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The Naturally Occurring P450 2B6 K262R Mutant of P450 2B6 Exhibits Alterations in Substrate Metabolism and Inactivation

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Abbreviations: P450, cytochrome P450; P450 Δ2B6, N-terminally truncated P450 2B6; P450 Δ2B6 K262R, N-terminally truncated P450 2B6 Lysine 262 Arginine mutant; tTEPA, N, N', N"-triethylenethiophosphoramide; 17EE, 17-α-ethynylestradiol; 7-EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; HPLC, high-performance liquid chromatography; LC-MS, liquid-chromatography mass-spectrometry; ESI, electrospray ionization.

ABSTRACT

The polymorphic human cytochrome P450 2B6 is primarily responsible for the metabolism of several clinically relevant drugs including bupropion, cyclophosphamide, propofol, and efavirenz. Although a number of single nucleotide polymorphisms (SNPs) have been found in the P450 *2B6* gene, the influence of these variants on the metabolism of substrates and on the response to known inactivators of P450 2B6 have not been examined. We have compared the metabolism of different substrates of P450 2B6 (P450 Δ2B6) and the effects of mechanism-based inactivators with that observed with the polymorphic P450 Δ2B6 K262R in a reconstituted monooxygenase system (reconstituted system). Metabolism of bupropion by P450 Δ2B6 K262R resulted in increased production of hydroxybupropion compared to P450 Δ2B6. However, production of formaldehyde from the metabolism of benzphetamine by the P450 Δ2B6 K262R mutant was significantly less than that of the wild-type isozyme. P450 Δ2B6 K262R formed less benzphetamine metabolites compared to the wild-type. *N,N',N"*-triethylenethiophosphoramide (tTEPA) and bergamottin decreased the ability of both enzymes to hydroxylate bupropion and to *O*-deethylate 7-hydroxy-4-(trifluoromethyl) coumarin (7EFC). Incubation with 17-α-ethynylestradiol (17EE) decreased bupropion hydroxylation and 7EFC O-deethylation with the wild type enzyme but had no effect on the mutant. The kinetics for inactivation of the variant by tTEPA and bergamottin were determined using 7EFC. The K_I values for inactivation of the variant were significantly greater than those determined for the wild-type enzyme. These data demonstrate a functional difference between P450 Δ2B6 and the allelic variant P450 Δ2B6 K262R.

The cytochrome P450 (P450) enzymes belong to a family of heme containing proteins that catalyze the metabolism of a wide range of endogenous and exogenous substrates. Although the polymorphic enzyme P450 2B6 is expressed in relatively low levels in the liver (Gervot et al., 1999), it has been shown to play an important role in the metabolism of a number of clinically relevant drugs including bupropion hydrochloride (Faucette et al., 2000, Hesse et al., 2000), cyclophosphamide (Chang et al., 1993), propofol (Oda et al., 2001) and efavirenz (Ward et al., 2003). A number of single nucleotide polymorphisms (SNPs) have been found in the P450 2B6 gene (Lang et al., 2001). However, the ability of these variants to metabolize other substrates and the response of these variants to known inactivators of P450 2B6 have not yet been examined. The P450 2B6 K262R (*2B6*4*, 785A>G, exon 5) is associated with increased clearance of bupropion and higher levels of the hydroxybupropion metabolite in German males (Kirchheiner et al., 2003). In this study we investigated the effect of this SNP on the metabolism of several P450 2B6 substrates including bupropion and the ability of P450 Δ2B6 K262R to become inactivated by three structurally unrelated mechanism-based inactivators of P450 2B6 (Figure 1a).

Bupropion is a widely used anti-depressant and smoking cessation aid that acts by inhibiting the reuptake of norepinephrine and dopamine (Ascher et al., 1995, Hurt et al., 1997). Bupropion has also been shown to be effective in the treatment of attention deficit/hyperactivity disorder (ADHD) in adults (Wilens et al., 2001). In humans, bupropion is extensively metabolized to give three primary metabolites: erythrohydrobupropion, threohydrobupropion, and hydroxybupropion (Figure 1b) (Schroeder, 1983). P450 2B6 catalyzes the hydroxylation of bupropion to form

hydroxybupropion, which is the pharmacologically active metabolite that plays a role in the antidepressant activity of bupropion (Ascher et al., 1995). Side effects of bupropion include seizures and even death (Wooltorton, 2002). It has been reported that approximately 1 in 1000 subjects treated with bupropion experience seizures (Johnston et al., 1991). Elevated plasma level concentrations of hydroxybupropion are thought to be associated with poor clinical outcomes and seizures (Golden et al., 1988, Preskorn, 1991, Wooltorton, 2002).

N, N', N" -triethylenethiophosphoramide (tTEPA), bergamottin and 17-α-ethynylestradiol (17EE) are all mechanism-based inactivators of P450 2B6 in a reconstituted system with reductase (Rae et al., 2002, Harleton et al., 2004, Guengerich, 1990a, Kent et al., 2002, Lin et al., 2005). Mechanism-based inactivation occurs when the enzyme converts the substrate to a reactive intermediate that binds covalently to a moiety in the active site and thereby inactivates the enzyme. tTEPA is an anti-neoplastic agent used in the treatment of breast, bladder and ovarian cancers (Maanen, 2000). Bergamottin, a furanocoumarin found in grapefruit juice, inactivates P450s 3A4 (He et al., 1998) 2B6, and 3A5 (Lin et al., 2005). 17EE, a major component of many oral contraceptives is also a mechanism-based inactivator of P450 2B6 (Guengerich, 1990b, Kent et al., 2002). Because these compounds all inactivate the wild-type form of P450 2B6, their use may be problematic in the clinic when given in combination with a drug that is primarily metabolized by this enzyme. The effects of these substrates and inactivators on the allelic variant P450 2B6 K262R reported here show significant differences in metabolism and in the ability to inactivate this mutant.

EXPERIMENTAL PROCEDURES

Materials. Bupropion hydrochloride, triprolidine hydrochloride, NADPH, BSA, benzphetamine, catalase and 17EE were purchased from Sigma Chemical Co. (St. Louis, MO). tTEPA was purchased from U.S. Pharmacopeia (Rockville, MD) and bergamottin from Indofine Chemical Co. (Hillsborough, NJ). 7-Ethoxy-4-(trifluoromethyl)coumarin (7-EFC) was obtained from Molecular Probes (Eugene, OR). Hydroxybupropion was purchased from BD Biosciences (San Diego, CA). The P450 Δ2B6 plasmid was a generous gift from Dr. James Halpert, University of Texas Medical Branch, Galveston, Texas. This P450 2B6 had amino acids 3-21 deleted and minor changes made to increase expression and solubility (Scott et al., 2001). Purified benzphetamine and D-norbenzphetamine were a gift from Dr. Haoming Zhang, Department of Anesthesiology, Veteran Affairs Health Service, Ann Arbor, Michigan.

