0090-9556/05/3311-1700–1707$20.00
DRUG METABOLISM AND DISPOSITION
Vol. 33, No. 11
5884/3058078
DMD 33:1700–1707, 2005
Printed in U.S.A.

PREDICTION OF CYP2C9-MEDIATED DRUG-DRUG INTERACTIONS: A COMPARISON USING DATA FROM RECOMBINANT ENZYMES AND HUMAN HEPATOCYTES

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Received June 1, 2005; accepted August 3, 2005

ABSTRACT:

The IC_{50} values of 14 drugs were determined in recombinantly expressed CYP2C9 (rCYP2C9) and human hepatocytes and the data used to simulate clinical area under the plasma concentration-time curve (AUC) changes upon coadministration with prototypic CYP2C9 substrates. There was an excellent correlation between $IC_{50,\ apparent}$ values determined using diclofenac and naproxen as CYP2C9 substrates ($r^2 = 0.82$, $p < 0.0001$), with values being generally higher in the naproxen assay. After correcting for nonspecific binding, the $IC_{50,\ unbound}$ values were similar between the assays, for the majority of compounds. Two compounds, amiodarone and benzbromarone, demonstrated substrate-specific differences, activating naproxen *O*-demethylase to ~250% of control activity at 1 mM and 1 μM, respectively, while inhibiting diclofenac 4′-hydroxylation with $IC_{50,\ apparent}$ values of 3 μM and 0.04 μM, respectively. CYP2C9 $IC_{50,\ apparent}$ values generated in human hepatocytes were systematically higher than those determined with rCYP2C9. After correcting for nonspecific binding, there was an excellent correlation of $IC_{50,\ unbound}$ values generated in the different milieu ($r^2 = 0.88$, $p < 0.0001$). The ratio of inhibitor concentration at the entrance to the liver to the inhibition constant ($[I]_{in}/K_i$) was used to simulate clinical δAUC changes and compared with that observed in vivo. Where $[I]_{in,\ total}/K_{i,\ apparent}$ was used, there were zero false negatives (observed δAUC ≥2, predicted δAUC <2), eight correct assignations, and seven false positives (observed δAUC ≤2, predicted δAUC >2. Where $[I]_{in,\ unbound}/K_{i,\ unbound}$ was used, there was one false negative, 14 correct assignations, and zero false positives. In summary, the data presented here suggest that for CYP2C9 interactions, the use of total liver inhibitor concentrations may indeed avoid false negatives, but more realistic predictions may be achieved using unbound liver inhibitor concentrations and unbound in vitro inhibition parameters.

Up to 2.8% of hospital admissions may be a consequence of drug-drug interactions (DDIs) (Jankel and Fitterman, 1993). Although metabolic DDIs have been reported for several enzyme families, it is clearly recognized that inhibition of cytochrome P450 (P450)-dependent metabolism is one of the more prevalent sources of such reactions and may have a serious outcome (Bertz and Granneman, 1997). Experiences with terfenadine (Honig et al., 1993), cisapride (Ahmad and Wolfe, 1995), and mibefradil (Krayenbuhl et al., 1999) have highlighted the importance of understanding the enzymology of drug metabolism to assess its impact on pharmacodynamics and toxicology.

An assessment of the potential of a new chemical entity to cause a DDI via inhibition of P450 metabolism is important early in the drug discovery process. Thus, a battery of automated in vitro screens to determine the degree of P450 inhibition are now routinely used in drug metabolism and pharmacokinetics departments across the pharmaceutical industry. Such screens are used both for the evaluation and optimization of potential candidate drugs and for prioritizing and designing suitable in vitro and clinical interaction studies in the drug development phase. The ability to predict in vivo DDIs from human in vitro assays would represent a major advance in drug discovery.

Approaches to predicting P450-mediated DDI potential have traditionally involved the use of human liver microsomes, although more recently, the use of recombinant cytochrome P450s (rP450s) has become widespread. More often than not, these assays focus on the five major drug-metabolizing P450s: CYP1A2, 2C9, 2C19, 2D6, and 3A4. Human hepatocytes in primary culture provide the closest in vitro model to human liver and have been excellent tools for elucidating the metabolic profile of drugs, for predicting hepatic metabolic clearance (McGinnity et al., 2004; Riley et al., 2005), for studying hepatotoxicity of drugs (Gomez-Lechon et al., 2001), and for the mechanistic understanding of DDIs mediated via P450 induction (LeCluyse et al., 2000). There are sporadic reports of the use of human hepatocytes to characterize P450 inhibition (Li et al., 1999; Cohen et al., 2000; Oleson et al., 2004; Zhao et al., 2005), but it is notable that investigations of inhibitory mechanisms remain under-represented compared with studies on metabolism and induction. This is likely due to the relative accessibility of recombinant P450s and their overall applicability in predicting clinically relevant DDIs. Availability of

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.
doi:10.1124/dmd.105.005884.

ABBREVIATIONS: DDI, drug-drug interaction; P450, cytochrome P450; rCYP2C9, recombinant cytochrome P450 2C9; HPLC, high-performance liquid chromatography; fu_{inc}, unbound fraction in incubation; fu_p, unbound fraction in plasma; RSP, robotic sample processor; $[I]$, inhibitor concentration; $[I]_{av}$, average systemic plasma concentration after repeated oral administration; $[I]_{max}$, maximum systemic plasma concentration after repeated oral administration; $[I]_{in}$, maximum hepatic input concentration; K_i, inhibition constant; AUC, area under the plasma concentration-time curve; fm_{CYP2C9}, fraction of total clearance metabolized by CYP2C9.

good quality fresh liver tissue limits human hepatocyte experiments, but, increasingly, cryopreservation technology is allowing hepatocytes to become an "off-the-shelf" reagent (Li, 1999). The end-points used for P450 inhibition screening in the pharmaceutical environment are typically one of the following; liquid scintillation counting of radioactivity liberated during site-specific metabolism (Moody et al., 1999), selective analysis of fluorescent metabolites (Crespi et al., 1998), and mass spectrometry (Ayrton et al., 1998; Weaver et al., 2003). In drug discovery, assays are routinely conducted at the K_m for the substrate since, under these conditions, for most inhibitors, $K_i = IC_{50}/2$ or IC_{50}.

Predicting the magnitude of an in vivo DDI via reversible enzyme inhibition from in vitro data is still fraught with uncertainty, yet there exists a common consensus as to the general principles underpinning such extrapolations. The increase in the AUC of a substrate when coadministered in the presence of an inhibitor of the substrate's elimination pathway is a function of the ratio of inhibitor concentration ([*I*]) to inhibition constant (K_i) (Bertz and Granneman, 1997; Ito et al., 1998b; Rostami-Hodjegan and Tucker, 2004). There still remains some uncertainty regarding how to measure or estimate the appropriate [*I*]. [*I*] values that are routinely used in such predictions include average systemic inhibitor concentration ($[I_{av}]$), maximum systemic inhibitor concentration ($[I_{max}]$), and maximum inhibitor concentration entering the liver after oral administration ($[I_{in}]$). In addition, despite the "free drug hypothesis" being a widely accepted tenet of pharmacokinetics, some investigators still use total rather than unbound plasma concentrations. This is usually due to pragmatic considerations, since, for some interactions, using $[I]_{total}$ results in better predictions of DDIs than $[I]_{unbound}$ (Venkatakrishnan et al., 2003). Although the determination and subsequent use of K_i values in predicting an in vivo DDI is less controversial than [*I*], there is still some variability in approaches taken in measuring K_i; for example, enzyme source, correction for nonspecific binding in the in vitro incubation, choice/level of cosolvent, and choice of substrate. These and other variables presumably account for the large degree of interlaboratory variation observed in K_i and IC_{50} measurements (Boobis et al., 1998). The choice of in vitro assay substrate should be particularly considered for interactions with CYP3A4 (Kenworthy et al., 1999) and increasingly for CYP2C9, where multisite kinetics and substrate-dependent interactions have been observed (Hutzler et al., 2001, 2005).

