Guidance for Industry

Drug Interaction Studies — Study Design, Data Analysis, and Implications for Dosing and Labeling

DRAFT GUIDANCE

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U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
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Clinical Pharmacology

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Guidance for Industry[1]

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I. INTRODUCTION

This guidance provides recommendations for sponsors of new drug applications (NDAs) and biologics license applications (BLAs) for therapeutic biologics[2] who are performing in vitro and in vivo drug metabolism, drug transport, and drug-drug interaction studies. The guidance reflects the Agency's current view that the metabolism of an investigational new drug should be defined during drug development and that its interactions with other drugs should be explored as part of an adequate assessment of its safety and effectiveness. For drug-drug interactions, the approaches considered in the guidance are offered with the understanding that the relevance of a particular study depends on the characteristics and proposed indication of the drug under development. Furthermore, not every drug-drug interaction is metabolism-based, but may arise from changes in pharmacokinetics caused by absorption, distribution, and excretion interactions. Drug-drug interactions related to transporters are being documented with increasing frequency and are important to consider in drug development. Although less well studied, drug-drug interactions may alter pharmacokinetic/pharmacodynamic (PK/PD) relationships. These important areas are not considered in detail in this guidance.

Discussion of metabolic and other types of drug-drug interactions is also provided in other guidances, including the International Conference on Harmonization (ICH) *E7 Studies in Support of Special Populations: Geriatrics,* and *E3 Structure and Content of Clinical Study Reports,* and FDA guidances for industry on *Studying Drugs Likely to be Used in the Elderly* and *Study and Evaluation of Gender Differences in the Clinical Evaluation of Drugs*.

[1] This guidance has been prepared by the Drug-Drug Interaction Working Group in the Clinical Pharmacology Section of the Medical Policy Coordinating Committee in the Center for Drug Evaluation and Research, with input from the Center for Biologics Evaluation and Research, at the Food and Drug Administration.

[2] For more information on what constitutes a therapeutic biologic product, please see Internet site http://www.fda.gov/cder/biologics/qa.htm.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in Agency guidances means that something is suggested or recommended, but not required.

II. BACKGROUND

A. Metabolism

The desirable and undesirable effects of a drug arising from its concentrations at the sites of action are usually related either to the amount administered (dose) or to the resulting blood concentrations, which are affected by its absorption, distribution, metabolism, and/or excretion. Elimination of a drug or its metabolites occurs either by metabolism, usually by the liver or gut mucosa, or by excretion, usually by the kidneys and liver. In addition, protein therapeutics may be eliminated through a specific interaction with cell surface receptors, followed by internalization and lysosomal degradation within the target cell. Hepatic elimination occurs primarily by the cytochrome P450 family (CYP) of enzymes located in the hepatic endoplasmic reticulum, but may also occur by non-P450 enzyme systems, such as N-acetyl and glucuronosyl transferases. Many factors can alter hepatic and intestinal drug metabolism, including the presence or absence of disease and/or concomitant medications, or even some foods, such as grapefruit juice. While most of these factors are usually relatively stable over time, concomitant medications can alter metabolism abruptly and are of particular concern. The influence of concomitant medications on hepatic and intestinal metabolism becomes more complicated when a drug, including a prodrug, is metabolized to one or more active metabolites. In this case, the safety and efficacy of the drug/prodrug are determined not only by exposure to the parent drug but by exposure to the active metabolites, which in turn is related to their formation, distribution, and elimination. Therefore, adequate assessment of the safety and effectiveness of a drug includes a description of its metabolism and the contribution of metabolism to overall elimination. For this reason, the development of sensitive and specific assays for a drug and its important metabolites is critical to the study of metabolism and drug-drug interactions.

B. Drug-Drug Interactions

1. Metabolism-Based Drug-Drug Interactions

Many metabolic routes of elimination, including most of those occurring through the P450 family of enzymes, can be inhibited or induced by concomitant drug treatment. Observed changes arising from metabolic drug-drug interactions can be substantial — an order of magnitude or more decrease or increase in the blood and tissue concentrations of a drug or metabolite — and can include formation of toxic and/or

active metabolites or increased exposure to a toxic parent compound. These large changes in exposure can alter the safety and efficacy profile of a drug and/or its active metabolites in important ways. This is most obvious and expected for a drug with a narrow therapeutic range (NTR), but is also possible for non-NTR drugs as well (e.g., HMG CoA reductase inhibitors).

It is important that metabolic drug-drug interaction studies explore whether an investigational agent is likely to significantly affect the metabolic elimination of drugs already in the marketplace and likely in medical practice to be taken concomitantly and, conversely, whether drugs in the marketplace are likely to affect the metabolic elimination of the investigational drug. Even drugs that are not substantially metabolized can have important effects on the metabolism of concomitant drugs. For this reason, metabolic drug-drug interactions should be explored, even for an investigational compound that is not eliminated significantly by metabolism.

Classical biotransformation studies are not a general requirement for the evaluation of therapeutic biologics (ICH guidance *S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals*), although certain protein therapeutics modify the metabolism of drugs that are metabolized by the P450 enzymes. Type I interferons, for example, inhibit CYP1A2 production at the transcriptional and post-translational levels, inhibiting clearance of theophylline. The increased clinical use of therapeutic proteins may raise concerns regarding the potential for their impacts on drug metabolism. Generally, these interactions cannot be detected by in vitro assessment. Consultation with FDA is appropriate before initiating metabolic drug-drug interaction studies involving biologics.

Identifying metabolic differences in patient groups based on genetic polymorphism, or on other readily identifiable factors, such as age, race, and gender, can aid in interpreting results. The extent of interactions may be defined by these variables (e.g., CYP2D6 genotypes). Further, in subjects who lack the major clearance pathway, remaining pathways become important and should be understood and examined.

A specific objective of metabolic drug-drug interaction studies is to determine whether the interaction is sufficiently large to necessitate a dosage adjustment of the drug itself or the drugs with which it might be used, or whether the interaction would require additional therapeutic monitoring.

In some instances, understanding how to adjust dose or dosage regimen in the presence of an interacting drug, or how to avoid interactions, may allow marketing of a drug that would otherwise have been associated with an unacceptable level of toxicity. Sometimes a drug interaction can be used intentionally to increase levels or reduce elimination of another drug (e.g., ritonavir and lopinavir). Rarely, the degree

of interaction caused by a drug, or the degree to which other drugs alter its metabolism, can be such that it cannot be marketed safely.

2. Transporter-Based Drug-Drug Interactions

Transporter-based interactions have been increasingly documented. Examples of these include the inhibition or induction of transport proteins, such as P-glycoprotein (P-gp), organic anion transporter (OAT), organic anion transporting polypeptide (OATP), organic cation transporter (OCT), multidrug resistance-associated proteins (MRP), and breast cancer resistant protein (BCRP). Examples of transporter-based interactions include the interactions between digoxin and quinidine, fexofenadine and ketoconazole (or erythromycin), penicillin and probenecid, and dofetilide and cimetidine. Of the various transporters, P-gp is the most well understood and may be appropriate to evaluate during drug development. Table 1 in Appendix A lists some of the major human transporters and known substrates, inhibitors, and inducers.

III. GENERAL STRATEGIES

To the extent possible, drug development should follow a sequence in which early in vitro and in vivo investigations can either fully address a question of interest or provide information to guide further studies. Optimally, a sequence of studies could be planned, moving from in vitro studies to in vivo human studies, including those employing special study designs and methodologies where appropriate. In many cases, negative findings from early in vitro and early clinical studies can eliminate the need for later clinical investigations. Early investigations should explore whether a drug is eliminated primarily by excretion or metabolism, with identification of the principal metabolic routes in the latter case. Using suitable in vitro probes and careful selection of interacting drugs for early in vivo studies, the potential for drug-drug interactions can be studied early in the development process, with further study of observed interactions assessed later in the process, as needed. These early studies can also provide information about dose, concentration, and response relationships in the general population, specific populations, and individuals, which can be useful in interpreting the consequences of a drug-drug interaction. Once potential drug-drug interactions have been identified, based on in vitro and/or in vivo studies, sponsors are encouraged to design and examine the safety and efficacy databases of larger clinical studies, as feasible, to (1) permit confirmation/discovery of the interactions predicted from earlier studies and/or (2) verify that dosage adjustments or other prescribing modifications made in response to the potential interaction(s) have been adequate to avoid undesired consequences of the drug-drug interaction.

A. In Vitro Studies

A complete understanding of the quantitative relationship between the in vitro findings and in vivo results of metabolism/drug-drug interaction studies is still emerging. Nonetheless, in vitro studies can frequently serve as a screening mechanism to rule out the importance of a

metabolic pathway and the drug-drug interactions that occur through this pathway so that subsequent in vivo testing is unnecessary. This opportunity should be based on appropriately validated experimental methods and rational selection of substrate/interacting drug concentrations.

For example, if suitable in vitro studies at therapeutic concentrations indicate that CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP3A enzyme systems do not metabolize an investigational drug, then clinical studies to evaluate the effect of CYP2D6 inhibitors or CYP1A2, CYP2C8, CYP2C9, CYP2C19, or CYP3A inhibitors/inducers on the elimination of the investigational drug will not be needed.

Similarly, if in vitro studies indicate that an investigational drug does not inhibit CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP3A metabolism, then corresponding in vivo inhibition-based interaction studies of the investigational drug and concomitant medications eliminated by these pathways are not needed. Figure 1 in Appendix B shows a decision tree on when in vivo interaction studies are indicated based on in vitro metabolism, inhibition, and induction and in vivo metabolism data.

The CYP2D6 enzyme has not been shown to be inducible. Recent data have shown co-induction of CYP2C, CYP2B and ABCB1 (P-gp) transporter with CYP3A. CYP3A appears to be sensitive to all known co-inducers. Therefore, to evaluate whether an investigational drug induces CYP1A2, CYP2C8, CYP2C9, CYP2C19, or CYP3A, the initial in vitro induction evaluation may include only CYP1A2 and CYP3A. If in vitro studies indicate that an investigational drug does not induce CYP3A metabolism, then in vivo induction-based interaction studies of the investigational drug and concomitant medications eliminated by CYP2C/CYP2B and CYP3A may not be needed.

Drug interactions based on CYP2B6 are emerging as important interactions. When appropriate, in vitro evaluations based on this enzyme can be conducted. Other CYP enzymes, including CYP2A6 and CYP2E1, are less likely to be involved in clinically important drug interactions, but should be considered when appropriate.

Appendix C describes general considerations in the in vitro evaluation of CYP-related metabolism and interactions. Appendices C-1, C-2, and C-3 provide considerations in the experimental design, data analysis, and data interpretation in drug metabolizing enzyme identification, including CYP enzymes (new drug as a substrate), CYP inhibition (new drug as an inhibitor), and CYP induction (new drug as an inducer), respectively. Appendix D describes general considerations in the in vitro evaluation of P-gp substrates and inhibitors. Figures 1 and 2 in Appendix D provide decision trees on when in vivo P-gp based interaction studies are indicated based on in vitro evaluation.

B. Specific In Vivo Clinical Investigations

In addition to in vitro metabolism and drug-drug interaction studies, appropriately designed

pharmacokinetic studies, usually performed in the early phases of drug development, can provide important information about metabolic routes of elimination, their contribution to overall elimination, and metabolic drug-drug interactions. Together with information from in vitro studies, these in vivo investigations can be a primary basis of labeling statements and can often help avoid the need for further investigations. Further recommendations about these types of studies appear in section IV of this guidance.

C. Population Pharmacokinetic Screens

Population pharmacokinetic analyses of data obtained from large-scale clinical studies with sparse or intensive blood sampling can be valuable in characterizing the clinical impact of known or newly identified interactions, and in making recommendations for dosage modifications. The results from such analyses can be informative and sometimes conclusive when the clinical studies are adequately designed to detect significant changes in drug exposure due to drug-drug interactions. Simulations can provide valuable insights into optimizing the study design. Population pharmacokinetic evaluations may detect unsuspected drug-drug interactions. Population analysis can also provide further evidence of the absence of a drug-drug interaction when this is supported by prior evidence and mechanistic data. However, 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 vivo studies specifically designed to assess a drug-drug interaction. To be optimally informative, population pharmacokinetic studies should have carefully designed study procedures and sample collections. A guidance for industry on population pharmacokinetics is available (Ref. 11).

IV. DESIGN OF IN VIVO DRUG-DRUG INTERACTION STUDIES

If in vitro studies and other information suggest that in vivo drug-drug interaction studies would be helpful (e.g., based on Figure 1 in Appendix B), the following general issues and approaches should be considered. Consultation with FDA regarding study protocols is recommended. In the following discussion, the term *substrate* (S) is used to indicate the drug studied to determine whether its exposure is changed by another drug, termed the *interacting drug* (I). Depending on the study objectives, the substrate and the interacting drug can be the investigational agents or approved products.