Statistical analysis. Graphs and the two-tailed unpaired t-test were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, California). K_m and V_{max} values were determined using EZ-Fit ™: Enzyme kinetic analysis (Perrella Scientific Inc., Amherst, NH). Data were fit using the Michaelis-Menten & unstable enzyme kinetics routine.

Site-Directed Mutagenesis and Purification of Enzymes: Construction of the P450 Δ2B6 K262R mutant was performed with Stratagene's Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) using primers: 5' GACCCCAGCGCCCCCAGGGACCTCATCGACACCTAC3' (upstream) and 5' GTAGGTGTCGATGAGGTCCCTGGGGGCGCTGGGTC3' (downstream). The

mutation was confirmed by DNA sequencing carried out at the University of Michigan Core Facility (Ann Arbor, MI).

Expression and Purification of P450s and NADPH-cytochrome P450 Reductase (Reductase). P450 Δ2B6, P450 Δ2B6 K262R and NADPH-P450 reductase were expressed in *E. coli* Topp 3 cells and purified according to published protocols (Scott et al., 2001, Hanna et al., 1998, Hanna et al., 2000) except that P450 Δ2B6 K262R was recovered from the cytosol rather than the microsomal fraction after the cell lysis step. Therefore, the cytosol was applied to the Ni^{++}-agarose column and the P450 was purified as previously described (Scott et al. 2001, Hanna et al., 2000).

Bupropion Metabolism. Purified P450s were reconstituted with reductase at a 1:2 ratio of P450 to reductase for 45 min at 4 °C. The reaction mixture consisted of 1 μM P450, 2 μM reductase, 110 U catalase and bupropion (concentrations ranging from 0 μM to 960 μM). NADPH was added to initiate the reactions and the mixtures were incubated for 30 min at 37 °C. The reaction was quenched by the addition of 125 μL of ice-cold acetonitrile containing 0.1% formic acid. The samples were then placed on ice and centrifuged at maximum speed for 10 min in an Eppendorf microcentrifuge at 4 °C. The method used to determine the concentration of hydroxybupropion was adapted from Faucette et al. (2000). Triprolidine (2 μl of a 20 mg/ml stock) was added as an internal standard and the samples were resolved on a 5 μm Waters Symmetry 15 x 3.9-mm C_{18} column (Millipore Corp., Milford, MA) at a flow rate of 1 ml/min, with the detector set at 214 nm. A gradient was generated with mobile phases A (0.25% triethylamine and 0.1% formic acid) and B (100% acetonitrile) that ranged from 13% B at 0 to 15.5 min, 25% B at 16 to 23 min, and 13% B at 23.5 to 35 min. The retention times were approximately

4.5 min for hydroxybupropion and 22 min for triprolidine. Hydroxybupropion formation was quantified from a standard curve generated by injecting increasing concentrations of authentic hydroxybupropion onto the HPLC column.

Benzphetamine Metabolism. The P450s were reconstituted as above and the formation of formaldehyde via *N*-demethylation of benzphetamine was measured as previously described (de Andrade et al., 1996). A saturating concentration of benzphetamine (2mM) was added to all samples. The amount of formaldehyde formed was determined using an excitation wavelength of 410 nm and an emission wavelength of 510 nm using a RF-5310 Spectrofluorophotometer (Shimadzu Scientific Instruments, Inc., Wood Dale, IL) and quantified from a standard curve. The individual metabolites of benzphetamine were also identified after adding the internal standard D-norbenzphetamine and after extraction of the metabolites with ethylacetate followed by separation and detection using ESI-LC-MS according to a previously published procedure (Kent et al., 2004). Because this ESI-LC-MS analysis did not allow for precise quantitation of each metabolite, the area under the peak of the metabolite was integrated and used for comparison purposes only between the two enzymes.

Inactivation of P450s Δ2B6 and Δ2B6 K262R. The purified P450s were reconstituted with reductase for 45 min at 4 °C. The primary reaction mixture contained 1 μM P450, 2 μM reductase, 110 U catalase and either tTEPA (100 μM), BG (10 μM) or 17EE (100 μM) in 50 mM potassium phosphate buffer, pH 7.4. The primary reaction mixtures were then incubated for 10 min at 30 °C prior to initiating the reactions by adding NADPH to a final concentration of 1.2 mM. After the addition of NADPH, 12 μL aliquots were removed from the primary reaction mixture at the times indicated and

transferred to 990 μL of the secondary reaction mixture which contained 100 μM 7-EFC, 1mM NADPH and 40 μg BSA/mL in 50 mM potassium phosphate buffer, pH 7.4. The reaction mixtures were incubated for 10 min at 30 °C, and then quenched with 334 μL of acetonitrile. The amount of 7-hydroxy-4-(trifluoromethyl) coumarin formed was measured at room temperature at an excitation wavelength of 410 nm and an emission wavelength of 510 nm using a RF-5310 Spectrofluorophotometer (Shimadzu Scientific Instruments, Inc., Wood Dale, IL). The amount of hydroxybupropion formed was determined as indicated previously for bupropion metabolism. For the tTEPA kinetic experiments, the primary reaction mixtures contained tTEPA concentrations ranging from 0 μM to 240 μM. The bergamottin kinetics experiments were performed using concentrations ranging from 0 μM to 12 μM. Aliquots (12 μL) were removed and added to the secondary reaction mixture at the indicated times.

17EE Metabolism. P450 Δ2B6 or P450 Δ2B6 K262R were reconstituted together with reductase as described above. The primary reaction mixtures contained 1 μM P450, 2 μM reductase, 200 μg/ml ascorbate, 110 U catalase, 40 μM 17EE and 50 mM potassium phosphate buffer, pH 7.4. The metabolites were resolved by reverse-phase HPLC according to a published protocol (Kent et al., 2002).

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RESULTS

Hydroxybupropion formation by P450 Δ2B6 and P450 Δ2B6 K262R.

