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CYP2C9 Inhibition: Impact of Probe Selection and Pharmacogenetics on *In Vitro*

Inhibition Profiles

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Running Title:

CYP2C9 Inhibition: Impact of Probe Selection and Pharmacogenetics

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CYP – Cytochrome P450

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Abstract

Drug-drug interactions may cause serious adverse events in the clinical setting and the cytochromes P450 are the enzyme system most often implicated in these interactions. Cytochrome P450 2C is the second most abundant sub-family of P450 enzymes and is responsible for metabolism of almost twenty percent of currently marketed drugs. The most abundant isoform of this sub-family is CYP2C9, which is the major clearance pathway for the low therapeutic index drugs warfarin and phenytoin. Considering the importance of CYP2C9 to drug-drug interactions, the *in vitro-in vivo* extrapolation of drug-drug interactions for CYP2C9 may be confounded by the presence of polymorphic variants and the possibility of multiple binding regions within the CYP2C9 active site, leading to the potential for genotype and substrate-dependent inhibition. To address the issues of genotype dependent enzyme inhibition as well as probe substrate correlations, the inhibitory potency (K_i) of twenty-eight effector molecules was assessed with five commonly used probes of CYP2C9 in both the CYP2C9.1 and CYP2C9.3 proteins. The inhibition of CYP2C9.1 and CYP2C9.3 by the battery of inhibitors with five substrate probes demonstrated not only differential inhibition potency between the two genotypes but also across substrate probes. Furthermore, the substrate probes fell into three distinct classes depending on genotype suggesting that multiple probes may be needed to fully assess inhibition of CYP2C9 *in vitro*. Thus, both genotype and choice of probe substrate must be considered when attempting to predict potential CYP2C9 drug-drug interactions from *in vitro* data.

Drug-drug interactions (DDI) are one of the primary causes of serious adverse events occurring in clinical practice (Dambro and Kallgren, 1988). The most commonly observed DDIs result from inhibition of target-drug metabolism by a co-administered drug. The cytochrome P450 enzymes are the primary family of oxidative drug metabolizing enzymes and as such, are implicated in a substantial number of DDIs. Cytochrome P450 2C is the second most abundant sub family of P450 enzymes and is responsible for metabolism of almost twenty percent of the drugs currently available in the market (Rendic and Di Carlo, 1997). CYP2C9 is an important member of the subfamily, serving as the primary metabolic pathway of the narrow therapeutic index drugs warfarin and phenytoin as well as numerous other therapeutic entities (Rettie and Jones, 2005). Interactions with warfarin or phenytoin metabolism are of substantial clinical concern and can result in serious adverse events.

With the increased mechanistic knowledge of P450 enzyme function and the role of P450s in drug metabolism, a more systematic approach has been taken by investigators and the pharmaceutical industry for predicting drug-drug interactions. The Food and Drug Administration as well as the Pharmaceutical Research Manufacturer's Association has defined guidelines for preclinical *in vitro* and *in vivo* studies for the prediction of DDIs (Bjornsson et al., 2003); http://www.fda.gov/cder/guidance/clin3.pdf). Oftentimes, for *in vitro* experiments one probe substrate is used for a single P450 enzyme to determine the drug interaction potential of new chemical entity (NCE) during the discovery screening phase. These results are then extrapolated to interaction potential with other compounds metabolized by the same P450. Unfortunately, this approach is not always successful. One factor that may play a role in unsatisfactory extrapolations is the

phenomenon of multiple binding regions within the enzyme active site. This phenomenon of multiple binding regions has been described for most of the P450s, but most frequently with CYP3A4 (Korzekwa et al., 1998; Shou et al., 1999; Schrag and Wienkers, 2001). With this in mind, Kenworthy and colleagues studied the correlation of inhibitory potential of thirty four drugs with ten commonly used in vitro probe substrates for CYP3A4 (Kenworthy et al., 1999). Their findings suggested three groupings of probe substrates and thus possibly three binding regions within the active site of CYP3A4. The authors suggested that to correctly predict the inhibition potential of CYP3A4 inhibitors, researchers should use three separate probe substrates for CYP3A4. As with CYP3A4, evidence suggests the presence of more than one binding region in the CYP2C9 active site (Williams et al., 2003), including the presence of atypical kinetic profiles (Korzekwa et al., 1998), heteroactivation (Hutzler et al., 2001) and NMR data (Hummel et al., 2004). Therefore, the possibility exists that multiple probe substrates of CYP2C9 might be needed to predict potential DDIs with CYP2C9.

Further confounding the issue of predicting *in vivo* DDIs from *in vitro* data is the occurrence of polymorphic variant alleles of CYP2C9. Twenty four variant alleles of CYP2C9 have been reported to date, with all variants displaying reduced substrate turnover compared to wild type enzyme (Lee et al., 2002). The most clinically significant variant allele that occurs with substantial frequency is the *CYP2C9*3* allele that results in a protein with markedly reduced substrate turnover as compared to the wild type enzyme (Higashi et al., 2002). However, it is unknown if the same enzyme features of variant enzyme proteins that result in decreased catalysis of substrate also alters the inhibitory potential of competing compounds. To address this issue of genotype

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dependent enzyme inhibition as well as probe substrate correlations the inhibitory

potency (K_i) of twenty-eight effector molecules was assessed with five commonly used

probes of CYP2C9 using both the CYP2C9.1 and CYP2C9.3 proteins. Correlations of

inhibition of probe substrate activity were compared against each other within the same

enzyme as well as between the two enzyme variants.

