1990s such that some well defined experimental approaches have become widely accepted. However, although experimental approaches are well defined for some specific P450 enzymes including CYP1A2, 2C9, 2C19, 2D6, and 3A, approaches for other P450 and non-P450 drug-metabolizing enzymes are still developing or remain to be developed. Also, although an appreciation of in vivo drug-drug interactions mediated by alterations in activities of CYP1A2, 2C9, 2C19, 2D6, and 3A exists, there is not as great an appreciation for CYP1A1, 1B1, 2A6, 2B6, 2C8, and other P450s, as well as other drug-metabolizing enzymes such as flavin-containing monooxygenases (FMOs), monoamine oxidases (MAOs), UDP-glucuronosyl-transferases (UGTs), sulfotransferases (STs), molybdenum-containing oxidases (Mo-CO), methyltransferases, acetyltransferases, and glutathione *S*-transferases (GSTs). As the underlying biochemistry of these enzymes and their impact on drug clearance in vivo becomes better understood, the principles applied for P450 reaction phenotyping could be applied to these enzymes as well. For example, ST and UGT enzymes are discussed below.

Initial Experiments and Enzyme Kinetics. Before P450 reaction phenotyping, it is important to first define the predominant clearance mechanisms for the investigational compound and the most appropriate in vitro system in which to study these clearance mechanisms. There is no rationale for performing P450 reaction phenotyping with an investigational compound if P450-mediated reactions only contribute a minor role in overall clearance (approximately <30%). Other clearance mechanisms include other enzymes (see above), renal clearance of unchanged drug, biliary clearance of unchanged drug, and other more obscure clearance mechanisms. Data obtained after administration of radiolabeled investigational compound to humans provides the most definitive information on the routes of drug clearance. In many cases, the human radiolabel study may not have been conducted by the time that P450 reaction phenotyping is to be commenced. Thus, major human clearance mechanisms must be predicted. In these cases, it is recommended that the metabolism of the NME be examined in as "complete" an in vitro system as is possible. It must be ensured that the in vitro system chosen to study the clearance mechanism for the investigational drug is appropriate in that the enzymes/proteins involved in the clearing process are present in abundance and are sufficiently functional. Thus, although P450 reaction phenotyping can be adequately done using human liver microsomes, examination of other clearance mechanisms requires other in vitro systems, e.g., hepatocytes, precision-cut liver slices, tissue homogenates, kidney membrane vesicles, etc.

If human in vivo data are available, the major initial routes of drug metabolism can be determined, or at least inferred, and defined as being potentially mediated by P450 enzymes, e.g., oxidations, or by

non-P450 enzymes. The data will also indicate the appropriate in vitro test systems for the study of the metabolic reactions. While most oxidative metabolites are due to the action of P450 enzymes, other enzymes could also be involved, depending on the specific reaction (Table 3). Metabolites arising via conjugation can be ascribed to the specific class of conjugating enzymes based on the structure of the metabolite. For oxidative reactions, the contribution of P450 versus non-P450 enzymes should be assessed before P450 reaction phenotyping. This can be accomplished in multiple ways, using specific inhibitors, or by altering experimental conditions. Some of these are listed in Table 4; however, the list is not exhaustive.

Experimental Approach Considerations. Although this document is not intended to be overly proscriptive, there are several experimental details that require attention and definition to avoid generating artifactual data and making erroneous conclusions.

Monitoring metabolite formation versus substrate depletion. In many cases, especially early in the drug development process, authentic standards of metabolites are not yet available or suitably radiolabeled NME. In these cases, monitoring the depletion of the investigational compound in selected in vitro systems is an approach that can be used to determine enzymes involved in metabolic clearance. However, such an approach can have shortcomings. First, conditions that provide linear initial reaction velocities, a requirement of appropriate enzyme kinetic investigations (as described previously), cannot be used since an adequate amount of substrate depletion must occur for reliable quantitation. In fact, reaction velocities are purposefully non-linear (first-order) in this approach. As total substrate depletion is measured, the consumption rates are likely going to be representative of multiple individual reactions, rather than single processes. Under some circumstances, the use of a substrate depletion approach in P450 phenotyping can be acceptable for compound registration dossiers. In these cases, the scientific justification for use of this approach should be clearly defined.

Measuring the formation of individual metabolites represents a scientifically superior approach in P450 reaction phenotyping. Individual reactions can be monitored, and conditions can be used such that linear initial reaction velocities can be measured. However, quantitative measurement of metabolite formation requires that either an authentic standard of the metabolite is available for construction of calibration curves or that radiolabeled material is available for radiometric HPLC quantitation.

Reaction velocity linearity. In P450 reaction phenotyping, in which metabolite formation is measured, it is of critical importance that in vitro conditions be defined that ensure reaction velocity linearity with regard to both time and enzyme concentration. Linearity of metabolite formation was mentioned previously.

For some investigational compounds, circumstances may exist such that it is not possible to conduct P450 reaction phenotyping experiments under strictly linear conditions. This can be due to very low reaction rates requiring greater enzyme concentrations, difficulties in analytical sensitivity, or autoinhibition. In such cases, it should be noted that P450 reaction phenotyping experiments were done under conditions of nonlinearity; the reasons why this was the case should be documented, and findings should be interpreted with appropriate caution.

In substrate depletion approaches, reaction velocity linearity, by the very intent of the approach, will not be obtained. In this approach, data needs to be obtained to ensure an accurate first-order decay curve. This includes the collection of at least three time points per incubation (in addition to $t = 0$). Typically >20% (for low concentrations) of the substrate is depleted by the final time point and regression of the

TABLE 3
Enzymes implicated in drug biotransformation/clearance

| Clearance Mechanism | Possible Enzymes/Proteins Involved |
|-----------------------------|---|
| Oxidative metabolism | P450, FMO, MAO, Mo-CO, peroxidases |
| Hydrolytic metabolism | Esterases, amidases, epoxide hydrolases |
| Conjugative metabolism | UGT, ST, methyltransferase, acetyltransferase |
| Excretion of unchanged drug | Transporters |

TABLE 4
Methods to identify pathways involved in the oxidative biotransformation of a compound

| In Vitro System | Condition | Tests |
|------------------|--------------------------|------------------------------------|
| HLM | ± NADPH | P450, FMO vs. other oxidases |
| HLM, hepatocytes | ± 1-Aminobenzotriazole | Broad specificity P450 inactivator |
| HLM | 45°C pretreatment | Inactivates FMO |
| S-9 | ± Pargyline | Broad MAO inactivator |
| S-9, cytosol | ± Menadione, allopurinol | Mo-CO inhibitors |

log-linear substrate declination plot should yield an acceptable correlation coefficient.

Substrate concentration. Perhaps one of the most critical experimental variables in P450 reaction phenotyping is the selection of the NME concentration used in the in vitro incubation mixture. As it is very common for multiple enzymes to catalyze the same metabolic transformation, selection of an inappropriate substrate concentration can yield results that are not reflective of the in vivo situation. Before conducting P450 reaction phenotyping, the kinetic parameters for NME metabolite formation must be established in pooled human liver microsomes from multiple individual donors. Microsomal preparations from individuals may be used if the microsomal activity of the P450 enzymes represents the average population. In P450 reaction phenotyping, a substrate concentration should be selected based on the following (with K_m representing the Michaelis-Menten constant associated with the enzyme activity of greatest intrinsic clearance if more than one kinetically distinguishable enzyme is observed). If $K_m > C_{in\ vivo}$, in which $C_{in\ vivo}$ represents a concentration associated with a projected efficacious dose (e.g., steady-state average circulating unbound concentration, steady-state C_{max} , concentration estimated during first-pass, etc., as the case best dictates), then the substrate concentration chosen for P450 reaction phenotyping should be $\leq K_m$. If $K_m < C_{in\ vivo}$ then the substrate concentration chosen should be proximal to $C_{in\ vivo}$. If $C_{in\ vivo}$ is unknown, then the substrate concentration chosen should be $< K_m$. However, when $C_{in\ vivo}$ becomes available, the substrate concentration may be reassessed and should be scientifically justifiable.

P450 Reaction Phenotyping. P450 reaction phenotyping can be performed when the aforementioned experimental considerations and initial experiments have been conducted. Conditions and basic procedures for ensuring optimal activities of P450 enzymes are well established and include incubations of liver microsomes at 37°C, open to air for adequate exposure to oxygen. For P450 reactions, incubations are often commenced by adding NADPH or an NADPH-regenerating system, and for FMO, the incubations are commenced by the addition of substrate.

At present, based on information in the scientific literature, the human P450 enzymes can be classified into three categories based on the importance played in the metabolism of drugs: P450s of major importance, emerging importance, and of low importance. P450 enzymes involved in the majority of P450-catalyzed drug biotransformation reactions are CYP1A2, 2C9, 2C19, 2D6, and 3A4. P450 enzymes of emerging importance are CYP2C8, 2B6, and 3A5. These P450 enzymes have recently received more attention in the scientific

literature due to involvement in the metabolism of specific drugs or classes of drugs or, in the case of CYP3A5, may be more involved in the metabolism of CYP3A substrates in vivo than previously thought. P450 enzymes that do not have a major role in drug metabolism are CYP1A1, 1B1, 2A6, 2E1, 4A11, etc. These P450s have been shown to be involved in the metabolism of few, if any, therapeutic agents. The thoroughness to which an investigational compound should be phenotyped versus these P450 enzymes should differ with regard to which of the three classes of P450s are being examined. At a minimum, P450 phenotyping should be applied for the "major" P450 enzymes involved in drug metabolism. Discretion should be applied for the other P450s and enzymes. Also, consideration should be made for the intended indication and target population, if it is anticipated that patients may possess different activities of specific P450 enzymes.

There are several literature reviews of P450 reaction phenotyping and substrate/inhibitor specificities of various P450 enzymes; this description does not attempt to reiterate this topic. P450 reaction phenotyping is performed using a combination of three basic approaches. The first approach is to examine the metabolic reaction of interest in the absence and presence of P450 isoform-specific chemical inhibitors and/or inhibitory antibodies. A second approach is to determine whether heterologously expressed recombinant human P450s will catalyze the reaction of interest. The third approach is to correlate the rate of the reaction of interest with a P450-specific marker activity across a panel of liver microsomal samples characterized for their activity levels of the various P450s from individual donors. Each of these three approaches are very well documented in the scientific literature and are amenable to creating standard procedural documents to outline specifics of experimental design, performance, and interpretation. It is important to note that neither of the approaches alone is adequate for assigning a biotransformation reaction to a specific P450. At least two should be used, provided the results of the two methods are similar.

Specific chemical inhibitors and inhibitory antibodies. Phenotyping experiments, utilizing specific chemical inhibitors or antibodies, may employ either a pool of human liver microsomes from multiple donors, or microsomes prepared from an individual donor, with the intent that all P450 activities are representative of an average in the population. The substrate concentration used should be as defined above. Inhibitors should be used at well established concentrations that ensure adequate selectivity and maximal potency, or alternatively a concentration range of inhibitor can be used to generate an IC_{50} . Inhibitory antibodies should have specificity characterized with P450-

specific marker activities. It should be noted that this approach requires inhibitors or antibodies; both are not needed. Also, specific and potent inhibitors have yet to be identified and characterized for some P450 enzymes. Lists of acceptable inhibitors have been compiled (Table 1) (Newton et al., 1995; Rodrigues, 1999; Ring and Wrighton, 2000; Tucker et al., 2001).

Recombinant enzymes. Over the past decade, human P450 enzymes have been cloned and heterologously expressed in various cell lines, with coexpression of NADPH:cytochrome P450 oxidoreductase and, in some cases, cytochrome b_5 . Due to the "artificial" nature of expressed enzymes, some differences in activity characteristics may exist with respect to P450s in their native membrane environment. However, such differences have not appeared to hinder the utility of these systems in P450 reaction phenotyping. In P450 reaction phenotyping, the investigational compound should be incubated with a battery of expressed enzymes at a substrate concentration as described above. For those P450 enzymes for which activity is observed above a recombinant control, an examination of the enzyme kinetics may be warranted to measure intrinsic clearance. The use of the "relative activity approach" has recently been described with some success and can be considered (Crespi and Miller, 1999; Stormer et al., 2000; Venkatakrisnan et al., 2001). Alternately, correction of intrinsic clearance values for relative abundance of specific P450 enzymes in liver microsomes can be made. Enzyme kinetic data from recombinant P450 systems can be useful in delineating low K_m (low capacity) and high K_m (high capacity) enzymes.

