Evaluation of CYP2C8 Inhibition In Vitro: Utility of Montelukast as a Selective CYP2C8 Probe Substrate

Brooke M. VandenBrink, Robert S. Foti, Dan A. Rock, Larry C. Wienkers, and Jan L. Wahlstrom

Pharmacokinetics and Drug Metabolism, Amgen, Inc., Seattle, Washington

Received February 22, 2011; accepted June 22, 2011

ABSTRACT:
Understanding the potential for cytochrome P450 (P450)-mediated drug-drug interactions is a critical step in the drug discovery process. Although in vitro studies with CYP3A4, CYP2C9, and CYP2C19 have suggested the presence of multiple binding regions within the P450 active site based on probe substrate-dependent inhibition profiles, similar studies have not been performed with CYP2C8. The ability to understand CYP2C8 probe substrate sensitivity will enable appropriate in vitro and in vivo probe selection. To characterize the potential for probe substrate-dependent inhibition with CYP2C8, the inhibition potency of 22 known inhibitors of CYP2C8 were measured in vitro using four clinically relevant CYP2C8 probe substrates (montelukast, paclitaxel, repaglinide, and rosiglitazone) and amodiaquine. Repaglinide exhibited the highest sensitivity to inhibition in vitro. In vitro phenotyping indicated that montelukast is an appropriate probe for CYP2C8 inhibition studies. The in vivo sensitivities of the CYP2C8 probe substrates cerivastatin, fluvastatin, montelukast, pioglitazone, and rosiglitazone were determined in relation to repaglinide on the basis of clinical drug-drug interaction (DDI) data. Repaglinide exhibited the highest sensitivity in vivo, followed by cerivastatin, montelukast, and pioglitazone. Finally, the magnitude of in vivo CYP2C8 DDI caused by gemfibrozil-1-O-β-glucuronide was predicted. Comparisons of the predictions with clinical data coupled with the potential liabilities of other CYP2C8 probes suggest that montelukast is an appropriate CYP2C8 probe substrate to use for the in vivo situation.

Introduction
The cytochrome P450 (P450) superfamily of drug-metabolizing enzymes is involved in the metabolism of a majority of currently prescribed drugs and new chemical entities (Wienkers and Heath, 2005). Within the P450 superfamily, CYP2C8 is responsible for 5 to 8% of P450-mediated metabolism and is involved in the metabolism of more than 60 drugs, including antimalarial drugs (amodiaquine), antidiabetes agents (pioglitazone, repaglinide, and rosiglitazone), statins (cerivastatin and fluvastatin), and anticancer agents (paclitaxel) (Lai et al., 2009). Because of the general importance of CYP2C8 in drug clearance, assessment of probe substrate dependence on CYP2C8 inhibition is a key part of the drug discovery and development process (Wahlstrom et al., 2006). The overall goal of studying CYP2C8 probe substrate-dependent inhibition is to gain a comprehensive understanding of in vitro and in vivo sensitivity, which will enable appropriate probe substrate selection.

The in vitro marker reactions recommended by the U.S. Food and Drug Administration (FDA) guidance for measuring CYP2C8 inhibition include paclitaxel 6α-hydroxylation (preferred), as well as amodiaquine N-deethylation and rosiglitazone para-hydroxylation (acceptable). Repaglinide and rosiglitazone are FDA-recommended in vivo probe substrates for studying CYP2C8 inhibition (Huang et al., 2007). To our knowledge, no studies have compared the inhibition profiles of these probe substrates in vitro. Our first aim was to measure the in vitro inhibition profiles of three clinically relevant CYP2C8 probe substrates (paclitaxel, repaglinide, and rosiglitazone) and amodiaquine (Fig. 1) versus a panel of 22 known CYP2C8 inhibitors to determine the relative in vitro sensitivities of each probe substrate. In addition, the in vitro inactivation profiles for each probe substrate versus gemfibrozil 1-O-β-glucuronide, a known mechanism-based inactivator of CYP2C8 (Ogilvie et al., 2006), were determined to evaluate the relative in vitro sensitivities toward irreversible inhibition.

