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Interactions of Two Major Metabolites of Prasugrel, a Thienopyridine Antiplatelet Agent, with the Cytochromes P450

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Interactions of metabolites of prasugrel with P450s

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Non-standard abbreviations: prasugrel, (\pm)-2-[2-Acetyloxy-6,7-dihydrothieno[3,2-c]pyridin-5(4*H*)-yl]-1-cyclopropyl-2-(2-fluorophenyl)ethanone hydrochloride; R-95913, 2-[2-oxo-6,7-dihydrothieno[3,2-c]pyridin-5(4*H*)-yl]-1-cyclopropyl-2-(2-fluorophenyl)ethanone; R-138727, 2-[1-2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-mercapto-3-piperidinylidene]acetic acid; P450, cytochrome P450; GSH, reduced glutathione; IS, internal standard; ACN, acetonitrile; BMAP, 2-bromo-3'-methoxyacetophenone; mAb, monoclonal antibody.

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Abstract

The biotransformation of prasugrel to R-138727 involves rapid de-esterification to R-95913 followed by cytochrome P450 (P450)-mediated formation of R-138727, the metabolite responsible for platelet aggregation. For identification of the P450s responsible for the formation of the active metabolite, the current studies were conducted with R-95913 as the substrate. Incubations required supplementation with reduced glutathione. Hyperbolic kinetics (K_m 21-30 μ M), consistent with a single enzyme predominating, were observed following incubations with human liver microsomes. Correlation analyses revealed a strong relationship between R-138727 formation and CYP3A-mediated midazolam 1'-hydroxylation ($r^2 = 0.98$; $p < .001$) in a bank of characterized human liver microsomal samples. The human lymphoblast-expressed enzymes capable of forming R-138727, in rank order of rates, were CYP3A4 > CYP2B6 > CYP2C19 \approx CYP2C9 > CYP2D6. A monoclonal antibody to CYP2B6 and the CYP3A inhibitor ketoconazole substantially inhibited R-138727 formation, while inhibitors of CYP2C9 (sulfaphenazole) and CYP2C19 (omeprazole) did not. Scaling of in vitro intrinsic clearance values from expressed enzymes to the whole liver using a relative abundance approach indicated that either CYP3A4 alone or CYP3A4 and CYP2B6 are the major contributors to R-138727 formation. R-95913 and R-138727 were also examined for their abilities to inhibit metabolism mediated by five P450s. R-138727 did not inhibit the P450s tested. In vitro, R-95913 inhibited CYP2C9, CYP2C19, CYP2D6, and CYP3A with K_i values ranging from 7.2 μ M to 82 μ M but did not inhibit CYP1A2. These K_i values exceed circulating concentrations in humans by 3.8- to 43-fold. Therefore, neither R-95913 nor R-138727 is expected to substantially inhibit the P450-mediated metabolism of co-administered drugs.

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Prasugrel (CS-747, LY640315; Figure 1) is a novel thienopyridine antiplatelet prodrug that is administered orally as a racemic mixture. It is rapidly metabolized in vivo to a potent ADP-receptor antagonist, designated R-138727 (Figure 1), which binds irreversibly to P2Y₁₂ receptors causing inhibition of platelet aggregation that persists for the life of the platelets (Kurihara et al. 2005, Niitsu et al. 2005). Prasugrel is rapidly hydrolyzed by carboxylesterases to yield the thiolactone, R-95913 (Figure 1), which circulates in human plasma, while prasugrel is not detected in human plasma (Farid et al. 2005b). Further metabolism of R-95913 yields a ring-opened form, R-138727 (Figure 1) (Sugidachi et al. 2000, 2001), which has two chiral centers and is actually a mixture of four stereoisomers that vary in potency of inhibition at P2Y₁₂ receptors (Kazui et al. 2001). Other drugs of the thienopyridine class, ticlopidine and clopidogrel, also require metabolic activation.

The active metabolites (both ring-opened forms similar to R-138727) of clopidogrel (Savi et al. 2000) and ticlopidine (Yoneda et al. 2004) have recently been identified. Some information as to the biotransformation routes of these two drugs is available, although only the surrogate endpoint of parent drug loss, rather than the more informative endpoint of metabolite formation, has been measured. For ticlopidine, the cytochromes P450 (P450s) most active in metabolizing the parent drug are CYP3A4 and CYP2C19 (Dalvie and O'Connell 2004). For clopidogrel, CYP1A2 has been found to be involved in the metabolism of parent drug in rat microsomes (Savi et al. 1994), but in a more recent study, the CYP3A enzymes were identified as the major enzymes involved in metabolism of parent drug in rat microsomes and a panel of expressed human P450s (Clarke and Waskell 2003).

For prasugrel, direct measurement of R-138727 in preliminary in vitro studies identified CYP3A4 and CYP2B6 as potential mediators of active metabolite formation (Kazui et al. 2000, 2001).

The current studies were performed at a more relevant range of concentrations, used a

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validated LC/MS/MS analytical method in which the unstable free thiol-containing R-138727 was stabilized by derivatization with 2-bromo-3'-methoxyacetophenone (BMAP), and used a scaling exercise to estimate the contributions of the relevant enzymes to R-138727 formation *in vivo*. The results of the current studies confirmed the roles of CYP3A and CYP2B6 and identified additional contributors to R-138727 formation.

For drugs such as the thienopyridine prodrugs, which require metabolism to form active compounds, identification of the enzymes responsible for biotransformation to the active metabolite helps determine whether therapeutic levels of active drug will be consistently achieved. Clopidogrel is the current standard of care for a variety of cardiovascular indications and has been administered to many thousands of patients, but there has been recent debate as to whether interpatient variability in CYP3A is related to the phenomenon of clopidogrel resistance and whether co-administration of CYP3A substrates with clopidogrel affects the formation of the active metabolite of clopidogrel (Clarke and Waskell 2003, Lau et al. 2003, Saw et al. 2003, Wienbergen et al. 2003, Lau et al. 2004, and Mitsios et al. 2004). The ability to measure the formation of the active metabolite of prasugrel allowed the results of the current *in vitro* studies to be used to 1) to evaluate possible variability in the pharmacokinetics of the major circulating metabolites of prasugrel, and 2) actually predict, rather than clarify retrospectively, the likelihood of potential metabolism-mediated drug-drug interactions that could affect the achievement of therapeutic levels of active drug. In addition, R-95913 and R-138727 were evaluated *in vitro* for their abilities to inhibit the P450s for which drug-drug interactions are of most concern: CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A.