The aims of this work were, first, to evaluate the power of predicting clinical δAUC changes of prototypic CYP2C9 substrates (*S*-warfarin, phenytoin, tolbutamide, and diclofenac) when coadministered with 14 drugs via K_i values determined in recombinantly expressed CYP2C9 and human hepatocytes. We have explored any substrate dependence of CYP2C9 inhibition by comparing IC_{50} values determined using diclofenac and naproxen as in vitro substrates, and the data are discussed in the context of predicting in vivo interactions for a number of different substrates. The importance of determining free concentration of inhibitor, both in vivo and in the in vitro assay, is also emphasized.

Materials and Methods

Materials. Amiodarone, benzbromarone, clotrimazole, diclofenac, 4′-hydroxydiclofenac, ibuprofen, ketoconazole, 4-methylimidazole (±)miconazole, (−)naproxen, piroxicam, quinine, sulfamethiazole, sulfaphenazole, (±)sulfinpyrazone, tolbutamide, (±)warfarin, and β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH) were purchased as the highest grade available from Sigma-Aldrich (Gillingham, UK). Fluconazole, fluvastatin, and sertraline were purchased from Sequoia Research Products Ltd. (Oxford, UK). Zafirlukast was synthesized at AstraZeneca R&D (Wilmington, DE). [*O*-methyl-^{14}C]naproxen (specific radioactivity 55 mCi/mmol, chemical purity >98.5%, radiochemical purity >99.4%) was synthesized as described previously (Moody et al., 1999). Dimethyl sulfoxide, acetonitrile, and trichloroacetic acid were purchased from Fisher Scientific (Loughborough, UK), and methanol was purchased from Romil Ltd. (Cambridge, UK).

Bactosomes prepared from *Escherichia coli* cells coexpressing recombinant human NADPH-P450 reductase and human CYP2C9 (CYP2C9R) were purchased from Cypex (Dundee, UK) Cryopreserved human hepatocytes (lot OCF) were purchased from In Vitro Technologies (Baltimore, MD).

Instrumentation. Inhibition assays using rP450s were performed on a robotic sample processor (RSP) (Genesis RSP 150; Tecan, Reading, UK). All HPLC-mass spectrometry was conducted on a Micromass Quattro Ultima triple quadrupole and an Alliance HT Waters 2790 HPLC system (Waters, Milford, MA). Scintillation counting used a TopCount NXT microplate scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA).

Inhibition Assays. Naproxen *O*-demethylation (Moody et al., 1999) and diclofenac 4-hydroxylation (Weaver et al., 2003) were used as probe reactions for CYP2C9 based on methods previously described. The P450 isoform selectivity of diclofenac (Weaver et al., 2003) and naproxen (Moody et al., 1999) have been previously established.

All assays used 100 mM phosphate buffer, pH 7.4, and were performed at a substrate concentration equivalent to the K_m of the CYP2C9 reaction, naproxen at 109 μM and diclofenac at 2 μM. All reactions were conducted under conditions shown to be linear with respect to time and protein concentration. IC_{50} determination was based on seven and five inhibitor concentrations for the rP450s and human hepatocytes, respectively.

Determination of IC_{50} Using rCYP2C9. The following volumes are used in each incubation well: 20 μl of 10 mM NADPH in 0.1 M phosphate buffer, pH 7.4, 178 μl of P450/substrate, and 2 μl of the predilution stocks of inhibitor are spiked directly into the incubation microtiter plate. The final incubation volume is 200 μl, giving 100-fold dilutions of the solvent stocks of the inhibitor generated in the predilution microtiter plate and a final concentration of 1 mM NADPH. The dilution of test compounds for a seven-point IC_{50} determination by the RSP were programmed as follows: the primary stock of each compound (e.g., 5 mM) was prepared manually in dimethyl sulfoxide and serially diluted by the RSP (using dimethyl sulfoxide) to give six secondary solutions (e.g., 5 mM to 20 μM). Each of these secondary solutions was further diluted 1:5 in 100 mM phosphate buffer, pH 7.4 to generate tertiary solutions (e.g., 1 mM to 4 μM). Finally, 10 μl of each of the tertiary solutions were spiked into the incubation mix (200-μl total volume to give final concentrations; e.g., 50 μM to 0.2 μM). An incubation containing vehicle alone allowed calculation of control activity. The final organic solvent concentration in all incubations was 1% (v/v).

Determination of IC_{50} Using Naproxen *O*-Demethylase Activity and rCYP2C9. The assay was carried out as described by Moody et al. (1999). The product of the [*O*-methyl-^{14}C]naproxen reaction is [^{14}C]formaldehyde, which is separated from the incubation mixture using reverse-phase C8 solid-phase extraction and quantified by liquid scintillation counting. Interaction with the respective P450 by an inhibitor results in a decrease in the amount of formaldehyde produced. Naproxen was used at a final concentration of 109 μM (0.1 μCi of [*O*-methyl-^{14}C]naproxen/incubation). An appropriate amount of naproxen was aliquoted and the solvent evaporated under nitrogen to dryness before addition of protein. The amount of rCYP2C9 used in the naproxen *O*-demethylation assay was 70 pmol/ml (0.14 mg of protein/ml of incubate). One batch of rCYP2C9 was used for all experiments, primarily to maintain a constant level of protein. In our experience, the variability of $IC_{50,\ unbound}$ values between batches of the expression system used is minimal. Incubations were conducted for 15 min and reactions were quenched with the addition of 50 μl of trichloroacetic acid (10% w/v).

Solid-phase extraction was used to separate [^{14}C]formaldehyde from [*O*-methyl-^{14}C]naproxen. A 100-mg C8 96-well plate (Supelco, Bellefonte, PA) and a 96-well, 2-ml round-bottom collection plate (Whatman, Clifton, NJ) using negative pressure were an improvement over early methodology using activated charcoal (Moody et al., 1999). A fully automated negative pressure sample preparation method was performed by the RSP. The 96 C8 columns were conditioned with 0.1 ml of methanol and two 0.2-ml water aliquots. All (250 μl) of the quenched incubation was applied to the column and eluted with two 200-μl aliquots of water. Aliquots (600 μl) were transferred to plates containing 750 μl of Ultima Gold liquid scintillant (PerkinElmer Life and

Analytical Sciences) and counted. A set of controls, which contains 178 μl of substrate/P450 incubation mixture, 400 μl of water, and 750 μl of scintillant, was used to calculate a control rate of reaction.

Determination of IC_{50} Using Diclofenac 4′-Hydroxylase Activity and rCYP2C9. The assay was carried out as described by Weaver et al. (2003). Using diclofenac as a substrate, the product of the reaction is 4′-hydroxydiclofenac, which is separated from the incubation mixture using HPLC. Interaction with the CYP2C9 by an inhibitor will result in a decrease in the amount of 4′-hydroxydiclofenac produced. Diclofenac was used at a final concentration of 2 μM. The amount of rCYP2C9 used in the diclofenac 4′-hydroxylation assay was 10 pmol/ml (0.02 mg of protein/ml of incubate; one batch of rCYP2C9 used for all experiments). Incubations were conducted for 10 min and reactions were quenched with the addition of 200 μl of methanol. Samples were chilled at −20°C for 2 h, spun at 3500 rpm for 15 min, and the supernatants were transferred to vials for analysis as described below.

Thawing of Cryopreserved Hepatocytes. Aliquots (20 ml) of hepatocyte suspension buffer (with no added albumin) were prewarmed to 37°C. Cryopreserved cells were removed from liquid N_2 and immediately immersed in a water bath that had been preheated to 37°C. The vials were shaken gently until the contents were completely free of ice crystals (~2 min) and were then emptied into the prewarmed hepatocyte suspension buffer. The cells were centrifuged at 40*g* for 5 min at 19°C, the supernatant was removed by aspiration, and the resultant pellet was suspended in hepatocyte suspension buffer. The concentration and viability of the hepatocytes was determined using trypan blue exclusion, and the cells were resuspended at a concentration of 2 million cells/ml.

