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Investigational small-molecule drug selectively suppresses constitutive CYP2B6 activity at the gene transcription level: PBPK-based assessment of clinical drug interaction risk

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Running Title: Xenobiotic selectively suppresses CYP2B6

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Abbreviations: CYP, cytochrome P450; GSK-3, glycogen synthase kinase-3; PBPK, physiologically-based pharmacokinetics; consititutive androstane receptor, CAR; pregnane X receptor, PXR; LY2090314, 3-[9-fluoro-2-(piperidin-1-ylcarbonyl)-1,2,3,4-tetrahydro[1,4]diazepino[6,7,1-hi]indol-7-yl]-4-imidazo[1,2-a]pyridin-3-yl-1H-pyrrole-2,5-dione; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b]1,3thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; PK11195, 1-(2-chlorophenyl-N-methylpropyl)-3-isoquinoline-carboxamide

Abstract

The glycogen synthase kinase-3 inhibitor, LY2090314, specifically impaired cytochrome P450 (CYP) 2B6 activity during *in vitro* evaluation of CYP enzyme induction in human hepatocytes. CYP2B6 catalytic activity was significantly decreased following 3-day incubation with 0.1-10μM LY2090314, on average by 64.3±5.0% at 10μM. 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 ≤10μM, 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 significantly reduced CYP2B6 mRNA levels (I_{max} = 61.9±1.4%; IC_{50} = 0.049±0.043μM), which were significantly correlated with catalytic activity (r^2 = 0.87, slope = 0.77; I_{max} = 57.0±10.8%, IC_{50} = 0.057±0.027μM). Direct inhibition of constitutive androstane receptor by LY2090314 is conceptually consistent with the observed CYP2B6 transcriptional suppression (I_{max} = 100.0±10.8% and 57.1±2.4%; IC_{50} = 2.5±1.2μM and 2.1±0.4μM, isoforms 1 and 3, respectively) and may be sufficiently extensive to overcome the weak but potent activation of PXR by ≤10μM LY2090314 (19.3±2.2% of maximal rifampin response, apparent EC_{50} = 1.2±1.1nM). The clinical relevance of these findings was evaluated through physiologically-based pharmacokinetic model simulations. CYP2B6 suppression by LY2090314 is not expected clinically, with projected <1% decrease in hepatic enzyme activity and <1% decrease in hydroxybupropion exposure following bupropion co-administration. However, simulations showed that observed CYP2B6 suppression could be clinically relevant for a drug with different pharmacokinetic properties than LY2090314.

Introduction

Cytochrome P450(CYP) 2B6 is a major drug metabolizing enzyme, which contributes to the elimination of 3-15% of marketed small-molecule medications and 2-10% of total hepatic CYP content (Wang and Tompkins, 2008). CYP2B6 is involved in the clearance of common drugs, spanning the chemotherapeutic, antidepressant, antiviral, opioid, anesthetic, and other important classes. As such, CYP2B6 modulation can be clinically important and is known to occur through enzyme induction and direct inhibition.

CYP2B6 induction occurs through activation of the consititutive androstane receptor (CAR; NR1I3) and/or pregnane X receptor (PXR; NR1I2), resulting in enhanced gene transcription, ultimately leading to increased enzyme level and activity (Wang and Tompkins, 2008; Tolson and Wang, 2010). While PXR activation is known to occur only through direct ligand binding, CAR can be stimulated by both direct interaction with ligand and indirect activation, for example by altered phosphorylation and enhanced nuclear translocation (Tolson and Wang, 2010). Prototypical CAR-mediated CYP2B6 induction by phenobarbital occurs by indirect activation, as phenobarbital is not a CAR ligand (Tolson and Wang, 2010). CYP2B6 can be highly induced, leading to increased clearance and diminished pharmacological activity of substrate drugs. For example, chronic rifampin treatment in humans decreased bupropion exposure 3-fold (Loboz et al., 2006).

CYP2B6 inhibition occurs commonly by mechanism-based enzyme inactivation (Richter et al., 2004), but potent competitive inhibitors have also been reported (Stiborova et al., 2002). Evaluation of 227 commonly-prescribed drugs identified thirty 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 hydoxybupropion/bupropion by 71-93% in humans (Turpeinen et al., 2005).

Based on its demonstrated clinical relevance, regulatory agencies recommend characterization of both perpetrator and victim drug interaction potential with CYP2B6 during development of new molecular entities (FDA 2012; EMA 2012; PMDA 2014). Characterization of perpetrator interactions involves *in vitro* evaluation of acute competitive and time-dependent inhibition in direct incubations with the enzyme (ex. 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 CYP enzyme activity at the transcriptional level is a relatively new area in development of small-molecule drugs. The FDA currently has no recommendation on evaluation of CYP suppression or follow-up assessment of clinical drug interaction risk; however, both the EMA and PMDA recommend attempting to understand potential clinical relevance of CYP suppression when mRNA levels are decreased in excess of 50% *in vitro* (EMA 2012; PMDA 2014). 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 (ex. 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 clotrimazole and 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 activation by enhancing phosphorylation and limiting nuclear translocation; however, while 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]indol-7-yl]-4-imidazo[1,2-a]pyridin-3-yl-1H-pyrrole-2,5-dione; structure is provided in Supplemental Figure 1) is an intravenous glycogen synthase kinase-3 (GSK-3) inhibitor (GSK-3α IC_{50} = 1.5 nM, GSK-3β = 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 CYP 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

Materials. 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 (Austin, TX); human liver microsomes (mixed sex pool of 150 donors) and Supersomes® for metabolic stability studies were purchased from BD Biosciences (Woburn, MA). LY2090314 and D_{10}-LY2090314 was provided by Eli Lilly and Company (Indianapolis, IN).

Hydroxybupropion was purchased from BD Gentest (Bedford, MA); phenobarbital, omeprazole, rifampin, bupropion, phenacetin, acetaminophen, testosterone, and 6β-hydroxytestosterone were purchased from Sigma Chemical Company (St. Louis, MO). Deuterated internal standards were from the following sources: D_6-hydroxybupropion (Toronto Research Chemicals, North York, Ontario), D_4-acetaminophen (CDN Isotopes, Pointe-Claire, Quebec), D_3-6β-hydroxytestosterone (Cerilliant Corp., Round Rock, TX). All noted hepatocyte culture reagents were purchased from Sigma Chemical Company (St. Louis, MO), except ITS+ (BD Biosceinces, Bedford, MA), Dulbecco's Modified Eagle's Medium Ham's F12 (CellGro, Manassas, VA), Biocoat® Collagen I Cellware 12-well plates (NUNC, Naperville, IL).

CYP Induction. The ability of LY2090314 (0.01-10 μM, 72 h) to induce CYP1A2, CYP2B6, and CYP3A activity was examined in primary cultures of fresh human hepatocytes (Hu877, Hu879, and Hu882). CYP2B6 activity and mRNA levels were characterized further in two additional hepatocyte preparations (Hu950 and Hu1022). Time course of CYP2B6 mRNA modulation was determined in hepatocytes from a single donor (Hu8088).

