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# 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### *DRAFT GUIDANCE*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)  
Center for Biologics Evaluation and Research (CBER)**

**September 2006  
Clinical Pharmacology**

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**U.S. Department of Health and Human Services  
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**Guidance for Industry<sup>1</sup>**

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

This draft guidance, when finalized, will represent the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

**I. INTRODUCTION**

This guidance provides recommendations for sponsors of new drug applications (NDAs) and biologics license applications (BLAs) for therapeutic biologics<sup>2</sup> 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*.

<sup>1</sup> 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.

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

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40  
41 FDA's guidance documents, including this guidance, do not establish legally enforceable  
42 responsibilities. Instead, guidances describe the Agency's current thinking on a topic and  
43 should be viewed only as recommendations, unless specific regulatory or statutory  
44 requirements are cited. The use of the word *should* in Agency guidances means that  
45 something is suggested or recommended, but not required.

46  
47

## 48 **II. BACKGROUND**

49

### 50 **A. Metabolism**

51

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

74

### 75 **B. Drug-Drug Interactions**

76

#### 77 *1. Metabolism-Based Drug-Drug Interactions*

78

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

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

89  
90 It is important that metabolic drug-drug interaction studies explore whether an  
91 investigational agent is likely to significantly affect the metabolic elimination of  
92 drugs already in the marketplace and likely in medical practice to be taken  
93 concomitantly and, conversely, whether drugs in the marketplace are likely to affect  
94 the metabolic elimination of the investigational drug. Even drugs that are not  
95 substantially metabolized can have important effects on the metabolism of  
96 concomitant drugs. For this reason, metabolic drug-drug interactions should be  
97 explored, even for an investigational compound that is not eliminated significantly by  
98 metabolism.

99  
100 Classical biotransformation studies are not a general requirement for the evaluation of  
101 therapeutic biologics (ICH guidance *S6 Preclinical Safety Evaluation of*  
102 *Biotechnology-Derived Pharmaceuticals*), although certain protein therapeutics  
103 modify the metabolism of drugs that are metabolized by the P450 enzymes. Type I  
104 interferons, for example, inhibit CYP1A2 production at the transcriptional and post-  
105 translational levels, inhibiting clearance of theophylline. The increased clinical use  
106 of therapeutic proteins may raise concerns regarding the potential for their impacts on  
107 drug metabolism. Generally, these interactions cannot be detected by in vitro  
108 assessment. Consultation with FDA is appropriate before initiating metabolic drug-  
109 drug interaction studies involving biologics.

110  
111 Identifying metabolic differences in patient groups based on genetic polymorphism,  
112 or on other readily identifiable factors, such as age, race, and gender, can aid in  
113 interpreting results. The extent of interactions may be defined by these variables  
114 (e.g., CYP2D6 genotypes). Further, in subjects who lack the major clearance  
115 pathway, remaining pathways become important and should be understood and  
116 examined.

117  
118 A specific objective of metabolic drug-drug interaction studies is to determine  
119 whether the interaction is sufficiently large to necessitate a dosage adjustment of the  
120 drug itself or the drugs with which it might be used, or whether the interaction would  
121 require additional therapeutic monitoring.

122  
123 In some instances, understanding how to adjust dose or dosage regimen in the  
124 presence of an interacting drug, or how to avoid interactions, may allow marketing of  
125 a drug that would otherwise have been associated with an unacceptable level of  
126 toxicity. Sometimes a drug interaction can be used intentionally to increase levels or  
127 reduce elimination of another drug (e.g., ritonavir and lopinavir). Rarely, the degree

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

130

#### 131 2. *Transporter-Based Drug-Drug Interactions*

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

142

143

### 144 **III. GENERAL STRATEGIES**

145

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

166

#### 167 **A. In Vitro Studies**

168

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

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

176

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

182

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

189

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

198

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

203

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

212

### **B. Specific In Vivo Clinical Investigations**

213

214  
215 In addition to in vitro metabolism and drug-drug interaction studies, appropriately designed



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

222

#### 223 **C. Population Pharmacokinetic Screens**

224

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

240

#### 241 **IV. DESIGN OF IN VIVO DRUG-DRUG INTERACTION STUDIES**

242

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

250

#### 251 **A. Study Design**

252

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

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

270

- 271 • When attainment of steady state is important and either the substrate or interacting  
272 drugs and/or their metabolites have long half-lives and a loading dose to reach steady  
273 state promptly cannot be used, special approaches may be needed. These include the  
274 selection of a one-sequence crossover or a parallel design, rather than a randomized  
275 crossover study design.  
276
- 277 • When it is important that a substrate and/or an interacting drug be studied at steady  
278 state because the effect of an interacting drug is delayed, as is the case for inducers  
279 and certain inhibitors, documentation that near steady state has been attained for the  
280 pertinent drug and metabolites of interest is critical. This documentation can be  
281 accomplished by sampling over several days prior to the periods when test samples  
282 are collected. This is important for both metabolites and the parent drug, particularly  
283 when the half-life of the metabolite is longer than the parent, and is especially  
284 important if both parent drug and metabolites are metabolic inhibitors or inducers.  
285
- 286 • Studies can usually be open label (unblinded), unless pharmacodynamic endpoints  
287 (e.g., adverse events that are subject to bias) are critical to the assessment of the  
288 interaction.  
289
- 290 • For a rapidly reversible inhibitor, administration of the interacting drug either just  
291 before or simultaneously with the substrate on the test day might increase sensitivity.  
292 For a mechanism-based inhibitor (a drug that requires metabolism prior to its  
293 inactivation of the enzyme; examples include erythromycin), administration of the  
294 inhibitor prior to the administration of the substrate drug can maximize the effect. If  
295 the absorption of an interacting drug (e.g., an inhibitor or an inducer) may be affected  
296 by other factors (e.g., the gastric pH), it may be appropriate to control the variables  
297 and confirm the absorption through plasma level measurements of the interacting  
298 drug.  
299
- 300 • When the effects of two drugs on one another are of interest, the potential for  
301 interactions can be evaluated in a single study or two separate studies. Some design  
302 options are randomized three-period crossover, parallel group, and one-sequence  
303 crossover.