Determination of IC_{50} Using Diclofenac 4′-Hydroxylase Activity and Human Hepatocytes. This assay was performed using manual pipetting in a shaking water bath at 37°C. Diclofenac was used at a final concentration of 2 μM. An appropriate amount of diclofenac was aliquoted and the solvent evaporated under nitrogen to dryness before resuspension in hepatocyte suspension buffer [2.2 g of $NaHCO_3$, 2.34 g of sodium-HEPES, 1 liter powder equivalent of Dulbecco's modified Eagle's medium (Sigma-Aldrich) diluted in 1 liter of water and adjusted to pH 7.4 with 1 M HCl] to give a concentration of 200 μM. Drug stocks were prepared in dimethyl sulfoxide at 100-fold incubation concentration (e.g., 5000, 1500, 100, 15, and 5 μM). Then, 10 ml of this 100× stock were added to a vial containing 490 μl of hepatocyte suspension buffer containing substrate. A 7-ml glass Bijou vial containing 250 μl of hepatocytes at a concentration of 2 million cells/ml was preincubated for 5 min in a shaking (80 oscillations/min) water bath at 37°C along with the vial containing the drug/buffer/substrate mix. Reactions were started by adding 250 μl of drug/buffer/substrate mix to the 250 μl of hepatocytes, giving, in the example shown, final inhibitor concentrations of between 50, 15, 1, 0.15, and 0.05 μM. The final concentration of organic solvent in all incubations was 1% (v/v). The samples were incubated for 15 min and quenched with an equivalent volume of ice-cold methanol before being frozen for 1 h at −20°C and then centrifuged at 3500 rpm for 15 min at 4°C. The supernatants were removed and transferred into vials and analyzed as described below. Assays were performed in triplicate.

Determination of fu_p and fu_{inc} in rCYP2C9 and Human Hepatocytes. Human blood was obtained from volunteers at AstraZeneca R&D Charnwood after local ethical approval and written informed consent. The extent of binding of compounds to rCYP2C9, human plasma, and human hepatocytes was determined using equilibrium dialysis at 37°C as described by Austin et al. (2002, 2005). Briefly, plasma was prepared by centrifugation of the blood, stored in EDTA tubes, at 350*g* for 15 min. The amount of rCYP2C9 used was the same as for the naproxen and diclofenac inhibition assays (0.14 and 0.02 mg protein/ml, respectively in 100 mM phosphate buffer, pH 7.4). The amount of human hepatocytes used was the same as for the inhibition assays [1 million cells (left to die for 24 h before use)/ml hepatocyte suspension buffer]. Plasma and microsomal binding are normally independent of compound concentration at typical therapeutic levels (0.1 μM to 50 μM) (Austin et al., 2002), and thus, binding was determined at a single concentration (10 μM for plasma and 1 μM for rP450 and hepatocytes). Compounds were solubilized in dimethyl sulfoxide, and the final organic solvent concentration was 1% (v/v). Plasma was dialyzed against Dulbecco's phosphate-buffered saline, pH 7.4 (Sigma-Aldrich), rP450 against 0.1 M phosphate buffer, pH 7.4, and hepatocytes against hepatocyte suspension buffer, overnight at 37°C.

Samples were quantified using HPLC-Mass Spectrometry with Symmetry C8 (5 μm × 3.9 mm × 20 mm columns; Waters) and a gradient of 1% acetonitrile/99% 0.05% aqueous ammonium acetate to 99% acetonitrile/1% 0.05% aqueous ammonium acetate at a flow rate of 2 ml/min over 3.5 min. The free fraction of each compound was determined from the ratio of buffer to sample concentrations, each interpolated from a six-point calibration curve.

Analysis of 4′-Hydroxydiclofenac. Aliquots (30 μl) were analyzed by HPLC-tandem mass spectrometry for 4′-hydroxydiclofenac appearance. Analysis used electrospray ionization and multiple reaction monitoring, mass transition 312.6 > 230.0, cone voltage 27 V, and collision energy 35 eV. A Devosil C30 column (Phenomenex, Cheshire, UK) and mobile phases of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) were used for the chromatography. The gradient was as follows: 97% A (0–0.3 min), 5% A (0.55–1.55 min), 97% A (1.6 min). The stop time was 2.5 min, the flow rate was 1.2 ml · min^{-1}, and column temperature was 40°C.

Data Analysis. Microsoft Excel (Microsoft, Redmond, WA) was used to calculate IC_{50} estimates by linear transformation of the raw data. The data were corrected for both background and control activities.

All assays were performed at a substrate concentration equivalent to the K_m of the CYP2C9 reaction since, under these conditions, irrespective of the type of reversible inhibition, IC_{50} should be within 2-fold of the K_i (Cheng and Prusoff, 1973). For competitive and uncompetitive inhibition, $IC_{50} = 2K_i$, and for noncompetitive and linear mixed-type inhibition, $IC_{50} = K_i$. The inhibition type has not been fully elucidated for all the inhibitors studied in this work; therefore, in the absence of this information, $K_i = IC_{50}/2$ was uniformly applied. As a generic strategy, this is appropriate, for in the drug discovery environment, IC_{50} and not K_i values are typically generated, and the type of reversible inhibition is usually unknown. Therefore, to minimize false-negative predictions, the lowest possible estimate of K_i is used for in vitro-in vivo predictions.

The theoretical basis underlying the quantitative predictions of drug interactions associated with reversible inhibition has been covered comprehensively in the literature (Bertz and Granneman, 1997; Ito et al., 1998b; Rostami-Hodjegan and Tucker, 2004).

In brief, for orally administered low clearance (enzyme-limited) drugs, which include *S*-warfarin, tolbutamide, phenytoin, and diclofenac, the ratio change of AUC in the presence or absence of a CYP2C9 inhibitor can be determined by eq. 1 (Ito et al., 1998b). In clinical situations, the substrate concentration is usually much lower than the K_m and so, eq. 1 is valid for all inhibitors, except for uncompetitive inhibitors, a rare inhibition type for drugs.

$$\delta AUC = \frac{AUC_{(inhib)}}{AUC_{(uninhib)}} = \frac{1}{\dfrac{fm_{CYP2C9}}{1 + \dfrac{[I]}{K_i}} + (1 - fm_{CYP2C9})} \quad (1)$$

where fm_{CYP2C9} is the fraction of total substrate clearance mediated by CYP2C9, K_i is the inhibition constant for CYP2C9, and $[I]$ is inhibitor concentration. For *S*-warfarin and tolbutamide, an estimate of fm_{CYP2C9} is 0.85 (Miners and Birkett, 1998), for phenytoin, 0.80 (Miners and Birkett, 1998), and for diclofenac, 0.75 (Shou, 2005).

The inhibitor concentration values used in the simulations were retrieved directly from Ito et al. (2004), Andersson et al. (2004), and Blanchard et al. (2004). Different inhibitor concentrations can be determined, including average systemic plasma concentration after repeated oral administration ($[I]_{av}$), maximum systemic plasma concentration after repeated oral administration ($[I]_{max}$), and maximum hepatic input concentration ($[I]_{in}$). $[I]_{in}$ can be calculated as follows:

$$[I]_{in} = [I]_{av} + \frac{\kappa_a F_a D}{Q_h} \quad (2)$$

where D is the dose of the inhibitor, and κ_a is the absorption rate constant. F_a is the fraction absorbed from the gastrointestinal tract, and Q_h is hepatic blood flow.

