Investigational Small-Molecule Drug Selectively Suppresses Constitutive CYP2B6 Activity at the Gene Transcription Level: Physiologically Based Pharmacokinetic Model Assessment of Clinical Drug Interaction Risk

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ABSTRACT
The glycogen synthase kinase-3 inhibitor LY2090314 specifically impaired CYP2B6 activity during in vitro evaluation of cytochrome P450 (P450) enzyme induction in human hepatocytes. CYP2B6 catalytic activity was significantly decreased following 3-day incubation with 0.1–10 μM LY2090314, on average by 64.3% ± 5.0% at 10 μM. These levels of LY2090314 exposure were not cytotoxic to hepatocytes and did not reduce CYP1A2 and CYP3A activities. LY2090314 was not a time-dependent CYP2B6 inhibitor, did not otherwise inhibit enzyme activity at concentrations ≤10 μM, and was not metabolized by CYP2B6. Thus, mechanism-based inactivation or other direct interaction with the enzyme could not explain the observed reduction in CYP2B6 activity. Instead, LY2090314 signifi-

CYP2B6 inhibition occurs commonly by mechanism-based enzyme inactivation (Richard et al., 2004), but potent competitive inhibitors have also been reported (Stiborova et al., 2002). Evaluation of 227 commonly prescribed drugs identified 30 CYP2B6 inhibitors (Walsky et al., 2006). The two specific clinical CYP2B6 inhibitors, clopidogrel and ticlopidine, significantly increased bupropion exposure by 60%–85% and decreased the ratio of hydroxybupropion/bupropion by 71%–93% in humans (Turpeinen et al., 2005).

perpetrator interactions involves in vitro evaluation of acute competitive and time-dependent inhibition in direct incubations with the enzyme (e.g. liver microsomes), as well as induction of activity following chronic exposure of human hepatocytes. Positive in vitro inhibition and induction results are then translated to humans using static and dynamic models to assess whether a drug interaction risk exists that necessitates clinical evaluation.

Suppression of P450 enzyme activity at the transcriptional level is a relatively new area in development of small-molecule drugs. The Food and Drug Administration currently has no recommendation on evaluation of P450 suppression or follow-up assessment of clinical drug interaction risk; however, both the European Medicines Agency and the Pharmaceutical and Medical Devices Agency recommend attempting to understand the potential clinical relevance of P450 suppression when mRNA levels are decreased in excess of 50% in vitro (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf; www.pmnda.go.jp/english). Suppression of constitutive CYP2B6 activity at the gene expression level has not been previously reported in the literature. Although CAR inhibition can occur by direct (inverse agonism) or indirect mechanisms (e.g. altered phosphorylation and diminished nuclear translocation), it is not known to elicit decreased constitutive CYP2B6 enzyme activity (Moore et al., 2000; Faucette et al., 2004; Li et al., 2008; Tolson and Wang, 2010; Yang et al., 2014). The lack of CYP2B6 functional suppression by known CAR inverse agonists, such as clomipramine and 1-(2-chlorophenyl-N-methylpropyl)-3-isooquinoline-carboxamide (PK11195), is attributed to their PXR activation, which overcompensates for decreased CAR activity yielding net CYP2B6 induction (Faucette et al., 2004; Li et al., 2008). Likewise, metformin was recently shown to indirectly inhibit CAR activity by enhancing phosphorylation and limiting nuclear translocation; however, although metformin suppressed CAR-mediated CYP2B6 induction, it did not decrease constitutive CYP2B6 levels (Yang et al., 2014).

LY2090314 (3-[9-fluoro-2-(piperidin-1-ylcarbonyl)-1,2,3,4-tetrahydro[1,4]diazepino[6,7,1-hi][1,2]pyrrole-2,5-dione; structure is provided in Supplemental Fig. 1) is an intravenous glycogen synthase kinase-3 (GSK-3) inhibitor (GSK-3α IC50 = 1.5 nM, GSK-3β IC50 = 0.9 nM) in clinical testing for the treatment of cancer. During in vitro evaluation of its drug interaction potential, LY2090314 demonstrated selective reduction of CYP2B6 activity in human hepatocytes at the level of gene transcription. Although CAR inhibition by a small-molecule drug is not unprecedented, the associated marked and selective suppression of CYP2B6 activity at clinically relevant concentrations is novel. We demonstrate how routine P450 enzyme induction studies can provide an alert to CYP2B6 suppression, and how a physiologically based pharmacokinetic (PBPK) approach can be used to evaluate the associated risk of clinical drug interactions.