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Materials and Methods

Materials. R-95913, R-138727, and $^2\text{H}_4$ -R-138727 were obtained from UBE Industries (Yamaguchi, Japan). Testosterone, 6β -hydroxytestosterone, 16-epiestriol, L-ascorbic acid, α -hydroxytriazolam, trolox, phenytoin, NADPH, reduced glutathione (GSH), ketoconazole, sulfaphenazole, phenacetin, acetaminophen, and BMAP were purchased from Sigma-Aldrich (St. Louis, MO). Midazolam was from Hoffmann-LaRoche (Nutley, NJ). The 4'-hydroxy metabolite of diclofenac was from BD Gentest (Woburn, MA). Deuterated acetaminophen was from C/D/N Isotopes (Pointe-Claire, Canada). Bufuralol, 1'-hydroxybufuralol, S-mephenytoin, 4'-hydroxy S-mephenytoin, and 1'-hydroxymidazolam were from Ultrafine (Manchester, UK). Omeprazole was obtained from RBI (Natick, MA). Inhibitory monoclonal antibody (mAb) to CYP2B6 (in ascites fluid) was obtained from Invitrogen Corporation (Carlsbad, CA). Control mouse ascites fluid was obtained from MP Biomedicals (Aurora, OH).

Microsomes. Human liver samples designated HLA through HLT were obtained from the Medical College of Wisconsin (Milwaukee, WI), Medical College of Virginia (Richmond, VA), or Indiana University School of Medicine (Indianapolis, IN), under protocols approved by the appropriate committee for the conduct of human research. Hepatic microsomes were prepared by differential centrifugation (van der Hoeven and Coon 1974) and characterized for relative levels of P450s through the use of form-selective catalytic activities or immunoquantification (Ekins et al. 1998, Ring et al. 2001). A mixture of equal microsomal protein concentrations from HLB, HLH, HLM, and HLP was used in the studies examining the abilities of R-95913 and R-138727 to inhibit various P450-mediated reactions. Microsomes prepared from a human β -lymphoblastoid cell line engineered to express the cDNA for the individual P450s were obtained from BD Gentest (Woburn, MA).

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Examination of R-138727 formation. Metabolism of R-95913 to R-138727 was examined in vitro in incubations (200 μ L) conducted under linear rate-of-formation conditions at 37°C. The final incubations contained varying concentrations of R-95913, 0.125 mg/mL human hepatic microsomal protein, 5 mM GSH, and 1 mM NADPH in 100 mM sodium phosphate buffer (pH 7.4). After three-minute pre-incubations at 37°C, reactions were initiated with NADPH. The mixtures were incubated for 15 minutes at 37°C. Reactions were stopped and samples treated as described in Analytical Conditions.

For estimation of apparent enzyme kinetic parameters for the formation of R-138727 by human liver microsomes, concentrations of R-95913 ranged from 0.5 to 75 μ M. For correlation analyses and incubations with selective chemical inhibitors or inhibitory mAb, the concentration of R-95913 (20 μ M) approximated the K_m for R-138727 formation. When P450 inhibitors were added to incubations, concentrations were: sulfaphenazole (CYP2C9-selective; Newton et al. 1995, Bourrié et al. 1996), 10 μ M; omeprazole (CYP2C19-selective; Ko et al. 1997), 10 μ M; and ketoconazole (CYP3A-selective; Newton et al. 1995, Bourrié et al. 1996), 2 μ M. When CYP2B6 mAb in ascites were included, modifications to the procedure were required. Microsomes with mAb (up to 8 μ L), in 100 mM sodium phosphate buffer (pH 7.4) with 5 mM GSH, were preincubated for five minutes at 37°C. Reactions (a total volume of 200 μ L) were initiated with addition of a solution of 1 mM NADPH plus 20 μ M R-95913. Reactions were stopped at the appropriate times and treated as described in Analytical Conditions.

Microsomes prepared from human β -lymphoblastoid cells engineered to express human P450s and P450 reductase were examined for their abilities to form R-138727 from R-95913. Incubations (200 μ L) contained 5 mM GSH, 2 mM NADPH, and 20 μ M R-95913 in 100 mM sodium phosphate buffer (pH 7.4). Following three-minute pre-incubations, microsomes (0.125

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mg/mL) from cells expressing CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A4 were added to start reactions, which continued for 15 minutes at 37°C. Apparent enzyme kinetic parameters were estimated for the formation of R-138727 by microsomes from β -lymphoblastoid cells expressing CYP2B6, CYP2C9, CYP2C19, and CYP3A4 under linear rate of formation conditions (0.125 mg/mL protein and 30 minutes for CYP2B6, CYP2C9, and CYP2C19; 0.125 mg/mL and 20 minutes for CYP3A4). Concentrations of R-95913 in these experiments ranged from 1 to 100 μ M.

Inhibition of P450-Selective Catalytic Activities by R-95913 and R-138727. All incubations were conducted under linear rate conditions and contained 100 mM sodium phosphate buffer at pH 7.4. A K_i value was determined when a preliminary experiment indicated substantial inhibition ($\geq 50\%$) of one of the P450s by a metabolite of prasugrel.

The *O*-deethylation of phenacetin (acetaminophen formation) was used as a marker of CYP1A2 activity (Ring et al. 2001). Incubations contained 0.5 mg/mL microsomal protein, 1 mM NADPH, 12.5 μ M phenacetin, and 0, 0.5, 2.5, 10, 50, and 100 μ M R-95913 or R-138727 as potential inhibitors.

The biotransformation of diclofenac to 4'-hydroxydiclofenac was used as the marker activity for CYP2C9 (Ring et al. 2001). Incubations contained 0, 10, 50, 100, and 200 μ M R-95913 and 2.5, 5, 10, 25 and 50 μ M diclofenac when R-95913 was the inhibitor. When R-138727 was the inhibitor, 0, 0.5, 2.5, 10, 50, and 200 μ M R-138727 was incubated with a concentration of diclofenac approximating the K_m (2.5 μ M) for the diclofenac 4'-hydroxylation. Additional components were 0.25 mg/mL microsomal protein and 1 mM NADPH.

