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**Prediction of CYP2C9 mediated drug-drug interactions: a comparison using data  
from recombinant enzymes and human hepatocytes**

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## Running Title

## Prediction of CYP2C9 mediated drug-drug interactions

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### Abbreviations

cytochrome P450 (CYP), recombinant cytochrome P450 2C9 (rCYP2C9), intrinsic clearance ( $CL_{int}$ ), HPLC/MS (high-performance liquid chromatography/mass spectrometry), metabolic clearance ( $CL_{met}$ ), unbound fraction in incubation ( $f_{u_{inc}}$ ), unbound fraction in plasma ( $f_{u_p}$ ), human serum albumin (HSA), bovine serum albumin (BSA), robotic sample processor (RSP), inhibitor concentration ( $[I]$ ), average systemic plasma concentration after repeated oral administration ( $[I]_{av}$ ), maximum systemic plasma concentration after repeated oral administration ( $[I]_{max}$ ), maximum hepatic input concentration ( $[I]_{in}$ ), inhibition constant ( $K_i$ ), area under the plasma concentration time curve (AUC), drug-drug interactions (DDIs), fraction of total clearance metabolised by CYP2C9 ( $f_{m_{CYP2C9}}$ )

## **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 co-administration with prototypic CYP2C9 substrates. There was an excellent correlation between  $IC_{50, \text{ apparent}}$  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 non-specific binding, the  $IC_{50, \text{ unbound}}$  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  $\mu$ M respectively, whilst inhibiting diclofenac 4'-hydroxylation with  $IC_{50, \text{ apparent}}$  values of 3  $\mu$ M and 0.04  $\mu$ M respectively. CYP2C9  $IC_{50, \text{ apparent}}$  values generated in human hepatocytes were systematically higher than those determined with rCYP2C9. After correcting for non-specific binding, there was an excellent correlation of  $IC_{50, \text{ unbound}}$  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 ( $[I]_{\text{in}}:K_i$ ) was used to simulate clinical  $\delta$ AUC changes and compared to that observed *in vivo*. Where  $[I]_{\text{in, total}}:K_{i, \text{ apparent}}$  was used there were 0 false negatives (observed  $\delta$ AUC  $\geq 2$ , predicted  $\delta$ AUC  $< 2$ ), 8 correct assignments and 7 false positives (observed  $\delta$ AUC  $\leq 2$ , predicted  $\delta$ AUC  $> 2$ ). Where  $[I]_{\text{in, unbound}}:K_{i, \text{ unbound}}$  was used there was 1 false negative, 14 correct assignments and 0 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 recognised that inhibition of cytochrome P450 (CYP) 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 in order 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 CYP metabolism is important early in the drug discovery process. Thus, a battery of automated *in vitro* screens to determine the degree of CYP inhibition are now routinely employed in DMPK departments across the pharmaceutical industry. Such screens are used both for the evaluation and optimisation of potential candidate drugs and for prioritising 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 CYP mediated DDI potential have traditionally involved the use of human liver microsomes though more recently the use of recombinant cytochrome P450s (rCYPs) has become widespread. More often than not, these assays focus on the five major drug metabolising CYPs: 1A2, 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; predicting hepatic metabolic clearance (McGinnity et al., 2004; Riley et al., 2005); studying hepatotoxicity of drugs (Gomez-Lechon et al., 2001) and for the mechanistic understanding of DDIs mediated via CYP induction (LeCluyse et al., 2000). There are sporadic reports of the use of human hepatocytes to characterise CYP inhibition (Cohen et al., 2000; Li et al., 1999; Oleson et al., 2004; Zhao et al., 2005) but it is notable that investigations of inhibitory mechanisms remain under represented compared to studies on metabolism and induction. This is likely

due to the relative accessibility of recombinant CYPs and their overall applicability in predicting clinically relevant DDI. Availability of 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 CYP 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 co-administered in the presence of an inhibitor of the substrates' elimination pathway is a function of the ratio of inhibitor concentration ([I]) to inhibition constant ( $K_i$ ) (Ito et al., 1998b; Bertz and Granneman, 1997; 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, as 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 non-specific binding in the *in vitro* incubation, choice/level of co-solvent and choice of substrate. These and other variables presumably

account for the large degree on inter-laboratory 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 multi-site kinetics and substrate dependent interactions have been observed (Hutzler et al., 2001, 2005).