Human hepatocytes (viability ≥ 85%; Supplemental Table 1) were isolated by collagenase perfusion and cultured in sandwich configuration (LeCluyse et al., 2005). Briefly,

isolated hepatocytes were resuspended in plating medium [0.75 million cells/mL; Dulbecco's Modified Eagle's Medium with 5% fetal bovine serum, insulin (4 μg/mL), and dexamethasone (1 μM)] and plated on 12-well plates (0.75 million cells/well; 1 mL/well). Following 3-6 hour incubation at 37ºC to allow cells to attach, plating medium containing unattached cells was aspirated and replaced with fresh ice-cold culture medium (serum-free Dulbecco's Modified Eagle's Medium Ham's F12 with 50 nM dexamethasone and ITS+) containing the extracellular matrix proteins (ECM®) in order to form the sandwich culture system upon incubation at 37ºC.

Following 36-48 h in culture, hepatocytes were treated daily with fresh culture medium containing LY2090314 (0.01, 0.1, 1, 10 μM), phenobarbital (1 mM), omeprazole (50 μM), or rifampin (10 μM) for 72 h or otherwise indicated duration (6, 24, or 48 h). CYP activity or CYP2B6 mRNA was determined at the end of the treatment period. All CYP modulation results are expressed relative to vehicle control (0.1% DMSO) from the same hepatocyte preparation.

For determination of CYP activity, hepatocytes were rinsed twice and incubated at 37°C with bupropion (500 μM, 40-min incubation), phenacetin (100 μM, 30-min incubation), or testosterone (200 μM, 15-min incubation). Reactions were quenched with organic solvent and cell supernatants were used for analysis of hydroxybuproprion, acetaminophen (from phenacetin), and 6β-hydroxytestosterone.

GAPDH-normalized CYP2B6 mRNA content in hepatocyte lysates was determined by reverse transcription and quantitative polymerase chain reaction using instrumentation and reagents from Applied Biosystems (Foster City, CA) per the manufacturer's instructions. Briefly, hepatocytes were lysed with Nucleic Acid Lysis Solution. Total RNA from lysates was isolated using a 6100 Prepstation using Absolute RNA Wash DNAse Reagent to remove any genomic DNA. Isolated RNA quantity and purity was characterized with a NanoDrop

spectrophotometer (NanoDrop, Wilmington, DE). RNA was reverse transcribed using the High Capacity cDNA Archive Kit; cDNA was then assayed for CYP2B6 and GAPDH using a 7900 HT instrument. Relative quantities of each gene product were determined using validated gene-specific TaqMan® primer/probe sets (Hs00167937_g1 for CYP2B6 and Hs99999905_m1 for GAPDH; Applied Biosystems, Foster City, CA).

Hepatocyte Cytotoxicity. Cell morphology was evaluated and photographed in a LY2090314 concentration range study (0.01-100 μM treatment for 3 days) in a pilot study in hepatocytes from a single donor; images of cell morphology also were captured in all subsequent studies. In addition, LY2090314 cytotoxicity was further evaluated using quantitative cytotoxicity endpoints. Specifically, following 72-hour incubation of sandwich-cultured human hepatocytes from a single donor with LY2090314 (0.1-100 μM), total cellular ATP was quantified using the Mitochondrial ToxGlo™ Assay (Promega, Madison, WI), leakage of lactate dehydrogenase into extracellular medium was quantified using the Cobas LDH assay (Roche Diagnostics, Indianapolis, IN), and NADPH/NADH levels were determined by MTS tetrazolium (MTT) conversion to formazan (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega).

CYP2B6 Inhibition. Bupropion (125 μM) was co-incubated with LY2090314 (0-50 μM with or without 15-min pre-incubation; with or without NADPH at 50 μM LY2090314) in human liver microsomes (0.25 mg/mL protein, 1 mM NADPH, 20 min, pH 7.4, 37ºC). 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 Supersomes® (0.25 mg/mL protein, 1 mM NADPH, pH 7.4, 37ºC).

Incubations were sampled 0-120 min, quenched with organic solvent, and supernatants were used to quantify 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 non-human mammalian host cells co-transfected with a chimeric plasmid containing the individual CAR ligand-binding domains in sequence with the GAL4 DNA-binding domain and a second plasmid with the GAL4-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 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 37ºC. 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 co-transfected with a chimeric plasmid containing the PXR ligand-binding domain in sequence with the GAL4 DNA-binding domain and a second plasmid with the GAL4-response element driving the expression of luciferase (reporter gene) were incubated with LY2090314 or rifampin (0.001-50 μM) for 24 hours at 37ºC. Following incubation, PXR activity was measured by emission of light from luciferase, normalized for light emission in vehicle-treated cells.

Bioanalysis. Supernatant from quenched CYP induction activity samples (spiked with internal standards) was evaporated, reconstituted in mobile phase, and analyzed by LC-MS/MS. 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, YMC-ODS-AQ, 4.0x50 mm, 3 μm (Waters Inc., Milford, MA); acetaminophen: Phenosphere-Next, 2.0x50 mm, 5 μm (Phenomenex, Torrance, CA)] with mobile phase optimized for each analyte [hydroxybupropion: isocratic, 40% Methanol, 60% 1 mM Ammonium Acetate Buffer, 0.1% Formic acid; 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 257 → 239, D_6-hydroxybupropion 263 → 245, acetaminophen 152 → 110, D_4-acetaminophen 156 → 114, 6β-hydroxytestosterone 305 → 269, D_3-6β-hydroxytestosterone 308 → 272 m/z.

Metabolic stability assay sample supernatants were directly analyzed for LY209014 and internal standard by LC-MS/MS. LY2090314 and D_{10}-LY2090314 were eluted from C18 column [Aquasil 2.1x20 mm, 5 μm, Javelin Guards (Thermo Fisher Scientific, Inc.; Waltham, MA)] with a mobile phase gradient that escalated from 10% B to 98% B [5 mM Ammonium Bicarbonate, 1% methanol, in water (A) or 5 mM Ammonium Bicarbonate in 100% methanol (B)]. Analytes were detected in positive ion mode using multiple reaction monitoring: LY2090314, 513 → 112 and D_{10}-LY2090314, 523 → 122 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 v. 6.3 (Certara, Cary, NC). The following equation was fitted to the inhibition-concentration data:

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

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

The Student's two-tailed t-test, appropriately corrected for multiple comparisons and unequal variance, was used to assess statistical significance. SigmaPlot v. 11.0 (Systat, Chicago, IL) was used for linear regression analysis. The minimal criterion for significance was $p < 0.05$. Data are reported as mean ± SEM with the associated n reported in all cases.

PBPK Simulations. Simcyp v. 13.1 (Certara) was used to simulate the plasma concentration-time course of LY2090314, bupropion, and hydroxybupropion. The LY2090314 model was constructed using previously reported human intravenous pharmacokinetic parameters (Zamek-Gliszczynski et al., 2013) and *in vitro* CYP2B6 suppression parameters from the present study (Supplemental Table 2). Simcyp default bupropion model [Supplemental Table 3 (Barter et al., 2013)] was used to simulate pharmacokinetics of the hydroxybupropion metabolite, which is a more sensitive marker of CYP2B6 activity than parent (Loboz et al., 2006). LY2090314 suppression of CYP2B6, and its impact on hydroxybupropion exposure, was simulated using the default Simcyp induction module. Analogous to induction, CYP2B6 suppression was simulated based on *in vitro* parameter estimates for mRNA, because the

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consensus opinion is that clinical translation of this endpoint is superior relative to enzyme activity (FDA, 2012). Suppression of CYP2B6 activity was modeled as decrease in enzyme synthesis, assuming that time-dependent unbound concentration of LY2090314 directly affects the synthesis rate:

$$\frac{dE}{dt} = k_{deg} \times E_{baseline} \times \left(1 + \frac{[(1 - I_{max}) - 1] \times C_{liver,u}}{IC_{50} + C_{liver,u}}\right) - k_{deg} \times E$$

where k_{deg} and $E_{baseline}$ are the Simcyp default hepatic CYP2B6 turnover rate and baseline levels, respectively, I_{max} is the maximal extent of CYP2B6 suppression expressed as a fraction, and $C_{liver,u}$ is the hepatic unbound LY2090314 concentration.