Correlation analysis. In the correlation analysis approach, the reaction velocity of the biotransformation pathway of interest is measured in liver microsomes from a panel of individual donors, using an appropriate substrate concentration. Velocities are subjected to multivariate correlation analysis with P450 isoform-specific marker activities. At least ten different liver samples are recommended with minimal covariance of the enzymes of interest. Acceptable P450 isoform-specific marker activities are listed (Table 1) (Ring and Wrighton, 2000; Tucker et al., 2001), but it should be noted that some of the less studied P450 enzymes do not have well established marker activities and that CYP3A4 and CYP3A5 have not been clearly distinguished from each other.

Use of P450 Phenotyping Information, Documentation, and Standardization. P450 phenotyping data can be used for the planning of clinical studies (drug interactions and special populations) and in support of claims in the product label. It should be emphasized that the relevance of in vitro findings to the in vivo situation should be carefully interpreted. When more than one P450 is involved in the metabolism of the NME, it may be of interest to use approaches involving relative activity factors and clearance values from recombinant systems to account for the relative contributions of specific P450s.

A minimum standard is needed regarding a cutoff for when a specific clearance mechanism (e.g., biotransformation reaction) becomes important enough to merit an investigation into identifying the enzyme(s)/protein(s) responsible. However, it is difficult to define a specific percentage that must be comprised by a single clearance mechanism in the absence of considerations of efficacy and clinical safety of the NME and the specific type of clearance mechanism. On average, for a population, a percentage of ~30% is proposed for P450-mediated metabolic reactions. Complete inhibition of a clearance mechanism, or lack of a clearance mechanism in a special population, that comprises ~30% of total clearance should, in theory, yield approximately a 40% increase in exposure, a value just in excess of bioequivalence standards. Thus, it is suggested that P450 reaction phenotyping be initiated if a sum total of 30% of compound clearance

appears to be related to the formation of primary oxidative metabolites.

In addition to understanding major clearance mechanisms for parent compound, information may be needed on the clearance mechanisms of major human circulating metabolites. Although major circulating metabolites may or may not arise via the major clearance mechanism for the parent drug, they are potentially important if associated with efficacy or safety of the compound. If the circulating metabolites contribute substantially to pharmacological activity, then criteria similar to that for the parent compound should be applied. For metabolites lacking pharmacological activity, but possessing safety issues, again a similar approach for P450 reaction phenotyping should be considered. For major circulating metabolites devoid of pharmacological activity, based on the most relevant preclinical model and exposure, or safety issues and not related to a major clearance pathway, P450 reaction phenotyping should be unnecessary.

In conclusion, P450 reaction phenotyping has progressed to a level of understanding that many practices and procedures can be standardized within laboratories. Information is still emerging for some P450 enzymes. However, for five of these enzymes that are considered major—CYP1A2, 2C9, 2C19, 2D6, and 3A4—it is an expectation that appropriate P450 reaction phenotyping approaches, as described above, will be applied when P450-mediated metabolism is determined to be a major clearance mechanism in humans. Such information is important to appropriate planning of clinical drug interaction studies, for pharmaceutical manufacturers and regulatory authorities in the assessment of risk and benefit for new drugs, and for use by the prescribing physician since these data are included on product labels.

Cytochrome P450 Induction

Drug interactions mediated by P450 induction are significantly less common than those mediated by P450 inhibition. The interaction is less likely to result in safety issues but may impact efficacy of one or more medications. The induction potential of NMEs is difficult to assess preclinically and is often inferred from animal studies, which are not necessarily predictive for humans. This ability is likely to increase with the availability of newer high throughput reporter gene assays, e.g., human pregnane X receptor, hPXR, activation assays, although the in vivo relevance of such assays remains to be fully established. Clinical induction is encountered more often in certain therapeutic areas, e.g., HIV, and the clinical impact can be complicated (sometimes to an advantage) by concurrent inhibition of P450-mediated clearance (Worboys and Carlile, 2001). Indications of when to measure induction in vitro may include apparent autoinduction in animals and humans as evidenced by, for example, changes in kinetic parameters such as decreased plasma AUC from single to multiple dosing regimens. The histopathology of hepatocytes, for instance, in toxicology animals may also indicate induction. It is less apparent, however, when to monitor for induction of genes not involved in the metabolism of the inducer. The well documented inability of animal data to routinely predict induction in humans has been offset in recent years by significant advances in human in vitro methods. The discussion below outlines these methods and how they may be applied in conjunction with clinical data, to obtain a more comprehensive understanding of an NME's induction properties.

In Vitro Test Systems. Primary hepatocytes. Cultured human hepatocytes are currently the most accepted method for studying P450 induction (LeCluyse, 2001; Worboys and Carlile, 2001). Availability of tissue is always a concern and limits the widespread use of human hepatocytes for in vitro applications. Although chances of getting high quality cells are probably greater with in-house perfusion, it is much simpler to use commercial suppliers. The quality of hepatocytes from

commercial suppliers varies drastically depending upon the quality of the tissue used for the perfusion and the success of the perfusion. For the experienced user, an idea of cell quality/viability for induction may be estimated using microscopic examination. Quality should be judged by the appropriate response to positive inducer controls.

A variety of matrix configurations are available for hepatocyte culturing. Plates and dishes must always be coated with an extracellular matrix (ECM), such as Matrigel or collagen, before plating. After the plating of hepatocytes, some researchers find it advantageous to overlay the hepatocytes with ECM. The latter procedure, termed sandwich culturing, appears to place the hepatocytes in a more physiological state mimicking *in vivo* conditions. Although a matrix overlay is not essential to obtain a good induction response in human hepatocytes, it is highly recommended, because it improves cell survival and morphology. Hepatocytes in sandwich culture have a cytoarchitecture, which closely mimics that found *in vivo* (this is not observed under conventional culture conditions). In addition, the overlay can overcome problems that may be encountered if the hepatocytes are under-seeded. Cell density can greatly affect the induction response, with a decrease in response observed with decreased cell density.

A P450 induction experiment will usually take 6 to 7 days. In a typical protocol, hepatocytes are isolated, plated, and allowed to recover for 48 to 72 h before treatment with the test compounds. This time is necessary for the hepatocytes to recover from the cell isolation procedure and adapt to the culture environment. Hepatocytes are then treated for 24 to 72 h. The shorter time points are suitable for mRNA studies, but for measuring enzyme activity or protein levels with Western blots, 72 h is generally required.

Cryopreserved hepatocytes. The methods for the cryopreservation of hepatocytes are still being refined and optimized. While cryopreserved hepatocytes can suffice for metabolic studies, in general, plating after thawing is not successful.

Liver slices. The human liver slice model has not historically been widely used for P450 induction assessment as compared with human hepatocytes. However, the successful use of human liver slices for induction of CYP1A, CYP2C, CYP2B, and CYP3A enzymes (enzymatic activities, apoprotein levels, and/or mRNA levels) is well documented (Lake et al., 1996, 1998, 1997; Draushuk et al., 1998; Glockner et al., 1999; Einolf et al., 2002; Edwards et al., 2003). The use of human liver slices offers the advantage of not having to isolate hepatocytes by digestive enzymes and does not require overlay of ECM; thereby, preparation is rapid and simple. The tissue slices inherently maintain cytoarchitecture, and in addition, organs other than the liver can be examined easily if needed. As with hepatocytes, P450 enzyme activities can be examined in the media or in prepared microsomes. Generally, incubation periods of up to or exceeding 72 h have typically been used for measurement of induction at the level of protein and changes in mRNA levels, in shorter periods of time. The challenge with human liver slices is the acquisition of liver and maintenance of slice cultures with ample viability for the length of the study. Improvements in tissue slice culture media and culturing methods to ensure morphology and cellular function for longer culturing times are being developed and offer progress in this area (Saulnier and Vickers, 2002).

Methods to Measure Inductive Response *In Vitro*. *Enzyme activity.* Measurement of changes in P450 activity is the classical method to determine whether enzyme induction has occurred and is probably the method of choice. Metabolism assays to determine P450 enzyme activities can be performed directly using the whole cells. Although some researchers advocate microsomal protein preparation first, metabolism in cell culture is a simpler approach. Microsomes

TABLE 5

Examples of common positive controls and suggested concentrations for *in vitro* human P450 enzyme induction (LeCluyse et al., 2000; Meunier et al., 2000; Gerbal-Chaloin et al., 2001; Goodwin et al., 2001)

| P450 | Inducer |
|--------|--|
| CYP1A2 | Omeprazole (25 μ M), β -naphthoflavone (10–50 μ M) |
| CYP2B6 | Rifampicin (10–50 μ M), phenobarbital (200–500 μ M) |
| CYP2C9 | Rifampicin (10–50 μ M), dexamethasone (10–50 μ M) |
| CYP3A4 | Rifampicin (10–50 μ M), dexamethasone (50 μ M) |

can, however, be used for Western blot assays and can be stored for replicates or further studies and may be necessary if the NME is an inhibitor of the P450 activity. However, this may not be feasible for multiwell plate formats due to the larger number of cells required. Several approaches have been useful such as using cocktails of substrates or serial incubation with different substrates in cell culture. To measure CYP3A activity, a probe substrate, such as testosterone or midazolam may be used. Multiple probe substrates for CYP3A induction should not be necessary. Establishment of an accurate and reproducible analytical method is required, which should include a defined standard curve, evaluation of interferences, and quality controls. The loss of enzyme activity upon exposure of the cultures to the NME may suggest that the NME is a mechanism-based inhibitor.

Protein levels. Western blot analyses are useful for determining gross changes in protein levels, but in general they are less quantitative. The value of Western blots, however, is the ability to detect induction by a compound that is a simultaneous P450 inhibitor and inducer, which can be difficult to determine enzymatically. Many P450 antibodies suitable for Western blotting are available commercially.

Gene expression. Changes in mRNA levels can be used to measure changes in the expression of P450 genes (Worboys and Carlile, 2001). One assumption is that a change in mRNA levels would reflect an eventual change in enzyme levels. In general, this is true for the P450s except, for example, CYP2E1 where protein stabilization plays a major role in the inductive response (Fuhr, 2000). Although this method does not let an investigator determine the actual change in enzyme activity, it is useful if a suitable enzymatic assay is not available, since all that is required is the cDNA sequence of the enzyme. This, coupled with higher throughput methods to determine mRNA levels, e.g., real-time polymerase chain reaction, make this an attractive approach for maximizing the amount of induction data that can be gained from hepatocytes or slices.

Positive controls. Examples of some positive controls for induction of P450 enzymes are listed in Table 5. The most commonly used positive control is rifampicin at \sim 10 μ M. It is suggested to use acetonitrile or \leq 0.1% dimethyl sulfoxide (DMSO) as a vehicle, as higher concentrations of DMSO may produce artifacts. For an acceptable quality liver, expect to see at least \sim 2-fold CYP3A4 activity enhancement with rifampicin (10 μ M). Significantly higher response levels may often be observed. Absolute enzymatic activity values must also be considered. Higher basal values may reduce the ability to enhance induction to this level but that does not mean that liver quality is poor or that induction is not occurring. There seems to be a maximum enzymatic activity level that is feasible for liver tissue, e.g., for CYP3A4 and testosterone at saturating substrate concentrations, the maximal levels is \sim 16,000 pmol \cdot min⁻¹ \cdot mg of microsomal protein; thus if activities are approaching this, relevant induction is occurring even if the “-fold induction relative to control” is not as substantial.

Variability in the inducibility of hepatocytes from different donors is commonplace, and the positive control can aid in comparing in-

duction data from different preparations of hepatocytes. As a guide, an induction of at least 40% of the positive control induction level would indicate a positive inductive response. In general, the concentration of test compound used should be based upon the human in vivo C_{max} and the dose.