The aims of this work were firstly to evaluate the power of predicting clinical  $\delta AUC$  changes of prototypic CYP2C9 substrates (S-warfarin, phenytoin, tolbutamide and diclofenac) when co-administered with 14 drugs via  $K_i$  values determined in recombinantly expressed CYP2C9 and human hepatocytes. We have explored any substrate dependency of CYP2C9 inhibition by comparing  $IC_{50}$  values determined using diclofenac and naproxen as *in vitro* substrates and the data is 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 the *in vitro* assay is also emphasised.

## **Materials and Methods**

### *Materials*

Amiodarone, benzbromarone, clotrimazole, diclofenac, 4'-hydroxydiclofenac, ibuprofen, ketoconazole, 4-methylimidazole ( $\pm$ )miconazole, (-)naproxen, piroxicam, quinine, sulphamethiazole, sulphaphenazole, ( $\pm$ )sulphinpyrazone, tolbutamide ( $\pm$ )warfarin and  $\beta$ -nicotinamide adenine dinucleotide phosphate reduced form ( $\beta$ -NADPH), were purchased as the highest grade available from Sigma-Aldrich Chemical (Gillingham, UK). Fluconazole, fluvastatin and sertraline were purchased from Sequoia Research Products Ltd. (Oxford, UK). Zafirlukast was synthesised at AstraZeneca R&D Wilmington. [O-methyl- $^{14}\text{C}$ ]-naproxen (specific radioactivity 55 mCi/mmol, chemical purity > 98.5%, radiochemical purity >99.4%) was synthesised as described previously (Moody et al., 1999). Dimethylsulfoxide, acetonitrile and trichloroacetic acid were purchased from Fisher Scientific (Loughborough, UK) and methanol was purchased from Romil Ltd. (Cambridge, UK).

Bactosomes prepared from *E.coli* cells co-expressing 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 rCYPs were performed on a robotic sample processor (RSP) (Genesis RSP 150, Tecan, Reading, UK). All HPLC/MS was conducted on a Micromass Quattro Ultima triple quadrupole and an Alliance HT Waters 2790 HPLC system. Scintillation counting used a TopCount NXT microplate scintillation counter (Packard, Meriden, CT).

### *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 CYP 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  $\mu$ M and diclofenac at 2  $\mu$ M. 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 rCYPs and human hepatocytes respectively.

### *Determination of $IC_{50}$ using rCYP2C9*

The following volumes are used in each incubation well; 20  $\mu$ l 10 mM NADPH in 0.1M phosphate buffer pH 7.4, 178  $\mu$ l CYP/substrate and 2  $\mu$ l of the pre-dilution stocks of inhibitor are spiked directly into the incubation micro-titre plate. The final incubation volume is 200  $\mu$ l giving 100 fold dilutions of the solvent stocks of the inhibitor generated in the pre-dilution 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 6 secondary solutions (e.g. 5 mM to 20  $\mu$ M). 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  $\mu$ M). Finally 10  $\mu$ l of each of the tertiary solutions were spiked into the incubation mix (200  $\mu$ l total volume to give final concentrations (e.g. 50  $\mu$ M to 0.2  $\mu$ M). 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 (SPE) and quantified by liquid scintillation counting. Interaction with the respective CYP by an inhibitor results in a decrease in the amount of formaldehyde produced. Naproxen was used at a final concentration of 109  $\mu M$  (0.1  $\mu Ci$  [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 protein/ml of incubate). One batch of rCYP2C9 was used for all experiments, primarily to maintain a constant level of protein. In the authors' experience, the variability of  $IC_{50, \text{unbound}}$  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  $\mu l$  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 96 well 2 ml round bottom collection plate (Whatman, Clifton, NJ) using negative pressure was 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 methanol and 2 x 0.2 ml water aliquots. All (250  $\mu l$ ) of the quenched incubation was applied to the column and eluted with 2 x 200  $\mu l$  aliquots of water. Aliquots (600  $\mu l$ ) were transferred to plates containing 750  $\mu l$  Ultima Gold liquid scintillant (Perkin-Elmer, Boston, MA) and counted. A set of controls, which contains 178  $\mu l$  of substrate/ CYP incubation mixture, 400  $\mu l$  of water and 750  $\mu l$  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 a inhibitor will result in a decrease in the amount of 4'-hydroxydiclofenac produced. Diclofenac was used at a final concentration of 2  $\mu$ M. The amount of rCYP2C9 used in the diclofenac 4'-hydroxylation assay was 10 pmol/ml (0.02 mg 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  $\mu$ l methanol. Samples were chilled at  $-20^{\circ}\text{C}$  for 2 h, spun at 3500 rpm for 15 min and the supernatants transferred to vials for analysis as described below.

