System-Dependent Outcomes during the Evaluation of Drug Candidates as Inhibitors of Cytochrome P450 (CYP) and Uridine Diphosphate Glucuronosyltransferase (UGT) Enzymes: Human Hepatocytes versus Liver Microsomes versus Recombinant Enzymes

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Summary: The ability of a drug to cause clinically significant drug-drug interactions due to direct or metabolism-dependent inhibition of cytochrome P450 (CYP) can generally be predicted from in vitro studies with human liver microsomes (HLM) or recombinant CYP enzymes, as recommended by the FDA and other regulatory agencies. This review highlights some examples of system-dependent inhibition of CYP and uridine diphosphate glucuronosyltransferase (UGT) enzymes. In the case of CYP enzymes, examples are presented where in vitro studies with HLM under-predict or over-predict the degree of inhibition observed in the clinic and where the correct prediction comes from studies with human hepatocytes. Studies with HLM under-predict the ability of gemfibrozil and bupropion to cause clinically significant inhibition of CYP2C8 and CYP2D6, respectively, and over-predict the ability of ezetimibe to cause clinically significant inhibition of CYP3A4. Gemfibrozil and bupropion represent examples of glucuronidation-dependent and reduction-dependent activation to metabolites that inhibit CYP2C8 and CYP2D6, respectively, whereas ezetimibe represents an example of glucuronidation-dependent protection against metabolism-dependent inhibition of CYP3A4. This article illustrates why, when drug candidates are extensively metabolized by non-CYP enzymes, it would be prudent to use human hepatocytes in addition to HLM or recombinant enzymes to evaluate their ability to inhibit CYP enzymes.

Keywords: cytochrome P450; hepatocytes; cell; microsomes; organelle; inhibition; UDP-glucuronosyltransferase; drug; xenobiotics

Introduction

In its draft guidance document Drug Interaction Studies—Study Design, Data Analysis and Implications for Dosing and Labeling and in a subsequent review article by Huang et al., the Food and Drug Administration (FDA) recommends in vitro studies with human liver microsomes (HLM) or recombinant cytochrome P450 (CYP) enzymes be conducted to evaluate the ability of drug candidates to inhibit the major human drug-metabolizing CYP enzymes, namely CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4.1) The FDA further recommends that such studies be designed to evaluate the ability of drug candidates to function as direct-acting inhibitors (to identify inhibitors such as ketoconazole) and as metabolism-dependent inhibitors (to identify inhibitors such as mibefradil). With relatively minor modifications, the FDA’s recommendations for the conduct of in vitro CYP inhibition studies have been reinforced in two consensus reviews (white papers) by the Pharmaceutical Research and Manufacturers of America (PhRMA).2,3) Figure 1 illustrates the results of a typical in vitro CYP inhibition study.
Fig. 1. Inhibition of CYP3A4 activity in human liver microsomes by the direct-acting inhibitor ketoconazole and the metabolism-dependent inhibitor mibefradil

This in vitro study was conducted as described by Ogilvie et al.6) and Paris et al.5) Briefly, NADPH-fortified pooled human liver microsomes (0.05 or 0.1 mg protein/mL) were incubated for zero or 30 min with ketoconazole (left) or mibefradil (right) prior to measuring CYP3A4 activity based on the 6β-hydroxylation of testosterone (70 μM) or the 1'-hydroxylation of midazolam (4 μM) with a 5-min incubation period with CYP marker substrate. Pooled human liver microsomes were from XenoTech LLC (Lenexa, KS).

study, which was designed to evaluate the ability of ketoconazole (Nizoral) and mibefradil (Posicor) to inhibit CYP3A4 activity in HLM. In this study, which was conducted as described by Ogilvie et al. and Paris et al., ketoconazole and mibefradil were incubated with NADPH-fortified HLM (≤ 0.1 mg protein/mL) for zero or 30 min prior to measuring CYP3A4 activity based on the 1'-hydroxylation of midazolam (4 μM) or the 6β-hydroxylation of testosterone (70 μM) with the CYP marker substrate concentrations set to Km (in the case of midazolam) or S50 (in the case of testosterone, which exhibits homotropic cooperativity with a Hill coefficient of 1.3).4,5) As shown in Figure 1, a 30-min preincubation with NADPH-fortified HLM does not enhance the ability of ketoconazole to inhibit CYP3A4 (in fact, it slightly decreases its inhibitory effect), whereas a 30-min preincubation with mibefradil markedly enhances its ability to inhibit CYP3A4, as evidenced by the ∼100-fold shift in IC50 value (from 5.5 μM to 0.063 μM).