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304

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

308

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

317

#### 318 **B. Study Population**

319

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

332

#### 333 **C. Choice of Substrate and Interacting Drugs**

334

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

336

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

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

357

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

374

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

386

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

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392 conducted in a sufficient number of subjects (see section IV.G). Negative results  
393 from a cocktail study can eliminate the need for further evaluation of particular CYP  
394 enzymes. However, positive results can indicate the need for further in vivo  
395 evaluation to provide quantitative exposure changes (such as AUC, C<sub>max</sub>), if the  
396 initial evaluation only assessed the changes in the urinary parent to metabolite ratios.  
397 The data generated from a cocktail study can supplement data from other in vitro and  
398 in vivo studies in assessing a drug's potential to inhibit or induce CYP enzymes.  
399

#### 400 2. *Investigational Drug as a Substrate of CYP Enzymes*

401

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

428

429 If an orally administered drug is a substrate of CYP3A and has low oral  
430 bioavailability because of extensive presystemic extraction contributed by enteric  
431 CYP3A, grapefruit juice may have a significant effect on its systemic exposure. Use  
432 of the drug with grapefruit juice may call for caution, depending on the drug's  
433 exposure-response relationship (see section V for labeling implications).  
434

435

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

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

440

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

447

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

467

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

476

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

478

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

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

481

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

483

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

489

#### 490 5. *Investigational Drug as a Substrate of other Transporters*

491

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

498

### 499 **D. Route of Administration**

500

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

515

### 516 **E. Dose Selection**

517

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

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

529

#### **F. Endpoints**

530

531

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

541

##### *1. Pharmacokinetic Endpoints*

542

543

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

562

##### *2. Pharmacodynamic Endpoints*

563

564

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



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

#### 576 577 **G. Sample Size and Statistical Considerations**

578  
579 The goal of the interaction study is to determine whether there is any increase or decrease in  
580 exposure to the substrate in the presence of the interacting drug. If there is, its implications  
581 must be assessed by an understanding of PK/PD relations both for C<sub>max</sub> and AUC.

582  
583 Results of drug-drug interaction studies should be reported as 90% confidence intervals  
584 about the geometric mean ratio of the observed pharmacokinetic measures with (S+I) and  
585 without the interacting drug (S alone). Confidence intervals provide an estimate of the  
586 distribution of the observed systemic exposure measure ratio of (S+I) versus (S alone) and  
587 convey a probability of the magnitude of the interaction. In contrast, tests of significance are  
588 not appropriate because small, consistent systemic exposure differences can be statistically  
589 significant ( $p < 0.05$ ) but not clinically relevant.

590  
591 When a drug-drug interaction of potential importance is clearly present (e.g., comparisons  
592 indicate twofold (or lower for certain NTR drugs) or greater increments in systemic exposure  
593 measures for (S+I)), the sponsor should provide specific recommendations regarding the  
594 clinical significance of the interaction based on what is known about the dose-response  
595 and/or PK/PD relationship for either the investigational agent or the approved drugs used in  
596 the study. For a new drug, the more difficult issue is the impact on the investigational drug  
597 as substrate. For inhibition or induction by the investigational drug, the main consequence of  
598 a finding will be to add the drug to the list of inhibitors or inducers likely already present in  
599 labeling of the older drug. This information can form the basis for reporting study results  
600 and for making recommendations in the package insert with respect to either the dose, dosing  
601 regimen adjustments, precautions, warnings, or contraindications of the investigational drug  
602 or the approved drug. FDA recognizes that dose-response and/or PK/PD information can  
603 sometimes be incomplete or unavailable, especially for an older approved drug used as S.

604  
605 The sponsor may wish to make specific claims in the package insert that no drug-drug  
606 interaction of clinical significance occurs. In these instances, it would be helpful for the  
607 sponsor to recommend specific *no effect* boundaries, or clinical equivalence intervals, for a  
608 drug-drug interaction. No effect boundaries represent the interval within which a change in a  
609 systemic exposure measure is considered not clinically meaningful.

610  
611 There are two approaches to defining no effect boundaries:

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

619

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

626

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

631

632

## 633 V. LABELING IMPLICATIONS

634

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

651

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

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

660

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

671

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

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### APPENDIX A- Tables

Table 1. Major human transporters <sup>(1,2)</sup>

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

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

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<sup>(1)</sup> 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>

<sup>(2)</sup> 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.

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Table 2. Examples of in vivo substrate, inhibitor, and inducer for specific CYP enzymes recommended for study (oral administration) <sup>(1,2)</sup>

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

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<sup>(1)</sup> 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**.

<sup>(2)</sup> 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>

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

<sup>(4)</sup> 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.

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704 Table 3. Examples<sup>(1)</sup> of sensitive CYP3A substrates or CYP3A substrates with  
705 narrow therapeutic range  
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Sensitive CYP3A substrates <sup>(2)</sup>	CYP3A Substrates with Narrow therapeutic range <sup>(3)</sup>
budesonide, buspirone, eplerenone, eletriptan, felodipine, fluticasone, lovastatin, midazolam, saquinavir, sildenafil, simvastatin, triazolam, vardenafil	alfentanil, astemizole(a), cisapride(a), cyclosporine, diergotamine, ergotamine, fentanyl, pimizide, quinidine, sirolimus, tacrolimus, terfenadine(a)

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

709 <sup>(2)</sup> *Sensitive CYP3A substrates* refers to drugs whose plasma AUC values have been shown to increase  
710 5-fold or higher when co-administered with a known CYP3A inhibitor.

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

714 (a) Not available in the United States.  
715

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716 Table 4. Examples<sup>(1)</sup> of sensitive CYP substrates or CYP substrates with narrow  
717 therapeutic range  
718

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

719 <sup>(1)</sup> 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 <sup>(2)</sup> *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 <sup>(3)</sup> *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).



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Table 5. Classification of CYP3A inhibitors<sup>(1)</sup>

727

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

728

<sup>(1)</sup> Please note the following:

729

- 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.

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742

Table 6. Classification of inhibitors of other CYP enzymes<sup>(1)</sup>

743

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

744

<sup>(1)</sup> Please note the following:

745

- A *strong inhibitor* is one that caused a  $\geq 5$ -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

746

747

748

- A *moderate inhibitor* is one that caused a  $\geq 2$ - 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.