A. Study Design

In vivo drug-drug interaction studies generally are designed to compare substrate concentrations with and without the interacting drug. Because a specific study can consider a number of questions and clinical objectives, many study designs for studying drug-drug interactions can be considered. A study can use a randomized crossover (e.g., S followed by S+I, S+I followed by S), a one-sequence crossover (e.g., S always followed by S+I or the reverse), or a parallel design (S in one group of subjects and S+I in another). The following possible dosing regimen combinations for a substrate and interacting drug can also be used:

single dose/single dose, single dose/multiple dose, multiple dose/single dose, and multiple dose/multiple dose. The selection of one of these or another study design depends on a number of factors for both the substrate and interacting drug, including (1) acute or chronic use of the substrate and/or interacting drug; (2) safety considerations, including whether a drug is likely to be an NTR (narrow therapeutic range) or non-NTR drug; (3) pharmacokinetic and pharmacodynamic characteristics of the substrate and interacting drugs; and (4) assessment of induction as well as inhibition. The inhibiting/inducing drugs and the substrates should be dosed so that the exposures of both drugs are relevant to their clinical use, including the highest doses likely to be used. Simulations can be helpful in selecting an appropriate study design. The following considerations may be useful:

- When attainment of steady state is important and either the substrate or interacting drugs and/or their metabolites have long half-lives and a loading dose to reach steady state promptly cannot be used, special approaches may be needed. These include the selection of a one-sequence crossover or a parallel design, rather than a randomized crossover study design.

- When it is important that a substrate and/or an interacting drug be studied at steady state because the effect of an interacting drug is delayed, as is the case for inducers and certain inhibitors, documentation that near steady state has been attained for the pertinent drug and metabolites of interest is critical. This documentation can be accomplished by sampling over several days prior to the periods when test samples are collected. This is important for both metabolites and the parent drug, particularly when the half-life of the metabolite is longer than the parent, and is especially important if both parent drug and metabolites are metabolic inhibitors or inducers.

- Studies can usually be open label (unblinded), unless pharmacodynamic endpoints (e.g., adverse events that are subject to bias) are critical to the assessment of the interaction.

- For a rapidly reversible inhibitor, administration of the interacting drug either just before or simultaneously with the substrate on the test day might increase sensitivity. For a mechanism-based inhibitor (a drug that requires metabolism prior to its inactivation of the enzyme; examples include erythromycin), administration of the inhibitor prior to the administration of the substrate drug can maximize the effect. If the absorption of an interacting drug (e.g., an inhibitor or an inducer) may be affected by other factors (e.g., the gastric pH), it may be appropriate to control the variables and confirm the absorption through plasma level measurements of the interacting drug.

- When the effects of two drugs on one another are of interest, the potential for interactions can be evaluated in a single study or two separate studies. Some design options are randomized three-period crossover, parallel group, and one-sequence crossover.

- To avoid variable study results because of uncontrolled use of dietary supplements, juices, or other foods that may affect various metabolizing enzymes and transporters during in vivo studies, it is important to exclude their use when appropriate.

  Examples of statements in a study protocol could include "Participants will be excluded for the following reasons: Use of prescription or over-the-counter medications, *including herbal products*, or alcohol within two weeks prior to enrollment," "For at least two weeks prior to the start of the study until its conclusion, volunteers will not be allowed to eat any food or drink any beverage containing *alcohol, grapefruit or grapefruit juice, apple or orange juice, vegetables from the mustard green family* (e.g., kale, broccoli, watercress, collard greens, kohlrabi, brussels sprouts, mustard) and *charbroiled meats."*

B. Study Population

Clinical drug-drug interaction studies can generally be performed using healthy volunteers. Findings in this population should predict findings in the patient population for which the drug is intended. Safety considerations may preclude the use of healthy subjects, however, and in certain circumstances, subjects drawn from the population of patients for whom the investigational drug is intended offer advantages, including the opportunity to study pharmacodynamic endpoints not present in healthy subjects. Performance of phenotype or genotype determinations to identify genetically determined metabolic polymorphisms is important in evaluating effects on enzymes with polymorphisms, notably CYP2D6, CYP2C19, and CYP2C9. The extent of drug interactions (inhibition or induction) may be different depending on the subjects' genotype for the specific enzyme being evaluated. Subjects lacking the major clearance pathway, for example, cannot show metabolism and remaining pathways can become important and should be understood and examined.

C. Choice of Substrate and Interacting Drugs

1. *Investigational Drug as an Inhibitor or an Inducer of CYP Enzymes*

In contrast to earlier approaches that focused mainly on a specific group of approved drugs (digoxin, hydrochlorothiazide) where co-administration was likely or the clinical consequences of an interaction were of concern, improved understanding of the mechanistic basis of metabolic drug-drug interactions enables more general approaches to and conclusions from specific drug-drug interaction studies. In studying an investigational drug as the interacting drug, the choice of substrates (approved drugs) for initial in vivo studies depends on the P450 enzymes affected by the interacting drug. In testing inhibition, the substrate selected should generally be one whose pharmacokinetics are markedly altered by co-administration of known specific inhibitors of the enzyme systems to assess the impact of the interacting investigational drug. Examples of substrates include (1) midazolam for CYP3A; (2)

theophylline for CYP1A2; (3) repaglinide for CYP2C8; (4) warfarin for CYP2C9 (with the evaluation of S-warfarin); (5) omeprazole for CYP2C19; and (6) desipramine for CYP2D6. Additional examples of substrates, along with inhibitors and inducers of specific CYP enzymes, are listed in Table 2 in Appendix A. If the initial study determines an investigation drug either inhibit or induce metabolism, further studies using other substrates, representing a range of substrates, based on the likelihood of co-administration, may be useful. If the initial study is negative with the most sensitive substrates (for sensitive substrates, see Tables 3 and 4 in Appendix A), it can be presumed that less sensitive substrates will also be unaffected.

CYP3A inhibitors can be classified based on their in vivo fold-change in the plasma AUC of oral midazolam or other CYP3A substrate, when given concomitantly. For example, if an investigational drug increases the AUC of oral midazolam or other CYP3A substrates by 5-fold or higher ($\geq$ 5-fold), it can be labeled as a *strong* CYP3A inhibitor. If an investigational drug, when given at the highest dose and shortest dosing interval, increases the AUC of oral midazolam or other sensitive CYP3A substrates by between 2- and 5-fold ($\geq$ 2- and <5-fold) when given together, it can be labeled as a *moderate* CYP3A inhibitor. Similarly, if an investigational drug, when given at the highest dose and shortest dosing interval, increases the AUC of oral midazolam or other sensitive CYP3A substrates by between 1.25- and 2-fold ($\geq$ 1.25- and < 2-fold), it can be labeled as a *weak* CYP3A inhibitor. When an investigational drug is determined to be an inhibitor of CYP3A, its interaction with sensitive CYP3A substrates or CYP3A substrates with narrow therapeutic range (see Table 3 in Appendix A for a list) can be described in various sections of the labeling, as appropriate. Similar classifications of inhibitors of other CYP enzymes are discussed in section V.

When an in vitro evaluation cannot rule out the possibility that an investigational drug is an inducer of CYP3A (see Appendix C-3), an in vivo evaluation can be conducted using the most sensitive substrate (e.g., oral midazolam, see Table 3 in Appendix A). When midazolam has been co-administered orally following administration of multiple doses of the investigational drug, as may have been done as part of an in vivo inhibition evaluation, and the results are negative, it can be concluded that the investigational drug is not an inducer of CYP3A (in addition to the conclusion that it is not an inhibitor of CYP3A). In vivo induction evaluation has often been conducted with oral contraceptives. However, as they are not the most sensitive substrates, negative data may not exclude the possibility that the investigational drug may be an inducer of CYP3A.

Simultaneous administration of a mixture of substrates of CYP enzymes in one study (i.e., a "cocktail approach") in human volunteers is another way to evaluate a drug's inhibition or induction potential, provided that the study is designed properly and the following factors are present: (1) the substrates are specific for individual CYP enzymes; (2) there are no interactions among these substrates; and (3) the study is

conducted in a sufficient number of subjects (see section IV.G). Negative results from a cocktail study can eliminate the need for further evaluation of particular CYP enzymes. However, positive results can indicate the need for further in vivo evaluation to provide quantitative exposure changes (such as AUC, Cmax), if the initial evaluation only assessed the changes in the urinary parent to metabolite ratios. The data generated from a cocktail study can supplement data from other in vitro and in vivo studies in assessing a drug's potential to inhibit or induce CYP enzymes.

2. Investigational Drug as a Substrate of CYP Enzymes

In testing an investigational drug for the possibility that its metabolism is inhibited or induced (i.e., as a substrate), selection of the interacting drugs should be based on in vitro or in vivo studies identifying the enzyme systems that metabolize the drug. The choice of interacting drug can then be based on known, important inhibitors of the pathway under investigation. For example, if the investigational drug is shown to be metabolized by CYP3A and the contribution of this enzyme to the overall elimination of this drug is either substantial (> 25% of the clearance pathway) or unknown, the choice of inhibitor and inducer could be ketoconazole and rifampin, respectively, because they are the most sensitive in identifying an effect of interest. If the study results are negative, then absence of a clinically important drug-drug interaction for the metabolic pathway would have been demonstrated. If the clinical study of the strong, specific inhibitor/inducer is positive and the sponsor wished to determine whether there is an interaction between the test drug and other less potent specific inhibitors or inducers, or to give advice on dosage adjustment, further clinical studies would generally be needed (see Table 2, Appendix A, for a list of CYP inhibitors and inducers; see Table 5, Appendix A, for additional 3A inhibitors). If a drug is metabolized by CYP3A and its plasma AUC is increased 5-fold or higher by CYP3A inhibitors, it is considered a *sensitive substrate* of CYP3A. The labeling can indicate that it is a "sensitive CYP3A substrate" and its use with strong or moderate inhibitors may call for caution, depending on the drug's exposure-response relationship. If a drug is metabolized by CYP3A and its exposure-response relationship indicates that increases in the exposure levels by the concomitant use of CYP3A inhibitors may lead to serious safety concerns (e.g., Torsades de Pointes), it is considered as a "CYP3A substrate with narrow therapeutic range" (see Table 3 of Appendix A for a list). Similar classifications of substrates of other CYP enzymes are discussed in section V and listed in Table 6, Appendix A.

If an orally administered drug is a substrate of CYP3A and has low oral bioavailability because of extensive presystemic extraction contributed by enteric CYP3A, grapefruit juice may have a significant effect on its systemic exposure. Use of the drug with grapefruit juice may call for caution, depending on the drug's exposure-response relationship (see section V for labeling implications).

If a drug is a substrate of CYP3A or P-gp and co-administration with St. John's wort

can decrease the systemic exposure and effectiveness, St John's wort may be listed in the labeling along with other known inducers, such as rifampin, rifabutin, rifapentin, dexamethasone, phenytoin, carbamazepine, or phenobarbital, as possibly decreasing plasma levels.

If a drug is metabolized by a polymorphic enzyme (such as CYP2D6, CYP2C9, or CYP2C19), the comparison of pharmacokinetic parameters of this drug in poor metabolizers versus extensive metabolizers may indicate the extent of interaction of this drug with strong inhibitors of these enzymes, and make interaction studies with such inhibitors unnecessary. When the above study shows significant interaction, further evaluation with weaker inhibitors may be necessary.

There may be situations when an evaluation of the effect of multiple CYP inhibitors on the drug can be informative. For example, it may be appropriate to conduct an interaction study with more that one inhibitor if all of the following conditions are met: (1) the drug exhibits blood concentration-dependent safety concerns; (2) multiple CYP enzymes are responsible for the metabolic clearance of the drug; (3) the residual or non-inhibitable drug clearance is low. Under these conditions, the effect of multiple, CYP-selective inhibitors on the blood AUC of a drug may be much greater than the product of the fold AUC changes observed when the inhibitors are given individually with the drug. The degree of uncertainty will depend on the residual fractional clearance (the smaller the fraction, the greater the concern) and the relative fractional clearances of the inhibited pathway. However, if results from a study with a single inhibitor trigger a safety concern (i.e., contraindication), no multiple inhibitor studies will be necessary. Additional considerations may include the likelihood of co-administration of the drug with multiple inhibitors. Before investigating the impact of multiple inhibitors on drug exposure, it is important to first characterize the individual effects of the CYP inhibitors and to estimate the combined effect of the inhibitors based on computer simulation. For safety concerns, lower doses of the investigational drug may be appropriate for evaluating the fold increase in systemic exposure when combined with multiple inhibitors.

The implications of simultaneous inhibition of a dominant CYP enzyme(s) and an uptake or efflux transporter that controls the availability of the drug to CYP enzymes can be just as profound as that of multiple CYP inhibitors. For example, the large effect of co-administration of itraconazole and gemfibrozil on the systemic exposure (AUC) of repaglinide may be attributed to collective effects on both enzyme and transporters. Unfortunately, current knowledge does not permit the presentation of specific guidance. The sponsor will need to use appropriate judgement when considering this situation.

