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Stereoselective bupropion hydroxylation by cytochrome P450 CYP2B6 and cytochrome P450 oxidoreductase genetic variants

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Running Title: Bupropion enantiomers metabolism by CYP2B6 variants

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Abstract

Bioactivation of the antidepressant and smoking cessation drug bupropion is catalyzed predominantly by cytochrome P4502B6 (CYP2B6). The metabolite hydroxybupropion derived from t-butylhydroxylation is considered to contribute to the antidepressant and smoking-cessation effects of the parent drug. Bupropion hydroxylation is the canonical *in vitro* and *in vivo* probe for CYP2B6 activity. P450 also requires obligate partnership with P450 oxidoreductase (POR). Human *CYP2B6* and *POR* genes are highly polymorphic. Some *CYP2B6* variants affect bupropion disposition. This investigation evaluated the influence of several human *CYP2B6* and *POR* genetic variants on stereoselective bupropion metabolism, using an insect cell coexpression system containing CYP2B6, POR and cytochrome b_5. Based on intrinsic clearances, relative activities for *S,S*-hydroxybupropion formation were in the order CYP2B6.4>CYP2B6.1>CYP2B6.17>CYP2B6.5>CYP2B6.6≈CYP2B6.26≈CYP2B6.19>CYP2B6.7> CYP2B6.9>>CYP2B6.16 and CYP2B6.18; relative activities for *R,R*-hydroxybupropion formation were in the order CYP2B6.17>CYP2B6.4>CYP2B6.1>CYP2B6.5≈CYP2B6.19≈CYP2B6.26>CYP2B6.6> CYP2B6.7≈ CYP2B6.9>>CYP2B6.16 and CYP2B6.18. Bupropion hydroxylation was not influenced by POR variants. CYP2B6-catalyzed bupropion hydroxylation is stereoselective. Though V_{max} and K_m varied widely among *CYP2B6* variants, stereoselectivity was preserved, reflected by similar Cl_{int}(*S,S*-hydroxybupropion)/Cl_{int}(*R,R*-hydroxybupropion) ratios (1.8 to 2.9), except CYP2B6.17, which was less enantioselective. Established concordance between human bupropion hydroxylation *in vitro* and *in vivo*, together with these new results, suggests additional *CYP2B6* variants may influence human bupropion disposition.

Significance

Bupropion pharmacokinetics, metabolism and clinical effects are affected by the *CYP2B6*6* polymorphism. Other expressed CYP2B6 polymorphisms had diminished (*5, *6, *7, *9, *19, *26) or defective (*16, *18) in vitro bupropion hydroxylation. P450 oxidoreductase genetic variants had no effect on metabolism, suggesting no clinical consequence of this polymorphism. These CYP2B6 polymorphisms may portend diminished *in vivo* bupropion hydroxylation and predict additional clinically important variant alleles.

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Introduction

Bupropion is FDA-approved to treat depression (Dhillon et al., 2008) and for smoking cessation therapy (Reid et al., 2016), and also used for obesity (Wilding, 2017), seasonal affective disorder (Cools et al., 2018), attention deficit hyperactivity disorder (Verbeeck et al., 2017), drug abuse (Lee et al., 2018), and chronic pain (Urits et al., 2019). Bupropion inhibits norepinephrine and dopamine reuptake transporters, increasing extracellular norepinephrine and dopamine concentrations, and is a nicotinic antagonist, blocking nicotinic acetylcholine receptors in the brain.

Bupropion is extensively metabolized by the liver, with less than 1% eliminated unchanged in urine (Welch et al., 1987). The major primary metabolic pathway is *t*-butyl hydroxylation to hydroxybupropion. Hydroxybupropion plasma exposure exceeds that of bupropion by an order of magnitude. Bupropion is used clinically as a racemate, and metabolism is stereoselective (Xu et al., 2007; Coles and Kharasch, 2008; Kharasch et al., 2008; Masters et al., 2016; Kharasch and Crafford, 2019).

Hydroxybupropion is pharmacologically active (Bondarev et al., 2003; Damaj et al., 2004), and is considered to contribute to the antidepressant and smoking-cessation effects of bupropion (Lee et al., 2007; Zhu et al., 2012; Laib et al., 2014; Malcolm et al., 2015). Hydroxylation of the t-butyl group and subsequent cyclic hemiketal formation creates a second chiral center. Therefore, there is the potential for four diastereomeric hydroxylated metabolites. However, only *2S,3S*- and *2R,3R*-hydroxybupropion have been found in plasma in humans, presumably due to steric hindrance precluding formation of *2S,3R*- and *2R,3S*-hydroxybupropion (Suckow et al., 1997). Plasma concentrations of *2R,3R*-hydroxybupropion are approximately 20-fold greater than *2S,3S*-hydroxybupropion. In contrast, *R*-bupropion concentrations are only about twofold greater than *S*-bupropion (Kharasch et al., 2008; Masters et al., 2016; Kharasch and Crafford, 2019). *2S,3S*-hydroxybupropion was deemed responsible for antidepressant activity and nicotine-cessation activities of bupropion, while *2R,3R*-hydroxybupropion was inactive toward inhibition of norepinephrine and dopamine reuptake (Lee et al., 2007; Zhu et al., 2012; Laib et al., 2014).

Human bupropion racemate and enantiomers hydroxylation is catalyzed exclusively by liver CYP2B6 (Faucette et al., 2000; Coles and Kharasch, 2008). Bupropion hydroxylation is the standard *in*

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vitro and *in vivo* probe to assess CYP2B6 activity, polymorphisms, and drug interactions (Faucette et al., 2000; Fahmi et al., 2016). The CYP2B6 gene is highly polymorphic, with at least 38 allelic variants have been described (https://www.pharmvar.org/gene/CYP2B6), of which 25 are considered important, and 8 are common or unique in at least one racial or ethnic population (Zhou et al., 2017). Carriers of certain *CYP2B6* allelic variants have altered *in vivo* hydroxylation of bupropion racemate (Kirchheiner et al., 2003; Chung et al., 2011; Zhu et al., 2012; Benowitz et al., 2013; Høiseth et al., 2015; Gao et al., 2016; Lv et al., 2016; Ma et al., 2018) and enantiomers (Kharasch and Crafford, 2019). *CYP2B6*4* and more so *CYP2B6*6* are the most studied common variants. *CYP2B6*6* homozygotes had decreased bupropion hydroxylation, while *CYP2B6*4* carriers had somewhat increased hydroxylation. These polymorphisms have been associated with clinical differences in bupropion-mediated smoking cessation rates (Tran et al., 2019).