749

750

751

- A *weak inhibitor* is one that caused a  $\geq 1.25$  - 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

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753

754

- This is not an exhaustive list. For an updated list, see the following link

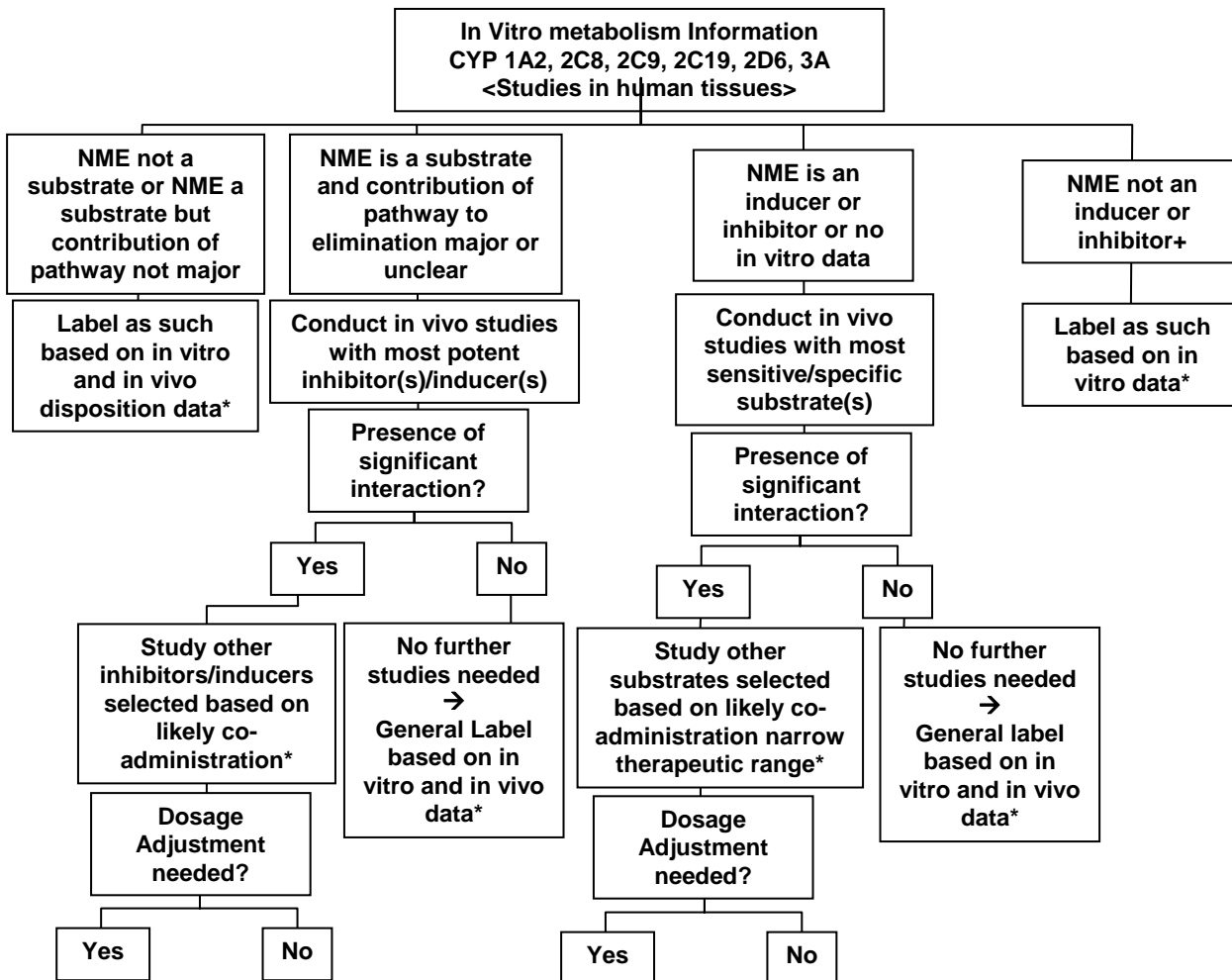
755

<http://www.fda.gov/cder/drug/drugInteractions/default.htm>

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761

Appendix B- Figures

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



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772

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.)

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### 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

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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°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

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855

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

863

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

867

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

878

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

881

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

887

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888

889 Table 2: Chemical inhibitors for in vitro experiments<sup>(7)</sup>

890

<b>CYP</b>	<b>Inhibitor <sup>(1)</sup> Preferred</b>	<b>Ki (<math>\mu</math>M)</b>	<b>Inhibitor <sup>(1)</sup> Acceptable</b>	<b>Ki (<math>\mu</math>M)</b>
1A2	furafylline <sup>(2)</sup>	0.6-0.73	$\alpha$ -naphthoflavone	0.01
2A6	tranlycypromine methoxsalen <sup>(2)</sup>	0.02-0.2 0.01-0.2	pilocarpine, tryptamine	4 1.7 <sup>(3)</sup>
2B6			3-isopropenyl-3-methyl diamantine, <sup>(4)</sup> 2-isopropenyl-2-methyl adamantine, <sup>(4)</sup> sertraline, phencyclidine, triethylenethiophosphoramidate (thiotepa), clopidogrel, ticlopidine	2.2 5.3 3.2 <sup>(5)</sup> 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	<sup>(6)</sup> 17 10, 24

891

892 (1) Substrates used for inhibition studies include: **CYP1A2**, phenacetin-o-deethylation, theophylline-N-  
893 demethylation; **CYP2A6**, coumarin-7-hydroxylation; **CYP2B6**, 7-pentoxeresorufin-O-depentylation,  
894 bupropion hydroxylation, 7-ethoxy-4-(trifluoromethyl)-coumarin O-deethylation, S-mephenytoin-N-  
895 demethylation; Bupropion-hydroxylation; **CYP2C8**, taxol 6- $\alpha$ -hydroxylation; **CYP2C9**, tolbutamide  
896 4-methylhydroxylation, S-warfarin-7-hydroxylation, phenytoin 4-hydroxylation; **2CYP2C19**, (S)-  
897 mephenytoin 4-hydroxylation **CYP2D6**, dextromethorphan O-demethylation, desbrisoquine hydroxylase;  
898 **CYP2E1**, chlorzoxazone 6-hydroxylation, aniline 4-hydroxylase; **CYP3A4/5**, testosterone-6 $\beta$ -  
899 hydroxylation, midazolam-1-hydroxylation; cyclosporine hydroxylase; nifedipine dehydrogenation.

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

902 (3) cDNA expressing microsomes from human lymphoblast cells.

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

904 (5) IC50 values.

905 (6) Specific time-dependent inhibitor.

906 (7) Note that this is not an exhaustive list. For an updated list, see the following link .