3. Investigational Drug as an Inhibitor or an Inducer of P-gp Transporter

In testing an investigational drug for the possibility that it may be an inhibitor/inducer

of P-gp, selection of digoxin or other known substrates of P-gp may be appropriate.

4. Investigational Drug as a Substrate of P-gp Transporter

In testing an investigational drug for the possibility that its transport may be inhibited or induced (as a substrate of P-gp), an inhibitor of P-gp, such as ritonavir, cyclosporine, or verapamil, or an inducer, such as rifampin should be studied. In cases where the drug is also a CYP3A substrate, inhibition should be studied by using a strong inhibitor of both P-gp and CYP3A, such as ritonavir.

5. Investigational Drug as a Substrate of other Transporters

In testing an investigational drug for the possibility that its disposition may be inhibited or induced (i.e., as a substrate of transporters other than or in addition to P-gp), it may be appropriate to use an inhibitor of many transporters (e.g., P-gp, OATP), such as cyclosporine. Recent interactions involving drugs that are substrates for transporters other than or in addition to P-gp include some HMG Co-A reductase inhibitors, rosuvastatin, and pravastatin.

D. Route of Administration

The route of administration chosen for a metabolic drug-drug interaction study is important. For an investigational agent, the route of administration should generally be the one planned for clinical use. When multiple routes are being developed, the need for metabolic drug-drug interaction studies by multiple routes depends on the expected mechanism of interaction and the similarity of corresponding concentration-time profiles for parent and metabolites. If only oral dosage forms will be marketed, studies with an intravenous formulation are not usually needed, although information from oral and intravenous dosings may be useful in discerning the relative contributions of alterations in absorption and/or presystemic clearance to the overall effect observed for a drug interaction. Sometimes certain routes of administration can reduce the utility of information from a study. For example, intravenous administration of a substrate drug may not reveal an interaction for substrate drugs where intestinal CYP3A activity markedly alters bioavailability. For an approved agent used either as a substrate or interacting drug, the route of administration will depend on available marketed formulations.

E. Dose Selection

For both a substrate (investigational drug or approved drug) and interacting drug (investigational drug or approved drug), testing should maximize the possibility of finding an interaction. For this reason, we recommend that the maximum planned or approved dose and shortest dosing interval of the interacting drug (as inhibitors or inducers) be used. For example, when using ketoconazole as an inhibitor of CYP3A, dosing at 400 mg QD for multiple days would be preferable to lower doses. When using rifampin as an inducer,

dosing at 600 mg QD for multiple days would be preferable to lower doses. In some instances, doses smaller than those to be used clinically may be recommended for substrates on safety grounds. In such instances, any limitations of the sensitivity of the study to detect the drug-drug interaction due to the use of lower doses should be discussed by the sponsor in the protocol and study report.

F. Endpoints

Changes in pharmacokinetic parameters can be used to assess the clinical importance of drug-drug interactions. Interpretation of findings from these studies will be aided by a good understanding of dose/concentration and concentration/response relationships for both desirable and undesirable drug effects in the general population or in specific populations. A CDER/CBER guidance for industry on *Exposure-Response Relationships — Study Design, Data Analysis, and Regulatory Applications* provides considerations in the evaluation of exposure-response relationships. In certain instances, reliance on endpoints in addition to pharmacokinetic measures/parameters may be useful. Examples include INR measurement (when studying warfarin interactions) or QT interval measurements.

1. Pharmacokinetic Endpoints

The following measures and parameters of substrate PK should be obtained in every study: (1) exposure measures such as AUC, Cmax, time to Cmax (Tmax), and others as appropriate; and (2) pharmacokinetic parameters such as clearance, volumes of distribution, and half-lives. In some cases, these measures may be of interest for the inhibitor or inducer as well, notably where the study is assessing possible effects on both study drugs. Additional measures may help in steady state studies (e.g., trough concentration) to demonstrate that dosing strategies were adequate to achieve near steady state before and during the interaction. In certain instances, an understanding of the relationship between dose, blood concentrations, and response may lead to a special interest in certain pharmacokinetic measures and/or parameters. For example, if a clinical outcome is most closely related to peak concentration (e.g., tachycardia with sympathomimetics), Cmax or another early exposure measure might be most appropriate. Conversely, if the clinical outcome is related more to extent of absorption, AUC would be preferred. The frequency of sampling should be adequate to allow accurate determination of the relevant measures and/or parameters for the parent and metabolites. For the substrate, whether the investigational drug or the approved drug, determination of the pharmacokinetics of important active metabolites is important.

2. Pharmacodynamic Endpoints

Pharmacokinetic measures are usually sufficient for drug-drug interaction studies, although pharmacodynamic measures can sometimes provide additional useful information. Pharmacodynamic measures may be indicated when a

pharmacokinetic/pharmacodynamic relationship for the substrate endpoints of interest is not established or when pharmacodynamic changes do not result solely from pharmacokinetic interactions (e.g., additive effect of quinidine and tricyclic antidepressants on QT interval). In most cases, when an approved drug is studied as a substrate, the pharmacodynamic impact of a given change in blood level (Cmax, AUC) caused by an investigational interaction should be known from other data. If a PK/PD study is needed, it will generally need to be larger than the typical PK study (e.g., a study of QT interval effects).

G. Sample Size and Statistical Considerations

The goal of the interaction study is to determine whether there is any increase or decrease in exposure to the substrate in the presence of the interacting drug. If there is, its implications must be assessed by an understanding of PK/PD relations both for Cmax and AUC.

Results of drug-drug interaction studies should be reported as 90% confidence intervals about the geometric mean ratio of the observed pharmacokinetic measures with (S+I) and without the interacting drug (S alone). Confidence intervals provide an estimate of the distribution of the observed systemic exposure measure ratio of (S+I) versus (S alone) and convey a probability of the magnitude of the interaction. In contrast, tests of significance are not appropriate because small, consistent systemic exposure differences can be statistically significant ($p < 0.05$) but not clinically relevant.

When a drug-drug interaction of potential importance is clearly present (e.g., comparisons indicate twofold (or lower for certain NTR drugs) or greater increments in systemic exposure measures for (S+I)), the sponsor should provide specific recommendations regarding the clinical significance of the interaction based on what is known about the dose-response and/or PK/PD relationship for either the investigational agent or the approved drugs used in the study. For a new drug, the more difficult issue is the impact on the investigational drug as substrate. For inhibition or induction by the investigational drug, the main consequence of a finding will be to add the drug to the list of inhibitors or inducers likely already present in labeling of the older drug. This information can form the basis for reporting study results and for making recommendations in the package insert with respect to either the dose, dosing regimen adjustments, precautions, warnings, or contraindications of the investigational drug or the approved drug. FDA recognizes that dose-response and/or PK/PD information can sometimes be incomplete or unavailable, especially for an older approved drug used as S.

The sponsor may wish to make specific claims in the package insert that no drug-drug interaction of clinical significance occurs. In these instances, it would be helpful for the sponsor to recommend specific *no effect* boundaries, or clinical equivalence intervals, for a drug-drug interaction. No effect boundaries represent the interval within which a change in a systemic exposure measure is considered not clinically meaningful.

There are two approaches to defining no effect boundaries:

Approach 1: No effect boundaries can be based on population (group) average dose and/or concentration-response relationships, PK/PD models, and other available information for the substrate drug to define a degree of difference caused by the interaction that is of no clinical consequence. If the 90% confidence interval for the systemic exposure measurement in the drug-drug interaction study falls completely within the *no effect* boundaries, the sponsor can conclude that no clinically significant drug-drug interaction was present.

Approach 2: In the absence of *no effect* boundaries defined in Approach 1, a sponsor can use a default *no effect* boundary of 80-125% for both the investigational drug and the approved drugs used in the study. When the 90% confidence intervals for systemic exposure ratios fall entirely within the equivalence range of 80-125%, standard Agency practice is to conclude that no clinically significant differences are present. This is, however, a very conservative standard and a substantial sample would need to be studied to meet it.

The selection of the number of subjects for a given drug-drug interaction study will depend on how small an effect is clinically important to detect or rule out, the inter- and intra-subject variability in pharmacokinetic measurements, and possibly other factors or sources of variability not well recognized.

V. LABELING IMPLICATIONS

It is important that all relevant information on the metabolic pathways and metabolites and pharmacokinetic interactions be included in the PHARMACOKINETICS subsection of the CLINICAL PHARMACOLOGY section of the labeling. The clinical consequences of metabolism and interactions should be placed in DRUG INTERACTIONS, WARNINGS AND PRECAUTIONS, BOXED WARNINGS, CONTRAINDICATIONS, or DOSAGE AND ADMINISTRATION sections, as appropriate. Information related to clinical consequences should not be included in detail in more than one section, but rather referenced from one section to other sections, as appropriate. When the metabolic pathway or interaction data results in recommendations for dosage adjustments, contraindications, or warnings (e.g., co-administration should be avoided) that are included in the BOXED WARNINGS, CONTRAINDICATIONS, WARNINGS AND PRECAUTIONS, or DOSAGE AND ADMINISTRATION sections, these recommendations should also be included in HIGHLIGHTS. Refer to the guidance for industry on *Labeling for Human Prescription Drug and Biological Products – Implementing the New Content and Format Requirements,* and *Clinical Pharmacology and Drug Interaction Labeling* for more information on presenting drug interaction information in labeling.

In certain cases, information based on clinical studies not using the labeled drug can be described, with an explanation that similar results may be expected for that drug. For example, if a drug has been determined to be a strong inhibitor of CYP3A, it does not need to be tested with all CYP3A substrates to warn about an interaction with sensitive CYP3A

substrates and CYP3A substrates with narrow therapeutic range. An actual test involving a single substrate would lead to labeling concerning use with all sensitive and NTR substrates. Table 3 in Appendix A lists examples of sensitive CYP3A substrates and CYP3A substrates with narrow therapeutic range.

Table 5 in Appendix A lists examples of strong, moderate, and weak CYP3A inhibitors. If a drug has been determined to be a sensitive CYP3A substrate or a CYP3A substrate with a narrow therapeutic range, it does not need to be tested with all strong or moderate inhibitors of CYP3A to warn about an interaction with strong or moderate CYP3A inhibitors, and it might be labeled in the absence of any actual study if its metabolism is predominantly by the CYP3A route. Similarly, if a drug has been determined to be a sensitive CYP3A substrate or a CYP3A substrate with a narrow therapeutic range, it does not need to be tested with all CYP3A inducers to warn about an interaction with CYP3A inducers. Examples of CYP3A inducers include *rifampin, rifabutin, rifapentin, dexamethasone, phenytoin, carbamazepine, phenobarbital, and St. John's wort.*

A similar classification system can be used for inhibitors of other CYP enzymes (Table 6 in Appendix A).

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APPENDIX A- Tables

Table 1. Major human transporters [1,2]

Gene	Aliases	Tissue	Drug Substrate	Inhibitor	Inducer
ABCB1	P-gp, MDR1	intestine, liver, kidney, brain, placenta, adrenal, testes	digoxin, fexofenadine, indinavir, vincristine, colchicine. topotecan, paclitaxel	ritonavir, cyclosporine, verapamil, erythromycin, ketocoanzole, itraconazole, quinidine, elacridar (GF120918) LY335979 valspodar (PSC833)	rifampin, St John's wort
ABCB4	MDR3	liver	digoxin, paclitaxel, vinblastine		
ABCB11	BSEP	liver	vinblastine		
ABCC1	MRP1	intestine, liver, kidney, brain	adefovir, indinavir		
ABCC2	MRP2, CMOAT	intestine, liver, kidney, brain	indinavir, cisplatin,	cyclosporine	
ABCC3	MRP3, CMOAT2	intestine, liver, kidney, placenta, adrenal	etoposide, methotrexate, tenoposide		
ABCC4	MRP4				
ABCC5	MRP5				
ABCC6	MRP6	liver, kidney	cisplatin, daunorubicin		
ABCG2	BCRP	intestine, liver, breast, placenta	daunorubicin, doxorubicin, topotecan, rosuvastatin, sulfasalazine	elacridar (GF120918), gefitinib	
SLCO1B1	OATP1B1, OATP-C OATP2	liver	rifampin, rosuvastatin, methotrexate, pravastatin, thyroxine	cyclosporine, rifampin	
SLCO1B3	OATP1B3, OATP8,	liver	digoxin, methotrexate, rifampin,		
SLCO2B1	SLC21A9, OATP-B	intestine, liver, kidney, brain	pravastatin		
SLC10A1	NTCP	liver, pancreas	rosuvastatin		

SLC10A2	ASBT	ileum, kidney, biliary tract			
SLC15A1	PEPT1	intestine, kidney	ampicillin, amoxicillin, captopril, valacyclovir		
SLC15A2	PEPT2	kidney	ampicillin, amoxicillin, captopril, valacyclovir		
SLC22A1	OCT-1	liver	acyclovir, amantadine, desipramine, ganciclovir metformin	disopyramide, midazolam, phenformin, phenoxy-benzamine quinidine, quinine, ritonavir, verapamil	
SLC22A2	OCT2	kidney, brain	amantadine, cimetidine, memantine	desipramine, phenoxy-benzamine quinine	
SLC22A3	OCT3	skeletal muscle, liver, placenta, kidney, heart	cimetidine	desipramine, prazosin, phenoxy-benzamine	
SLC22A4	OCTN1	kidney, skeletal muscle, placenta, prostate, heart	quinidine, verapamil		
SLC22A5	OCTN2	kidney, skeletal muscle, prostate, lung, pancreas, heart, small intestine, liver	quinidine, verapamil		
SLC22A6	OAT1	kidney, brain	acyclovir, adefovir, methotrexate, zidovudine	probenecid, cefadroxil, cefamandole, cefazolin,	
SLC22A7	OAT2	liver, kidney	zidovudine		
SLC22A8	OAT3	kidney, brain	cimetidine, methotrexate, zidovudine	probenecid, cefadroxil, cefamandole, cefazolin,	

[(1)] Note that this is not an exhaustive list. For an updated list, see the following link http://www.fda.gov/cder/drug/drugInteractions/default.htm

[(2)] ABC:ATP-binding cassette transporter superfamily; SLC: solute-linked carrier transporter family; SLCO: solute-linked carrier organic anion transporter family; MDR1: multi-drug resistance; MRP: multi-drug resistance related protein; BSEP:bile salt export pump; BCRP: breast cancer resistance protein; OAT: organic anion transporter; OCT: organic cation transporter; NTCP: sodium taurocholate co-transporting polypeptide; ASBT: apical sodium-dependent bile salt transporter.