Materials and Methods

Chemicals: Diclofenac, (*S*)-warfarin, phenytoin, tolbutamide, tenoxicam and *p*-hydroxy phenytoin were purchased from Sigma Aldrich (St. Louis, MO). The metabolites 4'-hydroxy diclofenac, 4-hydroxy tolbutamide, and 7-hydroxy warfarin were purchased from BD Gentest (Woburn, MA). (*S*)-flurbiprofen and 4'-hydroxyflurbiprofen were obtained from the Pfizer compound library. CYP2C9.1 and CYP2C9.3 Supersome® enzymes were purchased from BD Gentest (Woburn, MA). All other materials were purchased from commercial sources and were of the highest purity available.

Ki determination: The incubation times and protein concentrations used were within the linear range, with respect to time and protein, of each assay. Incubations were carried out using five common probe substrates of CYP2C9 [diclofenac, (*S*)-flurbiprofen, (*S*)-warfarin, phenytoin and tolbutamide]. Twenty-eight known inhibitors exhibiting a wide range of inhibition potencies were selected for study. Stock solutions of all the inhibitors were made in dimethylsulphoxide (DMSO) and then diluted 100 times with acetonitrile prior to addition to the incubation mixtures. Three concentrations of each substrate ($0.5K_m$, K_m, and $2K_m$) and four concentrations of each inhibitor (100 fold range) were used for determination of K_i in a 96 well plate format. Briefly, each reaction was carried out in duplicate and one pmol of CYP2C9.1 enzyme (2 pmol when phenytoin was the substrate) and 2 pmol of CYP2C9.3 (4 pmol in the case of phenytoin) was used per incubation. Each incubation reaction mixture contained enzyme, substrate and inhibitor suspended in phosphate buffer (50 mM, pH 7.4) and was preincubated for three minutes in an incubator-shaker at 37 °C. The reactions were initiated by the addition of NADPH

(1 mM final concentration). Organic solvent concentrations did not exceed 2% v/v. Solvent concentrations were the same for all experiments and turnover rates did not differ significantly from minimal solvent controls. The reaction was terminated with 50 μl of acetonitrile containing 1 μM of tenoxicam (internal standard) except for diclofenac in which 100 μl was used. Length of the incubations for diclofenac, (S)-flurbiprofen and tolbutamide was 10 min for CYP2C9.1 and 20 min for CYP2C9.3. For (S)-warfarin and phenytoin the incubations were carried out for 20 and 40 min for CYP2C9.1 and CYP2C9.3, respectively.

It is of note that to assure validity of the results and to allow comparison of inhibition profiles from different sets of experiments, a number of precautions were taken. To avoid batch to batch variability in enzyme, all samples of each variant were taken from the same batch provided by the manufacturer. The experiments were planned to minimize the amount of enzyme in each incubation to reduce the potential impact of non-specific binding of both substrate and inhibitor, and incubation times were limited to twenty minutes or less to avoid substrate or inhibitor depletion.

Liquid Chromatography/Tandem Mass Spectral Analysis. The LC/MS system consisted of an API 4000 triple quadropole mass spectrometer with an atmospheric pressure electrospray ionization source (MDS SCIEX, Concord, Ontario, Canada), and two LC-10ADvp pumps with a SCL-10ADvp controller (Shimadzu, Columbia, MD). A Thermo Electron Aquasil-C18 column (2.1 x 20 mm, 3.0 μm, Waltham, MA) was used for separation with initial conditions of 10% mobile phase B, followed by a gradient of 10% B to 90% B over 1 min (solvent A = 0.1% formic acid, solvent B = 100%

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acetonitrile) followed by an immediate return to initial conditions that were maintained for 1 min with a flow rate of 0.5 ml/min prior to the next injection.

The compounds were detected in negative ion mode. The deprotonated molecular ions were formed using an ion spray voltage of –3500 V, curtain gas of 10 V, collision gas of 8 eV, and source temperature of 600 °C for all compounds. Product ions were formed at collision energies of –16 eV (4'-hydroxydiclofenac, m/z 309.8→265.8), -12 eV (4-hydroxyflurbiprofen, m/z 258.9→214.9), -28 eV (7-hydroxywarfarin, m/z 322.9→176.6), –26 eV (4-hydroxytolbutamide, m/z 285.0→185.9), -18 eV (p-hydroxyphenytoin, m/z 266.8→224.0) and –14 eV (tenoxicam, m/z 335.9→271.8).

Data Analysis: The K_i of each inhibitor was calculated via non linear regression of the data to a competitive inhibition equation (Equation 1) using GraphPad Prism 4 (Graph pad software, San Diego, CA), except for amiodarone (all probe substrates and variants), (S)-ibuprofen [(S)-warfarin substrate probe, both variants], and quinine [(S)-flurbiprofen substrate probe, both variants], which were fit using a partial competitive inhibition equation (Equation 2). The goodness of the fit was determined by visual inspection of the data with the Dixon plot and r^2 values. Simple linear regression was used to determine the correlation between the K_i values of pairs of substrates using GraphPad Prism 4.

$$v = \frac{V_{max} \bullet [S]}{K_m (1 + \frac{[I]}{K_i}) + [S]} \qquad \text{Equation 1}$$

$$v = \frac{V_{max} \bullet [S]}{K_m \dfrac{(1 + \dfrac{[I]}{K_i})}{(1 + \dfrac{[I]}{\alpha K_i})} + [S]} \qquad \text{Equation 2}$$

Clustering Analysis: Statistical and clustering analysis of the inhibition potency data was performed using Spotfire DecisionSite 8.1 (Spotfire, Inc, Somerville, MA). An UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering algorithm was used to determine similarity between the inhibition data sets and form successively larger clusters using a Euclidean distance similarity measure. Data were entered as inhibition potency (K_i) values. Compounds that exhibited activation or K_i values above 100 µM were entered as a K_i of 100 µM. For instances where the modifier was also a substrate, a variant-specific average value of K_i for that modifier exhibited by the other four substrate probes was calculated and used.