Montelukast, a leukotriene receptor antagonist, is well characterized as a potent and selective inhibitor of CYP2C8 in vitro (Walsky et al., 2005). In vitro phenotyping studies (Chiba et al., 1997), based on high montelukast concentrations (100–500 μM), indicated that montelukast 21-hydroxylation (M5 formation) is catalyzed by CYP3A and montelukast 36-hydroxylation (M6 formation) by CYP2C9. In vivo, montelukast undergoes oxidative metabolism with the majority of the metabolites excreted in bile and less than 1% eliminated in the urine (Balani et al., 1997). After dosing of montelukast, the maximum plasma concentration is usually less than 1 μM (Karonen et al., 2010). The discrepancy in concentrations between the in vitro phenotyping experiments and the in vivo situation raises the possibility that other P450 isoforms could be involved. In addition, an in vivo study using gemfibrozil, whose glucuronide metabolite is a mechanism-based inactivator of CYP2C8, indicated marked inhibition of montelukast in vivo.
vivo and suggested a previously unknown role for CYP2C8 in montelukast metabolism (Karonen et al., 2010). More recent data indicate that CYP2C8 plays a role in the in vitro metabolism of montelukast at clinically relevant concentrations (Filippula et al., 2011). Our second aim was to further evaluate the contribution of CYP2C8 in the metabolism of montelukast in vitro. Given that montelukast demonstrated CYP2C8 selectivity at clinically relevant concentrations, we also characterized montelukast for its potential as an in vitro probe substrate versus the panel of 22 known CYP2C8 inhibitors.

The ability to predict changes in the exposure levels of a given drug in the presence of an inhibitor for the in vivo situation is useful (Rostami-Hodjegan and Tucker, 2007). The magnitude of a DDI depends on the fraction metabolized (fm) of a probe substrate for the inhibited pathway. However, to our knowledge, no studies have compared the in vivo sensitivity of CYP2C8 probe substrates on the basis of DDI data from the literature, as has been demonstrated for CYP3A4 (Ragueneau-Majlessi et al., 2007; Foti et al., 2010). Our final aim was to mine the literature for clinical DDI data and to correlate the DDI sensitivity of six probe substrates (cerivastatin, paclitaxel, repaglinide, and rosiglitazone) with known contribution to clearance from CYP2C8.

Materials and Methods

Materials. Pooled human liver microsomes (HLMs, 15 individual donors) and individual HLMs were purchased from CellzDirect (Durham, NC) or Xenotech, LLC (Lenexa, KS). Recombinant human P450 isoforms (SuperSomes) containing cytochrome b5 protein (except for CYP1A2 and CYP2D6) and recombinant flavin-containing monoxygenase isoforms were purchased from BD Biosciences (San Diego, CA). CYP2C9 monoclonal inhibitory antibody was purchased from Xenotech, LLC, and all other P450 selective monoclonal inhibitory antibodies (with ascites fluid) were purchased from BD Biosciences. Ammonium formate, high-performance liquid chromatography-grade methanol were obtained from Alfa Aesar (Ward Hill, MA). NADPH was purchased from EMD Biosciences (San Diego, CA). Montelukast and rosiglitazone were purchased from Cayman Chemical (Ann Arbor, MI). Montelukast metabolites (M2, M5, and M6) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The metabolite N-desethylrosmadiquine was purchased from BD Biosciences. The metabolites N-desmethylrosiglitazone, hydroxypioglitazone, and 3'-hydroxyrepaglinide were purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). All other chemicals used were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available.

Ki Determination. Incubations were performed using five probe substrates of CYP2C8: amodiaquine, montelukast, paclitaxel, repaglinide, and rosiglitazone. Twenty-two known inhibitors of CYP2C8 exhibiting a wide range of inhibition potencies were selected for the in vitro studies. Stock solutions of all the inhibitors were made and diluted in acetonitrile-dimethyl sulfoxide (90:10) to minimize organic solvent content. Four concentrations of each probe substrate (0.5×Km, 1×Km, 2×Km, and 4×Km; Km = 0.80 μM for amodiaquine, Km = 0.014 μM for montelukast, Km = 7.1 μM for paclitaxel, Km = 3.4 μM for repaglinide, and Km = 1.1 μM for rosiglitazone) and five concentrations of each inhibitor (spanning a 10-fold range of the expected Ki) were used for determination of Ki in a 96-well plate format. In brief, each reaction was performed in duplicate, containing 0.1 mg/ml human liver microsomal protein per incubation. Each incubation reaction mixture contained enzyme, probe substrate, and inhibitor suspended in phosphate buffer (100 mM, pH 7.4) containing 3 mM MgCl2, and was preincubated for 3 min in an incubator-shaker at 37°C. The reactions were initiated by the addition of NADPH (1 mM final concentration). Dimethyl sulfoxide concentrations did not exceed 0.1% v/v, and total organic solvent concentrations did not exceed 0.5% v/v. Solvent concentrations were the same for all experiments, and turnover rates did not differ significantly from minimal solvent controls. The reactions were terminated by addition of 100 μl of acetonitrile containing 0.1 μM tolbutamide (internal standard). Lengths of the incubations were 5 min, with the exception of paclitaxel, which was run for 10 min to increase analytical sensitivity. The incubation time and protein concentrations used were within the linear range for each respective P450 probe reaction. In addition, depletion of the substrate and inhibitor was less than 10% over the reaction time.