CYP2B6 metabolizes nearly 8% of marketed drugs, including for example methadone, ketamine, efavirenz, cyclophosphamide, and artemisinin. Previous studies showed that the activity of CYP2B6 allelic variants is allele-, substrate-, and expression system-dependent, and cannot be extrapolated across substrates, or expression systems, and requires substrate-specific evaluation (Coles and Kharasch, 2008; Ariyoshi et al., 2011; Honda et al., 2011; Zhang et al., 2011; Xu et al., 2012; Radloff et al., 2013; Gadel et al., 2015; Wang et al., 2018; Wang et al., 2019). Bupropion hydroxylation *in vitro* by CYP2B6 variants has been evaluated, including CYP2B6.4, CYP2B6.5, CYP2B6.6, CYP2B6.7, and CYP2B6.9 (Zhang et al., 2011; Xu et al., 2012; Radloff et al., 2013). Nevertheless, expression systems have variably used *E. coli*, SF9 and COS-1 cells. Therefore the first purpose of this investigation was to characterize stereoselective bupropion hydroxylation, and by a greater number of clinically relevant *CYP2B6* variants, and using a standard expression system for all.

CYP catalysis requires an obligate partnership with P450 oxidoreductase (POR), which transfers electrons from NADPH to CYP. Cytochrome b_5 can also function electively in this capacity. POR plays important roles in metabolism of drugs and steroid hormones. Genetic variation in POR is associated with altered metabolism of endogenous steroids (Burkhard et al., 2017) and xenobiotics (Riddick et al.,

2013). Therefore the second purpose of the present investigation was to determine the influence of POR variants on bupropion hydroxylation by wild-type CYP2B6.

Materials and Methods

Materials

Racemic bupropion hydrochloride was purchased from Sigma Aldrich (St. Louis, MO). The standards *rac*-hydroxybupropion was purchased from Cerilliant (Round Rock, Texas), and *rac*-hydroxybupropion-d6 from Toronto Research Chemicals (TRC, Toronto, ON, Canada). *Spodoptera frugiperda* (Sf9) cells and Sf-900 III SFM culture media were from ThermoFisher (Waltham, MA). *Trichoplusia ni* (Tni) cells and ESF AF culture media were from Expression Systems (Davis, CA). All other reagents were from Sigma Aldrich (St. Louis, MO).

Generation of baculovirus constructs and recombinant proteins expression

Production of recombinant proteins CYP2B6 variants, P450 reductase (POR) variants and cytochrome b_5 was carried out in insect cells by triple infection as described previously (Gadel et al., 2015; Wang et al., 2018; Wang et al., 2019). All CYP2B6 variants were coexpressed with P450 reductase (POR) and cytochrome b_5. For each variant, protein contents of CYP2B6, POR and b_5 were measured as described previously (Wang et al., 2018; Wang et al., 2019).

Bupropion metabolism

All incubations were carried out in triplicate in 96-well PCR plates with raised wells in 100 mM potassium phosphate buffer (pH 7.4), as adapted from published protocols with modifications (Coles and Kharasch, 2008). *RS*-bupropion substrate (final racemate concentrations of 0, 2 ,5, 10, 25, 50, 100, 250, 500, and 1000 µM) was mixed with CYP2B6/POR/b_5. Final CYP2B6 concentration was 2.5 pmol/ml and total reaction volume is 200 µL. After preincubation for 5 min at 37°C, the reaction was initiated by adding NADPH regenerating system (final concentrations: 10 mM glucose 6-phosphate, 1 mM ß-NADP, 1 U/ml glucose-6-phosphate dehydrogenase, and 5 mM magnesium chloride, preincubated at 37°C for 10 min). The reaction was terminated after 10 min by adding 40 µL 20% TCA containing 75 ng/mL internal standard *rac* hydroxybupropion-d6. The plate was centrifuged at 2500 rpm for 5 min to remove precipitated proteins, and the supernatant was transferred to a 96-well shallow well plate for LC/MSMS

analysis. Preliminary experiments showed that under the assay conditions bupropion hydroxylation was linear with time and CYP2B6 concentration.

Analysis of bupropion hydroxylation by HPLC/tandem mass spectrometry

Calibration samples were prepared using standard of *rac* hydroxybupropion, which is made up of equal amount of the two enantiomers (*R*,*R*)- and (*S*,*S*)-hydroxybupropion. Aqueous working stock solutions of *rac* hydroxybupropion were prepared at concentrations of 0.1, 1, and 100 µg/mL, by diluting the certified methanolic stock solution (1 mg/mL) in deionized water. The calibration standards were prepared at 2, 5, 10, 50, 100, 500, 1000 and 2000 ng/mL *rac* hydroxybupropion from the working stock solutions. 200 µL calibration samples were treated with 40 µL 20% TCA containing 75 ng/mL internal standard *rac* hydroxybupropion-d6, and followed by centrifugation at 2500 rpm for 5 min. The supernatant was transferred to a 96-well shallow well plate for LC/MSMS analysis.

LC–MS/MS analysis was performed on a Shimadzu HPLC system composed of two LC-20AD XR pumps, DGU20A5R degasser, CBM-20A system controller, CTO-20AC column oven, FCV-11AL solvent selection valve, and a SIL-20AC XR temperature regulated autosampler. The LC system was coupled to an API6500 triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) operated with Analyst 1.6.2. MultiQuant 3.0.1(AB Sciex) was utilized for peak integration, generation of calibration curves, and data analysis.