Metabolism of the P450 2B6 specific substrate bupropion to hydroxybupropion was examined using HPLC. Figure 2 shows the rate of formation of hydroxybupropion produced by P450 Δ2B6 and P450 Δ2B6 K262R at substrate concentrations ranging from 0 μM-960 μM. Buproprion was poorly soluble at concentrations higher than 960 μM. The K_m value for P450 Δ2B6 was approximately 8.8 μM while the K_m value for P450 Δ2B6 K262R was approximately 54 μM. The V_{max} of the variant was approximately 6.9 nmol hydroxybupropion/nmol P450/min whereas the V_{max} of the wild-type was 2.6 nmol hydroxybupropion/nmol P450/min. The V_{max}/ K_m for P450 Δ2B6 was approximately 0.30 while the V_{max}/ K_m for P450 Δ2B6 K262R was approximately 0.13. Therefore, the catalytic efficiency ($V_{max}/ K_{m)}$ of the variant for buproprion was approximately 40% less than that of the wild-type enzyme.

Benzphetamine metabolism by P450 Δ2B6 and P450 Δ2B6 K262R. The enzymatic activities of P450 Δ2B6 and P450 Δ2B6 K262R were compared using benzphetamine as a substrate. The ability of each enzyme to metabolize benzphetamine to formaldehyde was determined first. P450 Δ2B6 K262R *N*-demethylated benzphetamine to produce 9.4 ± 0.9 pmol formaldehyde/pmol P450/min while the wild-type P450 Δ2B6 generated 16.3 ± 1.3 pmols formaldehyde/pmol P450/min (Figure 3). The individual metabolites norbenzphetamine (*N*-demethylation), amphetamine (*N*-demethylation and *N*-debenzylation), methamphetamine (*N*-debenzylation), hydroxynorbenzphetamine (*N*-demethylation and aromatic hydroxylation) and

hydroxybenzphetamine (aromatic hydroxylation) were also separated and the amounts estimated by ESI-LC-MS and the results are shown in Table 1. It can be seen that the mutation caused a decrease of approximately 50% or greater in the formation of most of the metabolites except for OH-norbenzphetamine, where it's formation by the mutant was less than 20% of that formed by the wild-type enzyme.

Inactivation of P450s Δ2B6 and Δ2B6 K262R by tTEPA, bergamottin and 17EE. The inactivation of P450 Δ2B6 K262R by these three mechanism-based inactivators was performed as described in the Experimental Methods. P450 Δ2B6 K262R was inactivated by both tTEPA (Figure 4) and bergamottin (Figure 5). The inactivation was time- and concentration-dependent with both compounds and displayed an absolute requirement for NADPH. The approximate K_I value for the tTEPA-mediated inactivation of the variant determined from the inset of Figure 4 was 210 μM with a $t_{1/2}$ of 18.6 min and a rate of inactivation of 0.04 min^{-1} as measured using the 7-EFC *O*-deethylation assay. The approximate K_I value for the inactivation of P450 Δ2B6 K262R by bergamottin determined from the inset of Figure 5 was 8.2 μM, the rate of inactivation was 0.23 min^{-1} with a $t_{1/2}$ of 3.01 min as determined using the 7-EFC *O*-deethylation activity assay. 17EE has previously been shown to be a mechanism-based inactivator of the P450 2B6 wild-type enzyme (Kent et al., 2002). In contrast to the wild-type enzyme, 17EE had no effect on the 7-EFC activity of the P450 Δ2B6 K262R mutant. In order to see if the loss in enzymatic activity observed with 7-EFC was substrate-dependent, each of the samples incubated with the three inactivators and NADPH was also analyzed simultaneously using the bupropion hydroxylation assay (Table 2). Bergamottin had the greatest effect on both P450 Δ2B6 and P450 Δ2B6 K262R leaving approximately 30%

and 31% bupropion hydroxylation activity remaining, respectively, and similar effects were seen using both assays. tTEPA inactivated the wild-type enzyme to a greater extent than the variant. There was a very significant difference between the inactivation of the mutant enzyme by tTEPA as determined by the bupropion assay when compared to the 7-EFC assay (p=.0028), although no significant difference was seen with the wild-type enzyme. 17EE inactivated the wild-type P450 Δ2B6 leaving 61% activity remaining with bupropion as the probe substrate while the P450 Δ2B6 K262R was not inactivated at all by 17EE. There was also a significant difference between the inactivation of the wild-type enzyme by 17EE when measured by the bupropion assay as compared to the 7-EFC assay (p=.0002).

Metabolism of 17EE by P450s Δ2B6 and Δ2B6 K262R. In order to see if the lack of inactivation of 2B6 K262R by 17EE was due to an inability of the enzyme to catalyze the metabolism of 17EE, the metabolism of 17EE by the two P450s was investigated. 17EE was incubated with the reconstituted P450s in the presence or absence of NADPH and the metabolites analyzed using reverse phase HPLC as shown in Figure 6. P450 Δ2B6 metabolized 17EE to give a number of major metabolites denoted as A, C, D and E as well as numerous other minor metabolites as previously described (Kent et al., 2002) (Panel A). However, as shown in Panel B, P450 Δ2B6 K262R did not produce any metabolite of 17EE above the levels of the control incubations incubated in the absence of NADPH.

DISCUSSION

These studies comparing the metabolic activities of purified P450 Δ2B6 and P450 Δ2B6 K262R in the reconstituted system show that a single mutation at position 262 to give the K262R variant results in a dramatically different ability of the mutant to metabolize a number of P450 2B6 specific drugs compared to the wild-type enzyme. Though regarded as a relatively minor component of the P450 family in the liver, P450 2B6 has been shown to play a significant role in the metabolism of many xenobiotics and in the activation of a number of pro-carcinogens including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Code et al., 1997, Rendic et al., 1997, Gervot et al., 1999). A number of chemotherapeutic drugs such as tTEPA are substrates for 2B6 and they are often given in combination with other drugs (Maanen et al., 2000, Rae et al., 2002, Harleton et al., 2004). As a result, there is a significant potential for interactions with other drugs that are also metabolized by this enzyme [particularly in instances where this isoform is induced by other xenobiotics.] Because P450 2B6 is polymorphic, drug interactions may be more detrimental for certain patients than for others, depending on the genotype. P450 2B6 has previously been shown to be responsible for the interindividual variability of propofol hydroxylation in liver microsomes (Court et al., 2001). A recent study demonstrated higher mean plasma concentrations of efavirenz in patients homozygous for P450 *2B6*6* (Q172H, K262R) when compared to wild-type (Tsuchiya et al., 2004). The K262R SNP is thought to be particularly important as it was found to have an allele frequency of approximately 5% in German males and a SNP frequency of 30% since it is present in three different P450 2B6 allelic variants (*2B6*4*, *2B6*6* and *2B6*7*) (Kirchheiner et al., 2003, Lang et al., 2001).

