THE EFFECTS OF A NATURALLY OCCURRING GENETIC POLYMORPHISM ON THE CATALYTIC PROPERTIES OF HUMAN CYTOCHROME P450 2B6

by

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TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

CHAPTER 1

INTRODUCTION

The Cytochromes P450

The cytochromes P450 (P450) are a superfamily of heme-containing monooxygenases that play a major role in the oxidative metabolism of a number of exogenous and endogenous compounds [1]. Substrates of P450s include xenobiotics, pesticides, vitamins and hormones [2]. P450s catalyze a variety of reactions; however, the most common is substrate oxidation by the insertion of one oxygen atom. Most P450-mediated reactions result in the detoxification of exogenous compounds via the formation of hydrophilic metabolites that can be readily excreted from the body. Because metabolism by P450s leads to a decrease in plasma concentration of the parent drug, P450s play an important role in drug bioavailability. Poor bioavailability is a major reason why compounds generated in the discovery phase fail to make it to the market. Thus, there is considerable interest in better prediction of clinical outcomes by using *in vitro* approaches to understand P450-catalyzed reactions.

The completion of the sequencing of the human genome has revealed that there are 57 human P450s [3], which are grouped into families (1, 2, 3, …) and subfamilies (A, B, C, …) based upon sequence homology. Individual P450s are then given a second

number, resulting in a unique name for each enzyme, e.g., 1A1, 1B1 or 2C9. The majority of P450s in humans are believed to be involved in the biosynthesis of endogenous compounds. The remaining P450s, in families 1-3, are involved the metabolism of xenobiotics. Figure 1.1 illustrates the relative contributions of P450s in drug metabolism overall. The majority of drug-metabolizing P450s are found in the liver embedded in the membrane of the endoplasmic reticulum; however, there are a number of P450s that are also expressed in extrahepatic tissues [4].

Unlike many enzymes which have strict substrate selectivity, P450s can bind and metabolize a diverse group of substrates which differ in size, shape and stereochemistry. A major challenge is to understand how individual P450s accommodate structurally unrelated substrates and oxidize these substrates in a stereo- and regio-specific manner [5]. Individual P450s have been shown to adopt multiple conformations in response to various ligands [6]. The structures of a number of mammalian and bacterial P450s have been solved and reveal that P450s contain several common structural elements including a series of helices denoted by letters A through L [7]. The A helix is closest to the N-terminus of the catalytic domain [7]. Site-directed mutagenesis studies have indicated that residues in helices B, F and I contact the substrate [8]. There are also six well-conserved substrate recognition sequences that are present in most P450s [9].

The major events involved in the P450 catalytic cycle are shown in Figure 1.2. At the beginning of the cycle, the P450 heme iron is in the ferric state (A). In the first step, the substrate binds to the P450, resulting in the transition of the iron from a low-spin to a high-spin state (B). An electron is then transferred from NADPH via NADPH-P450 reductase (reductase) and the iron is reduced to the ferrous state (C). The ferrous

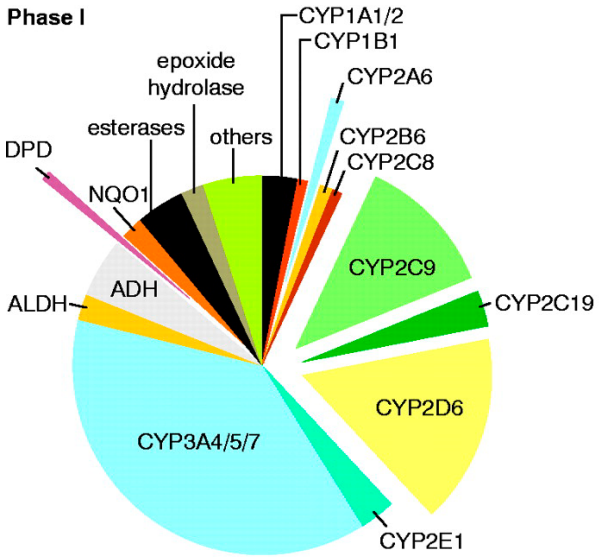

Figure 1.1 – Phase I metabolism of xenobiotics [10]. The percentage of phase I metabolism of drugs that each enzyme contributes is estimated by the relative size of each section of the corresponding chart. P450s (denoted as CYPs in the figure) constitute the majority of phase I metabolism of xenobiotics.

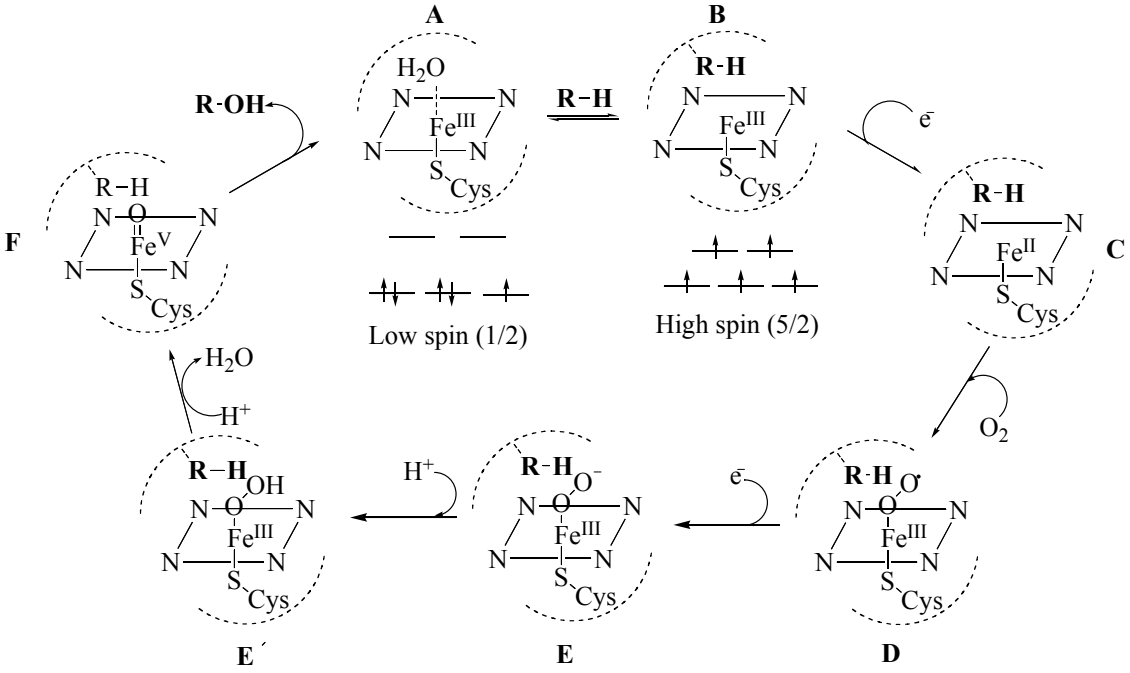

Figure 1.2 – The P450 catalytic cycle. The steps of the catalytic cycle are shown as described in the text including heme spin state in the substrate-free and substrate-bound form.

iron can then bind O_2 (D). However, this complex is unstable and can lead to the formation of superoxide anion and ferric iron. A second electron is then transferred from reductase, or, in certain instances, it has also been shown that this electron can come from cytochrome b_5. A proton is then added (E'), followed by cleavage of the O-O bond, generating H_2O and the Fe^{V}=O complex. Activated oxygen is then inserted into the substrate and the product is released (F) [8].

P450 Redox Partners

Figure 1.3 shows the electron transfer pathway from redox partners reductase and cytochrome b_5. The reductase transfers two electrons derived from a hydride ion of NADPH via FAD and FMN to P450s in one-electron transfer steps. In the liver, P450s are present in large excess over reductase, with the ratios ranging from 10:1-20:1. Despite this ratio, P450s have been shown to form 1:1 complexes with reductase [11]. Metabolism studies can be carried out *in vitro* by reconstituting P450 and reductase in a 1:1 or 1:2 ratio depending upon the P450 being utilized in the study [12, 13]. In certain instances, cytochrome b_5 has been shown to donate the second electron to the P450. Cytochrome b_5 is thought to be involved only in the transfer of the second electron, because it is very inefficient at delivering the first electron. This may be due to the fact that the second reduction is more thermodynamically favorable than the first [14].