Results

Comparison of $IC_{50,\ apparent}$ and $IC_{50,\ unbound}$ Values in rCYP2C9 Using Naproxen and Diclofenac as Substrates. Figure

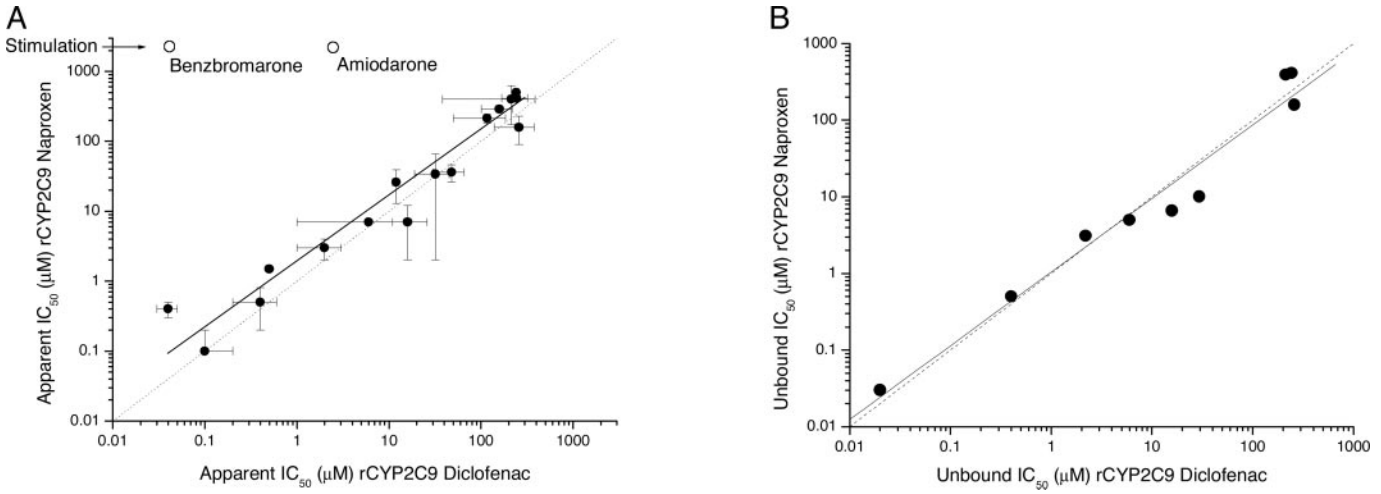

FIG. 1. A, $IC_{50,\ apparent}$ comparisons using naproxen and diclofenac as substrates for rCYP2C9. The IC_{50} determinations were conducted as described under *Materials and Methods*. Results were determined for amiodarone, benzbromarone, clotrimazole, fluconazole, fluvastatin, ibuprofen, ketoconazole, 4-methylimidazole, (±)miconazole, (−)naproxen, piroxicam, quinine, sulfamethiazole, sulfaphenazole, (±)sulfinpyrazone, tolbutamide, (±)warfarin, and zafirlukast. Assays were performed in triplicate; the data points represent the mean IC_{50} values and error bars reflect the standard deviation from the mean. The dotted line is unity. The solid line indicates linear regression of the data ($r^2 = 0.82$, $p < 0.0001$). B, $IC_{50,\ unbound}$ comparisons using naproxen and diclofenac as substrates for rCYP2C9. fu_{inc} was determined under the conditions used for the naproxen and diclofenac inhibition assays (0.14 and 0.02 mg protein/ml, respectively, in 100 mM phosphate buffer, pH 7.4) as described under *Materials and Methods*. Results were determined for fluconazole, fluvastatin, ketoconazole, (±)miconazole, sulfamethiazole, sulfaphenazole, (±)sulfinpyrazone, tolbutamide, and zafirlukast using the mean $IC_{50,\ apparent}$ values and measured fu_{inc} in each assay. The dotted line is unity. The solid line indicates linear regression of the data ($r^2 = 0.77$, $p < 0.002$).

1A shows a correlation of the mean $IC_{50,\ apparent}$ values determined in rCYP2C9 for a set of marketed drugs, amiodarone, benzbromarone, clotrimazole, fluconazole, fluvastatin, ibuprofen, ketoconazole, 4-methylimidazole, (±)miconazole, (−)naproxen, piroxicam, quinine, sulfamethiazole, sulfaphenazole, (±)sulfinpyrazone, tolbutamide, (±)warfarin, and zafirlukast, using naproxen *O*-demethylation and diclofenac 4′-hydroxylation as prototypic reactions ($r^2 = 0.82$, $p < 0.0001$). Assays were carried as outlined under *Materials and Methods*. Substrates were incubated at the apparent K_m for CYP2C9. IC_{50} values could not be determined for amiodarone and benzbromarone using naproxen as substrate due to marked activation of naproxen *O*-demethylase activity. Figure 1B shows a correlation of the mean $IC_{50,\ unbound}$ values ($r^2 = 0.77$, $p < 0.002$) for a subset of compounds shown in Fig. 1A, where fu_{inc} was determined at the protein concentrations used in each assay.

Figure 2 shows representative plots of CYP2C9 activity using naproxen and diclofenac as substrates with increasing concentrations of amiodarone and benzbromarone. Amiodarone activated naproxen *O*-demethylase to ~250% of control activity at 1 mM. Insolubility restricted the use of higher concentrations. Benzbromarone activated CYP2C9-mediated naproxen *O*-demethylase activity to a maximum of ~250% of control activity at approximately 1 μM. Using diclofenac as the CYP2C9 substrate, no activation was observed for amiodarone and benzbromarone at any concentration tested. Indeed, potent inhibition was observed and mean $IC_{50,\ apparent}$ values of 3 μM and 0.04 μM, respectively, were determined.

All compounds were assessed for time or NADPH-dependent inhibition of CYP2C9 (data not shown). None was observed, allowing the assumption that the interaction of all compounds and CYP2C9 was of a reversible nature.

Comparison of IC_{50} Values in Human Hepatocytes and rCYP2C9. Table 1 shows the $IC_{50,\ apparent}$ values determined using diclofenac 4′-hydroxylase activity in human hepatocytes and rCYP2C9 for 11 compounds: amiodarone, benzbromarone, fluconazole, fluvastatin, (±)miconazole, sertraline, sulfamethiazole, sulfaphenazole, (±)sulfinpyrazone, (±)warfarin, and zafirlukast. All of the $IC_{50,\ apparent}$ values generated in human hepatocytes were higher than those determined using rP450s. Four compounds showed a >10-fold higher $IC_{50,\ apparent}$ using hepatocytes compared with rCYP2C9: amiodarone ($IC_{50,\ apparent}$ using hepatocytes of 500 μM versus $IC_{50,\ apparent}$ using rCYP2C9 of 3 μM), benzbromarone (1.2 μM versus 0.04 μM), miconazole (19 μM versus 0.04 μM), and zafirlukast (29 μM versus 0.5 μM). These four compounds are highly bound to hepatocytes relative to rCYP2C9 (Table 1), amiodarone (fu_{inc} in human hepatocytes of 0.001 versus fu_{inc} in rCYP2C9 of 0.01), benzbromarone (0.03 versus 0.95), miconazole (0.03 versus 0.52), and zafirlukast (0.04 versus 1.0). $IC_{50,\ apparent}$ values were converted to $IC_{50,\ unbound}$ values, thus correcting for nonspecific binding, by multiplying fu_{inc} determined in the appropriate milieu. Figure 3 and Table 1 show the correlation of $IC_{50,\ unbound}$ values determined in human hepatocytes and rCYP2C9 ($r^2 = 0.88$, $p < 0.0001$). Of the four compounds that showed a high degree of nonspecific binding to hepatocytes relative to rCYP2C9, the $IC_{50,\ unbound}$ values of zafirlukast ($IC_{50,\ unbound}$ using hepatocytes of 1.1 μM versus $IC_{50,\ unbound}$ using rCYP2C9 of 0.5 μM) and benzbromarone (0.04 μM versus 0.04 μM) were comparable between the two enzyme sources. There were still significant differences in $IC_{50,\ unbound}$ values, albeit less than for $IC_{50,\ apparent}$ values, for amiodarone (0.5 μM versus 0.03 μM) and miconazole (0.6 μM versus 0.02 μM).