#### *Thawing of cryopreserved hepatocytes*

Aliquots (20 ml) of hepatocyte suspension buffer (with no added albumin) were pre-warmed to  $37^{\circ}\text{C}$ . Cryopreserved cells were removed from liquid  $\text{N}_2$  and immediately immersed in a water bath that had been pre-heated to  $37^{\circ}\text{C}$ . The vials were shaken gently until the contents were completely free of ice crystals ( $\sim 2$  min) and were then emptied into the pre-warmed hepatocyte suspension buffer. The cells were centrifuged at 40 g for 5 min at  $19^{\circ}\text{C}$ , 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 re-suspended at a concentration of 2 million cells/ml.

#### *Determination of $\text{IC}_{50}$ using diclofenac 4'-hydroxylase activity and human hepatocytes*

This assay was performed using manual pipetting in a shaking water bath at  $37^{\circ}\text{C}$ . Diclofenac was used at a final concentration of 2  $\mu$ M. An appropriate amount of diclofenac was aliquoted and the solvent evaporated under nitrogen to dryness before re-suspension in hepatocyte suspension buffer (2.2 g  $\text{NaHCO}_3$ , 2.34 g Na HEPES, 1 L powder equivalent of DMEM (Sigma, Gillingham, UK) diluted in 1 L of water and adjusted to pH 7.4 with 1 M HCl) to give a concentration of 200  $\mu$ M. Drug stocks were prepared in dimethyl sulfoxide

at 100-fold incubation concentration (e.g. 5000, 1500, 100, 15, 5  $\mu$ M). 10  $\mu$ l of this x100 stock were added to a vial containing 490  $\mu$ l of hepatocyte suspension buffer containing substrate. A 7 ml glass bijou vial containing 250  $\mu$ l of hepatocytes at a concentration of 2 million cells/ml was pre-incubated 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  $\mu$ l of drug/buffer/substrate mix to the 250  $\mu$ l of hepatocytes giving, in the example shown, final inhibitor concentrations of between 50, 15, 1, 0.15, 0.05  $\mu$ M. 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 for 3500 rpm for 15 min at 4 °C. The supernatants were removed and transferred into vials and analysed as described below. Assays were performed in triplicate.

#### *Determination of $f_{up}$ and $f_{uinc}$ 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 were 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  $\mu$ M – 50  $\mu$ M) (Austin et al., 2002) and so binding was determined at a single concentration (10  $\mu$ M for plasma and 1  $\mu$ M for rCYP and hepatocytes). Compounds were solubilised in dimethyl sulfoxide and the final organic solvent concentration was 1% (v/v). Plasma was

dialysed against Dulbecco's phosphate buffered saline pH 7.4 (Sigma, Gillingham, UK), rCYP against 0.1M phosphate buffer pH 7.4 and hepatocytes against hepatocyte suspension buffer, overnight at 37 °C.

Samples were quantified using HPLC/MS with Symmetry C8 (5 µm x 3.9 mm x 20 mm columns, Waters, Milford, MA) 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 analysed by HPLC-MS/MS 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, (Phenomenx, 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<sup>-1</sup> and column temperature 40°C.

#### *Data Analysis*

Microsoft Excel (Redmond, WA) was used to calculate IC<sub>50</sub> 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<sub>m</sub> of the CYP2C9 reaction as under these conditions, irrespective of the type of reversible inhibition, IC<sub>50</sub> should be within 2-fold of the K<sub>i</sub> (Cheng and Prusoff, 1973). For competitive and uncompetitive inhibition IC<sub>50</sub> = 2K<sub>i</sub> and for non-competitive and linear mixed-type inhibition IC<sub>50</sub> = K<sub>i</sub>. The inhibition type has not been fully elucidated for all the inhibitors

studied in this work and so 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 minimise 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 have 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 includes S-Warfarin, tolbutamide, phenytoin and diclofenac, the ratio change of AUC in the presence or absence of a CYP2C9 inhibitor can be determined by equation 1 (Ito et al., 1998b). In clinical situations, the substrate concentration is usually much lower than the  $K_m$  and so equation 1 is valid for all inhibitors, except for uncompetitive inhibitors, a rare inhibition type for drugs.

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

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 concentrations 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 ( $[I]_{\text{in}}$ ).  $[I]_{\text{in}}$  can be calculated as follows:

$$[I]_{\text{in}} = [I]_{\text{av}} + \frac{\kappa_a F_a D}{Q_h}$$

where  $D$  and  $\tau$  are the dose and dosing interval respectively of the inhibitor,  $\kappa$  and  $\kappa_a$  are the elimination and absorption rate constants respectively.  $F_a$  is the fraction absorbed from the gastrointestinal tract and  $Q_h$  is hepatic blood flow.