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CYP2C19 activity was studied with the formation of 4'-hydroxy S-mephenytoin from S-mephenytoin as the form-selective activity (Ring et al. 2001). Incubations contained 0.5 mg/mL microsomal protein, 1 mM NADPH, and at least a single concentration (5 μ M) of S-mephenytoin. When R-95913 was the inhibitor, R-95913 concentrations were 0, 0.5, 2.5, 10, and 20 μ M, and S-mephenytoin concentrations were 5, 10, 25, 50, and 100 μ M. When R-138727 was the inhibitor, R-138727 concentrations were 0, 0.5, 2.5, 10, 50, and 100 μ M.

The biotransformation of bufuralol to 1'-hydroxybufuralol was used as the marker activity for CYP2D6 (Ring et al. 1996). Incubations contained 0, 10, 50, 100, and 200 μ M R-95913 and 5, 10, 25, 50, and 100 μ M bufuralol when R-95913 was the inhibitor. When R-138727 was the inhibitor, 0, 0.7, 3.3, 13, 67, and 267 μ M R-138727 was incubated with a concentration of bufuralol approximating the K_m (5 μ M) for the bufuralol 1'-hydroxylation. Additional components were 0.1 mg/mL microsomal protein and 1 mM NADPH.

The biotransformation of midazolam to 1'-hydroxymidazolam was used as one of the marker activities for CYP3A (Ring et al. 2001). Incubations contained 0, 10, 20, 40, and 60 μ M R-95913 and 2.5, 5, 10, 25, and 50 μ M midazolam when R-95913 was the inhibitor. When R-138727 was the inhibitor, 0, 0.5, 2.5, 10, 50, and 200 μ M R-138727 was incubated with a concentration of midazolam approximating the K_m (2.5 μ M) for the midazolam 1'-hydroxylation. Additional components were 0.5 mg/mL microsomal protein and 1 mM NADPH.

The biotransformation of testosterone to 6 β -hydroxytestosterone was employed as the second marker activity for CYP3A. Incubation conditions were as previously described (Fayer et al. 2001). Incubations contained 0, 2.5, 100, 150, and 200 μ M R-95913; 5, 25, 50, 150, and 250 μ M testosterone; 0.375 mg/mL microsomal protein; and 1 mM NADPH. Co-elution of R-138727

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with 6 β -hydroxytestosterone prevented inclusion of R-138727 as an inhibitor of testosterone 6 β -hydroxylation.

Analytical conditions. A variation of the method of Pang et al. (2005) was used for the analyses of samples containing R-138727 (Table 1). R-138727 was stabilized by derivatization with BMAP (10 mM; in excess of the combined R-138727 and GSH concentrations), which was considered to have proceeded to completion in all samples.

Conditions for LC/MS/MS analyses of samples containing acetaminophen, 4'-hydroxy S-mephenytoin, and 1'-hydroxymidazolam are also found in Table 1. HPLC sample analyses for 4'-hydroxydiclofenac (Ring et al. 2001) and 1'-hydroxybufuralol (Ring et al. 1996) formation were described previously. All LC/MS/MS analyses were conducted with a Sciex API 3000 equipped with a Shimadzu LC10 AD pump, a Shimadzu SCL 10 AD VP Controller, and a HTS PAL Leap Autosampler. For analysis of 6 β -hydroxytestosterone formation, HPLC with UV detection was as described in Analytical Method A from Fayer et al. (2001) when R-95913 was the inhibitor.

Calculations. Enzyme kinetics of the formation of R-138727 from R-95913 were initially evaluated by visual examination of Eadie-Hofstee plots to assess whether one or more enzymes were involved (Enzyme Kinetics Module version 1.1 for SigmaPlot, SPSS, Chicago, IL). Nonlinear regression analysis (Ring et al. 2001) was used to fit the Michaelis-Menten model (Segel 1975; equation 1) to the formation rate data (WinNonlin, Version 3.1, Pharsight Corporation, Mountain View, California) to determine apparent K_m and V_{max} parameters.

$$v = (V_{max} * S)/(K_m + S) \quad (1)$$

Intrinsic clearance (Cl_{int}) was determined with equation 2:

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$$Cl_{int} = V_{max}/K_m \quad (2)$$

Using equations 3 and 4, relative contributions of various P450s to total P450-mediated clearance were determined by scaling up Cl_{int} determined for expressed P450s based on abundance of P450s in the human liver (Hijazi and Boulieu 2002):

$$\text{Scaled Clearance}_{P4501} = (\text{Liver Abundance}_{P4501} * CL_{int,expressed P4501}) \quad (3)$$

$$\text{Contribution}_{P4501} = \text{Scaled Clearance}_{P4501} / \sum \text{Scaled Clearance}_{\text{all P450s}} \quad (4)$$

Correlation analyses were performed (JMP, Version 4.0.2, SAS Institute, Inc., Cary, NC) between the rates of R-138727 formation during incubation with 20 μ M R-95913 and enzymatic activities or immunoquantified levels of various P450s in a human liver microsomal bank of up to 20 samples as described previously (Ekins et al. 1998, Ring et al. 2001). The form-selective catalytic activities or immunoquantified levels for CYP1A2 (phenacetin O-deethylation); CYP2A6 (coumarin 7-hydroxylation); CYP2B6 (immunoquantified levels); CYP2C8 (taxol 6-hydroxylation); CYP2C9 (diclofenac 4'-hydroxylation); CYP2C19 (S-mephenytoin 4'-hydroxylation); CYP2D6 (bufuralol 1'-hydroxylation); CYP2E1 (chlorzoxazone 6-hydroxylation); and CYP3A (midazolam 1'-hydroxylation) were used as possible coregressors. For inhibition studies, the apparent kinetic parameters V_{max} , K_m , and K_i and the standard errors of the parameter estimates were determined using conventional relationships for reversible inhibition (Segel 1975). The predicted in vivo inhibition by R-95913 of the catalytic activities of the examined P450s was calculated as follows (Segel 1975, Ring 2002):

$$\% \text{ inhibition} = ([I] / ([I] + K_i)) * 100 \quad (5)$$

The K_i terms entered into this formula are those generated in this study. The inhibitor concentration (I) was the highest individual C_{max} value observed for R-95913 in a clinical study following a 60 mg loading dose of prasugrel: 616 ng/mL (1.9 μ M; unpublished observations).

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Results

Identification of the enzymes responsible for the formation of R-138727 from R-95913.