Ketoconazole and mibefradil both inhibit the metabolism of a large number of drugs whose clearance is largely determined by their rate of metabolism by CYP3A4, such as terfenadine, cisapride and astemizole, three victim drugs that have been withdrawn from the US market or which carry black box warnings due to adverse events (potentially life-threatening cardiotoxicity characterized by QT prolongation) observed in the presence of a CYP3A4 inhibitor. Ketoconazole carries a black box warning for its ability to inhibit the CYP3A4-dependent metabolism of the aforementioned drugs, and mibefradil was withdrawn from the US market largely on the basis of its ability to cause both extensive and prolonged inhibition of CYP3A4 (prolonged because mibefradil is an irreversible metabolism-dependent inhibitor of CYP3A4 and, as such, the reversal of its inhibitory effect requires both the discontinuation of drug treatment and the synthesis of CYP3A4, which takes several days).3,4)

For drug candidates that are primarily metabolized by CYP enzymes, HLM and recombinant CYP enzymes are appropriate in vitro test systems to evaluate their inhibitory potential. Accordingly, in many cases, an assessment of a drug’s inhibitory potential is not dependent on the choice of in vitro test system, as illustrated in Figure 2 for the inhibition of CYP2C19 by omeprazole with HLM, recombinant CYP2C19 and human hepatocytes.6) Both in vivo and, at pharmacologically relevant concentrations, in vitro, omeprazole is primarily metabolized by CYP2C19, and it is also a weak metabolism-dependent inhibitor of this enzyme. A shift to a lower IC50 value is observed when omeprazole is preincubated with NADPH-fortified HLM, NADPH-fortified recombinant CYP2C19 or human hepatocytes, and the IC50 value following a 30-min preincubation is roughly the same in all three test systems (IC50 = 1.3–1.7 μM). Results similar to those represented in Figure 2 were obtained when omeprazole was evaluated as both an in vitro and in vivo inhibitor of diazepam metabolism in rats.7) In this study, Zomorodi and Houston were able to determine an in vivo Ki value because in rats it is possible to infuse a probe or victim drug directly into the hepatic portal vein to accurately determine its clearance, and subsequently to administer a wide range of bolus intravenous doses of a perpetrator drug in order to achieve a range of steady-state plasma concentrations. This approach was used in rats to determine that omeprazole inhibits the metabolism of diazepam with an in vivo Ki value of 21 μM, which is comparable to the in vitro Ki values determined with both rat liver microsomes and rat hepatocytes by a variety of experimental approaches (e.g., substrate depletion, individual metabolite formation and Ki determination).7)

From the results in Figure 2, it is apparent that
Fig. 2. Inhibition of CYP2C19 by omeprazole in human liver microsomes, recombinant CYP2C19 and human hepatocytes
This in vitro study was conducted essentially as described by Ogilvie et al.4) and Parisi et al.5,6) CYP2C19 activity was measured based on the rate of 4′-hydroxylation of S-mephenytoin (40 μM), which was assessed immediately after the addition of omeprazole (zero preincubation) or following a 30-min preincubation of omeprazole with NADPH-fortified pooled human liver microsomes (0.1 mg protein/mL), NADPH-fortified recombinant human CYP2C19 (15 pmol/mL) or pooled human hepatocytes (1.0 × 10⁶ cells/mL). Pooled human liver microsomes and pooled human hepatocytes were from XenoTech LLC (Lenexa, KS); recombinant CYP2C19 was from Cynex (Dundee, UK).

omeprazole does not exhibit system-dependent inhibition of CYP2C19 inasmuch as the same results are observed regardless of whether CYP2C19 inhibition is evaluated in HLM, the recombinant enzyme or in human hepatocytes. However, system-dependent inhibition can occur when the inhibitory drug is extensively metabolized by a non-CYP pathway, such as glucuronidation, as illustrated below for the lipid-lowering drug gemfibrozil (Lopid) and the cholesterol-absorption inhibitor ezetimibe (Zetia).

Gemfibrozil: a system-dependent inhibitor of CYP2C8

Gemfibrozil causes clinically significant inhibition of CYP2C8. It inhibits the metabolism of pioglitazone, rosiglitazone and repaglinide (the latter being an FDA-approved in vivo CYP2C8 probe drug),8) and it inhibits the metabolism [as well as the organic anion transporting polypeptide-1B1 (OATP1B1)-mediated hepatic uptake] of the potent cholesterol-lowering drug cerivastatin.9–14) Cerivastatin (Baycol) was withdrawn from the US, European and Japanese markets because, compared with other statins, it caused a relatively high incidence of rhabdomyolysis, an adverse effect characterized by muscle weakness and potentially life-threatening kidney failure secondary to the release of myoglobin from damaged skeletal muscle. In approximately one third of the fatal cases of cerivastatin-induced rhabdomyolysis, cerivastatin was coadministered with gemfibrozil (www.emea.europa.eu/pdfs/human/referral/Cerivastatin/081102en.pdf), and this interaction was shown to be pharmacokinetic in nature.9) Gemfibrozil causes a marked increase (up to eight-fold) in the systemic exposure to cerivastatin [based on the area under the plasma concentration-time curve (AUC)], which directs cerivastatin away from its pharmacological target (the liver) and towards the target of toxicity (skeletal muscle).