Predicting the Magnitude of Clinical Drug-Drug Interactions from in Vitro IC_{50} Values. Table 2 shows the mean $K_{i,\ apparent}$ and $K_{i,\ unbound}$ values for all the inhibitors using rCYP2C9. Since the assays were performed at a substrate concentration equivalent to the K_m of the CYP2C9 reaction, the experimentally derived IC_{50} should be within 2-fold of the K_i values; assuming competitive inhibition, then $K_i = IC_{50}/2$. Both $[I]_{in}$ values, estimates of inhibitor concentrations at the entrance to the liver, and observed δAUC for CYP2C9 substrates in the presence and absence of the respective inhibitor, were retrieved from Andersson et al. (2004), Blanchard et al. (2004), Ito et al. (2004) and references therein. Fraction unbound in plasma (fu_p) for all the inhibitors was determined as described under *Materials and Methods* and, together with $[I]_{in,\ total}$ values, used to estimate $[I]_{in,\ unbound}$. The predicted in vivo effects are determined using eq. 1. Simulations used both the ratio of $[I]_{in,\ total}/K_{i,\ apparent}$ and

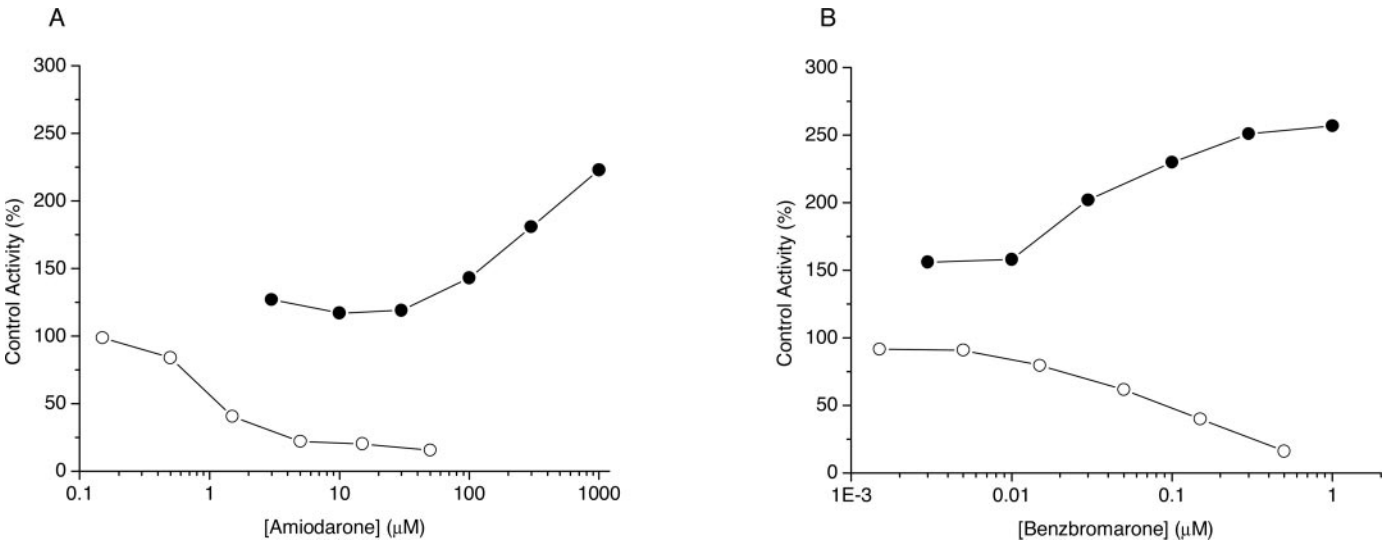

FIG. 2. Effect of amiodarone and benzbromarone on the activity of rCYP2C9 using diclofenac and naproxen as substrates. The IC_{50} determinations of amiodarone (A) and benzbromarone (B) were conducted as described under *Materials and Methods* using naproxen (closed circles) and diclofenac (open circles) as substrates. Using diclofenac as a substrate, the $IC_{50,\ apparent}$ values for amiodarone and benbromarone were 3 μM and 0.04 μM, respectively.

TABLE 1

fu_{inc}, $IC_{50,\ apparent}$, and $IC_{50,\ unbound}$ values determined in rCYP2C9 and human hepatocytes

Results are expressed as the mean IC_{50} of triplicate determinations. The IC_{50} measurements were carried out using diclofenac as the substrate as described under *Materials and Methods*. fu_{inc} was determined under the same conditions as for the inhibition experiment.

Compound	$IC_{50,\ apparent}$		fu_{inc}		$IC_{50,\ unbound}$	
	rCYP2C9	Human Hepatocytes	rCYP2C9	Human Hepatocytes	rCYP2C9	Human Hepatocytes
	μM				μM	
Amiodarone	4	500	0.01	0.001	0.04	0.5
Benzbromarone	0.04	1.2	0.95	0.03	0.04	0.04
Clotrimazole	0.1					
Diclofenac	12[a]		1	0.44	12	
Fluconazole	16	60	1	0.86	16	52
Fluvastatin	2	6.7	1	0.2	2	1.3
Ibuprofen	159		1	0.62	159	
Ketoconazole	6		0.95	0.35	11	
4-Methylimidazole	117					
Miconazole	0.04	19	0.52	0.03	0.02	0.6
Piroxicam	32					
Quinine	243	500				
Sertraline	48	118	0.62	0.22	30	26
Sulfaphenazole	0.4	0.6	1	1	0.4	0.6
Sulfamethiazole	259	463	1	1	259	463
Sulfinpyrazone	213	1000	1	0.19	213	190
Tolbutamide	244		1	1	244	
Warfarin	12	13				
Zafirlukast	0.5	29	1	0.04	0.5	1.1

[a] IC_{50} determined using naproxen as substrate.

$[I]_{in,\ unbound}/K_{i,\ unbound}$. Where $[I]_{in,\ total}/K_{i,\ apparent}$ was used to predict δAUC, there were zero false negatives (defined as observed δAUC $\geq$2, predicted δAUC $<$2), eight correct assignations, and seven false positives (defined as observed δAUC $\leq$2, predicted δAUC $>$2; Table 2). Where $[I]_{in,\ unbound}/K_{i,\ unbound}$ was used to predict δAUC, there was one false negative (benzbromarone), 14 correct assignations, and zero false positives.

Discussion

Over the last decade or so, a relatively thorough mechanistic understanding of the DDI that led to the withdrawal of compounds such as terfenadine and cisapride, so-called "victim drugs," and mibefradil, a "perpetrator drug," has been achieved. As a result, preclinical screens assessing the potential of DDIs, typically via P450 inhibition, are routinely used by drug discovery scientists. However, accurate, quantitative predictions of DDIs from preclinical data still remain a challenge.

Most approaches used to predict the extent of a DDI, caused by reversible P450 inhibition, rely on the use of the $[I]/K_i$ ratio. There is no consensus, however, as to which value of I or, indeed, K_i to use. Despite several reports, stretching over 40 years, emphasizing the need to correct for nonspecific binding (Gillette, 1963; Riley and Grime, 2004; Rostami-Hodjegan and Tucker, 2004), there remains widespread use of apparent or total K_i values for this purpose. In this study, for several CYP2C9 inhibitors, estimates of K_i were determined in our laboratory, a prudent approach for establishing in vitro-in vivo correlations after considering the extent of interlaboratory variability in inhibition parameters (Boobis et al., 1998). This variability likely

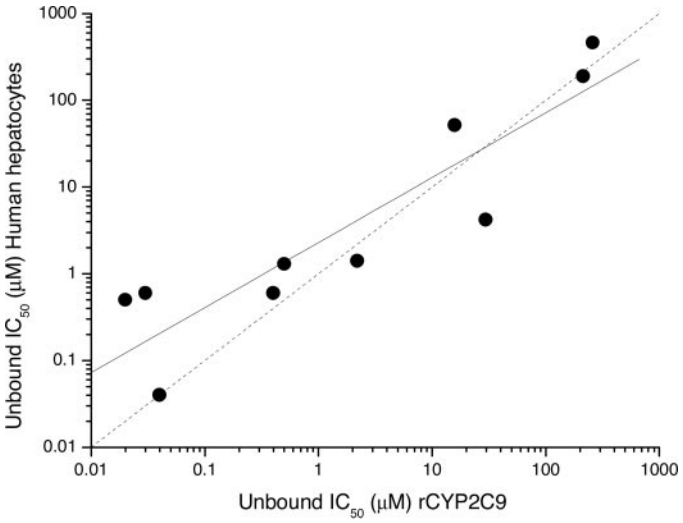

FIG. 3. $IC_{50,\ unbound}$ comparisons using diclofenac as a CYP2C9 substrate in recombinant enzyme and human hepatocytes. The $IC_{50,\ apparent}$ values were corrected for fu determined in rCYP2C9 and human hepatocytes to generate $IC_{50,\ unbound}$ values as outlined under *Materials and Methods*. Results were determined for amiodarone, benzbromarone, fluconazole, fluvastatin, (±)miconazole, sertraline, sulfamethiazole, sulfaphenazole, (±)sulfinpyrazone, and zafirlukast. fu determinations were performed in triplicate. The dotted line is unity. The solid line indicates linear regression of the data ($r^2 = 0.88$, $p < 0.0001$). The data from this plot are tabulated in Table 1.

arises from the use of different probe substrates, substrate concentrations, and enzyme sources.