Estimation of Potential In Vivo Inhibition: To estimate the potential *in vivo* effects of inhibition of CYP2C9.1 and CYP2C9.3 with each of the substrate probes, data were computed using an equation for competitive inhibition (Equation 1) to estimate velocities in the presence and absence of inhibitor. Values for K_m and V_{max} in the absence of inhibitor were determined experimentally for each substrate, the estimated K_i was used and literature derived values of C_{max} (total plasma concentrations) for inhibitor and substrate were inserted into the equation to determine the predicted velocity of the reaction.

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Results

For each of the substrate probes [*(S)*-flurbiprofen, *(S)*-warfarin, tolbutamide, phenytoin and diclofenac] inhibition profiles and the resulting inhibition constant (K_i) were determined with a set of 28 inhibitors in both the CYP2C9.1 and CYP2C9.3 enzymes (Tables 1 and 2, respectively). Potency of inhibition across the twenty-eight inhibitors spanned several orders of magnitude for each substrate. A number of interesting trends were noted upon examination of the data.

Comparing within only the CYP2C9.1 enzyme results using a recently proposed system of binning inhibition potency [$K_i < 1$ µM (high concern), K_i 1-10 µM (moderate concern), $K_i > 10$ µM (low concern)] (Obach et al., 2006), it is noted that 21 of the 28 inhibitors exhibited a substantially lower K_i value (increased inhibition potency) against *(S)*-warfarin hydroxylation than against any of the other probe substrates. In fact, the estimated K_i value was < 1 µM for sixteen of the twenty-eight inhibitors of *(S)*-warfarin hydroxylation. In contrast, only eight, six, nine and nine of the inhibitors exhibited K_i values < 1 µM against *(S)*-flurbiprofen hydroxylation, phenytoin hydroxylation, tolbutamide hydroxylation and diclofenac hydroxylation, respectively. An additional eight compounds exhibited K_i values between 1 and 10 µM toward (*S*)-warfarin hydroxylation resulting in 24 (of 28) compounds exhibiting K_i values less than 10 µM toward this reaction. For the other four probe substrates, the majority of inhibitors fell within this 1-10 µM K_i range. There was some switching of "bins" (i.e., $K_i < 1$ µM vs. K_i 1-10 µM vs. $K_i > 10$ µM) of inhibitors across the five substrates. Notably, Vivid Green® inhibited the metabolism of (*S*)-warfarin, phenytoin, tolbutamide and diclofenac relatively potently ($K_i = 0.5$-1.7 µM) but was a much weaker inhibitor of (*S*)-flurbiprofen

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metabolism (K_i = 8.2 µM). In contrast, quinine was a relatively potent inhibitor of (*S*)-flurbiprofen metabolism (K_i = 1.1µM) but was a very poor inhibitor of the metabolism of the other four probe substrates (K_i = 20 to > 100 µM). Indomethacin was a very potent (K_i = 0.7 µM) inhibitor of (*S*)-warfarin hydroxylation but a relatively weak (K_i > 10 µM) inhibitor of all other probe substrates. Finally, (*S*)-ibuprofen was a poor (K_i > 40 µM) inhibitor of (*S*)-warfarin hydroxylation but a relatively potent (K_i ~ 4 µM) inhibitor of the other four probe substrates. Closer analysis of Table 1 indicates that for a number of inhibitors, the K_i values varied 10-fold across substrates.

Another method used to determine whether K_i values assessed with CYP2C9 are substrate-dependent was to calculate the number of inhibitors that showed a greater than three-fold difference in inhibition potency when compared to a standard. Due to its prevalent use in industry as a CYP2C9 substrate probe, diclofenac was chosen as the standard. Eighteen inhibitors of (*S*)-warfarin hydroxylation exhibited greater than a three-fold difference in inhibition potency (K_i values) when compared to inhibition of diclofenac hydroxylation in CYP2C9.1. Except for amiodarone and quercetin, the inhibitory potency was always greater toward (*S*)-warfarin hydroxylation than diclofenac hydroxylation. Interestingly, for five compounds (mibefradil, indomethacin, benzbromarone, ketoconazole and dapsone) the K_i value was at least 10-fold lower toward (*S*)-warfarin hydroxylation as compared to diclofenac hydroxylation.

When comparing inhibition of (*S*)-flurbiprofen and diclofenac metabolism, twelve inhibitors of (*S*)-flurbiprofen hydroxylation exhibited greater than three-fold differences in K_i values when compared to diclofenac as a substrate probe with CYP2C9.1. In five of these cases (omeprazole, quinine, quercitin, Vivid Green® and dapsone) the difference

was at least ten-fold. In contrast to the results with *(S)*-warfarin, in four of the five cases (omeprazole, quercitin, Vivid Green® and dapsone), the K_i values were ten-fold lower toward diclofenac hydroxylation than *(S)*-flurbiprofen metabolism.