Interpretation and in Vivo Extrapolation. In vitro induction data would not be required for the registration of any new chemical entity. However, the results of the in vitro induction studies can help design appropriate clinical trials to determine whether induction is likely to be observed at therapeutic doses and what impact this may have on drug-drug interactions. Due to the variability of human hepatocytes, reproducible negative results in at least three human hepatocyte preparations could be used for labeling purposes, without the need for further clinical data. If induction is observed in vitro, then one can design multiple dose studies to specifically look for induction, e.g., by studying the pharmacokinetics (PK) of the probe substances in vivo. The PK after multiple dosing can also be evaluated to identify potential autoinduction. This is discussed later in this article. The type of induction information obtained should depend on what is known about the enzymology of the compound and may not be the same in every case. Metabolic activity determinations should be the primary mode of assessing in vitro induction, with protein and gene expression levels examined in addition, whenever possible.

Phase II Drug-Metabolizing Enzymes

For a majority of the drug candidates, conjugative metabolism is usually preceded by Phase I biotransformation. However, for compounds bearing polar functional groups, direct conjugative metabolism can sometimes be a major metabolic pathway. If the Phase II conjugation pathway is significant based upon human ADME or human tissue culture studies, a more detailed investigation may be warranted to understand the role of these pathway(s) in the disposition of the drug candidate. As with the P450 enzymes, a significant contribution of Phase II conjugation to the clearance of a drug was estimated to be approximately >30%. Compared with P450 enzymes, however, there are fewer reported clinical interactions with Phase II conjugation reactions, possibly due in part to the nature of the enzymatic reaction (high V_{max} and moderate to high K_m values). Although the enzymes involved in the conjugative metabolism, e.g., UGT and ST, are less well understood, several advances have been made in the recent past. Recombinant enzyme systems are available for many UGT and ST enzymes; however, phenotyping of the Phase II reaction is only qualitative at this time due to the lack of relativity/scaling factors to predict in vivo hepatic clearance. The lack of specific probe substrates and inhibitors is one of the major issues. Systematic in vitro drug-drug interaction studies for Phase II enzymes are not necessary; however, they could be performed on a case-by-case basis.

Glucuronidation. Glucuronidation reactions represent one of the most important Phase II conjugation reactions. UGTs are involved in the clearance of many drugs as the result of the ubiquitous nature of the enzyme and the direct conjugation of many different functional groups to produce more polar and inactive metabolites, for the most part (with exception of the morphine glucuronide conjugate) (Liston et al., 2001).

Glucuronidation can be measured in microsomes with uridine diphosphate glucuronic acid (UDPGA) using the microsomal pore-forming reagent, alamethicin. Due to the reasonably high in vivo UDPGA cofactor concentrations in the liver ($\sim 250 \mu\text{M}$), cofactor depletion is probably not an issue. Glucuronidation can also be measured in whole cell systems, such as hepatocytes and tissue slices. In this case, issues regarding competing pathways, such as P450, are addressed and may be physiologically more relevant. Kinetics of

glucuronidation reactions can be determined in microsomes or by recombinant enzymes. Generally the reactions require high substrate concentrations due to typically high K_m values, in which solubility limitation can be an issue for hydrophobic compounds.

There are likely polymorphisms in coding or noncoding regions for all UGT enzymes. However there are only five UGT genes described as being polymorphic to date (Mackenzie et al., 2000). Those are UGT1A1, 1A6, 2B4, 2B7, and 2B15. Clinical toxicity has been reported in individuals with polymorphisms in UGT1A1 (Gilbert syndrome) when treated with irinotecan (CPT-11) (Ando et al., 2000). The clinical relevance of the other UGT enzyme polymorphisms is unknown. Thus, knowledge of genetically based interindividual variability may be useful when designing clinical trials.

Inhibition of glucuronidation. In the evaluation of a drug candidate as an inhibitor of UGT catalyzed reactions, the drug candidate does not need to be a UGT substrate for it to be an inhibitor. Typically, IC_{50} or K_i values can range from 10 to >300 μM for UGT inhibition. Thus the therapeutic plasma concentrations are likely to be much lower than K_i or IC_{50} values, with an $[I]/K_i$ ratio of <1. Hence it is not likely that the potential for drug-drug interactions via inhibition of a UGT pathway by a coadministered drug will be a major concern. In that respect, there is very limited clinical data on UGT-dependent drug-drug interactions [valproate on zidovudine (ZDV), lorazepam, and lamotrigine]. The inhibition of a drug candidate's clearance by inhibition of glucuronidation would be similar to that described above for a drug candidate as a P450 inhibitor. The magnitude of interactions is likely to be low and may be of significance only if the drug candidate has a low therapeutic index. Furthermore, existence of parallel clearance pathways, such as renal elimination of parent, oxidative metabolism, and enterohepatic recirculation are likely to lessen the significance of such a drug-drug interaction. As mentioned above, if only one enzyme is involved with known clinically relevant polymorphisms, it may be of importance to examine potential clinical adverse effects.

Evaluation of the effect of UGT inducers on the drug candidate. Glucuronidation reactions become important if this pathway is the primary route of elimination or if a UGT-dependent minor pathway becomes a major pathway upon induction. UGT inducers need not be substrates of UGT enzymes. Like for P450 enzymes, UGT induction can be measured in hepatocyte cell cultures. Typically, UGTs are co-induced with P450 enzymes by, e.g., rifampicin and phenobarbital, yet the molecular basis of UGT induction is not yet well understood. Hence it will be very difficult to prospectively explore the potential for UGT induction by an NME in vitro. This decision has to be made based on careful evaluation of multiple-dose pharmacokinetic data in animals and if the NME induces P450s. There are some examples in the literature describing the alteration of PK profiles of drugs when coadministered with rifampicin, e.g., ZDV or azidodeoxythymidine (AZT), and phenobarbital, presumably via UGT-induction (Burger et al., 1993; Gallicano et al., 1999). Another important consequence of UGT induction can be the effect on glucuronidation of endogenous UGT substrates such as bilirubin, thyroxine (T4), and steroids.

Sulfation. Sulfotransferases represent a large family of enzymes that catalyze the transfer of the sulfuryl group of 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to phenols, amines, primary and secondary alcohols, and other oxygen-containing functional groups. Model systems for measuring sulfate conjugation are liver S-9 homogenates or cytosol with additional cofactor, PAPS, or in whole cell systems, such as hepatocytes and slices. Recombinant enzyme systems are also available for some of the STs, but as with UGTs, there is a lack of an ability to correlate in vitro/in vivo clearance, at the present time. This is due primarily to lack of specific substrates/

TABLE 6
Drug-drug interactions due to the inhibition of transport proteins (modified from Ayrton and Morgan, 2001)

| Substrate | Inhibitor | Effect | Potential Transport Protein |
|--|--|--|-----------------------------|
| Digoxin | Quinidine, verapamil, itraconazole | ↑ Plasma concentrations, ↓ renal clearance | P-Glycoprotein; OATP |
| Fexofenadine | Ketoconazole, erythromycin, azithromycin | ↑ Plasma concentrations | P-Glycoprotein; OATP |
| Talinolol | Verapamil | ↑ or ↓ plasma concentrations; ↓ intestinal secretion | P-Glycoprotein |
| Loperamide | Quinidine | ↑ Central adverse events | P-Glycoprotein |
| Dofetilide, procainamide, levofloxacin | Cimetidine | ↑ in AUC; ↓ renal clearance | OCT; OAT; OATP |
| Penicillins, ACE inhibitors, antiviral drugs | Probenecid | ↓ Renal clearance; prolong $t_{1/2}$ | OAT |
| Paclitaxel | Valsopodar | ↑ Plasma concentrations | P-Glycoprotein |

OCT, organic cation transporter; OAT, organic anion transporter; OATP, organic anion transport protein.

inhibitors for the ST enzymes. There is very limited information pertaining to the effect of ST inducers and inhibitors on the disposition of compounds that are cleared via the sulfation pathway. Only recently, potent ST inhibitors (mefanamic acid, quercetin) have been identified. Several P450 inducers—phenobarbital, rifampicin, glucocorticoids—can also induce ST. It is not certain if there will be clinically significant drug-drug interactions due to either induction or inhibition of sulfation of a drug candidate. In the limited amount of data published so far (acetaminophen versus fenoldopam), interactions appear to be more related to cofactor depletion rather than direct inhibitory effect on sulfotransferases. Due to the low in vivo cofactor concentrations ($\sim 75 \mu\text{M}$), cofactor depletion is more likely an issue for sulfate conjugation compared with glucuronidation reactions. Functionally significant polymorphisms of ST may have more of an implication in the clinic for those compounds eliminated by a specific ST. For example, significant genetic polymorphisms in *SULT1A3* have been implicated in changes of the disposition or action of levosalbutamol (Boulton and Fawcett, 2001).

Drug Transporters

The disposition of drugs by transport proteins has been recognized to a greater extent more recently in drug development. A comprehensive review has recently been published and focuses on the relevance to drug discovery and to the development process (Ayrton and Morgan, 2001). However, at present, no accurate prediction of transporter involvement in humans is possible from nonclinical studies.

Some drug interactions, previously believed to be P450-mediated, are now considered at least in part due to the inhibition of transport proteins (Table 6). Nevertheless it is often difficult to distinguish between in vivo P450 inhibition and inhibition of transport processes. For example for talinolol, which undergoes little metabolism, the increased bioavailability in the presence of verapamil, is most likely due to inhibition of P-glycoprotein (Pgp) by verapamil. On the other hand, the results for cyclosporin A (CsA) are controversial. Using in vitro Caco-2 monolayer cells, the flux of CsA from basolateral to apical was inhibitable by Pgp inhibitors. Indicative of active transport, inhibition was greater from apical to basolateral at low micromolar concentrations (Augustijns et al., 1993). However, CsA does not stimulate ATPase activity although it inhibits ATPase activity stimulated by verapamil (Rao and Scarborough, 1994), indicating that it is an inhibitor but not a substrate. Controversial conclusions were also drawn from in vivo studies. Pgp was implicated in the absorption of CsA (Wu et al., 1995); however, the absence of significant Pgp versus P450 involvement in CsA disposition is implied by insignificant amounts of unchanged CsA into the bile (Christians et al., 1988, 1991). Furthermore, high CsA bioavailability using improved vehicles was not mediated by inhibition of Pgp (Choc et al., 1998).

Although molecular biology of transport proteins has greatly advanced, a better understanding of the quantitative contribution of the

transport proteins to clearance vis-à-vis to metabolism is needed to attribute the effects observed in vivo to the action of transport or metabolizing proteins and to make accurate predictions based on in vitro systems. Although there are in vitro systems to test the potential of an NME to inhibit relevant transporters and to characterize possible involvement of transporters in the disposition, these in vitro systems are not standardized nor are they all readily available. Furthermore, most if not all cell-based systems presently available contain multiple transporter proteins, which can lead to confounding results. A quantitative prediction of the in vivo relevance from in vitro studies is not possible at this time. This is in part due to the shear fact that transporter phenomena have been an emerging area, but also because the contribution of transporters to clearance cannot be readily assessed from in vivo studies. The contribution of drug-metabolizing enzymes for clearance can be estimated by measuring metabolites in excreta, whereas transport proteins are complex because they are involved in several processes such as absorption, tissue distribution, and excretion. A simple determination of unchanged drug in the excreta may only be useful if changes in tissue distribution, e.g., liver uptake, can be excluded.

The effect of the NME on the transport of a substrate marker is determined in cellular systems; however, no strong correlation between NME binding to the transporter protein and active transport has been found. Hence, binding studies are not accurate predictors of in vivo membrane transport and thus direct transport measurements in monolayer cell systems are typically performed. In vitro test systems include isolated cells and represent the organ of origin, or cell lines that were selected and/or cDNA transfected to express a certain transporter protein. Expression of transport proteins in cDNA-transfected *Xenopus laevis* oocytes is another potentially useful technique. For ABC transport proteins, the determination of ATPase activity in vesicular systems may provide an alternative test system for transport activity. However, inconsistencies have been observed compared with cellular systems where transport of the NME is measured directly (Polli et al., 2001). It appears that the efflux assay is more reliable when the compound has low/moderate permeability values (P_{app}). Since transport is most important when P_{app} is low, the efflux assay is suggested to be the method of choice. Commonly used efflux monolayer systems include the human cells Caco-2, HT-29, and T84 as well as cDNA-transfected Madin-Darby canine kidney cells (MDCK) and LLC-PK1 pig kidney cells. It appears that Caco-2 cell monolayers are the most commonly used in vitro technique. For 9 of 10 efflux proteins from the ABC transporter family investigated, Caco-2 cells have been reported to resemble human jejunum in transcript levels (Taipalensuu et al., 2001). In addition, the peptide transporter PEPT1, which transports many β -lactam antibiotics, is expressed in Caco-2 cells (Bretschneider et al., 1999). However, without the use of specific well defined inhibitors, it is difficult to assess which transporters are involved in the Caco-2 transport.