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Results

Effect of LY2090314 (0.01-10 μM, 3 days) on CYP2B6, 1A2, and 3A activity in human hepatocytes is presented in Figure 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 not clinically relevant. In these hepatocyte preparations, phenobarbital induced CYP2B6 activity 21.9 ± 3.4 fold; omeprazole induced CYP1A2 79.0 ± 17.2 fold; rifampin induced CYP3A 30.9 ± 2.2 fold.

In a concentration range study (0.01-100 μM) in hepatocytes from a single donor, no morphological changes associated with cytotoxicity were observed following 3-day incubation with LY2090314 concentrations ≤10 μM (data not shown). In contrast, exposure to100 μM LY2090314 for 3 days resulted in cell death, and so ≤10 μM concentrations were used in subsequent CYP induction studies. Due to consistently decreased CYP2B6 activity, cell morphology was further inspected for signs of cytotoxicity in recorded images from CYP 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 (Figure 2). Likewise, NADPH/NADH levels determined by MTS tetrazolium (MTT) 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 (Figure 3A). However, CYP2B6

activity was 45.3 ± 2.2% decreased by co-incubation with 50 μM LY2090314. There was no shift in the inhibition profile with LY2090314 pre-incubation. Furthermore, pre-incubation with 50 μM LY2090314 and 1mM NADPH did not decrease CYP2B6 activity to a greater extent than pre-incubation 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 h), whose metabolic stability profile resembled vector control (no CYP) membranes (Figure 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-min time point.

LY2090314 concentration-dependent suppression of CYP2B6 mRNA levels is presented in Figure 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 μM 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 non-canonical mechanism of inhibiting CYP2B6 catalytic activity. The time course of change in mRNA level is presented in Figure 4B. CYP2B6 mRNA steadily declined over time of LY2090314 treatment, reaching a plateau by 48 h; thereafter mRNA level was on average decreased by 58.2 ± 4.8%.

Effects of LY2090314 on expression of CAR and PXR downstream reporter genes are presented in Figure 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 μM and 2.1 ± 0.4 μM, respectively). In contrast, LY2090314 (2.5 nM-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 E_{max} 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. LY2090314 model was qualified by establishing predictability of reported pharmacokinetics following 60-min intravenous infusion of a 40-mg dose [Figure 6A (Zamek-Gliszczynski et al., 2013)]. The bupropion PBPK model simulated hydroxybupropion $AUC_{0\text{-}inf}$ and C_{max} values of 11.5 [95%CI 10.0-13.3] μg*h /mL and 242 [209-279] ng/mL, which were comparable to the reported values of 14.7 [12.7-18.4] μg*h /mL and 395 [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-min intravenous infusion once weekly for 3 weeks) and hydroxybupropion following concomitant administration of bupropion (Figure 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 than LY2090314. A hypothetical perpetrator drug was constructed, which shared all inhibitory and pharmacokinetic parameters with LY2090314, except clearance was reduced by 10-fold to 4.2 L/h, and plasma fraction unbound was increased by 4-fold to 10%. The model predicted a low drug interaction with this hypothetical drug when administered 40 mg once daily as a 60-min intravenous infusion for 20 days (9 days prior to and 10 days post bupropion dosing; Figure 7A). The predicted hydroxybupropion AUC and C_{max} 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 (Figure 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 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 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 been 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 LY2090134 was not associated with cytotoxicity or suppression of other CYP 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 IC_{50} ~ 0.05 μM vs. CAR IC_{50} ~ 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 (EC_{50} ~ 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 is not available for other GSK-3 inhibitors, so it is not possible to investigate whether this may be 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 CYP enzyme activity at the transcriptional level is a relatively new field in development of small-molecule drugs, but nonetheless recommend attempting to understand potential clinical relevance of CYP suppression when mRNA levels are decreased in excess of 50% *in vitro* (EMA 2012; PMDA 2014).

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 reduced by 10-fold and plasma fraction unbound increased by 4-fold, clinical suppression of CYP2B6 is expected to result in altered pharmacokinetics of CYP2B6-susbtrate drugs (Figure 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 CYP 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 (EMA 2012; PMDA 2014), 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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Authorship Contributions

Participated in research design: Zamek-Gliszczynski, Mohutsky, Rehmel, Ke

Contributed new reagents: N/A

Conducted experiments: Zamek-Gliszczynski, Mohutsky, Rehmel, Ke

Performed data analysis: Zamek-Gliszczynski, Mohutsky, Rehmel, Ke

Wrote or contributed to the writing of the manuscript: Zamek-Gliszczynski, Mohutsky, Rehmel, Ke

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Figure Legends

Figure 1. Effect of LY2090314 on activity of inducible CYP enzymes. Following 3-day incubation of human hepatocytes, CYP activity was determined with specific marker reactions: bupropion hydroxylation for CYP2B6 (A), phenacetin *O*-deethylation for CYP1A2 and testosterone 6β-hydroxylation for CYP3A (B). Mean ± S.E.M. of hepatocyte preparations from three donors (triplicate measurements per donor), *$p < 0.05$ versus vehicle control.

Figure 2. LY2090314 cytotoxicity in sandwich-cultured human hepatocytes. Total ATP levels (A) and lactate dehydrogenase leakage into cellular media (B) following 3-day incubation of hepatocytes with LY2090314 (0.1-100 μM). Mean ± S.E.M., n = 3-6.

Figure 3. Direct interactions of LY2090314 with CYP2B6 enzyme. Bupropion hydroxylation in human liver microsomes in the presence of LY2090314 with or without 15-min pre-incubation (A). Metabolic stability of LY2090314 in incubations with human liver microsomes, as well as vector-control (no CYP), recombinant CYP2B6, CYP3A4, and CYP3A5 supersome membranes (B). Mean ± S.E.M., n = 2-6.

Figure 4. Association between CYP2B6 mRNA and catalytic activity. Concentration-dependent effect of LY2090314 on CYP2B6 transcript and activity levels (A). Lines represent the simulated inhibition relationship using mean parameters estimated in individual hepatocyte donors. Correlation between CYP2B6 activity and mRNA (A inset). Mean ± S.E.M. of hepatocyte preparations from five (activity) or two (mRNA) donors (triplicate measurements per

donor), *p < 0.05 versus vehicle control; correlation or slope ≠ 0. Effect of incubation time on CYP2B6 mRNA level (B). Time course was determined in hepatocytes from a single donor (two donors at 48 h) and at three LY2090314 concentrations (0.1, 1, 10 μM). As all tested LY2090314 concentrations were in excess of the CYP2B6 mRNA and activity IC_{50} (0.05-0.06 μM), time trend did not show concentration dependence in this range approaching maximal inhibition. As such, data from the three concentrations were combined for clarity. Mean ± S.E.M. of triplicate measurements per donor per concentration, *p < 0.05 versus vehicle control.