Materials and Methods

Primary cultures of fresh human hepatocytes were prepared from livers of six male and female donors by CellzDirect (Austin, TX; Supplemental Table 1). Human liver microsomes for CYP2B6 inhibition studies (mixed sex pool of 15 donors) were from CellzDirect; human liver microsomes (mixed sex pool of 15 donors) were from CellzDirect; human liver microsomes (mixed sex pool of 15 donors) were from CellzDirect; human liver microsomes (mixed sex pool of 15 donors) were from CellzDirect; human liver microsomes (mixed sex pool of 15 donors) were from CellzDirect (Austin, TX; Supplemental Table 1). Human liver microsomes for CYP2B6 inhibition studies (mixed sex pool of 15 donors by CellzDirect (Austin, TX; Supplemental Table 1). Human liver microsomes for CYP2B6 inhibition studies (mixed sex pool of 15 donors by CellzDirect (Austin, TX; Supplemental Table 1). Human liver microsomes for CYP2B6 inhibition studies (mixed sex pool of 15 donors by CellzDirect (Austin, TX; Supplemental Table 1). Human liver microsomes for CYP2B6 inhibition studies (mixed sex pool of 15 donors by CellzDirect (Austin, TX; Supplemental Table 1). Human liver microsomes for CYP2B6 inhibition studies (mixed sex pool of 15 donors by CellzDirect (Austin, TX; Supplemental Table 1). Human liver microsomes for CYP2B6 inhibition studies (mixed sex pool of 15 donors by CellzDirect (Austin, TX; Supplemental Table 1).

CYP2B6 Inhibition. Bupropion (125 μM) was coincubated with LY2090314 (0.01–10 μM with or without 15-minute preincubation; with or without 15-minute preincubation) in human liver microsomes (0.25 mg/ml protein, 1 mM NADPH, 20 minutes, pH 7.4, 37°C). Reactions were quenched with organic solvent, and supernatants were used for analysis of hydroxybupropion.

CYP2B6 Metabolic Stability. LY2090314 (30 and 60 nM) was incubated with human liver microsomes or Supernatants (0.25 mg/ml protein, 1 mM NADPH, 20 minutes, pH 7.4, 37°C). Reactions were quenched with organic solvent, and supernatants were used for analysis of hydroxybupropion.
NADPH, pH 7.4, 37°C). Incubations were sampled between 0–120 minutes and quenched with organic solvent, and supernatants were used to quantify the remaining LY2090314.

**CAR Activity.** Activities of CAR (NR1I3) isoforms 1 and 3 were determined using the CAR1 and CAR3 reporter assay systems per the manufacturer’s instructions (Indigo Biosciences, State College, PA). Proprietary nonhuman mammalian host cells were cotransfected with a chimeric plasmid containing the individual CAR ligand-binding domains in sequence with the GALA DNA-binding domain and a second plasmid with the GALA response element driving the expression of luciferase (reporter gene). CAR1-expressing cells were incubated with LY2090314 (0.01–100 μM) and separately with the known inverse agonist PK11195 (0.015–60 μM; Indigo Biosciences). Unlike CAR1, CAR3 is not constitutively active (Auerbach et al., 2005), so potential CAR3 antagonism was investigated with LY2090314 (0.01–100 μM) in the presence of the known agonist 6-(4-chlorophenyl)imidazo[2,1-b]1,3thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) (0.4 μM; Indigo Biosciences). In addition, the CAR3 cells were exposed to CITCO (0.02–5 μM) to demonstrate concentration-dependent CAR3 agonist activity. All cells were incubated for 24 hours at 37°C. Following incubation, CAR activity was measured by emission of light from luciferase normalized for light emission in vehicle-treated cells.

**PXR Activity.** HuH7 human hepatoma cells cotransfected with a chimeric plasmid containing the PXR ligand-binding domain in sequence with the GALA DNA-binding domain and a second plasmid with the GALA-response element driving the expression of luciferase (reporter gene) were incubated with LY2090314 or rifampin (0.001–50 μM) for 24 hours at 37°C. Following incubation, PXR activity was measured by emission of light from luciferase, normalized for light emission in vehicle-treated cells.