Km and kact Determination. Time-dependent inactivation experiments were conducted using gemfibrozil 1-O-β-glucuronide as the inactive and
five probe substrates of CYP2C8: amodiaquine, montelukast, paclitaxel, repaglinide, and rosiglitazone. Concentrations of gemfibrozil 1-O-β-glucuronide were used to 50 μM for the primary reactions. In Brief, each primary reaction was performed in duplicate, containing 1.0 mg/ml human liver microsomal protein per incubation. Each primary incubation reaction mixture contained enzyme and inhibitor suspended in phosphate buffer (100 mM, pH 7.4) containing 3 mM MgCl₂ and was preincubated for 3 min in an incubator-shaker at 37°C. The reactions were initiated by the addition of NADPH (1 mM final concentration). At time points of 0, 1, 2, 3, and 5 min, 20-μl aliquots were removed and transferred to a secondary incubation (final volume 200 μl) containing probe substrate and 1 mM NADPH in phosphate buffer (100 mM, pH 7.4) containing 3 mM MgCl₂. Concentrations of probe substrate used were approximately 5-fold above K<sub>m</sub> for amodiaquine, 0.1 μM for montelukast, 55 μM for paclitaxel, 15 μM for repaglinide, and 5 μM for rosiglitazone) for the secondary reaction. The secondary reactions were terminated by adding 100 μl of acetonitrile containing 0.1 μM tolbutamide (internal standard). Length of the secondary reactions was 5 min in the presence of paclitaxel, which was run for 10 min to increase analytical sensitivity. The incubation time and protein concentrations used were within the linear range for each respective P450 probe reaction.

Montelukast Kinetics. Michaelis-Menten kinetic parameters K<sub>m</sub> and V<sub>max</sub> were determined over a range of montelukast concentrations (0–0.25 μM) in HLMs and CYP2C8, CYP2C9, and CYP3A4 Supersomes for the M2 (sulfoxidation), M5 (21-hydroxylation), and M6 (36-hydroxylation) metabolites. Each incubation reaction mixture contained HLMs (0.1 mg/ml or 0.05 μM Supersomes) and montelukast, suspended in phosphate buffer (100 mM, pH 7.4) containing 3 mM MgCl₂ and was preincubated for 3 min in an incubator-shaker at 37°C. The reactions were initiated by the addition of NADPH (1 mM final concentration). The reactions were terminated by addition of 100 μl of acetonitrile containing 0.1 μM ketocaconazole (internal standard). Length of the incubations was 5 min. Montelukast metabolites were measured by LC-MS/MS as described below.

Montelukast Phenotyping. To determine which P450 isoforms were responsible for montelukast metabolism, we used three in vitro approaches: 1) immunochromatographic inhibition in HLMs, 2) metabolism by P450 Supersomes, and 3) a correlation analysis in HLMs. Immunochromatographic inhibition studies were performed with antihuman CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 as recommended previously (Gelboin et al., 1995) and per vendor specifications. In brief, 5 μl of pooled human liver microsomes (20 mg/ml) were incubated with 10 μl of the antibody (10 mg/ml) plus 5 μl of Tris buffer for 30 min on ice. The final concentration of antibody was 100 μg/ml HLMs. The incubation was then diluted to 0.2 mg/ml microsomal protein in 100 mM potassium phosphate buffer (pH 7.4). Ketoconazole (500 nM) was added to the CYP3A4 incubation (Rock et al., 2008). Montelukast was added to the incubation mixture at a final concentration of 0.1 μM. Control P450 substrate marker reactions, as reported previously (Walsky and Obach, 2004), were run to verify the effectiveness of the P450 antibodies. Each incubation reaction mixture contained HLMs, probe substrate, or montelukast, suspended in phosphate buffer (100 mM, pH 7.4) containing 3 mM MgCl₂ and was preincubated for 3 min in an incubator-shaker at 37°C. The reactions were initiated by the addition of NADPH (1 mM final concentration). The reactions were terminated by addition of 100 μl of acetonitrile containing 0.1 μM tolbutamide (internal standard). Length of the incubations was 60 min. Montelukast metabolites were measured by LC-MS/MS as described below.