The rates of R-138727 formation over a range of R-95913 concentrations were determined in incubations containing human liver microsomal samples HLC, HLG, and HLH, each of which possessed a full complement of P450s (Ekins et al. 1998, Ring et al. 2001). Eadie-Hofstee plots (not shown) were monophasic for all three samples, consistent with a single enzyme predominating in the formation of R-138727. Samples HLC, HLG, and HLH exhibited apparent K_m values of 21, 30, and 26 μM , respectively, for the formation of R-138727 (Table 2). The corresponding CL_{int} values in these microsomal samples, calculated from equation 2, ranged from 5.8 to 12 $\mu\text{L}/\text{minute}/\text{mg}$ protein (Table 2).

When the R-138727 formation rates were analyzed for correlation with the rates of form-selective catalytic activities or immunoquantified levels of the P450s (Table 3), the only regressor that exhibited statistical significance in relation to R-138727 formation was the activity associated with CYP3A, 1'-hydroxymidazolam formation, ($r^2 = 0.98$, $p < .001$), suggesting that CYP3A predominates in R-138727 formation.

The abilities of cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 to form R-138727 were evaluated following incubations containing 20 μM R-95913. In rank order of rates, $\text{CYP3A4} > \text{CYP2B6} > \text{CYP2C19} \approx \text{CYP2C9} > \text{CYP2D6}$ were all able to form R-138727 (Figure 2). Michaelis-Menten kinetic parameters were determined (using equation 1) for the four most active of these enzymes under linear rate conditions (Table 2). As demonstrated by the low K_m value, 2.3 μM , R-95913 had the highest affinity for CYP2B6. The K_m value for R-95913 interaction with CYP3A4 (21 μM) was the highest of the K_m values for the four P450s and similar to the K_m value observed for human liver

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microsomal samples (21 μM to $\sim 30 \mu\text{M}$). The corresponding V_{max} for CYP3A4-mediated R-138727 formation from R-95913 was higher than for the other three P450s. Considering the K_m and V_{max} for CYP2B6 and CYP3A4, CYP2B6 appears to be a high affinity, low capacity enzyme for R-138727 formation, while CYP3A4 appears to be a low affinity, high capacity enzyme.

The Cl_{int} values from the in vitro expressed P450 system were then scaled according to the relative abundances of each P450 in human liver microsomes determined in five different studies (Shimada et al. 1994, Lasker et al. 1998, Rodrigues 1999, Shimada et al. 1999, Hijazi and Boulieu 2002), and the potential contributions from the respective enzymes to the clearance of R-95913 via the R-138727 formation route were estimated (Table 4). Overall, the projected contribution of CYP2B6 varied 18-fold, from 2% - 36%, depending on the source of the relative abundance data (Table 4). Estimates of the contributions of CYP2C9 (14% -19%) and CYP2C19 (8% - 11%) varied only slightly and indicated relatively minor involvement in overall R-138727 formation by these enzymes (Table 4). The contribution of CYP3A4 was consistently projected to be the highest of the four enzymes, ranging from 38-70% (Table 4).

Specific inhibitors of CYP2B6 (8 μL inhibitory mAb), CYP2C9 (10 μM sulfaphenazole), CYP2C19 (10 μM omeprazole), and CYP3A4 (2 μM ketoconazole) were examined to determine their effects on the formation of R-138727 from R-95913 (20 μM) (Figure 3). The effects of adding up to 8 μL of the CYP2B6 mAb to incubations were evaluated in comparison to control incubations containing the same volume of vehicle (ascites fluid). Although a range of volumes of mAb were examined, maximal inhibition occurred in the presence of 8 μL of CYP2B6 mAb, yielding a consistent 48% to 52% inhibition in the four microsomal samples tested (Figure 3). Sulfaphenazole produced only minor (12-32%) inhibition (Figure 3). The effects of omeprazole varied from 29% inhibition to a 36% increase relative to controls without omeprazole (Figure 3).

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Ketoconazole inhibited R-138727 formation activity 33% to at least 86% (Figure 3). The maximal inhibition ($\geq 86\%$), observed for HLL, was calculated based on the lower limit of quantitation (LLOQ) for R-138727, although the level of R-138727 formed was actually below the LLOQ.

Inhibition of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A by R-95913 and R-138727

In vitro, R-95913 was found to inhibit CYP2C9, CYP2C19, CYP2D6, and CYP3A. The inhibition of CYP2C9-mediated diclofenac 4'-hydroxylation, CYP2C19-mediated S-mephenytoin 4'-hydroxylation, and CYP3A-mediated midazolam 1'-hydroxylation by R-95913 best fit the competitive inhibition model, yielding respective apparent K_i values of 82 μM , 7.2 μM , and 13 μM (Table 5). The non-competitive inhibition model was the best fit to the data for inhibition of CYP2D6-mediated bufuralol 1'-hydroxylation and CYP3A-mediated testosterone 6 β -hydroxylation by R-95913, yielding apparent K_i values of 41 μM and 56 μM , respectively (Table 5). Using these K_i values and a plasma concentration of 1.9 μM (as described in Methods) in equation 5 yielded estimates of predicted in vivo inhibition ranging from 2% to 21% (Table 5).

The abilities of R-95913 to inhibit CYP1A2-mediated phenacetin O-deethylation and of R-138727 to inhibit all the P450s were examined in incubations containing substrate concentrations approximating the K_m values for the respective reactions. Inhibition at the highest concentration of inhibitor (200 μM to 267 μM) did not exceed 50% (data not shown). Thus, inhibition with these substrate/inhibitor combinations was deemed non-substantial, and full K_i determinations were not performed. Due to interference in the HPLC chromatograms, R-138727 could not be tested as an inhibitor of testosterone 6 β -hydroxylation.

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Discussion

After oral administration to humans, prasugrel is not detected in plasma because of rapid de-esterification to R-95913 (Farid et al. 2005b). The active metabolite of prasugrel, R-138727, is then generated from R-95913 via P450s. In the current study, the P450s involved in the biotransformation of R-95913 to R-138727 were identified and the relative contributions of those enzymes to R-138727 formation were projected. The second series of experiments explored the potential for R-95913 and R-138727 to cause drug-drug interactions resulting from inhibition of the major P450s involved in drug metabolism. This information, collectively, can potentially be used to evaluate 1) possible pharmacokinetic variability in the formation of prasugrel metabolites within the human population; 2) pharmacokinetic/pharmacodynamic relationships for prasugrel metabolites; and 3) the effects of co-administered drugs on R-138727 formation and the effects of the circulating metabolites of prasugrel on the metabolism of co-administered drugs.