Characterization of Test System. As indicated previously, most test systems are not standardized, and good scientific practice needs to be applied when these systems are used. The best characterized are cell monolayer efflux systems. Cells have been grown on various membranes such as nitrocellulose or polycarbonate. Some compounds bind to these membranes and data for nonspecific binding of the NME need to be determined to assess the validity of the experiment. Monolayer integrity can be checked by transepithelial electrical resistance (TEER) measurements or by passive paracellular markers such as radiolabeled PEG 4000 or lucifer yellow. In addition, mannitol and propranolol are typically used as reference compounds for a low and highly permeable compound, respectively. Characterization of the transport system through determination of gene expression and/or activities of characteristic substrates is also desirable. The expression of transporters in cell lines is dependent on the passage number, and it is therefore necessary to limit this variability. Many transport studies are currently performed at pH 7.4 on both the apical and basolateral site. The pH in the intestine is, however, more acidic, and a lower pH on the apical site is more representative of physiological conditions for studies modeling intestinal transport (Yamashita et al., 2000). In addition, transporters such as PEPT1 work in a proton gradient-dependent manner (Matsumoto et al., 1994). Some investigators add serum albumin to the basolateral side to improve sink conditions. An initial lag phase to reach steady state is usually observed during transport experiments and can be minimized by stirring or shaking.

Study Design. In many studies, the main goal is to determine the ability of the compound to inhibit a specific transporter and also to characterize the NME as a substrate of a specific transport protein. For these purposes, the same principles apply as described for metabolizing enzymes (see above), e.g., the experiment should be performed under linear conditions and the substrate concentration should be below or equal to K_m for inhibition studies and should span K_m for kinetic investigations. Since multiple transporters exist in most systems, the use of specific inhibitors can be useful in identifying the transporters involved. However, at present there are few specific transporters characterized. When the objective is to determine the in vivo relevance of efflux proteins to offset passive paracellular and transcellular absorption of drugs, some investigators prefer to use for an apical concentration the clinical dose dissolved in 250 ml or less depending on the solubility of the compound (Rege et al., 2001).

In Vivo Prediction. As mentioned earlier, transporter involvement in humans cannot be accurately predicted from nonclinical studies. When predictions are made from cell-based systems such as Caco-2, active transport may be of no relevance for compounds with a high intrinsic permeability and also the transporter may be saturated at the high intestinal concentrations achieved during absorption. Thus, in the absence of good physiologically based pharmacokinetic models for the prediction of in vivo relevance of transporters, some basic assumptions may be most practical. For instance, bidirectional transport studies in Caco-2 cells using concentrations of the clinical dosage in 250 ml or less would provide a first estimate of the relevance of active transport contributions during absorption. Transporter kinetic values could be correlated with compounds known to possess clinically relevant transport processes, e.g., digoxin, vinblastine, talinolol, fexofenadine, etc. High absorption in animals or humans would suggest a low potential for intestinal efflux transporter related drug-drug interactions, although the rate of absorption may be affected. Finally, the relevance of efflux transport proteins to clearance may be assessed by determining unchanged NME in the excreta from animals, but preferably from human studies.

In Vivo Drug-Drug Interactions

Clinical Pharmacokinetic Drug-Drug Interaction Studies—Basic Principles

Pharmacokinetic Interactions Must be Evaluated within the Context of Clinical Relevance. The fact that two drugs share a common metabolic pathway does not guarantee that they will have a clinically significant pharmacokinetic interaction when coadministered to a patient. Whether the two coadministered drugs will interact in humans will depend on various factors, including the relative affinities of each drug for the binding site on the metabolizing enzyme as well as the effective free drug concentrations available locally for binding. In addition, parallel pathways for elimination of one or both drugs would tend to reduce the potential for a significant pharmacokinetic interaction.

The magnitude of the elevation in systemic levels of a drug recipient as a result of inhibition of a P450 isoform responsible for its metabolism by a coadministered inhibitor (precipitant) will depend on the degree of inhibition of the relevant P450 isoform by the precipitant. A documented and statistically significant pharmacokinetic interaction may not necessarily lead to a clinical consequence. For example, when a drug is coadministered with a known inhibitor of the P450 isoform responsible for its metabolism, the resulting increased systemic exposure to the recipient drug, and its metabolites may not necessarily result in a clinically detectable increase in toxicity. Whether a given magnitude of effect of an interacting inhibitory drug on plasma levels of a recipient drug results in an increased risk of adverse events depends to a great extent on the therapeutic index of the recipient drug. Even small pharmacokinetic interactions can result in significant pharmacodynamic adverse effects for drugs of narrow therapeutic index. However, small to moderate pharmacokinetic interactions may not necessarily result in detectable and clinically significant consequences for drugs of wider therapeutic index.

Clinical data regarding the safety of coadministration of a drug with another potentially interacting drug, when available from clinical trials and from post-marketing surveillance, are always more relevant and definitive in assessing the clinical relevance of a proven or potential pharmacokinetic drug interaction than the pharmacokinetic data in itself, i.e., when sufficient clinical experience exists, clinical data always takes precedence over pharmacokinetic data in terms of establishing the clinical significance of drug-drug interactions.

Clinically Relevant Pharmacokinetic Interactions Should be Described in Product Labels. Whenever clinical data are available, allowing for the assessment of the clinical significance of pharmacokinetic interactions for a given drug, only those pharmacokinetic interactions expected to be (or known to be) of clinical relevance should be noted in the drug's label. The complete absence of a possibly relevant pharmacokinetic interaction based on the results of a clinical pharmacokinetic study could also be appropriately reflected in the product's label. If sufficient clinical data are available that do not provide evidence that a detected (statistically significant) pharmacokinetic interaction is clinically relevant, then inclusion of pharmacokinetic data regarding this interaction without its clinical context would be confusing, not interpretable, and ultimately not useful to health care providers and to their patients. Whenever possible, any proven or potential pharmacokinetic interaction noted in labeling should be anchored in the context of its clinical relevance and its "real life" implications to health care providers and patients. The reflexive inclusion in product labels of quantitative pharmacokinetic data without mention of their clinical relevance is not helpful to physicians or patients, who cannot be expected to have the technical expertise to appropriately interpret such data. If there are insufficient data to

TABLE 7
Preferred *in vivo* probe P450 substrates

| P450 | Substrate | |
|---------|--|--|
| | November 1999 FDA Guidance for Industry <i>In Vivo Drug Metabolism/Drug Interaction Studies...</i> | Basel Conference (November 2000), sponsored by the FDA, EUFEPS, and AAPS (Tucker et al., 2001) |
| CYP1A2 | Theophylline | Caffeine |
| CYP2B6 | | Bupropion |
| CYP2C9 | S-Warfarin | Tolbutamide |
| CYP2C19 | | S-Mephenytoin, omeprazole |
| CYP2D6 | Desipramine | Debrisoquine (not available in the United States) dextromethorphan |
| CYP2E1 | | Chlorzoxazone |
| CYP3A | Midazolam, buspirone, felodipine, simvastatin, lovastatin | Midazolam (i.v. ± p.o.), p.o. midazolam + i.v. erythromycin, simvastatin, atorvastatin |

interpret the potential clinical significance of a statistically significant pharmacokinetic interaction, then the pharmacokinetic findings may be reflected in labeling with wording indicating that the observed interaction might not be clinically relevant. Only by achieving clarity in the communication of the clinical consequences of a product's pharmacokinetic drug interactions through labeling, will regulatory agencies and pharmaceutical sponsors achieve the shared goal of promoting the effective and safe use of pharmaceutical agents.

Many physicians and other health care providers may have limited training in the area of drug-drug interactions. It is thus essential to improve clarity in labeling and continue efforts to place drug interaction wording in product circulars into clinical context to promote the safe and effective use of drugs. It is also crucial to improve the awareness by practitioners of the principles and the clinical consequences associated with drug interactions.

Standardization of P450 Probe Substrates

Regulatory agencies, academic investigators, and the pharmaceutical industry have increasingly recognized the importance of trying to achieve greater consistency in the choice of P450 probe substrates for clinical pharmacokinetic drug-drug interaction studies. Standardization of *in vivo* P450 probe substrates, doses, and sampling procedures would allow for more useful comparison across studies and across compounds. Characteristics of an ideal probe P450 substrate include: 1) substrate for a single P450 pathway; 2) highly sensitive to changes in status/activity of the respective P450 enzyme; 3) unaffected by P-glycoprotein or other known transporters; 4) negligible pharmacodynamic (especially adverse) effects at dose of probe used; 5) commercially available; and 6) a readily measured pharmacokinetic endpoint.

The November 1999 FDA Guidance for Industry *In Vivo Drug Metabolism/Drug Interaction Studies—Study Design, Data Analysis, and Recommendations for Dosing and Labeling* suggested that the most sensitive drugs to clinical inhibition of the respective P450 isoform should be selected as the preferred P450 probe substrates. This guidance suggested preferred probe substrates for the respective P450 pathways (Table 7). The table also lists the preferred *in vivo* probe P450 substrates recommended at a conference held in Basel (November, 2000) that was sponsored jointly by the FDA, EUFEPS, and AAPS (Tucker et al., 2001).

A Paradigm for Classifying CYP3A Inhibitors

Orally administered midazolam is metabolized exclusively via CYP3A and possesses all of the attributes of an ideal P450 probe substrate noted above. In addition, the metabolism of oral midazolam relies, to a comparable extent, on intestinal CYP3A and on hepatic CYP3A, thus allowing for quantitation of effects on CYP3A activity

in both the gut and the liver. A single dose of 2 mg of oral midazolam has been used safely in many drug-drug interaction studies and is recommended (Lin et al., 2001).

The use of orally administered midazolam as a preferred CYP3A probe substrate allows for the development of a classification system for CYP3A inhibitors. This system represents a paradigm that, in the future, may be applied more widely to pharmacokinetic drug-drug interactions mediated via other P450 pathways and possibly to interactions at the level of Phase II reactions or by various transporter systems. The usefulness of this and similar classification systems for labeling will require further discussion with regulatory agencies. Tables 8 through 11 summarize available data on the effects of various known or potential CYP3A inhibitors on plasma levels (plasma AUC) of midazolam following oral administration of midazolam. Of note, the dose and dosing regimen of the probe CYP3A inhibitor can influence the quantitative result in terms of the observed elevation in the plasma AUC of midazolam. This concept is illustrated by the varying results reported for ketoconazole (Table 8), with elevations in the plasma AUC of midazolam ranging from 5- to 16-fold, depending on the dosing regimen of ketoconazole used. The recommended starting dose of oral ketoconazole is 200 mg daily; a dose of 400 mg daily is recommended for serious infections and for lack of response to the lower dose. Therefore, since the regimen of 400 mg of ketoconazole daily is recommended for certain clinical situations and since it has the largest reported effect (after 4 days of treatment) on midazolam pharmacokinetics, this dose level of ketoconazole is recommended to optimally assess the effect of the most potent clinical inhibition of CYP3A on the pharmacokinetics of a CYP3A substrate. The extent of pharmacokinetic interaction may also depend on the dose of P450 probe drug, i.e., lower inhibitory effect can be observed with increased dose. In the case of midazolam use as a CYP3A probe, a dose of 2 mg p.o. could be used to investigate the maximal inhibitory effect *in vivo*. Based on their *in vivo* effect on the plasma AUC of orally administered midazolam, drugs being tested for possible CYP3A inhibitory activity can be generally classified as described in Table 12. Although the current classification system is based on effects on the plasma AUC of orally administered midazolam, it may be possible in the future to base a similar system on effects on a single-point determination (at 4 h post dose) of midazolam plasma concentration (Lin et al., 2001).