Figure 5. Effect of LY2090314 on constitutive androstane receptor (CAR) and pregnane X receptor (PXR) activity. CAR isoform 1 activity in the presence of LY2090314 (A). Effect of the positive control inverse agonist, PK11195 on CAR1 activity (A inset). CAR isoform 3 activity following co-incubation the known agonist CAR3, CITCO (0.4 μM), with LY2090314 (B). CAR3 activity in the presence of CITCO (B inset). Lines represents fit of the simple IC_{50} or EC_{50} model to the data (A-B). Effect of LY2090314 on PXR activity expressed as percentage of maximal activation by rifampin determined in the same run (C). Mean ± S.E.M., n = 4.

Figure 6. LY2090314 PBPK model qualification and drug interaction potential prediction. (A) Predicted (solid line) and observed (symbols) LY2090314 plasma concentrations following 60-min intravenous infusion of a 40-mg dose (Zamek-Gliszczynski et al., 2013). The predicted LY2090314 $AUC_{0\text{-inf}}$ [1131 (31% CV) ng*h /mL] and C_{max} [447 (32% CV) ng/mL] were comparable to the observed $AUC_{0\text{-inf}}$ [976 (43% CV) ng*h /mL] and C_{max} [603 (48% CV) ng/mL]. (B) Simulated systemic exposures to hydroxybupropion following once weekly oral

dosing of bupropion 150 mg in the absence (solid line) or presence (dashed line) of once weekly LY2090314 (40 mg, 60-min intravenous infusion) for 3 weeks.

Figure 7. Drug interaction prediction using a hypothetical perpetrator drug with input parameters like LY2090314, except clearance reduced 10-fold to 4.2 L/h and plasma fraction unbound increased 4-fold to 10%. (A) Simulated hydroxybupropion pharmacokinetics following bupropion doing (150 mg PO) in the absence (solid line) or presence (dashed line) of concurrent treatment with the hypothetical perpetrator drug given once daily as a 60-min intravenous infusion for 20 days (9 days prior to and 10 days post bupropion dosing). The predicted hydroxybupropion AUC and C_{max} ratios were 0.73 (95% CI: 0.71-0.75) and 0.72 (95% CI: 0.69-0.74), respectively. (B) Predicted hepatic CYP2B6 activity in the absence (solid line) or presence (dashed line) of concurrent treatment with the hypothetical perpetrator drug.

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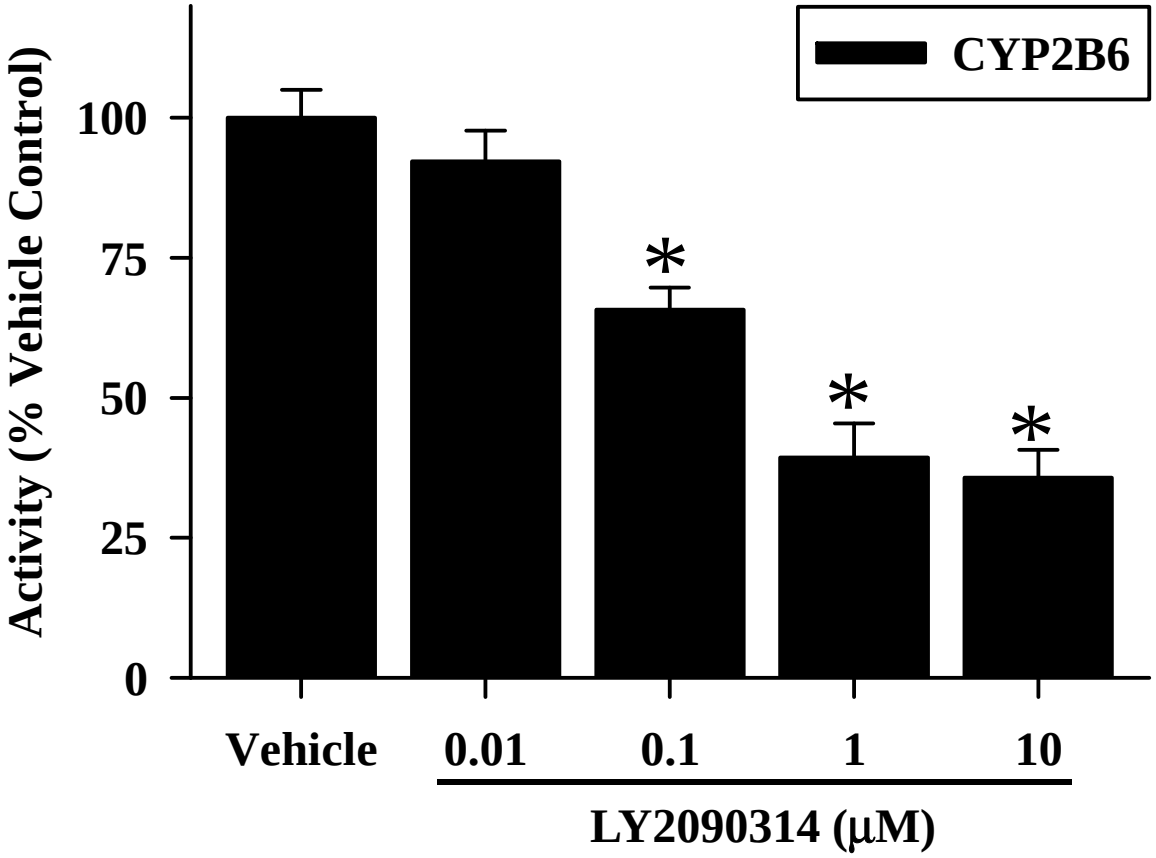
Fig. 1

A.



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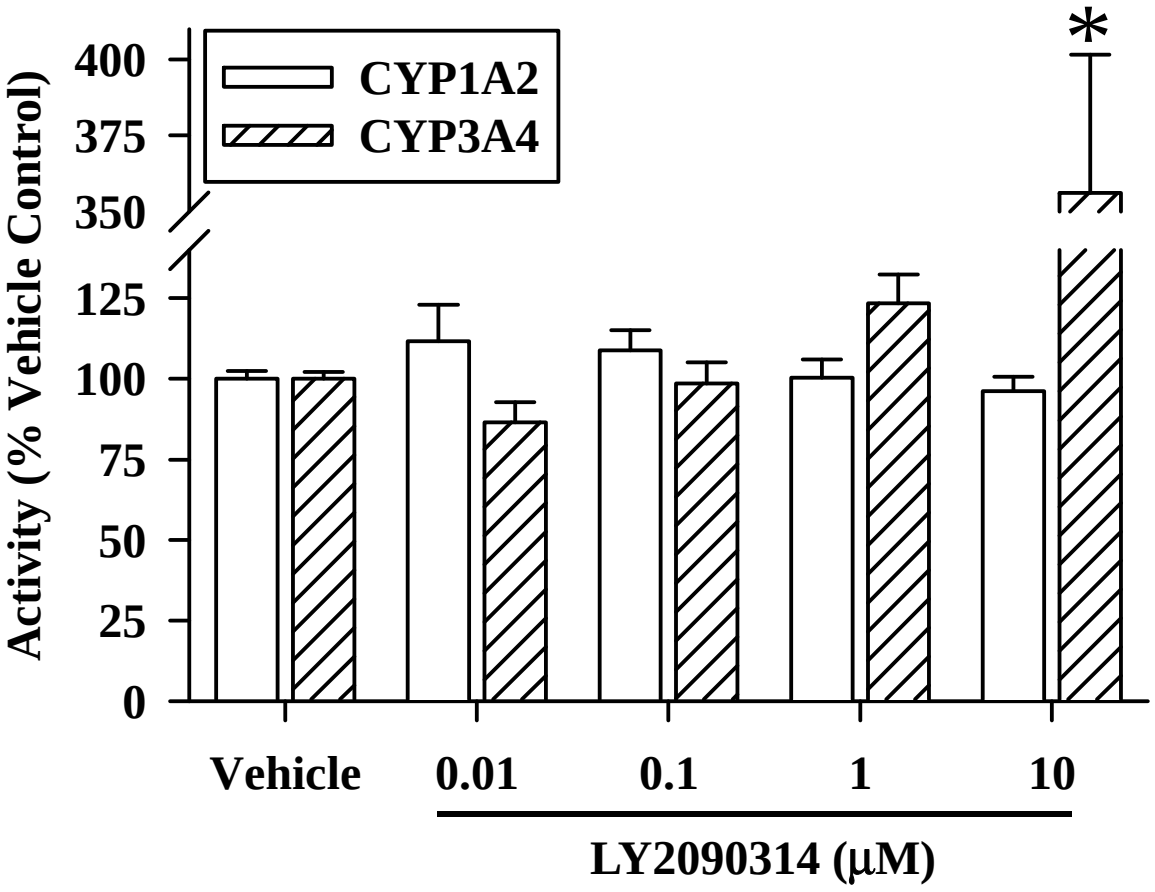