Cerivastatin is actively transported into the liver (its pharmacological site of action) by OATP1B1, and in the liver it is metabolized by CYP2C8 and, to a lesser extent, by CYP3A4.11,15) To account for the pharmacokinetic interaction between gemfibrozil and cerivastatin, gemfibrozil was evaluated as an inhibitor of OATP1B1 and both CYP2C8 and CYP3A4, but the potency with which gemfibrozil inhibited this transporter and these enzymes was too weak to account for its effect on cerivastatin pharmacokinetics. Gemfibrozil is extensively conjugated with glucuronic acid to form a relatively stable (non-toxic) acyl glucuronide, and studies by Shitara et al.13) identified gemfibrozil glucuronide as an inhibitor of OATP1B1, whereas subsequent studies by Ogilvie et al.16) identified gemfibrozil glucuronide (but not gemfibrozil itself) as an irreversible, metabolism-dependent inhibitor of CYP2C8. The in vitro finding that, following its conversion to an acyl glucuronide, gemfibrozil is an irreversible inhibitor of CYP2C8 is supported by a subsequent clinical drug interaction study by Tornio et al.,14) who observed that gemfibrozil causes a prolonged inhibition of repaglinide metabolism in vivo.

Due to marked differences in the interaction of gemfibrozil and gemfibrozil glucuronide with CYP2C8, the ability of gemfibrozil to inhibit CYP2C8 in HLM is markedly different from its ability to inhibit CYP2C8 in human hepatocytes. This system-dependent inhibition is shown in Figure 3. In HLM, gemfibrozil is a weak inhibitor of CYP2C8 (IC₅₀ > 100 μM) when examined either as a direct-acting inhibitor (no preincubation) or a metabolism-dependent inhibitor (30-min preincubation). In hepatocytes, however, gemfibrozil is a potent inhibitor of CYP2C8 and, in contrast to the situation in HLM, it is a time-dependent inhibitor of CYP2C8 in human hepatocytes. Following a 30-min preincubation with gemfibrozil, the IC₅₀ value for CYP2C8 inhibition in hepato-
Fig. 3. System-dependent inhibition of CYP2C8 by gemfibrozil in human liver microsomes and human hepatocytes
This *in vitro* study was conducted essentially as described by Ogilvie *et al.*4,5) and Paris *et al.*16) CYP2C8 activity was measured based on the rate of 6α-hydroxylation of paclitaxel (15 μM) or the N-dealkylation of amodiaquine (15 μM), which was assessed immediately after the addition of gemfibrozil (zero preincubation) or following a 30-min preincubation of gemfibrozil with NADPH-fortified pooled human liver microsomes (0.05 mg protein/mL) or pooled human hepatocytes (0.3 x 10^6 cells/mL). Pooled human liver microsomes and pooled human hepatocytes were from XenoTech LLC (Lenexa, KS).

![Diagram of gemfibrozil metabolism](image)

Fig. 4. Mechanism of the irreversible metabolism-dependent inhibition of CYP2C8 by gemfibrozil
The metabolism-dependent inhibition of CYP2C8 by gemfibrozil involves the conversion of gemfibrozil to an acyl glucuronide which is then metabolized by CYP2C8 to a benzylic radical that covalently binds to the heme moiety and irreversibly inhibits the enzyme.16,17)
cytes (IC_{50} = 1.7 μM) is almost two orders of magnitude lower than the corresponding IC_{50} value in HLM (IC_{50} = 150 μM). Furthermore, the IC_{50} value for CYP2C8 inhibition in hepatocytes following a 30-min preincubation with gemfibrozil (IC_{50} = 1.7 μM) is nearly identical to the published IC_{50} value for CYP2C8 inhibition in human liver microsomes following a 30-min preincubation with gemfibrozil glucuronide (IC_{50} = 1.8 μM).16)

The system-dependent inhibition of CYP2C8 by gemfibrozil can be understood from the metabolic scheme shown in Figure 4. Gemfibrozil is glucuronidated prior to its metabolism by CYP2C8, which catalyzes the benzylic-hydroxylation of gemfibrozil glucuronide (i.e., methyl-hydroxylation on the 2',5'-dimethylphenoxy moiety).16) In the process of doing so, CYP2C8 converts gemfibrozil glucuronide to a carbon-centered radical (a benzylic intermediate) that alkylates the heme moiety and thereby irreversibly inactivates CYP2C8.17) This entire metabolic pathway is operational in human hepatocytes, which can both glucuronidate gemfibrozil and oxidize gemfibrozil glucuronide, but it is not operational in NADPH-fortified HLM [which cannot glucuronidate gemfibrozil in the absence of added uridine diphosphate (UDP)-glucuronic acid]. The irreversible, metabolism-dependent inhibition of CYP2C8 can be detected in HLM with gemfibrozil itself, provided the microsomes are fortified with the cofactors for both glucuronidation and oxidation (i.e., if HLM are incubated with both UDP-glucuronic acid and NADPH).16)