## Results

### *Comparison of $IC_{50, \text{ apparent}}$ and $IC_{50, \text{ unbound}}$ values in rCYP2C9 using naproxen and diclofenac as substrates*

Figure 1a shows a correlation of the mean  $IC_{50, \text{ apparent}}$  values determined in rCYP2C9, for a set of marketed drugs, amiodarone, benzbromarone, clotrimazole, fluconazole, fluvastatin, ibuprofen, ketoconazole, 4-methylimidazole, ( $\pm$ )miconazole, (-)naproxen, piroxicam, quinine, sulphamethiazole, sulphaphenazole, ( $\pm$ )sulphinpyrazone, tolbutamide, ( $\pm$ )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 in 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  $IC_{50, \text{ unbound}}$  values ( $r^2 = 0.77$ ,  $p < 0.002$ ), for a subset of compounds shown in Figure 1a where  $f_{u, \text{ inc}}$  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  $\mu$ M. 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  $IC_{50, \text{ apparent}}$  values of 3  $\mu$ M and 0.04  $\mu$ M were respectively 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<sub>50</sub> values in human hepatocytes and rCYP2C9*

Table 1 shows the IC<sub>50, apparent</sub> values determined using diclofenac 4'-hydroxylase activity in human hepatocytes and rCYP2C9 for eleven compounds; amiodarone, benzbromarone, fluconazole, fluvastatin, (±)miconazole, sertraline, sulphamethiazole, sulphaphenazole, (±)sulphinpyrazone, (±)warfarin and zafirlukast. All of the IC<sub>50, apparent</sub> values generated in human hepatocytes were higher than those determined using rCYPs. Four compounds showed a >10 fold higher IC<sub>50, apparent</sub> using hepatocytes compared with rCYP2C9; amiodarone (IC<sub>50, apparent</sub> using hepatocytes of 500 µM versus IC<sub>50, apparent</sub> 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<sub>inc</sub> in human hepatocytes of 0.001 versus fu<sub>inc</sub> 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<sub>50, apparent</sub> values were converted to IC<sub>50, unbound</sub> values, thus correcting for non-specific binding, by multiplying fu<sub>inc</sub> determined in the appropriate milieu. Figure 3 and Table 1 show the correlation of IC<sub>50, unbound</sub> values determined in human hepatocytes and rCYP2C9 ( $r^2 = 0.88$ ,  $p < 0.0001$ ). Of the four compounds that showed a high degree of non-specific binding to hepatocytes relative to rCYP2C9 the IC<sub>50, unbound</sub> values of zafirlukast (IC<sub>50, unbound</sub> using hepatocytes of 1.1 µM versus IC<sub>50, unbound</sub> using rCYP2C9 of 0.5 µM) and benzbromarone (0.04 µM v 0.04 µM) were comparable between the two enzyme sources. There were still significant differences in IC<sub>50, unbound</sub> values, albeit less than for IC<sub>50, apparent</sub> values, for amiodarone (0.5 µM v 0.03 µM) and miconazole (0.6 µM v 0.02 µM).

### *Predicting the magnitude of clinical drug-drug interactions from in vitro IC<sub>50</sub> values*

Table 2 shows the mean K<sub>i, apparent</sub> and K<sub>i, unbound</sub> values for all the inhibitors using rCYP2C9. As the assays were performed at a substrate concentration equivalent to the K<sub>m</sub> of the

CYP2C9 reaction, the experimentally derived  $IC_{50}$  should be within two-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  $\delta AUC$  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 refs therein. Fraction unbound in plasma ( $f_{up}$ ) for all the inhibitors were determined as described in Materials and Methods and together with  $[I]_{in, total}$  values, used to estimate  $[I]_{in, unbound}$ . The predicted *in vivo* effects are determined using equation 1. Simulations used both the ratio of  $[I]_{in, total}:K_{i, apparent}$  and  $[I]_{in, unbound}:K_{i, unbound}$ . Where  $[I]_{in, total}:K_{i, apparent}$  was used to predict  $\delta AUC$  there were 0 false negatives (defined as observed  $\delta AUC \geq 2$ , predicted  $\delta AUC < 2$ ), 8 correct assignments and 7 false positives (defined as observed  $\delta AUC \leq 2$ , predicted  $\delta AUC > 2$ ) (Table 2). Where  $[I]_{in, unbound}:K_{i, unbound}$  was used to predict  $\delta AUC$  there was 1 false negative (benzbromarone), 14 correct assignments and 0 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 DDI, typically *via* CYP inhibition, are routinely employed by drug discovery scientists. However accurate, quantitative predictions of DDI from preclinical data still remain a challenge.