P450-Mediated Pharmacokinetic Drug Interaction Studies—Study Design

The design of a clinical pharmacokinetic drug-drug interaction study should allow for the assessment of the drug interaction liability of an NME as either the recipient of a drug interaction, when coadministered with an inhibitor or inducer of a relevant P450 pathway, or

TABLE 8
Strong CYP3A inhibitors (oral midazolam used as probe substrate)

| Drug | Dose/Regimen | Midazolam Dose | Fold Elevation in Midazolam AUC (GMR) ^a | Reference |
|----------------|-----------------------------|-------------------|--|-----------------------|
| Ketoconazole | 400 mg q.d. × 4 days | 7.5 mg p.o. | 16 | Olkkola et al., 1994 |
| Ketoconazole | 3 doses of 200 mg | 6 mg p.o. | 11 | Tsunoda et al., 1999 |
| Itraconazole | 200 mg q.d. × 4 days | 7.5 mg p.o. | 11 | Olkkola et al., 1994 |
| Mibefradil | 100 mg p.o. | 2 mg p.o. | 8.9 | Veronese et al., 1999 |
| Ketoconazole | 200 mg q.d. × 11 days | Not specified | 8.7 | Lam et al., 1999 |
| Itraconazole | 200 mg q.d. × 4 days | 15 or 7.5 mg p.o. | 8 | Backman et al., 1998 |
| Clarithromycin | 500 mg b.i.d. × 7 days | 3–4 mg p.o. | 7.1 | Gorski et al., 1999 |
| Clarithromycin | 500 mg b.i.d. × 7 days | 4 mg p.o. | 7 | Gorski et al., 1998 |
| Ketoconazole | 200 mg given simultaneously | 2 mg p.o. | 6.2 | Olkkola et al., 1994 |
| Itraconazole | 100 mg q.d. × 4 days | 7.5 mg p.o. | 6 | Ahonen et al., 1995 |
| Ketoconazole | 200 mg given 2 h before | 2 mg p.o. | 5 | Olkkola et al., 1994 |

^a GMR, geometric mean ratio.

TABLE 9
Moderate CYP3A inhibitors (oral midazolam used as probe substrate)

| Drug | Dose/Regimen | Midazolam Dose | Fold Elevation in Midazolam AUC (GMR) | Reference |
|------------------|------------------------------|----------------|---------------------------------------|---|
| Erythromycin | 500 mg t.i.d. × 7 days | 15 mg p.o. | 4.4 | Olkkola et al., 1993 |
| Erythromycin | 500 mg t.i.d. × 5 days | 15 mg p.o. | 3.8 | Zimmermann et al., 1996 |
| Diltiazem | 60 mg t.i.d. × 2 days | 15 mg p.o. | 3.7 | Backman et al., 1994b (Versed package circular, 2001) |
| Clarithromycin | 250 mg b.i.d. × 5 days | 15 mg p.o. | 3.6 | Yeates et al., 1996 |
| Fluconazole | 400 mg p.o. | 7.5 mg p.o. | 3 | Ahonen et al., 1997 |
| Fluconazole | 400 mg i.v. | 7.5 mg p.o. | 3 | Ahonen et al., 1997 |
| Verapamil | 80 mg t.i.d. × 2 days | 15 mg p.o. | 2.9 | Backman et al., 1994b (Versed package circular, 2001) |
| Grapefruit Juice | 8 oz with breakfast × 4 days | 2 mg p.o. | 2.4 | Rogers et al., 1999 |
| Grapefruit Juice | 8 oz with breakfast × 4 days | 2 mg p.o. | 2.4 | Blum et al., 1999 |

TABLE 10
Weak CYP3A inhibitors (oral midazolam used as probe substrate)

| Drug | Dose/Regimen | Midazolam Dose | Fold Elevation in Midazolam AUC (GMR) | Reference |
|------------------|---|----------------|---------------------------------------|---|
| Grapefruit juice | 200 ml, normal strength, 1 h before | 15 mg p.o. | 1.5 | Kupferschmidt et al., 1995 |
| Roxithromycin | 300 mg q.d. × 6 days | 15 mg p.o. | 1.5 | Backman et al., 1994a (Versed package circular, 2001) |
| Fentanyl | 200 µg with nitrous oxide and isoflurane/(n = 15/group) | 0.2 mg/kg p.o. | 1.4 | Hase et al., 1997 |

TABLE 11
Drugs without CYP3A inhibitory activity (oral midazolam used as probe substrate)

| Drug | Dose/Regimen | Midazolam Dose | Fold Elevation in Midazolam AUC (GMR) | Reference |
|--------------|-------------------------------------|-----------------|---------------------------------------|------------------------------|
| Azithromycin | 400 mg q.d. × 3 days | 15 mg p.o. | 1.3 | Zimmermann et al., 1996 |
| Cimetidine | 800 mg p.o. × 1 day | Not noted | 1.3 | Fee et al., 1987 |
| Simvastatin | 80 mg q.d. × 7 days | 2 mg p.o. | 1.1 | Prueksaritanont et al., 2000 |
| Fluoxetine | 60 mg q.d. × 5 days, then 20 mg/day | Not specified | 0.8 | Lam et al., 1999 |
| Rifampicin | 600 mg q.d. × 5 days | 15 mg p.o. | 0.023 | Backman et al., 1998 |
| Azithromycin | 500 mg q.d. × 3 days | 15 mg p.o. | NSD ^a | Yeates et al., 1996 |
| Terbinafine | 250 mg q.d. × 4 days | 7.5 mg p.o. | NSD | Olkkola et al., 1994 |
| Azithromycin | 500 mg day 1250 mg days 2–5 | 15 mg p.o. days | NSD | Backman et al., 1995 |

^a NSD, no significant difference in midazolam plasma AUC, with vs. without the interacting drug (GMR ≈ 1.0).

as the precipitant of a drug interaction by either inducing or inhibiting the P450-based metabolism of a coadministered probe substrate. The crucial drug interaction studies, necessary in the clinical development of an NME, can be rationally determined based on in vitro data, as described elsewhere in this document. The study designs for exclusively pharmacodynamic drug-drug interaction studies are not considered here.

Three study design scenarios are generally feasible for human pharmacokinetic drug interaction studies: 1) single-dose NME and

TABLE 12
Classification of CYP3A in vivo inhibitory activity

| Inhibitory Class | Fold Increase in Plasma AUC of Midazolam |
|---|--|
| Strong | ≥5 |
| Moderate | >2 but <5 |
| Weak | ≤2 |
| Drugs without CYP3A inhibitory activity | ~1 |

TABLE 13
Guidelines for selecting multiple-dose/multiple-dose design trials

| Multiple-Dose/Multiple-Dose Design Conditions |
|---|
| 1. Specific safety issues are possible that depend on achieving steady-state exposure of both compounds. |
| 2. The interaction is not strictly competitive, e.g., mechanism-based or suicidal inhibition occurs or may occur. |
| 3. A claim that widens the absolute bioequivalency criteria is desired. |
| 4. The time course of induction or inhibition needs to be determined. |
| 5. First dose effects require titration to a standard steady-state dose for either compound. |
| 6. One drug shows a significant accumulation ratio and a single-dose regimen that achieves comparable steady-state concentrations is not feasible. |
| 7. One of the drugs at steady-state concentrations forms a metabolite that produces additive induction or inhibition, which cannot be demonstrated through the administration of larger single doses. |
| 8. Dose-dependent or time-dependent pharmacokinetics, e.g., phenytoin or omeprazole, respectively, leading to at least a 50 to 100% accumulation of parent. |

single-dose probe administered in combination and each compound given alone in a three-way crossover trial; 2) single-dose P450 probe followed by multiple dose NME + single-dose probe OR single-dose NME followed by multiple-dose precipitant drug (inhibitor or inducer) + single-dose NME in a sequential two-way crossover; and 3) multiple-dose probe and multiple-dose NME in one three-way crossover trial or two two-way crossover trials.

The following guidelines should be considered for the design of clinical studies. For single-dose/single-dose trial designs, the half-lives of inhibitor and substrate should be similar; most critically, the inhibitor should be present throughout the elimination phase of the probe compound. If a significant disparity in T_{max} is anticipated between the two compounds, dosing should be timed so as that the T_{max} for both compounds coincides if a competitive and reversible interaction is being studied. If noncompetitive inhibition or induction is the basis of the drug-drug interaction under study, then coincidence of T_{max} may not be necessary. Some inhibitors of CYP3A have a propensity to form a metabolic complex with this P450 isoform. The formation of the complex may be time-dependent and generally requires multiple dosing *in vivo*; thus, inhibitory potential may be underestimated after single dosing study designs.

Although not mandatory, the single-dose three-way crossover study could be used to confirm the lack of a significant interaction if the *in vitro* interaction results suggest that no interaction is likely ($[I]/K_i < 0.1$). Single-dose trials would be especially useful to explore a dose range for the NME and inhibitor or as a pilot study to avoid a multiple-dosing trial in which one of the interacting agents could potentially involve safety risks for healthy volunteers. The single-dose probe and multiple-dose NME would meet regulatory needs if the interaction were in doubt but possible. The multiple-dose/multiple-dose design would be used in the same situation but under the conditions described in Table 13. Examples of typical study designs are listed in Tables 14 and 15. For studies which determine whether an NME is a specific inhibitor of a P450 isoform (Table 14), the study design is valid only if the probe substrate does not inhibit the metabolism of the NME. In some cases, if the NME inhibits a genetically polymorphic P450 isoform, then a drug interaction study may not be needed for the P450 pathway involved if it is assumed that the worst case would be that an extensive metabolizer (EM) becomes a poor metabolizer (PM) and that substrates of narrow therapeutic index (requiring dose adjustment for EM versus PM) are not affected.

Cocktails

The first simultaneous administration of two or more probe substrates, a cocktail, to characterize changes in pharmacokinetics was performed more than 20 years ago. Mixtures of probe substrates have been used to estimate the degree of renal (Lanchote et al., 1996) or hepatic dysfunction (Brockmoller and Roots, 1994) and to characterize drug-metabolizing enzyme inhibition and induction. Recently, verified cocktails have been proposed for the confirmation of *in vitro*

predictions for drug-drug interactions before more definitive single-probe substrate studies (Tucker et al., 2001). The statistical multiplicity penalty resulting from multiple simultaneous comparisons in a single cocktail study must be recognized as a weakness of this approach compared with conducting several single-probe substrate studies. Furthermore, to validly assess the PK changes after administration of several probe substrates at once, the selectivity and sensitivity of each probe for a single target P450 pathway must be rigorously established. The potential for the probe substrates to interact with each other has engendered the greatest concern about the use of cocktails. It must be assured that, at the higher concentration range of the probe in the presence of an inhibitor, specificity is maintained and inhibition of other P450s by the probe does not occur. A five substrate combination—caffeine (CYP1A2), chlorzoxazone (CYP2E1), debrisoquine (CYP2D6), dapsone (CYP3A), and mephenytoin (CYP2C19)—was initially studied without evidence of probe substrate interactions when coadministered without inhibitors or inducers (Frye et al., 1997; Zhu et al., 2001). Using this probe substrate mixture, specific suppression of CYP2D6 activity with chloroquine and selective induction of CYP2E1 and *N*-acetyltransferase (NAT2) with all-*trans*-retinoic acid were observed (Adedoyin et al., 1998a,b). Using a combination of quinidine and rifampin with the five P450 probe substrate mixture, selective enzyme inhibition and induction of multiple enzymes were demonstrated (Branch et al., 2000). Despite these initial promising results, subsequent studies identified an interaction between chlorzoxazone (CYP2E1) and midazolam (CYP3A) (Palmer et al., 2001).

Additional probe substrate mixtures—midazolam (CYP3A), dextromethorphan (CYP2D6) caffeine (CYP1A2), and omeprazole (CYP2C19)—have been developed partly in the response to probe substrate interactions and the limited availability of five-substrate constituents (Streetman et al., 2000). These probe substrate mixtures have been investigated to meet the requirements of enzyme selectivity, favorable pharmacokinetic profile, acceptable safety profile, bio-analytical assay availability, and limited sample volume. Probe substrate mixture screening for P450 induction is potentially useful when *in vitro* methods may not be predictive (e.g., nuclear receptor modulators). Presently, limited *in vivo* experience with P450 probe substrate mixtures and the difficulties in establishing high P450 selectivity under a variety of inhibitory and enzyme inductive conditions limits the potential utility of “cocktails” for assessing drug-drug interactions *in vivo*.