In the case of gemfibrozil, the degree of CYP2C8 inhibition is markedly greater in hepatocytes than in HLM due to glucuronidation-dependent activation (i.e., due to the conversion of gemfibrozil to gemfibrozil glucuronide which, in contrast to gemfibrozil itself, is an irreversible, metabolism-dependent inhibitor of CYP2C8). The opposite outcome (i.e., glucuronidation-dependent protection against irreversible, metabolism-dependent inhibition) is observed with ezetimibe.

**Ezetimibe: a system-dependent inhibitor of CYP3A4**

Ezetimibe (Zetia) is a cholesterol-absorption inhibitor.18) It is often coadministered with a statin (a cholesterol synthesis inhibitor) such as simvastatin (Zocor). In fact this particular combination of hypolipidemic drugs (i.e., ezetimibe and simvastatin) is marketed as Vytorin. As shown in Figure 5, ezetimibe contains two sites for direct conjugation, an aliphatic alcohol and a phenol. The phenolic group is rapidly and extensively glucuronidated both in the small intestine and liver (by several UGT enzymes, including UGT1A1, 1A3 and 2B15) and is returned to the intestine by biliary excretion [by P-glycoprotein (MDR1 or ABCB1) and MRP2 (ABCC2)].18,19) Although glucuronidation typically terminates the pharmacological activity of a drug, ezetimibe is an exception: its phenolic glucuronide is pharmacologically active.18)

In HLM, ezetimibe is an irreversible, metabolism-dependent inhibitor of CYP3A4.18) Following a 30-min preincubation of ezetimibe with NADPH-fortified HLM, the IC_{50} value for CYP3A4 inhibition shifts about 100-fold from 31 to 0.34 μM, as shown in Figure 6. The magnitude of the IC_{50} shift with ezetimibe is comparable to that observed with mibefradil (Fig. 2), the drug withdrawn from the US market due to its extensive and prolonged inhibition of CYP3A4. Over the same range of concentrations, ezetimibe does not inhibit the CYP3A4-dependent metabolism of midazolam in human hepatocytes even after a 30-min preincubation. In fact, high concentrations of ezetimibe (10–30 μM) actually increase the concentration of 1'-hydroxymidazolam, either due to increased metabolite formation or decreased secondary metabolism, as shown in Figure 6. Interestingly, whereas, ezetimibe does not inhibit midazolam 1'-hydroxylation with either a zero- or 30-min preincubation in human hepatocytes, it does inhibit the 6β-hydrox-
This in vitro study was conducted essentially as described previously. CYP3A4 activity was measured based on the rate of 1'-hydroxylation of midazolam (4 μM), which was assessed immediately after the addition of ezetimibe (zero preincubation) or following a 30-min preincubation of ezetimibe with NADPH-fortified pooled human liver microsomes (0.1 mg protein/mL) or pooled human hepatocytes (0.5 × 10⁶ cells/mL). Pooled human liver microsomes and pooled human hepatocytes were from XenoTech LLC (Lenexa, KS).

CYP3A4 activity was measured based on the rate of 1'-hydroxylation of midazolam (4 μM), which was assessed following a 30-min preincubation of ezetimibe with pooled human liver microsomes (0.05 mg protein/mL) in the presence or absence of NADPH (1 mM) and in the presence or absence of UDP-glucuronic acid (8 mM). Pooled human liver microsomes were from XenoTech LLC (Lenexa, KS).

The system-dependent inhibition of CYP3A4 by ezetimibe is likely due to glucuronidation-dependent protection, which would be expected to occur in hepatocytes but not in NADPH-fortified HLM. This interpretation is supported by the results in Figure 7, which show that the presence of UDP-glucuronic acid decreases the potency with which ezetimibe functions as both a direct-acting and metabolism-dependent inhibitor of CYP3A4 in HLM. When examined as a direct-acting inhibitor (i.e., with no preincubation period), the presence of UDP-glucuronic acid increases the IC₅₀ value for CYP3A4 inhibition about threefold (from 12 μM to 37 μM), and when examined as a metabolism-dependent inhibitor (i.e., with a 30-min preincubation period with NADPH), the presence of UDP-glucuronic acid increases the IC₅₀ value for CYP3A4 inhibition by about ninefold (from 0.24 μM to 2.1 μM).