Most approaches used to predict the extent of a DDI, caused by reversible CYP 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 forty years, emphasizing the need to correct for non-specific binding (Gillette, 1963; Rostami-Hodjegan and Tucker, 2004; Riley and Grime, 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 inter laboratory variability in inhibition parameters (Boobis et al., 1998). This variability likely 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 (Figure 1a,  $r^2 = 0.82$ ,  $p < 0.0001$ ). On average, the  $IC_{50, \text{apparent}}$  values generated using naproxen are  $\sim 1.5 \times$  those determined using diclofenac. This however appears to be a result of greater non-specific binding in the naproxen assay which uses seven times more protein than the diclofenac assay. For several compounds a comparison of  $IC_{50, \text{unbound}}$  values results in a 1:1 correlation (Figure 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 analogues are amongst the most potent reported CYP2C9

inhibitors (Locuson et al., 2004). However, the two analogues studied here, benzbromarone and amiodarone, show dramatically different effects depending on whether diclofenac or naproxen is the substrate (Figure 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 DA 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 DDI. There is, however, little published comparative data of inhibition constants generated in rCYP and human hepatocytes and the use of such data for predicting DDI. Our dataset demonstrates  $IC_{50, \text{ apparent}}$  values generated in human hepatocytes, using diclofenac 4'-hydroxylase activity, were all higher than those determined using rCYP2C9. This appears predominantly due to greater non-specific binding in hepatocytes compared to rCYPs (Table 1), as there was greater concordance between the respective  $IC_{50, \text{ unbound}}$  values (Figure 3 & Table 1,  $r^2 = 0.88$ ,  $p < 0.0001$ ). The higher  $IC_{50, \text{ unbound}}$  values for amiodarone and miconazole in hepatocytes versus rCYPs was likely a result of not fully accounting for the non-specific 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  $K_i$  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 CYP inhibition screening assays, this laboratory

routinely uses rCYPs expressed in *E.coli* at the lowest acceptable protein concentration where for compounds of moderate or less lipophilicity,  $f_{inc}$  approaches unity.

In this dataset,  $IC_{50, \text{ unbound}}$  values were broadly similar between rCYPs and human hepatocytes, yet a case can be made for using both rCYPs and human hepatocytes in a CYP inhibition screening strategy. In addition to the pragmatic considerations of availability and cost, rCYPs have the advantage of being able to use non-selective probes such as naproxen O-demethylation for CYP2C9. However CYP 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 to microsomes were observed for a range of 2D inhibitors (Di Marco et al., 2003) and the authors speculated that cell associated inhibitor concentrations were higher. As hepatocytes contain a comprehensive set of cofactors and drug-metabolising enzyme pathways, metabolites of one pathway may lead to inhibition of another, a phenomenon indiscernible using single rCYPs.

In order 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  $\delta AUC$  and simulated  $\delta AUC$  determined using equation 1 using the ratio of  $[I]_{in, \text{ total}}:K_{i, \text{ apparent}}$  and  $[I]_{in, \text{ unbound}}:K_{i, \text{ unbound}}$ . Both approaches ranked the observed  $\delta AUC$  reasonably well. The FDA ([www.fda.gov](http://www.fda.gov)) describe a clinically relevant drug-drug interaction as being clearly present when systemic exposure measures such as AUC are  $\geq 2$  for substrate plus inhibitor compared to the substrate control. Thus, for this dataset, the only clinically relevant CYP2C9 inhibitors are sulphaphenazole (observed  $\delta AUC$  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  $[I]_{in, \text{ total}}:K_{i, \text{ apparent}}$  to predict  $\delta AUC$  resulted in 8 correct assignments and 7 false positives whereas using  $[I]_{in, \text{ unbound}}:K_{i, \text{ unbound}}$  resulted in 14 correct assignments and 1 false negative. The use of  $[I]_{in, \text{ total}}$  in the

simulations results in a broadly systematic overestimation of the observed  $\delta\text{AUC}$ , whilst  $[I]_{\text{in, unbound}}$  appears to afford more quantitative predictions of the effect. Based on this analysis of CYP2C9 interactions, the use of  $[I]_{\text{in, total}}:K_i$  ratio within a drug discovery screening cascade would likely avoid false negatives, but more accurate predictions may result using  $[I]_{\text{in, unbound}}$ , albeit with an increased risk of false negative outcomes. As  $[I]_{\text{max}}$  and  $[I]_{\text{av}}$  are always lower than  $[I]_{\text{in}}$ , then the use of  $[I]_{\text{max, unbound}}$  and  $[I]_{\text{av, unbound}}$  would clearly increase the risk of false negative predictions versus  $[I]_{\text{in, unbound}}$ .