**Bioanalysis.** Supernatant from quenched P450 induction activity samples (spiked with internal standards) was evaporated, reconstituted in mobile phase, and analyzed by liquid chromatography–tandem mass spectrometry. The bioanalytical method outlined for hydroxybupropion was also used to analyze reconstituted supernatant from human liver microsomes in the CYP2B6 inhibition studies. Analytes were eluted from C18 columns (hydroxybupropion and 6β-hydroxytestosterone: AQ12, YM-ODS-AQ, 4.0 × 50 mm, 3 μm (Waters Inc., Milford, MA); acetaminophen: Phenosphere-Nexxt, 2.0 × 50 mm, 5 μm (Phenomenex, Torrance, CA)) with mobile phase optimized for each analyte (hydroxybupropion: isocratic, 40% methanol, 60% 1 mM ammonium acetate buffer; acetaminophen: isocratic, 50% methanol, 50% 1 mM ammonium acetate buffer, 0.1% trifluoroacetic acid; 6β-hydroxytestosterone: gradient, A: 1 mM ammonium acetate buffer, pH 2.5, B: ethanol). Analytes were detected in positive ion mode using multiple reaction monitoring: hydroxybupropion → 272 m/z, D6-hydroxybupropion → 295 m/z, D6-acetaminophen → 319 m/z, and 6β-hydroxytestosterone → 272 m/z.

**Data Analysis.** Inhibitory potency of LY2090314 toward CYP2B6 activity and mRNA, as well as CAR1 and CAR3, was estimated by nonlinear least-squares regression using Phoenix WinNonlin version 6.3 (Certara, Cary, NC). The following equation was fitted to the inhibition-concentration data:

$$E = E_0 - \frac{I_{\text{max}} \times C}{I_{C_{50}} + C}$$

where $E$ is the remaining CYP2B6 activity or mRNA at a given LY2090314 concentration ($E_0$ is the remaining CYP2B6 activity or mRNA without LY2090314 (i.e., 100%)), $I_{\text{max}}$ is the maximal extent of CYP2B6 inhibition, and $I_{C_{50}}$ is the LY2090314 concentration eliciting 50% of $I_{\text{max}}$. The $I_{C_{50}}$ equation with a Hill coefficient ($γ$) was also evaluated, but the simple $I_{C_{50}}$ relationship was selected based on superior model performance. Likewise, the simple maximal effect relationship was used to describe CAR3 and PXR activation.

**Results**

The effect of LY2090314 (0.01–10 μM, 3 days) on CYP2B6, 1A2, and 3A activity in human hepatocytes is presented in Fig. 1. LY2090314 (≥0.1 μM) significantly decreased CYP2B6 bupropion hydroxylation, on average by 64.3% ± 5.0% at 10 μM. In contrast, CYP1A2 oxidation of phenacetin to acetaminophen was unaltered. CYP3A testosterone 6β-hydroxylation was not decreased and was even significantly increased by 10 μM LY2090314; however, this increase was only 11.5% ± 1.3% of rifampin positive control, and thus was not clinically relevant. In these hepatocyte preparations, phenobarbital induced CYP2B6 activity 21.9 ± 3.4-fold, omeprazole induced CYP1A2 79.0 ± 17.2-fold; and rifampin induced CYP3A 30.9 ± 2.2-fold.

In a concentration range study (0.01–100 μM) in hepatocytes from a single donor, no morphologic changes associated with cytotoxicity were observed following 3-day incubation with LY2090314 concentrations ≤10 μM (data not shown). In contrast, exposure to 100 μM LY2090314 for 3 days resulted in cell death, and so ≤10 μM concentrations were used in subsequent P450 induction studies. Due to consistently decreased CYP2B6 activity, cell morphology was further inspected for signs of cytotoxicity in recorded images from P450 induction studies, but none were observed. These empirical cytotoxicity observations were further confirmed on a quantitative level. Exposure to LY2090314 concentrations ≤10 μM for 3 days did not result in decreased total ATP levels or increased leakage of intracellular lactate dehydrogenase into cell media (Fig. 2). Likewise, NADPH/NADH levels determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazolium conversion to formazan confirmed the absence of cytotoxicity at incubation concentrations ≤10 μM (data not shown).

LY2090314 did not inhibit CYP2B6 bupropion hydroxylation in human liver microsomes at the ≤10 μM concentration range tested in hepatocytes (Fig. 3A). However, CYP2B6 activity was 45.3% ± 2.2%
decreased by coincubation with 50 μM LY2090314. There was no shift in the inhibition profile with LY2090314 preincubation. Furthermore, preincubation with 50 μM LY2090314 and 1 mM NADPH did not decrease CYP2B6 activity to a greater extent than preincubation in the absence of cofactor (50.3% ± 0.8% and 50.2% ± 0.8%, respectively). Taken together, these findings rule out mechanism-based CYP2B6 inactivation.

LY2090314 was not metabolized in incubations with recombinant CYP2B6 (99.8% ± 0.9% of initial concentration remaining at 2 hours), whose metabolic stability profile resembled vector control (no P450) membranes (Fig. 3B). For comparison, in incubations with matrices that metabolized LY2090314 (human liver microsomes, recombinant CYP3A4 and CYP3A5), concentrations declined to below the limit of quantification by the 30-minute time point.