The system-dependent inhibition of CYP3A4 by ezetimibe raises the question: Which system (HLM or hepatocytes) best predicts the clinical effects of ezetimibe on CYP3A4? The answer is: hepatocytes. Ezetimibe does not cause clinically significant inhibition of CYP3A4 as evidenced by its lack of effect on the pharmacokinetics (plasma Cmax and AUC) of several drugs whose disposition is determined, at least in part, by CYP3A4-dependent metabolism, including midazolam, simvastatin, atorvastatin and lovastatin. The lack of a pharmacokinetic interaction between ezetimibe and simvastatin is significant not only because it supports the view that ezetimibe fails to cause clinically significant inhibition of CYP3A4 but also because it supports the combined use of these two particular drugs in Vytorin.

In the case of both gemfibrozil and ezetimibe, glucuronidation is the basis for the observed system-dependent inhibition of CYP enzymes because both these drugs are extensively glucuronidated, and because glucuronidation occurs in hepatocytes but not in NADPH-fortified HLM. This begs the question: Are there examples of clinically relevant, system-dependent inhibition of CYP enzymes due to the metabolism of an inhibitory drug by enzymes other than UDP-glucuronyltransferases? And the answer is yes: bupropion is a system-dependent inhibitor of CYP2D6 due, at least in part, to its carbonyl reduction to a pair of hydrobupropion metabolites.
Bupropion: a system-dependent inhibitor of CYP2D6

Although bupropion (Wellbutrin, Zyban) is primarily metabolized by CYP2B6,20 it causes clinically significant inhibition of the metabolism of several CYP2D6 substrates including desipramine,21–23 dextromethorphan24,25 and venlafaxine.26 For example, Reese et al. recently reported that bupropion causes a marked (5.2-fold) increase in the plasma AUC of desipramine, an FDA-approved in vivo probe of CYP2D6 activity.8,22 Hesse et al. reported that bupropion and hydroxybupropion are relatively weak inhibitors of CYP2D6 in HLM (IC50 = 58 and 78 μM, respectively).27) Recently, Reese et al. reported that the ability of bupropion to inhibit CYP2D6 depends, at least in part, on its reduction to erythro- and threo-hydrobupropion (formed by reduction of a keto group to an enantiomeric pair of secondary alcohols, presumably by carbonyl reductase or aldo-keto reductase) as shown in Figure 8.22) Not only is bupropion rapidly converted to these reduced metabolites, such that the estimated hepatic concentrations of erythro-hydrobupropion and threo-hydrobupropion are greater than that of bupropion itself, but both reduced metabolites inhibit CYP2D6 more potently than does bupropion (the Ki values for CYP2D6 inhibition by threo-hydrobupropion and erythro-hydrobupropion are 5.4 and 1.7 μM, respectively).22)

As shown in Figure 9, bupropion exhibits system-dependent inhibition of CYP2D6. In HLM, bupropion is a relatively weak direct-acting inhibitor of CYP2D6 (IC50 = 79 μM), and a 30-min preincubation of bupropion with NADPH-fortified HLM slightly decreases its inhibitory effect (the IC50 value increases from 79 to 150 μM). These results are consistent with those reported by Hesse et al., who demonstrated that hydroxybupropion is a less potent inhibitor of CYP2D6 than is bupropion.27) In human hepatocytes, however, bupropion is a relatively potent inhibitor of CYP2D6 (IC50 = 18 μM), and a 30-min preincubation of bupropion with human hepatocytes increases its inhibitory effect (the IC50 value decreases from 18 to 9.2 μM). Following a 30-min preincubation period, bupropion inhibits CYP2D6 about 16 times more potently in human hepatocytes compared with HLM.

Fig. 8. Metabolic scheme for the oxidative and reductive metabolism of bupropion

Fig. 9. System-dependent inhibition of CYP2D6 by bupropion in human liver microsomes and human hepatocytes

This in vitro study was conducted essentially as described previously.4,5 CYP2D6 activity was measured based on the rate of O-demethylation of dextromethorphan (7.5 μM), which was assessed following a 30-min preincubation of bupropion with NADPH-fortified pooled human liver microsomes (0.1 mg protein/mL) or pooled human hepatocytes (0.5 × 10⁶ cells/mL). Pooled human liver microsomes and pooled human hepatocytes were from XenoTech LLC (Lenexa, KS).
The observation that bupropion is a much more potent inhibitor of CYP2D6 in human hepatocytes compared with HLM is consistent with the experimental findings of Reese et al.\textsuperscript{22} In other words, the results in Figure 9 are consistent with the proposal that bupropion undergoes carbonyl reduction in human hepatocytes to erythro- and threo-hydrobupropion, both of which are more potent inhibitors of CYP2D6 than bupropion is itself. However, we cannot exclude the possibility that other factors (such as active hepatocellular uptake) may account for the greater CYP2D6 inhibitory effect of bupropion in hepatocytes over HLM.

When should hepatocytes be used for CYP inhibition studies?