P450 Supersomes (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5) at a concentration of 0.05 μM were suspended in phosphate buffer (100 mM, pH 7.4) containing 3 mM MgCl₂ and montelukast at a final concentration of 0.1 μM. The incubation mixtures were initiated, quenched, and analyzed as described above. Human liver microsomes obtained from 18 individual subjects were incubated with 0.1 μM montelukast and 0.5 μM midazolam, 40 μM testosterone, 10 μM paclitaxel, or 20 μM diclofenac to measure montelukast metabolism and P450 marker activities (Walsky and Obach, 2004). The same reaction mixtures and incubation conditions as described above were used. The fraction metabolized (f<sub>metabolized</sub>) of montelukast was calculated from the HLM phenotyping experiments by dividing the total amount of M6 formed by the total sum of oxidative metabolism, where the total amount of M6 formation was corrected by the percentage contribution of CYP2C8.

Liquid Chromatography/Tandem Mass Spectral Analysis. All analytical methods were conducted using LC-MS/MS technology. For P450 substrate probe reactions, the LC-MS/MS system comprised an Applied Biosystems 4000 Q-Trap (operated in triple quadrupole mode) equipped with an electro spray ionization source (Applied Biosystems, Foster City, CA). The MS/MS system was coupled to two LC-20AD pumps with an in-line C18-20A controller and DGU-20A, solvent degasser (Shimadzu, Columbia, MD) and a LEAP CTC HTS PAL autosampler equipped with a dual-solvent self-washing system (CTC Analytics, Carrboro, NC). The injection volume was 10 μl for each sample. LC separation was achieved using a Gemini C18 2.0 × 50 mm 5-μm column (Phenomenex, Torrance, CA). Gradient elution (flow rate = 500 μl/min) was performed using a mobile phase system consisting of (A) 5 mM ammonium formate with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The solvent flow was diverted from the MS/MS system for the first 20 s to remove any nonvolatile salts. MS/MS conditions were optimized for individual analytes, accordingly. Generic mass spectrometer parameters included the curtain gas (10 arbitrary units), collisionally activated dissociation gas (medium), ionspray voltage (4500 V), source temperature (450°C), and ion source gas 1 and gas 2 (40 arbitrary units each). Interface heaters were kept on for all analytes. Analysis masses were (positive ionization mode): N-desethylamodiaquine, m/z 328.1 → 283.1; N-desmethylrosiglitazone, m/z 344.2 → 121.2; p'-3'-hydroxy-paclitaxel m/z 870.1 → 105.3; 6a-hydroxy-paclitaxel, m/z 870.1 → 286.2; hydroxygliptazone, m/z 413.1 → 178.1; 3'-hydroxypioglitazone, m/z 469.1 → 246.1, and tolbutamide, m/z 271.2 → 91.1. For identification of montelukast metabolites, the LC-MS/MS system comprised a TSQ Vantage Triple Stage Quadrupole Mass Spectrometer equipped with a heated electrospray ionization probe (Thermo Fisher Scientific, Waltham, MA). The MS/MS system was coupled to an Accela high-speed LC pump and a Thermo Fisher Scientific autosampler. The injection volume for each sample was 20 μl. LC separation was achieved using a Kinex C18 2.10 × 50 mm 2.6 μm column (Phenomenex). Gradient elution (flow rate = 700 μl/min) was performed using a mobile phase system consisting of (A) 5 mM ammonium formate with 0.1% formic acid plus 1% isopropanol and (B) acetonitrile with 0.1% formic acid. LC flow was diverted from the MS/MS system for the first 2 min to remove any nonvolatile salts. MS/MS conditions were optimized for individual analytes, accordingly. Generic mass spectrometer parameters included the curtain gas (10 arbitrary units), collisionally activated dissociation gas (high), ionspray voltage (4500 V), source temperature (400°C), and ion source gas 1 and gas 2 (40 arbitrary units each). Analysis masses were treated as global and ion source gas 1 and gas 2 (40 arbitrary units each). Interface heaters were kept on for all analytes. Analysis masses were (positive ionization mode): montelukast, m/z 586.2 → 423.3; M6 (36-hydroxylation), m/z 602.2 → 438.3; M5 (21-hydroxylation), m/z 602.2 → 147.1; M2 (sulfoxidation), m/z 602.2 → 422.3; and ketoconazole, m/z 531.2 → 82.0.