With respect to the inhibition of phenytoin hydroxylation by CYP2C9.1 as compared to inhibition of diclofenac hydroxylation, fewer differences were noted in K_i values. Only four inhibitors differed by more than three-fold between phenytoin and diclofenac hydroxylation inhibition (benzbromarone, nicardipine, omeprazole and piroxicam). Only one inhibitor differed in potency by ten-fold (nicardipine). In every case, the inhibition constant was lower (greater inhibition) with respect to diclofenac hydroxylation as compared to the inhibition of phenytoin. Finally, for CYP2C9.1, in only one instance did the inhibition of tolbutamide hydroxylation differ by more than three-fold as compared to the inhibition of diclofenac hydroxylation and this was with the compound piroxicam.

These same 28 inhibitors were analyzed for their ability to inhibit metabolism of the same five probe substrates in the CYP2C9.3 enzyme. Relatively few changes in inhibition potency were noted with respect to (*S*)-warfarin hydroxylation when compared to the K_i values obtained in CYP2C9.1 enzyme. Only two inhibitors differed by more than three-fold with respect to K_i (indomethacin and benzbromarone). The largest number of compounds whose K_i estimates differed by more than three-fold between the two enzymes occurred with the substrate probe flurbiprofen. For sixteen inhibitors, the K_i values differed by more than three-fold between the CYP2C9.1 and CYP2C9.3 enzymes. For three compounds (benzbromarone, piroxicam and dapsone) the inhibition constant varied more than 40-fold. Even more intriguing is that in the cases of quercitin

and Vivid Green® greater inhibition was noted in the CYP2C9.3 enzyme. With respect to inhibition of phenytoin hydroxylation, only two compounds (benzbromarone and nicardipine) exhibited K_i values that differed between the variants, but the differences were substantial (benzbromarone was ~10-fold less potent and nicardipine was ~10-fold more potent). For inhibition of tolbutamide hydroxylation, only three compounds (diclofenac, (*S*)-ibuprofen and sulfamethizole) were less potent inhibitors (~three-fold less potent) in the CYP2C9.3 enzyme. Finally, five compounds differed in inhibition potency more than three-fold with respect to their inhibition of diclofenac hydroxylation (benzbromarone, tolbutamide, (*S*)-warfarin, (*S*)-ibuprofen and gemfibrozil) between the CYP2C9 variants and in all cases became less potent inhibitors in CYP2C9.3.

Log transformed inhibition data was analyzed for correlation between the substrate probes for CYP2C9.1 (Table 3 and Figure 1), between the substrate probes for CYP2C9.3 (Table 4 and Figure 2), and for each substrate probe comparing inhibition profiles between the CYP2C9.1 and CYP2C9.3 variants (Table 5). The inhibition profiles of diclofenac, phenytoin, and tolbutamide were highly correlated for CYP2C9.1 ($r^2 > 0.92$), while *(S)*-flurbiprofen and *(S)*-warfarin showed lower correlations with each other ($r^2 = 0.69$) and with the three aforementioned probes ($r^2 < 0.76$). The inhibition profiles of diclofenac, phenytoin, and tolbutamide were also highly correlated for CYP2C9.3 ($r^2 > 0.88$). The probes *(S)*-flurbiprofen and *(S)*-warfarin showed lower correlations with each other ($r^2 = 0.69$) and with diclofenac, phenytoin, and tolbutamide ($r^2 \cong 0.69 - 0.84$). When comparing differences in inhibition profiles for each probe between the CYP2C9.1 and CYP2C9.3 variants, diclofenac, tolbutamide, and (*S*)-

warfarin were highly correlated ($r^2 > 0.88$), phenytoin exhibited a lower correlation ($r^2 = 0.73$), and (S)-flurbiprofen exhibited the lowest correlation ($r^2 = 0.55$).

Hierarchical clustering analysis was performed on the non-transformed inhibition potency data using an UPGMA clustering algorithm and a Euclidean distance similarity measure. Results from the clustering analysis for the CYP2C9.1 and CYP2C9.3 data were visualized as a dendrogram (Figures 3 and 4, respectively) where the horizontal axis of the dendrogram represents the Euclidean linkage distance between substrate clusters. For the panel of modifiers, the vertical axis of the dendrogram represents the Euclidean linkage distance between effector clusters. The clustering analysis for CYP2C9.1 probe substrate inhibition data suggested three distinct groupings of probe substrate similarities: diclofenac, tolbutamide and phenytoin (Euclidean linkage distance 13.5); (S)-warfarin (Euclidean linkage distance 27.2); and (S)-flurbiprofen (Euclidean linkage distance 76.6). These results correlated well with the linear regression analysis of probe substrate correlation mentioned above. However, the clustering analysis for the CYP2C9.3 inhibition data for probe substrate similarity differed from the groupings obtained by linear regression analysis of inhibition data (and from that observed with CYP2C9.1), although three distinct groupings were still obtained: (S)-warfarin and (S)-phenytoin (Euclidean linkage distance 42.9), diclofenac and tolbutamide (Euclidean linkage distance 51.7), and (S)-flurbiprofen (Euclidean linkage distance 79.1). Inhibition potency results were also visualized as a heat map (Figures 3 and 4). Heat map coloration indicates relative closeness of inhibition potency data to maximal (< 1 µM, red), moderate (1-10 µM, yellow) or minimal (> 10 µM, green) inhibition. Again, confirming

the K_i rankings discussed previously, compounds exhibited lower K_i values toward (*S*)-

warfarin as a probe substrate as compared to the other probes, in general.