Population Pharmacokinetic Approach to Characterize Drug-Drug Interactions

Another approach to characterize the possible drug-drug interactions associated with an NME is to apply population pharmacokinetic techniques. As suggested in the November, 1999 FDA Guidance for Industry *In Vivo Drug Metabolism/Drug Interaction Studies—Study Design, Data Analysis, and Recommendations for Dosing and Label-*

TABLE 14

Typical clinical study designs to determine if the NME is an inhibitor or inducer of a specific P450 enzyme^a

| Order of Drug Administration and Data Collected | Dose |
|--|---|
| 1. Administer probe compound as an SD ^b regimen; collect PK information | 1. Safe and well tolerated with minimal pharmacodynamic effects, allowing for adequate sensitivity in detecting effects on the activity of the relevant P450 pathway; common referenced doses |
| 2. Administer NME as an MD ^c regimen until steady-state achieved (for inhibition) or at least 5 days for induction to occur (this may extend to 10–14 days, depending on the properties of the NME) | 2. For inhibition: standard (relevant therapeutic dose) or highest approved dose (or highest dose in clinical development) ^d ; for induction: highest dose |
| 3. Administer probe compound as an SD + NME on the last few days (≈ 5 half-lives of NME) or last day (if half-lives of the two compounds are similar), collect PK information for the probe | 3. Same as in 1., dosing of the NME should continue until the PK information of the probe is collected |

^a This type of induction study is not mandatory, in particular, if the NME is both a substrate and an inducer of a given P450 pathway, evidence of auto-induction from a multiple oral dose study may be sufficient.

^b Single dose (SD), e.g. oral midazolam for CYP3A, if the NME is an inhibitor or inducer of several P450s, it is possible to combine several probe substrates in a cocktail to reduce the studies.

^c Multiple dosing (MD) may be especially needed if a metabolite of the NME is a P450 inhibitor or if there is dose accumulation (e.g., >2-fold) with NME MD.

^d If the NME has a potentially narrow therapeutic index, then a low but clinically relevant dose may be appropriate.

TABLE 15

Typical clinical study designs to determine whether a P450 inhibitor or inducer (marketed drug) would affect the PK of the NME

| Order of Drug Administration and Data Collected | Dose |
|--|--|
| 1. Administer NME as SD regimen and collect PK information | 1. Standard therapeutic dose or high dose |
| 2. Administer the known P450 inhibitor ^a or inducer as an MD regimen until steady-state is achieved or maximal induction has occurred | 2. Depends upon the inhibitor or inducer dose reported in the literature |
| 3. Administer an SD of the NME + known inducer or inhibitor on the last days or final day, collect PK information of the NME (and perhaps of major active metabolites) | 3. Same as in 1. |

^a It is possible that a single dose of the known inhibitor may be sufficient; in this case a single dose study where the NME is given with and without the known P450 inhibitor with an appropriate washout period may be sufficient.

ing, a population PK approach may be useful in detecting unsuspected drug-drug interactions. Population PK can also provide further evidence of the absence of a pharmacokinetic drug-drug interaction when this is suggested by in vitro data. Population pharmacokinetic analyses conducted in the later phase of clinical drug development might also be valuable in characterizing the clinical impact of known or newly identified interactions and in making recommendations for dosage modifications. Since the power of a sparse sampling strategy to detect drug-drug interactions is not yet well established, it is unlikely that population analysis can be used to prove the absence of an interaction that is strongly suggested by information arising from in vitro or in vivo studies specifically designed to assess such a drug-drug interaction.

Population pharmacokinetic analyses may be most valuable if designed as an add-on to efficacy/safety trials. In general, population pharmacokinetics can be applied to two types of study designs to characterize drug-drug interactions. The first scenario is that in which the NME is developed to be coadministered with another marketed drug. In such a case, a typical efficacy/safety trial is designed to use a combination therapy with the NME coadministered with the other marketed drug in one or more arms versus a single agent in another arm. Therefore, population pharmacokinetic analyses can be applied to examine the possible influences between the two drugs by comparing the data from the controlled arm or from the existing historical information. In a second scenario, the NME is usually given as a single agent in efficacy/safety trials but the target patients are likely to have a medical need for one or more coadministered drugs. With carefully designed study procedures and sample collection, the influence of coadministered medications on the pharmacokinetics of the NME can be detected by comparison with its pharmacokinetics in the absence of the coadministered drug. It is also possible to detect the influence of the NME on these medications, if samples were obtained and analyzed for the concentrations of those medications.

To be optimally informative, the study procedures and samples collections for population pharmacokinetic studies should be carefully designed and prespecified (February, 1999 FDA Guidance for Industry *Population Pharmacokinetics*). Key points to be considered in the design and preparation of such studies include: a) prespecify the assessment of drug-drug interactions using a population PK approach as one of the objectives of the study; b) predefine the coadministered marketed drugs of interest for the analyses; c) dosing information should be collected for both the NME and for the coadministered marketed drugs; and d) PK data on the coadministered marketed drugs should also be considered at the time of data analysis.

Clinical Drug Interactions Due to Modulation of Transport Processes

Active transport of drugs and their metabolites has now been recognized as an important determinant in their absorption, tissue distribution, and elimination. Numerous transporters are being characterized for their structure-function, tissue distribution, and regulation. Transporters have been identified and characterized in the intestine, liver, kidney, lung, and at the blood-brain barrier. At these sites they serve diverse endogenous functions—such as transport of sugars, amino acids, peptides, and hormones—and are also involved in the uptake and efflux of exogenous drugs and their metabolites. For example transporters in the gastrointestinal tract influence the bioavailability of orally administered drugs whereas those in the liver affect hepatic uptake and biliary secretion. Transporters in the kidney impact on the renal secretion of various drugs and those at the blood-brain barrier can determine access to the central nervous system (CNS). The tissue uptake and efflux transport functions in drug disposition have the potential of being inhibited and, in some cases, induced during drug treatment, and therefore effects at the level of transporters may be involved in drug-drug interactions. Because there is overlap in substrate specificity and distribution of drug transporters and metabolizing enzymes, drug disposition and the extent of drug-

drug interactions may be determined by a complex interplay between the activities of these proteins.

Pgp is currently the most well understood drug transporter with respect to its regulation, tissue-specific expression, substrate specificity, and modulation of its function by inhibitors (Cvetkovic et al., 1999; Schwarz et al., 1999, 2000; Drewe et al., 2000; Westphal et al., 2000a; Chiou et al., 2001; Lin et al., 2001; Tayrouz et al., 2001) or inducers (Westphal et al., 2000b; Hamman et al., 2001; Niemi et al., 2001) both in vitro and in vivo. Pgp has been shown to be an important drug efflux transporter in the intestine, liver, brain, and other epithelial tissues and is documented to be involved in clinically significant drug interactions. One of the most common and serious interactions demonstrated in clinical practice is between the narrow therapeutic index drug, digoxin, and drugs that reduce its clearance. Digoxin is not extensively metabolized and is eliminated unchanged by Pgp and possibly organic anion transport protein (OATP) transport in the kidney. Elevated plasma concentrations of digoxin have been observed, not only with the antiarrhythmic agent, quinidine, but also during concomitant therapy with several other drugs—verapamil, nifedipine, propafenone, CsA, itraconazole, and amiodarone. Quinidine and many of these drugs have been shown to inhibit Pgp, which is believed to be the mechanism responsible for increased exposure to digoxin. Reported clinical drug interactions with the common opioid anti-diarrheal agent loperamide serve to illustrate the difficulties in generalizing clinical transporter interactions. Loperamide is not normally associated with CNS side effects due to the effective barrier provided by Pgp efflux at the blood-brain barrier. When loperamide is coadministered with ritonavir, there is a change in systemic exposure to loperamide, which does not cause adverse CNS effects, whereas when quinidine is given with loperamide, respiratory depression results that cannot be explained by changes in systemic loperamide exposure.

While examples of pharmacokinetic drug interactions involving drug transporters exist in the literature, relatively few clinically significant drug interactions affecting efficacy or safety have been demonstrated to be based on an effect of a single transporter mechanism. Because knowledge of the function, substrate specificities, inhibitors, and inducers of specific transporters is still emerging and current in vitro tools do not model the complexities of the function of transport systems in vivo where uptake, efflux, passive permeability, metabolism, and protein binding processes may occur simultaneously, it will be difficult to predict transport level clinical drug interactions qualitatively or quantitatively based on in vitro assays. In addition, apparent differences in substrate specificity between humans and preclinical species also generally preclude the use of animal data to confidently predict the potential for clinical drug interactions related to transport processes. At present, it is difficult to design clinical trials in a rational way to study drug-drug interactions involving individual transporters based on information generated in vitro. However, in vitro transporter interaction studies may be highly valuable to help rationalize pharmacokinetic drug interactions that are found not to involve modulation (inhibition or induction) of metabolizing enzymes or other mechanisms.

Drugs that are Pgp substrates, such as digoxin, fexofenadine, talinolol, or loperamide, or inhibitors (e.g., valsopodar, verapamil, or formulation excipients such as Cremophor and Pluronic P85) are being evaluated to determine their clinical utility as probes for the function of this transporter in vivo. However, a number of issues exist in establishing their common use as standard probes for transport-related drug-drug interactions: safety, different concentrations, and relevance for the blood-brain barrier versus the gastrointestinal tract, and unknown predictive value due to substrate dependence and dif-

ferences in the in vivo disposition of other “interactant” drugs with respect to membrane permeability and other poorly understood effects.

Genetic Polymorphisms—Relevance to Drug Interaction Studies

Differences in the safety and efficacy of drugs may be caused by genetic polymorphisms of drug metabolizing enzymes, drug transporters, and drug receptors. Five P450 enzymes—CYP1A2, 2C9, 2C19, 2D6, and 3A4—are thought to be responsible for metabolizing most commonly prescribed drugs. The CYP3A4 enzyme accounts for the metabolic routes in ~50% of these drugs. CYP2C9, CYP2C19, and CYP2D6 enzymes account for approximately another 40% of drug metabolism by P450s and exhibit a genetically based polymorphic phenotype (Wong et al., 2000). Genetic variation for these enzymes results in poor metabolizers with a potentially unacceptable frequency of adverse events and in ultra-rapid metabolizers (CYP2D6), with a potentially inadequate efficacy.

The major allelic variants of CYP2C9, CYP2C19, and CYP2D6, which are associated with impaired xenobiotic clearance, have been identified and screening assays are available for use in research and clinical practice (Linder et al., 1997). Over 50 different mutations in the CYP2D6 gene have been identified, with 20 variant alleles associated with changes in metabolism. A limited subset of the CYP2D6 variant alleles accounts for the incidence of poor metabolizers found in Caucasians. Two polymorphisms of CYP2C19 (CYP2C19*2 and CYP2C19*3) account for >90% of the alleles associated with the poor metabolism phenotype (Chang et al., 1995; Xie et al., 1999). Similarly two major CYP2C9 polymorphisms (2C9*2 and 2C9*3) account for >90% of alleles associated with reduced activity (Takahashi and Echizen, 2001). Patients with these genetic variants have been shown to require lower maintenance doses of warfarin.

Significant differences in allelic frequency for both CYP2C9 and CYP2C19 exist among ethnic groups. Caucasians have an 8 to 13% frequency of CYP2C9*2 and a 6 to 10% frequency of CYP2C9*3. In contrast, Asians very rarely have the CYP2C9*2 allele with only 2 to 5% frequency of the CYP2C9*3 allele (Xie et al., 1999). For CYP2C19, the ethnic differences are reversed with a 3 to 6% frequency in Caucasians and a 13 to 23% frequency in Asians (Kaneko et al., 1999). Isolated Pacific Islanders have a far greater prevalence of CYP2C9*2 or CYP2C9*3 (77%). Further characterization of the variation of the drug pharmacokinetics and of drug interactions with genotype should facilitate early studies in racially distinct populations and may potentially facilitate early clinical *bridging* studies.

Genotyping to screen individuals for altered drug metabolism has become more common in clinical practice and in research. Screening for CYP2C9, CYP2C19 and CYP2D6 genotypes may be valuable in selecting study populations or providing insights into the variation and outliers in pharmacokinetic studies. Conversely, phenotypic characterization for the low incidence of altered metabolizers among subjects homozygous for the normal alleles would be unlikely to be necessary. Other potential uses of genotyping, e.g., in combination with population PK, have not been reported and would require rigorous evaluation, such as a statistical approach, before more general use.