The studies presented here have focused on the potential effect of the K262R mutation in substrate metabolism and inactivation of this mutant in a reconstituted system by drugs that have been well characterized with the wild-type enzyme. Bupropion, a drug that is widely used to treat depression and aid in smoking cessation, is hydroxylated primarily by cytochrome P450 2B6 (Faucette et al., 2000, Hesse et al., 2000). Our findings suggest that P450 Δ2B6 K262R produced the hydroxylated product at a rate that was significantly greater than the wild-type enzyme. The K_m of wild-type P450 Δ2B6 for bupropion in this study is one-tenth that previously reported in human liver microsomes (Hesse et al., 2000). This observation may be due to the differences in the protein or lipid composition between the reconstituted system employed in these studies and liver microsomes. It was not possible to use lower concentrations of bupropion in the kinetics studies because the amount of hydroxybupropion produced from lower bupropion concentrations was below the limits of detection of our assay. Because of the variability in expression of wild type P450 2B6 or that of the naturally occurring mutant, it is difficult to extrapolate our *in vitro* data directly and to draw clinical implications. However, our results with bupropion are consistent with the findings in a population of German males, where subjects expressing the P450 *2B6*4* allele displayed higher levels of hydroxybupropion as well as moderately increased clearance of bupropion (Kirchheiner et al., 2003).

Benzphetamine was readily metabolized by both P450 Δ2B6 and P450 Δ2B6 K262R with the wild type enzyme generating approximately twice the amount of formaldehyde seen with the mutant. When individual metabolites of benzphetamine were analyzed by ESI-LC-MS, norbenzphetamine was found to be the primary metabolite

produced by both enzymes, however the wild-type enzyme produced norbenzphetamine at levels approximately 1.7-fold greater than what was observed with P450 Δ2B6 K262R. This observation is consistent with what was found using the formaldehyde assay, because norbenzphetamine is generated via *N*-demethylation with the release of formaldehyde, suggesting that *N*-demethylation is the primary route of metabolism of benzphetamine by the mutant as well. Amphetamine, which is the result of *N*-demethylation and *N*-debenzylation, was formed in small quantities by the mutant and wild-type. However, the wild-type enzyme produced amphetamine at a rate that was 2.9-fold greater than the variant. Interestingly, neither P450 Δ2B6 or P450 Δ2B6 K262R metabolized benzphetamine to methamphetamine in the reconstituted system. This result, along with the low amounts of amphetamine produced, suggests that the *N*-debenzylation pathway is compromised in both of these enzymes. This is not due to truncation, as the full-length P450 also did not metabolize benzphetamine to methamphetamine (data not shown). In contrast, rat enzyme P450 2B1, produces significant amounts of methamphetamine and amphetamine (Kent et al., 2004). These results demonstrate that there is a marked difference in specificity between the human and rat enzyme and that previous data obtained with the rat isofom may not be applicable for the human enzyme. P450 Δ2B6 K262R also preferentially metabolized benzphetamine via *N*-demethylation rather than aromatic hydroxylation. Hydroxynorbenzphetamine formed as a result of both *N*-demethylation and aromatic hydroxylation was produced at higher levels than hydroxybenzphetamine, which is generated solely by aromatic hydroxylation.

The decrease in the ability of both enzymes to catalyze bupropion hydroxylation as well as 7-EFC O-deethylation when inactivated by tTEPA is shown in Table 2. This

finding is consistent with a recent study that demonstrated that tTEPA inhibits bupropion hydroxylation in human liver microsomes (Turpeinen et al., 2004). The estimated K_I value for the inactivation of P450 2B6 K262R by tTEPA as measured using the 7-EFC activity assay was approximately 4-fold greater than the value previously reported for full-length P450 2B6 (Harleton et al., 2004). The rate of inactivation of the mutant was approximately 3-fold less than what has been reported for the full-length wild-type enzyme (Harleton et al., 2004). tTEPA inactivated the variant and reduced hydroxylation of bupropion by 40% and *O*-deethylation of 7-EFC by 20% at 100 µM. The estimated K_I value for the bergamottin-mediated inactivation of P450 Δ2B6 K262R and the rate of inactivation were approximately 2-fold greater and 3-fold greater, respectively, than what was observed with the wild-type enzyme (Lin et al., 2005), but the K_I value of the mutant is similar to the value previously determined for P450 3A4 of 7.7 µM (He et al., 1998). 17EE is metabolized by P450 Δ2B6 to give several metabolites and has been shown to be a mechanism-based inactivator for 2B enzymes (Kent et al., 2002). Surprisingly, in contrast to other inactivators or the wild type enzyme, P450 Δ2B6 K262R was not inactivated by 17EE when incubated under identical conditions. Our inability to observe metabolites of 17EE suggests that the binding of this particular substrate to the mutated protein may be compromised by the mutation. The single mutation may have resulted in a significant structural alteration of the enzyme as may be suspected from the observation that this mutant was localized in the bacterial cytosol in contrast to the wild type enzyme of P450 2B6 which is membrane-bound.

Significant differences in the levels of inactivation were observed for both the wild-type P450 Δ2B6 and the P450 Δ2B6 K262R mutant when different probe substrates

were used to determine catalytic activity remaining. For example, P450 Δ2B6 was inactivated to ~80% when activity was measured using the 7-EFC O-deethylation assay whereas only 40% inactivation was observed using the bupropion hydroxylation assay. P450 Δ2B6 K262R was inactivated ~40% by tTEPA as determined using the 7-EFC assay but only ~20% based on the bupropion assay. Thus, the levels of inactivation differed not only between the wild-type and mutant enzymes, but also depended significantly on the substrate that was used to assay activity remaining. The differences in the levels of inactivation when the same protein is assayed using different substrates may be due to the fact that the covalently bound inactivator in the active site interferes more with the binding of one substrate than with the binding of another. This may be due to differences in the sizes of the substrates, their binding orientations in the active site, or the presence of multiple potential binding regions in the active site having preferred binding for different substrates. The differences observed between wild-type and mutant enzyme may be due to differences in the active site architectures of the two proteins. It is of interest that bergamottin and tTEPA have greater effects on bupropion metabolism whereas 17EE exhibited a greater effect on 7-EFC metabolism.

In this study we have shown that P450 Δ2B6 K262R in the reconstituted system metabolized bupropion to hydroxybupropion at a faster rate than P450 Δ2B6. The P450 Δ2B6 K262R mutant was inactivated by tTEPA and bergamottin similarly to the wild type enzyme. In contrast, 17EE was not metabolized by the mutant under identical conditions and did not inactivate it. Our studies with this single P450 2B6 variant underscore the importance of investigating the functional consequences of genetic polymorphisms at the level of the proteins in order to be able to predict the potential

consequences to the patient. The results of these types of functional studies are of critical importance for the development of a comprehensive database for predictive genotyping in the clinic that could be used to increase the efficacy of some treatment regimens and decrease the extent and severity of adverse drug reactions.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1 A) Chemical structures of the mechanism-based inactivators N,N',N" triethylenethiophosphoramide (tTEPA), bergamottin, and 17-α-ethynylestradiol (17EE), B) Chemical structures of bupropion and the primary metabolites of bupropion.