The potential *in vivo* significance of differential inhibition of probe substrate

metabolism in the CYP2C9.1 and CYP2C9.3 variants is depicted in Table 6. It can be

noted that the estimated percent remaining activity, in general, was greater in the

CYP2C9.3 enzyme suggesting a reduced inhibition of metabolism in the CYP2C9.3

genotype. However, in some cases such as with nicardipine inhibition of phenytoin

metabolism, a substantially greater inhibition is predicted to occur with the CYP2C9.3

genotype. Not unexpectedly, these differences were both substrate and inhibitor

independent.

Discussion

Prediction of *in vivo* enzyme inhibition (and potential drug-drug interactions) from *in vitro* data remains an important area of investigation among drug metabolism researchers. Though several studies have been conducted to assess correlations of *in vitro* predictions of drug-drug inhibition interactions and their potential extrapolation to *in vivo* data, none has assessed the potential impact of genetic variants on these predictions. Furthermore, correlation analysis of inhibition potency for multiple substrate probes had previously been assessed in the CYP3A4 enzyme (Kenworthy et al., 1999), but not for CYP2C9. The P450 isoform CYP2C9 shares many characteristics with CYP3A4, such as substrate-dependent atypical kinetics profiles, heteroactivation, and large active site volume that all suggest the possibility for multiple binding regions within the same active site. Additionally, CYP2C9 is a polymorphically expressed enzyme and the CYP2C9.3 variant, in particular, exhibits substantially reduced substrate turnover, which may further confound predictions of drug-drug interaction potential.

For the CYP2C9.1 enzyme, both correlation analysis and Euclidean linkage analysis indicated that the substrate probes diclofenac, phenytoin and tolbutamide were highly correlated suggesting that they could be used interchangeably as substrate probes for CYP2C9.1. However, the substrate probes (*S*)-flurbiprofen and (*S*)-warfarin were not correlated with each other, or with the other three probes suggesting that they may represent different binding regions (or orientations) within the active site and exhibit different susceptibilities to inhibition. Together, these data suggest multiple binding modes or regions in the CYP2C9.1 active site. Thus, it appears that as with CYP3A4 (Kenworthy et al., 1999), the use of multiple probes might be required to accurately

assess the potential for drug-drug inhibition interactions. One combination of probes that could be considered would be to use the commonly employed probe diclofenac in combination with (*S*)-warfarin as a probe for inhibition studies. This would allow obtainment of information regarding the two CYP2C9 substrates (phenytoin and (S)-warfarin) that possess the lowest therapeutic index and thus are most worrisome in terms of potential drug-drug interactions. If one were to choose a single probe, as has more recently been suggested with CYP3A4 (Galetin et al., 2005), then (*S*)-warfarin would seem the most logical choice since the K_i values of the inhibitors toward this substrate are the lowest and the drug is commonly prescribed and requires close monitoring. One must however be cognizant that (*S*)-warfarin, is turned over very slowly and thus excess consumption of inhibitor must be monitored, potential for shunting to other metabolites is possible in human liver microsomal preparations and non-specific binding of inhibitor to the microsomes must be checked since higher protein concentrations may be required, due again to the slow turnover.

Interestingly, for the CYP2C9.3 variant, correlation analysis suggested the same grouping of probe substrates as observed with CYP2C9.1, but Euclidean linkage analysis suggested that (*S*)-warfarin and phenytoin were correlated, tolbutamide and diclofenac were correlated and that *(S)*-flurbiprofen was not correlated with any of the other substrate probes. Thus, the difference as compared to CYP2C9.1 is that phenytoin now tracks with *(S)*-warfarin in the CYP2C9.3 enzyme. The lack of flexibility of the probes (*S*)-warfarin and phenytoin may make them more sensitive to active site alterations than the more flexible probes. Despite the relatively conservative change in the CYP2C9.3

enzyme (I359L), some effect on binding orientation is potentially occurring in addition to substantially reduced metabolic rate.

With respect to the inhibitors, hierarchical clustering resulted in similar groupings between the CYP2C9.1 and CYP2C9.3 variants based upon variant-specific average values for each modifier. Eight of the ten most potent inhibitors were shared between the variants. For CYP2C9.1, four inhibitors exhibited Euclidean linkage distances furthest from the rest of the cluster (indomethacin, dapsone, thiobendazole, and quinine, Euclidean linkage distance > 48). Seven inhibitors for the CYP2C9.3 variant (phenytoin, dapsone, indomethacin, flurbiprofen, sulphamethizole, thiobendazole, and quinine, Euclidean linkage distance > 41) exhibited Euclidean linkage distances that were furthest from the rest of the cluster. Both the least potent and most potent inhibitors were shared by CYP2C9.1 and CYP2C9.3.