Concluding Remarks

This document represents a consensus view of participating PhRMA member companies for the conduct of in vitro and in vivo drug-drug interaction studies involving Phase I (P450) and Phase II (UGT, ST) metabolic and transporter interactions. This document outlines a means to achieve consistency in quality and validity of the data among laboratories. Such “best practice” will be an ever-evolving

process as advances in techniques and new discoveries are made. Several topics within the report are not as well defined as others and are the topic of future direction. Advancements in the utility of “cocktails” for assessing drug-drug interactions in vivo, and defining in vivo relevance from some in vitro studies, such as time-dependent P450 inhibition, non-Michaelis-Menten kinetic P450 characteristics, and prediction of transporter involvement, remain topics of potential significance.

References

- Adedoyin A, Frye RF, Mauro K, and Branch RA (1998a) Chloroquine modulation of specific metabolizing enzymes activities: investigation with selective five drug cocktail. *Br J Clin Pharmacol* **46**:215–219.
- Adedoyin A, Stiff DD, Smith DC, Romkes M, Bahnsen RC, Day R, Hofacker J, Branch RA, and Trump DL (1998b) All-trans-retinoic acid modulation of drug-metabolizing enzyme activities: investigation with selective metabolic drug probes. *Cancer Chemother Pharmacol* **41**:133–139.
- Ahonen J, Olkkola KT, and Neuvonen PJ (1995) Effect of itraconazole and terbinafine on the pharmacokinetics and pharmacodynamics of midazolam in healthy volunteers. *Br J Clin Pharmacol* **40**:270–272.
- Ahonen J, Olkkola KT, and Neuvonen PJ (1997) Effect of route of administration of fluconazole on the interaction between fluconazole and midazolam. *Eur J Clin Pharmacol* **51**:415–419.
- Ando Y, Saka H, Ando M, Sawa T, Muro K, Ueoka H, Yokoyama A, Saitoh S, Shimokata K, and Hasegawa Y (2000) Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis. *Cancer Res* **60**:6921–6926.
- Augustijns PF, Bradshaw TP, Gan LS, Hendren RW, and Thakker DR (1993) Evidence for a polarized efflux system in CACO-2 cells capable of modulating cyclosporin A transport. *Biochem Biophys Res Commun* **197**:360–365.
- Ayrton A and Morgan P (2001) Role of transport proteins in drug absorption, distribution and excretion. *Xenobiotica* **31**:469–497.
- Backman JT, Aranko K, Himberg JJ, and Olkkola KT (1994a) A pharmacokinetic interaction between roxithromycin and midazolam. *Eur J Clin Pharmacol* **46**:551–555.
- Backman JT, Kivisto KT, Olkkola KT, and Neuvonen PJ (1998) The area under the plasma concentration-time curve for oral midazolam is 400-fold larger during treatment with itraconazole than with rifampicin. *Eur J Clin Pharmacol* **54**:53–58.
- Backman JT, Olkkola KT, Aranko K, Himberg JJ, and Neuvonen PJ (1994b) Dose of midazolam should be reduced during diltiazem and verapamil treatments. *Br J Clin Pharmacol* **37**:221–225.
- Backman JT, Olkkola KT, and Neuvonen PJ (1995) Azithromycin does not increase plasma concentrations of oral midazolam. *Int J Clin Pharmacol Ther* **33**:356–359.
- Baillie TA, Cayen MN, Fouda H, Gerson RJ, Green JD, Grossman SJ, Klunk LJ, LeBlanc B, Perkins DG, and Shipley LA (2002) Drug metabolites in a smelter setting. *Toxicol Appl Pharmacol* **182**:188–196.
- Blum RA, Rogers JD, Zhao J, Liu L, Amin RD, Gagliano KD, Stepanavage M, Wilson MF, and Vega JM (1999) The effect of grapefruit juice (GFJ) on plasma levels of simvastatin HMG-CoA reductase inhibitors. 71st Congress of the European Atherosclerosis Society, May 28–29, Athens, Greece.
- Boulton DW and Fawcett JP (2001) The pharmacokinetics of levosalbutamol: what are the clinical implications? *Clin Pharmacokinet* **40**:23–40.
- Branch RA, Adedoyin A, Frye RF, Wilson JW, and Romkes M (2000) In vivo modulation of CYP enzymes by quinidine and rifampin. *Clin Pharmacol Ther* **68**:401–411.
- Bretschneider B, Brandsch M, and Neubert R (1999) Intestinal transport of beta-lactam antibiotics: analysis of the affinity at the H⁺/peptide symporter (PEPT1), the uptake into Caco-2 cell monolayers and the transepithelial flux. *Pharm Res* **16**:55–61.
- Brockmoller J and Roots I (1994) Assessment of liver metabolic function. Clinical implications. *Clin Pharmacokinet* **27**:216–248.
- Burger DM, Meenhors PL, Koks CH, and Beijnen JH (1993) Pharmacokinetic interaction between rifampin and zidovudine. *Antimicrob Agents Chemother* **37**:1426–1431.
- Chang M, Dahl ML, Tybring G, Gotharson E, and Bertilsson L (1995) Use of omeprazole as a probe drug for CYP2C19 phenotype in Swedish Caucasians: comparison with S-mephenytoin hydroxylation phenotype and CYP2C19 genotype. *Pharmacogenetics* **5**:358–363.
- Chiou WL, Chung SM, Wu TC, and Ma C (2001) A comprehensive account on the role of efflux transporters in the gastrointestinal absorption of 13 commonly used substrate drugs in humans. *Int J Clin Pharmacol Ther* **39**:93–101.
- Choc MG, Mueller EA, Robinson WT, Kumle A, Smith HT, and Charnick SB (1998) Relative bioavailability of Neoral versus Sandimmune in the presence of a P450IIIa and P-glycoprotein inhibitor. *Transplant Proc* **30**:1664–1665.
- Christians U, Schlitt HJ, Bleck JS, Schiebel HM, Kownatzki R, Maurer G, Strohmeier SS, Schottmann R, Wonigeit K, Pichlmayr R, et al. (1988) Measurement of cyclosporine and 18 metabolites in blood, bile and urine by high-performance liquid chromatography. *Transplant Proc* **20**:609–613.
- Christians U, Strohmeier S, Kownatzki R, Schiebel HM, Bleck J, Greipel J, Kohlhaw K, Schottmann R, and Sewing KF (1991) Investigations on the metabolic pathways of cyclosporine: I. Excretion of cyclosporine and its metabolites in human bile—isolation of 12 new cyclosporine metabolites. *Xenobiotica* **21**:1185–1198.
- Crespi CL and Miller VP (1999) The use of heterologously expressed drug metabolizing enzymes—state of the art and prospects for the future. *Pharmacol Ther* **84**:121–131.
- Cvetkovic M, Leake B, Fromm MF, Wilkinson GR, and Kim RB (1999) OATP and P-glycoprotein transporters mediate the cellular uptake and excretion of fexofenadine. *Drug Metab Dispos* **27**:866–871.
- Drahushuk AT, McGarrigle BP, Larsen KE, Stegeman JJ, and Olson JR (1998) Detection of CYP1A1 protein in human liver and induction by TCDD in precision-cut liver slices incubated in dynamic organ culture. *Carcinogenesis* **19**:1361–1368.
- Drewe J, Ball HA, Beglinger C, Peng B, Kemmler A, Schachinger H, and Haefeli WE (2000) Effect of P-glycoprotein modulation on the clinical pharmacokinetics and adverse effects of morphine. *Br J Clin Pharmacol* **50**:237–246.
- Edwards RJ, Price RJ, Watts PS, Renwick AB, Tredger JM, Boobis AR, and Lake BG (2003) Induction of cytochrome P450 enzymes in cultured precision-cut human liver slices. *Drug Metab Dispos* **31**:282–288.
- Einolf HJ, Bedi-Singh S, and Fischer V (2002) Precision-cut human liver slices as a model to examine changes in cytochrome P450 expression to predict or preclude potential drug-drug interactions. *The Toxicologist* **46**:19.
- Fee JP, Collier PS, Howard PJ, and Dundee JW (1987) Cimetidine and ranitidine increase midazolam bioavailability. *Clin Pharmacol Ther* **41**:80–84.
- Frye RF, Matzke GR, Adedoyin A, Porter JA, and Branch RA (1997) Validation of the five-drug “Pittsburgh cocktail” approach for assessment of selective regulation of drug-metabolizing enzymes. *Clin Pharmacol Ther* **62**:365–376.
- Fuhr U (2000) Induction of drug metabolizing enzymes: pharmacokinetic and toxicological consequences in humans. *Clin Pharmacokinet* **38**:493–504.
- Gallicano KD, Sahai J, Shukla VK, Seguin I, Pakuts A, Kwok D, Foster BC, and Cameron DW (1999) Induction of zidovudine glucuronidation and amination pathways by rifampicin in HIV-infected patients. *Br J Clin Pharmacol* **48**:168–179.
- Gerbal-Chaloin S, Pascucci JM, Pichard-Garcia L, Daujat M, Waechter F, Fabre JM, Carrere N, and Maurel P (2001) Induction of CYP2C genes in human hepatocytes in primary culture. *Drug Metab Dispos* **29**:242–251.
- Glockner R, Steinmetzer P, Drobner C, and Muller D (1999) Use of fresh and cryopreserved human liver slices in toxicology with special reference to in vitro induction of cytochrome P450. *Toxicol In Vitro* **13**:531–535.
- Goodwin B, Moore LB, Stoltz CM, McKee DD, and Kliever SA (2001) Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor. *Mol Pharmacol* **60**:427–431.
- Gorski JC, Jones DR, Haehner-Daniels BD, Hamman MA, O'Mara EM Jr, and Hall SD (1998) The contribution of intestinal and hepatic CYP3A to the interaction between midazolam and clarithromycin. *Clin Pharmacol Ther* **64**:133–143.
- Gorski JC, Liu Y, Hall SD, and Haehner-Daniels BD (1999) The effect of clarithromycin on human cytochrome P450 (CYP) enzymes. *Clin Pharmacol Ther* **65**:136.
- Hamman MA, Bruce MA, Haehner-Daniels BD, and Hall SD (2001) The effect of rifampin administration on the disposition of fexofenadine. *Clin Pharmacol Ther* **69**:114–121.
- Hase I, Oda Y, Tanaka K, Mizutani K, Nakamoto T, and Asada A (1997) I. v. fentanyl decreases the clearance of midazolam. *Br J Anaesth* **79**:740–743.
- Houston JB and Kenworthy KE (2000) In vitro-in vivo scaling of CYP kinetic data not consistent with the classical Michaelis-Menten model. *Drug Metab Dispos* **28**:246–254.
- Kaneko A, Lum JK, Yaviong L, Takahashi N, Ishizaki T, Bertilsson L, Kobayakawa T, and Bjorkman A (1999) High and variable frequencies of CYP2C19 mutations: medical consequences of poor drug metabolism in Vanuatu and other Pacific islands. *Pharmacogenetics* **9**:581–590.
- Kupferschmidt HH, Ha HR, Ziegler WH, Meier PJ, and Krahenbuhl S (1995) Interaction between grapefruit juice and midazolam in humans. *Clin Pharmacol Ther* **58**:20–28.
- Lake BG, Ball SE, Renwick AB, Tredger JM, Kao J, Beaman JA, and Price RJ (1997) Induction of CYP3A isoforms in cultured precision-cut human liver slices. *Xenobiotica* **27**:1165–1173.
- Lake BG, Charzat C, Tredger JM, Renwick AB, Beaman JA, and Price RJ (1996) Induction of cytochrome P450 isoenzymes in cultured precision-cut rat and human liver slices. *Xenobiotica* **26**:297–306.
- Lake BG, Tredger JM, Renwick AB, Barton PT, and Price RJ (1998) 3,3'-Diindolylmethane induces CYP1A2 in cultured precision-cut human liver slices. *Xenobiotica* **28**:803–811.
- Lam Y, Ereshefsky L, Alfaro C, and Miller M (1999) In vivo inhibition of midazolam disposition by ketoconazole and fluoxetine and comparison to in vitro prediction. *Clin Pharmacol Ther* **65**:143.
- Lanchote VL, Ping WC, and Santos SR (1996) Influence of renal failure on cytochrome P450 activity in hypertensive patients using a “cocktail” of antipyrine and nifedipine. *Eur J Clin Pharmacol* **50**:83–89.
- LeCluyse EL (2001) Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. *Eur J Pharm Sci* **13**:343–368.
- LeCluyse E, Madan A, Hamilton G, Carroll K, DeHaan R, and Parkinson A (2000) Expression and regulation of cytochrome P450 enzymes in primary cultures of human hepatocytes. *J Biochem Mol Toxicol* **14**:177–188.
- Lin YS, Lockwood GF, Graham MA, Brian WR, Loi CM, Dobrinska MR, Shen DD, Watkins PB, Wilkinson GR, Kharasch ED, and Thummel KE (2001) In-vivo phenotyping for CYP3A by a single-point determination of midazolam plasma concentration. *Pharmacogenetics* **11**:781–791.
- Linder MW, Prough RA, and Valdes R Jr (1997) Pharmacogenetics: a laboratory tool for optimizing therapeutic efficiency. *Clin Chem* **43**:254–266.
- Liston HL, Markowitz JS, and DeVane CL (2001) Drug glucuronidation in clinical psychopharmacology. *J Clin Psychopharmacol* **21**:500–515.
- Ludden LK, Ludden TM, Collins JM, Pentikis HS, and Strong JM (1997) Effect of albumin on the estimation, in vitro, of phenytoin V_{max} and K_m values: implications for clinical correlation. *J Pharmacol Exp Ther* **282**:391–396.
- Mackenzie PI, Miners JO, and McKinnon RA (2000) Polymorphisms in UDP glucuronosyltransferase genes: functional consequences and clinical relevance. *Clin Chem Lab Med* **38**:889–892.
- Matsumoto S, Saito H, and Inui K (1994) Transcellular transport of oral cephalosporins in human intestinal epithelial cells. Caco-2: interaction with dipeptide transport systems in apical and basolateral membranes. *J Pharmacol Exp Ther* **270**:498–504.
- Mayhew BS, Jones DR, and Hall SD (2000) An in vitro model for predicting in vivo inhibition of cytochrome P450 3A4 by metabolic intermediate complex formation. *Drug Metab Dispos* **28**:1031–1037.
- Meunier V, Bourrie M, Julian B, Marti E, Guillouf F, Berger Y, and Fabre G (2000) Expression and induction of CYP1A1/1A2, CYP2A6 and CYP3A4 in primary cultures of human hepatocytes: a 10-year follow-up. *Xenobiotica* **30**:589–607.
- Murray M (1997) Drug-mediated inactivation of cytochrome P450. *Clin Exp Pharmacol Physiol* **24**:465–470.
- Newton DJ, Wang RW, and Lu AY (1995) Cytochrome P450 inhibitors. Evaluation of specificities in the in vitro metabolism of therapeutic agents by human liver microsomes. *Drug Metab Dispos* **23**:154–158.
- Niemi M, Backman JT, Neuvonen M, Neuvonen PJ, and Kivisto KT (2001) Effects of rifampin on the pharmacokinetics and pharmacodynamics of glyburide and glipizide. *Clin Pharmacol Ther* **69**:400–406.