LY2090314 concentration-dependent suppression of CYP2B6 mRNA levels is presented in Fig. 4A. The extent of decline in activity and mRNA levels was comparable (I max = 57.0% ± 10.8% and 61.9% ± 1.4%; IC 50 = 0.057 ± 0.027 and 0.049 ± 0.043 μM, respectively), and a significant linear correlation was established between CYP2B6 activity and mRNA (r 2 = 0.87, slope = 0.77), thus establishing that CYP2B6 suppression is a noncanonical mechanism of inhibiting CYP2B6 catalytic activity. The time course of change in mRNA level is presented in Fig. 4B. CYP2B6 mRNA steadily declined over time of LY2090314 treatment, reaching a plateau by 48 hours; thereafter, mRNA level was decreased on average by 58.2% ± 4.8%.

Effects of LY2090314 on expression of CAR and PXR downstream reporter genes are presented in Fig. 5. LY2090314 directly inhibited CAR isoforms 1 and 3 (I max = 100.0% ± 10.8% and 57.1% ± 2.4%; IC 50 = 2.5 ± 1.2 and 2.1 ± 0.4 μM, respectively). In contrast, LY2090314 (2.5 nM to 5.6 μM) weakly activated PXR, except at the two highest concentrations tested (16.7–50 μM). Excluding the two highest LY2090314 concentrations, the apparent PXR activation maximal effect was 19.3% ± 2.2% of maximal rifampin response, and the apparent EC 50 was 1.2 ± 1.1 nM.

PBPK model simulations were used to predict the clinical relevance of observed CYP2B6 suppression. The LY2090314 model was qualified by establishing predictability of reported pharmacokinetics following 60-minute intravenous infusion of a 40-mg dose (Fig. 6A; Zamek-Gliszczynski et al., 2013). The bupropion PBPK model simulated hydroxybupropion area under the curve (AUC 0–inf) and C max values of 11.5 μg·h/ml [95% confidence interval (CI) 10.0–13.3 μg·h/ml] and 242 ng/ml [209–279 ng/ml], which were comparable to the reported values of 14.7 μg·h/ml [12.7–18.4 μg·h/ml] and 395 ng/ml [341–497 ng/ml] following a single 150-mg oral dose, respectively (Loboz et al., 2006). The model predicted the absence of a drug interaction between LY2090314 (40 mg, 60-minute intravenous infusion once weekly for 3 weeks) and hydroxybupropion following concomitant administration of bupropion (Fig. 6B); the predicted decrease in hydroxybupropion exposure was <1%, consistent with negligible decrease in hepatic CYP2B6 activity (<1%). Even if LY2090314 were administered once daily for 3 weeks, which it is not clinically, there would be no interaction with CYP2B6; the predicted decrease in hydroxybupropion exposure was 1% (95% CI 0.7%–1.2%).

PBPK simulations were used to evaluate whether the observed in vitro CYP2B6 suppression could be important for a drug with different pharmacokinetic and dosing properties from LY2090314. A hypothetical perpetrator drug was constructed, which shared all inhibitory and pharmacokinetic parameters with LY2090314, except...
clearance was reduced 10-fold to 4.2 l/h, and plasma fraction unbound was increased 4-fold to 10%. The model predicted a low drug interaction with this hypothetical drug when administered 40 mg once daily as a 60-minute intravenous infusion for 20 days (9 days prior to and 10 days post bupropion dosing; Fig. 7A). The predicted hydroxybupropion AUC and Cmax ratios were 0.73 (95% CI 0.71–0.75) and 0.72 (95% CI 0.69–0.74), respectively. Hepatic CYP2B6 activity was decreased by 41% following multiple dosing of this hypothetical drug (Fig. 7B).

Discussion

The present studies demonstrated previously unreported specific suppression of constitutive CYP2B6 activity by a small-molecule drug. These findings could be explained by competitive or time-dependent direct inhibition of the CYP2B6 enzyme (Stiborova et al., 2002; Richter et al., 2004), but LY2090314 did not directly interact with CYP2B6 as inhibitor or substrate. Instead, LY2090314 specifically suppressed constitutive expression of the CYP2B6 gene, which is at least partially explained by direct CAR inhibition.