Recently proposed guidelines indicate that compounds with K_i values < 1 µM are of high concern for drug-drug interactions, compound with K_i values between 1 and 10 µM are of moderate concern, and compounds with K_i values greater than 10 µM tend to be of lesser concern (Obach et al., 2006). Using the substrate diclofenac as the comparator (as it is the most commonly used CYP2C9 substrate probe), when the inhibition of each compound was compared for the other substrates, inhibitors of *(S)*-warfarin metabolism showed the greatest number of changes in classification in both CYP2C9.1 and CYP2C9.3 enzymes (i.e., were now classified as being in a category of greater concern). With *(S)*-warfarin as substrate probe, twelve inhibitors of CYP2C9.1 and ten inhibitors of CYP2C9.3 became classified as being of more concern and eight of the twelve in CYP2C9.1 and four of the inhibitors of CYP2C9.3 moved from the

moderate to the high concern classification, as compared to when diclofenac was used as the probe. Again, comparing to results with diclofenac, changes in classification of inhibitors were mixed for the other three probe substrates (phenytoin, tolbutamide and *(S)*-flurbiprofen) as some inhibitors became classified as being of more concern and some becoming of less concern depending on the probe substrate. Thus, only with the probe *(S)*-warfarin were the inhibitors almost universally classified as being of greater concern. With the other probe substrates, it was more difficult to predict the relative inhibitory potency of an inhibitor when comparing to diclofenac.

The inhibition profiles for *(S)*-flurbiprofen and *(S)*-warfarin differ from the other substrate probes in several ways. Inhibition of *(S)*-flurbiprofen by the studied compounds exhibits substantial variability in the degree to which it is inhibited by the studied compounds when compared across genotypes, particularly when compared to a standard such as diclofenac. For the inhibition of (*S*)-flurbiprofen, the inhibition potency of a compound may be significantly increased or reduced depending upon the particular substrate-inhibitor combination when compared between the two CYP2C9 variants. This is further exemplified by the low correlation of the inhibition profile of *(S)*-flurbiprofen between the two CYP2C9 variants. The active site residues Arg-108 and Phe-114 have been reported to be important in the binding, orientation, and metabolism of NSAIDS such as flurbiprofen and diclofenac in the CYP2C9 active site, forming an anionic binding site and a *pi*-stacking region, respectively (Davies et al., 2004; Dickmann et al., 2004; Wester et al., 2004). While the recently published crystal structure of CYP2C9 with *(S)*-flurbiprofen bound as a ligand indicates interaction with the Arg-108 and Phe-114 residues (Wester et al., 2004), the rigid biphenyl structural core of *(S)*-flurbiprofen

may not allow orientation to the same binding modes or full access to a hydrophobic pocket that are exhibited by less conformationally restrained probes such as diclofenac and tolbutamide. Thus, use of (S)-flurbiprofen as a substrate probe may allow analysis of a different set of interactions than would be assessed using the probes that correlate well together such as phenytoin, tolbutamide and diclofenac.

The substrate probe *(S)*-warfarin also exhibits a unique inhibition profile. Not only is the inhibition profile different from any of the other probes, but the potency of inhibitors is generally increased when *(S)*-warfarin is used as a substrate probe. It should be noted that *(S)*-warfarin may exist in several distinct tautomer conformations in solution (Heimark and Trager, 1984; He et al., 1999). While previous studies indicate that the ring-closed form of *(S)*-warfarin is the preferred conformation for metabolism, perhaps other tautomers contribute to non-productive binding of *(S)*-warfarin. Differential or allosteric binding modes and non-productive binding may in part explain the differences in inhibition potency and profile observed with *(S)*-warfarin (Seifert et al., 2006).

In summary, inhibition of CYP2C9.1 and CYP2C9.3 by a battery of inhibitors with five substrate probes demonstrated not only differential inhibition potency between the two genotypes but also across substrate probes. Furthermore, the substrate probes fell into three distinct classes depending on genotype suggesting that multiple probes may be needed to fully assess inhibition of CYP2C9 *in vitro*. A combination of inhibition studies using diclofenac and (S)-warfarin may provide the broadest "chemical space" with respect to CYP2C9 inhibition but use of (S)-warfarin as a single probe may also provide acceptable results, with recognition of the aforementioned potential caveats.

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Footnotes

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Figure legends

Figure 1

CYP2C9.1 Linear Correlation Graphs of the Log Inhibition Data: A) Diclofenac vs (*S*)-Flurbiprofen, B) Phenytoin vs (*S*)-Flurbiprofen, C) Tolbutamide vs (*S*)-Flurbiprofen, D) (*S*)-Warfarin vs (*S*)-Flurbiprofen, E) Diclofenac vs Phenytoin, F) Tolbutamide vs Phenytoin, G) Diclofenac vs Tolbutamide H) Diclofenac vs (*S*)-Warfarin, I) Tolbutamide vs (*S*)-Warfarin, J) Phenytoin vs (*S*)-Warfarin

Figure 2

CYP2C9.3 Linear Correlation Graphs of the Log Inhibition Data: A) Diclofenac vs (*S*)-Flurbiprofen, B) Phenytoin vs (*S*)-Flurbiprofen, C) Tolbutamide vs (*S*)-Flurbiprofen, D) (*S*)-Warfarin vs (*S*)-Flurbiprofen, E) Diclofenac vs Phenytoin, F) Tolbutamide vs Phenytoin, G) Diclofenac vs Tolbutamide H) Diclofenac vs (*S*)-Warfarin, I) Tolbutamide vs (*S*)-Warfarin, J) Phenytoin vs (*S*)-Warfarin

Figure 3

CYP2C9.1 Inhibitor Hierarchical Clustering Dendrogram and Heat Map [(red < 1 µM); (1 µM < yellow < 10 µM); (green > 10 µM)]

Figure 4

CYP2C9.3 Inhibitor Hierarchical Clustering Dendrogram and Heat Map [(red < 1 µM); (1 µM < yellow < 10 µM); (green > 10 µM)]

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Table 1 K_i values (μM) obtained in five probes substrates i.e. (*S*)-flurbiprofen, (*S*)-warfarin, phenytoin, tolbutamide and diclofenac using CYP2C9.1[a]