Inclusion of GSH in incubations of R-95913 was required, consistent with *in vitro* studies in which the active thiol metabolites of clopidogrel (Savi et al. 2000; Pereillo et al. 2002) and ticlopidine (Yoneda et al. 2004) have been detected. To measure the chemically unstable, free thiol-containing R-138727, stabilization of R-138727 in the reduced form also required post-incubation derivatization with 10 mM BMAP. The ratio of GSH in the incubations to BMAP in the stop solution was optimized and the derivatization was found to be relatively specific. Glutathione:BMAP mixed disulfides were observed, but in relatively small amounts. Additionally, low levels of glutathione:R-138727 mixed disulfides were observed, but R-138727 homodimers were never detected. Therefore, with the addition of excess BMAP, the derivatization of R-138727 with BMAP was considered to have proceeded to completion in all samples.

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The formation of R-138727 by five of nine expressed P450s established that multiple P450s may contribute to R-138727 formation in vivo. Upon gauging the relative efficiency of this conversion by four of these P450s with the kinetic parameter CL_{int} , CYP2B6 was the most efficient enzyme involved, followed by CYP2C19, CYP3A, and CYP2C9. However, to account for the varying quantities of the relevant P450s in the liver, an exercise was conducted where each CL_{int} estimate was adjusted using the relative hepatic abundances of the P450s (Table 4). Because the P450 contents reported in the literature vary extensively, multiple determinations were made, and a range of potential in vivo contributions was then projected (Table 4). The CYP2B6 levels reported in the literature varied 39-fold, with the low estimate (1 pmol/mg) from Shimada et al. (1994), and the high (39 pmol/mg) estimate from Rodrigues (1999). CYP2B6 expression has been shown to vary with genotype, sex, and ethnicity (Lamba et al. 2003), but a more likely explanation for the extensive variability in reported CYP2B6 abundance has to do with the antibodies used for protein detection (reviewed in Stresser and Kupfer 1999). CYP2B6 antibody sensitivity and specificity have improved in recent years, with later studies (for instance, Code et al. 1997 and Ekins et al. 1998) indicating measurable CYP2B6 in all samples and higher maximal and average levels than reported in earlier studies. As the wide-ranging estimates of CYP2B6 contribution to R-138727 formation (2% - 36%, Table 4) show, the ability to robustly measure CYP2B6 greatly influences the projected in vivo outcome, and, in the case presented here, use of the higher abundance value for CYP2B6 (determinations B and E; Table 4) resulted in a compensatory reduction in the projected contributions for CYP3A. In fact, in Determination E (Table 4), the projected contributions from CYP2B6 and CYP3A are similar. With CYP2B6 being the more efficient catalyst of the R-138727 reaction ($Cl_{int} = 0.170$ vs. 0.065 ; Table 2), there may be cases where individuals possess high levels of CYP2B6 versus CYP3A, and CYP2B6-mediated formation of R-138727 predominates. The inherent uncertainty in these scaling exercises is a major reason why a multi-pronged in vitro approach is used for a

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comprehensive picture of metabolism and drug-drug interaction potential (Ring and Wrighton 2000).

In the earliest reports of the relative abundances of the P450s in the human liver (Shimada et al., 1994, Shimada et al. 1999), the individual contributions of CYP2C9 and CYP2C19 were not reported. Instead, a total CYP2C content was indicated. Therefore, in two of the five determinations of the contributions of CYP2C9 and CYP2C19 to the formation of R-138727, the total CYP2C content was adjusted using the results of Lasker et al. (1998) to reflect the content of the individual CYP2C enzymes (Table 4). Even after these adjustments, CYP2C9 and CYP2C19 abundance estimates varied only 1.9-fold, much less than the reported CYP2B6 abundances. The reported average CYP3A abundances were consistently high and also varied only 1.9-fold. Upon scale-up, the ranges of projected contributions from each enzyme varied 19-fold for CYP2B6, but only 1.4- to 1.8-fold for CYP2C9, CYP2C19, and CYP3A. Contributions to R-138727 formation from CYP2C9 and CYP2C19 were projected to be relatively minor in all cases, while substantial CYP3A contributions were consistently predicted.

Inhibition of R-138727 formation by the chemical inhibitors selective for CYP2C9 and CYP2C19 was minimal and consistent with the results of the expressed enzymes experiment and the abundance-scaling exercises. Inhibition by the mAb to CYP2B6 was consistently near 50% (Figure 3) across the four microsomal samples tested, confirming a substantial involvement of CYP2B6 in R-138727 formation. Additionally, ketoconazole, the selective CYP3A inhibitor, inhibited R-138727 substantially (> approximately 50%) for three of the four microsomal samples tested, confirming the involvement of CYP3A in R-138727 formation. In summary, the overall results for the formation of R-138727 indicate substantial contributions of CYP3A and CYP2B6, with CYP3A predominating in most cases and CYP2B6 predominating in certain individuals.

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The inhibitory potentials of the major circulating metabolites of prasugrel, R-95913 and R-138727, towards CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A were also measured. Inhibition by R-138727 did not exceed the $\geq 50\%$ threshold for K_i determinations for any of the form-selective activities in vitro. Consequently, R-138727 would not be predicted to be an inhibitor in vivo. On the other hand, R-95913, in vitro, inhibited the catalytic activities for all the P450s tested except CYP1A2. To put these results into perspective, a conservative estimate of a R-95913 concentration in human plasma ($1.9 \mu\text{M}$, the highest individual C_{max} achieved after a 60-mg prasugrel loading dose) was used to relate the in vitro K_i values to inhibition in vivo. Inhibition of CYP2C19, the enzyme most potently affected in vitro, was predicted to affect metabolism of other CYP2C19 substrates in vivo by only 21%. Additionally, because CYP3A contributes to the clearance of so many drugs, potential inhibition of CYP3A-mediated metabolism by R-95913 was a concern. However, CYP3A was predicted to be affected only a maximum of 13% by R-95913 in vivo. CYP2C9 and CYP2D6 were predicted not to be affected by R-95913 in vivo. Thus, neither R-95913 nor R-138727 is expected to have a clinically meaningful impact on the P450-mediated metabolic clearance of other drugs.