Bupropion metabolites were analyzed utilizing a Chiralpak α_1-acid glycoprotein (AGP) analytical column (100 x 2.0 mm, 5 µm, Chiral Technologies, Westchester, PA) equipped with a chiral AGP guard cartridge (10 x 2.0 mm, Chiral Technologies). A 0.25 µm inline filter was placed prior to the sample entering the column. The column oven was at ambient temperature and the autosampler was at 4 °C. The flow rate was 0.22 mL/min with a mobile phase consisting of 20 mM aqueous ammonium formate, pH 5.7 (A) and methanol (B). The sample injection volume was 5 µL and total run time is 12 min. The time program to achieve the separation was as follows: 10% B for 0.5 min, linear gradient to 20% B until 1 min, held at 20% B until 5 min, linear gradient to 50% B until 8 min, and then re-equilibrated to initial conditions until 12 min. Flow was directed into the mass spectrometer at 2.5 min and diverted to waste at

11.5 min. Under these conditions, the retention time was 6.7 min for (*R,R*)-hydroxybupropion, 4.4 min for (*S,S*)-hydroxybupropion, 6.4 min for (*R,R*)-hydroxybupropion-d6, and 4.4 min for (*S,S*)-hydroxybupropion-d6. The mass spectrometer was operated with a turbo spray ion source in the positive mode (ESI+) with multiple reaction monitoring (MRM). Analytes were detected with the MRM transition of *m/z* 256.2 > 238.2 for both (*R,R*)- and (*S,S*)-hydroxybupropion, and *m/z* 262.2 > 244.2 for (*R,R*)- and (*S,S*)-hydroxybupropion-d6. Operating conditions for analysis on the mass spectrometer were as previously described (27). Global parameters were optimized as follows: curtain gas 20 psig, ion spray voltage 5000 V, source temperature 600 °C, Gas 1 40 psig, and Gas 2 40 psig. Dwell times were 500 ms. Interday coefficients of variation for hydroxybupropion were 5% or less at all concentrations.

Data Analysis

Hydroxybupropion formation by enzyme variants was analyzed by ANOVA with *post hoc* Dunnet's test (SigmaPlot 12.5; Systat, USA). The results are the mean ± 95% confidence interval. Statistical significance was assigned at $P<0.05$. Hydroxybupropion formation *versus* substrate concentration data were analyzed by nonlinear regression analysis using Michaelis-Menten model. The results are reported as the parameter estimate ± standard error of the estimate. *In vitro* intrinsic clearance (Cl_{int}) was V_{max}/K_m.

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Results

Metabolism of bupropion by CYP2B6 was evaluated first at approximately peak therapeutic concentrations. Based on previous publication (Coles and Kharasch, 2008), 2μM *RS*-bupropion (1 μM enantiomers) was considered to be in the range of clinically relevant concentrations. As shown in Fig 1 for *R,R*- and *S,S*-hydroxybupropion formation at 2 μM *RS*-bupropion substrate, activities of CYP2B6 variants vary over a wide range. For *S,S*-hydroxybupropion formation, CYP2B6.4 showed similar activity with the wild type, while all other variants had diminished activity compared with CYP2B6.1, and CYP2B6.16 and CYP2B6.18 were essentially catalytically inactive. At clinically relevant concentrations, hydroxylation rates were of the order CYP2B6.1 ≈ CYP2B6.4 > CYP2B6.17 ≈ CYP2B6.26 > CYP2B6.5 ≈ CYP2B6.6 ≈ CYP2B6.19 > CYP2B6.7 ≈ CYP2B6.9 >> CYP2B6.16 ≈ CYP2B6.18. For *R,R*-hydroxybupropion formation, the order is similar except that CYP2B6.17 was more active than CYP2B6.1.

Racemic bupropion hydroxylation catalyzed by wild-type CYP2B6.1 and the catalytically active CYP2B6 variants with co-expressed POR and b_5 was determined as a function of bupropion enantiomer concentrations (Fig 2). For CYP2B6.1 and all CYP2B6 variant proteins, *S,S*-hydroxyburopion formation exceeded that of *R,R*-hydroxybupropion. Eadie-Hosfstee plots were generally linear (not shown). Michaelis-Menten kinetic parameters were determined for all active enzymes (Table 1). There was a 3-fold (*S*-bupropion) and 4-fold (*R*-bupropion) range in V_{max}, and a 5-fold range in K_m parameters for both *R*- and *S*-bupropion. CYP2B6.19 had the highest V_{max}, which was about 2-fold greater than that of CYP2B6.1 (for both *S*- and *R*-bupropion), but the K_m was also higher (4-fold), so the net effect was a lower *in vitro* intrinsic clearance (Cl_{int}) compared with CYP2B6.1. Similarly, CYP2B6.6 V_{max} was also greater than that of CYP2B6.1 for S-bupropion, but the K_m was also higher, so Cl_{int} was lower than that of CYP2B6.1. All variants had Cl_{int} values lower than that of CYP2B6.1, except CYP2B6.4 (*R*- and *S*-bupropion) and CYP2B6.17 (*R*-bupropion). Based on intrinsic clearances, relative activities for *S,S*-hydroxybupropion formation were in the order CYP2B6.4≥CYP2B6.1>CYP2B6.17>

CYP2B6.5>CYP2B6.6≈CYP2B6.26≈CYP2B6.19>CYP2B6.7>CYP2B6.9>>CYP2B6.16≈CYP2B6.18, and relative activities for *R,R*-hydroxybupropion formation were in the order CYP2B6.17≈CYP2B6.4≈CYP2B6.1>CYP2B6.5≈CYP2B6.19≈CYP2B6.26>CYP2B6.6>CYP2B6.7≈ CYP2B6.9>>CYP2B6.16≈CYP2B6.18.

Bupropion hydroxylation by CYP2B6.1 is stereoselective, with *S,S*-hydroxybupropion formation greater than that of *R,R*-hydroxybupropion. The ratios of V_{max}(*S,S*-hydroxybupropion)/V_{max}(*R,R*-hydroxybupropion) and Cl_{int}(*S,S*-hydroxybupropion)/Cl_{int}(*R,R*-hydroxybupropion) for CYP2B6.1 were 2.9 and 1.8, respectively, consistent with previous report.(Coles and Kharasch, 2008) All CYP2B6 variants showed similar stereoselectivity, with the V_{max}(*S,S*-hydroxybupropion)/V_{max}(*R,R*-hydroxybupropion) ratio 1.9 to 4.2. The Cl_{int}(*S,S*-hydroxybupropion)/Cl_{int}(*R,R*-hydroxybupropion) ratio for most 2B6 variants was also similar to the wild type, 1.8 to 2.9, except that CYP2B6.17 (1.2) was lower.