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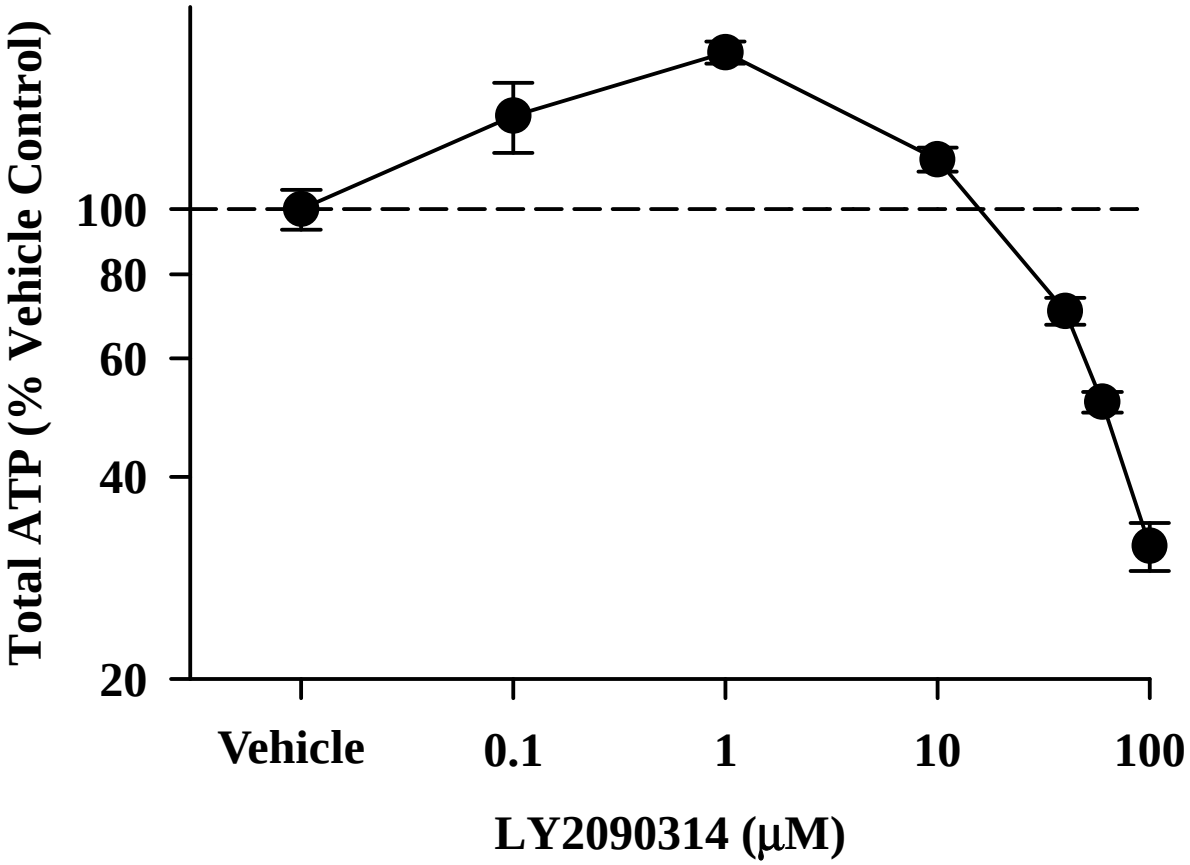
Fig. 2

A.



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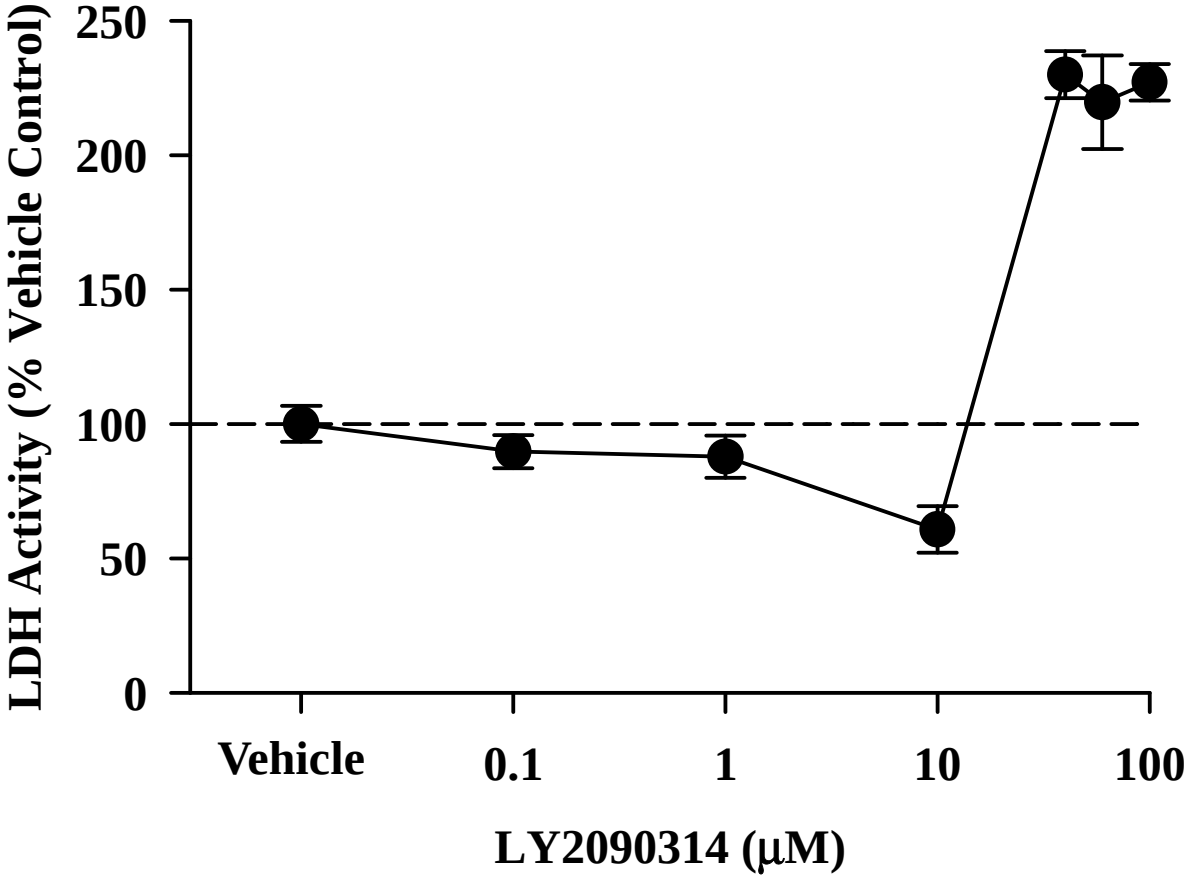

