THE NATURALLY OCCURRING CYTOCHROME P450 (P450) 2B6 K262R MUTANT OF P450 2B6 EXHIBITS ALTERATIONS IN SUBSTRATE METABOLISM AND INACTIVATION

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Received January 18, 2005; accepted March 11, 2005

ABSTRACT:
The polymorphic human cytochrome P450 (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 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 has 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 2B6 K262R in a reconstituted mono-oxygenase system (reconstituted system). Metabolism of bupropion by P450 Δ2B6 K262R resulted in increased production of hydroxybupropion compared with P450 Δ2B6. However, production of formaldehyde from the metabolism of benzylationine by the P450 Δ2B6 K262R mutant was significantly less than that of the wild-type isozyme. P450 Δ2B6 K262R formed fewer benzphetamine metabolites compared with the wild type. N,N,N′,N′-Triethyl-ethiophosphoramide (tTEPA) and bergamottin decreased the ability of both enzymes to hydroxylate bupropion and to O-deethyl- late 7-hydroxy-4-(trifluoromethyl)coumarin (7-EFC). Incubation with 17-α-ethylestradiol decreased bupropion hydroxylation and 7-EFC 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 7-EFC. The K1 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.

This study was supported in part by National Institutes of Health Grants CA 16954 and T32 GM007767.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.105.003749.

ABBREVIATIONS: P450, cytochrome P450; P450 Δ2B6, N-terminally truncated P450 2B6; P450 Δ2B6 K262R, N-terminally truncated P450 2B6 lysine 262 arginine mutant; SNP, single nucleotide polymorphism; tTEPA, N,N,N′,N′-triethyl-ethiophosphoramide; 17EE, 17-α-ethylestradiol; 7-EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; HPLC, high-performance liquid chromatography; ESI-LC/MS, electrospray ionization-liquid chromatography/mass spectrometry.

Bupropion is a widely used antidepressant 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 in adults (Wilens et al., 2001). In humans, bupropion is extensively metabolized to give three primary metabolites: ethyrohydrobupropion, threo-hydrobupropion, and hydroxybupropion (Fig. 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; Woolorton, 2002). N,N′,N′-Triethyl-ethiophosphoramide (tTEPA), bergamottin, and 17-α-ethylestradiol (17EE) are all mechanism-based inactivators of P450 2B6 in a reconstituted system with reductase (Guengerich, 1990a; Kent et al., 2002; Rae et al., 2002; Harleton et al., 2004; 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 antineoplastic agent used in the treatment of breast,
bladder, and ovarian cancers (Maanen et al., 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.

Materials and Methods

Materials. Bupropion hydrochloride, triprolidine hydrochloride, NADPH, bovine serum albumin, benzphetamine, catalase, and 17EE were purchased from Sigma-Aldrich (St. Louis, MO). tTEPA was purchased from U.S. Pharmacopoeia (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 PharMingen (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 to 21 deleted and minor changes made to increase expression and solubility (Scott et al., 2001). Purified benzphetamine and t-norbenzphetamine were a gift from Dr. Haoming Zhang, Department of Anesthesiology, Veteran Affairs Health Service (Ann Arbor, MI).

Statistical Analysis. Graphs and the two-tailed unpaired t test were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software Inc., San Diego, CA). \(K_m\) and \(V_{max}\) values were determined using EZ-Fit Enzyme kinetic analysis (Perrella Scientific, Inc., Amherst, NH). Data were fit using the Michaelis-Menten and 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’-GACCCCAGCGCCCCAGGGACCTCATCGACACCTAC-3’ (upstream) and 5’-GTAGGTGTCGATGAGGTCCCTGGGGGCGCTGGGTC-3’ (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 Escherichia coli Topp 3 cells and purified according to published protocols (Hanna et al., 1998, 2000; Scott et al., 2001), except that P450 Δ2B6 K262R was recovered from the cytosol rather than the...
as an internal standard, and the samples were resolved on a 5-
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consisted of 1
(Hanna et al., 2000; Scott et al., 2001).
were also identified after adding the internal standardD-norbenzphetamine and
orophotometer (Shimadzu Scientific Instruments, Inc., Wood Dale, IL) and
410 nm and an emission wavelength of 510 nm using a RF-5310 spectroflu-
of formaldehyde formed was determined using an excitation wavelength of
concentration of benzphetamine (2 mM) was added to all samples. The amount
measured as previously described (de Andrade et al., 1996). A saturating
N
the formation of formaldehyde via
by injecting increasing concentrations of authentic hydroxybupropion onto the
16 to 23 min, and 13% B at 23.5 to 35 min. The retention times were
generated with mobile phases A (0.25% triethylamine and 0.1% formic acid)
integrated and used for comparison purposes only between the two enzymes.
Inactivation of P450s
Mt o9 6 0
-agarose column and the P450 was purified as previously described
2B6 and
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). Tripropidine (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 × 3.9 mm C18 column (Millipore Corporation, Billerica, 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 tripropidine. 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 (2 mM) 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 ethyl acetate, 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 quantification of each metabolite, the area under the peak of the metabolite was integrated and used for comparison purposes only between the two enzymes.