- Olkkola KT, Aranko K, Luurila H, Hiller A, Saarnivaara L, Himberg JJ, and Neuvonen PJ (1993) A potentially hazardous interaction between erythromycin and midazolam. *Clin Pharmacol Ther* **53**:298–305.
- Olkkola KT, Backman JT, and Neuvonen PJ (1994) Midazolam should be avoided in patients receiving the systemic antimycotics ketoconazole or itraconazole. *Clin Pharmacol Ther* **55**:481–485.
- Palmer JL, Scott RJ, Gibson A, Dickins M, and Pleasance S (2001) An interaction between the cytochrome P450 probe substrates chlorzoxazone (CYP2E1) and midazolam (CYP3A). *Br J Clin Pharmacol* **52**:555–561.
- Polli JW, Wring SA, Humphreys JE, Huang L, Morgan JB, Webster LO, and Serabjit-Singh CS (2001) Rational use of in vitro P-glycoprotein assays in drug discovery. *J Pharmacol Exp Ther* **299**:620–628.
- Pruksaritanont T, Vega JM, Rogers JD, Gagliano K, Greenberg HE, Gillen L, Brucker MJ, McLoughlin D, Wong PH, and Waldman SA (2000) Simvastatin does not affect CYP3A activity, quantified by the erythromycin breath test and oral midazolam pharmacokinetics, in healthy male subjects. *J Clin Pharmacol* **40**:1274–1279.
- Rao US and Scarborough GA (1994) Direct demonstration of high affinity interactions of immunosuppressant drugs with the drug binding site of the human P-glycoprotein. *Mol Pharmacol* **45**:773–776.
- Rege BD, Yu LX, Hussain AS, and Polli JE (2001) Effect of common excipients on Caco-2 transport of low-permeability drugs. *J Pharm Sci* **90**:1776–1786.
- Ring BJ and Wrighton SA (2000) Industrial viewpoint: Application of in vitro drug metabolism in various phases of drug development, in *Metabolic Drug Interactions* (Levy RH, Thummel KE, Trager WF, Hansten PD, and Eichelbaum M, eds) pp 29–39, Lippincott Williams & Wilkins, Philadelphia.
- Rodrigues AD (1999) Integrated cytochrome P450 reaction phenotyping: attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochem Pharmacol* **57**:465–480.
- Rogers JD, Zhao J, Liu L, Amin RD, Gagliano KD, Porras AG, Blum RA, Wilson MF, Stepanavage M, and Vega JM (1999) Grapefruit juice has minimal effects on plasma concentrations of lovastatin-derived 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Clin Pharmacol Ther* **66**:358–366.
- Saulnier MJ and Vickers AE (2002) Optimization of organ slice longevity in culture to link gene expression with altered morphology. *The Toxicologist* **46**:247.
- Schwarz UI, Gramatte T, Krappweis J, Berndt A, Oertel R, von Richter O, and Kirch W (1999) Unexpected effect of verapamil on oral bioavailability of the beta-blocker talinolol in humans. *Clin Pharmacol Ther* **65**:283–290.
- Schwarz UI, Gramatte T, Krappweis J, Oertel R, and Kirch W (2000) P-glycoprotein inhibitor erythromycin increases oral bioavailability of talinolol in humans. *Int J Clin Pharmacol Ther* **38**:161–167.
- Silverman RB (1995) Mechanism-based enzyme inactivators. *Methods Enzymol* **249**:240–283.
- Stormer E, von Moltke LL, and Greenblatt DJ (2000) Scaling drug biotransformation data from cDNA-expressed cytochrome P-450 to human liver: a comparison of relative activity factors and human liver abundance in studies of mirtazapine metabolism. *J Pharmacol Exp Ther* **295**:793–801.
- Streetman DS, Bleakley JF, Kim JS, Nafziger AN, Leeder JS, Gaedigk A, Gotschall R, Kearns GL, and Bertino JS Jr (2000) Combined phenotypic assessment of CYP1A2, CYP2C19, CYP2D6, CYP3A, N-acetyltransferase-2 and xanthine oxidase with the “Cooperstown cocktail”. *Clin. Pharmacol Ther* **68**:375–383.
- Stresser DM, Blanchard AP, Turner SD, Erve JC, Dandeneau AA, Miller VP, and Crespi CL (2000) Substrate-dependent modulation of CYP3A4 catalytic activity: analysis of 27 test compounds with four fluorometric substrates. *Drug Metab Dispos* **28**:1440–1448.
- Taipalensuu J, Tornblom H, Lindberg G, Einarsson C, Sjoqvist F, Melhus H, Garberg P, Sjöstrom B, Lundgren B, and Artursson P (2001) Correlation of gene expression of ten drug efflux proteins of the ATP-binding cassette transporter family in normal human jejunum and in human intestinal epithelial Caco-2 cell monolayers. *J Pharmacol Exp Ther* **299**:164–170.
- Takahashi H and Echizen H (2001) Pharmacogenetics of warfarin elimination and its clinical implications. *Clin Pharmacokinet* **40**:587–603.
- Tang W and Stearns RA (2001) Heterotropic cooperativity of cytochrome P450 3A4 and potential drug-drug interactions. *Curr Drug Metab* **2**:185–198.
- Tang W, Stearns RA, Kwei GY, Iliff SA, Miller RR, Egan MA, Yu NX, Dean DC, Kumar S, Shou M, et al. (1999) Interaction of diclofenac and quinidine in monkeys: stimulation of diclofenac metabolism. *J Pharmacol Exp Ther* **291**:1068–1074.
- Tayrouz Y, Ganssmann B, Ding R, Klingmann A, Aderjan R, Burhenne J, Haefeli WE, and Mikus G (2001) Ritonavir increases loperamide plasma concentrations without evidence for P-glycoprotein involvement. *Clin Pharmacol Ther* **70**:405–414.
- Tsunoda SM, Velez RL, and Greenblatt DJ (1999) Ketoconazole (K) inhibition of intestinal and hepatic cytochrome P450 3A4 (CYP3A4) activity using midazolam (M) as an in vivo probe. *Clin Pharmacol Ther* **65**:172.
- Tucker GT, Houston JB, and Huang SM (2001) Optimizing drug development: strategies to assess drug metabolism/transporter interaction potential—toward a consensus. *Pharm Res* **18**:1071–1080.
- Venkatakrishnan K, von Moltke LL, and Greenblatt DJ (2001) Application of the relative activity factor approach in scaling from heterologously expressed cytochromes P450 to human liver microsomes: studies on amitriptyline as a model substrate. *J Pharmacol Exp Ther* **297**:326–337.
- Veronese M, Gillen L, Dorval E, Hauck W, Waldman S, and Greenberg H (1999) Inhibition of hepatic and intestinal CYP3A4 by mibefradil measured by the erythromycin breath test (EBT) and oral midazolam pharmacokinetics (PK) (Abstract). *Clin Pharmacol Ther* **65**:131.
- Westphal K, Weinbrenner A, Giessmann T, Stuhr M, Franke G, Zschiesche M, Oertel R, Terhaag B, Kroemer HK, and Siegmund W (2000a) Oral bioavailability of digoxin is enhanced by talinolol: evidence for involvement of intestinal P-glycoprotein. *Clin Pharmacol Ther* **68**:6–12.
- Westphal K, Weinbrenner A, Zschiesche M, Franke G, Knoke M, Oertel R, Fritz P, von Richter O, Warzok R, Hachenberg T, et al. (2000b) Induction of P-glycoprotein by rifampin increases intestinal secretion of talinolol in human beings: a new type of drug/drug interaction. *Clin Pharmacol Ther* **68**:345–355.
- Wong JY, Seah ES, and Lee EJ (2000) Pharmacogenetics: the molecular genetics of CYP2D6 dependent drug metabolism. *Ann Acad Med Singapore* **29**:401–406.
- Worboys PD and Carlile DJ (2001) Implications and consequences of enzyme induction on preclinical and clinical drug development. *Xenobiotica* **31**:539–556.
- Wu CY, Benet LZ, Hebert MF, Gupta SK, Rowland M, Gomez DY, and Wacher VJ (1995) Differentiation of absorption and first-pass gut and hepatic metabolism in humans: studies with cyclosporine. *Clin Pharmacol Ther* **58**:492–497.
- Xie HG, Stein CM, Kim RB, Wilkinson GR, Flockhart DA, and Wood AJ (1999) Allelic, genotypic and phenotypic distributions of S-mephenytoin 4'-hydroxylase (CYP2C19) in healthy Caucasian populations of European descent throughout the world. *Pharmacogenetics* **9**:539–549.
- Yamashita S, Furubayashi T, Kataoka M, Sakane T, Sezaki H, and Tokuda H (2000) Optimized conditions for prediction of intestinal drug permeability using Caco-2 cells. *Eur J Pharm Sci* **10**:195–204.
- Yeates RA, Laufen H, and Zimmermann T (1996) Interaction between midazolam and clarithromycin: comparison with azithromycin. *Int J Clin Pharmacol Ther* **34**:400–405.
- Zhu B, Ou-Yang DS, Chen XP, Huang SL, Tan ZR, He N, and Zhou HH (2001) Assessment of cytochrome P450 activity by a five-drug cocktail approach. *Clin Pharmacol Ther* **70**:455–461.
- Zimmermann T, Yeates RA, Laufen H, Scharpf F, Leitold M, and Wildfeuer A (1996) Influence of the antibiotics erythromycin and azithromycin on the pharmacokinetics and pharmacodynamics of midazolam. *Arzneim-Forsch* **46**:213–217.