907 <http://www.fda.gov/cder/drug/drugInteractions/default.htm>

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908

909 The effectiveness of competitive inhibitors is dependent on concentrations of the drug  
910 and inhibitor. Experiments designed to identify and quantitate the relative importance of  
911 individual CYP enzymes mediating a drug's metabolism should use drug concentrations  
912  $\leq K_m$ . The experiments should include the inhibitor at concentrations that ensure  
913 selectivity and adequate potency. It is also acceptable to use a range of inhibitor  
914 concentrations.

915

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

919

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

922

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

924

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

929

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

938

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

940

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

947

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

949

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



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

953

954 The set of characterized microsomes should include microsomes prepared from at least  
955 10 individual donor livers. The variation in metabolic activity for each CYP enzyme  
956 should be sufficient between individual donor livers to ensure adequate statistical power.  
957 Enzyme activities in the set of microsomes used for correlation studies should be  
958 determined using appropriate probe substrates and experimental conditions.

959

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

964

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### APPENDIX C-2

#### **In Vitro Evaluation of CYP Inhibition**

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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.

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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

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\* 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.

1000

## 2. Design Considerations for In Vitro CYP Inhibition Studies

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(a) Typical experiments for determining IC<sub>50</sub> values involve incubating the substrate, if the metabolic rate is sufficient, at concentrations below its K<sub>m</sub> to more closely relate the inhibitor IC<sub>50</sub> to its K<sub>i</sub>. For K<sub>i</sub> determinations, both the substrate and inhibitor concentrations should be varied to cover ranges above and below the drug's K<sub>m</sub> and inhibitor's K<sub>i</sub>.

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

(c) Because buffer strength, type, and pH can all significantly affect V<sub>max</sub> and K<sub>m</sub>, standardized assay conditions are recommended.

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

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1017 (e) We suggest a linear relationship between time and amount of product formed.

1018

1019 (f) We recommend a linear relationship between amount of enzyme and product formation.

1020

1021 (g) Any solvents should be used at low concentrations ( $\leq 1\%$  (v/v) and preferably  $< 0.1\%$ ).  
1022 Some of the solvents inhibit or induce enzymes. The experiment can include a no-  
1023 solvent control and a solvent control.

1024

1025 (h) Use of an active control (known inhibitor) is optional.

1026

### 1027 3. **Determining Whether an NME is a Reversible Inhibitor**

1028

1029 Theoretically, significant enzyme inhibition occurs when the concentration of the inhibitor  
1030 present at the active site is comparable to or in excess of the  $K_i$ . In theory, the degree of  
1031 interaction (R, expressed as fold-change in AUC) can be estimated by the following  
1032 equation:  $R = 1 + [I]/K_i$ , where [I] is the concentration of inhibitor exposed to the active site  
1033 of the enzyme and  $K_i$  is the inhibition constant.

1034

1035 Although the  $[I]/K_i$  ratio is used to predict the likelihood of inhibitory drug interactions,  
1036 there are factors that affect selection of the relevant [I] and  $K_i$ . Factors that affect [I] include  
1037 uncertainty regarding the concentration that best represents concentration at the enzyme  
1038 binding site (at the gastrointestinal versus liver) and uncertainty regarding the impact of first-  
1039 pass exposure. Factors that affect  $K_i$  include substrate specificity, binding to components of  
1040 incubation system, and substrate and inhibitor depletion.

1041

### 1042 **Current recommended approach**

1043

1044 The likelihood of an in vivo interaction is projected based on the  $[I]/K_i$  ratio where [I]  
1045 represents the mean steady-state  $C_{max}$  value for total drug (bound plus unbound) following  
1046 administration of the highest proposed clinical dose. As the ratio increases, the likelihood of  
1047 an interaction increases. The following table suggests the likelihood of in vivo interaction  
1048 based on estimated  $[I]/K_i$  ratios. **An estimated  $[I]/K_i$  ratio of greater than 0.1 is**  
1049 **considered positive and a follow-up in vivo evaluation is recommended.**

1050

1051 Table 4. Prediction of clinical relevance of competitive CYP inhibition

1052

$[I]/K_i$	Prediction
$[I]/K_i > 1$	Likely
$1 > [I]/K_i > 0.1$	Possible
$0.1 > [I]/K_i$	Remote

1053

1054 Although quantitative predictions of in vivo drug-drug interactions from in vitro studies are  
1055 not possible, rank order across the different CYP enzymes for the same drug may help

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1056 prioritize in vivo drug-drug interaction evaluations. When various [I]/K<sub>i</sub> ratios are obtained  
1057 with the major CYP enzymes (CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and  
1058 CYP3A), an in vivo study starting with the CYP with the largest [I]/K<sub>i</sub> (or smallest K<sub>i</sub>) may  
1059 be appropriate. If the CYP with the largest [I]/K<sub>i</sub> (or smallest K<sub>i</sub>) shows no interaction in  
1060 vivo, in vivo evaluation of the other CYPs with smaller [I]/K<sub>i</sub> (or larger K<sub>i</sub>) will not be  
1061 needed. For CYP3A inhibition, two structurally unrelated substrates should be evaluated. If  
1062 one of the two evaluations suggests a potential interaction (i.e., [I]/K<sub>i</sub> more than 0.1), an in  
1063 vivo evaluation should be carried out.

1064

#### 1065 4. **Determining Whether an NME is a Mechanism-Based Inhibitor**

1066

1067 Time-dependent inhibition should be examined in standard in vitro screening protocols,  
1068 because the phenomenon cannot be predicted with complete confidence from chemical  
1069 structure. A 30-minute pre-incubation of a potential inhibitor before the addition of substrate  
1070 is recommended. Any time-dependent and concentration-dependent loss of initial product  
1071 formation rate indicates mechanism-based inhibition. For compounds containing amines,  
1072 metabolic intermediate complex formation can be followed spectroscopically. Detection of  
1073 time-dependent inhibition kinetics in vitro indicates follow-up with in vivo studies in  
1074 humans.

1075

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### 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 <sup>(1)</sup> -Preferred	Inducer Concentrations (µM)	Fold Induction	Inducer <sup>(1)</sup> -Acceptable	Inducer Concentrations (µM)	Fold Induction
1A2	omeprazole β-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.