In this work, for the majority of the compounds studied, there was an excellent correlation between the IC_{50} values determined using diclofenac and naproxen as CYP2C9 substrates (Fig. 1, left, $r^2 = 0.82$, $p < 0.0001$). On average, the $IC_{50,\ apparent}$ values generated using naproxen are ~1.5 times those determined using diclofenac. This, however, appears to be a result of greater nonspecific binding in the naproxen assay, which uses 7 times more protein than the diclofenac assay. For several compounds, a comparison of $IC_{50,\ unbound}$ values results in a 1:1 correlation (Fig. 1B, $r^2 = 0.77$, $p < 0.002$). A weaker correlation has been reported between IC_{50} values obtained using diclofenac and the fluorogenic substrate 7-methoxy-4-trifluoromethyl-coumarin ($r^2 = 0.59$) (Cohen et al., 2003).

Benzbromarone and close analogs are among the most potent reported CYP2C9 inhibitors (Locuson et al., 2004). However, the two analogs studied here, benzbromarone and amiodarone, show dramatically different effects depending on whether diclofenac or naproxen is the substrate (Fig. 2). IC_{50} values could not be determined for amiodarone and benzbromarone due to marked activation (~250%) of naproxen *O*-demethylase activity. Hutzler et al. (2005) recently reported unpublished observations from D. A. Rock and colleagues that benzbromarone activates the dealkylation of the fluorescent probe 7-methoxy-4-trifluoromethyl-coumarin up to 350%. However, potent inhibition was observed using both (*S*)-warfarin and Vivid Green, another CYP2C9 fluorescent substrate. K_i values for benzbromarone using diclofenac (20 nM, this laboratory) and (*S*)-warfarin (19 nM) (Locuson et al., 2004) are comparable. Thus, it appears that for benzbromarone, at least, naproxen and 7-methoxy-4-trifluoromethyl-coumarin may bind in a similar manner, causing activation, whereas diclofenac, (*S*)-warfarin, and Vivid Green bind in a distinct orientation, resulting in potent inhibition.

Hepatocytes in primary culture provide the closest in vitro model to human liver and so may have advantages when predicting clinical DDIs. There are, however, little published comparative data of inhibition constants generated in rP450 and human hepatocytes and the use of such data for predicting DDIs. Our dataset demonstrates that $IC_{50,\ apparent}$ values generated in human hepatocytes, using diclofenac 4′-hydroxylase activity, were all higher than those determined using rCYP2C9. This appears predominantly due to greater nonspecific binding in hepatocytes compared with rP450s (Table 1), as there was greater concordance between the respective $IC_{50,\ unbound}$ values (Fig. 3; Table 1, $r^2 = 0.88$, $p < 0.0001$). The higher $IC_{50,\ unbound}$ values for amiodarone and miconazole in hepatocytes versus rP450s was likely a result of not fully accounting for the nonspecific binding from these extremely lipophilic compounds (log P >6), although the possibility of the inhibitors being actively effluxed from the hepatocytes cannot be discounted. The influence of protein concentration on the apparent

TABLE 2

Simulated δAUC for CYP2C9 mediated drug-drug interactions

K_i values were determined as described under *Materials and Methods* using rCYP2C9 and diclofenac as substrate. $[I]_{in,\ total}$ values and observed δAUC for amiodarone, benzbromarone, fluconazole (both clinical studies), fluvastatin, ketoconazole, miconazole, sertraline, sulfamethiazole, sulfaphenazole, and sulfinpyrazone were retrieved directly from Ito et al. (2004). $[I]_{in,\ total}$ values and observed δAUC for diclofenac, ibuprofen, and tolbutamide were retrieved from Andersson et al. (2004). $[I]_{max,\ total}$ values and observed δAUC for zafirlukast were retrieved from Blanchard et al. (2004). $[I]_{unbound}$ values were calculated by multiplying the $[I]_{total}$ by the fu_p determined in this laboratory. $[I]$ and K_i values together with the estimated fm_{CYP2C9} for the respective in vivo substrates were used in eq. 1 to simulate the δAUC.

In Vivo Substrate	Inhibitor	CYP2C9		$[I]_{in,\ total}$	fu_p	$[I]_{in,\ unbound}$	δAUC		
		$K_{i,\ apparent}$	$K_{i,\ unbound}$				Observed	Simulated using $[I]_{in,\ total}/K_{i,\ apparent}$	Simulated using $[I]_{in,\ unbound}/K_{i,\ unbound}$
		μM		*μM*		*μM*			
Tolbutamide	Sulfaphenazole	0.2	0.2	170	0.0059	1.02	5.3	6.6	3.3
S-Warfarin	Miconazole	0.02	0.01	17	0.0023	0.038	4.7	6.6	3.0
S-Warfarin	Fluconazole	8	8	128	0.8600	110	2.8	5.0	4.8
S-Warfarin	Benzbromarone	0.02	0.02	12	0.0005	0.006	2.2	6.6	1.2
S-Warfarin	Amiodarone	2	0.01	22	0.0027	0.059	2.1	4.7	3.0
Tolbutamide	Fluconazole	8	8	32	0.8600	27.5	2.1	3.1	2.9
Tolbutamide	Ketoconazole	3	3	28	0.0063	0.178	1.8	4.2	1.1
S-Warfarin	Sulfinpyrazone	106	106	35	0.0068	0.235	1.7	1.3	1.0
Tolbutamide	Sulfamethiazole	130	130	233	0.0654	15.2	1.6	2.2	1.1
S-Warfarin	Zafirlukast	0.25	0.25	1[a]	0.0002	0.0002	1.6	3.1	1.0
Diclofenac	Fluvastatin	1	1	6	0.0048	0.027	1.3	2.7	1.0
Phenytoin	Sertraline	24	15	36	0.0116	0.42	1.2	1.9	1.0
Tolbutamide	Diclofenac	6[b]	6	17	0.0032	0.053	1.1	2.7	1.0
Diclofenac	Ibuprofen	80	80	275	0.0046	1.27	1.0	2.4	1.0
Diclofenac	Tolbutamide	122	122	322	0.0171	5.5	1.0	2.2	1.0

[a] $[I_{max}]$.
[b] K_i determined using naproxen as substrate.