Cytochrome b_5 is a 17-kDa heme-containing protein that is located in the membrane of the endoplasmic reticulum [15], and it functions as an electron donor in a number of reactions, including cholesterol biosynthesis and certain P450-catalyzed reactions [16]. Depending upon the P450 isoform and the substrate being investigated,

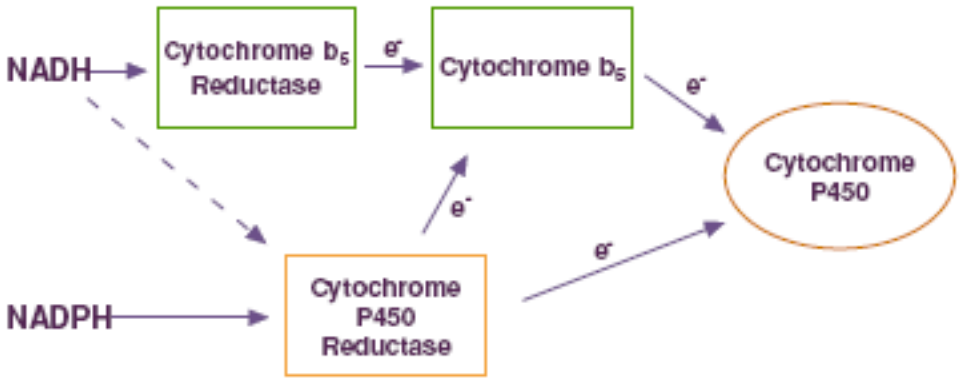

Figure 1.3 – Electron transfer pathways to the P450 in the endoplasmic reticulum [15]. *In vivo*, cytochrome b_5 can receive an electron from NADH via cytochrome b_5 reductase. In the reconstituted system, the P450 is incubated with reductase and NADPH is the sole source of electrons. In the presence of cytochrome b_5, the second electron in the catalytic cycle can be transferred from reductase to reduce cytochrome b_5, then donated to the P450.

cytochrome b_5 has been shown to increase, inhibit, or have no effect on P450 activity [17]. Studies on the effects of the b_5 protein on P450 2B enzymes began in the early 1980's with the observation by Canova-Davis and Waskell that metabolism of methoxyflurane by P450 2B4 in the reconstituted system exhibited an absolute requirement for cytochrome b_5 [18, 19]. Interestingly, cytochrome b_5 had no effect on P450 2B4-mediated metabolism of benzphetamine [20]. There are two primary hypotheses to explain the stimulatory effect of cytochrome b_5 on some P450-mediated reactions. The first is that reduced cytochrome b_5 donates the second electron in the catalytic cycle to the P450 [21-23]. This is supported by studies showing electron transfer from cytochrome b_5 to P450 as well as the observation that "uncoupling" is decreased in the presence of the b_5 protein [24]. The second hypothesis is that cytochrome b_5 physically interacts with the P450 causing a conformational change that facilitates interaction with the substrate or reductase. This notion is supported by studies where the apo-cytochrome b_5, which cannot be reduced and donate the second electron, was able to stimulate P450-catalyzed reactions [25]. However, the exact role of cytochrome b_5 in P450-dependent hydroxylation and oxidations is unclear. Again, it appears that the effect is highly dependent upon the substrate and the isoforms being studied.

Mechanism-Based Inactivation

In the clinical setting, P450 inhibition can result in elevated plasma levels of other drugs that are primarily metabolized by the particular P450 being inhibited. As a result, inhibition of P450s has the potential to cause severe adverse events. For instance, studies with patients that are receiving warfarin along with 5-fluorouracil (a P450 inhibitor) to

treat a cancer have exhibited altered coagulation parameters, bleeding, and even death in some cases [26]. P450 inhibition may also result in decreased metabolism of prodrugs, such as cyclophosphamide, that have to be metabolically activated [27]. Alternatively, P450 inhibition can be used to increase the bioavailablity of a compound. For example, ritonavir, a potent inhibitor of P450 3A4 [28], has been co-formulated with lopinavir, a novel protease inhibitor with relatively low bioavailability [29]. The combined drug formulation was shown to significantly improve the pharmacokinetic properties and hence the activity of lopinavir against HIV-1 protease [30]. *In vitro*, P450 inhibition is a useful tool to help elucidate the relative contributions of particular P450s in the metabolism of a substrate.

There are three steps in the P450 catalytic cycle that appear to be susceptible to inhibition: 1) substrate binding, 2) binding of molecular oxygen to the ferrous enzyme, and 3) transfer of activated oxygen from the heme iron to the substrate [31]. Inhibitors can then be divided into three categories based upon their mechanism of action: 1) reversible, 2) quasi-irreversible, and 3) irreversible. Reversible inhibition, or competitive inhibition, most often occurs when there is structural similarity between the inhibitor and the substrate(s) of the P450 [31]. The inhibitor itself may be a substrate for the P450; however, this is not necessarily required. Competitive inhibition is considered to be solely dependent upon concentration, as opposed to quasi-irreversible inhibition and irreversible inhibition, which are both time- and concentration-dependent.

Quasi-irreversible inhibition occurs when a compound is metabolized to an intermediate that then binds tightly to the heme or the P450, resulting in inhibition of enzymatic activity. This phenomenon has been described for a variety of compounds,

including erythromycin where a metabolite intermediate (MI) complex is formed with the P450 heme iron, resulting in inhibition. The formation of this complex can be observed spectrally at a maximum absorbance of 455 nm [32-34]. Blobaum et al. [35, 36] have described a second type of reversible intermediate (maximum absorption at 485 nm) that can be observed during inactivation of P450 2E1 T303A by *tert*-butyl acetylene.

Irreversible inhibition in which metabolism of the compound leads to covalent binding to the heme or protein, is also termed "mechanism-based inactivation". Mechanism-based inactivation is defined as the process in which a substrate is metabolized and converted to a reactive intermediate that binds irreversibly to the active site of the P450, thereby rendering it inactive [37].

The criteria employed to identify a mechanism-based inactivator *in vitro* are as follows [38]:

1) the inhibition is time-, concentration- and NADPH-dependent;
2) addition of exogenous nucleophiles, such as glutathione, does not protect the enzyme from inactivation;
3) the inactivation is irreversible, and the activity cannot be recovered after dialysis or gel filtration;
4) the decrease in activity exhibits pseudo-first order kinetics;
5) addition of a substrate with high affinity for the P450 can protect against inactivation;
6) total P450 content is reduced;
7) 1:1 stoichiometry is observed with complete inhibition of enzymatic activity.

Investigation of mechanism-based inactivators can lead to useful information for understanding the architecture of the P450 active site. Further, increased knowledge about mechanism-based inactivators has the potential to aid in the prevention of certain drug-drug interactions.

Cytochromes P450 Polymorphisms

Inter-individual variability in P450 activity is a major reason for failure of drug therapy [39]. Variability in P450 activity can be caused by a number of factors, including age, gender, morbidity and disease state, as well as concurrent medications, diet, alcohol and smoking. However, genetic polymorphisms appear to play the largest role in variability observed in patients [40]. Mutations have been found in all genes encoding P450 enzymes in families 1-3, although the functional importance of the variant alleles differs along with the frequencies of their distribution [41]. P450 activity for certain metabolic reactions has been shown to vary up to fifty-fold between individuals [42]. The wild-type alleles of P450s where genetic mutations have been found are denoted as "*1" and the allelic variants (containing one or more single nucleotide polymorphism(s)) are sequentially numbered as they are identified (i.e., *2, *3). Polymorphic P450s play a large role in adverse drug reactions. It has been estimated that 56% of drugs that are cited in adverse drug reaction studies are primarily metabolized by polymorphic phase I enzymes, of which 86% are P450s [43]. The costs associated with treating patients who express variant forms of P450s are significantly greater than those required to treat patients expressing wild-type P450s [44]. The majority of investigations into the effects of P450 polymorphisms are done by genotyping patients and correlating the genetic

differences to phenotypic differences. Ingelman-Sundberg has suggested that information gained from fundamental studies of the effects of P450 polymorphisms could result in a 10-20% improvement of clinical efficacy of all drug therapy and a 10-15% reduction of the frequency of adverse drug reactions [41]. With this in mind, studies investigating the mechanisms behind phenotypic differences, which are currently lacking, are necessary to aid in safer and more efficacious drug development.

Cytochromes P450 2B6 and P450 2B6 K262R

P450 family 2 enzymes collectively catalyze over 54% of all phase I oxidations of drug substrates [45]. P450 2B6 is a polymorphic human P450 that is expressed in a variety of tissues including the liver, skin [46], kidney [47], heart [48], brain [49] and larynx [50]. P450 2B6 is involved in the metabolism of about 3% of clinically used drugs including sertraline, a selective serotonin reuptake inhibitor [51, 52]; cyclophosphamide, a prodrug that is an anticancer agent [27]; and propofol, an anesthetic [53, 54]. P450 2B6-specific substrates include efavirenz, a non-nucleoside transcriptase inhibitor [55] and bupropion, an anti-depressant and smoking cessation aid [56, 57]. This enzyme has been shown to play a role in the activation of a number of procarcinogens, including aflatoxin and tobacco-specific nitrosamines [58, 59]. In addition, P450 2B6 can activate certain centrally active drugs, such as cocaine [60] and amphetamines [61], to neurotoxic intermediates. P450 2B expression is induced by a number of compounds, including phenobarbital and nicotine, which primarily increases P450 2B expression in the brain [62].