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#### 1105 2. **Design of In Vitro Drug Induction Studies**

1106

1107 At this time, the most reliable method to study a drug's induction potential is to quantify the  
1108 enzyme activity of primary hepatocyte cultures following treatments including the potential  
1109 inducer drug, a positive control inducer drug (see Table 5), and vehicle-treated hepatocytes  
1110 (negative control), respectively. Freshly isolated human hepatocytes or cryopreserved  
1111 hepatocytes that can be thawed and cultured are the preferred liver tissue for these studies;  
1112 immortalized liver cells are acceptable if it can be demonstrated with positive controls that  
1113 CYP3A4 and CYP1A2 are inducible in these cell lines.

1114

1115 (a) Test drug concentrations should be based on the expected human plasma drug  
1116 concentrations be used. At least three concentrations spanning the therapeutic range  
1117 should be studied, including at least one concentration that is an order of magnitude  
1118 greater than the average expected plasma drug concentration. If this information is not  
1119 available, concentrations ranging over at least two orders of magnitude should be studied.

1120

1121 (b) Following treatment of hepatocytes for 2 to 3 days, the resulting enzyme activities can be  
1122 determined using appropriate CYP-specific probe drugs (see Table 3, Appendix C-2).  
1123 Either whole cell monolayers or isolated microsomes can be used to monitor drug-  
1124 induced enzyme changes; however, the former tissue is the simplest and the most direct  
1125 method.

1126

1127 (c) When conducting experiments to determine enzyme activity, the experimental conditions  
1128 listed in section Appendix C-2 are relevant.

1129

1130 (d) When using freshly isolated human or cryopreserved hepatocytes for induction studies,  
1131 experiments should be conducted with hepatocytes prepared from at least three individual  
1132 donor livers because of the inter-individual differences in induction potential.

1133

1134 (e) Experiments should be carried out in triplicate when using immortalized human liver  
1135 cells for induction studies.

1136

#### 1137 3. **Endpoints for Subsequent Prediction of Enzyme Induction**

1138

1139 When analyzing the results of experiments to determine whether a drug induces an enzyme  
1140 activity, the following issues are relevant.

1141

1142 (a) A drug that produces a change that is equal to or greater than 40% of the positive control  
1143 can be considered as an enzyme inducer in vitro and in vivo evaluation is warranted.

1144

1145 % positive control =  $\frac{\text{activity of test drug treated cells} - \text{activity of negative control}}{\text{activity of positive control} - \text{activity of negative control}} \times 100$   
1146

1147

1148 (b) An alternative endpoint is the use of an EC50 (effective concentration at which 50%  
1149 maximal induction occurs) value, which represents a potency index that can be used to

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1150 compare the potency of different compounds.

1151

1152 (c) Based on our present knowledge of cellular mechanisms leading to CYP enzyme  
1153 induction, if induction studies with a test drug confirm that it is not an inducer of  
1154 CYP3A4 then it can be concluded that the test drug is also not an inducer of CYP2C8,  
1155 CYP2C9, or CYP2C19.

1156

#### 1157 4. **Other Methods Proposed for Identifying In Vitro Enzyme Induction**

1158

1159 Although the most reliable method for quantifying a drug's induction potential is  
1160 measurement of enzyme activities after incubation of the drug in primary cultures of human  
1161 hepatocytes, other methods are being evaluated. Several of these methods are described  
1162 briefly below.

1163

1164 (a) Western immunoblotting or immunoprecipitation probed with specific polyclonal  
1165 antibodies.

1166

1167 Relative quantification of specific P450 enzyme protein requires that the  
1168 electrophoretic system clearly resolve the individual enzymes and/or that the  
1169 primary antibodies be specific for the enzyme quantified. Enzyme antibody  
1170 preparations are highly variable.

1171

1172 (b) Measurement of mRNA levels using reverse transcriptase-polymerase chain  
1173 reaction (RT-PCR).

1174

1175 RT-PCR can quantify mRNA expression for a specific CYP enzyme but is not  
1176 necessarily informative of enzyme activities. Measurement of mRNA levels is  
1177 helpful when both enzyme inhibition and induction are operative.

1178

1179 (c) Receptor gene assays for receptors mediating induction of P450 enzymes.

1180

1181 Cell receptors mediating CYP1A, CYP2B, and CYP3A induction have been  
1182 identified. Higher throughput AhR (aromatic hydrocarbon receptor) and PXR  
1183 (pregnane X receptor) binding assays and cell-based reporter gene assays have  
1184 been developed and used to screen for compounds that have CYP1A and CYP3A  
1185 induction potential. Although results of these assays provide supportive evidence  
1186 for a compound's induction potential, they do not necessarily reflect the enzyme  
1187 activities.

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## APPENDIX D

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

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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.

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

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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.

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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 MDR1 cells	Net drug flux ratio of B to A and A to B	<ul style="list-style-type: none"><li>• Directly measure efflux across cell barrier</li><li>• Evaluation of P-gp transport and inhibition</li><li>• Allow for localization/identification of the transporters within the apical or basolateral side of the membrane</li></ul>
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	<ul style="list-style-type: none"><li>• Cannot distinguish substrate from inhibitor</li><li>• Tends to fail to identify substrate and/or inhibitor with low permeability</li></ul>
ATPase	membrane vesicles from various tissues or cells expressing P-gp, Reconstituted P-gp	ATPase stimulation	<ul style="list-style-type: none"><li>• Same comments as uptake/efflux assay</li><li>• Do not always show good correlation with functional assay for P-gp</li></ul>

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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

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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

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- (1) Selective for the P-gp transporter
- (2) Exhibits low to moderate passive membrane permeability ( $2-30 \times 10^{-6}$  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)

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1251  
1252 Unfortunately, a P-gp substrate that meets all of the above criteria has not been  
1253 identified, due to overlapping substrate selectivity between transporter/transporter  
1254 and transporter /metabolizing enzymes. Table 2 lists examples of acceptable P-gp  
1255 substrates that meet the majority of the above mentioned criteria. These P-gp  
1256 substrates serve as positive controls to ensure the cell systems have functional P-gp  
1257 expression (see section (d) below) when used for transport experiments.  
1258

1259 Table 2. Acceptable P-gp Substrates

1260

Drug	Conc. Used ( $\mu\text{M}$ )	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 <sup>a</sup>	0.004-10	2-18	> 9 <sup>b</sup>	3
Talinolol	30	26		

1261

1262 Note that this is not an exhaustive list. For an updated list, see the following link  
1263 <http://www.fda.gov/cder/drug/drugInteractions/default.htm>

1264

1265 \*  $P_{\text{app, B-A}} / P_{\text{app, A-B}}$ ;  $P_{\text{app}}$  = apparent permeability

1266 \*\* Data for MDR1-MDCK and MDR1-LLCPK are the ratio observed in transfected  
1267 cells relative to the ratio observed in respective wild-type cells.