**Results**

**Hydroxybupropion Formation by P450 2B6 and P450 2B6 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 2B6 and P450 2B6 K262R at substrate concentrations ranging from 0 
μM to 960 
μM. Bupropion was poorly soluble at concentrations higher than 960 
μM. The 
Km value for P450 2B6 was approximately 8.8 
μM, whereas the 
Km value for P450 2B6 K262R was approximately 54 
μM. The 
Vmax of the variant was approximately 6.9 nmol of hydroxybupropion/nnmol P450/min, whereas the 
Vmax of the wild-type was 2.6 nmol of hydroxybupropion/nnmol P450/min. The 
Vmax/

![Figure 2](image-url)
K262R was approximately 0.13. Therefore, the catalytic efficiency ($V_{\text{max}}/K_m$) of the variant for bupropion was approximately 40% less than that of the wild-type enzyme.

**Benzphetamine Metabolism by P450 2B6 and P450 2B6 K262R.** The enzymatic activities of P450 2B6 and P450 2B6 K262R were compared using benzphetamine as a substrate. The ability of each enzyme to metabolize benzphetamine to formaldehyde was determined first. P450 2B6 K262R N-demethylated benzphetamine to produce 9.4 ± 0.9 pmol of formaldehyde/pmol P450/min, whereas the wild-type P450 2B6 generated 16.3 ± 1.3 pmol of formaldehyde/pmol P450/min (Fig. 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 hydroxynorbenzphetamine, where its formation by the mutant was less than 20% of that formed by the wild-type enzyme.

**Inactivation of P450s 2B6 and 2B6 K262R by tTEPA, Bergamottin, and 17EE.** The inactivation of P450 2B6 K262R by these three mechanism-based inactivators was performed as described under Materials and Methods. P450 2B6 K262R was inactivated by both tTEPA (Fig. 4) and bergamottin (Fig. 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 Fig. 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 2B6 K262R by bergamottin, determined from the inset of Fig. 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-deethyl 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 2B6 K262R mutant. To see whether 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 2B6 and P450 2B6 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 did 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 with the 7-EFC assay (p = 0.0002), although no significant difference was seen with the wild-type enzyme. 17EE inactivated the wild-type P450 2B6, leaving 61% activity remaining with bupropion as the probe substrate, whereas the P450 2B6 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 with the 7-EFC assay (p = 0.0002).

**Metabolism of 17EE by P450s 2B6 and 2B6 K262R.** To see whether 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 were analyzed using reverse-phase HPLC as shown in Fig. 6. P450 2B6 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) (Fig. 6A). However, as shown in Fig. 6B, P450 2B6 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 2B6 and P450 2B6 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 with the wild-type enzyme. Although 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 procarcinogens including 4-(methyleneamino)-1-(3-pyridyl)-1-butanone (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 the 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 with wild-type (Tsuchiya et al., 2004). The K262R SNP is thought to be particularly important, since it was found to have an allele frequency of approximately 5% in German males and a SNP frequency of 30% because it is present in three different P450 subtypes, each of which has a SNP frequency of 30% (Lang et al., 2001; Kirchheiner et al., 2003). 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 2B6 K262R produced the hydroxylated product at a rate that was significantly greater than that
of the wild-type enzyme. The $K_m$ of wild-type P450 $\Delta 2B6$ 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 used 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 $\Delta 2B6$ 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 $\Delta 2B6$ and P450 $\Delta 2B6$ 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 $\Delta 2B6$ 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 that of the variant. Interestingly, neither P450 $\Delta 2B6$ nor P450 $\Delta 2B6$ 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, since 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 isofrom may not be applicable for the human enzyme. P450 $\Delta 2B6$ K262R also preferentially metabolized benzphetamine via N-demethylation rather than aromatic hydroxylation. Hydroxybenzphetamine was formed as a result of both N-demethylation and aromatic hydroxylation was produced at higher levels than was 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
M. Bergamottin, tTEPA, and 17EE were present in the primary reaction mixture at concentrations of 10 μM, 1 μM, 2 μM, 4 μM, 8 μM, and 12 μM. The data show the means and standard deviations from three separate experiments using duplicate samples. The inset depicts the double reciprocal plot of the rates of inactivation as a function of the bergamottin concentrations.

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, 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 not only differed 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.

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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 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.

Acknowledgments. We thank Dr. James Halpert for the generous gift of the P450 Δ2B6 plasmid and Hsia-lien Lin for the expression and purification of the P450 reductase.

References


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