Statistical Analysis. Standard curve-fitting was performed using AnalyS (version 1.4; Applied Biosystems). In general, standard curves were weighted using 1/x. Substrate saturation curves and inhibition data were plotted and analyzed using GraphPad Prism (version 4.01; GraphPad Software Inc., San Diego, CA). Data were then fit to a competitive (eq. 1), noncompetitive (eq. 2), or linear-mixed inhibition model (eq. 3).

\[
\nu = \frac{V_{\text{max}}}{K_m + [S]} + [I]
\]

\[
\nu = \frac{V_{\text{max}}}{K_m' + [I]/[K_i'] + [S]} + [I]/[K_i']
\]

\[
\nu = \frac{V_{\text{max}}}{K_m' + [S]} + [I]/[K_i']
\]
parameters. The mechanism of inhibition was determined by visual inspection of the data using Dixon ([I] versus 1/v) plots and comparative model assessment using the Akaike information criterion. For the inactivation experiments, the natural logarithm decrease in plots and comparative model assessment using the Akaike information criterion was made using eq. 5 (Mayhew et al., 2000):

\[ k_\text{obs} = k_\text{obs} - k_\text{inact} \frac{[I]}{K_I + [I]} \]  

(4)

In the preceding equation, \( k_\text{obs} \) is equal to the rate of inactivation by the inactivator, \( k_{\text{obs} - 0} \) is equal to the rate of inactivation by the inactivator at time \( t = 0 \), \( k_\text{inact} \) is the maximal rate of inactivation, \( K_I \) is equal to the inactivator concentration at the half-maximal rate of inactivation, and \( [I] \) is the concentration of inactivator.

**Correlation Analysis of In Vivo Drug Interaction Potential.** Literature data for AUC/AUC were obtained using the University of Washington Metabolism and Transport Drug Interaction Database, where AUC is defined as the area under the plasma concentration-time curve for a given probe substrate in the absence of an inhibitor and AUC is defined as the area under the plasma concentration-time curve for a given probe substrate in the presence of an inhibitor. Studies were considered comparable if they had a similar dose regimen for both inhibitor and probe substrate. For instances in which multiple AUC/AUC values were available in the literature, the AUC/AUC values were averaged.

**Prediction of In Vivo Drug Interactions.** Prediction of inactivation DDIs was made using eq. 5 (Mayhew et al., 2000):

\[ \Delta \text{AUC} = \frac{\text{AUC}_{\text{final}}}{\text{AUC}_{\text{initial}}} = 1 \left( \frac{f_m \text{CYP}}{1 + (K_{I_{\text{max}}}/K_I) \cdot f_{\text{m}} \text{CYP}} \right) + (1 - f_m \text{CYP}) \]  

(5)

where \( f_m \text{CYP} \) is the fraction of probe substrate cleared by the indicated P450, \( [I]_{\text{max}} \) is the maximum unbound hepatic input concentration, \( K_{I_{\text{max}}} \) is the maximal rate of inactivation, \( K_I \) is the concentration of inactivator at the half-maximal rate of inactivation, and \( f_{\text{m}} \text{CYP} \) is the degradation rate of CYP2C8 [0.0005 days (Lai et al., 2009)]. For the predictions using gemfibrozil-1-O-β-glucuronide as inhibitor, the following inputs were used: \( f_m \text{CYP} \) values of 0.81, 0.63, 0.49, and 0.50 for montelukast, paclitaxel, repaglinide, and rosiglitazone, respectively (Hinton et al., 2008).

**Results**

The inhibition constants (\( K_I \)) for a set of 22 effectors were determined for the probe substrates amodiaquine, montelukast, paclitaxel, repaglinide, and rosiglitazone in vitro (Table 1 and structures in Fig. 1). Competitive inhibition profiles were observed in all cases. Repaglinide was the most sensitive probe substrate, followed by amodiaquine, rosiglitazone, and montelukast, whereas paclitaxel exhibited marked differences in observed inhibition potency from those of the other probe substrates. In addition to the set of competitive inhibition constants, \( K_I \) and \( K_{I_{\text{max}}} \) for gemfibrozil-1-O-β-glucuronide, a mechanism-based inhibitor of CYP2C8, were determined for the five probe substrates. There was a range of values observed among the five probe substrates, with the \( K_I \) ranging from 10 to 49 μM and the \( K_{I_{\text{max}}} \) ranging from 0.02 to 0.08 mm⁻¹ (Table 2). The magnitude of in vivo DDI caused by gemfibrozil-1-O-β-glucuronide was predicted for the clinically relevant probe substrates paclitaxel, repaglinide, rosiglitazone, and montelukast (Table 2).