1268 a Vinblastine is also a substrate for MRP2 that is constitutively expressed in Caco-2, and wild type  
1269 MDCK and LL-CPK1 cells.

1270 b Data are derived from net B to A flux in the absence of GF120918, a potent P-gp inhibitor,  
1271 relative to that observed in the presence of GF120918.

1272

1273 Acceptable P-gp substrates are not limited to compounds listed in Table 2. Selection of  
1274 other compounds as probe P-gp substrates may be appropriate based on scientific  
1275 justification.

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

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(1) Selective for P-gp transporter

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(2) Inhibit P-gp with low  $K_i$  or  $IC_{50}$  values (e.g.,  $IC_{50} < 10 \mu M$ )

1281

(3) No significant metabolism of the inhibitor occurs in the cells (optional)

1282

(4) Commercially available (optional)

1283

(5) May be used as an in vivo P-gp inhibitor (optional)

1284

1285

Most P-gp substrates with high affinity are also potent competitive inhibitors.

1286

Examples of compounds extensively studied and reported in the literature as

1287

potent P-gp inhibitors are listed in Table 3. The table includes  $IC_{50}$  or  $K_i$

1288

values determined using bi-directional transport assays. Some inhibitors may

1289

inhibit multiple transporters, because of overlap among transporters. For

1290

example, in addition to being potent inhibitors for P-gp, cyclosporine A is also

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a potent inhibitor for MRP2 and OATP-C, and quinidine and verapamil are

1292

also potent inhibitors for various organic cation transporters. Because of the

1293

lack of inhibitor specificity, the use of multiple inhibitors is recommended to

1294

determine whether the efflux activity observed in vitro is related to P-gp.

1295

1296

Acceptable P-gp inhibitors are not limited to compounds listed in Table 3. Selection of

1297

other compounds as probe P-gp inhibitors may be appropriate based on scientific

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justification.

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Table 3. In Vitro P-gp Inhibitors

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Inhibitor	IC50 (μM)	Ki (μM)		
		Caco-2*		MDCK-
		Caco-2*	MDR1*	LLC-PK1 MDR1**
Cyclosporine A <sup>a</sup>	1.3	0.5	2.2	1.3
Ketoconazole <sup>a</sup>	1.2			5.3
LY335979	0.024			
Nelfinavir <sup>a</sup>	1.4			
Quinidine <sup>b</sup>	2.2	3.2	8.6	
Ritonavir <sup>a</sup>	3.8			
Saquinavir <sup>a</sup>	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	

1303

Note that this is not an exhaustive list. For an updated list, see the following link

1304

<http://www.fda.gov/cder/drug/drugInteractions/default.htm>

1305

1306

\* Digoxin as a P-gp substrate

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\*\* Vinblastine as a P-gp substrate

1308

<sup>a</sup> also CYP3A inhibitor

1309

<sup>b</sup> also CYP2D6 inhibitor

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### **(c) Tissue culture considerations to ensure functionally polarized cells**

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1314

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

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1316 Caco-2, transfected LLC-PK1-MDR1, and transfected MDCK-MDR1. LLC-PK1 and  
1317 MDCK wild type cells are used as negative controls.

1318

1319 (1) Caco-2 cells should be seeded at a density of approximately  $0.5-5 \times 10^5$  cells/cm<sup>2</sup>  
1320 on polycarbonate microporous membrane filters and allowed to grow to  
1321 confluence (typically 18-21 days).

1322 (2) LLC-PK1 and LLC-PK1-MDR1, MDCK, and MDCK-MDR1 cells should be  
1323 seeded at a density of approximately  $0.05-5.0 \times 10^6$  cells/cm<sup>2</sup> on polycarbonate  
1324 microporous membrane filters and allowed to grow to confluence (typically 3-5  
1325 days).

1326 (3) The transepithelial electrical resistance (TEER) of the polarized cells should be  
1327 determined before each experiment (typical values are 100-800  $\Omega$  cm<sup>2</sup>).

1328 (4) A paracellular marker such as [<sup>14</sup>C] mannitol can be used as an additional integrity  
1329 marker (typical permeability values are  $< 0.2-2 \times 10^{-6}$  cm/sec).

1330

1331 **(d) Design of bi-directional experiments conducted to determine whether the drug**  
1332 **is a P-gp substrate**

1333

1334 After selection of the cell type and P-gp substrate positive control, bi-directional  
1335 substrate experiments are typically performed using polycarbonate filter inserts and  
1336 side-side diffusion chambers as follows:

1337

1338 (1) The efflux of the investigational drug should be studied over a range of  
1339 concentrations (e.g., 1, 10 and 100  $\mu$ M).

1340 (2) Before initiating bidirectional experiments, the medium in the donor and receiver  
1341 chambers is removed, replaced with fresh medium, and pre-incubated for 30  
1342 minutes.

1343 (3) Bi-directional permeability studies are initiated by adding an appropriate volume of  
1344 buffer containing a known drug probe P-gp substrate or the test drug to either the  
1345 apical (for apical to basolateral transport, A/B) or the basolateral (for basolateral to  
1346 apical, B/A) side of the monolayer.

1347 (4) Samples are incubated at 37°C. At selected times (typically 1, 2, 3, 4 hours),  
1348 aliquots from the receiver compartment are collected for determination of the test  
1349 compound concentrations. The volume removed is replaced immediately with  
1350 buffer.

1351 (5) A known P-gp substrate (see Table 2) should be run as a positive control.

1352 (6) When using LLC-PK1-MDR1 or MDCK-MDR1 cells for bi-directional studies,  
1353 LLC-PK1 and MDCK cells, respectively, should be included as negative controls.

1354 (7) Each experiment should be performed at least in triplicate on different days to allow  
1355 for assessment of intra- and inter-day variations.

1356 (8) Optimal experiments should determine recovery of substrate, to allow estimation of  
1357 metabolism and non-specific binding.