The activity of POR variants was determined with co-expressed wild-type CYP2B6 and cytochrome b_5. In contrast to CYP, POR variants caused minor, albeit statistically significant changes to bupropion (2 μM) hydroxylation compared with wild type POR (Fig 3). Bupropion hydroxylation by POR variants over the full substrate concentration range is shown in Figure 4 and Table 2. The kinetic parameters for the three POR variants POR.5, POR.28 and PORP228L are similar to the wild type.

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Discussion

The first major observation was that *CYP2B6* allelic variants have altered and diverse activities towards racemic bupropion hydroxylation compared with wild type CYP2B6, for both *R*- and *S*-bupropion. CYP2B6 variants selection was based on allele frequency and consequence. The 516G>T (Q172H) and 785G>T (K262R) polymorphisms are common, and variants with these mutations (*2B6*4, *6, *7, *9, *16, *19, *26*) were studied, even though some have minor population frequency. The other three variants *(*5, *17, *18*) have relatively high allele frequency. At clinically relevant concentrations, CYP2B6.5, CYP2B6.6, CYP2B6.7, CYP2B6.9, CYP2B6.19 and CYP2B6.26 were less active than wild type CYP2B1.1. CYP2B6.4 had activity similar to CYP2B1.1, while Cl_{int} was slightly higher than wild type for both enantiomers. CYP2B6.7 and CYP2B6.9 had the lowest activities of the enzymatically active variants, with 20-35% of wild type Cl_{int}. CYP2B6.16 and CYP2B6.18 were essentially inactive.

Bupropion hydroxylation by CYP2B6 is stereoselective. Although V_{max} and K_m vary over a wide range among the variants, stereoselectivity was retained in most variants, reflected by similar ratios of V_{max}(*S,S*-hydroxybupropion)/V_{max}(*R,R*-hydroxybupropion) and Cl_{int}(*S,S*-hydroxybupropion)/Cl_{int}(*R,R*-hydroxybupropion). CYP2B6.17 was the only variant with a moderate difference in enantioselectivity compared with CYP2B6.1.

Only limited kinetic data are available on bupropion hydroxylation by *CYP2B6* variants, and none report on enantiomer metabolism. Table 3 summarizes the previously reported activity of expressed *CYP2B6* variants towards *RS*-bupropion hydroxylation (Zhang et al., 2011; Xu et al., 2012; Radloff et al., 2013), and the present results. For CYP2B6.6, our results are comparable with previous studies, and lower Cl_{int} for CYP2B6.6 is consistently reported. CYP2B6.6 had both increased V_{max} (1.5-fold for *S,S*-hydroxybupropion and 1.2-fold for *R,R*- hydroxybupropion) and increased K_m (2.9-fold for *S,S*-hydroxybupropion and 3.4-fold for *R,R*-hydroxybupropion) compared with CYP2B6.1. Zhang *et al* reported that *E. coli*-expressed CYP2B6.6 had 1.8-fold greater k_{cat} versus wild type, and 4-fold greater K_m, although the protein expression system was different than used herein (Zhang et al., 2011). In

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contrast, the one previous investigation of CYP2B6.4 reported substantially lower Cl_{int} while we found normal to slightly greater activity (Zhang et al., 2011).

CYP2B6 is an unusual P450 isoform, in that activity of variants is clearly substrate-dependent, and generalizations about catalytic consequence or clinical implications of *CYP2B6* polymorphisms are not possible. CYP2B6.4 (785G>T, K262R) had greater activity in metabolism of methadone (Gadel et al., 2015), efavirenz (Wang et al., 2019), artemether (Honda et al., 2011), and selegiline (Watanabe et al., 2010), and decreased activity with cyclophosphamide (Ariyoshi et al., 2011; Raccor et al., 2012), ifosfamide (Zanger and Klein, 2013; Calinski et al., 2015), ketamine (Wang et al., 2018), and nicotine (Bloom et al., 2019). Bupropion metabolism in the present study, using the CYP2B6.4 expression system used to study many of these other substrates, was slightly (10%) but not substantially greater than CYP2B6.1. CYP2B6.5 (1459C>T, R487C) was less active than CYP2B6.1 in metabolizing bupropion, as well as methadone (Gadel et al., 2015), ketamine (Wang et al., 2018), cyclophosphamide (Raccor et al., 2012), artmether (Honda et al., 2011) and nicotine (Bloom et al., 2019). It had moderately decreased activity in *S*-efavirenz hydroxylation and slightly increased activity in *R*-efavirenz hydroxylation (Wang et al., 2019). CYP2B6.9 (516G>T, Q172H) was substantially less active than CYP2B6.1 in bupropion metabolism, as well as methadone, ketamine, efavarenz, and nicotine, and moderately less (30%) with artemether, but more active than CYP2B6.1 in ifosfamide bioactivation (Honda et al., 2011; Calinski et al., 2015; Gadel et al., 2015; Wang et al., 2018; Bloom et al., 2019; Wang et al., 2019). CYP2B6.6 (516G>T-785A>G, Q172H-K262R) has been studied extensively *in vitro* and *in vivo* due to its high frequency of occurrence and therapeutic significance. CYP2B6.6 was less active than wild-type in metabolism of bupropion, as well as methadone, ketamine, efavirenz, bupropion, ifosfamide, and nicotine, but more active in metabolizing cyclophosphamide and artemether (Ariyoshi et al., 2011; Honda et al., 2011; Calinski et al., 2015; Gadel et al., 2015; Wang et al., 2018; Bloom et al., 2019; Wang et al., 2019). CYP2B6.16 and CYP2B6.18, which were essentially inactive towards buproiopion, are also inactive with methadone, ketamine, and efavirenz (Gadel et al., 2015; Wang et al., 2018; Wang et al., 2019). These two variants are expressed as apoproteins only, without detectable cofactor heme bound.

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The mutation shared in the two variants, 983T>C (I328T), is located in the J-helix, and the structural change caused by this mutation is relayed to C- and I-helices, which are directly involved in ligand/heme recognition (Kobayashi et al., 2014). As a result heme binding is disrupted, leading to abolition of catalytic activity. The common polymorphisms of CYP2B6 include 516G>T, 785G>T, 983T>C and 1459C>T. Based on *in vitro* studies with substrates evaluated so far, 516G>T, 983T>C and 1459C>T can generally be considered loss of function variants.