Inhibitors	Flurbiprofen	Warfarin	Phenytoin	Tolbutamide	Diclofenac
Mibefradil	11.10	1.04	6.75	6.63	13.79
Indomethacin	53.41	0.66	15.76	14.24	14.47
Benzbromarone	0.004	0.001	0.04	0.02	0.01
Sulphaphenazole	0.06	0.12	0.23	0.22	0.15
Nicardipine	0.07	0.01	0.33	0.03	0.03
(*S*)-Ibuprofen	4.27	3.06	4.02	3.95	4.46
Amiodarone	2.10	2.99	4.03	0.69	1.89
Sulphamethizole	17.90	2.22	13.08	7.17	14.69
Omeprazole	5.33	0.64	2.16	0.92	0.41
Clozapine	4.13	3.46	12.88	11.00	11.43
Gemfibrozil	12.43	0.79	2.38	2.83	3.64
Ketoconazole	0.38	0.08	1.81	1.79	1.52
Progesterone	1.72	1.41	4.30	4.01	5.20
Nifedipine	1.14	0.34	1.35	0.84	0.57
Thiobendazole	>100	17.22	36.21	33.09	41.08
Quinine	3.45	19.80	85.53	76.35	>100
Quercitin	1.18	0.25	0.27	0.14	0.13
Fluvoxamine	0.63	0.58	2.46	2.74	4.12
Tamoxifen	3.44	0.66	3.77	3.32	4.67
Miconazole	0.03	0.01	0.11	0.05	0.04
α–Napthoflavone	0.78	0.29	0.75	0.34	0.41
Diclofenac	1.60	1.24	1.64	0.75	
Flurbiprofen		3.27	16.38	19.86	11.35
Warfarin	4.17		4.35	2.13	3.52
Phenytoin	5.58	4.04		6.65	9.02
Tolbutamide	1.54	1.00	4.70		3.41
Piroxicam	0.47	0.92	18.27	15.18	4.02
Vivid Green	8.21	0.53	1.72	1.05	0.84
Dapsone	Activator	0.09	7.10	9.49	5.29

[a]Global standard error for data fitting was less than 15% and $r^2 > 0.9$ for each effector

Table 2 K_i values (μM) obtained in five probes substrates i.e (*S*)-flurbiprofen, (*S*)-warfarin, phenytoin, tolbutamide and diclofenac using CYP2C9.3[a]

Inhibitors	Flurbiprofen	Warfarin	Phenytoin	Tolbutamide	Diclofenac
Mibefradil	13.08	1.80	6.90	6.90	14.30
Indomethacin	38.74	5.04	12.66	16.87	25.62
Benzbromarone	1.54	0.01	0.71	0.03	0.04
Sulphaphenazole	0.72	0.15	0.39	0.33	0.36
Nicardipine	0.29	0.01	0.02	0.02	0.02
(*S*)-Ibuprofen	9.78	3.15	5.50	14.03	14.68
Amiodarone	3.64	1.66	1.87	0.81	2.10
Sulphamethizole	35.04	3.87	24.74	25.93	31.86
Omeprazole	16.24	0.90	1.60	1.46	0.30
Clozapine	10.49	2.80	5.07	5.92	8.04
Gemfibrozil	14.33	1.65	4.95	7.21	14.41
Ketoconazole	1.51	0.08	0.69	0.70	0.79
Progesterone	11.03	1.97	3.27	2.58	7.91
Nifedipine	1.53	0.28	0.61	0.28	1.18
Thiobendazole	>100	11.02	26.92	24.54	69.85
Quinine	21.2	18.89	39.68	86.52	96.70
Quercitin	0.25	0.11	0.29	0.08	0.13
Fluvoxamine	4.19	0.67	2.37	1.57	2.53
Tamoxifen	9.77	0.58	6.69	2.72	8.36
Miconazole	0.10	0.02	0.12	0.06	0.07
α–Napthoflavone	1.13	0.18	0.39	0.24	0.44
Diclofenac	5.34	1.16	3.32	2.58	
Flurbiprofen		5.27	23.15	33.49	23.66
Warfarin	2.70		11.12	5.89	12.21
Phenytoin	24.20	5.93		6.99	16.53
Tolbutamide	12.91	2.30	5.51		9.54
Piroxicam	19.38	1.27	8.67	7.40	6.05
Vivid Green	1.59	0.44	0.70	2.53	0.84
Dapsone	>100	0.26	10.47	12.76	15.62

[a]Global standard error for data fitting was less than 15% and $r^2 > 0.9$ for each effector

Table 3 r^2 values obtained from the linear regression of the K_i values for two probes using CYP2C9.1

	Diclofenac	Flurbiprofen	Warfarin	Phenytoin	Tolbutamide
Substrate					
Diclofenac	1				
Flurbiprofen	0.66	1			
Warfarin	0.76	0.69	1		
Phenytoin	0.92	0.62	0.73	1	
Tolbutamide	0.95	0.61	0.68	0.92	1

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Table 4 r^2 values obtained from the linear regression of the K_i values for two probes

using CYP2C9.3

	Diclofenac	Flurbiprofen	Warfarin	Phenytoin	Tolbutamide
Substrate					
Diclofenac	1				
Flurbiprofen	0.74	1			
Warfarin	0.84	0.69	1		
Phenytoin	0.88	0.80	0.78	1	
Tolbutamide	0.92	0.77	0.83	0.88	1