1358

1359 Because Caco-2 cells, wild-type MDCK, and wild-type LLC-PK1 cells may also express

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1360 efflux transporters other than P-gp, data interpretation of data from bi-directional transport  
1361 studies using the test drug as a substrate should be viewed with caution. To strengthen the  
1362 results from bi-directional transport studies, it is recommended that additional experiments  
1363 be conducted in the presence of potent P-gp inhibitors (at least 2-3 potent P-gp inhibitors; see  
1364 Table 3 for examples). If the test drug efflux is inhibited by these P-gp inhibitors, it is likely  
1365 that the efflux activity is related to P-gp. Finally, experiments that compare efflux activity  
1366 observed in overexpressed-MDR1 cells to that observed in their respective wild-type cells  
1367 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**

1371 The apparent permeability of compounds across the monolayer cells used for  
1372 transporter studies is calculated using the following equation:  
1373

$$1374 \quad P_{\text{app}} = (V_r/C_0)(1/S)(dC/dt) \quad (1)$$

1376 Where  $P_{\text{app}}$  = apparent permeability,  $V_r$  is the volume of medium in the receiver  
1377 chamber,  $C_0$  is the concentration of the test drug in the donor chamber,  $S$  is the  
1378 surface area of monolayer,  $dC/dt$  is the is the linear slope of the drug concentration in  
1379 the receptor chamber with time after correcting for dilution.  
1380

1381 Flux through the monolayer must be linear with time ( $dC/dt$  is constant) for accurate  
1382 determination of  $P_{\text{app}}$ .  
1383

1384 The efflux ratio ( $R_E$ ) for basolateral to apical and apical to basolateral transport is  
1385 defined by the following equation:  
1386

$$1387 \quad R_E = P_{B/A} / P_{A/B} \quad (2)$$

1389 where  $P_{B/A}$  and  $P_{A/B}$  represent the apparent permeability of test compound from the  
1390 basal to apical and apical to basal side of the cellular monolayer, respectively.  
1391

1392 When using Caco-2 cells, the ratio ( $R_E$ ) is calculated directly. However, for the LLC-  
1393 PK1-MDR1 or MDCK-MDR1 cells, an  $(R) = (R_T) / (R_w)$  is calculated where ( $R_T$ ) and  
1394 ( $R_w$ ) are the permeability ratios for the transfected and the non-transfected lines (used  
1395 for negative controls), respectively.  
1396

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

1397 After selection of the cell type, probe P-gp substrate, and known P-gp inhibitors,  
1398 experiments designed to evaluate whether a test drug is an inhibitor of P-gp are  
1399 performed using polycarbonate filter inserts and side-side diffusion chambers, as  
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1404 follows:

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- (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):

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

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.  $I_{max}$  represents maximal inhibitory effect, and (c) is the Hill Plot exponent. The IC50 is the inhibitor concentration (test drug) achieving half maximal inhibition effect.



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

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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.

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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.

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- 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.

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- 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.

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- 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.

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1463

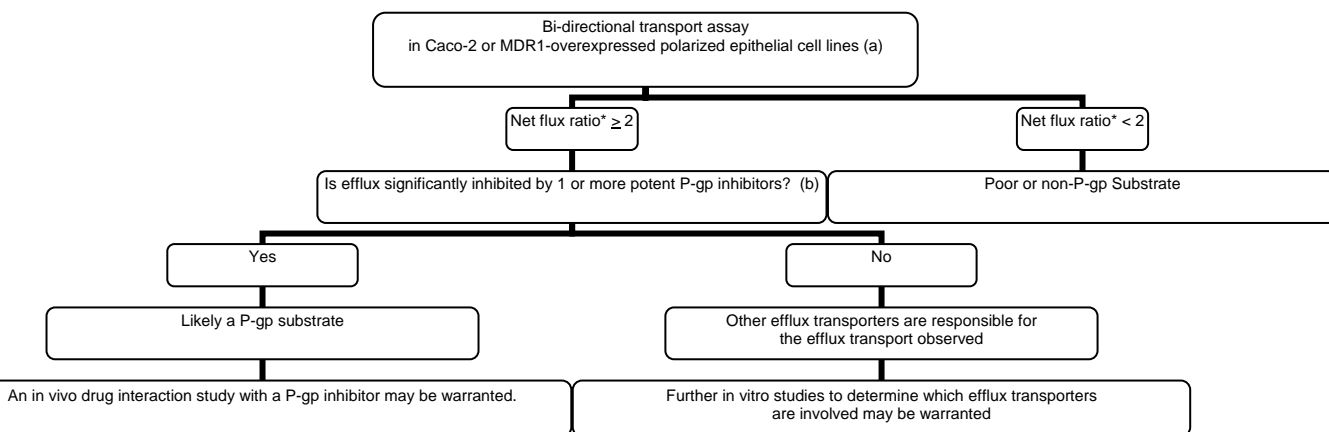
1464

- 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.

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1465 **Figure 1. Decision tree to determine whether an investigational drug is a substrate**  
1466 **for P-gp and whether an in vivo drug interaction study with a P-gp**  
1467 **inhibitor is needed**  
1468  
1469  
1470



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1484

1485 \*For Caco-2 cells, net flux ratio is calculated as  $(\text{Permeability}_{\text{app, B-A}}/\text{Permeability}_{\text{app, A-B}})$ ; For MDR1-

1486 overexpressed cell lines, net flux ratio is calculated as ratio of  $(\text{Permeability}_{\text{app, B-A}}/\text{Permeability}_{\text{app, A-B}})_{\text{MDR1}}$  to

1487  $(\text{Permeability}_{\text{app, B-A}}/\text{Permeability}_{\text{app, A-B}})_{\text{wild-type}}$ .

1488 (a) An acceptable system produces net flux ratios of probe substrates similar to the literature values. A net

1489 flux ratio  $\geq 2$  for the investigational drug is a positive signal for further evaluation. Note: there is a concern that

1490 this value is too liberal and will lead to too many positive results. An alternative is to use a % value (net flux of

1491 investigation drug relative to a probe substrate, such as digoxin).

1492 (b) reduction of the flux ratio significantly ( $> 50\%$ ) or to unity

1493

#### 1494 **4. Criteria for Determining Whether a Test Compound (Investigational Drug) is an**

#### 1495 **Inhibitor of P-gp, and Whether an In Vivo Interaction Study is Needed**

1496

1497 Before evaluating data regarding a test compound's status as a P-gp inhibitor, it is

1498 important to determine whether the cell system used for the experiments is sufficient.