Fig. 2. Metabolism of bupropion to hydroxybupropion by P450 Δ2B6 and P450 Δ2B6 K262R. Samples were reconstituted as described in Experimental Procedures and incubated with bupropion ranging from 0 μM to 960 μM. Hydroxybupropion formation by P450 Δ2B6 K262R (■) and P450 Δ2B6 (▲) was measured by integrating the area under the HPLC peak and comparison to areas from a standard curve generated by injecting different amounts of authentic hydroxybupropion on the HPLC column. The data represent the means and standard deviations of 3 separate experiments using duplicate samples.

Fig. 3. Metabolism of benzphetamine by P450 Δ2B6 and P450 Δ2B6 K262R. *N*-demethylation of benzphetamine to formaldehyde was measured as described in the Experimental Procedures.

Fig. 4. Inactivation of P450 Δ2B6 K262R by tTEPA. The time- and concentration-dependent inactivation of P450 Δ2B6 K262R was measured by determining the 7-EFC *O*-deethylation activity. After initiation of inactivation by the addition of NADPH,

aliquots were removed from the primary reaction mixture at 0, 5, 10, 16 and 21 min. The concentrations of tTEPA were (■) 0 μM, (▲) 80 μM, (▼) 120 μM, (◆) 160 μM, (●) 200 μM and (□) 240 μM. The data show the means and standard deviations from 4 separate experiments using duplicate samples. The inset represents the double reciprocal plot of the rates of inactivation as a function of the tTEPA concentrations.

Fig. 5. Inactivation of P450 Δ2B6 K262R by bergamottin. The time- and concentration-dependent inactivation of P450 Δ2B6 K262R was measured by determining the 7-EFC *O*-deethylation activity. After the addition of NADPH, aliquots were removed from the primary reaction mixture at 0, 2, 4, 6, and 8 min. The concentrations of bergamottin were (■) 0 μM, (▲) 1 μM, (▼) 2 μM, (◆) 4 μM, (●) 8 μM, and (□) 12 μM. The data show the means and standard deviations from 3 separate experiments using duplicate samples. The inset depicts the double reciprocal plot of the rates of inactivation as a function of the bergamottin concentrations.

Fig. 6. Metabolism of 17EE by P450s Δ2B6 and Δ2B6 K262R. Samples were reconstituted and incubated with 17EE in the presence or absence of NADPH as described in Experimental Procedures. The metabolites of 17EE produced by P450 Δ2B6 (Panel A) and P450 Δ2B6 K262R (Panel B) were separated as described in Experimental Procedures. The identities of metabolites labeled A_1, A_2, and C have not yet been determined. Metabolite D corresponds to 2-hydroxy-17EE, metabolite E corresponds to estrone and F corresponds to the substrate, 17EE (Kent et al., 2002).

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TABLE 1

Metabolism of benzphetamine by P450s Δ2B6 and Δ2B6 K262R.

P450s were reconstituted in the presence of reductase as described in Experimental Procedures. A saturating concentration of benzphetamine was used and samples were incubated for 30 min at 30°C. Metabolites were isolated and analyzed by ESI-LC-MS. Standard error of the mean (SEM) is shown.

	Δ2B6	***Δ2B6 K262R***	***Δ2B6/Δ2B6 K262R***
	area under the peak		
Norbenzphetamine	13 ± 0.13	7.6 ± 0.12	1.7
Amphetamine	0.17 ± 0.01	.06 ± 0.001	2.9
Methamphetamine	N.D.[1]	N.D.	-
OH-norbenzphetamine	5.8 ± 0.03	1.0 ± 0.02	5.8
OH-benzphetamine	0.72 ± 0.02	0.31 ± 0.01	2.3

[1]Not detected

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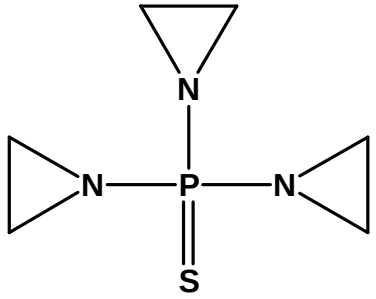
TABLE 2

Effect of bergamottin, tTEPA, and 17EE on the bupropion hydroxylation and 7-EFC activities of P450s Δ2B6 and Δ2B6 K262R.

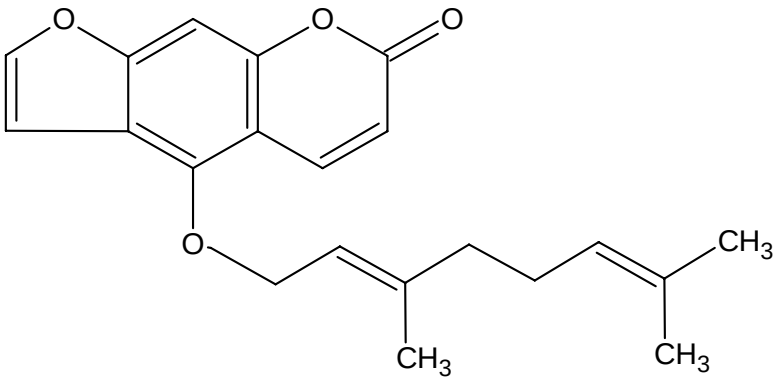
P450s Δ2B6 and Δ2B6 K262R were reconstituted with reductase as described in Experimental Procedures. Bergamottin, tTEPA and 17EE were present in the primary reaction mixture at concentrations of 10 μM, 100 μM, and 100 μM, respectively. NADPH was added to the primary reaction mixture to initiate the reaction.