These studies demonstrate that R-138727, the active metabolite of prasugrel, is produced from R-95913 by multiple P450s. CYP3A appears to be the enzyme most predominant in R-138727 formation, based on the high degree of correlation between R-138727 formation activity and midazolam-1'hydroxylation activity, the substantial inhibition of R-138727 formation by ketoconazole in human liver microsomal samples, and the consistently high (38-70%) contribution predicted from the abundance scaling exercise. CYP2B6 may also contribute substantially to R-138727 formation in some individuals, as evidenced by consistent substantial inhibition of R-138727 formation in the presence of mAb to CYP2B6 and the moderate to low contribution predicted from the abundance scaling exercise. The scale-up exercises reinforced

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the finding that CYP3A and possibly CYP2B6 play substantial roles in R-138727 formation. In addition, CYP2C9, CYP2C19, and CYP2D6 were capable of forming R-138727, and as estimated by scaling, may contribute as much as 10% to 20% to R-138727 formation. Therefore, in vivo, it was predicted that should any single P450 involved in R-138727 formation be absent or inhibited by a co-administered drug, other P450s would remain capable of forming R-138727. Thus, therapeutic failure due to lack of formation of the active metabolite of prasugrel would be unlikely. This hypothesis was proven in a clinical drug-drug interaction study where the potent CYP3A inhibitor ketoconazole was co-administered with prasugrel but affected neither the exposure to active R-138727 over 24 hours nor the antiplatelet effects of prasugrel (Farid et al. 2005a). Furthermore, predictions of in vivo inhibition potential indicate that neither R-95913 nor R-138727 is expected to substantially inhibit the CYP1A2-, CYP2C9-, CYP2C19-, CYP2D6-, or CYP3A-mediated metabolism of co-administered drugs. In total, this series of in vitro studies indicates that drug-drug interaction liability with prasugrel therapy appears minimal.

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Footnotes

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Figure Legends

Figure 1. *Prasugrel and the biotransformation pathway to the inactive intermediate R-95913, followed by conversion to active R-138727.*

Figure 2. *Formation of R-138727 by human lymphoblast-expressed P450s.* Results are presented as means of triplicate results \pm SD (except for CYP3A4, where the mean of duplicate results is shown).

Figure 3. *Effects of an inhibitory mAb to CYP2B6 and chemical inhibitors selective for CYP2C9 (sulfaphenazole), CYP2C19 (omeprazole), and CYP3A (ketoconazole) on R-138727 formation by four human liver microsomal samples.* The CYP2B6 inhibition study was conducted with human liver samples HLB, HLD, HLG, and HLP, while the CYP2C9, CYP2C19, and CYP3A inhibition study was conducted with human liver samples HLB, HLD, HLG, and HLL.

Table 1. LC/MS/MS analytical methods.

Catalytic activity	P450-selective activity	Internal Standard (IS)	Sample prep ^a	Column and Flow rate	Mobile Phases	Gradient conditions	Ionization mode	Ions monitored (m/z)
R-95913 → R-138727	not applicable	² H ₄ -R-138727	A	Monochrome C18, 5 μm, 100 x 2.0 mm (Metachem); 0.2 mL/min	A: 5% methanol in 0.2% formic acid B: 95% methanol in 0.2% formic acid	6 minutes: escalation from 10% B to 95% B and back to 10% B	Positive Electrospray	498 → 348 for derivatized R-138727 502 → 111 for derivatized IS
Phenacetin → acetaminophen	CYP1A2	² H ₄ -acetaminophen	B	Monochrome C18, 5 μm, 50 x 2.0 mm (Metachem) 0.2 mL/min	A: 5% methanol in 5 mM ammonium acetate B: 95% methanol in 5 mM ammonium acetate	3.6 minutes: escalation from 10% B to 80% B and back to 10% B	Positive Electrospray	152 → 110 for acetaminophen 155.95 → 114.05 for IS
S-mephenytoin → 4'-hydroxy S-mephenytoin	CYP2C19	phenytoin	B	Monochrome C18, 5 μm, 50 x 2.0 mm (Metachem) 0.2 mL/min	A: 5% methanol in 5 mM ammonium acetate B: 95% methanol in 5 mM ammonium acetate	3.6 minutes: escalation from 10% B to 80% B and back to 10% B	Negative Electrospray	233 → 189.9 for 4'-hydroxy S-mephenytoin 250.9 → 102.1 for IS
Midazolam → 1'-hydroxy-midazolam	CYP3A	α-hydroxy-triazolam	C	YMC-AQ, C18, 5 μm, 100 x 2.0 mm (Waters) 0.22 mL/min	A: 5% methanol in 50 mM ammonium acetate B: 95% methanol in 50 mM ammonium acetate	6.0 minutes: escalation from 65% B to 95% B and back to 65% B	Positive Electrospray	342 → 324 for 1'-hydroxy-midazolam 359 → 331 for IS

^aSample Preparation Methods

A: Internal standard (IS) and 10 mM BMAP (derivatizing agent) added in an equal volume of acetonitrile (ACN) to stop reactions; samples centrifuged and 30 μL supernatant diluted with 170 μL deionized water.

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B: IS added in equal volume ACN to stop reactions; samples centrifuged and 30 μ L supernatant diluted with 170 μ L deionized water.

C: Equal volume ACN added to stop reactions. IS (20 μ L) added, samples centrifuged, and 30 μ L supernatant diluted with 170 μ L deionized water.

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Table 2. Kinetic parameters for the formation of R-138727 by human liver microsomal samples and expressed P450s^a

Microsomal Sample	K_m μM	V_{max} $pmol/min/mg$	Cl_{int} $\mu L/min/mg$
HLC	21 ± 4	122 ± 12	5.8
HLG ^b	30 (27 – 33)	318 (290 – 346)	11 (10 – 11)
HLH	26 ± 2	300 ± 16	12
Expressed P450	K_m μM	V_{max} $pmol/min/pmol P450$	Cl_{int} $\mu L/min/pmol P450$
CYP2B6	2.3 ± 0.4	0.39 ± 0.02	0.170
CYP2C9	11 ± 2	0.37 ± 0.02	0.032
CYP2C19	3.8 ± 0.7	0.35 ± 0.01	0.092
CYP3A4	21 ± 3	1.4 ± 0.1	0.065

^aThe conversion rates of R-95913 to R-138727 were determined in triplicate incubations as indicated in Methods. Values for K_m and V_{max} are reported as parameter estimate ± the standard error of the parameter estimate.

^bValues for HLG are reported as average value (range) from two separate experiments.