A number of single nucleotide polymorphisms have been found in the *P450 2B6* gene that encode variant alleles currently denoted as *2B6*2* through *2B6*25*. *In vivo* studies have suggested that these variant alleles exhibit phenotypic differences when compared to the wild-type [63-65]. P450 2B6 polymorphisms have differential effects on the metabolism of P450 2B6 substrates. The P450 2B6 lysine 262 to arginine mutant [*2B6*4* (2B6.4 when referring to the purified enzyme; K262R), 785A>G, exon 5] is of particular interest because it has been reported to have a 5-9% allele frequency and up to an overall 50% mutation frequency since it is present in multiple variant alleles [66-68]. In a clinical study, *P450 2B6*4* was associated with an increased clearance of bupropion and higher plasma levels of the hydroxybupropion metabolite in German males [69]. Bupropion, a widely used anti-depressant and smoking cessation aid, acts by inhibiting the reuptake of norepinephrine and dopamine [70, 71]. P450 2B6 is the primary enzyme catalyzing the hydroxylation of bupropion to form hydroxybupropion, the pharmacologically active metabolite that plays a role in the antidepressant and, presumably, anti-smoking activity of bupropion [70]. Elevated plasma concentrations of hydroxybupropion are thought to be associated with seizures, the most severe side effect of bupropion treatment, occurring in 1 in 1000 patients [72]. Therefore, patients expressing this variant allele who take the standard therapeutic dose of bupropion to treat depression or nicotine addiction may have an increased risk of side effects. Since this mutation occurs with such a high frequency, a better understanding of the mechanisms of its effects are critical to understanding and predicting adverse drug reactions.

The x-ray crystal structure of P450 2B6 has not yet been solved, however the structure of the rabbit isoform is available in both an open and closed confirmation [73,

74]. Since P450 2B4 and P450 2B6 are members of the same subfamily, the x-ray crystal structure of P450 2B4 can be used a model to help understand the architecture of P450 2B6. The P450 2B4 structure reveals a large open cleft, approximately 15Å wide, between the α-helical and β-rich domains that extends directly to the heme and originates from the distal surface of the protein [73]. The cleft is composed of helices F, F', G' and G on one side and by the B'/C loop and the C helix on the opposite side. The C helix is on the proximal side of the protein and contains a strong electropositive potential that can interact with the negatively charged surface of redox partners, reductase and/or cytochrome b_5. Mutation of residues R122 and F126 in helix C have been shown to effect association with both redox partners [75]. When the open structure of P450 2B4 is superimposed with the closed structure (Figure 1.4), one of the regions with the highest root mean square difference is the G/H loop, the site of residue K262. Substrate binding has been demonstrated to enhance redox partner binding and P450 2B4 reduction [76]. In the substrate bound structure of P450 2B4, portions of the G/H loop interact with residues in the C/D loop, which causes a 4Å shift when compared to the open conformation. This interaction significantly alters the orientation of helix C, suggesting that, when the substrate is bound, the G/H loop may provide a physical mechanism for conformational change that facilitates electron transfer from redox partners [74]. The G/H loop of the closed structure is relatively small consisting of only eight residues.

With this in mind, it is reasonable to hypothesize that mutating lysine 262 in the G/H loop to an arginine, which is slightly larger, could result in altered interaction with redox partners. Further, since the interaction with redox partners is effected by substrates, the consequences of this mutation may be substrate dependent. A similar phenomenon

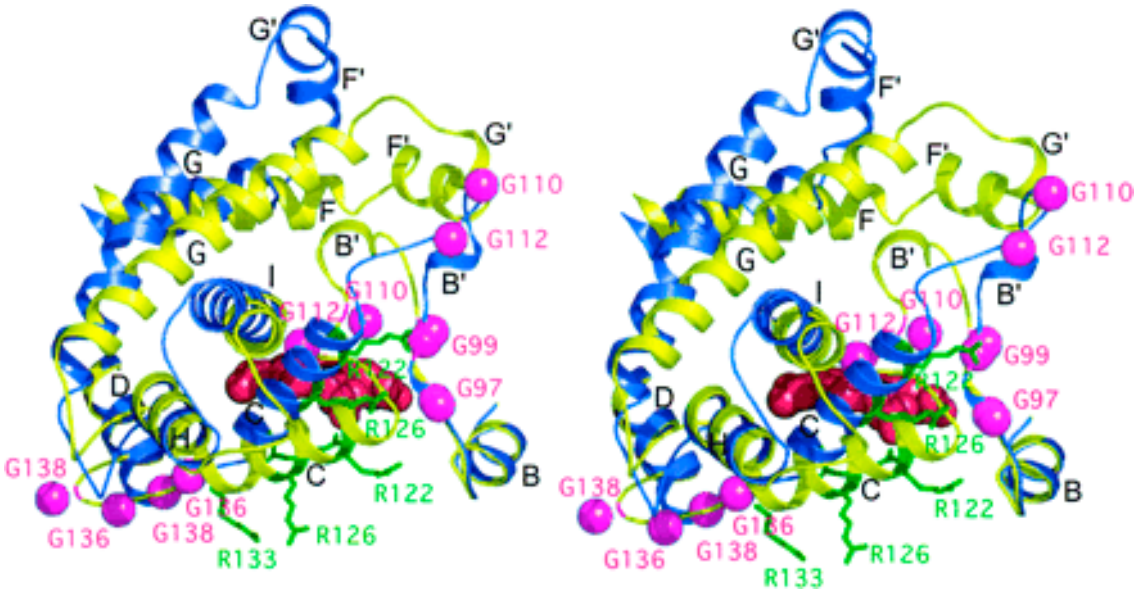

Figure 1.4 - Spatial relationships between the open P450 2B4 (*blue*) and closed P450 2B4 (*yellow*) [74]. The G/H and C/D loops are regions with high root mean square differences between the two structures. The interaction of these loops with other areas of the protein and the flexibility of these loops facilitates the repositioning of the C helix, which is involved in redox partner binding. Residues shown by site-directed mutagenesis to be involved in redox partner binding are R133, R126 and R122 (*green sticks*).

has been described where a lysine to arginine mutation of the estrogen receptor-α on the border of the hinge-region of the hormone binding domain affects the binding of co-activators [77]. Strobel and colleagues have reported that mutating a lysine residue in the G/H loop of P450 1A1 altered the ability of the P450 to metabolize substrates in the presence of reductase [78]. In subsequent studies, the use of cumene hydroperoxide as an oxidant in a reductase-free system resulted in the ability of the mutant to metabolize the substrate similar to the wild-type enzyme, suggesting that the mutation interrupted electron-transfer [78]. The studies in this thesis were designed to test the functional consequence of the P450 2B6 K262R mutation *in vitro* on the catalytic activity of the enzyme, with a particular focus on mechanism-based inactivation. Because of the location of the mutation, the effects of the presence of cytochrome b_5 have also been examined in order to test the hypothesis that the K262R mutation exhibits differences in catalytic activity as a result of altered interaction with redox partners when compared to the wild-type enzyme.

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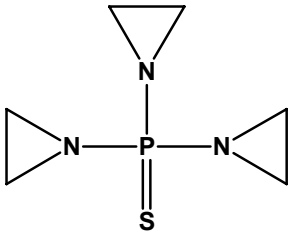
CHAPTER 2

THE NATURALLY OCCURRING P450 2B6.4 MUTANT OF P450 2B6 EXHIBITS ALTERATIONS IN SUBSTRATE METABOLISM AND INACTIVATION

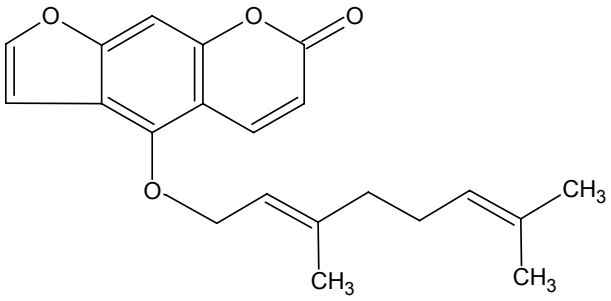
Introduction

A number of single nucleotide polymorphisms (SNPs) have been found in the *P450 2B6* gene [1]. 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. P450 2B6 K262R (*2B6*4*, 785A>G, exon 5; 2B6.4) is associated with increased clearance of bupropion and higher levels of the hydroxybupropion metabolite in German males [2]. This chapter details our investigations of the effects of this SNP on the metabolism of several P450 2B6 substrates including bupropion and the ability of P450 2B6.4 to become inactivated by three structurally unrelated mechanism-based inactivators of P450 2B6 (Figure 2.1).

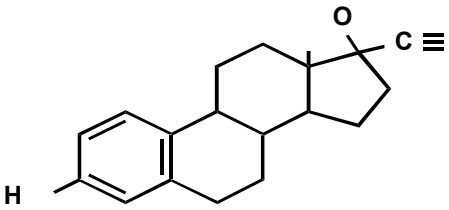
Bupropion is a widely used anti-depressant and smoking cessation aid that acts by inhibiting the reuptake of norepinephrine and dopamine [3, 4]. Bupropion has also been



tTEPA



Bergamottin



17EE

Figure 2.1 - Chemical structures of the mechanism-based inactivators N,N',N" triethylenethiophosphoramide (tTEPA), bergamottin, and 17-α-ethynylestradiol (17EE)

shown to be effective in the treatment of attention deficit/hyperactivity disorder (ADHD) in adults [5]. In humans, bupropion is extensively metabolized to give three primary metabolites: erythrohydrobupropion, threohydrobupropion, and hydroxybupropion (Figure 2.2) [6]. 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 [3]. Side effects of bupropion include seizures and even death [7]. It has been reported that approximately 1 in 1000 subjects treated with bupropion experience seizures [8]. Elevated plasma level concentrations of hydroxybupropion are thought to be associated with poor clinical outcomes and seizures [7, 9, 10].