Human hepatocytes contain a more complete and intact hepatic drug-metabolizing system than do HLM or recombinant human CYP enzymes. For this reason, it is sometimes argued that hepatocytes are superior to pooled human liver microsomes or recombinant human CYP enzymes for the conduct of in vitro CYP inhibition studies, and therefore provide better predictive value than do the other in vitro test systems. Intuitively, it would appear that hepatocytes would yield superior data (i.e., better IC\textsubscript{50} and K\textsubscript{i} values) with which to make in vivo predictions, but, with the exception of the examples cited in this review, there is surprisingly little experimental evidence to support this viewpoint. In many cases, as illustrated in Figure 2 for CYP2C19 inhibition by omeprazole, the results of CYP inhibition studies are consistent across the various in vitro test systems (i.e., HLM, recombinant enzymes and human hepatocytes). Zhao \textit{et al.}, and McGinnity \textit{et al.} have similarly observed that human hepatocytes produce K\textsubscript{i} and k\textsubscript{inact} values for mechanism-based inhibitors of CYP enzymes that are generally comparable to those determined with HLM or recombinant CYP enzymes.\textsuperscript{28,29} Furthermore, human hepatocytes do not offer many of the advantages afforded by HLM. In contrast to HLM, human hepatocytes are difficult to pool in sufficiently large quantities to permit a detailed analysis of the kinetics of each marker substrate. Consequently, the measurement of CYP activity in hepatocytes is often measured under non-Michaelis-Menten conditions or, if they are, with substrate concentrations that greatly exceed Km (contrary to the FDA’s and PhRMA’s recommendations that the concentration of CYP marker substrate be equal to or less than Km). In hepatocytes, a portion of the metabolite formed from various marker substrates may be conjugated, which further complicates the analysis of enzyme kinetics.

Inasmuch as the use of human hepatocytes for in vitro CYP inhibition studies has not been shown to improve the general prediction of in vivo inhibitory interactions, there is no compelling reason to propose that all CYP inhibition studies be conducted with human hepatocytes rather than with HLM or recombinant enzymes. However, in this article we have provided three examples where the results of CYP inhibition studies conducted with human hepatocytes do in fact provide a better prediction of clinical outcome than the corresponding studies conducted with HLM. In addition to showing system-dependent CYP inhibition, these three examples – gemfibrozil, ezetimibe and bupropion – have one thing in common: they are all drugs that are rapidly and extensively metabolized by non-CYP enzymes (by UDP-glucuronosyltransferases in the case of gemfibrozil and ezetimibe and by carbonyl reductase in the case of bupropion).

Based on these findings, we recommend that drug candidates be examined for their ability to function as direct-acting and metabolism-dependent inhibitors of the major drug-metabolizing CYP enzymes in HLM in accordance with recommendations published by the FDA and PhRMA.\textsuperscript{1-3,8} Additionally, when the drug candidate is rapidly and extensively metabolized by a non-CYP enzyme, we further recommend that studies also be conducted in human hepatocytes to explore the possibility that metabolism of the drug candidate by a non-CYP enzyme might lead to clinically significant CYP inhibition (as in the case of gemfibrozil and bupropion) or prevent clinically significant CYP inhibition (as in the case of ezetimibe).

System-dependent inhibition of CYP enzymes: HLM versus recombinant enzymes

The FDA guidance document and the PhRMA consensus papers advocate the use of either HLM or recombinant human CYP enzymes to assess the inhibitory potential of drug candidates.\textsuperscript{2,3,8} The advantages and disadvantages of each of these in vitro test systems were reviewed recently by Ogilvie \textit{et al.}.\textsuperscript{41} The two test systems differ in their interaction with inhibitory drugs.\textsuperscript{41} In general, regardless of which CYP enzyme is examined, the IC\textsubscript{50} values determined with HLM are lower than those determined with recombinant CYP enzymes.\textsuperscript{8} Kumar and colleagues determined the K\textsubscript{i} values for inhibition of CYP2C9 by 12 competitive inhibitors in Supersomes\textsuperscript{TM}, RECO\textsuperscript{®} CYP2C9, and HLM. K\textsubscript{i} values in RECO\textsuperscript{®} CYP2C9 were approximately 3- to 14-fold higher than those determined with HLM, with the exception of fluvoxamine, ketoconazole, phenytoin, piroxicam and tolbutamide (the latter two were 30–40\% lower).\textsuperscript{30} The K\textsubscript{i} values in Supersomes\textsuperscript{TM} were found to be within a factor of 3 of the values for human liver microsomes with the exception of fluvoxamine, ketoconazole and piroxicam (in which case the K\textsubscript{i} values were 9-, 5.5- and 21-fold higher with human liver microsomes) and gemfibrozil and indomethacin (in which case the K\textsubscript{i} values were 12.7- and 4.2-fold higher with recombinant CYP2C9).\textsuperscript{30}