Table 2. Examples of in vivo substrate, inhibitor, and inducer for specific CYP enzymes recommended for study (oral administration) [1,2]

CYP	Substrate	Inhibitor	Inducer
1A2	theophylline, caffeine	fluvoxamine	smokers versus non-smokers[3]
2B6	efavirenz		rifampin
2C8	repaglinide, rosiglitazone	gemfibrozil	rifampin
2C9	warfarin, tolbutamide	fluconazole, amiodarone (use of PM versus EM subjects) [4]	rifampin
2C19	omeprazole, esoprazole, lansoprazole, pantoprazole	omeprazole, fluvoxamine, moclobemide (use of PM versus EM subjects) [4]	rifampin
2D6	desipramine, dextromethorphan, atomoxetine	paroxetine, quinidine, fluoxetine (use of PM versus EM subjects) [4]	none identified
2E1	chlorzoxazone	disulfirum	ethanol
3A4/ 3A5	midazolam, buspirone, felodipine, lovastatin, eletriptan, sildenafil, simvastatin, triazolam	atazanavir, clarithromycin, indinavir, itraconazole, ketoconazole, nefazodone, nelfinavir, ritonavir, saquinavir, telithromycin	rifampin, carbamazepine

[1] Substrates for any particular CYP enzyme listed in this table are those with plasma AUC values increased by **2-fold or higher** when co-administered with inhibitors of that CYP enzyme; for CYP3A, only those with plasma AUC increased by **5-fold or higher** are listed. Inhibitors listed are those that increase plasma AUC values of substrates for that CYP enzyme by 2-fold or higher. For CYP3A inhibitors, only those that increase AUC of CYP3A substrates by 5-fold or higher are listed. Inducers listed are those that decrease plasma AUC values of substrates for that CYP enzyme by **30% or higher**.

[2] Note that this is not an exhaustive list. For an updated list, see the following link http://www.fda.gov/cder/drug/drugInteractions/default.htm

[3] A clinical study can be conducted in smokers as compared to non-smokers (in lieu of an interaction study with an inducer), when appropriate.

[4] A clinical study can be conducted in poor metabolizers (PM) as compared to extensive metabolizers (EM) for the specific CYP enzyme (in lieu of an interaction study with an inhibitor), when appropriate.

Table 3. Examples[1] of sensitive CYP3A substrates or CYP3A substrates with narrow therapeutic range

Sensitive CYP3A substrates[2]	CYP3A Substrates with Narrow therapeutic range[3]
budesonide, buspirone, eplerenone, eletriptan, felodipine, fluticasone, lovastatin, midazolam, saquinavir, sildenafil, simvastatin, triazolam, vardenafil	alfentanil, astemizole(a), cisapride(a), cyclosporine, diergotamine, ergotamine, fentanyl, pimozide, quinidine, sirolimus, tacrolimus, terfenadine(a)

[1] Note that this is not an exhaustive list. For an updated list, see the following link http://www.fda.gov/cder/drug/drugInteractions/default.htm

[2] *Sensitive CYP3A substrates* refers to drugs whose plasma AUC values have been shown to increase 5-fold or higher when co-administered with a known CYP3A inhibitor.

[3] CYP3A *substrates with narrow therapeutic range* refers to drugs whose exposure-response indicates that increases in their exposure levels by the concomitant use of CYP3A inhibitors may lead to serious safety concerns (e.g., Torsades de Pointes).

(a) Not available in the United States.

716 Table 4. Examples[(1)] of sensitive CYP substrates or CYP substrates with narrow
717 therapeutic range
718

Sensitive CYP1A2 substrates[(2)]	CYP1A2 substrates with narrow therapeutic range[(3)]
duloxetine, alosetron	theophylline, tizanidine
Sensitive CYP2C8 substrates[(2)]	**CYP2C8 substrates with narrow therapeutic range[(3)]**
repaglinide	paclitaxel
Sensitive CYP2C9 substrates[(2)]	**CYP2C9 substrates with narrow therapeutic range[(3)]**
	warfarin, phenytoin
Sensitive CYP2C19 substrates[(2)]	**CYP2C19 substrates with narrow therapeutic range[(3)]**
omeprazole	s-mephenytoin
Sensitive CYP2D6 substrates[(2)]	**CYP2D6 substrates with narrow therapeutic range[(3)]**
desipramine	thioridazine

719 [(1)] Note that this is not an exhaustive list. For an updated list, see the following link
720 http://www.fda.gov/cder/drug/drugInteractions/default.htm
721 [(2)] *Sensitive CYP substrates* refers to drugs whose plasma AUC values have been shown to increase 5-
722 fold or higher when co-administered with a known CYP inhibitor.
723 [(3)] CYP *substrates with narrow therapeutic range* refers to drugs whose exposure-response indicates that
724 increases in their exposure levels by the concomitant use of CYP inhibitors may lead to serious safety
725 concerns (e.g., Torsades de Pointes).

Table 5. Classification of CYP3A inhibitors[(1)]

Strong CYP3A inhibitors	Moderate CYP3A inhibitors	Weak CYP3A inhibitors
≥ 5-fold increase in AUC	≥ 2 but <5-fold increase in AUC	≥ 1.25 but <2-fold increase in AUC
atazanavir, clarithromycin, indinavir, itraconazole, ketoconazole, nefazodone, nelfinavir, ritonavir, saquinavir, telithromycin	amprenavir, aprepitant, diltiazem, erythromycin, fluconazole, fosamprenavir, grapefruit juice(a), verapamil	cimetidine

(1) Please note the following:

- A *strong inhibitor* is one that caused a ≥ 5-fold increase in the plasma AUC values or more than 80% decrease in clearance of CYP3A substrates (not limited to midazolam, a sensitive CYP3A substrate) in clinical evaluations
- A *moderate inhibitor* is one that caused a ≥ 2- but < 5-fold increase in the AUC values or 50-80% decrease in clearance of sensitive CYP3A substrates when the inhibitor was given at the highest approved dose and the shortest dosing interval in clinical evaluations.
- A *weak inhibitor* is one that caused a ≥ 1.25 - but < 2-fold increase in the AUC values or 20-50% decrease in clearance of sensitive CYP3A substrates when the inhibitor was given at the highest approved dose and the shortest dosing interval in clinical evaluations
- This is not an exhaustive list. For an updated list, see the following link http://www.fda.gov/cder/drug/drugInteractions/default.htm

(a) The effect of grapefruit juice varies widely.

Table 6. Classification of inhibitors of other CYP enzymes[(1)]

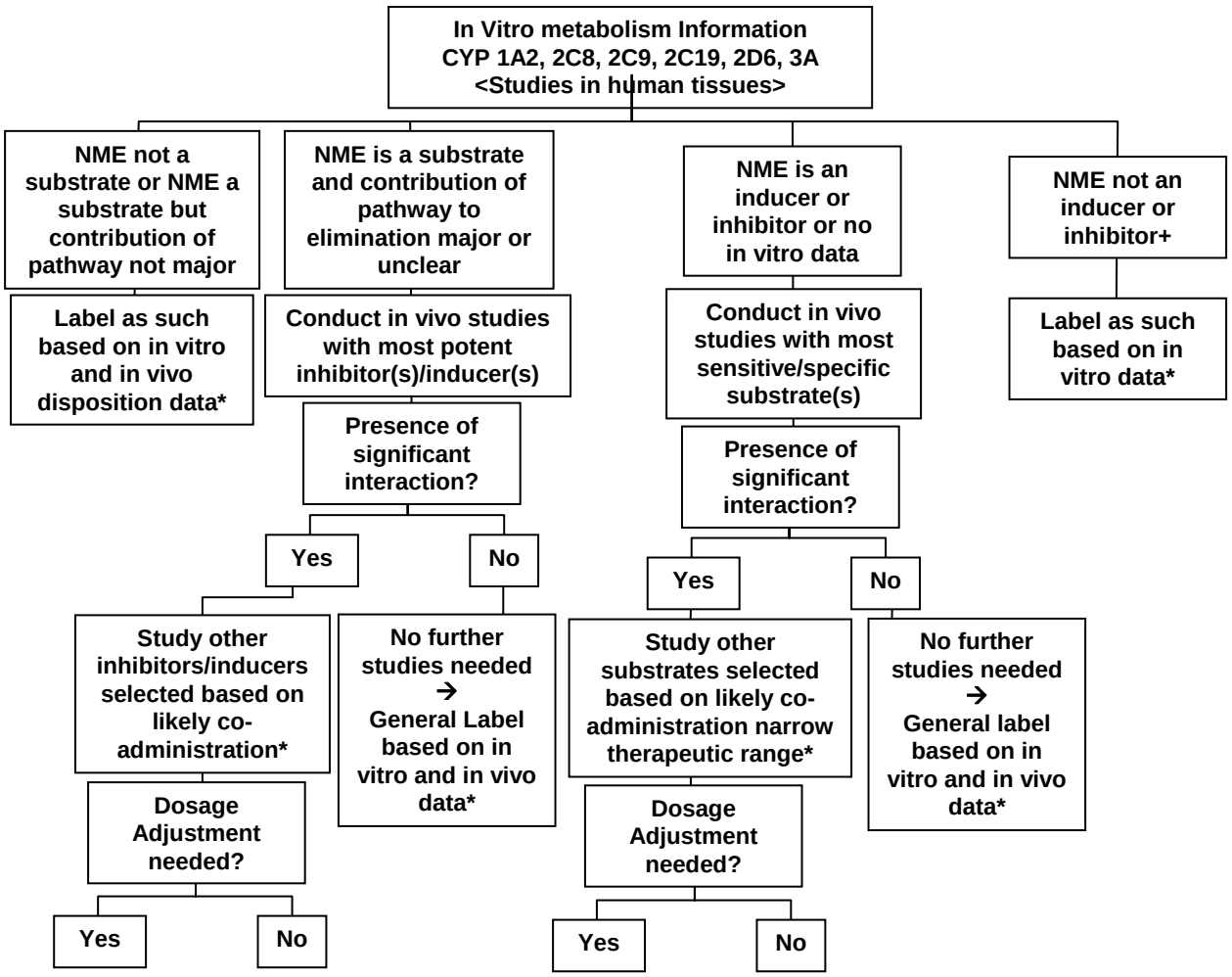
Strong CYP1A2 inhibitors	Moderate CYP1A2 inhibitors	Weak CYP1A2 inhibitors
fluvoxamine	ciprofloxacin, mexiletine, propafenone, zileuton	acyclovir, cimetidine, famotidine, norfloxacin, verapamil
Strong CYP2C8 inhibitors	Moderate CYP2C8 inhibitors	Weak CYP2C8 inhibitors
gemfibrozil		trimethoprim
Strong CYP2C9 inhibitors	Moderate CYP2C9 inhibitors	Weak CYP2C9 inhibitors
	amiodarone, fluconazole, oxandrolone	sulfinpyrazone
Strong CYP2C19 inhibitors	Moderate CYP2C19 inhibitors	Weak CYP2C19 inhibitors
omeprazole		
Strong CYP2D6 inhibitors	Moderate CYP2D6 inhibitors	Weak CYP2D6 inhibitors
fluoxetine, paroxetine, quinidine	duloxetine, terbinafine	amiodarone, sertraline

(1) Please note the following:

- A *strong inhibitor* is one that caused a ≥ 5-fold increase in the plasma AUC values or more than 80% decrease in clearance of CYP substrates (not limited to sensitive CYP substrate) in clinical evaluations
- A *moderate inhibitor* is one that caused a ≥ 2- but < 5-fold increase in the AUC values or 50-80% decrease in clearance of sensitive CYP substrates when the inhibitor was given at the highest approved dose and the shortest dosing interval in clinical evaluations.
- A *weak inhibitor* is one that caused a ≥ 1.25 - but < 2-fold increase in the AUC values or 20-50% decrease in clearance of sensitive CYP substrates when the inhibitor was given at the highest approved dose and the shortest dosing interval in clinical evaluations
- This is not an exhaustive list. For an updated list, see the following link http://www.fda.gov/cder/drug/drugInteractions/default.htm

Appendix B- Figures

Figure 1. CYP-Based Drug-Drug Interaction Studies — Decision Tree



NME: New molecular entity

* Additional population pharmacokinetic analysis may assist the overall evaluation.