The second major observation in this investigation was that the three POR polymorphisms evaluated had very minor influence on bupropion metabolism. POR transfers electrons from NADPH to the P450 heme, an essential step in P450-catalysed metabolism. Influence of POR polymorphism on drug metabolism is substrate- and P450 isoform-dependent. POR.5 (859G>C) had minimal or no effect on bupropion metabolism, and 25% less activity in a previous bupropion study (Chen et al., 2012). In contrast, POR.5 had substantially lower activity with CYP3A4 and testosterone, and with CYP17A1 and androgen biosynthesis (Chen et al., 2012; Pandey and Sproll, 2014). POR.28 had a small (*R*-bupropion) or no (*S*-bupropion) influence on CYP2B6.1-catalyzed bupropion metabolism.

There is high concordance between *POR* and *CYP2B6* genetic variant effects on bupropion metabolism *in vitro* and *in vivo*. *POR*28* had little effect on bupropion hydroxylation *in vitro*, or *in vivo*, based on plasma hydroxybupropion/bupropion area under the curve (AUC) ratios and urine hydroxybupropion formation clearance (Gao et al., 2016; Lv et al., 2016; Kharasch and Crafford, 2019). Accordingly, the present results would also suggest little influence of *POR*5* and *P228L* polymorphisms on bupropion hydroxylation *in vivo*. The influence of *CYP2B6*6*, which consistently results in diminished bupropion hydroxylation *in vitro* (Table 3), is highly concordant *in vivo*. Plasma racemic hydroxybupropion/bupropion AUC ratios after single-dose bupropion were lower in *CYP2B6*6* carriers than noncarriers (Chung et al., 2011), lower in *CYP2B6*6/*6* and *CYP2B6*1/*6* versus *CYP2B6*1/*1* genotypes (Gao et al., 2016; Lv et al., 2016), and hydroxybupropion concentrations were lower in *CYP2B6*6* heterozygotes than wild-types (Ma et al., 2018). Assessed stereoselectively, plasma AUC ratios and urine hydroxybupropion formation clearances for both bupropion enantiomers, after a single

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bupropion dose, were lower in *CYP2B6*6/*6* but not *CYP2B6*1/*6* compared with *CYP2B6*1/*1* genotypes (Kharasch and Crafford, 2019). At steady-state, plasma racemic hydroxybupropion/bupropion AUC ratios in CYP2B6 slow (**6/*6, *18/*18* genotypes) and intermediate (**6* and **18* heterozygotes) metabolizers were 34% and 54%, respectively, of those in normal metabolizers (**1/*1*, **1/*4*, **1/*5*, **1/*22*, **22/*22*), and hydroxybupropion plasma concentrations were 40% and 20% lower in slow and intermediate than normal metabolizers (Zhu et al., 2012). Steady-state ratios were also lower in *CYP2B6*6* carriers than wild types (Benowitz et al., 2013). Steady-state ratios were also lower than wild-types in *CYP2B6*6/*6* and *CYP2B6*1/*6* genotypes, and plasma hydroxybupropion concentrations were less in *CYP2B6*6/*6* but not *CYP2B6*1/*6* genotypes (Høiseth et al., 2015). The influence of *CYP2B6* polymorphisms on steady-state hydroxylation of bupropion enantiomers has not been reported. *CYP2B6*18* coded for markedly deficient activity *in vitro*, and at steady state, hydroxybupropion/bupropion AUC ratios were lower in *CYP2B6*18* carriers than wild types, and even less than in *CYP2B6*6* carriers (Benowitz et al., 2013). *In vitro-in vivo* concordance is less consistent for *CYP2B6*4* than for decreased metabolism polymorphisms, which may be influenced by the rarity of *CYP2B6*4*. CYP2B6.4 had minimally increased bupropion hydroxylation *in vitro*, and numerically but not significantly higher plasma hydroxybupropion/bupropion AUC ratios in *CYP2B6*4* hererozygotes for the racemate (Kirchheiner et al., 2003; Zhu et al., 2012; Ma et al., 2018; Kharasch and Crafford, 2019) and enantiomers (Kharasch and Crafford, 2019). Based on these patterns, and the present *in vitro* results, it could be expected that other *CYP2B6* polymorphisms coding for diminished (**5, *6, *7, *9, *19, *26*) or defective (**16*) *in vitro* bupropion hydroxylation might result in diminished *in vivo* bupropion hydroxylation.

There are acknowledged potential limitations to this investigation. First, the purpose was only to test singular CYP and POR variants, not combinations; hence CYP and POR variants coexpressed in combination was not evaluated. Second, racemic bupropion was used as the substrate, rather than single enantiomers, because bupropion is used clinically as a racemate and results were intended to more accurately reflect and inform on clinical metabolism. Third, for the CYP variants, the CYP:POR ratio

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varied, ranging from 1:1.3 to 1:4.5. CYP and POR form a 1:1 complex, and if POR content equals or exceeds CYP content all constructs should be fully catalytically competent and extra POR beyond 1:1 should not further increase activity. For the POR variants, the CYP:POR ratio exceeded 1, except for *POR*5* (1:0.6). While it is possible that POR.5 expression affected apparent activity, it was not different than wild type for *S*-bupropion, and only marginally less with *R*-bupropion. In addition, there are several types of CYP expression systems, including yeast, bacteria, mammalian, and insect cells, using transient or stable transfection, with or without co-expressed POR and b_5, and expression systems can influence apparent catalytic activity (Wang et al., 2018). Yeast and bacteria are straightforward but require exogenous POR and b_5, and bacterial expression usually requires sequence modification of mammalian CYPs. Mammalian systems (e.g. monkey kidney COS cells and human embryonic kidney HEK cells) contain POR and b_5, and allow easy CYP expression, protein expression and integrity can vary and thus influence apparent catalytic activity. Baculovirus-mediated expression in insect cells is a mature technology and allows simultaneous and flexible expression of individual genetic variants, using separate or combination multi-protein virus cassettes.