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 [11-16]. 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 [17]. Bergamottin, a furanocoumarin found in grapefruit juice, inactivates P450s 3A4 [18], 2B6, and 3A5 [15]. 17EE, a major component of many oral contraceptives is also a mechanism-based inactivator of P450 2B6 [12, 14]. 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 2B6.4 reported here show significant differences in metabolism and in the ability to inactivate this mutant.

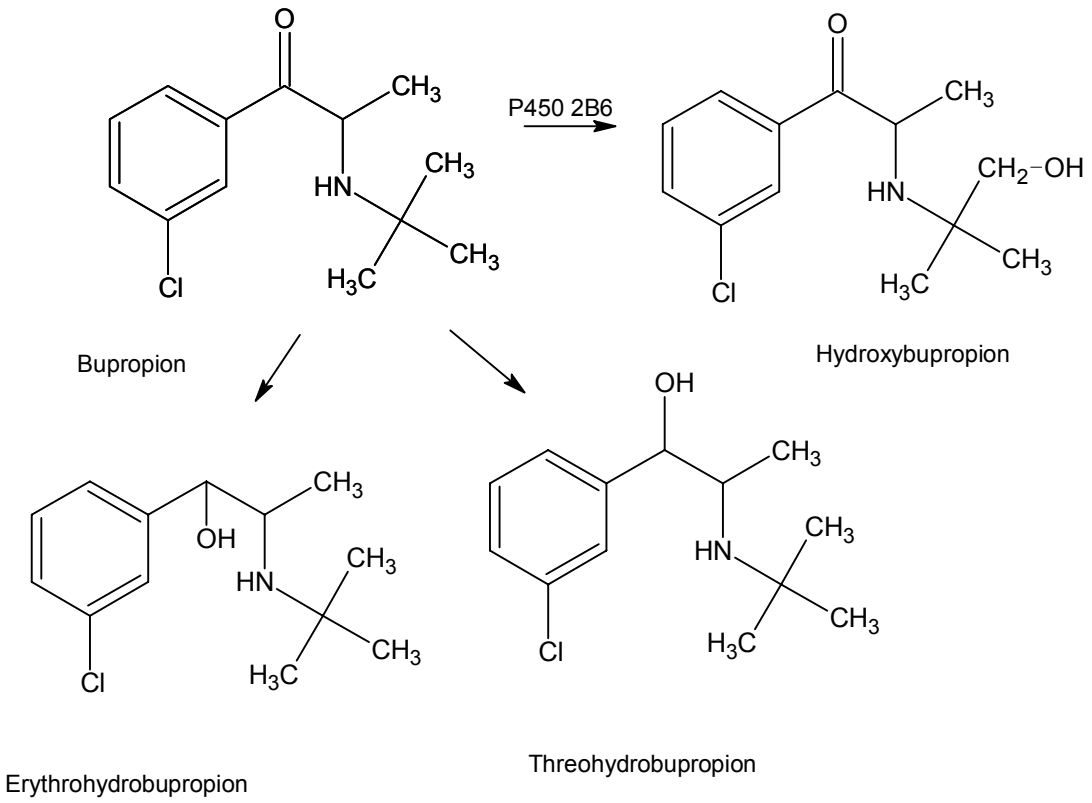


Figure 2.2 - Chemical structures of bupropion and the primary metabolites of bupropion.

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 2B6 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 [19]. 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. Construction of the P450 2B6.4 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 2B6, P450 2B6.4 and NADPH-P450 reductase were expressed in *E. coli* Topp 3 cells and purified according to published protocols [19-21] except that P450 2B6.4 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 [19, 20].

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. [22]. 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 [23]. 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 [24]. 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 2B6 and 2B6.4. 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 2B6 or P450 2B6.4 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 [14].

Results

Hydroxybupropion formation by P450 2B6 and P450 2B6.4. Metabolism of the P450 2B6 specific substrate bupropion to hydroxybupropion was examined using HPLC. Figure 2.3 shows the rate of formation of hydroxybupropion produced by P450 2B6 and P450 2B6.4 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 2B6 was approximately 8.8 μM while the K_m value for P450 2B6.4 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 2B6 was approximately 0.30 while the V_{max}/ K_m for P450 2B6.4 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 2B6 and P450 2B6.4. The enzymatic activities of P450 2B6 and P450 2B6.4 were compared using benzphetamine as a substrate. The ability of each enzyme to metabolize benzphetamine to formaldehyde was determined first. P450 2B6.4 *N*-demethylated benzphetamine to produce 9.4 ± 0.9 pmol formaldehyde/pmol P450/min while the wild-type P450 2B6 generated 16.3 ± 1.3 pmols formaldehyde/pmol P450/min (Figure 2.4). 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 LC-MS and the results are shown in Table 2.1.

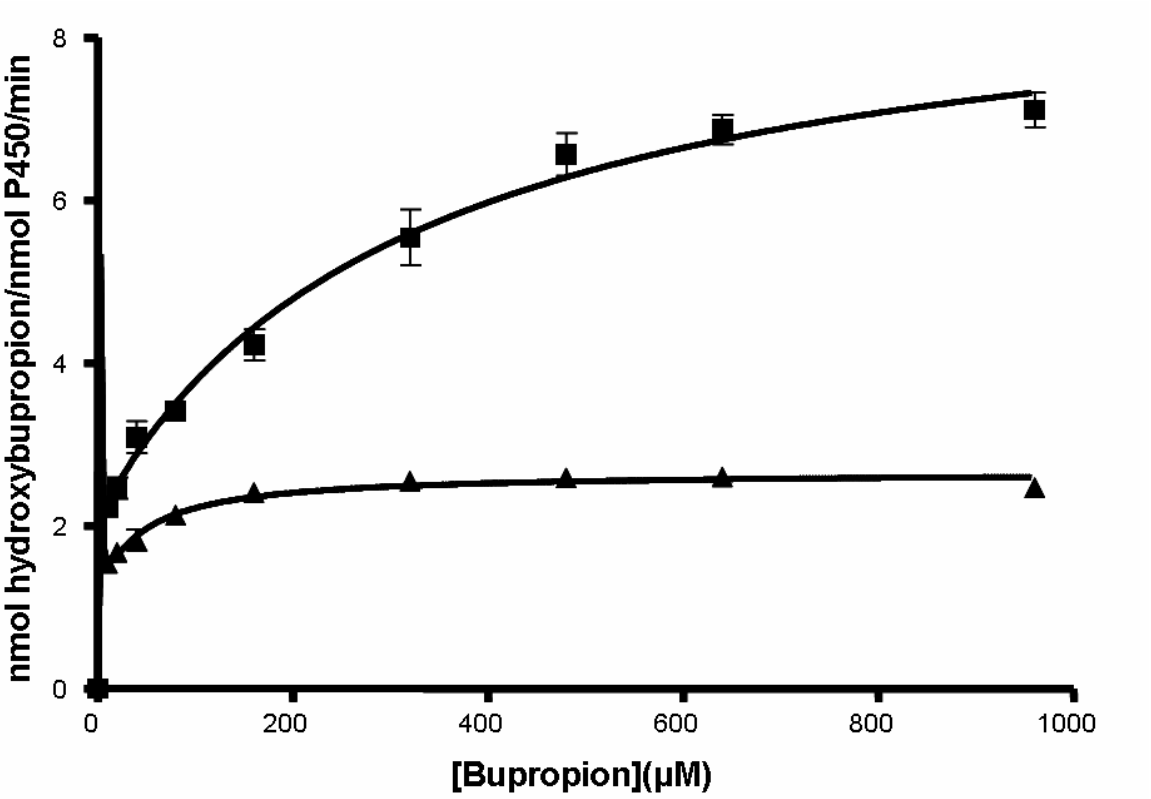


Figure 2.3 - Metabolism of bupropion to hydroxybupropion by P450 2B6 and P450 2B6.4. Samples were reconstituted as described in Experimental Procedures and incubated with bupropion ranging from 0 μM to 960 μM. Hydroxybupropion formation by P450 2B6.4 (■) and P450 2B6 (▲) was measured by integrating the 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.