In pooled human liver microsomes, the oxidation of montelukast resulted in the formation of three major metabolites (M2, M5, and M6; Fig. 2). Michaelis-Menten kinetic parameters (\( K_m \) and \( V_{\text{max}} \)) were determined for the oxidative metabolism of montelukast over a concentration range of 0 to 0.25 μM in HLMs and CYP2C8, CYP3A4, and CYP2C9 Supersomes (Fig. 3). Over the range of concentrations, formation of M6 (36-hydroxylation) was linear in both CYP2C9 and CYP3A4 Supersomes. In CYP2C8 Supersomes and HLMs, the formation of M6 (36-hydroxylation) followed Michaelis-Menten kinetics, and the \( K_m \) was determined to be 14 and 65 nM, respectively. A panel of nine P450 enzymes (Supersomes) was incubated with montelukast (Fig. 4, A–C). The formation of M2 (sulfoxidation) and M5 (21-hydroxylation) was inhibited with the CYP3A inhibitor ketocazole (500 nM) plus a CYP3A-selective antibody, a combination known to produce a more complete and selective inhibition than either alone (Rock et al., 2008) (Fig. 4, E and F). Formation of M6 (36-hydroxylation) was inhibited by a selective CYP2C8 antibody (Fig. 4D). Both results revealed similar profiles, with CYP3A4 and CYP2C8 as the major P450 enzymes involved in the turnover of montelukast. Montelukast did not appear to be metabolized by flavin-containing monoxygenase, in agreement with previous results (Chiba et al., 1997). In addition, correlation analysis across a bank of 18 individual human liver microsomes showed a significant correlation coefficient between the CYP2C8-selective formation of 6α-hydroxypaclitaxel and the M6 (36-hydroxylation) oxidative metabolite of montelukast (0.1 μM) with an \( r^2 = 0.89 \) (Fig. 5). The CYP3A metabolism of 1'-hydroxymidazolam and 6-hydroxytestosterone also demonstrated a correlation coefficient with the M2 (sul-
foxidation) and M5 (21-hydroxylation) oxidative metabolite of montelukast with $r^2$ values greater than 0.84 (data not shown).

The metabolism of cerivastatin, fluvastatin, montelukast, pioglitazone, repaglinide, and rosiglitazone is partially mediated by CYP2C8 in vivo. In vivo DDI results for those compounds were collected from the literature and compiled (Table 3). A linear correlation analysis was performed on the nontransformed data relative to repaglinide, the CYP2C8 probe substrate with the largest number of comparative DDI studies. All probe substrates exhibited lower sensitivity relative to that of repaglinide. Of the currently prescribed probe substrates, the rank in order of decreasing sensitivity was montelukast, pioglitazone, rosiglitazone, and fluvastatin.

**Discussion**

One of the primary causes of serious adverse events occurring in clinical practice is drug-drug interactions (Huang et al., 2007). A crucial part of the drug discovery and development paradigm is screening for and predicting the magnitude of P450-mediated DDIs. Examples of drugs withdrawn from the market due to drug interactions include mibefradil (Po and Zhang, 1998) and cerivastatin (Davidson, 2002). Cerivastatin was withdrawn from the market after 500 adverse events; approximately half of the events were directly correlated with coadministration of the CYP2C8 inhibitor gemfibrozil (Farmer, 2001; Backman et al., 2002).

In vitro inhibition studies serve as a basis for in vivo DDI predictions. Probe substrate-dependent inhibition has been demonstrated for CYP2C9 (Kumar et al., 2006), CYP2C19 (Foti and Wahlstrom, 2008), and CYP3A4 (Kenworthy et al., 1999; Stresser et al., 2000; Foti et al., 2010) and may confound predictions of the in vivo situation. Selection of a probe substrate that is applicable to both the in vitro and in vivo situations may reduce the impact of probe substrate-dependent inhibition on prediction accuracy. Therefore, a comprehensive under-

![Fig. 2. The chemical structures of montelukast and the major P450-mediated metabolites M2 (sulfoxidation), M5 (21-hydroxylation), and M6 (36-hydroxylation).](image)

![Fig. 3. The rate of 36-hydroxylation (M6) versus montelukast concentration in CYP2C8 Supersomes and human liver microsomes.](image)
standing of in vitro and in vivo applicability and sensitivity enables appropriate probe substrate selection.