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Table 3. Rates of formation of R-138727 from R-95913 by a bank of human liver microsomal samples

Microsomal Sample	R-138727 formation ^a <i>pmol/min/mg</i>
HLA	139
HLB	119
HLC	73
HLD	96
HLE	509
HLF	254
HLG	151
HLH	99
HLI	695
HLJ	191
HLK	169
HLL	112
HLM	133
HLN	164
HLO	801
HLP	414
HLQ	98
HLR	105
HLS	220
HLT	610

^aRates are averages of triplicate results.

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Table 4. Scaling of in vitro Cl_{int} to project contributions of CYP2B6, CYP2C9, CYP2C19, and CYP3A to in vivo clearance of R-95913 via R-138727.

Scaling Determination	CYPs	Literature References	Abundance	Contribution to R-95913 clearance ^a
			<i>pmol/mg</i>	%
A	CYP2B6	Hijazi and Boulieu 2002	4.8	6
	CYP2C9	Hijazi and Boulieu 2002, Lasker et al. 1998	89	19
	CYP2C19	Lasker et al. 1998	18	11
	CYP3A	Hijazi and Boulieu 2002	146	64
B	CYP2B6	Rodrigues et al. 1999	39	32
	CYP2C9	Hijazi and Boulieu 2002, Lasker et al. 1998	89	14
	CYP2C19	Lasker et al. 1998	18	8
	CYP3A	Hijazi and Boulieu 2002	146	46
C	CYP2B6	Shimada et al. 1994	1.0	2
	CYP2C9	Shimada et al. 1994, Lasker et al. 1998 ^b	50	18
	CYP2C19	Shimada et al. 1994, Lasker et al. 1998 ^b	10	10
	CYP3A	Shimada et al. 1994	96	70
D ^c	CYP2B6	Shimada et al. 1999	7.8	14
	CYP2C9	Shimada et al. 1999, Lasker et al. 1998 ^b	55	19
	CYP2C19	Shimada et al. 1999, Lasker et al. 1998 ^b	11	11
	CYP3A	Shimada et al. 1999	78	55
E	CYP2B6	Rodrigues et al. 1999	39	36
	CYP2C9	Rodrigues et al. 1999	96	17
	CYP2C19	Rodrigues et al. 1999	19	9
	CYP3A	Rodrigues et al. 1999	108	38
			P450s	Range of contributions (%)
			CYP2B6	2 - 36
			CYP2C9	14 - 19
			CYP2C19	8 - 11
			CYP3A4	38 - 70

^a Cl_{int} from Table 2 were scaled according to equations 3 and 4 in Methods to determine contributions.

^b The abundances of total CYP2C from Shimada et al. 1994 and Shimada et al. 1999 were adjusted by the proportions of CYP2C9 and CYP2C19 (5 parts to 1 part) reported in Lasker et al. 1998.

^c Shimada et al. 1999 for the livers of Caucasian origin only.

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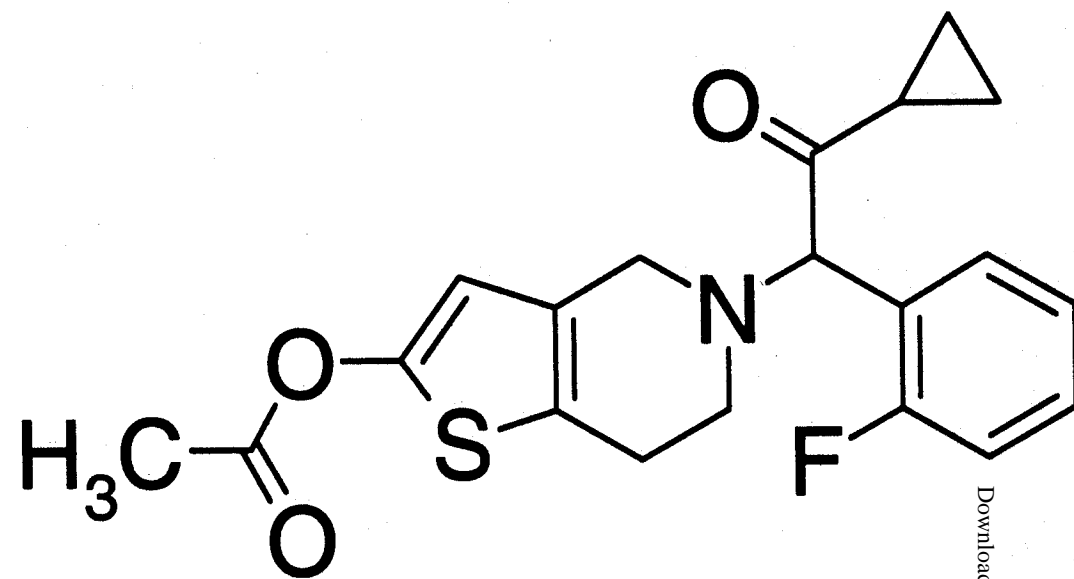
Table 5. In vitro inhibition of P450 form-selective catalytic activities and predicted in vivo inhibition of P450s by R-95913

P450	Form-selective activity	Inhibition type	Apparent K_i value	Predicted inhibition of P450 in vivo (%)
			μM	
CYP2C9	diclofenac 4'-hydroxylation	competitive	82 ± 10	2
CYP2C19	S-mephenytoin 4'-hydroxylation	competitive	7.2 ± 0.9	21
CYP2D6	bufuralol 1'-hydroxylation	non-competitive	41 ± 4	4
CYP3A	midazolam 1'-hydroxylation	competitive	13 ± 2	13
CYP3A	testosterone 6 β -hydroxylation	non-competitive	56 ± 4	3

^aValues for K_m and V_{max} are reported as parameter estimate \pm the standard error of the parameter estimate.

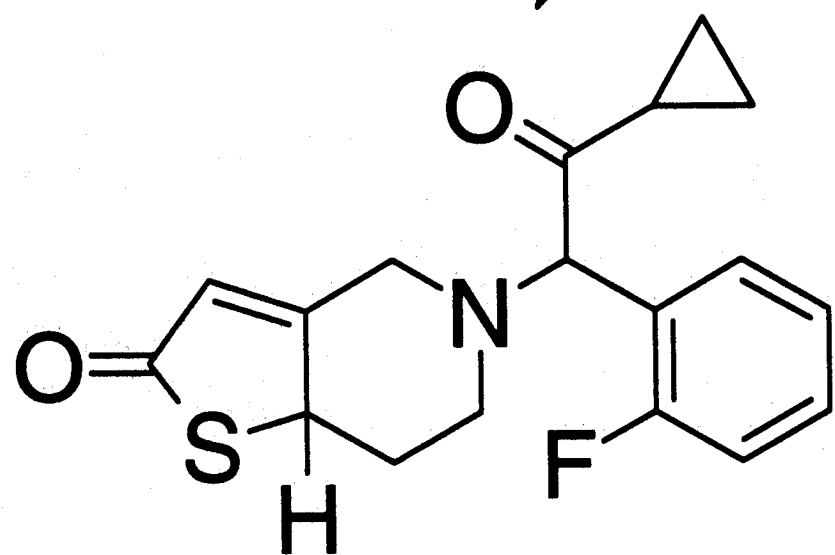
Figure 1.