\+ See Appendix C for criteria to determine whether an NME is an inhibitor (Appendix C-2) or an inducer (Appendix C-3) of a specific CYP enzyme; negative results from a cocktail study would preclude further evaluation to determine whether an NME is an inhibitor or an inducer of a particular CYP enzyme (see IV.C.1). (Reference: *Journal of Clinical Pharmacology*, 39:1006-1014, 1999.)

APPENDIX C-1

In Vitro Drug Metabolizing Enzyme Identification

Drug metabolizing enzyme identification studies, often referred to as reaction phenotyping studies, are a set of experiments that identify the specific enzymes responsible for metabolism of a drug. Oxidative and hydrolytic reactions involve cytochrome P450 (CYP) and non-CYP enzymes. For many drugs, transferase reactions are preceded by oxidation or hydrolysis of the drug. However, direct transferase reactions may represent a major metabolic pathway for compounds containing polar functional groups.

An efficient approach is to determine the metabolic profile (identify metabolites formed and their quantitative importance) of a drug and estimate the relative contribution of CYP enzymes to clearance before initiating studies to identify specific CYP enzymes that metabolize the drug. Identification of CYP enzymes is warranted if CYP enzymes contribute > 25% of a drug's total clearance. In vitro identification of drug metabolizing CYP enzymes helps predict the potential for in vivo drug-drug interactions, the impact of polymorphic enzyme activity on drug disposition, and the formation of toxic or active metabolites. There are few documented cases of clinically significant drug-drug interactions related to non-CYP enzymes, but the identification of drug metabolizing enzymes of this kind (i.e., glucuronosyltransferases, sulfotransferases, and N-acetyl transferases) is encouraged. Although classical biotransformation studies are not a general requirement for the evaluation of therapeutic biologics, certain protein therapeutics modify the metabolism of drugs that are metabolized by CYP enzymes. Given their unique nature, consultation with FDA is appropriate before initiating drug-drug interaction studies involving biologics.

1. Metabolic Pathway Identification Experiments (Determination of Metabolic Profile)

(a) Rationale and Goals

Data obtained from in vitro drug metabolic pathway identification experiments help determine whether experiments to identify drug metabolizing enzymes are warranted, and guide the appropriate design of any such experiments. The metabolic pathway identification experiments should identify the number and classes of metabolites produced by a drug and whether the metabolic pathways are parallel or sequential.

(b) Tissue Selection for Metabolic Pathway Identification Experiments

Human tissues, including freshly prepared hepatocyte, cryopreserved hepatocytes, and freshly isolated liver slices, provide cellular integrity with respect to enzyme architecture and contain the full complement of drug metabolizing enzymes. Subcellular liver tissue fractions, fractions that include microsomes, S9, cytosol (adding appropriate co-factors as necessary), or recombinant enzymes can be used in combination with the tissues

817 mentioned above to identify the individual drug metabolites produced and classes of
818 enzyme involved.
819
820 (c) Design of Metabolic Pathway Identification Experiments
821
822 One approach to metabolic pathway identification is to incubate the drug with
823 hepatocytes or liver slices, followed by chromatographic analysis of the incubation
824 medium and intracellular content by HPLC-MS/MS. This type of experiment leads to the
825 direct identification of metabolites formed by oxidative, hydrolytic, and transferase
826 reactions, and provides information concerning parallel versus sequential pathways.
827 Another approach is to analyze the incubation medium by HPLC using UV, fluorescent,
828 or radiochemical detection.
829
830 In view of the known multiplicity and overlapping substrate specificity of drug
831 metabolizing enzymes and the possibility of either parallel or sequential metabolic
832 pathways, experiments should include several drug concentrations and incubation times.
833 Expected steady state in vivo plasma drug concentrations may be helpful in determining
834 the range of drug concentrations used for these experiments.
835
836 (d) In Vitro Systems and Study Conditions
837
838 As indicated in the PhRMA position paper on drug-drug interactions (Bjornsson TD et
839 al., 2003), the methods listed in Table 1 can be used to identify CYP and non-CYP
840 oxidative pathways responsible for the observed metabolites.
841
842 Table 1. Methods to identify pathways involved in the oxidative biotransformation of a drug
843

In vitro System	**Condition**	**Tests**
microsomes	+/- NADPH	CYP, FMO versus other oxidases
microsomes, hepatocytes	+/- 1-aminobenzotriazole	broad specificity CYP inactivator
microsomes	45^{o}C pretreatment	inactivates FMO
S-9	+/- pargyline	broad MAO inactivator
S-9, cytosol	+/- menadione, allopurinol	Mo-CO (oxidase) inhibitors

844
845
846 2. **Studies Designed to Identify Drug Metabolizing CYP Enzymes**
847
848 If human in vivo data indicate CYP enzymes contribute > 25% of a drug's clearance, studies
849 to identify drug metabolizing CYP enzymes in vitro should be conducted. This
850 recommendation includes cases in which oxidative metabolism is followed by transferase
851 reactions, because a drug-drug interaction that inhibits oxidation of the parent compound can
852 result in elevated levels of the parent compound**.**
853
854 (a) General Experimental Methods for Identifying Drug Metabolizing CYP Enzymes

There are three well-characterized methods for identifying the individual CYP enzymes responsible for a drug's metabolism. The respective methods use (1) specific chemical or antibodies as specific enzyme inhibitors; (2) individual human recombinant CYP enzymes; or (3) a bank of human liver microsomes characterized for CYP activity prepared from individual donor livers. We recommend that at least two of the three methods be performed to identify the specific enzyme(s) responsible for a drug's metabolism.

Either pooled human liver microsomes or microsomes prepared from individual liver donors can be used for the methods described in (a.1). For correlation analysis (a.3), a bank of characterized microsomes from individual donor livers should be used.

Whenever possible, experiments to identify the CYP enzymes responsible for a drug's metabolism should be conducted with drug concentrations deemed appropriate by kinetic experiments. Enzyme identification experiments should be conducted under initial rate conditions (linearity of metabolite production rates with respect to time and enzyme concentrations). In some cases, the experiments are conducted under nonlinear conditions because of analytical sensitivity; results of these experiments should be interpreted with caution. Thus, reliable analytical methods, based upon a sound scientific rationale, should be developed to quantitate each metabolite produced by individual CYP enzymes selected for identification. For racemic drugs, individual isomers should be evaluated separately

(b) The use of **Specific Chemical Inhibitors** to Identify Drug Metabolizing CYP Enzymes

Most chemical inhibitors are not absolutely specific for an individual CYP enzyme, but a valuable attribute of chemical inhibitors is their commercial availability. Although not all-inclusive, the chemical inhibitors listed in Table 2 can be used to identify individual CYP enzymes responsible for a drug's metabolism, and to determine the relative contribution of an individual CYP enzyme.

Table 2: Chemical inhibitors for in vitro experiments(7)

CYP	Inhibitor (1) Preferred	Ki (µM)	Inhibitor (1) Acceptable	Ki (µM)
1A2	furafylline (2)	0.6-0.73	α-naphthoflavone	0.01
2A6	tranylcypromine methoxsalen (2)	0.02-0.2 0.01-0.2	pilocarpine, tryptamine	4 1.7 (3)
2B6			3-isopropenyl-3-methyl diamantine, (4) 2-isopropenyl-2-methyl adamantine, (4) sertraline, phencyclidine, triethylenethiophosphoramide (thiotepa), clopidogrel, ticlopidine	2.2 5.3 3.2 (5) 10 4.8 0.5 0.2
2C8	montelukast quercetin	1.1	trimethoprim, gemfibrozil, rosiglitazone, pioglitazone	32 69-75 5.6 1.7
2C9	sulfaphenazole	0.3	fluconazole, fluvoxamine, fluoxetine	7 6.4-19 18-41
2C19			ticlopidine, nootkatone	1.2 0.5
2D6	quinidine	0.027-0.4		
2E1			diethyldithiocarbamate, clomethiazole, diallyldisulfide	9.8-34 12 150
3A4/5	ketoconazole itraconazole	0.0037- 0.18 0.27, 2.3	azamulin, troleandomycin, verapamil	(6) 17 10, 24

(1) Substrates used for inhibition studies include: **CYP1A2**, phenacetin-o-deethylation, theophylline-N-demethylation; **CYP2A6**, coumarin-7-hydroxylation; **CYP2B6**, 7-pentoxyresorufin-O-depentylation, bupropion hydroxylation, 7-ethoxy-4-(trifluoromethyl)-coumarin O-deethylation, S-mephenytoin-N-demethylation; Bupropion-hydroxylation; **CYP2C8**, taxol 6-alpha-hydroxylation; **CYP2C9**, tolbutamide 4-methylhydroxylation, S-warfarin-7-hydroxylation, phenytoin 4-hydroxylation; **2CYP2C19**, (S)-mephenytoin 4-hydroxylation **CYP2D6**, dextramethorphan O-demethylation, desbrisoquine hyddroxylase; **CYP2E1**, chlorzoxazone 6-hydroxylation, aniline 4-hydroxylase; **CYP3A4/5**, testosterone-6ß-hydroxylation, midazolam-1-hydroxylation; cyclosporine hydroxylase; nifedipine dehydrogenation.

(2) Furafylline and methoxsalen are mechanism-based inhibitors and should be pre-incubated before adding substrate.

(3) cDNA expressing microsomes from human lymphoblast cells.

(4) Supersomes, microsomal isolated from insect cells transfected with baculovirus containing CYP2B6.

(5) IC50 values.

(6) Specific time-dependent inhibitor.

(7) Note that this is not an exhaustive list. For an updated list, see the following link.
http://www.fda.gov/cder/drug/drugInteractions/default.htm

The effectiveness of competitive inhibitors is dependent on concentrations of the drug and inhibitor. Experiments designed to identify and quantitate the relative importance of individual CYP enzymes mediating a drug's metabolism should use drug concentrations ≤ Km. The experiments should include the inhibitor at concentrations that ensure selectivity and adequate potency. It is also acceptable to use a range of inhibitor concentrations.

Noncompetitive and mechanism-based inhibitors are not dependent on the drug (substrate) concentration. When using a mechanism-based inhibitor, it is advisable to pre-incubate the inhibitor for 15 to 30 minutes.

For additional information concerning inhibition experiments see the Inhibition section (Appendix C-2).

(c) The use of **Recombinant Enzymes** to Identify Drug Metabolizing CYP Enzymes

When a drug is metabolized by only one recombinant human CYP enzyme, interpretation of the results is relatively straightforward. However, if more than one recombinant CYP enzyme is involved, measurement of enzyme activity alone does not provide information on the relative importance of the individual pathways.

Recombinant CYP enzymes are not present in their native environment and are often overexpressed. Accessory proteins (NADPH-CYP reductase and cytochrome b5) or membrane lipid composition may differ from native microsomes. Several approaches have been reported to quantitatively scale metabolic activity obtained using recombinant CYP enzymes to activities expected in the human liver microsomes. These techniques can be helpful for determining the relative importance of each of the enzymes in the overall metabolite formations but may not reflect absolute formation rates in human liver microsomes in vitro.

(d) The use of **Specific Antibodies** to Identify Drug Metabolizing CYP Enzymes

The inhibitory effect of an inhibitory antibody should be tested at sufficiently low and high concentrations to establish the titration curve. If only one CYP enzyme is involved in the drug's metabolism, > 80% inhibition is expected in a set of pooled or individual microsomes. If the extent of inhibition is low, it is difficult to determine whether the partial inhibition is the result of the involvement of other CYPs in metabolism of the drug or whether the antibody has poor potency.

(e) The use of **Correlation Analyses** to Identify Drug Metabolizing CYP Enzymes

This approach relies on statistical analyses to establish a correlation between the production rate of an individual metabolite and activities determined for each CYP

enzyme in a set of microsomes prepared from individual donor livers.

The set of characterized microsomes should include microsomes prepared from at least 10 individual donor livers. The variation in metabolic activity for each CYP enzyme should be sufficient between individual donor livers to ensure adequate statistical power. Enzyme activities in the set of microsomes used for correlation studies should be determined using appropriate probe substrates and experimental conditions.

Results are suspect when a single outlying point dictates the correlation coefficient. If the regression line does not pass through or near the origin, it may indicate that multiple CYP enzymes are involved or it may reflect a set of microsomes that are inherently insensitive.