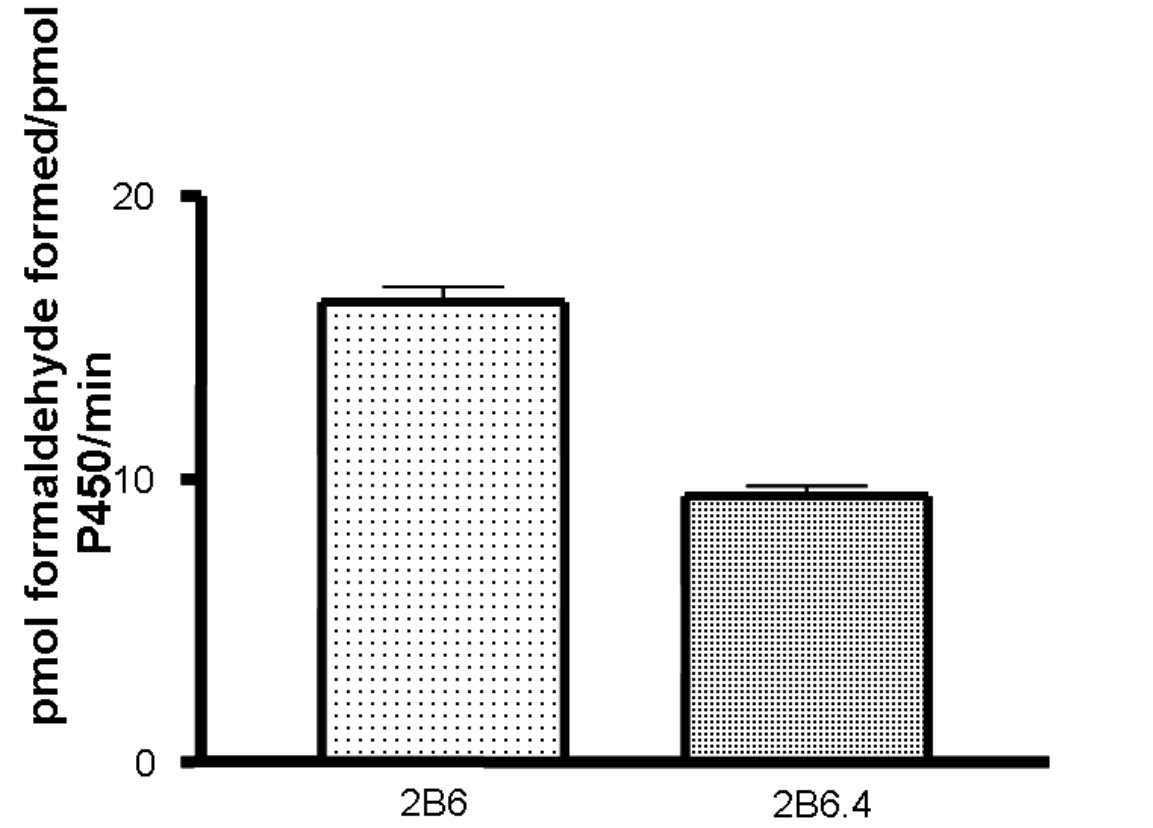


Figure 2.4 - Metabolism of benzphetamine by P450 2B6 and P450 2B6.4. *N*-demethylation of benzphetamine to formaldehyde was measured as described in the Experimental Procedures.

Table 2.1 - Metabolism of benzphetamine by P450s 2B6 and 2B6.4. 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.

	2B6	**2B6.4**	**2B6/2B6.4**
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

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 2B6 and 2B6.4 by tTEPA, bergamottin and 17EE. The inactivation of P450 2B6.4 by these three mechanism-based inactivators was performed as described in the Experimental Methods. P450 2B6.4 was inactivated by both tTEPA (Figure 2.5) and bergamottin (Figure 2.6). 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 2B6.4 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 [14]. In contrast to the wild-type enzyme, 17EE had no effect on the 7-EFC activity of the P450 2B6.4 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 2.2). Bergamottin had the greatest effect on both P450 2B6 and P450 2B6.4 leaving approximately 30% and 31% bupropion hydroxylation activity remaining, respectively,

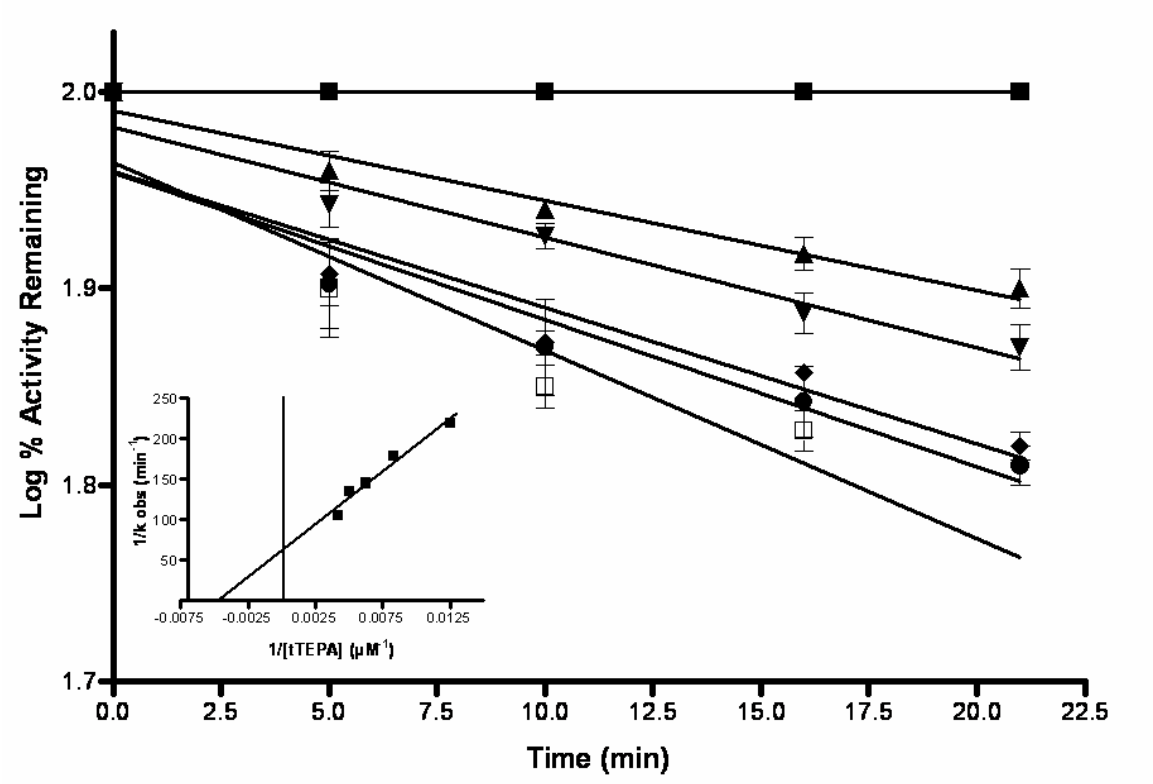


Figure 2.5 - Inactivation of P450 2B6.4 by tTEPA. The time- and concentration-dependent inactivation of P450 2B6.4 was measured by determining the 7-EFC *O*-deethylation activity. After initiation of inactivation by the addition of NADPH, aliquots were removed from the primary reaction mixture at 0, 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.

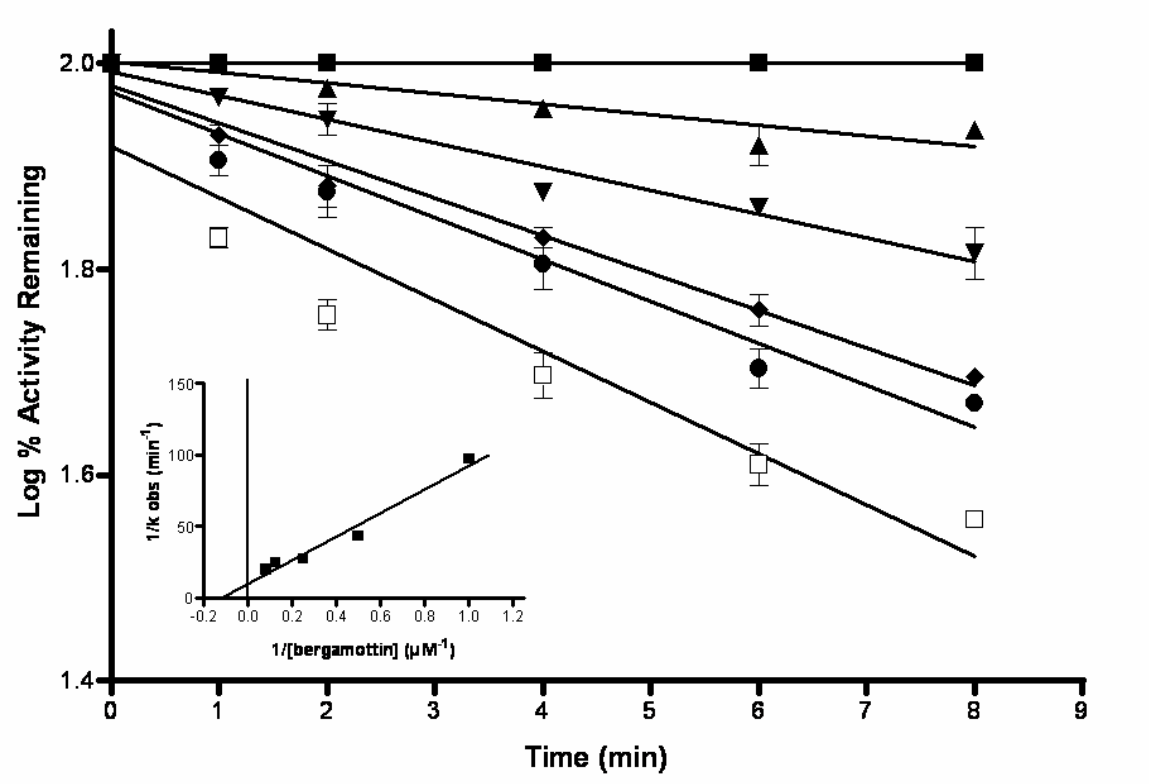


Figure 2.6 - Inactivation of P450 2B6.4 by bergamottin. The time- and concentration-dependent inactivation of P450 2B6.4 was measured by determining the 7-EFC *O*-deethylation activity. After the addition of NADPH, aliquots were removed from the primary reaction mixture at 0, 2, 4, 6, and 8 min. The 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.