Table 5 r^2 values obtained from the linear regression of the K_i values for probes using CYP2C9.1 and CYP2C9.3

Substrate	r^2
Diclofenac	0.94
Flurbiprofen	0.55
Warfarin	0.88
Phenytoin	0.73
Tolbutamide	0.90

Table 6 Potential *in vivo* significance of differential inhibition of probe substrate

metabolism in CYP2C9.1 and CYP2C9.3 variants

		Ratio of Predicted In Vivo % Activity Remaining for CYP2C9.1/ Predicted In Vivo % Activity Remaining CYP2C9.3[a]				
Inhibitors	**Estimated In Vivo C_{max} (μM)[b]**	**Flurbiprofen**	**Warfarin**	**Phenytoin**	**Tolbutamide**	**Diclofenac**
Mibefradil	1.0	1.0*	0.8	1.0*	1.0*	1.0*
Indomethacin	15.4	1.1*	0.2	1.2	1.1	1.0*
Benzbromarone	2.9	0.0	0.1	0.1	0.7	0.4
Sulphaphenazole	169	0.1	0.8	0.8	0.9	0.7
Nicardipine	0.49	0.4	1.2	12.4	2.2	1.9
(s)-Ibuprofen	172	0.5	1.0	1.0	0.4	0.5
Amiodarone	5.4	1.0*	5.4	2.0	1.1	1.4
Sulphamethizole	74.0	0.7	0.6	0.8	0.5	0.8
Omeprazole	3.0	0.9*	0.8	1.4	0.9	1.8
Clozapine	1.4	0.9*	1.1*	1.1*	1.1*	1.0*
Gemfibrozil	118	1.0	0.5	0.7	0.6	0.5
Ketoconazole	7.0	0.4	1.1	2.8	2.7	2.2
Progesterone	0.02	1.0*	1.0*	1.0*	1.0*	1.0*
Nifedipine	0.39	1.0*	1.1	1.3	1.6	1.0*
Thiobendazole	N/A	N/A	N/A	N/A	N/A	N/A
Quinine	13.3	0.2	1.0	1.1*	1.0*	1.0*
Quercitin	4.5	4.2	2.3	1.3	2.2	1.5
Fluvoxamine	0.15	0.9*	1.0*	1.0*	1.0*	1.0*
Tamoxifen	0.54	1.0*	1.1	1.0*	1.1*	1.0*
Miconazole	14.9	0.3	0.7	1.4	1.2	0.8
α–Napthoflavone	N/A	N/A	N/A	N/A	N/A	N/A
Diclofenac	6.3	0.6	1.1	0.8	0.5	---
Flurbiprofen	17.2	---	0.7	1.0	0.9	0.9
Warfarin	1.8	1.2*	---	0.9*	0.8	0.9*
Phenytoin	14.4	0.6	0.8	---	1.2	1.0
Tolbutamide	189	0.2	0.4	1.2	---	0.6
Piroxicam	7.5	0.1	0.8	1.3	1.4	1.0
Vivid Green	N/A	N/A	N/A	N/A	N/A	N/A
Dapsone	10.0	Activator	0.4	1.0	1.0	0.8

[a] Values less than 1.0 indicate greater inhibition of CYP2C9.1 mediated metabolism of the probe substrate. Values greater than 1.0 indicate greater inhibition of CYP2C9.3 mediated metabolism of the probe substrate. In both cases, the farther the deviation is from 1.0, the greater the difference in inhibition of the two isoforms. The ratio values represent the worst case scenario, individuals homozygous for CYP2C9.3.

[b] Values are the estimated C_{max} for each inhibitor and were obtained from the literature. Individuals who are homozygous or heterozygous for CYP2C9*3 would likely exhibit higher C_{max} values.

[c] Not applicable. Literature values for C_{max} were not identified.

* Values marked with an asterisk indicate that the percent inhibition predicted would be less than 30% for both isoforms and thus, not likely to be clinically relevant.

Figure #1

Figure #2

Figure 3

	(S)-Warfarin	Tolbutamide	Phenytoin	Diclofenac	(S)-Flurbiprofen
Benzbromarone					
Miconazole					
Nicardipine					
Sulphaphenazole					
Quercitin					
Alpha-napthoflavone					
Nifedipine					
Ketoconazole					
Diclofenac					
Fluvoxamine					
Tolbutamide					
Progesterone					
Tamoxifen					
Amiodarone					
(S)-Warfarin					
(S)-Ibuprofen					
Omeprazole					
Vivid Green					
Gemfibrozil					
Piroxicam					
Phenytoin					
Mifebradil					
Clozapine					
Sulphamethizole					
(S)-Flurbiprofen					
Indomethacin					
Dapsone					
Thiobendazole					
Quinine					

Figure 4

Rows (top to bottom):
Nicardipine
Miconazole
Quercitin
Sulphaphenazole
Alpha-napthoflavone
Benzbromarone
Ketoconazole
Nifedipine
Vivid Green
Amiodarone
Fluvoxamine
Diclofenac
Omeprazole
(S)-Warfarin
Progesterone
Tamoxifen
Clozapine
Tolbutamide
Gemfibrozil
Mifebradil
Piroxicam
(S)-Ibuprofen
Phenytoin
Dapsone
Indomethacin
(S)-Flurbiprofen
Sulphamethizole
Thiobendazole
Quinine

Columns: (S)-Warfarin, Phenytoin, Tolbutamide, Diclofenac, (S)-Flurbiprofen