APPENDIX C-2

In Vitro Evaluation of CYP Inhibition

A drug that inhibits a specific drug-metabolizing enzyme can decrease the metabolic clearance of a co-administered drug that is a substrate of the inhibited pathway. A consequence of decreased metabolic clearance is elevated blood concentrations of the co-administered drug, which may cause adverse effects or enhanced therapeutic effects. On the other hand, the inhibited metabolic pathway could also lead to decreased formation of an active metabolite of the co-administered drug, resulting in decreased efficacy of that drug.

1. Probe Substrates

In vitro experiments conducted to determine whether a drug inhibits a specific CYP enzyme involve incubation of the drug with probe substrates for the CYP enzymes.

There are two scientific criteria for selection of a probe substrate. The substrate (1) should be selective (predominantly metabolized by a single enzyme in pooled human liver microsomes or recombinant P450s) and (2) should have a simple metabolic scheme (ideally, no sequential metabolism). There are also some practical criteria — commercial availability of substrate and metabolite(s); assays that are sensitive, rapid, and simple; and a reasonable incubation time.

Preferred substrates listed in Table 3 meet a majority of the criteria listed above. Acceptable substrates meet some of the criteria, and are considered acceptable by the scientific community.

Table 3. Preferred and acceptable chemical substrates for in vitro experiments*

CYP	Substrate Preferred	Km (μM)	Substrate Acceptable	Km (μM)
1A2	phenacetin-O-deethylation	1.7-152	7-ethoxyresorufin-O-deethylation theophylline-N-demethylation caffeine-3-N-demethylation tacrine 1-hydroxylation	0.18-0.21 280-1230 220-1565 2.8, 16
2A6	coumarin-7-hydroxylation nicotine C-oxidation	0.30-2.3 13-162		
2B6	efavirenz hydroxylase bupropion-hydroxylation	17-23 67-168	propofol hydroxylation S-mephenytoin-N-demethylation	3.7-94 1910
2C8	Taxol 6-hydroxylation	5.4-19	amodiaquine N-deethylation rosiglitazone para-hydroxylation	2.4, 4.3-7.7
2C9	tolbutamide methyl-hydroxylation S-warfarin 7-hydroxylation diclofenac 4'-hydroxylation	67-838 1.5-4.5 3.4-52	flurbiprofen 4'-hydroxylation phenytoin-4-hydroxylation	6-42 11.5-117
2C19	S-mephenytoin 4'-hydroxylation	13-35	omeprazole 5-hydroxylation fluoxetine O-dealkylation	17-26 3.7-104
2D6	(±)-bufuralol 1'-hydroxylation dextromethorphan O-demethylation	9-15 0.44-8.5	debrisoquine 4-hydroxylation	5.6
2E1	chlorzoxazone 6-hydroxylation	39-157	p-nitrophenol 3-hydroxylation lauric acid 11-hydroxylation aniline 4-hydroxylation	3.3 130 6.3-24
3A4/5**	midazolam 1-hydroxylation testosterone 6β-hydroxylation	1-14 52-94	erythromycin N-demethylation dextromethorphan N-demethylation triazolam 4-hydroxylation terfenadine C-hydroxylation nifedipine oxidation	33 – 88 133-710 234 15 5.1- 47

* Note that this is not an exhaustive list. For an updated list, see the following link http://www.fda.gov/cder/drug/drugInteractions/default.htm

** Recommend use of 2 structurally unrelated CYP3A4/5 substrates for evaluation of in vitro CYP3A inhibition. If the drug inhibits at least one CYP3A substrate in vitro, then in vivo evaluation is warranted.

2. Design Considerations for In Vitro CYP Inhibition Studies

(a) Typical experiments for determining IC50 values involve incubating the substrate, if the metabolic rate is sufficient, at concentrations below its Km to more closely relate the inhibitor IC50 to its Ki. For Ki determinations, both the substrate and inhibitor concentrations should be varied to cover ranges above and below the drug's Km and inhibitor's Ki.

(b) Microsomal protein concentrations used are usually less than 1 mg/ml.

(c) Because buffer strength, type, and pH can all significantly affect Vmax and Km, standardized assay conditions are recommended.

(d) Preferably no more than 10-30% substrate or inhibitor depletion should occur. However, with low Km substrates, it may be difficult to avoid > 10% substrate depletion at low substrate concentrations.

(e) We suggest a linear relationship between time and amount of product formed.

(f) We recommend a linear relationship between amount of enzyme and product formation.

(g) Any solvents should be used at low concentrations (≤ 1% (v/v) and preferably < 0.1%). Some of the solvents inhibit or induce enzymes. The experiment can include a no-solvent control and a solvent control.

(h) Use of an active control (known inhibitor) is optional.

3. Determining Whether an NME is a Reversible Inhibitor

Theoretically, significant enzyme inhibition occurs when the concentration of the inhibitor present at the active site is comparable to or in excess of the Ki. In theory, the degree of interaction (R, expressed as fold-change in AUC) can be estimated by the following equation: $R = 1 + [I]/Ki$, where [I] is the concentration of inhibitor exposed to the active site of the enzyme and Ki is the inhibition constant.

Although the [I]/Ki ratio is used to predict the likelihood of inhibitory drug interactions, there are factors that affect selection of the relevant [I] and Ki. Factors that affect [I] include uncertainty regarding the concentration that best represents concentration at the enzyme binding site (at the gastrointestinal versus liver) and uncertainty regarding the impact of first-pass exposure. Factors that affect Ki include substrate specificity, binding to components of incubation system, and substrate and inhibitor depletion.

Current recommended approach

The likelihood of an in vivo interaction is projected based on the [I]/Ki ratio where [I] represents the mean steady-state Cmax value for total drug (bound plus unbound) following administration of the highest proposed clinical dose. As the ratio increases, the likelihood of an interaction increases. The following table suggests the likelihood of in vivo interaction based on estimated [I]/Ki ratios. **An estimated [I]/Ki ratio of greater than 0.1 is considered positive and a follow-up in vivo evaluation is recommended.**

Table 4. Prediction of clinical relevance of competitive CYP inhibition

[I]/Ki	Prediction
[I]/Ki > 1	Likely
1 > [I]/Ki > 0.1	Possible
0.1 > [I]/Ki	Remote

Although quantitative predictions of in vivo drug-drug interactions from in vitro studies are not possible, rank order across the different CYP enzymes for the same drug may help

prioritize in vivo drug-drug interaction evaluations. When various [I]/Ki ratios are obtained with the major CYP enzymes (CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A), an in vivo study starting with the CYP with the largest [I]/Ki (or smallest Ki) may be appropriate. If the CYP with the largest [I]/Ki (or smallest Ki) shows no interaction in vivo, in vivo evaluation of the other CYPs with smaller [I]/Ki (or larger Ki) will not be needed. For CYP3A inhibition, two structurally unrelated substrates should be evaluated. If one of the two evaluations suggests a potential interaction (i.e., [I]/Ki more than 0.1), an in vivo evaluation should be carried out.

4. Determining Whether an NME is a Mechanism-Based Inhibitor

Time-dependent inhibition should be examined in standard in vitro screening protocols, because the phenomenon cannot be predicted with complete confidence from chemical structure. A 30-minute pre-incubation of a potential inhibitor before the addition of substrate is recommended. Any time-dependent and concentration-dependent loss of initial product formation rate indicates mechanism-based inhibition. For compounds containing amines, metabolic intermediate complex formation can be followed spectroscopically. Detection of time-dependent inhibition kinetics in vitro indicates follow-up with in vivo studies in humans.

APPENDIX C-3

In Vitro Evaluation of CYP Induction

A drug that induces a drug-metabolizing enzyme can increase the rate of metabolic clearance of a co-administered drug that is a substrate of the induced pathway. A potential consequence of this type of drug-drug interaction is sub-therapeutic blood concentrations. Alternatively, the induced metabolic pathway could lead to increased formation of an active compound, resulting in an adverse event.

1. Chemical Inducers as a Positive Control

In evaluating the potential for a drug to induce a specific CYP enzyme, the experiment should include an acceptable enzyme inducer as a control, such as those listed in Table 5. The use of a positive control accounts for the variability in catalytic enzyme activity between hepatocyte preparations from individual donor livers. The positive controls should be potent inducers (> 2-fold increase in enzyme activity of probe substrate at inducer concentrations < 500 μM). The selection of probe substrates is discussed in Appendix C-2.

Table 5. Chemical Inducers for In Vitro Experiments*

CYP	Inducer [(1)] -Preferred	Inducer Concentrations (μM)	Fold Induction	Inducer [(1)] -Acceptable	Inducer Concentrations (μM)	Fold Induction
1A2	omeprazole ß-naphthoflavone(2) 3-methylcholanthrene	25-100 33-50 1,2	14-24 4-23 6-26	lansoprazole	10	10
2A6	dexamethasone	50	9.4	pyrazole	1000	7.7
2B6	phenobarbital	500-1000	5-10	phenytoin	50	5-10
2C8	rifampin	10	2-4	phenobarbital	500	2-3
2C9	rifampin	10	3.7	phenobarbital	100	2.6
2C19	rifampin	10	20			
2D6	none identified					
2E1	none identified					
3A4	rifampin(3)	10-50	4-31	phenobarbital(3) phenytoin rifapentine troglitazone taxol dexamethasone(4)	100-2000 50 50 10-75 4 33-250	3-31 12.5 9.3 7 5.2 2.9- 6.9

*Note that this is not an exhaustive list. For an updated list, see the following link
http://www.fda.gov/cder/drug/drugInteractions/default.htm

(1) Except for the cases noted below, the following test substrates were used: CYP1A2, 7-ethoxyresorufin; CYP 2A6, coumarin; CYP2C9, tolbutamide, CYP2C19, S-mephenytoin; CYP3A4, testosterone.

(2) CYP1A2: 1 of 4 references for β-naphthoflavone used phenacetin.

(3) CYP3A4: 2 of 13 references for rifampin and 1 of 3 references for phenobarbital used midazolam.

(4) CYP3A4: 1 of the 4 references for dexamethasone used nifedipine.

2. Design of In Vitro Drug Induction Studies

At this time, the most reliable method to study a drug's induction potential is to quantify the enzyme activity of primary hepatocyte cultures following treatments including the potential inducer drug, a positive control inducer drug (see Table 5), and vehicle-treated hepatocytes (negative control), respectively. Freshly isolated human hepatocytes or cryopreserved hepatocytes that can be thawed and cultured are the preferred liver tissue for these studies; immortalized liver cells are acceptable if it can be demonstrated with positive controls that CYP3A4 and CYP1A2 are inducible in these cell lines.

(a) Test drug concentrations should be based on the expected human plasma drug concentrations be used. At least three concentrations spanning the therapeutic range should be studied, including at least one concentration that is an order of magnitude greater than the average expected plasma drug concentration. If this information is not available, concentrations ranging over at least two orders of magnitude should be studied.

(b) Following treatment of hepatocytes for 2 to 3 days, the resulting enzyme activities can be determined using appropriate CYP-specific probe drugs (see Table 3, Appendix C-2). Either whole cell monolayers or isolated microsomes can be used to monitor drug-induced enzyme changes; however, the former tissue is the simplest and the most direct method.

(c) When conducting experiments to determine enzyme activity, the experimental conditions listed in section Appendix C-2 are relevant.

(d) When using freshly isolated human or cryopreserved hepatocytes for induction studies, experiments should be conducted with hepatocytes prepared from at least three individual donor livers because of the inter-individual differences in induction potential.

(e) Experiments should be carried out in triplicate when using immortalized human liver cells for induction studies.

3. Endpoints for Subsequent Prediction of Enzyme Induction

When analyzing the results of experiments to determine whether a drug induces an enzyme activity, the following issues are relevant.

(a) A drug that produces a change that is equal to or greater than 40% of the positive control can be considered as an enzyme inducer in vitro and in vivo evaluation is warranted.

$$\% \text{ positive control} = \frac{(\text{activity of test drug treated cells} - \text{activity of negative control}) \times 100}{(\text{activity of positive control} - \text{activity of negative control})}$$

(b) An alternative endpoint is the use of an EC50 (effective concentration at which 50% maximal induction occurs) value, which represents a potency index that can be used to

compare the potency of different compounds.

(c) Based on our present knowledge of cellular mechanisms leading to CYP enzyme induction, if induction studies with a test drug confirm that it is not an inducer of CYP3A4 then it can be concluded that the test drug is also not an inducer of CYP2C8, CYP2C9, or CYP2C19.

4. Other Methods Proposed for Identifying In Vitro Enzyme Induction

Although the most reliable method for quantifying a drug's induction potential is measurement of enzyme activities after incubation of the drug in primary cultures of human hepatocytes, other methods are being evaluated. Several of these methods are described briefly below.

(a) Western immunoblotting or immunoprecipitation probed with specific polyclonal antibodies.

Relative quantification of specific P450 enzyme protein requires that the electrophoretic system clearly resolve the individual enzymes and/or that the primary antibodies be specific for the enzyme quantified. Enzyme antibody preparations are highly variable.