Our selection of in vitro probe substrates was based on recommendations from the FDA guidance and the availability of clinical DDI data. Amodiaquine, paclitaxel, and rosiglitazone have been used as CYP2C8 in vitro probe substrates (Walsky et al., 2005). Repaglinide was selected because of the availability of clinical DDI results (Huang et al., 2007), whereas montelukast was selected on the basis of a recent report of CYP2C8-mediated metabolism in vivo (Karonen et al., 2010). Repaglinide exhibited the greatest sensitivity as an in vitro probe. Relative to repaglinide, a 3-fold average decrease in inhibition potency was observed for rosiglitazone and amodiaquine and a 4-fold decrease for montelukast. Paclitaxel displayed a 15-fold average decrease in observed inhibition potency relative to that of repaglinide. On the basis of these results, CYP2C8 exhibited probe substrate-dependent inhibition.

Plausible mechanisms for probe substrate-dependent inhibition observed in vitro include the presence of multiple binding regions within the P450 active site, metabolic switching, and the involvement of more than one enzyme in metabolite formation (Kenworthy et al., 1999; Regal et al., 2005). The elucidation of the crystal structure of CYP2C8 has provided useful information on how ligands interact with this enzyme and how CYP2C members determine probe substrate specificity. In the ligand-free structure of CYP2C8, the large active site cavity exhibits architecture that approximates a T or Y shape with branches of differing lengths, widths, and chemical properties (Schoch et al., 2004, 2008). The crystal structures of CYP2C8 with felodipine, montelukast, or troglitazone bound demonstrate distinct binding interactions to the CYP2C8 active site: montelukast fills the entire structure of CYP2C8, felodipine occupies the active site cavity near the heme, and troglitazone fills the upper portion of the active site (Schoch et al., 2004, 2008). Paclitaxel produces two metabolites, 6α-hydroxyplaclitaxel (CYP2C8) and p-3'-hydroxypaclitaxel (CYP3A4), such that metabolic switching is another plausible explanation for the distinctive decrease in sensitivity to inhibition. However, formation of p-3'-hydroxypaclitaxel did not increase upon addition of the inhibitors, indicating that metabolic switching did not occur (data not shown). In addition, the formation of 6α-hydroxyplaclitaxel was
demonstrated to be selective for CYP2C8 (Rahman et al., 1994). Taken together, the CYP2C8 results suggest that specific probe substrate-P450 active site interactions may have a marked effect on enzyme inhibition and distinguish between probe substrates that share some overlap in binding modes.

The probe substrate montelukast is a leukotriene receptor antagonist and a known inhibitor of CYP2C8. Although montelukast is a potent and selective inhibitor of CYP2C8 in vitro (Walsky et al., 2005), it does not alter the clearance of pioglitazone or repaglinide in vivo (Jaakkola et al., 2006; Kajosaari et al., 2006). More recently, gemfibrozil was shown to markedly increase the plasma concentrations of montelukast, implying that CYP2C8 may contribute to the elimination of montelukast (Karonen et al., 2010). Experiments were performed to further evaluate which P450 enzymes were responsible for the in vitro metabolism of montelukast. Three P450-mediated metabolites of montelukast are M2 (sulfoxidation), M5 (21-hydroxylation), and M6 (36-hydroxylation) as shown in Fig. 2 (Chiba et al., 1997).