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Fig. 3

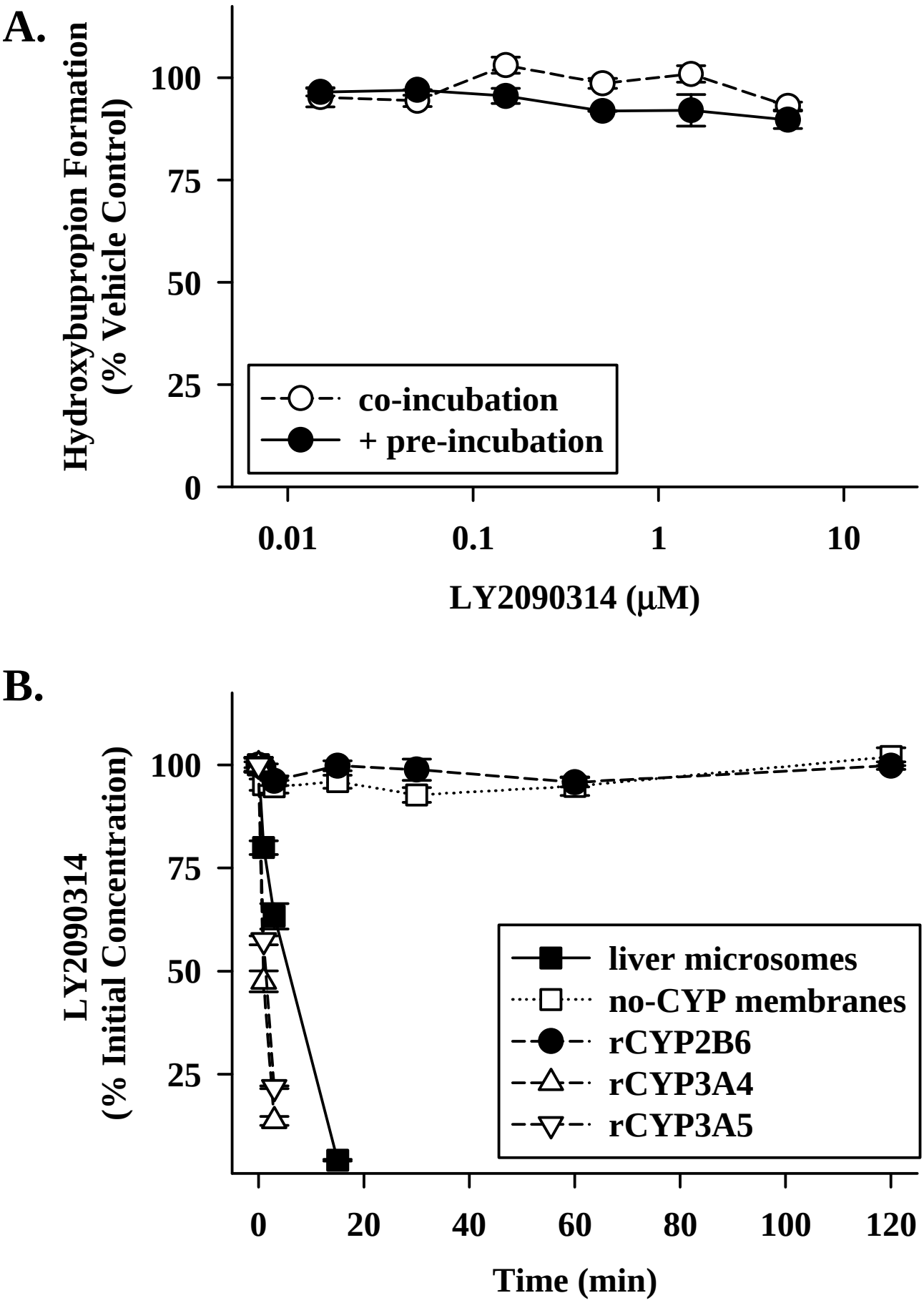

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Fig. 4

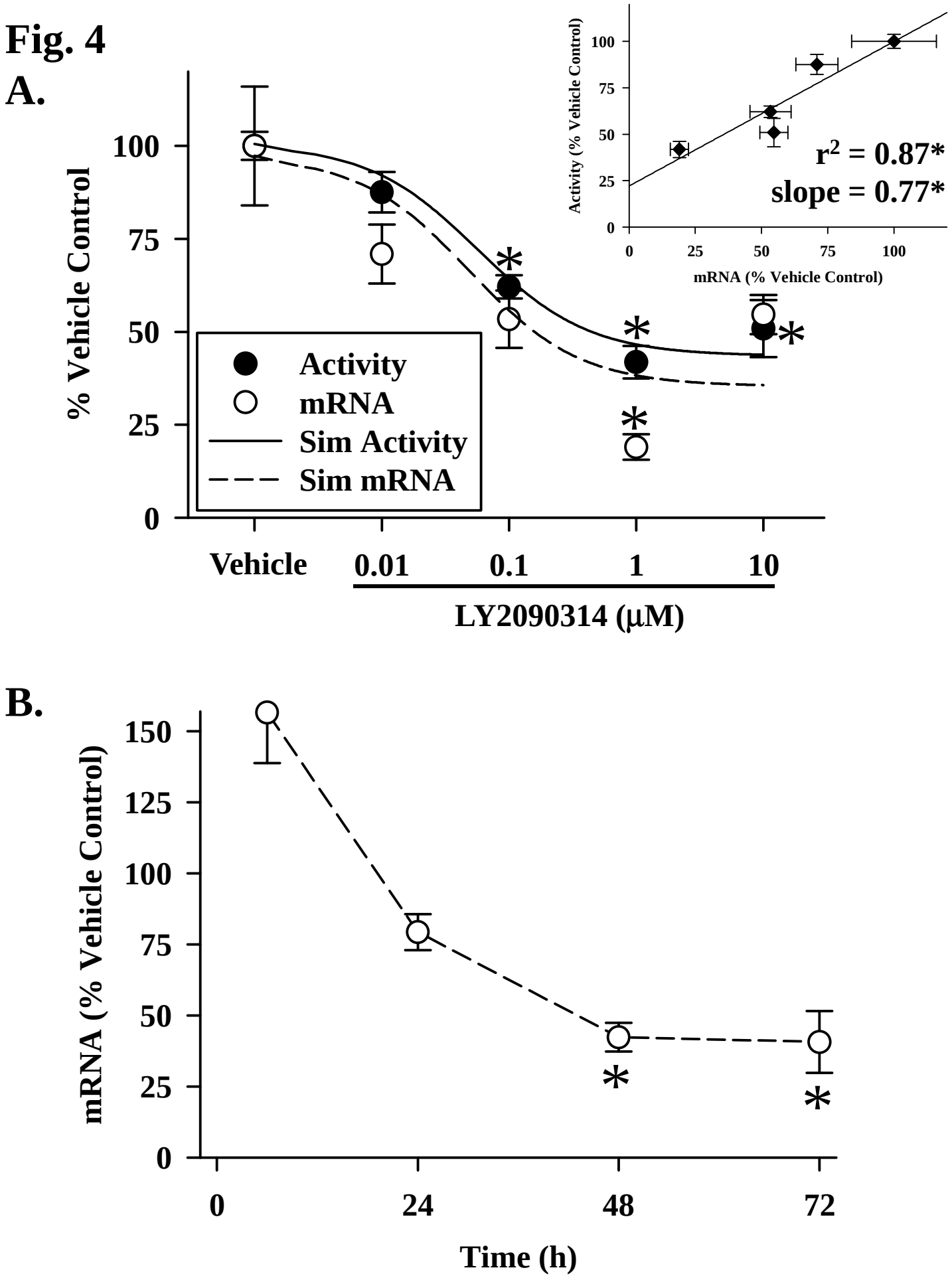