	Percentage of control activity remaining	
	Bupropion assay	**7-EFC assay**
Δ2B6 Bergamottin	30 ± 1	34 ± 2
Δ2B6 K262R Bergamottin	31 ± 1	40 ± 3
Δ2B6 tTEPA	51 ± 2	55 ± 3
Δ2B6 K262R tTEPA	62 ± 3	81 ± 4
Δ2B6 17EE	61 ± 4	23 ± 3
Δ2B6 K262R 17EE	100 ± 0	100 ± 0

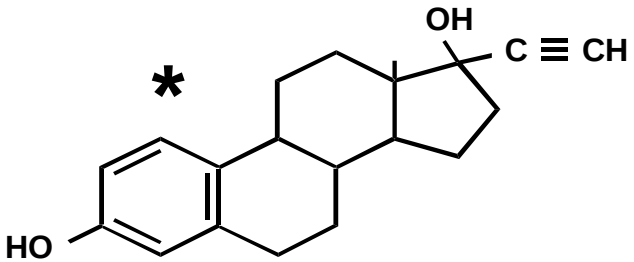
Figure 1a



tTEPA



Bergamottin



17EE

Figure 1B

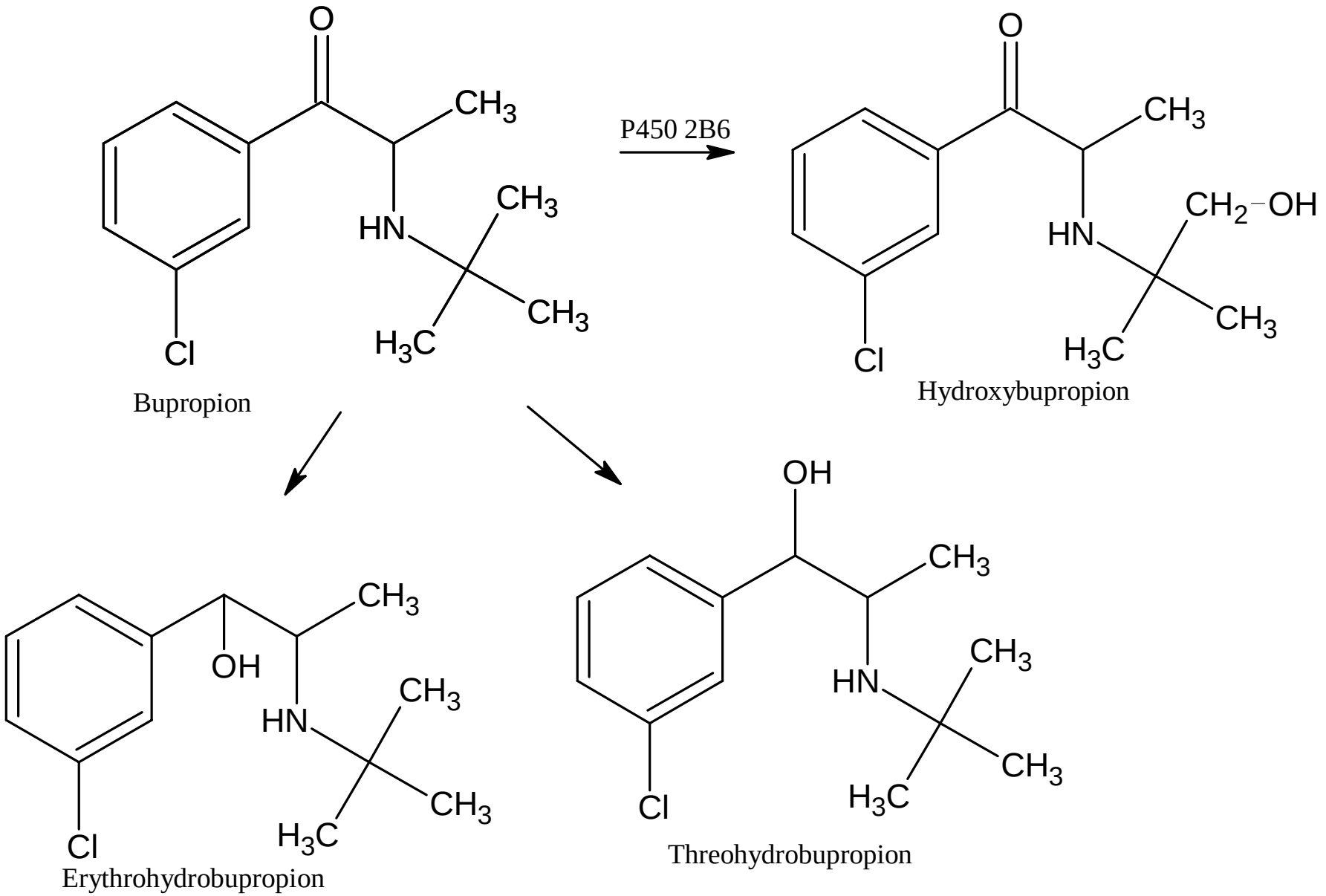


Figure 2

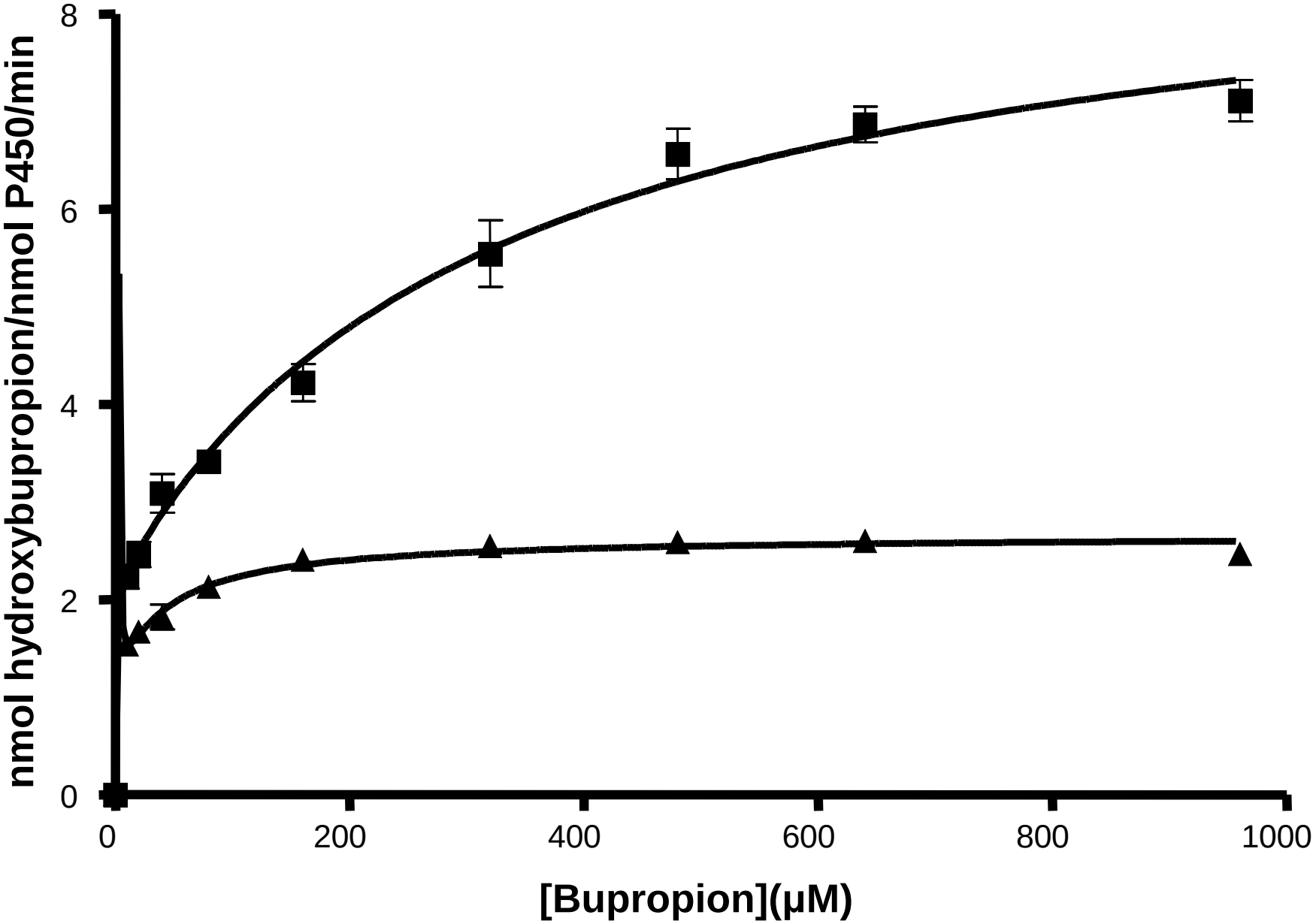