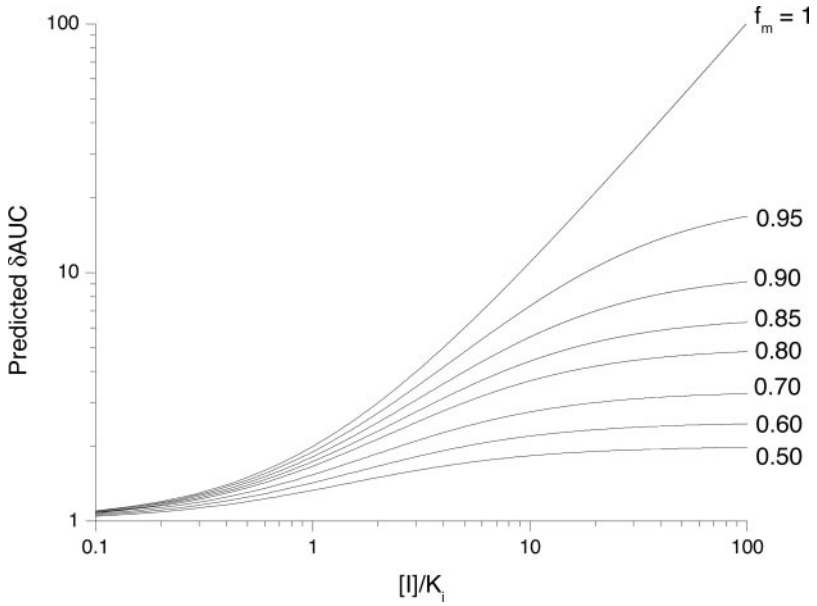

FIG. 4. Simulation of predicted δAUC as a function of $[I]/k_i$ with different fm values. The simulation is based on eq. 1, where fm is the fraction of substrate clearance mediated by the inhibited metabolic pathway. The solid lines represent fm values of 0.50, 0.60, 0.70, 0.80, 0.85, 0.90, 0.95 and 1. An analogous simulation has recently been published by Ito et al. (2005).

K_i or IC_{50} has been well documented for the CYP3A4 inhibitor ketoconazole (Riley, 2001) and the CYP2D6 inhibitor buprenorphine (Umeda et al., 2005). To this end, in P450 inhibition screening assays, this laboratory routinely uses rP450s expressed in *E. coli* at the lowest acceptable protein concentration where, for compounds of moderate or less lipophilicity, fu_{inc} approaches unity.

In this dataset, $IC_{50,\ unbound}$ values were broadly similar between rP450s and human hepatocytes, yet a case can be made for using both rP450s and human hepatocytes in a P450 inhibition screening strategy. In addition to the pragmatic considerations of availability and cost, rP450s have the advantage of being able to use nonselective probes such as naproxen *O*-demethylation for CYP2C9. However, P450 inhibition in intact hepatocytes may be warranted for compounds that concentrate in the liver as a result of cellular transport. Lower IC_{50} values in rat hepatocytes compared with microsomes were observed for a range of CYP2D inhibitors (Di Marco et al., 2003), and the authors speculated that cell-associated inhibitor concentrations were higher. Because hepatocytes contain a comprehensive set of cofactors and drug-metabolizing enzyme pathways, metabolites of one pathway may lead to inhibition of another, a phenomenon indiscernible using single rP450s.

To avoid false-negative predictions of in vivo interactions due to underestimation of the relevant inhibitor concentration in vivo, the use of inhibitor concentration at the entrance to the liver $[I]_{in}$ has been proposed (Ito et al., 1998a). Table 2 compares the observed δAUC and simulated δAUC determined using eq. 1, using the ratio of $[I]_{in,\ total}/K_{i,\ apparent}$ and $[I]_{in,\ unbound}/K_{i,\ unbound}$. Both approaches ranked the observed δAUC reasonably well. The U.S. Food and Drug Administration (www.fda.gov) describes a clinically relevant drug-drug interaction as being clearly present when systemic exposure measures such as AUC are ≥2 for substrate plus inhibitor compared with the substrate control. Thus, for this dataset, the only clinically relevant CYP2C9 inhibitors are sulfaphenazole (observed δAUC of 5.3), miconazole (4.7), fluconazole (2.8 and 2.1 in two studies at different doses), benzbromarone (2.2), and amiodarone (2.1). On this basis, using $[I]_{in,\ total}/K_{i,\ apparent}$ to predict δAUC resulted in 8 correct assignations and 7 false positives, whereas using $[I]_{in,\ unbound}/K_{i,\ unbound}$ resulted in 14 correct assignations and 1 false negative. The use of $[I]_{in,\ total}$ in the simulations results in a broadly systematic overestimation of the observed δAUC, whereas $[I]_{in,\ unbound}$ appears to afford more quantitative predictions of the effect. Based on this analysis of CYP2C9 interactions, the use of $[I]_{in,\ total}/K_i$ ratio within a drug discovery screening cascade would likely avoid false negatives, but more accurate predictions may result using $[I]_{in,\ unbound}$, albeit with an increased risk of false-negative outcomes. Since $[I]_{max}$ and $[I]_{av}$ are always lower than $[I]_{in}$, then the use of $[I]_{max,\ unbound}$ and $[I]_{av,\ unbound}$ would clearly increase the risk of false-negative predictions versus $[I]_{in,\ unbound}$.

The K_i values used in these simulations were generated using diclofenac as the in vitro substrate. Intuitively, using the same inhibitor-drug pair in vitro and in vivo should allow for better predictions. Diclofenac was the in vivo probe for fluvastatin, ibuprofen, and tolbutamide (Table 2). K_i values for benzbromarone using diclofenac (20 nM, this laboratory) and (*S*)-warfarin (19 nM) (Locuson et al., 2004) are comparable, and this is likely to be true of analogs such as amiodarone. For the remaining inhibitors, there was no evidence of substrate dependence using diclofenac and naproxen, providing some reassurance that the K_i values would be similar using the in vivo substrate. In the absence of a K_i value against the in vivo substrate, it is advocated to screen using multiple CYP2C9 probes, akin to those established for CYP3A4 (Kenworthy et al., 1999).

Mean values of fm_{CYP2C9} for the common probes used in studying CYP2C9 interactions have been estimated. For *S*-warfarin and tolbutamide, fm_{CYP2C9} is estimated as 0.85 (Miners and Birkett, 1998), for phenytoin, 0.80 (Miners and Birkett, 1998), and for diclofenac, 0.75 (Shou, 2005). Figure 4 shows a simulation of the effect of fm on predicted δAUC using eq. 1. If $fm > 0.5$, the predicted δAUC is very sensitive to the exact value of fm at high $[I]/K_i$ ratios. This sensitivity highlights the importance of using a precise and accurate fm value for in vitro-in vivo extrapolations, and this has recently been reinforced by Ito et al. (2005). Approaches such as that exemplified by eq. 1 allow prediction of a mean δAUC but do not facilitate a straightforward way of defining the variability in response one would undoubtedly observe in a human volunteer trial or the patient population as a

whole. As has been emphasized by Rostami-Hodjegan and Tucker (2004), predicting outcomes for specific population subgroups is important as a way of identifying those individuals most at risk from a DDI. To this end, there exists Simcyp, a population-based prediction tool that is worthy of further validation.

This study has demonstrated that, in drug discovery, screening CYP2C9 inhibitors with multiple substrates may be warranted. Consideration should also be given to screening a discreet number of late-phase discovery candidates using human hepatocytes. Correcting inhibition parameters for nonspecific binding is a prerequisite to making in vitro-in vivo extrapolations, especially when using relatively high protein concentrations required with microsomes and hepatocytes. Inhibition data from simple and rapid rCYP2C9 screens has demonstrated promise in extrapolating to the in vivo situation.

There is a need to distinguish between attempting quantitative predictions of in vivo interactions versus a process that aims to avoid false-negative outcomes, potentially leading to the termination of a clinical program at a more advanced and costly stage. In particular, the data presented here suggest that for CYP2C9 interactions, the use of total liver inhibitor concentrations may indeed avoid false negatives, but more realistic *predictions* may be achieved using unbound liver inhibitor concentrations and unbound in vitro inhibition parameters. This analysis will be extended to other P450s to assess the overall success and accuracy of this approach to predicting DDIs in humans.

Acknowledgments. We thank Nigel Waters and Roger Fessey for technical assistance.

References

Ahmad S and Wolfe S (1995) Cisapride and torsades de pointes. *Lancet* **345:**508.

Andersson TB, Bredberg E, Ericsson H, and Sjoeberg H (2004) An evaluation of the in vitro metabolism data for predicting the clearance and drug-drug interaction potential of CYP2C9 substrates. *Drug Metab Dispos* **32:**715–721.

Austin RP, Barton P, Cockroft SL, Wenlock MC, and Riley RJ (2002) The influence of nonspecific microsomal binding on apparent intrinsic clearance and its prediction from physicochemical properties. *Drug Metab Dispos* **30:**1497–1503.