1499 This assessment considers the net flux ratio of the probe substrates. A sufficient system

1500 produces net flux ratios of the probe substrates similar to values reported in the literature

1501 (a minimum net flux ratio of 2 is recommended). The probe substrate concentration used

1502 should be below its apparent  $K_m$  for P-gp. Two to three known potent inhibitors of P-gp

1503 should be included in the study as positive controls. Initially, a high concentration (e.g.,

1504  $>100 \mu\text{M}$  or as high as solubility of the compound allows) can be used to determine

1505 whether the efflux of the probe P-gp substrate is affected by the investigational drug.

1506

1507 If the cell system is acceptable, the following items (and Figure 2) describe the process

1508 for determining whether a test drug is a P-gp inhibitor and whether in vivo interaction

1509 studies with P-gp substrates are recommended.

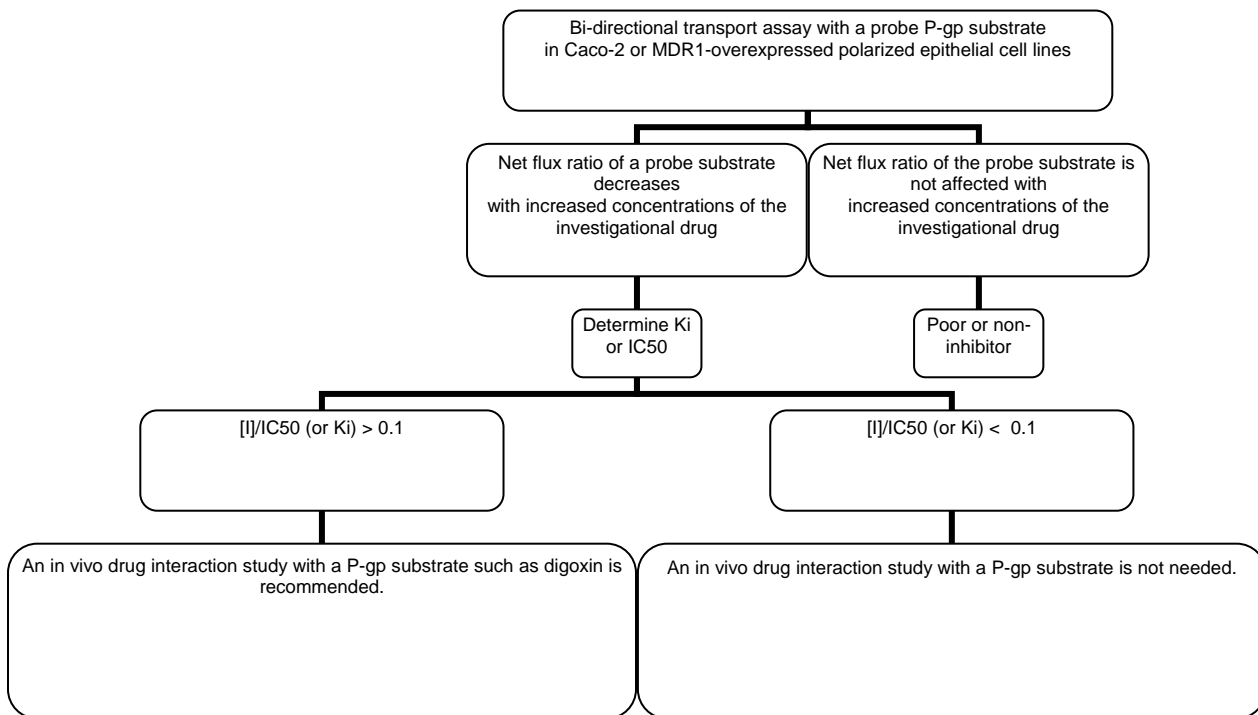
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- 1511 • If the efflux of the probe substrate is not inhibited by the investigational drug, then  
1512 the investigational drug is likely a poor or non-inhibitor of P-gp.  
1513
- 1514 • If the efflux of the probe substrate is inhibited by the investigational drug, then the  
1515 inhibition should be studied over a range of concentrations to determine IC<sub>50</sub> or K<sub>i</sub>.  
1516 IC<sub>50</sub> or K<sub>i</sub> values may be experiment-dependent. Therefore, the obtained IC<sub>50</sub> or K<sub>i</sub>  
1517 values should be compared to IC<sub>50</sub> or K<sub>i</sub> values obtained for 2-3 known potent P-gp  
1518 inhibitors (positive controls).  
1519
- 1520 • If [I]/ IC<sub>50</sub> (or K<sub>i</sub>) is > 0.1, then the investigational drug is likely a P-gp inhibitor.  
1521 An in vivo drug interaction study with a P-gp substrate such as digoxin should be  
1522 conducted.  
1523
- 1524 • If [I]/IC<sub>50</sub> (or K<sub>i</sub>) is < 0.1, then the investigational drug is likely a weak P-gp  
1525 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  $(\text{Permeability}_{\text{app, B-A}}/\text{Permeability}_{\text{app, A-B}})$ ; For MDR1-overexpressed cell lines, net flux ratio is calculated as ratio of  $(\text{Permeability}_{\text{app, B-A}}/\text{Permeability}_{\text{app, A-B}})_{\text{MDR1}}$  to  $(\text{Permeability}_{\text{app, B-A}}/\text{Permeability}_{\text{app, A-B}})_{\text{wild-type}}$ . Note that [I] represents the mean steady-state C<sub>max</sub> 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-resistant (LS 180/AD 50) or vinblastine-resistant (LS

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1571 180/V) sublimes have been used to study induction for both P-gp and CYP3A.  
1572  
1573 Methods for in vitro evaluation for P-gp induction are not well understood. Thus, the P-gp  
1574 induction potential of an investigational drug can only be evaluated in vivo. Because of  
1575 similarities in the mechanism of CYP3A and P-gp induction, information from test of  
1576 CYP3A inducibility can inform decisions about P-gp. As stated previously, if an  
1577 investigational drug is found not to induce CYP3A in vitro, no further tests of CYP3A and P-  
1578 gp induction in vivo are necessary. If a study of the investigational drug's effect on CYP3A  
1579 activity in vivo is indicated from a positive in vitro screen, but the drug is shown not to  
1580 induce CYP3A in vivo, then no further test of P-gp induction in vivo is necessary. However,  
1581 if the in vivo CYP3A induction test is positive, then an additional study of the investigation  
1582 drug's effect on a P-gp probe substrate is recommended.

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