Table 2.2 - Effect of bergamottin, tTEPA, and 17EE on the bupropion hydroxylation and 7-EFC activities of P450s 2B6 and 2B6.4. P450s 2B6 and 2B6.4 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**
2B6 Bergamottin	30 ± 1	34 ± 2
2B6.4 Bergamottin	31 ± 1	40 ± 3
2B6 tTEPA	51 ± 2	55 ± 3
2B6.4 tTEPA	62 ± 3	81 ± 4
2B6 17EE	61 ± 4	23 ± 3
2B6.4 17EE	101 ± 2	103 ± 1

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 2B6 leaving 61% activity remaining with bupropion as the probe substrate while the P450 2B6.4 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 2B6 and 2B6.4. In order to see if the lack of inactivation of 2B6.4 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 2.7. 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 [14] (Panel A). However, as shown in Panel B, P450 2B6.4 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 2B6.4 in the reconstituted system show that a single mutation at position 262 to give the

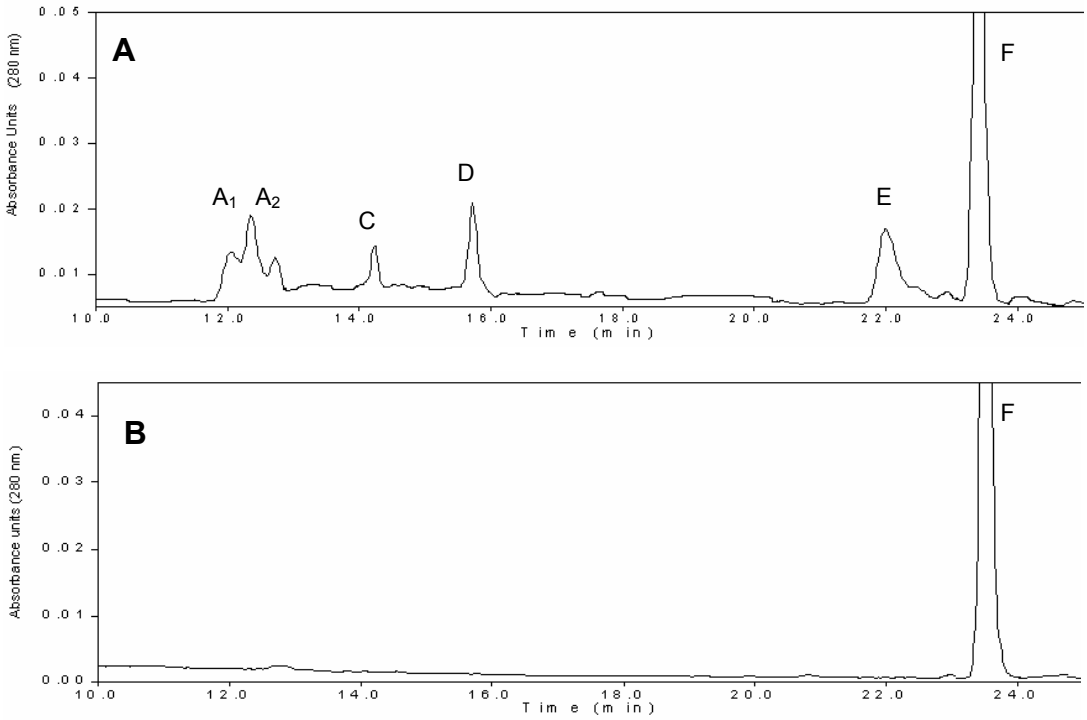


Figure 2.7 - Metabolism of 17EE by P450s 2B6 and 2B6.4. Samples were reconstituted and incubated with 17EE in the presence or absence of NADPH as described in Experimental Procedures. The metabolites of 17EE produced by P450 2B6 (Panel A) and P450 2B6.4 (Panel B) were separated as described in Experimental 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 [14].

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) [25-27]. A number of chemotherapeutic drugs such as tTEPA are substrates for 2B6 and they are often given in combination with other drugs [13, 16, 17]. 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 [28]. A recent study demonstrated higher mean plasma concentrations of efavirenz in patients homozygous for P450 *2B6*6* (Q172H, K262R) when compared to wild-type [29]. 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*) [1, 2].

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 [22, 30]. Our findings suggest that P450 2B6.4

produced the hydroxylated product at a rate that was significantly greater than the wild-type enzyme. The K_m of wild-type P450 2B6 for bupropion in this study is one-tenth that previously reported in human liver microsomes [30]. 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 [2].

Benzphetamine was readily metabolized by both P450 2B6 and P450 2B6.4 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 2B6.4. 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 2B6 or P450 2B6.4 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 [24]. 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 2B6.4 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 [31]. The estimated K_I value for the inactivation of P450 2B6.4 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 [13]. The rate of inactivation of the mutant was approximately 3-fold less than what has been reported for the full-length wild-type enzyme [13]. 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 2B6.4 and the rate of inactivation were approximately 2-fold greater and 3-fold greater, respectively, than what was observed with the wild-type enzyme [15], but the K_I value of the mutant is similar to the value previously determined for P450 3A4 of 7.7 μM [18]. 17EE is metabolized by P450 2B6 to give several metabolites and has been shown to be a mechanism-based inactivator for 2B enzymes [14]. Surprisingly, in contrast to other inactivators or the wild type enzyme, P450 2B6.4 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 2B6 and the P450 2B6.4 mutant when different probe substrates were used to determine catalytic activity remaining. For example, P450 2B6 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 2B6.4 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 2B6.4 in the reconstituted system metabolized bupropion to hydroxybupropion at a faster rate than P450 2B6. The P450 2B6.4 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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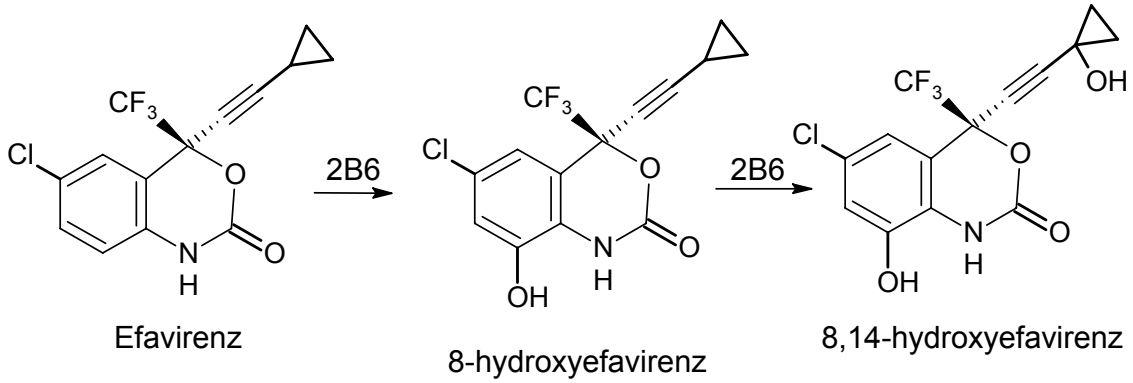
CHAPTER 3

METABOLISM OF EFAVIRENZ AND 8-HYDROXYEFAVIRENZ BY P450 2B6 LEADS TO INACTIVATION BY TWO DISTINCT MECHANISMS

Introduction

Efavirenz is a non-nucleoside reverse transcriptase inhibitor used in the treatment of human immunodeficiency virus-1 (HIV-1). Efavirenz is prescribed as part of a combination therapy and is particularly effective due to its long half-life of 40-55 hours following multiple doses [1]. P450 2B6 has been shown to be primarily responsible for the hydroxylation of efavirenz to 8-hydroxyefavirenz and 8,14-hydroxyefavirenz (Figure 3.1) [2]. *In vivo* and *in vitro* studies have shown that 8-hydroxyefavirenz is formed rapidly and is the major metabolite formed [2, 3]. Polymorphisms of P450 2B6 may have a significant effect on efavirenz metabolism since it has been shown that patients genotyped as P450 *2B6 *6/*6* (Q172H and K262R) have significantly higher mean plasma efavirenz concentrations than patients that are **6* heterozygous or that do not have **6* alleles [4]. Efavirenz has also been shown to inhibit bupropion hydroxylation in human liver microsomes [5].

In this study, we used recombinant *N*-terminally truncated P450 2B6 and P450 2B6.4 to 1) evaluate the effect of the K262R mutation on the hydroxylation of efavirenz to 8-hydroxyefavirenz, 2) to investigate the ability of efavirenz to inactivate both



Efavirenz

8-hydroxyefavirenz

8,14-hydroxyefavirenz

Figure 3.1 - Chemical structures of efavirenz and the two primary metabolites formed through hydroxylation by P450 2B6.

enzymes, and 3) to investigate the ability of 8-hydroxyefavirenz , a major metabolite of efavirenz, to act as a mechanism-based inactivator of both enzymes. We found that the mutant was able to metabolize efavirenz to 8- hydroxyefavirenz. In addition, efavirenz inactivated the wild-type 2B6 but not the mutant and the inactivation of 2B6 was reversible after dialysis. In contrast to the results observed with the parent compound, incubations with the 8-hydroxy metabolite resulted in the irreversible inactivation of both enzymes. These studies provide valuable information regarding the effect of the K262R mutation on P450 2B6 catalytic activity. In addition, these results show that hydroxylation of a substrate can lead to marked differences in the mechanism of inactivation. These studies also suggest that efavirenz and 8-hydroxyefavirenz may be useful tools for studying the structure of the active site of P450 2B6.