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 analogues such as amiodarone. For the remaining inhibitors, there was no evidence of substrate dependency 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  $f_{\text{mCYP2C9}}$  for the common probes used in studying CYP2C9 interactions have been estimated. For S-Warfarin and tolbutamide,  $f_{\text{mCYP2C9}}$  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  $f_{\text{m}}$  on predicted  $\delta\text{AUC}$  using equation 1. If  $f_{\text{m}} > 0.5$ , the predicted  $\delta\text{AUC}$  is very sensitive to the exact value of  $f_{\text{m}}$  at high  $[I]/K_i$  ratios. This sensitivity highlights the importance of using a precise and accurate  $f_{\text{m}}$  value for *in vitro-in vivo* extrapolations and this has recently been reinforced by Ito et al., 2005. Approaches such as that exemplified by Equation 1 allow prediction of a mean  $\delta\text{AUC}$  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 emphasised 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 non-specific 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 programme 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 CYPs to assess the overall success and accuracy of this approach to predicting DDIs in humans.

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## Footnotes

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## Legends for Figures

Figure 1a.  $IC_{50, \text{ apparent}}$  comparisons using naproxen and diclofenac as substrates for rCYP2C9

The  $IC_{50}$  determinations were conducted as described in Materials and Methods. Results were determined for amiodarone, benzbromarone, clotrimazole, fluconazole, fluvastatin, ibuprofen, ketoconazole, 4-methylimidazole, ( $\pm$ )miconazole, (-)naproxen, piroxicam, quinine, sulphamethiazole, sulphaphenazole, ( $\pm$ )sulphinpyrazone, tolbutamide, ( $\pm$ )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 ( $r^2 = 0.82$ ,  $p < 0.0001$ ).

Figure 1b.  $IC_{50, \text{ unbound}}$  comparisons using naproxen and diclofenac as substrates for rCYP2C9

$Fu_{\text{inc}}$  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 in Materials and Methods. Results were determined for fluconazole, fluvastatin, ketoconazole, ( $\pm$ )miconazole, sulphamethiazole, sulphaphenazole, ( $\pm$ )sulphinpyrazone, tolbutamide and zafirlukast using the mean  $IC_{50, \text{ apparent}}$  values and measured  $f_{u, \text{inc}}$  in each assay. The dotted line is unity. The solid line indicates linear regression of the data ( $r^2 = 0.77$ ,  $p < 0.002$ ).

Figure 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 in Materials and Methods using naproxen (closed circles) and diclofenac (open circles) as substrates. Using diclofenac as a substrate, the  $IC_{50, \text{ apparent}}$  values for amiodarone and benzbromarone were 3  $\mu\text{M}$  and 0.04  $\mu\text{M}$  respectively.

Figure 3.  $IC_{50, \text{ unbound}}$  comparisons using diclofenac as a CYP2C9 substrate in recombinant enzyme and human hepatocytes

The  $IC_{50, \text{ apparent}}$  values were corrected for  $f_u$  determined in rCYP2C9 and human heps to generate  $IC_{50, \text{ unbound}}$  values as outlined in Materials and Methods. Results were determined for amiodarone, benzbromarone, fluconazole, fluvastatin ( $\pm$ )miconazole, sertraline, sulphamethiazole, sulphaphenazole, ( $\pm$ )sulphinpyrazone, and zafirlukast.  $f_u$  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.

Figure 4. Simulation of predicted  $\delta\text{AUC}$  as a function of  $[I]/k_i$  with different  $f_m$  values.

The simulation is based on equation 1 where  $f_m$  is the fraction of substrate clearance mediated by the inhibited metabolic pathway. The solid lines represent  $f_m$  values of 0.50, 0.60, 0.70, 0.80, 0.85, 0.90, 0.85 and 1. A analogous simulation has been recently published by (Ito et al., 2005).

Table 1  $F_{u,inc}$ ,  $IC_{50, apparent}$  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 in Materials and Methods.  $F_{u,inc}$  was determined under the same conditions as for the inhibition experiment.

Compound	$IC_{50, apparent}$ ( $\mu M$ )		$f_{u,inc}$		$IC_{50, unbound}$ ( $\mu M$ )	
	rCYP2C9	Human hepatocytes	rCYP2C9	Human hepatocytes	rCYP2C9	Human hepatocytes
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*	-	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
Sulphamethiazole	259	463	1	1	259	463
Sulphinpyrazone	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

\* $IC_{50}$  determined using naproxen as substrate.

Table 2. Simulated  $\delta$ AUC for CYP2C9 mediated drug-drug interactions.