According to prescribing information and in vitro studies, CYP3A4 and CYP2C9 are the main P450 enzymes involved in the formation of M2 (sulfoxidation)/M5 (21-hydroxylation) and M6 (36-hydroxylation), respectively. However, at the time of the montelukast phenotyping experiments, the in vitro tools to evaluate CYP2C8 were lacking. More so, the high montelukast concentrations (100–500 μM) used in the experiments (100–1000-fold higher than therapeutic total plasma concentrations) would have saturated the CYP2C8-mediated metabolism and confounded the results. More recently, in vivo administration of gemfibrozil greatly impaired the elimination of M6 (Karonen et al., 2010). In addition, CYP2C8 has been shown to be crucial in the in vitro metabolism of montelukast at clinically relevant concentrations (Filppula et al., 2011). Here, additional in vitro experiments were performed, including correlation analysis, phenotyping with selective antibodies, and in vitro kinetics to further characterize the in vitro metabolism of montelukast by CYP2C8. Montelukast concentrations in plasma range between 0.05 and 0.80 μM (Karonen et al., 2010); therefore, to approximate these conditions, low montelukast concentrations were used in our microsomal incubation. At low montelukast concentrations (0–0.25 μM), the formation of M6 was predominantly mediated by CYP2C8, not CYP2C9 or CYP3A4 (Fig. 4). In contrast, administration of gemfibrozil did not reduce the formation of M5 (21-hydroxylation), in agreement that CYP3A4 is responsible for its formation (Chiba et al., 1997). Together, these results indicate that at clinically relevant concentrations, metabolism by CYP2C8 is a major determinant in the elimination of montelukast.

The mechanism of CYP2C8 inactivation by gemfibrozil-1-O-β-glucuronide is the formation of a benzyl radical intermediate that binds to the γ-meso position of the prosthetic heme and renders CYP2C8 catalytically inactive (Baer et al., 2007). The inactivation kinetic parameters (K_i and k_inact) across five probe substrates (Table 2) were determined. As expected for mechanisms that inactivate through heme modification, the kinetic inactivation parameters between probe substrates were similar. These differences observed also did not play a role in changing the in vivo predictions, because a key characteristic affecting DDI predictions is the fraction metabolized by the specific P450 (fm CYP) enzyme. Predictions for inhibition by gemfibrozil-1-O-β-glucuronide were driven by the fm CYP2C8 (Hinton et al., 2008) and were insensitive to substrates with fm CYP2C8 values less than 0.65. Using our in vitro kinetic parameters for inactivation by gemfibrozil-1-O-β-glucuronide (Table 2) and the fm CYP2C8 of montelukast (0.81), our predicted AUCI/AUC was within 10% of the observed value. Collectively, the P450 phenotyping and DDI predictions indicate that CYP2C8 is the primary enzyme involved in the oxidation of montelukast at clinically relevant substrate concentrations.

The in vivo sensitivity of the CYP2C8 probe substrates may influence selection of CYP2C8 probe substrate for a clinical study. Retrospective analysis of clinical DDI studies from the literature has been used to correlate the relative sensitivity of probe substrates in vivo on the basis of results from multiple CYP3A4 inhibitors (Ragueneau-Majlessi et al., 2007; Foti et al., 2010). The availability of clinical DDI studies relative to repaglinide and a known contribution of CYP2C8 to
clearance were our selection criteria for inclusion in the in vivo correlation analysis. The DDI results were averaged for instances in which multiple clinical studies for the same inhibitor and probe substrate combination were available and performed using similar conditions. All of the probe substrates displayed reduced sensitivity relative to that of repaglinide. Although cerivastatin and repaglinide were sensitive to inhibition by gemfibrozil in vivo, both are substrates of OATP1B1; observed DDI in vivo may be due to a combination of metabolism and transporter inhibition (Hinton et al., 2008). Gemfibrozil-1-O-glucuronide, a key component for the in vivo sensitivity analysis presented here, is an inhibitor of both CYP2C8 and OATP1B1 (Shitara et al., 2004). Therefore, consideration of confounding factors such as transporter-mediated clearance may aid in selecting an appropriate probe substrate for a clinical DDI study.

The selection of a CYP2C8 probe substrate for in vitro experiments and clinical DDI studies affects both the measured inhibition potency in vitro and the observed magnitude of DDI in vivo. The results presented here indicate that probe selection for CYP2C8 is important. Amodiaquine is a sensitive CYP2C8 in vitro probe substrate but lacks marketing approval in the United States; testing an unusual in vitro substrate is an inhibitor of both CYP2C8 and OATP1B1 (Shitara et al., 2004). Therefore, consideration of confounding factors such as transporter-mediated clearance may aid in selecting an appropriate probe substrate for a clinical DDI study.

Authorship Contributions
Participated in research design: VandenBrink, Foti, Rock, Wienkers, and Wahlstrom.
Conducted experiments: VandenBrink.

Performed data analysis: VandenBrink, Foti, and Wahlstrom.
Wrote or contributed to the writing of the manuscript: VandenBrink, Foti, Rock, Wienkers, and Wahlstrom.

References