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Fig. 5

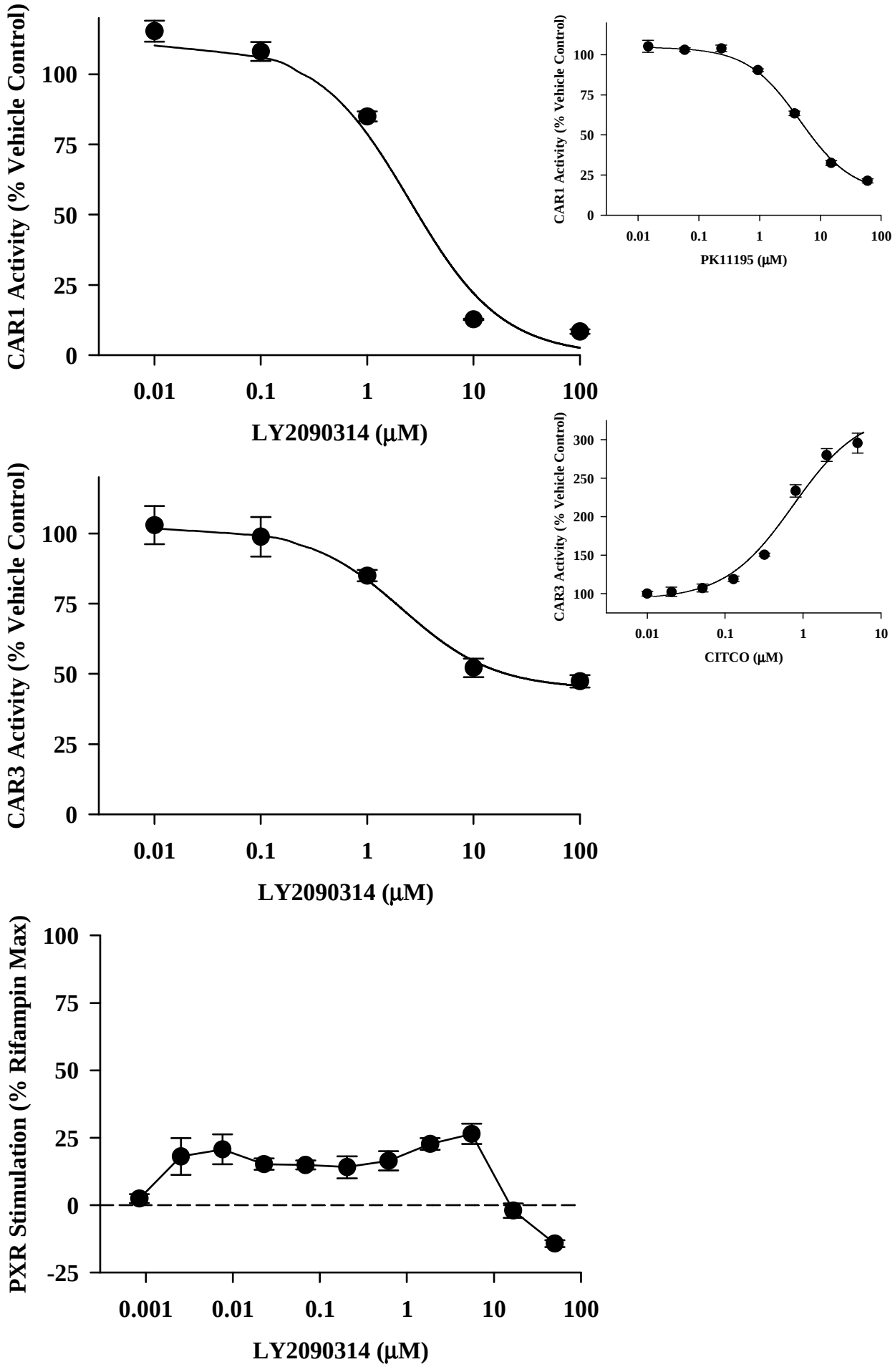

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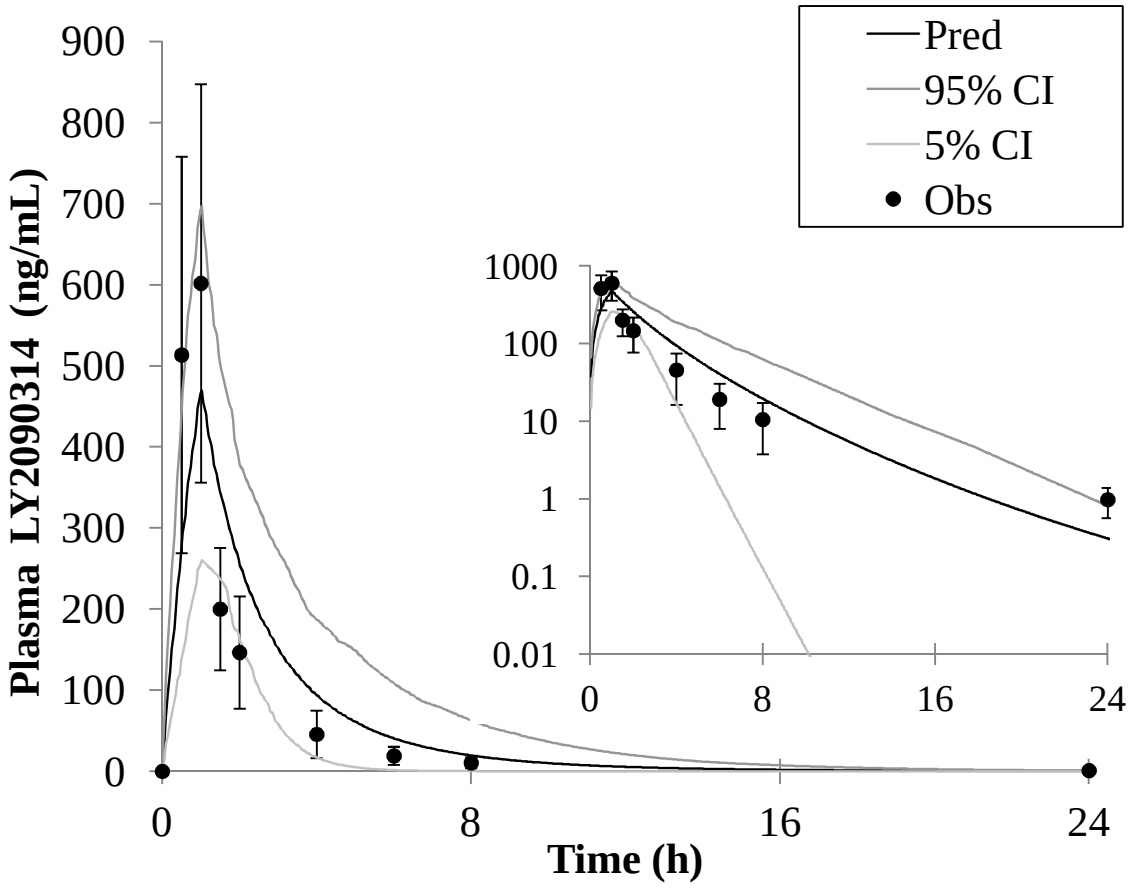
Fig. 6

A.