Nonspecific pan-CYP suppression in hepatocyte studies can be caused by cytotoxicity, and has also been reported in cytokine-mediated inflammation (Aitken et al., 2006) and by cytoskeleton disrupting agents (Dvorak et al., 2003). Notably, the selective suppression of CYP2B6 by LY2090314 was not associated with cytotoxicity or suppression of other P450 enzymes. Furthermore, GSK-3 inhibitors exhibit anti-inflammatory activity (Jope et al., 2007) and are not cytoskeleton-disrupting agents. As such, the present observation of specific suppression of constitutive CYP2B6 transcription and activity by a small-molecule drug is a previously unreported phenomenon in the literature.

LY2090314 was approximately 40-fold more potent in suppression of CYP2B6 mRNA and activity than in direct inhibition of CAR (CYP2B6 activity and mRNA IC50 ~0.05 μM vs. CAR IC50 ~2 μM). One explanation for this discrepancy may be that CYP2B6 transcriptional suppression requires less extensive CAR inhibition. However, the difference between inhibitory potency toward CYP2B6 suppression versus CAR inhibition may be even larger considering that these potencies are calculated based on nominal incubation concentrations, and LY2090314 metabolism in hepatocytes is likely higher than in CAR host cells (Zamek-Gliszczynski et al., 2013). Likewise, weak but potent PXR agonism (EC50 ~1 nM) may partially counteract CAR inhibition (Tolson and Wang, 2010), further highlighting the disparity between potency for CYP2B6 suppression versus CAR inhibition. This difference in inhibitory potency suggests that LY2090314 suppression of CYP2B6 activity may be more complex than just direct inhibition of CAR.

Recently, metformin was shown to inhibit CAR-mediated CYP2B6 induction by indirect CAR inhibition at the level of phosphorylation
and nuclear translocation (Yang et al., 2014). LY2090314 potency toward inhibition of GSK-3 (IC$_{50}$ ~1 nM) is approximately 50-fold more potent than CYP2B6 suppression in hepatocytes (IC$_{50}$ ~50 nM). GSK-3 is a serine/threonine kinase that regulates numerous pathways involved in protein synthesis (Doble and Woodgett, 2003). This raises the possibility that, in addition to direct CAR inhibition, LY2090314 may also impair CAR function by indirect mechanism(s), a question that merits further future investigation. Unfortunately, at present, CYP2B6 modulation data are not available for other GSK-3 inhibitors, so it is not possible to investigate whether this is an on-target class effect.

Suppression of constitutive CYP2B6 activity at the gene expression level has not been previously reported in the literature for small-molecule drugs. Although CAR inhibitors have been described, they do not elicit decreased CYP2B6 enzyme activity due to concomitant PXR activation (Moore et al., 2000; Faucette et al., 2004; Li et al., 2008; Tolson and Wang, 2010; Yang et al., 2014). Regulatory agencies recognize that suppression of P450 enzyme activity at the transcriptional level is a relatively new field in the development of small-molecule drugs, but nonetheless recommend attempting to understand potential clinical relevance of P450 suppression when mRNA levels are decreased in excess of 50% in vitro (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/ WC500129606.pdf; www.pmda.go.jp/english).

In the present study, PBPK model simulations were used to assess the clinical risk of drug interactions associated with the observed CYP2B6 suppression. Due to the unique clinical pharmacokinetic and dosing properties of LY2090314 (Supplemental Table 2; Zamek-Gliszczynski et al., 2013), the observed in vitro CYP2B6 suppression does not pose a clinical drug interaction risk. However, in a hypothetical scenario of a drug dosed daily with the same inhibitory and pharmacokinetic parameters as LY2090314, except clearance is reduced 10-fold and plasma fraction unbound is increased 4-fold, clinical suppression of CYP2B6 is expected to result in altered pharmacokinetics of CYP2B6-substrate drugs (Fig. 7).
Since constitutive CYP2B6 suppression by a small-molecule drug is a new finding, ultimately, clinical translation of this phenomenon remains to be established. The present work demonstrated how hepatocyte P450 induction assays provide an alert to CYP2B6 suppression, and how PBPK model simulations can be used for assessment of the clinical drug interaction risk. With greater attention paid to this issue in drug development (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf; www.pmda.go.jp/english), clinical translation of CYP2B6 suppression, or lack thereof, is likely to be directly established in future human studies.

In conclusion, the present studies provided evidence for extensive suppression of constitutive CYP2B6 activity at the level of gene transcription by an investigational small-molecule drug. PBPK model simulations were subsequently used for clinical translation of these findings, and they showed that, in this particular case, a clinical drug interaction risk did not exist. As CYP2B6 suppression becomes more closely scrutinized in drug development, clinical translation of this phenomenon is likely to be directly examined in future clinical drug interaction studies.

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References


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