Experimental Procedures

Materials. Efavirenz was purchased from Toronto Research Chemicals (Ontario, Canada). 8-hydroxyefavirenz was a generous gift from Bristol-Myers Squibb. Bupropion hydrochloride, triprolidine hydrochloride, NADPH, BSA and catalase were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Ethoxy-4-(trifluoromethyl)coumarin (7-EFC) was obtained from Molecular Probes (Eugene, OR). Barium hydroxide, 3-aminophenol and hydroxylamine hydrochloride were purchased from Aldrich Chemical Co. (Milwaukee, WI). The P450 2B6 plasmid was a generous gift from Dr. James Halpert, University of Texas Medical Branch, Galveston, Texas. All other chemicals were of the highest grade commercially available.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, California).

Site-directed mutagenesis, expression and purification of P450s and NADPH-cytochrome P450 Reductase (reductase). Construction of the P450 2B6.4 mutant was performed as described previously [6]. P450 2B6, P450 2B6.4 and NADPH-P450 reductase were expressed in *E. coli* Topp 3 cells and purified as previously described [7-9] except that P450 2B6.4 was recovered from the cytosol rather than the membrane pellet after the cell lysis step. Therefore, the cytosol was applied to the Ni^{++}-agarose column and the P450 was then eluted and purified as previously described.

Efavirenz metabolism. The method used to determine efavirenz metabolism was adapted from Ward et al.[2]. The purified P450s were reconstituted with reductase at a ratio of 1:2 of P450 to reductase for 45 minutes at 4 °C. The reaction mixtures consisted of 1 μM P450, 2 μM reductase, 110 U catalase and efavirenz (concentrations ranging from 0 μM to 60 μM). NADPH was added to initiate the reactions and the mixtures were incubated for 30 min at 37 °C. The reactions were quenched by adding 500 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 supernatants were placed in clean tubes and 500 μL of 0.5M NaOH, pH 10, was added. Testosterone (2 μL of a 20 μM stock) was added as an internal standard. The samples were extracted twice with ethyl acetate and dried under a stream of nitrogen. 200 μL of mobile phase was added and the samples were resolved on a Varian Microsorb-MV 250 x

4.6-mm C_{18} column (Varian Inc., Palo Alto, CA) at a flow rate of 0.8 ml/min with the detector set at 247 nm. Isocratic conditions were used consisting of 55% mobile phase A (water, 0.1% trifluoroaceticacid) and 45 % mobile phase B (acetonitrile, 0.1% trifluoroaceticacid). The retention times were approximately 13 min for the internal standard testosterone, 23.5 min for 8-hydroxyefavirenz, and 42 min for efavirenz.

Inactivation of P450s 2B6 and 2B6.4. The purified P450s were reconstituted with reductase for 45 minutes at 4 °C. The primary reaction mixtures contained 1 μM P450, 2 μM reductase, 110 U catalase and efavirenz (0-50 μM) or 8-hydroxyefavirenz (0-120 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 mixtures at the times indicated and transferred to 990 μL of the secondary reaction mixtures which contained 100 μM 7-EFC, 1 mM NADPH, and 40 μg BSA/mL in 50 mM potassium phosphate buffer, pH 7.4. The secondary reaction mixtures were incubated for 10 min at 30 °C, and then quenched by the addition of 334 μL of acetonitrile. The amount of 7-hydroxy-4-(trifluoromethyl) coumarin formed was measured at room temperature 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). The amount of 8-hydroxybupropion formed was determined as previously described [6]. To measure the effect of efavirenz on the cyclophosphamide (CPA) hydroxylation activity of the P450s, the primary reaction mixtures were incubated with 20 μM efavirenz at 37°C. After the addition of NADPH,

aliquots were removed at the times indicated. The secondary reaction mixtures contained 100 μM CPA, 1 mM NADPH, and 40 μg BSA/mL in 50 mM potassium phosphate buffer, pH 7.4. CPA hydroxylation was determined using the procedure of Roy et al. [10].

Reversibility of inactivation of P450s 2B6 and 2B6.4 by efavirenz. P450s were reconstituted and incubated with 50 μM efavirenz or 20 μM 8-hydroxyefavirenz (80 μM for P450 2B6.4) in the presence or absence of NADPH as described above. Aliquots were removed at 0 and 20 minutes to determine the amount of 7-EFC *O*-deethylation activity remaining as described above. Each sample was further analyzed for P450 remaining using the reduced CO spectral assay and intact heme by HPLC as described by Harleton et al. [11]. The remainder of each of the control and inactivated samples was dialyzed separately for 24 hours at 4°C in Slide-A-Lyzer cassettes (Pierce Chemical, Rockford, IL) against 2 x 500 mL dialysis buffer (50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 100 μM EDTA). After dialysis, the samples were incubated with or without fresh reductase at 4°C for 15 minutes and catalytic activity, heme and reduced CO spectra analysis were again carried out as described above.

Determination of spectral intermediate formation. P450s were reconstituted as described above and incubated with 10 μM efavirenz at 30°C for 10 min. NADPH (0.6 mM) was added to the sample cuvette and an equal amount of water was added to the reference cuvette. Difference spectra were recorded from 350 to 700 nm using a DW2 UV-Vis spectrophotometer (SLM Aminco, Urbana, IL) that was equipped with an OLIS

spectroscopy operating system (On-Line Instrument Systems, Inc., Bogart, GA). Scans were taken continuously at 2 minute intervals.

Results

Formation of 8-hydroxyefavirenz by P450 2B6 and P450 2B6.4. The metabolism of efavirenz to 8-hydroxyefavirenz by 2B6 and 2B6.4 was measured by HPLC. The rates of formation of 8-hydroxyefavirenz by P450 2B6 and P450 2B6.4 using concentrations of efavirenz ranging from 0 μM to 100 μM are shown in Figure 3.2. The kinetic constants for these reactions are shown in Table 3.1. The approximate K_m value for wild-type P450 2B6 (14.3 μM) was very similar to the approximate K_m for the variant (15.9 μM). The V_{max} for 8-hydroxyefavirenz formation by P450 2B6 was approximately 4.3 pmol formed/pmol P450/min, whereas the V_{max} of P450 2B6.4 was approximately 2-fold higher (8.1 nmol formed/nmol P450/min). The catalytic efficiency (V_{max}/K_m) of P450 2B6.4 was approximately 66% greater than that of P450 2B6 (approximately 0.3 for 2B6 as compared to approximately 0.5 for the mutant).

Inactivation of P450 2B6 by efavirenz. Inactivation of the wild-type enzyme was measured using the 7-EFC *O*-deethylation assay. The wild-type enzyme was inactivated by efavirenz (Figure 3.3) in a time- and concentration-dependent manner and the inactivation exhibited an absolute requirement for NADPH. The activity loss followed pseudo first order kinetics. Linear regression analysis was performed and the kinetic constants for the efavirenz- mediated inactivation of the wild-type enzyme were determined from the inset of Figure 3.3. The K_I was approximately 30 μM and the K_{inact}

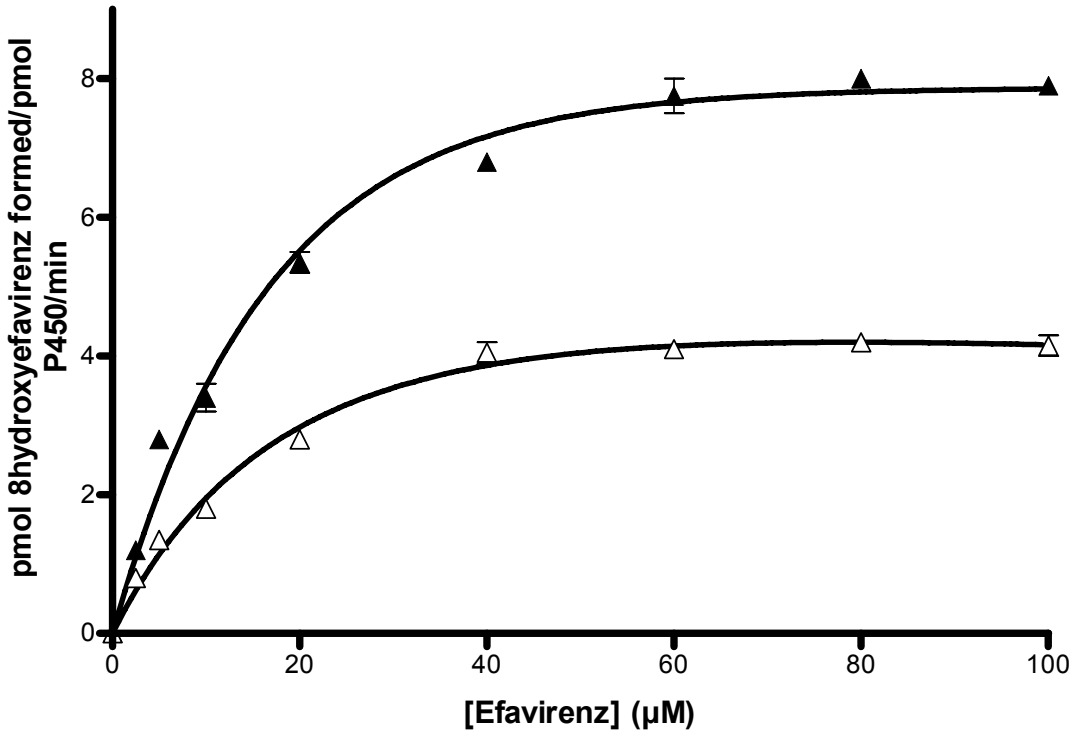


Figure 3.2 - Metabolism of efavirenz to 8-hydroxyefavirenz by P450 2B6 and P450 2B6.4. Samples were reconstituted as described in Methods and incubated with efavirenz at concentrations ranging from 0 μM to 100 μM. 8-Hydroxyefavirenz formation by P450 2B6 (△) and P450 2B6.4 (▲) was measured by HPLC by integrating the area under the metabolite peak and comparing it to a standard curve generated by injecting known amounts of authentic 8-hydroxyefavirenz onto the HPLC. The data shown are representative of the means and standard deviations from 4 separate experiments done in duplicate.