The newly identified CYP2B6 genetic polymorphisms in bupropion hydroxylation may also portend clinical consequence, because there are important relationships between *CYP2B6* genetics and clinical outcomes of bupropion pharmacotherapy. Bupropion hydroxylation is a bioactivation pathway, and hydroxybupropion, specifically *S,S*-hydroxybupropion, is considered to contribute to bupropion anti-smoking (Bondarev et al., 2003; Damaj et al., 2004; Lee et al., 2007; Zhu et al., 2012; Carroll et al., 2014; Malcolm et al., 2015) and antidepressant effects (Carroll et al., 2014; Laib et al., 2014). Bupropion-treated smokers who were CYP2B6 1459C>T carriers (**5, *7*) reported greater cravings for cigarettes and had higher relapse rates compared to non-carriers.(Lerman et al., 2002) Lower plasma racemic hydroxybupropion concentrations, related to *CYP2B6* slow and intermediate metabolizer status, were associated with a lesser incidence of smoking abstinence (Zhu et al., 2012), although not all studies agree.(Lee et al., 2007) Among depressed patients, lower plasma racemic hydroxybupropion concentrations were associated with lesser improvement in depression (Laib et al., 2014). The incidence

of other *CYP2B6* polymorphisms coding for deficient (**5, *6, *7, *9, *19, *26*) or defective (**16*) *in vitro* bupropion hydroxylation on clinical bupropion effectiveness is unknown.

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none

Authorship Contributions

Participated in research design: Wang, Kharasch

Conducted experiments: Wang, Neiner

Contributed new reagents or analytic tools: none

Performed data analysis: Wang, Neiner, Kharasch

Wrote or contributed to the writing of the manuscript: Wang, Kharasch

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Footnotes

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Figure Legends

Figure 1. Metabolism of *RS*-bupropion catalyzed by coexpressed recombinant CYP2B6 variants/POR.1/b_5 at concentration of 2 µM *RS*-bupropion (1 µM *R*- or *S*-bupropion). Results are the mean ± 95% confidence interval of triplicate determinations. Asterisks denote rates significantly different from the wild-type CYP2B6.1 (P<0.001). (A) *R,R*-hydroxybupropion formation from *RS*-bupropion. (B) *S,S*-hydroxybupropion formation from *RS*-bupropion.

Figure 2. Hydroxybupropion formation from *RS*-bupropion catalyzed by co-expressed CYP2B6 variants, wild-type P450 oxidoreductase and cytochrome b_5. (●) *S,S*-hydroxybupropion from *RS*-bupropion, (○) *R,R*-hydroxybupropion from *RS*-bupropion. Results are the mean ± 95% confidence interval of triplicate determinations. Lines are predicted concentrations based on kinetic parameters obtained by nonlinear regression analysis of measured concentrations

Figure 3. Metabolism of *RS*-bupropion catalyzed by coexpressed recombinant CYP2B6.1/POR variants/b_5 at concentration of 2 µM *RS*-bupropion (1 µM *R*- or *S*-bupropion). Results are the mean ± 95% confidence interval of triplicate determinations. Asterisks denote rates significantly different from the wild-type POR (P<0.001 for *R*-bupropion and P=0.002 for *S*-bupropion). (A) *R,R*-hydroxybupropion formation from *RS*-bupropion. (B) *S,S*-hydroxybupropion formation from *RS*-bupropion.

Figure 4. Hydroxybupropion formation from *RS*-bupropion catalyzed by co-expressed CYP2B6.1, P450 oxidoreductase variants and cytochrome b_5. (●) *S,S*-hydroxybupropion from *RS*-bupropion, (○) *R,R*-hydroxybupropion from R-bupropion. Results are the mean ± 95% confidence interval of triplicate determinations. Lines are predicted concentrations based on kinetic parameters obtained by nonlinear regression analysis of measured concentrations.

Table 1. Kinetic parameters for (*S*,*S*)- and (*R*,*R*)-hydroxybupropion formation from *RS*-bupropion by CYP2B6 variants

CYP2B6 variant	(*S*,*S*)-hydroxybupropion formation from *RS*-bupropion			(*R*,*R*)-hydroxybupropion formation from *RS*-bupropion		
	V_{max} (pmol/min/pmol)	K_m (μM)	Cl_{int} [a] (ml/min/nmol)	V_{max} (pmol/min/pmol)	K_m (μM)	Cl_{int} [a] (ml/min/nmol)
CYP2B6.1	26.4 ± 0.5	18.2 ± 1.5	1.45 (100)	9.0 ± 0.1	11.4 ± 0.8	0.79 (100)
CYP2B6.4	23.5 ± 0.4	14.5 ± 1.0	1.62 (112)	8.0 ± 0.1	9.3 ± 1.6	0.86 (109)
CYP2B6.5	14.4 ± 0.2	17.3 ± 0.8	0.83 (57)	5.1 ± 0.1	12.2 ± 0.7	0.42 (53)
CYP2B6.6	39.0 ± 0.6	53.0 ± 2.7	0.74 (51)	10.8 ± 0.2	39.3 ± 2.0	0.27 (34)
CYP2B6.7	15.6 ± 0.2	31.1 ± 1.5	0.50 (34)	3.7 ± 0.1	21.4 ± 1.5	0.17 (22)
CYP2B6.9	20.7 ± 0.2	53.3 ± 2.1	0.39 (27)	6.0 ± 0.6	37.7 ± 1.3	0.16 (20)
CYP2B6.16[b]	0.74 ± 0.03			0.72 ± 0.02		
CYP2B6.17	22.3 ± 1.1	21.3 ± 2.5	1.05 (72)	11.8 ± 0.2	13.1 ± 0.8	0.90 (114)
CYP2B6.18[b]	0.91 ± 0.03			0.78 ± 0.01		
CYP2B6.19	49.2 ± 4.1	75.6 ± 11.0	0.65 (45)	16.2 ± 0.2	45.4 ± 2.1	0.36 (46)
CYP2B6.26	31.7 ± 2.1	41.5 ± 5.3	0.76 (52)	8.5 ± 0.1	22.0 ± 1.4	0.39 (49)

[a]Values in brackets represent percentages of CYP2B6.1 Cl_{int}.

[b]For CYP2B6.16 and CYP2B6.18, rates were measured at a fixed substrate concentration of 1000 μM *RS*-bupropion.