(b) Measurement of mRNA levels using reverse transcriptase-polymerase chain reaction (RT-PCR).

RT-PCR can quantify mRNA expression for a specific CYP enzyme but is not necessarily informative of enzyme activities. Measurement of mRNA levels is helpful when both enzyme inhibition and induction are operative.

(c) Receptor gene assays for receptors mediating induction of P450 enzymes.

Cell receptors mediating CYP1A, CYP2B, and CYP3A induction have been identified. Higher throughput AhR (aromatic hydrocarbon receptor) and PXR (pregnane X receptor) binding assays and cell-based reporter gene assays have been developed and used to screen for compounds that have CYP1A and CYP3A induction potential. Although results of these assays provide supportive evidence for a compound's induction potential, they do not necessarily reflect the enzyme activities.

APPENDIX D

In Vitro Evaluation of P-glycoprotein (P-gp, MDR1) Substrates and Inhibitors

The P-glycoproteins MDR1 and MDR3, are expressed by two genes, *ABCB1* and *ABCB4*, respectively. They are members of the ATP-binding cassette transporters. MDR3 has been identified in various human tissues, but there is little evidence that it plays a major role in the transport of drugs. Therefore, P-glycoprotein (P-gp) here refers to MDR1, the most studied member of the ABC transporters. It is generally accepted that co-administration of drugs that interact with this transporter (as a substrate, inhibitor, or inducer) can result in drug-drug interactions that affect the pharmacokinetics and pharmacodynamics of the co-administered drugs. This P-gp efflux transporter is mainly, although not exclusively, present on the apical side of epithelial cells. Specific locations of the P-gp transporter include brush border membrane of small intestine enterocytes, canalicular membrane of hepatocytes, brush border membrane of proximal tubule cells in the kidney, and capillary endothelial cells in the blood brain barrier. Modulation of this transporter can affect the oral bioavailability, biliary and renal clearance, and brain uptake of drugs. In addition, modulation of MDR1 expression in other tissues can affect access of chemical to the respective tissues. For example, modulation of MDR1 expression in tumor tissues can affect access to the tumor, and modulation of expression in the placenta can affect access to the fetus.

1. In Vitro Models Used for Identifying Whether a Drug is a P-gp Substrate and/or Inhibitor

There are several in vitro methods that can evaluate whether a drug candidate is a substrate or inhibitor of the P-gp efflux transporter. The most commonly used methods are listed in Table 1.

Table 1. In vitro methods for identifying whether a drug is a P-gp substrate and/or inhibitor

Assay Type	Tissues	Parameters	Comments
Bi-Directional Transport	Caco-2 cells; MDCK-MDR1 cells; LLC-PK1 MDR1cells	Net drug flux ratio of B to A and A to B	• Directly measure efflux across cell barrier • Evaluation of P-gp transport and inhibition • Allow for localization/identification of the transporters within the apical or basolateral side of the membrane
Uptake/efflux	tumor cells, cDNA transfected cells, oocytes injected with cRNA of transporters	Inhibition of uptake or efflux of fluorescent probe Calcein-AM or rhodamine-123	• Cannot distinguish substrate from inhibitor • Tends to fail to identify substrate and/or inhibitor with low permeability
ATPase	membrane vesicles from various tissues or cells expressing P-gp, Reconstituted P-gp	ATPase stimulation	• Same comments as uptake/efflux assay • Do not always show good correlation with functional assay for P-gp

The bi-directional transport assay is regarded as the definitive assay for identifying P-gp substrates and inhibitors because it measures drug efflux in a more direct manner than other methods.

The ATPase activity assay and the uptake/efflux assay can screen compounds rapidly, but they are not designed to distinguish P-gp substrates from inhibitors. Moreover, literature data suggest that both ATPase and fluorescent indicator assays often fail to identify P-gp substrates with relatively low permeability. Although the bi-directional transport assay may fail to identify highly permeable compounds as P-gp substrates, the failure to identify high permeable compounds would not be a concern because in this situation, P-gp is not likely to be a significant barrier for these compounds to cross membrane. Thus, the transcellular transport assay should be used as a definitive method for identifying P-gp substrates and inhibitors.

2. Bi-Directional Transport Assays Using Polarized Monolayer Cells

Bi-directional transport methodology is the preferred functional assay used to identify drugs as substrates and/or inhibitors of P-gp. These experiments require the use of known P-gp substrates and inhibitors.

(a) Criteria for preferred in vitro P-gp probe substrates

(1) Selective for the P-gp transporter
(2) Exhibits low to moderate passive membrane permeability (2-30 x 10^{-6} cm/sec)
(3) No significant metabolism of the substrate occurs (optional)
(4) Commercially available (optional)
(5) May be used as an in vivo P-gp probe substrate (optional)

Unfortunately, a P-gp substrate that meets all of the above criteria has not been identified, due to overlapping substrate selectivity between transporter/transporter and transporter /metabolizing enzymes. Table 2 lists examples of acceptable P-gp substrates that meet the majority of the above mentioned criteria. These P-gp substrates serve as positive controls to ensure the cell systems have functional P-gp expression (see section (d) below) when used for transport experiments.

Table 2. Acceptable P-gp Substrates

Drug	Conc. Used (µM)	Ratio*		
		Caco-2	MDR1-MDCK**	MDR1-LLCPK**
Digoxin	0.01-10	4-14	4	4
Loperamide	1-10	2-5		3.4
Quinidine	0.05	3		5
Vinblastine [a]	0.004-10	2-18	> 9 [b]	3
Talinolol	30	26		

Note that this is not an exhaustive list. For an updated list, see the following link http://www.fda.gov/cder/drug/drugInteractions/default.htm

* $P_{app, B-A} / P_{app, A-B}$; P_{app} = apparent permeability

** Data for MDR1-MDCK and MDR1-LLCPK are the ratio observed in transfected cells relative to the ratio observed in respective wild-type cells.

a Vinblastine is also a substrate for MRP2 that is constitutively expressed in Caco-2, and wild type MDCK and LL-CPK1 cells.

b Data are derived from net B to A flux in the absence of GF120918, a potent P-gp inhibitor, relative to that observed in the presence of GF120918.

Acceptable P-gp substrates are not limited to compounds listed in Table 2. Selection of other compounds as probe P-gp substrates may be appropriate based on scientific justification.

(b) Criteria for preferred in vitro P-gp inhibitors

(1) Selective for P-gp transporter
(2) Inhibit P-gp with low Ki or IC50 values (e.g., IC50 < 10 μM)
(3) No significant metabolism of the inhibitor occurs in the cells (optional)
(4) Commercially available (optional)
(5) May be used as an in vivo P-gp inhibitor (optional)

Most P-gp substrates with high affinity are also potent competitive inhibitors. Examples of compounds extensively studied and reported in the literature as potent P-gp inhibitors are listed in Table 3. The table includes IC50 or Ki values determined using bi-directional transport assays. Some inhibitors may inhibit multiple transporters, because of overlap among transporters. For example, in addition to being potent inhibitors for P-gp, cyclosporine A is also a potent inhibitor for MRP2 and OATP-C, and quinidine and verapamil are also potent inhibitors for various organic cation transporters. Because of the lack of inhibitor specificity, the use of multiple inhibitors is recommended to determine whether the efflux activity observed in vitro is related to P-gp.

Acceptable P-gp inhibitors are not limited to compounds listed in Table 3. Selection of other compounds as probe P-gp inhibitors may be appropriate based on scientific justification.

Table 3. In Vitro P-gp Inhibitors

Inhibitor	IC50 (μM)	Ki (μM)		
	Caco-2*	Caco-2*	MDCK-MDR1*	LLC-PK1 MDR1**
Cyclosporine A[a]	1.3	0.5	2.2	1.3
Ketoconazole[a]	1.2			5.3
LY335979	0.024			
Nelfinavir[a]	1.4			
Quinidine[b]	2.2	3.2	8.6	
Ritonavir[a]	3.8			
Saquinavir[a]	6.5			
Tacrolimus	0.74			
Valspodar (PSC833)	0.11			
Verapamil	2.1	8	15	23
Elacridar (GF120918) (GG 918)		0.4	0.4	
Reserpine		1.4	11.5	

Note that this is not an exhaustive list. For an updated list, see the following link http://www.fda.gov/cder/drug/drugInteractions/default.htm

* Digoxin as a P-gp substrate
** Vinblastine as a P-gp substrate
[a] also CYP3A inhibitor
[b] also CYP2D6 inhibitor

(c) Tissue culture considerations to ensure functionally polarized cells

Cells used for bi-directional transport studies should form a functionally polarized cell monolayer, complete with tight junctions. At present, the preferred cells lines include

Caco-2, transfected LLC-PK1-MDR1, and transfected MDCK-MDR1. LLC-PK1 and MDCK wild type cells are used as negative controls.

(1) Caco-2 cells should be seeded at a density of approximately 0.5-5 x 10^5 cells/cm^2 on polycarabonate microporous membrane filters and allowed to grow to confluence (typically 18-21 days).
(2) LLC-PK1 and LLC-PK1-MDR1, MDCK, and MDCK-MDR1 cells should be seeded at a density of approximately 0.05-5.0 x 10^6 cells/cm^2 on polycarbonate microporous membrane filters and allowed to grow to confluence (typically 3-5 days).
(3) The transepithelial electrical resistance (TEER) of the polarized cells should be determined before each experiment (typical values are 100-800 Ω cm^2).
(4) A paracellular marker such as [^{14}C] mannitol can be used as an additional integrity marker (typical permeability values are < 0.2-2 x 10^{-6}cm/sec).

(d) Design of bi-directional experiments conducted to determine whether the drug is a P-gp substrate

After selection of the cell type and P-gp substrate positive control, bi-directional substrate experiments are typically performed using polycarbonate filter inserts and side-side diffusion chambers as follows:

(1) The efflux of the investigational drug should be studied over a range of concentrations (e.g., 1, 10 and 100 μM).
(2) Before initiating bidirectional experiments, the medium in the donor and receiver chambers is removed, replaced with fresh medium, and pre-incubated for 30 minutes.
(3) Bi-directional permeability studies are initiated by adding an appropriate volume of buffer containing a known drug probe P-gp substrate or the test drug to either the apical (for apical to basolateral transport, A/B) or the basolateral (for basolateral to apical, B/A) side of the monolayer.
(4) Samples are incubated at 37°C. At selected times (typically 1, 2, 3, 4 hours), aliquots from the receiver compartment are collected for determination of the test compound concentrations. The volume removed is replaced immediately with buffer.
(5) A known P-gp substrate (see Table 2) should be run as a positive control.
(6) When using LLC-PK1-MDR1 or MDCK-MDR1 cells for bi-directional studies, LLC-PK1 and MDCK cells, respectively, should be included as negative controls.
(7) Each experiment should be performed at least in triplicate on different days to allow for assessment of intra- and inter-day variations.
(8) Optimal experiments should determine recovery of substrate, to allow estimation of metabolism and non-specific binding.

Because Caco-2 cells, wild-type MDCK, and wild-type LLC-PK1 cells may also express

efflux transporters other than P-gp, data interpretation of data from bi-directional transport studies using the test drug as a substrate should be viewed with caution. To strengthen the results from bi-directional transport studies, it is recommended that additional experiments be conducted in the presence of potent P-gp inhibitors (at least 2-3 potent P-gp inhibitors; see Table 3 for examples). If the test drug efflux is inhibited by these P-gp inhibitors, it is likely that the efflux activity is related to P-gp. Finally, experiments that compare efflux activity observed in overexpressed-MDR1 cells to that observed in their respective wild-type cells can help determine the extent of P-gp contribution to the efflux activity.

(e) Calculation of the apparent permeability of drugs through the cell monolayer

The apparent permeability of compounds across the monolayer cells used for transporter studies is calculated using the following equation:

$$P_{app} = (V_r/C_0)(1/S)(dC/dt) \qquad (1)$$

Where P_{app} = apparent permeability, V_r is the volume of medium in the receiver chamber, C_0 is the concentration of the test drug in the donor chamber, S is the surface area of monolayer, dC/dt is the is the linear slope of the drug concentration in the receptor chamber with time after correcting for dilution.

Flux through the monolayer must be linear with time (dC/dt is constant) for accurate determination of $P_{app.}$

The efflux ratio (R_E) for basolateral to apical and apical to basolateral transport is defined by the following equation:

$$R_E = P_{B/A} / P_{A/B} \qquad (2)$$

where $P_{B/A}$ and $P_{A/B}$ represent the apparent permeability of test compound from the basal to apical and apical to basal side of the cellular monolayer, respectively.

When using Caco-2 cells, the ratio (R_E) is calculated directly. However, for the LLC-PK1-MDR1 or MDCK-MDR1 cells, an (R) = (R_T) / (R_w) is calculated where (R_T) and (R_W) are the permeability ratios for the transfected and the non-transfected lines (used for negative controls), respectively.