An important limitation of recombinant human CYP
enzymes for CYP inhibition studies is that this test system fails to detect cases in which metabolites generated by one CYP enzyme inhibit another “bystander” CYP enzyme (unless the two appropriate recombinant CYP enzymes are co-incubated). CYP enzymes do in fact form metabolites that inhibit other CYP enzymes, and in some cases this occurs to a clinically significant extent. For example, fluoxetine is converted to norfluoxetine by CYP2C9, 2C19 and 2D6, and norfluoxetine reaches plasma concentrations of ~0.6 μM with a half-life of ~16 days, whereas fluoxetine reaches plasma concentrations of ~1.5 μM with a half-life of ~2.2 days. Although fluoxetine and norfluoxetine both cause clinically significant inhibition of CYP2D6, norfluoxetine also causes clinically significant inhibition of CYP3A4 and CYP2C19.

Amiodarone is another drug that is converted to metabolites that inhibit CYP enzymes other than those that form the inhibitory metabolites. Amiodarone is metabolized by CYP3A4 and CYP2C8 to desethylamiodarone (http://www.wyeth.com/content/getfile.asp?id=93). In recombinant human CYP enzymes, amiodarone inhibits CYP2D6 (Ki = 45 μM) followed by CYP2C9 (Ki = 95 μM) and CYP3A4 (Ki = 272 μM). In HLM, however, amiodarone most potently inhibits CYP3A4 (IC50 = 15 μM), followed by CYP2C9 (IC50 = 25 μM) and CYP1A2 (IC50 = 86 μM). Amiodarone is an irreversible metabolism-dependent inhibitor of recombinant human CYP2C8 (Ki = 1.5 μM, k inact = 0.079 min⁻¹) and recombinant human CYP3A4 (Ki = 13.4 μM, k inact = 0.06 min⁻¹). Amiodarone also inactivates CYP2C8 (Ki = 51.2 μM, k inact = 0.029 min⁻¹) and CYP3A4 (Ki = 10.2 μM, k inact = 0.032 min⁻¹) in pooled HLM. Ohyama and colleagues reported that, compared with amiodarone, desethylamiodarone is a more potent direct-acting inhibitor of recombinant CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6 and 3A4, with Ki values ranging from 2.3 μM for CYP2C9 to 18.8 μM for CYP1A2. Moreover, whereas amiodarone inactivates only recombinant human CYP3A4, desethylamiodarone also inactivates recombinant CYP1A2, 2B6 and 2D6. Thus, desethylamiodarone is not only a more potent direct-acting inhibitor of more CYP enzymes than the parent drug, but it is also a metabolism-dependent inhibitor of more CYP enzymes than the parent drug. These effects were observed in HLM with the parent drug, but they were not observed with recombinant human CYP enzymes without directly examining the inhibitory effects of the metabolite. The lower IC50 or Ki values observed in human liver microsomes as compared with recombinant CYP enzymes for CYP1A2, CYP2C9 and CYP3A4 reflects the conversion of amiodarone to desethylamiodarone by CYP2C8 and CYP3A4 in human liver microsomes.

Overall, head-to-head comparisons of CYP inhibition studies with HLM and recombinant CYP enzymes suggest that the two in vitro systems both have utility in evaluating the inhibitory potential of drug candidates but that there are cases where studies with HLM provide more clinically relevant information than studies with recombinant CYP enzymes, especially when the metabolites of a drug produced by one CYP enzyme are capable of causing clinically significant inhibition of another “bystander” CYP enzyme, as illustrated above.

**System-dependent inhibition of UGT enzymes:**

HLM versus recombinant enzymes

During drug discovery, the metabolic stability of drug candidates is often evaluated in NADPH-fortified HLM. Those drug candidates that are rapidly metabolized in this in vitro system are generally dropped from further development, as are drug candidates that strongly inhibit
System-dependent CYP/UGT Inhibition

CYP2D6 or CYP3A4, the two enzymes that quantitatively dominate the metabolism of approved drugs. These criteria bias the selection of drug candidates away from CYP enzymes and towards other drug-metabolizing enzymes, such as the UDP-glucuronosyltransferase (UGT) enzymes. Accordingly, there is growing interest in the ability of drug candidates to inhibit UGT activity in HLM.