Table 2. Kinetic parameters for (*S*,*S*)- and (*R*,*R*)-hydroxybupropion formation from *RS*-bupropion by POR variants

POR variant	(*S*,*S*)-hydroxybupropion formation from *RS*-bupropion			(*R*,*R*)-hydroxybupropion formation from *RS*-bupropion		
	V_{max} (pmol/min/pmol)	K_m (μM)	Cl_{int} [a] (ml/min/nmol)	V_{max} (pmol/min/pmol)	K_m (μM)	Cl_{int} [a] (ml/min/nmol)
POR.1	26.4 ± 0.5	18.2 ± 1.5	1.45 (100)	9.0 ± 0.1	11.4 ± 0.8	0.79 (100)
POR.5	21.0 ± 1.6	15.5 ± 3.1	1.35 (93)	9.1 ± 0.1	10.5 ± 0.7	0.87 (110)
POR.28	21.4 ± 0.7	13.3 ± 1.1	1.61 (111)	10.5 ± 0.1	8.9 ± 0.5	1.18 (149)
POR P228L	21.3 ± 0.8	18.8 ± 1.8	1.13 (78)	10.5 ± 0.1	13.0 ± 0.5	0.81 (103)

[a]Values in brackets represent percentages of CYP2B6.1 Cl_{int}.

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Table 3. Summary of reported bupropion hydroxylation. Relative activities are shown as percentage of CYP2B6 variants Cl_{int} compared to the wild type (100 x Cl_{int}(variant)/Cl_{int}(wt)).

Reference	Zhang et al (Zhang et al., 2011)	Xu et al (Xu et al., 2012)	Radloff et al (Radloff et al., 2013)	This study	
Expression system	*E. coli*	SF9	COS-1	*T. ni*	
	RS	*RS*	*RS*	*S,S*	*R,R*
CYP2B6.1	100	100	100	100	100
CYP2B6.4	35			112	109
CYP2B6.5	47		44	57	53
CYP2B6.6	43	65	81	51	34
CYP2B6.7	79			34	22
CYP2B6.9	38			27	20
CYP2B6.17				72	114
CYP2B6.19				45	46
CYP2B6.26				52	49