Austin RP, Barton P, Mohmed S, and Riley RJ (2005) The binding of drugs to hepatocytes and its relationship to physicochemical properties. *Drug Metab Dispos* **33:**419–425.

Ayrton J, Plumb R, Leavens WJ, Mallett D, Dickins M, and Dear GJ (1998) Application of a generic fast gradient liquid chromatography tandem mass spectrometry method for the analysis of cytochrome P450 probe substrates. *Rapid Commun Mass Spectrom* **12:**217–224.

Bertz RJ and Granneman GR (1997) Use of in vitro and in vivo data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clin Pharmacokinet* **32:**210–258.

Blanchard N, Richert L, Coassolo P, and Lave T (2004) Qualitative and quantitative assessment of drug-drug interaction potential in man, based on Ki, IC50 and inhibitor concentration. *Curr Drug Metab* **5:**147–156.

Boobis AR, McKillop D, Robinson DT, Adams DA, and McCormick DJ (1998) Interlaboratory comparison of the assessment of P450 activities in human hepatic microsomal samples. *Xenobiotica* **28:**493–506.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Cohen LH, Remley MJ, Raunig D, and Vaz ADN (2003) In vitro drug interactions of cytochrome P450: an evaluation of fluorogenic to conventional substrates. *Drug Metab Dispos* **31:**1005–1015.

Cohen LH, van Leeuwen RE, van Thiel GC, van Pelt JF, and Yap SH (2000) Equally potent inhibitors of cholesterol synthesis in human hepatocytes have distinguishable effects on different cytochrome P450 enzymes. *Biopharm Drug Dispos* **21:**353–364.

Crespi CL, Miller VP, and Penman BW (1998) High throughput screening for inhibition of cytochrome P450 metabolism. *Med Chem Res* **8:**457–471.

Di Marco A, Yao D, and Laufer R (2003) Demethylation of radiolabelled dextromethorphan in rat microsomes and intact hepatocytes. *Eur J Biochem* **270:**3768–3777.

Gillette J (1963) Drug metabolism by enzyme mechanisms. *Prog Drug Res* **6:**55–57.

Gomez-Lechon MJ, Ponsoda X, Bort R, and Castell JV (2001) The use of cultured hepatocytes to investigate the metabolism of drugs and mechanisms of drug hepatotoxicity. *ATLA* **29:**225–231.

Honig PK, Wortham DC, Zamani K, Conner DP, Mullin JC, and Cantilena LR (1993) Terfenadine-ketoconazole interaction. Pharmacokinetic and electrocardiographic consequences. *J Am Med Assoc* **269:**1513–1518.

Hutzler JM, Hauer MJ, and Tracy TS (2001) Dapsone activation of CYP2C9-mediated metabolism: evidence for activation of multiple substrates and a two-site model. *Drug Metab Dispos* **29:**1029–1034.

Hutzler JM, Messing DM, and Wienkers LC (2005) Predicting drug-drug interactions in drug discovery: where are we now and where are we going? *Curr Opin Drug Discov Dev* **8:**51–58.

Ito K, Brown HS, and Houston JB (2004) Database analyses for the prediction of in vivo drug-drug interactions from in vitro data. *Br J Clin Pharmacol* **57:**473–486.

Ito K, Hallifax D, Obach RS, and Houston JB (2005) Impact of parallel pathways of drug elimination and multiple cytochrome P450 involvement on drug-drug interactions: CYP2D6 paradigm. *Drug Metab Dispos* **33:**837–844.

Ito K, Iwatsubo T, Kanamitsu S, Nakajima Y, and Sugiyama Y (1998a) Quantitative prediction of in vivo drug clearance and drug interactions from in vitro data on metabolism, together with binding and transport. *Annu Rev Pharmacol Toxicol* **38:**461–499.

Ito K, Iwatsubo T, Kanamitsu S, Ueda K, Suzuki H, and Sugiyama Y (1998b) Prediction of pharmacokinetic alterations caused by drug-drug interactions: metabolic interaction in the liver. *Pharmacol Rev* **50:**387–412.

Jankel CA and Fitterman LK (1993) Epidemiology of drug-drug interactions as a cause of hospital admissions. *Drug Safety* **9:**51–59.

Kenworthy KE, Bloomer JC, Clarke SE, and Houston JB (1999) CYP3A4 drug interactions: correlation of 10 in vitro probe substrates. *Br J Clin Pharmacol* **48:**716–727.

Krayenbuhl JC, Vozeh S, Kondo-Oestreicher M, and Dayer P (1999) Drug-drug interactions of new active substances: mibefradil example. *Eur J Clin Pharmacol* **55:**559–565.

LeCluyse E, Madan A, Hamilton G, Carroll K, DeHaan R, and Parkinson A (2000) Expression and regulation of cytochrome P450 enzymes in primary cultures of human hepatocytes. *J Biochem Mol Toxicol* **14:**177–188.

Li AP (1999) Overview: hepatocytes and cryopreservation—a personal historical perspective. *Chem -Biol Interact* **121:**1–5.

Li AP, Lu C, Brent JA, Pham C, Fackett A, Ruegg CE, and Silber PM (1999) Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability and drug-drug interaction potential. *Chem-Biol Interact* **121:**17–35.

Locuson CW, Rock DA, and Jones JP (2004) Quantitative binding models for CYP2C9 based on benzbromarone analogues. *Biochemistry* **43:**6948–6958.

McGinnity DF, Soars MG, Urbanowicz RA, and Riley RJ (2004) Evaluation of fresh and cryopreserved hepatocytes as in vitro drug metabolism tools for the prediction of metabolic clearance. *Drug Metab Dispos* **32:**1247–1253.

Miners JO and Birkett DJ (1998) Cytochrome P4502C9: an enzyme of major importance in human drug metabolism. *Br J Clin Pharmacol* **45:**525–538.

Moody GC, Griffin SJ, Mather AN, McGinnity DF, and Riley RJ (1999) Fully automated analysis of activities catalysed by the major human liver cytochrome P450 (CYP) enzymes: assessment of human CYP inhibition potential. *Xenobiotica* **29:**53–75.

Oleson FB, Berman CL, and Li AP (2004) An evaluation of the P450 inhibition and induction potential of daptomycin in primary human hepatocytes. *Chem-Biol Interact* **150:**137–147.

Riley RJ (2001) The potential pharmacological and toxicological impact of P450 screening. *Curr Opin Drug Discov Dev* **4:**45–54.

Riley RJ and Grime K (2004) Metabolic screening in vitro: metabolic stability, CYP inhibition and induction. *Drug Discov Today* **1:**365–372.

Riley RJ, McGinnity DF, and Austin RP (2005) A unified model for predicting human hepatic, metabolic clearance from in vitro intrinsic clearance data in hepatocytes and microsomes. *Drug Metab Dispos* **33:**1304–1311.

Rostami-Hodjegan A and Tucker G (2004) 'In silico' simulations to assess the 'in vivo' consequences of 'in vitro' metabolic drug-drug interactions. *Drug Discov Today* **1:**441–448.

Shou M (2005) Prediction of pharmacokinetics and drug-drug interactions from in vitro metabolism data. *Curr Opin Drug Discov Dev* **8:**66–77.

Umeda S, Harakawa N, Yamamoto M, and Ueno K (2005) Effect of nonspecific binding to microsomes and metabolic elimination of buprenorphine on the inhibition of cytochrome P4502D6. *Biol Pharm Bull* **28:**212–216.

Venkatakrishnan K, von Moltke LL, Obach RS, and Greenblatt DJ (2003) Drug metabolism and drug interactions: application and clinical value of in vitro models. *Curr Drug Metab* **4:**423–459.

Weaver R, Graham KS, Beattie IG, and Riley RJ (2003) Cytochrome P450 inhibition using recombinant proteins and mass spectrometry/multiple reaction monitoring technology in a cassette incubation. *Drug Metab Dispos* **31:**955–966.

Zhao P, Kunze KL, and Lee CA (2005) Evaluation of time-dependent inactivation of CYP3A in cryopreserved human hepatocytes. *Drug Metab Dispos* **33:**853–861.

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