$K_i$  values were determined as described in Materials and Methods using rCYP2C9 and diclofenac as substrate.  $[I]_{in, total}$  values and observed  $\delta$ AUC for amiodarone, benzbromarone, fluconazole (both clinical studies), fluvastatin, ketoconazole, miconazole, sertraline, sulphamethiazole, sulphaphenazole, sulphinpyrazone were retrieved directly from Ito et al., 2004.  $[I]_{in, total}$  values and observed  $\delta$ AUC for diclofenac, ibuprofen and tolbutamide were retrieved from Andersson et al., 2004.  $[I]_{max, total}$  values and observed  $\delta$ AUC for zafirlukast was retrieved from Blanchard et al., 2004.  $[I]_{unbound}$  values were calculated by multiplying the  $[I]_{total}$  by the  $F_{up}$  determined in this laboratory.  $[I]$  and  $K_i$  values together with the estimated  $f_{mCYP2C9}$  for the respective *in vivo* substrates were used in Equation 1 to simulate the  $\delta$ AUC.

							$\delta$ AUC		
<i>In vivo</i> Substrate	Inhibitor	CYP2C9 $K_i$ apparent ( $\mu$ M)	CYP2C9 $K_i$ unbound ( $\mu$ M)	$[I]_{in, total}$ ( $\mu$ M)	$F_{up}$	$[I]_{in, unbound}$ ( $\mu$ M)	Observed	Simulated using $[I]_{in, total} : K_{i, apparent}$	Simulated using $[I]_{in, unbound} : K_{i, unbound}$
Tolbutamide	Sulphaphenazole	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	Sulphinpyrazone	106	106	35	0.0068	0.235	1.7	1.3	1.0
Tolbutamide	Sulphamethiazole	130	130	233	0.0654	15.2	1.6	2.2	1.1
S-Warfarin	Zafirlukast	0.25	0.25	1 <sup>#</sup>	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*	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

\* $K_i$  determined using naproxen as substrate. <sup>#</sup> $[I]_{max}$

Figure 1a, 1b

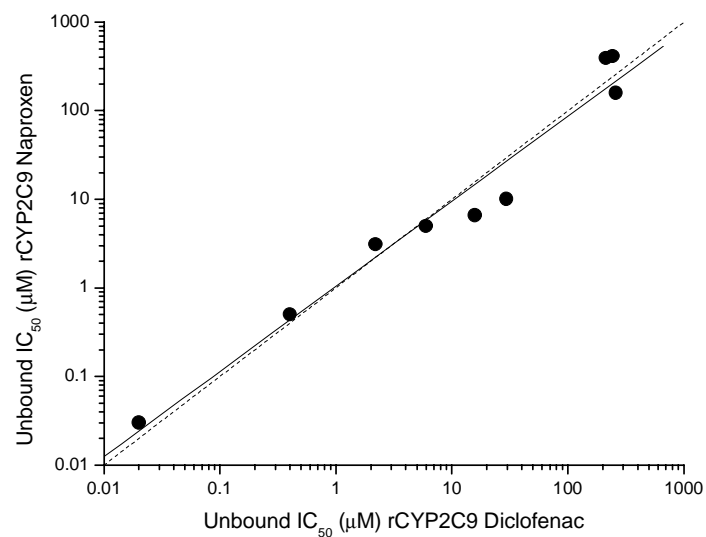
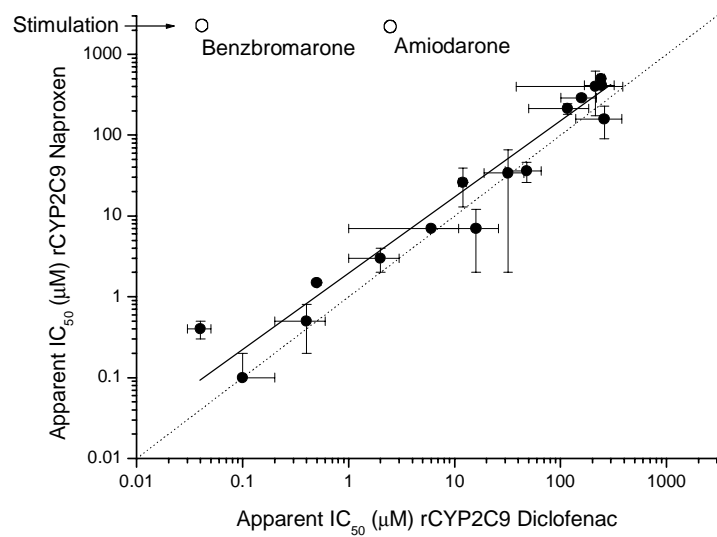