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Figure 3

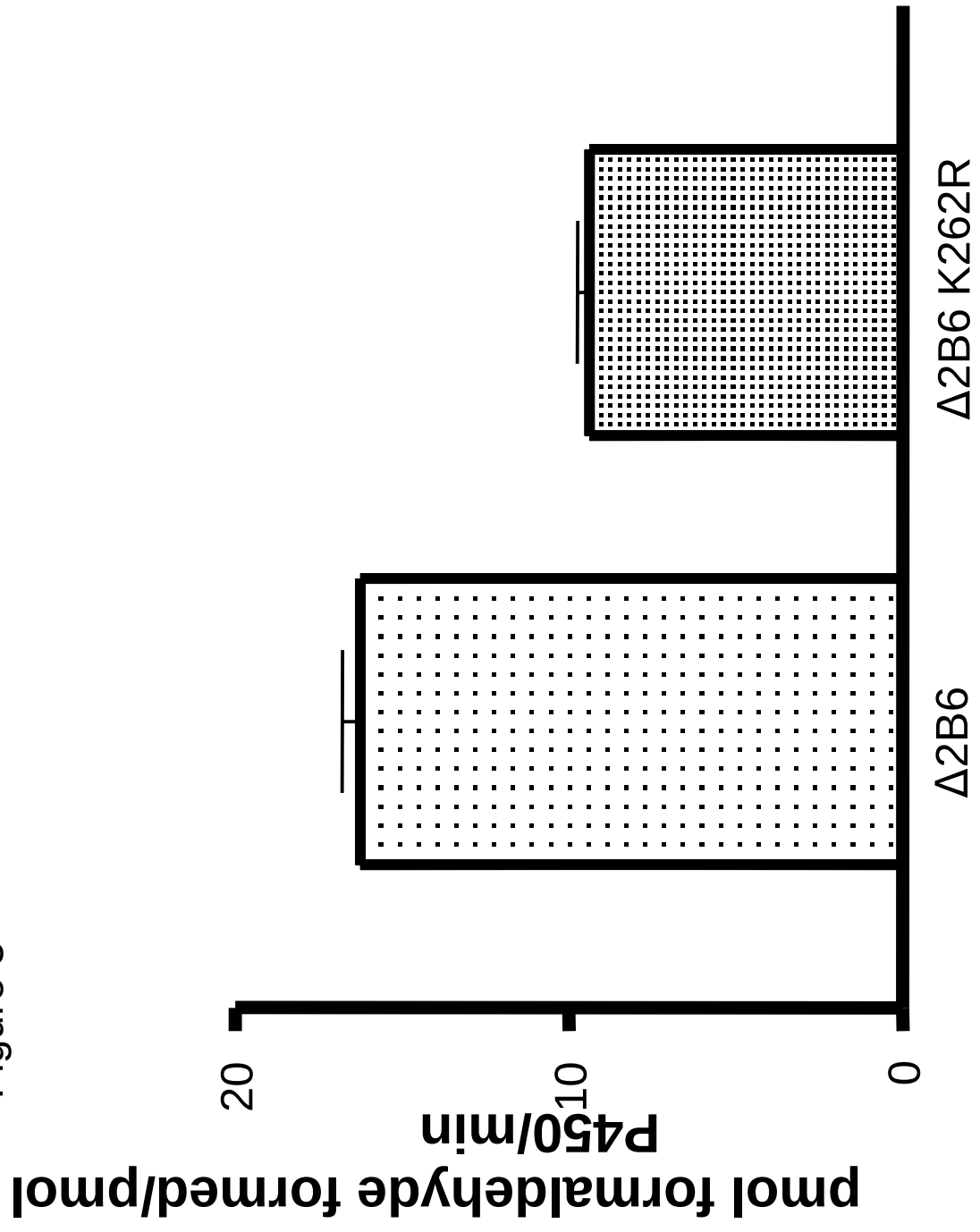

Figure 4

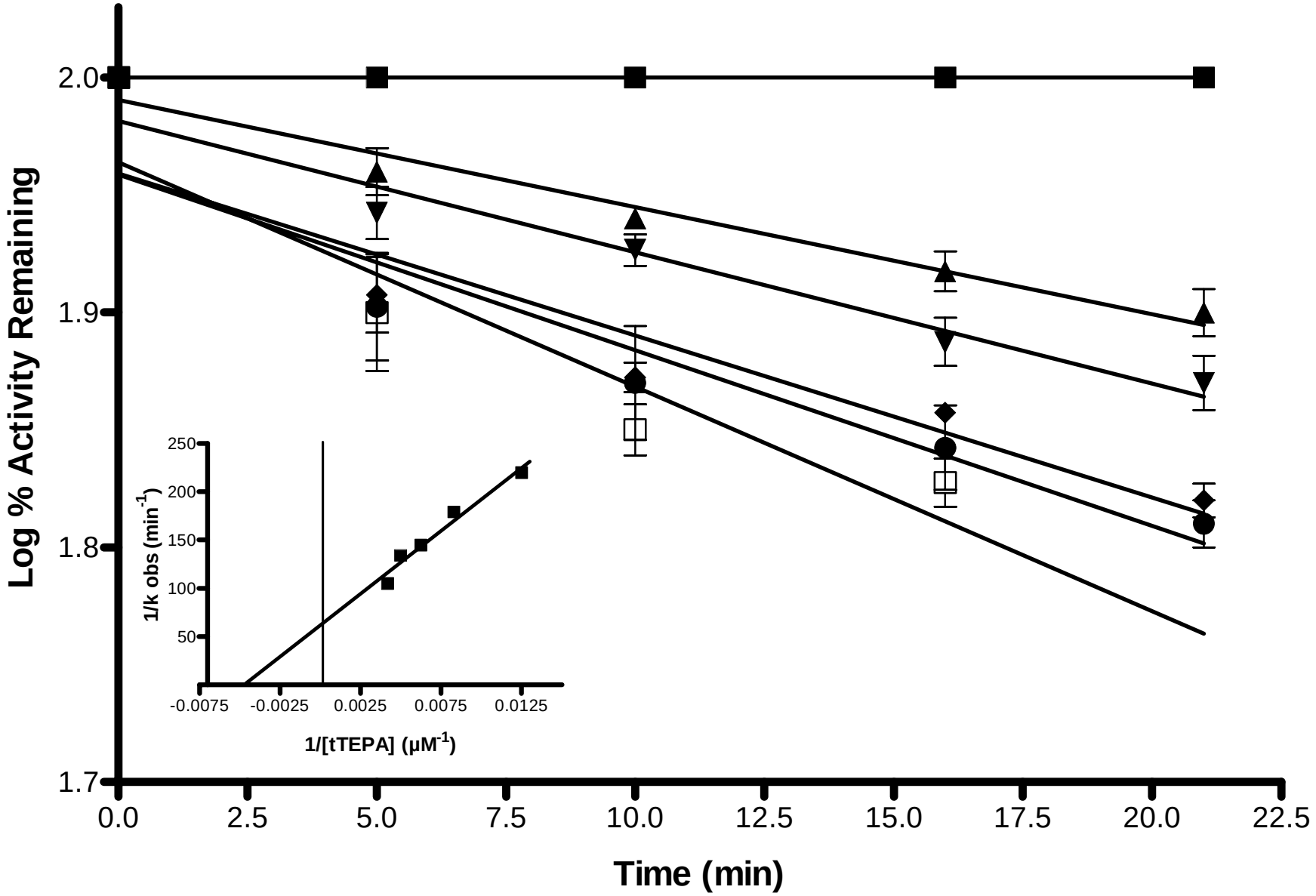

Figure 5

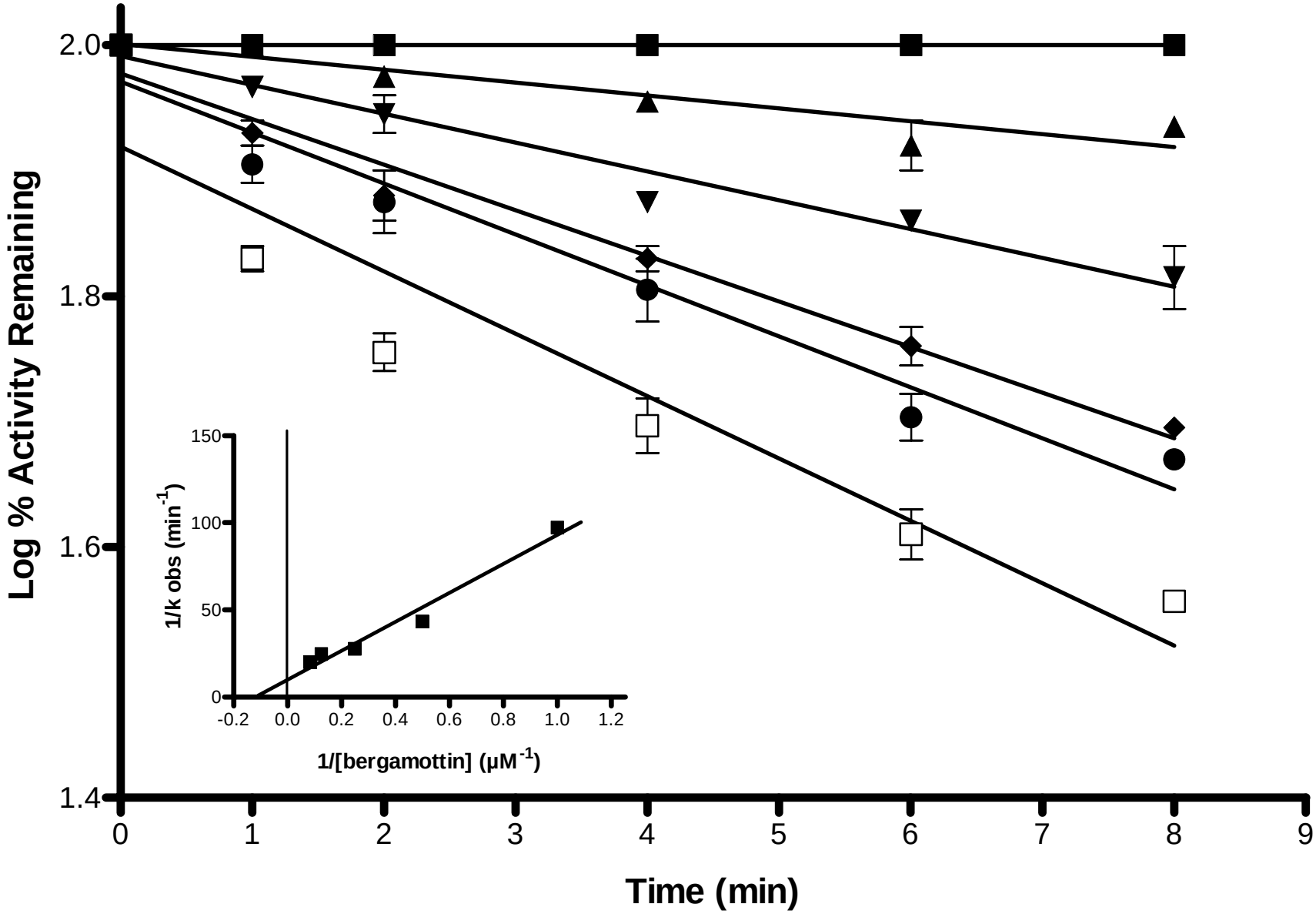

Figure 6