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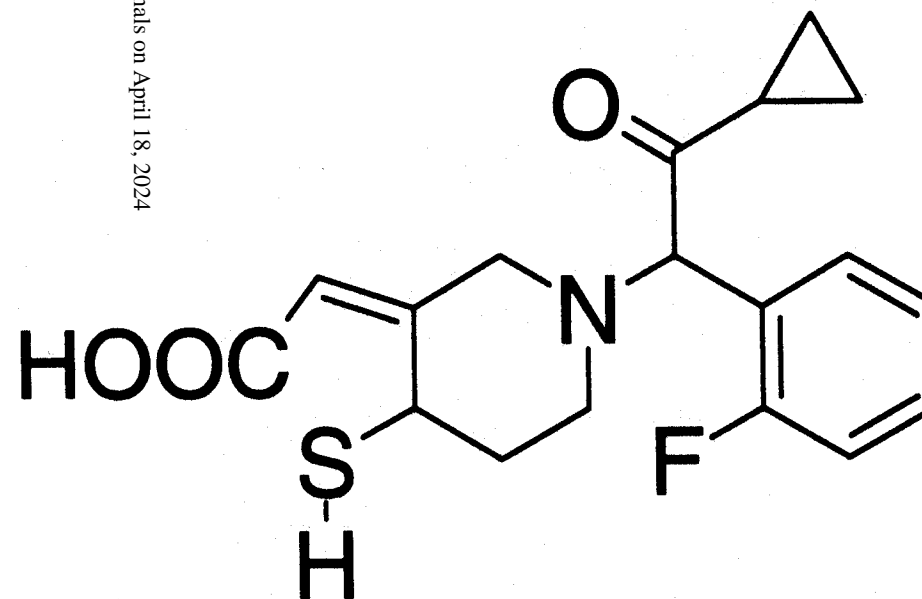
Prasugrel

esterases



R-95913 (inactive)

P450s



R-138727 (active)

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Figure 2.

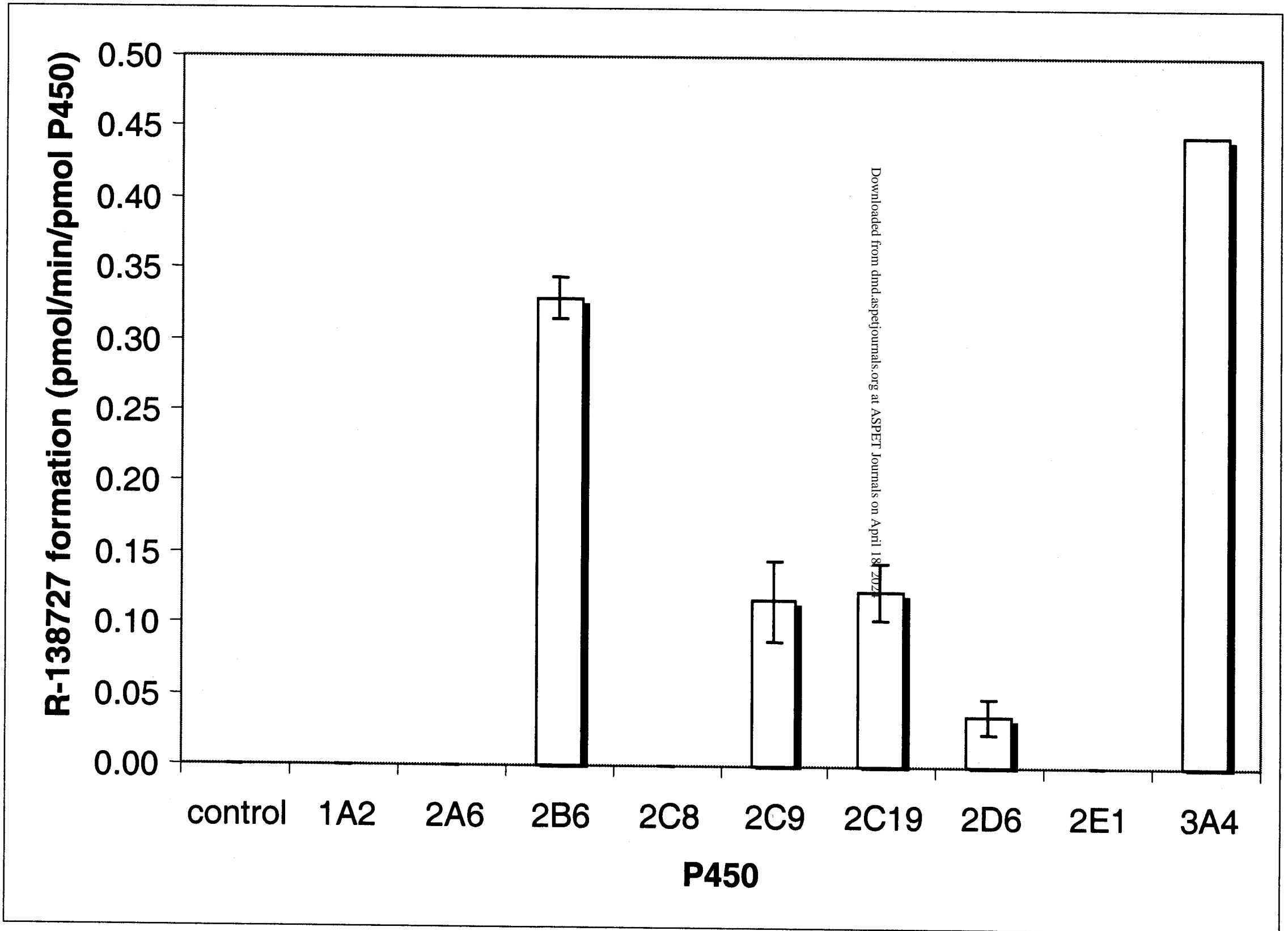


Figure 3.