Figure 2a, 2b

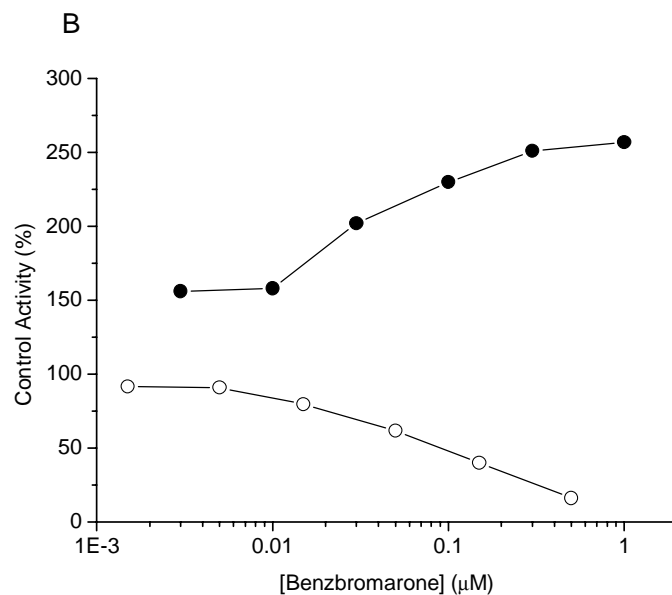
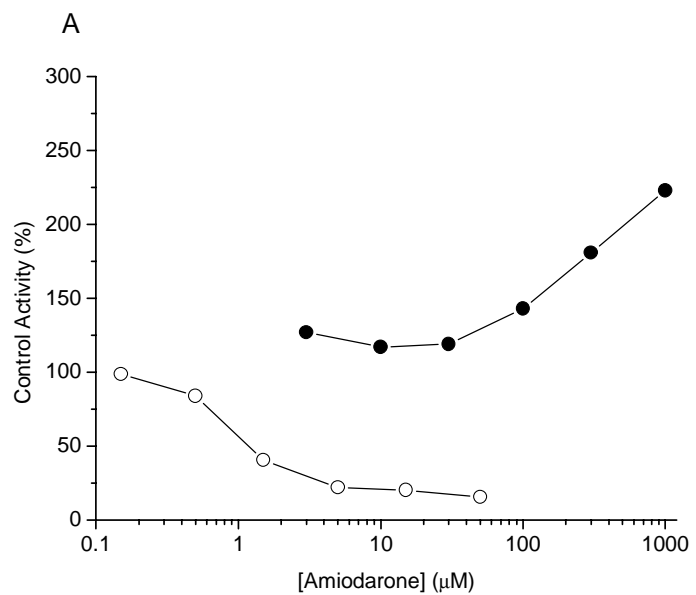


Figure 3

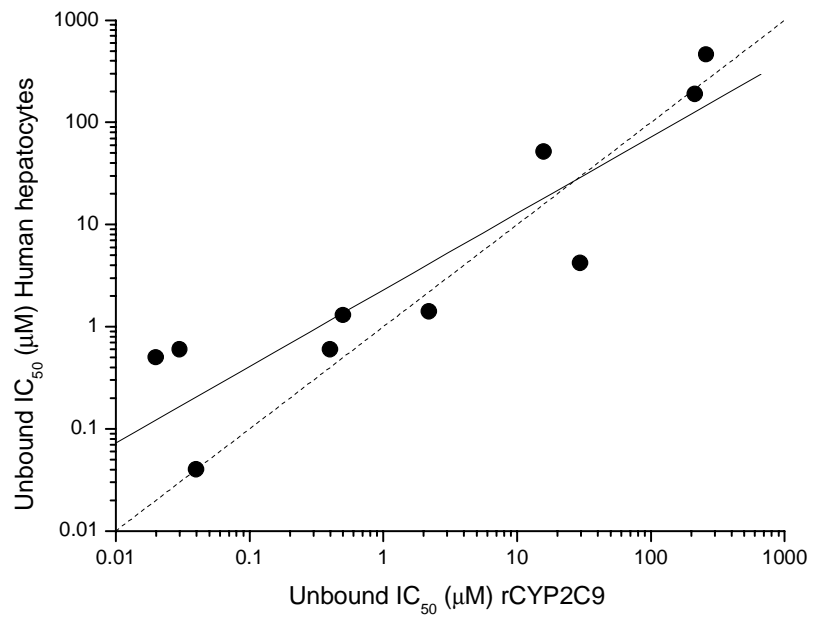


Figure 4