Fujiwara et al. recently drew attention to the potential for marked differences between the results of UGT inhibition studies conducted with HLM versus those conducted with recombinant UGT enzymes. System-dependent differences are observed with UGT inhibitors that are themselves rapidly glucuronidated, as illustrated in Figure 10 for 1-naphthol. 1-Naphthol is a substrate for UGT1A6 and an inhibitor of UGT1A9. In native HLM (i.e., microsomes that are not activated with the pore-forming peptide alamethicin or the zwitterionic detergent CHAPS), UGT1A6 glucuronidates 1-naphthol with high affinity (Km ~ 1 µM) and high capacity (Vmax 10–15 nmol/mg protein/min). The rapid glucuronidation of 1-naphthol by microsomal UGT1A6 protects microsomal UGT1A9 from the inhibitory effect of 1-naphthol (because 1-naphthol inhibits UGT1A9 whereas 1-naphthyl-glucuronide does not). However, the glucuronidation-dependent protection observed in HLM does not occur when 1-naphthol is evaluated as an inhibitor of recombinant UGT1A9 (because in this system there is no UGT1A6 to glucuronidate 1-naphthol). Consequently, as shown in Figure 10, 1-naphthol is 20 times more potent as an inhibitor of recombinant UGT1A9 (IC₅₀ = 0.23 µM) than of microsomal UGT1A9 (IC₅₀ = 4.6 µM).

The converse is observed when 1-naphthol is evaluated for its ability to inhibit microsomal and recombinant UGT1A4. In this case, 1-naphthol is a more potent inhibitor of microsomal UGT1A4 than of recombinant UGT1A4, as shown in Figure 10. Why does 1-naphthol inhibit microsomal UGT1A4 but not recombinant UGT1A4? It inhibits microsomal UGT1A4 by undergoing rapid and extensive glucuronidation by microsomal UGT1A6 (something that does not occur with recombinant UGT1A4), and this results in both a decrease in the levels of UDP-glucuronic acid and an increase in the levels of UDP. The latter is a competitive inhibitor of UDP-glucuronic acid. Consequently, in HLM, the glucuronidation of 1-naphthol removes an inhibitor of UGT1A9 and, through the formation of UDP, forms an inhibitor of UGT1A4 (and other UGT enzymes). In other words, as an inhibitor of UGT activity, 1-naphthol undergoes glucuronidation-dependent protection (in the case of UGT1A9) and glucuronidation-dependent activation (in the case of UGT1A4), and these events occur in HLM (which contain UGT1A6) but not with individual recombinant UGT enzymes, which gives rise to system-dependent inhibition.

Whereas CYP-dependent reactions can be supported with an NADPH-generating system to maintain relatively constant levels of NADPH, there is no simple UDP-glucuronic acid-generating system to maintain relatively constant levels of UDP-glucuronic acid. Therefore, UGT assays with HLM and recombinant enzymes are conducted with a fixed initial concentration of UDP-glucuronic acid, one that declines over time in a system-dependent manner. As shown in Figure 11, the initial concentration of UDP-glucuronic acid influences the potency with which 1-naphthol inhibits microsomal UGT1A4 and UGT1A9. Increasing the concentration of UDP-glucuronic acid from 0.2 to 20 mM decreases the potency with which 1-naphthol inhibits both UGT1A4 and UGT1A9. However, the higher initial concentration of UDP-glucuronic acid decreases the inhibition of UGT1A4 by 1-naphthol by maintaining a high ratio of UDP-glucuronic acid to UDP (which decreases the inhibitory effect of UDP), whereas it decreases the inhibition of UGT1A9 by...
increasing the rate of conversion of 1-naphthol to the non-inhibitory metabolite 1-naphthyl-glucuronide.

As reported by Fujiwara et al., system-dependent inhibition of UGT activity can occur with HLM and recombinant enzymes when a potential inhibitor of one UGT enzyme is rapidly glucuronidated by another UGT enzyme. The difference between HLM and recombinant enzymes can be due to glucuronidation-dependent protection (in which the recombinant enzyme is inhibited more potently than the corresponding microsomal enzyme) or glucuronidation-dependent activation due to the formation of inhibitory concentrations of UDP (in which case the recombinant enzyme is inhibited less potently than the corresponding microsomal enzyme). The latter effect (i.e., the inhibition of UGT activity due to the formation of UDP) likely represents an in vitro artifact because UDP will be converted back to UDP-glucuronic acid in vivo. This artifact can be minimized by conducting UGT inhibition studies in HLM with a high concentration (20 mM) of UDP-glucuronic acid.

Conclusions

System-dependent differences in the in vitro inhibition of CYP enzymes have been observed, and these differences impact the outcome of in vitro to in vivo extrapolations. In particular, studies with HLM under-predict the ability of gemfibrozil and bupropion to cause clinically significant inhibition of CYP2C8 and CYP2D6, respectively, but over-predict the ability of ezetimibe to cause clinically significant inhibition of CYP3A4. Gemfibrozil and bupropion represent examples of glucuronidation-dependent and reduction-dependent activation to metabolites that inhibit CYP2C8 and CYP2D6, respectively, whereas ezetimibe represents an example of glucuronidation-dependent protection against metabolism-dependent inhibition of CYP3A4. We have provided examples that illustrate why, when drug candidates are extensively metabolized by non-CYP enzymes, it may be necessary to use human hepatocytes in addition to HLM or recombinant enzymes to fully evaluate their ability to inhibit CYP enzymes.

References


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