(f) Design of bi-directional experiments conducted to determine whether the drug is a P-gp inhibitor

After selection of the cell type, probe P-gp substrate, and known P-gp inhibitors, experiments designed to evaluate whether a test drug is an inhibitor of P-gp are performed using polycarbonate filter inserts and side-side diffusion chambers, as

follows:

(1) When using Caco-2 cells, the experiment is started by adding fresh medium to both sides of the monolayer. The medium contains no drug (control sample) or appropriate concentrations of the test drug.
(2) When using LLC-PK1-MDR1 or MDCK-MDR1 cells for bi-directional studies, the wild type LLC-PK1 MDCK cells, respectively, should be included as negative controls.
(3) After incubation of the cells for 0.5-1 hour at 37°C, the medium is removed from the apical or basolateral side of the monolayer and replaced with the appropriate concentration of the selected probe P-gp substrate (see Table 2).
(4) Following incubation of the cells for 1-3 hours, the receiver side is sampled and the concentration of the probe P-gp substrate is determined.
(5) Each experiment should be performed at least in triplicate on different days, and at least three filters should be used for each condition at each time point.

(g) Calculation of inhibition constant IC50 for the test drug as a P-gp inhibitor

IC50 values for the test drug can be determined after non linear regression of the data using the Hill equation (3):

$$(R_{Ei}/R_{Ea}) = 1 - [(Imax * I^c) / (I^c + IC50^c)] \qquad (3)$$

where (R_{Ei}/R_{Ea}) represents the efflux ratio of the probe P-gp substrate in the presence of inhibitor concentration (I) relative to that for the control without inhibitor. Imax represents maximal inhibitory effect, and (c) is the Hill Plot exponent. The IC50 is the inhibitor concentration (test drug) achieving half maximal inhibition effect.

3. Criteria for Determining Whether a Test Drug is a Substrate for P-gp, and Whether an In Vivo Interaction Study is Needed

Before evaluating data regarding a test compound's status as a P-gp substrate, it is important to determine whether the cell system used for the experiments is sufficient. This assessment considers the net flux ratio of the probe substrate (positive control). An acceptable cell system produces net flux ratios of the probe substrates similar to values reported in the literature (a minimum net flux ratio of 2 is recommended). For cell systems that show low functional P-gp efflux activity for the probe substrates (e.g., net flux ratio < 2), the system is not sufficient to determine whether an investigational drug is a substrate of P-gp.

If the cell system is sufficient, the following items (and Figure 1) describe the process for determining whether a test drug is a P-gp substrate and whether in vivo interaction studies with P-gp inhibitors are recommended.

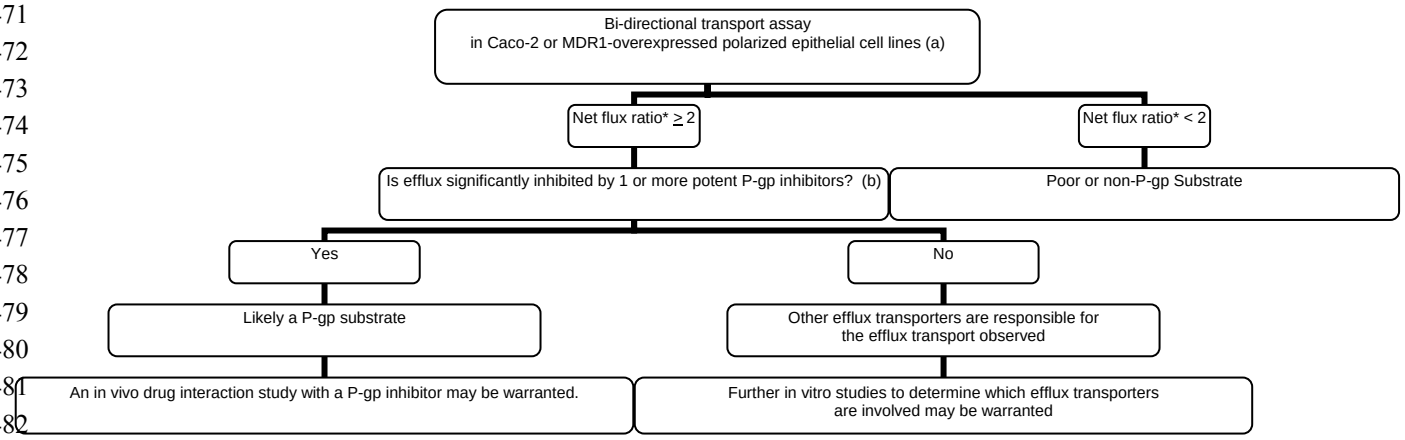
- A net flux ratio over 2 is considered a positive result. To further confirm whether the efflux activity observed is due to P-gp, inhibition studies with one or more potent P-gp inhibitors are needed.

- If the addition of known P-gp inhibitors to the experiment reduces the net flux ratio by a significant amount (more than 50% reduction or reduces the ratio to close to unity), it is likely that the investigational drug is a P-gp substrate.

- If an investigational drug is a P-gp substrate in vitro, evaluation of available in vivo data can help determine whether an in vivo drug interaction study that explores the drug interaction potential with co-administered drugs that are P-gp inhibitors is recommended.

- If a significant amount of efflux activity is not inhibited by the inhibitors studied, then other efflux transporters may contribute to the efflux activity. Further studies to determine which efflux transporters are involved may be warranted.

Figure 1. Decision tree to determine whether an investigational drug is a substrate for P-gp and whether an in vivo drug interaction study with a P-gp inhibitor is needed



*For Caco-2 cells, net flux ratio is calculated as (Permeability $_{app, B-A}$/Permeability $_{app, A-B}$); For MDR1-overexpressed cell lines, net flux ratio is calculated as ratio of (Permeability $_{app, B-A}$/Permeability $_{app, A-B}$)$_{MDR1}$ to (Permeability $_{app, B-A}$/Permeability $_{app, A-B,}$)$_{wild-type}$.
(a) An acceptable system produces next flux ratios of probe substrates similar to the literature values. A net flux ratio ≥2 for the investigational drug is a positive signal for further evaluation. Note: there is a concern that this value is too liberal and will lead to too may positive results. An alternative is to use a % value (net flux of investigation drug relative to a probe substrate, such as digoxin).
(b) reduction of the flux ratio significantly (> 50%) or to unity

4. Criteria for Determining Whether a Test Compound (Investigational Drug) is an Inhibitor of P-gp, and Whether an In Vivo Interaction Study is Needed

Before evaluating data regarding a test compound's status as a P-gp inhibitor, it is important to determine whether the cell system used for the experiments is sufficient. This assessment considers the net flux ratio of the probe substrates. A sufficient system produces net flux ratios of the probe substrates similar to values reported in the literature (a minimum net flux ratio of 2 is recommended). The probe substrate concentration used should be below its apparent Km for P-gp. Two to three known potent inhibitors of P-gp should be included in the study as positive controls. Initially, a high concentration (e.g., >100 µM or as high as solubility of the compound allows) can be used to determine whether the efflux of the probe P-gp substrate is affected by the investigational drug.

If the cell system is acceptable, the following items (and Figure 2) describe the process for determining whether a test drug is a P-gp inhibitor and whether in vivo interaction studies with P-gp substrates are recommended.

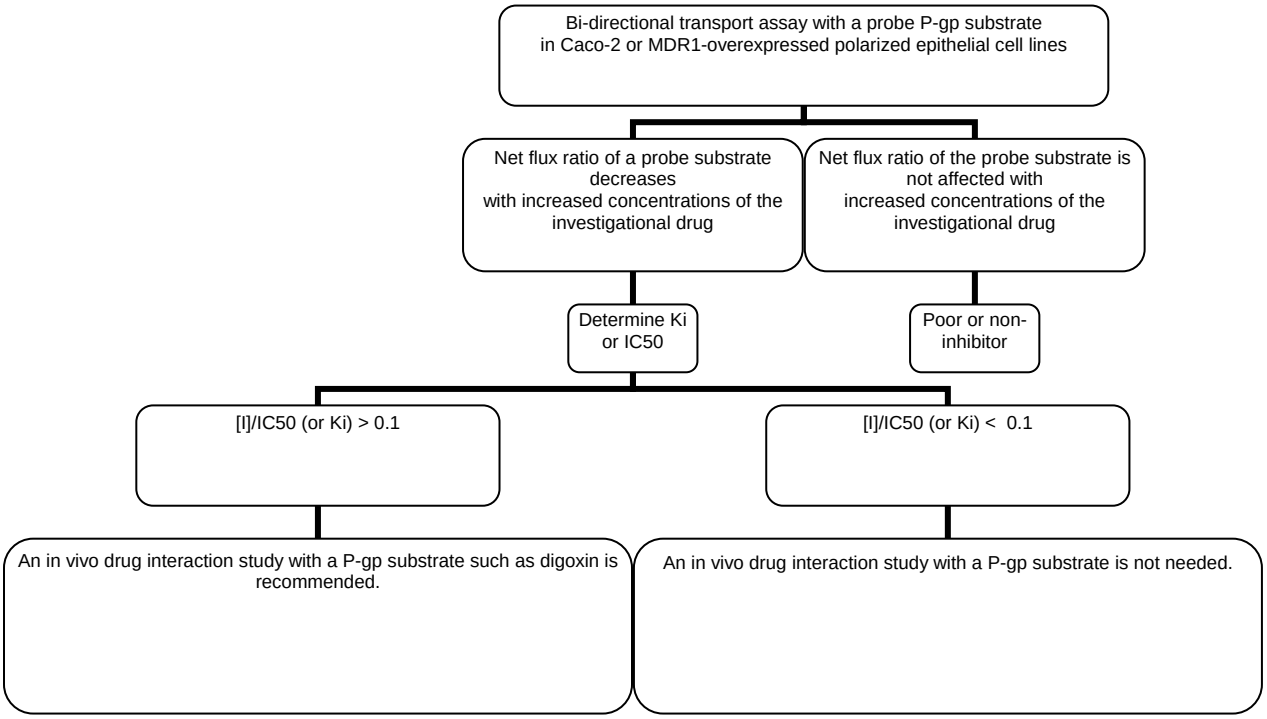
- If the efflux of the probe substrate is not inhibited by the investigational drug, then the investigational drug is likely a poor or non-inhibitor of P-gp.

- If the efflux of the probe substrate is inhibited by the investigational drug, then the inhibition should be studied over a range of concentrations to determine IC50 or Ki. IC50 or Ki values may be experiment-dependent. Therefore, the obtained IC50 or Ki values should be compared to IC50 or Ki values obtained for 2-3 known potent P-gp inhibitors (positive controls).

- If [I]/ IC50 (or Ki) is > 0.1, then the investigational drug is likely a P-gp inhibitor. An in vivo drug interaction study with a P-gp substrate such as digoxin should be conducted.

- If [I]/IC50 (or Ki) is < 0.1, then the investigational drug is likely a weak P-gp inhibitor. Further in vivo drug interaction study would not be needed.

Figure 1. Decision tree to determine whether an investigational drug is an inhibitor for p-gp and whether an in vivo drug interaction study with a P-gp substrate such as digoxin is needed



* For Caco-2 cells, net flux ratio is calculated as (Permeability $_{app, B-A}$/Permeability $_{app, A-B}$); For MDR1-overexpressed cell lines, net flux ratio is calculated as ratio of (Permeability $_{app, B-A}$/Permeability $_{app, A-B}$)$_{MDR1}$ to (Permeability $_{app, B-A}$/Permeability $_{app, A-B,}$)$_{wild-type}$. Note that [I] represents the mean steady-state Cmax value for total drug (bound plus unbound) following administration of the highest proposed clinical dose.

5. Evaluation of a Test Drug as a Potential P-gp Inducer

The expression of P-gp is inducible. Known P-gp inducers include rifampin and St. John's wort. Like CYP enzymes, species differences in inductive response to P-gp inducers are observed. Therefore, animal models may not be valuable for the evaluation of P-gp induction.

Co-induction of P-gp and CYP3A is possible because P-gp, like CYP3A, is also regulated by PXR.

The Caco-2 cell line is not a suitable model for the in vitro evaluation of P-gp induction, possibly due to lack of expression of PXR. In the literature, human colon adenocarcinoma cell LS180/WT, and its adriamycin-resistent (LS 180/AD 50) or vinblastine-resistent (LS

180/V) sublines have been used to study induction for both P-gp and CYP3A.

Methods for in vitro evaluation for P-gp induction are not well understood. Thus, the P-gp induction potential of an investigational drug can only be evaluated in vivo. Because of similarities in the mechanism of CYP3A and P-gp induction, information from test of CYP3A inducibility can inform decisions about P-gp. As stated previously, if an investigational drug is found not to induce CYP3A in vitro, no further tests of CYP3A and P-gp induction in vivo are necessary. If a study of the investigational drug's effect on CYP3A activity in vivo is indicated from a positive in vitro screen, but the drug is shown not to induce CYP3A in vivo, then no further test of P-gp induction in vivo is necessary. However, if the in vivo CYP3A induction test is positive, then an additional study of the investigation drug's effect on a P-gp probe substrate is recommended.

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