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Figure 1

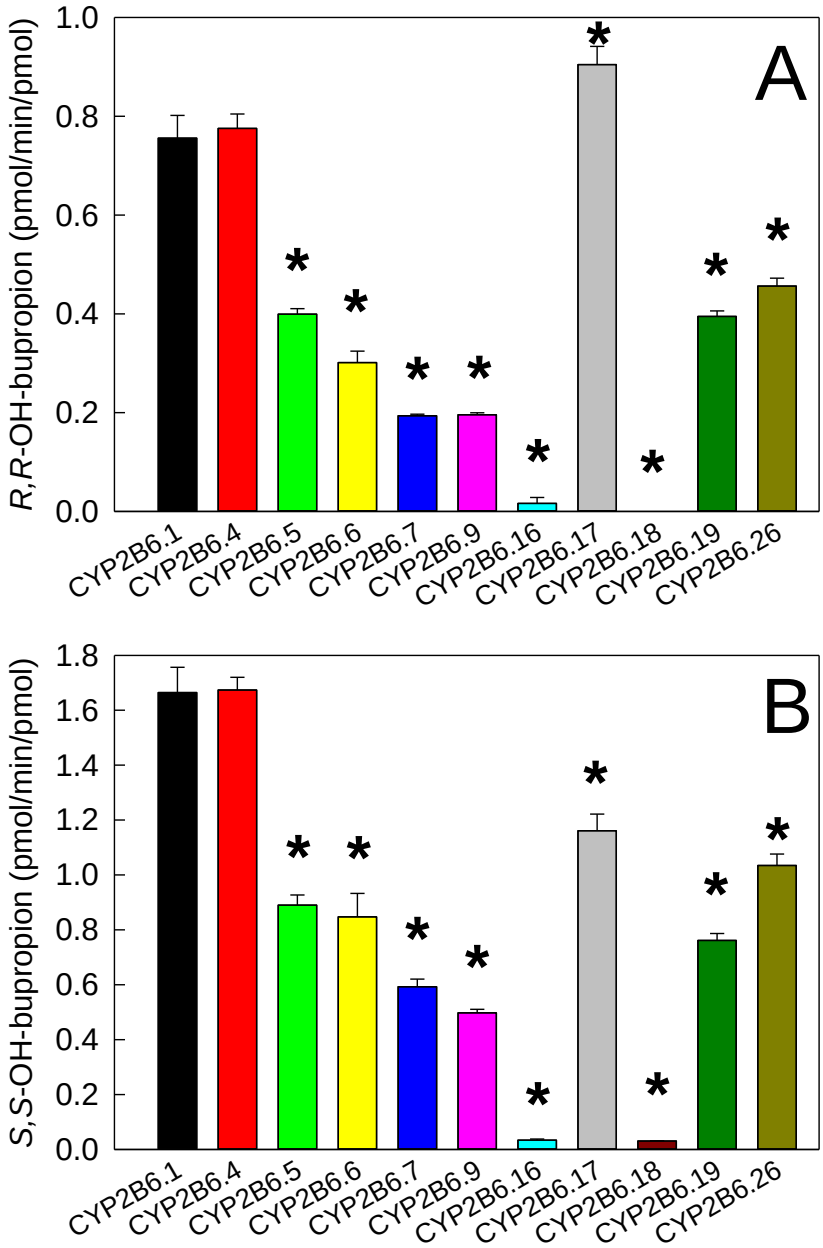

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Figure 2

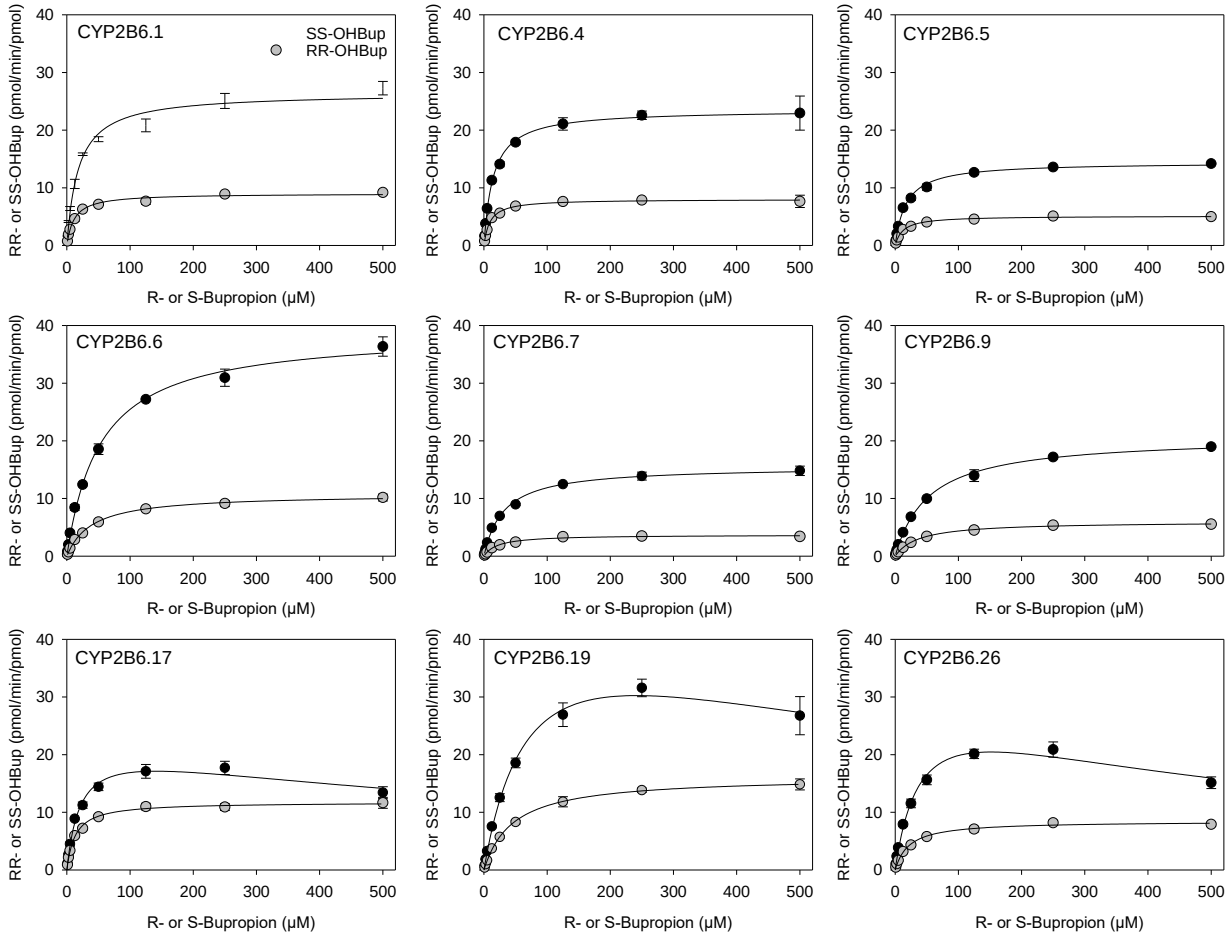

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Figure 3

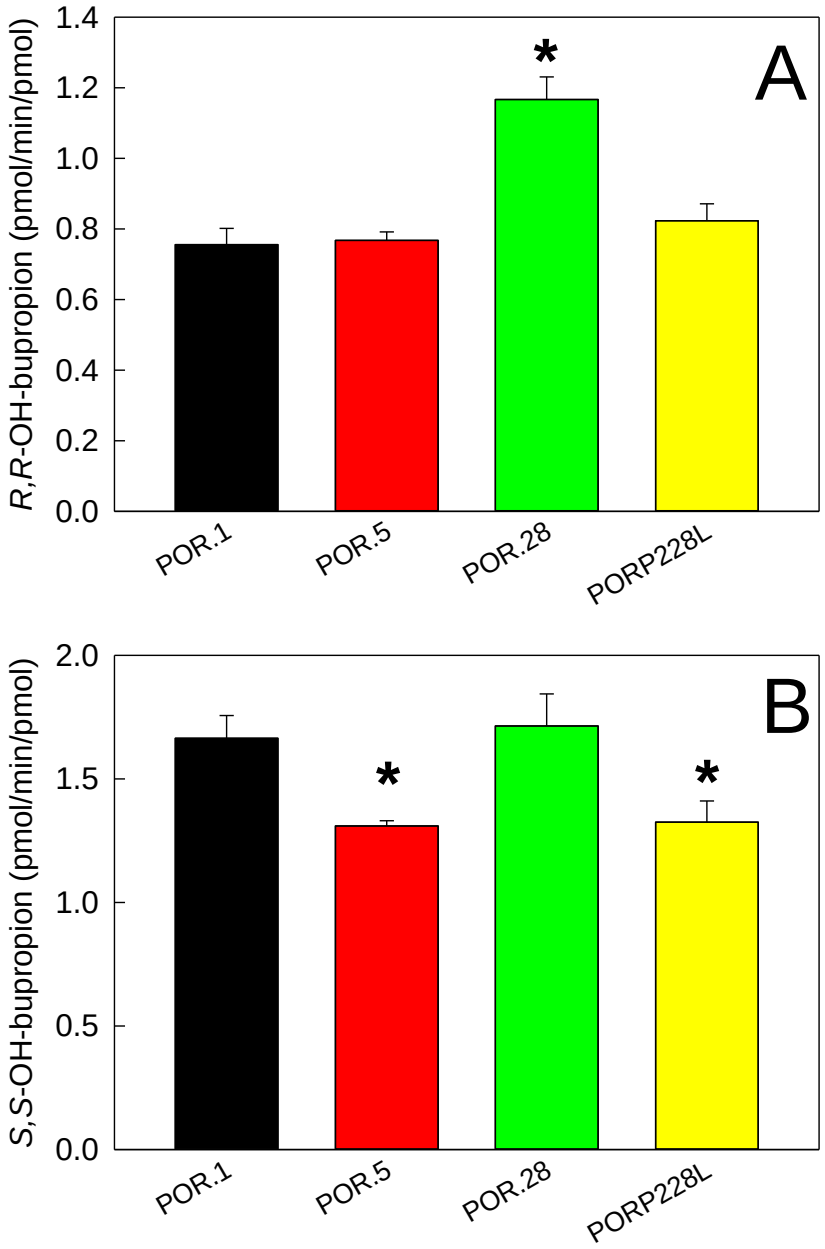

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Figure 4