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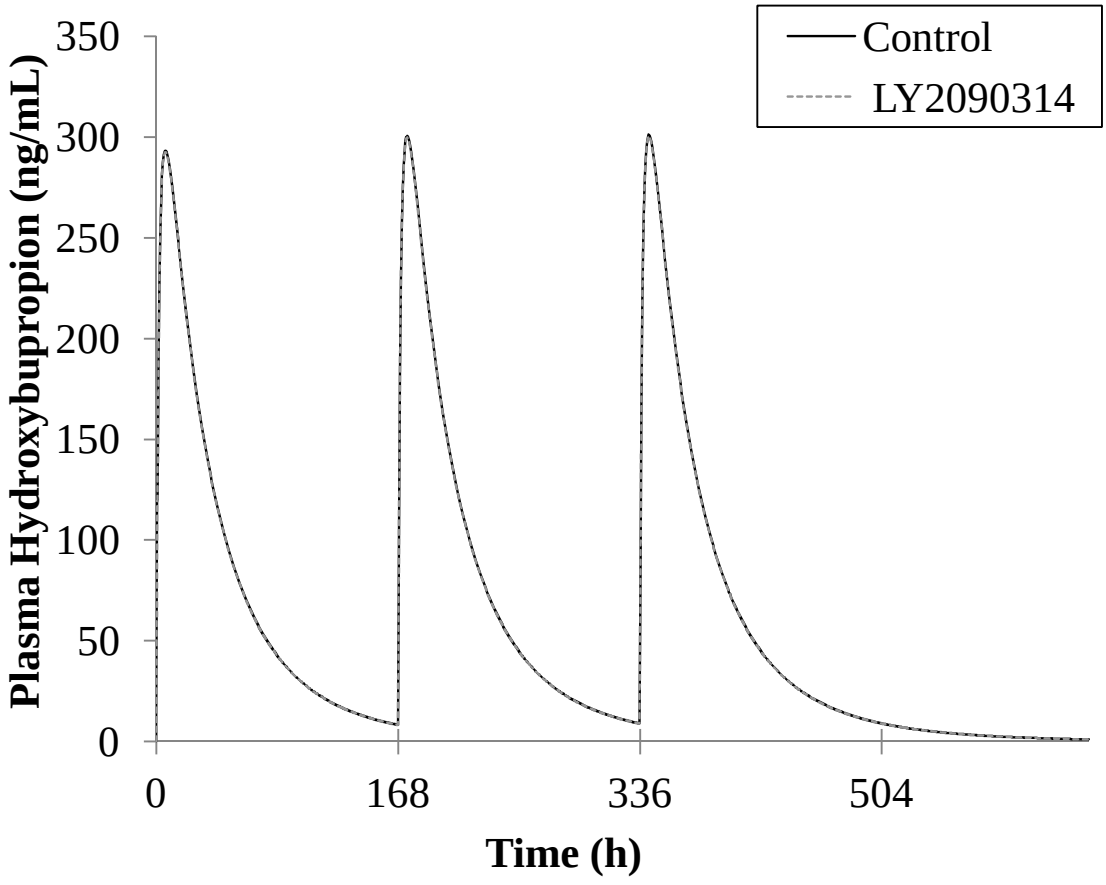

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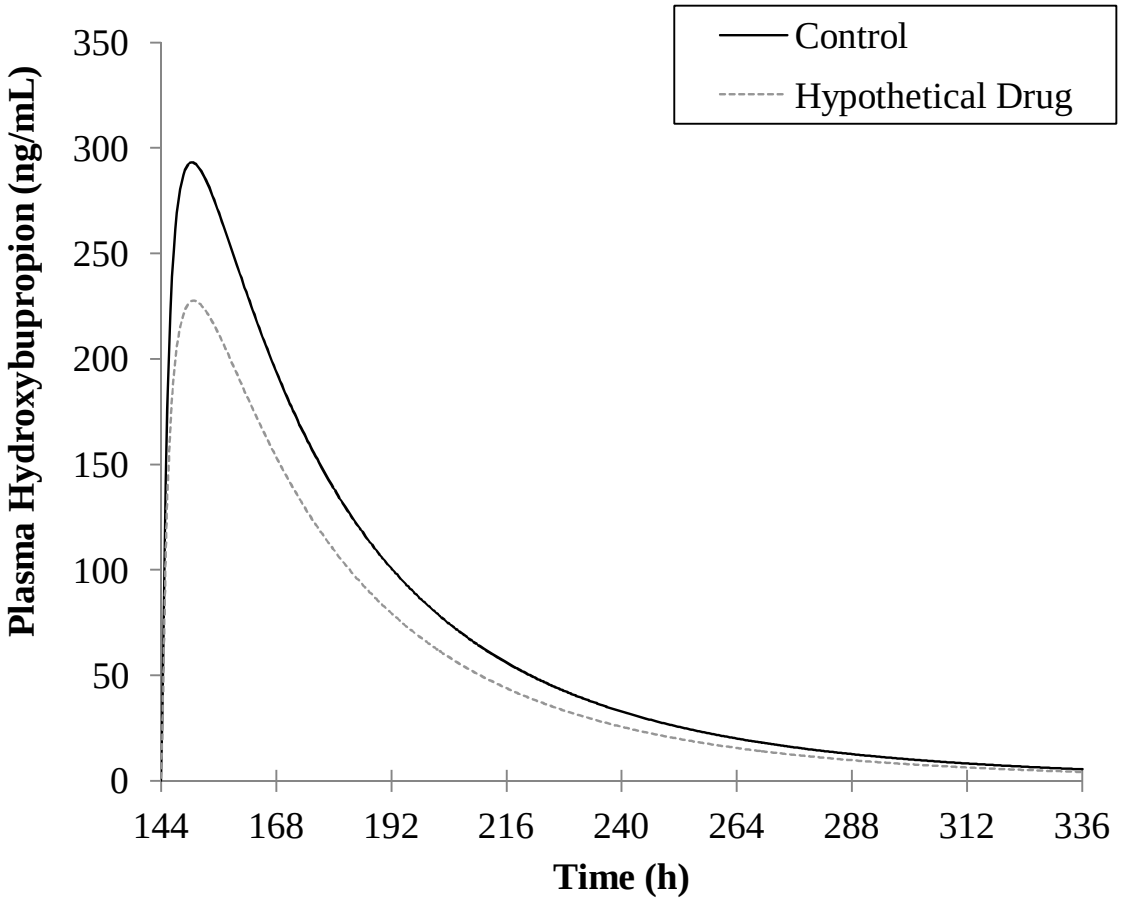
Fig. 7

A.



B.