Table 3.1 - Kinetic constants for the formation of 8-hydroxyefavirenz by recombinant P450 2B6 and P450 2B6.4. Kinetic constants were determined from the data shown in Figure 3.2 as described in Experimental Procedures.

Kinetic Constant	Enzyme	
	P450 2B6	P450 2B6.4
K_m (μM)	14.3	15.9
V_{max} (pmol/pmol P450/min)	4.3	7.9
V_{max}/K_m	.30	.51

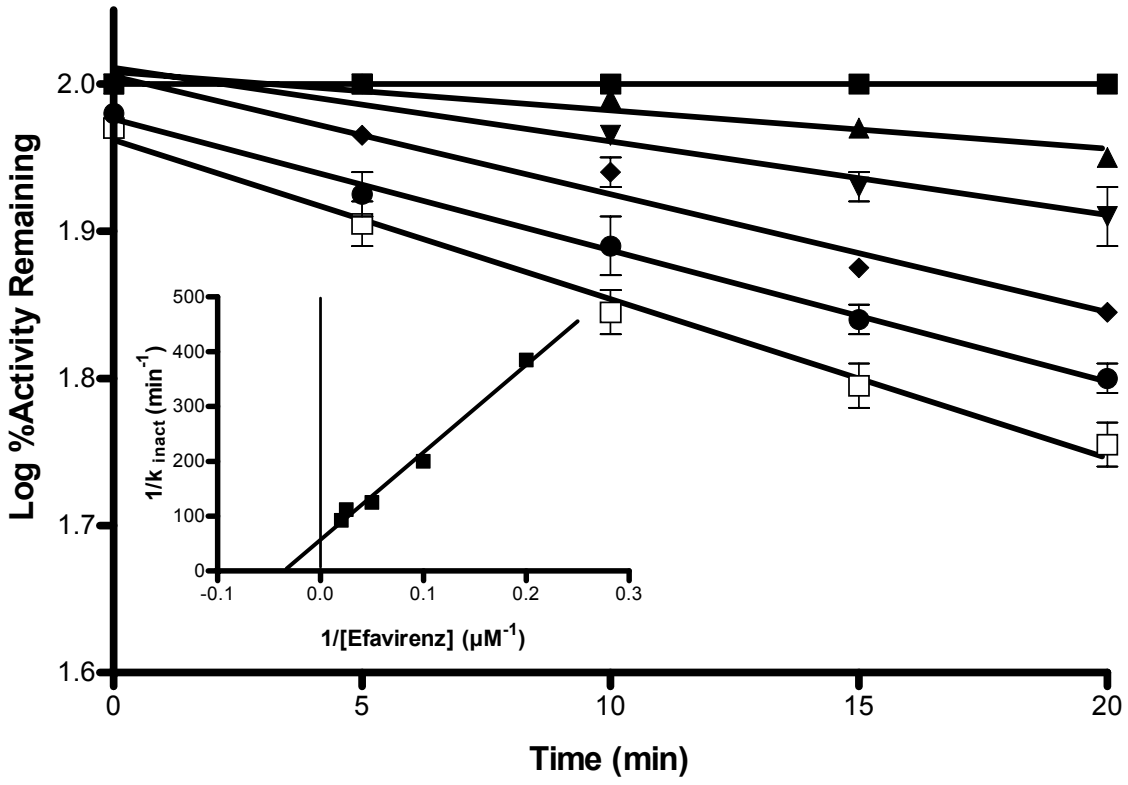


Figure 3.3 - Inactivation of P450 2B6 by efavirenz. The time- and concentration-dependent inactivation of P450 2B6 was measured by determining the 7-EFC *O*-deethylation activity as described in Methods. After initiation of inactivation by the addition of NADPH, aliquots were removed from the primary reaction mixture at 0, 5, 10, 15 and 20 min. The concentrations of efavirenz were (■) 0 μM, (▲) 5 μM, (▼) 10 μM, (◆) 20 μM, (●) 40 μM and (□) 50 μM. The data show the means and standard deviations from 4 separate experiments done in duplicate. In some cases the standard deviations were less than the size of the symbols. The inset shows the double reciprocal plot of the rates of inactivation as a function of the efavirenz concentrations.

was 0.04 min^{-1} giving a $t_{1/2}$ of 16 min. In contrast to the wild-type enzyme, the variant form of 2B6 was not inactivated by efavirenz at concentrations up to 200 μM as measured by the 7-EFC *O*-deethylation activity of the variant enzyme (data not shown). The effect of mechanism-based inactivation by efavirenz on the metabolism of other structurally unrelated P450 2B6 substrates was also determined to investigate the possibility that the inactivation of wild-type 2B6 and the observed lack of inactivation of the variant might be due to the 7-EFC substrate that was chosen to measure activity. P450 2B6 samples that had been pre-incubated with efavirenz and NADPH were assayed for bupropion and cyclophosphamide hydroxylation activities (Table 3.2). Pre-incubation with efavirenz decreased the ability of the wild type P450 2B6 to hydroxylate both substrates. Approximately 45% of the initial bupropion hydroxylation activity and 42% of the CPA activity remained following incubation with 20 μM efavirenz. These values were similar to the activity remaining when 7-EFC was used as the substrate (Figure 3.2, and data not shown). As seen with 7-EFC, pre-incubation of the variant enzyme with efavirenz did not result in inactivation as measured by the hydroxylation of bupropion or CPA (data not shown).

Reversibility of efavirenz-mediated inactivation of P450 2B6. As shown in Table 3.3, the inactivation of P450 2B6 by efavirenz was reversible after overnight dialysis and the enzymatic activity, reduced CO spectra and heme remaining were completely restored. Control and efavirenz inactivated samples were analyzed for activity, CO spectral and heme loss before and after 24 hours of dialysis at 4°C. Table 3.3 shows that prior to

Table 3.2 - Effect of pre-incubation with efavirenz on the bupropion and cyclosphosphamide hydroxylation activities of P450 2B6. The P450s were reconstituted with reductase as described under Experimental Procedures. The primary reaction mixture contained the concentrations of efavirenz indicated. NADPH was added to the primary reaction mixture to initiate the reaction.

Primary Reaction Conditions	Percentage of Control Activity Remaining	
	Bupropion	Cyclophosphamide
-NADPH + 10 μM Efavirenz	100	100
+NADPH + 10 μM Efavirenz	62 ± 3	67 ± 5
-NADPH + 20 μM Efavirenz	100	100
+NADPH + 20 μM Efavirenz	45 ± 2	42 ± 4

Table 3.3 - Effect of efavirenz on P450 2B6 catalytic activity, P450 content as measured by the reduced CO spectrum and heme, before and after 24 hr dialysis. The assays were performed as described under Experimental Procedures. Samples were incubated for 15 min in the presence or absence of NADPH and the data are represented as percentages of the control samples incubated in the absence of efavirenz. Samples were then dialyzed for 24 hrs. The data represent the means and standard deviations of 3 separate experiments.

Primary Reaction Conditions	Percentage of Control		
	Activity Remaining	Reduced CO Remaining	HPLC Heme
Before dialysis			
-Efavirenz + NADPH	100	100	100
+Efavirenz + NADPH	32 ± 3	35 ± 4	31 ± 2
After dialysis			
-Efavirenz + NADPH	100	100	100
+Efavirenz + NADPH	98 ± 5	96 ± 4	101 ± 3

dialysis there was a 68% loss in activity, a 65% CO spectral loss and 69% heme loss. After dialysis for 24 hours, catalytic activity, reduced CO spectrum and the heme remaining had all returned to levels commensurate with the control samples. To determine if the reversibility observed was the result of MI complex formation, as has been observed with other reversible inactivators [12], difference spectra for efavirenz-inactivated versus control samples were determined. As shown in Figure 3.4, there is a peak with a maximum absorbance at 435 nm in the difference spectrum. This peak does not appear to be representative of a MI complex since MI complexes normally exhibit a characteristic peak absorbance at 455 nm, not at 435 nm. The addition of ferricyanide results in the loss of the peak that we observe at 435 nm suggesting that it is an intermediate. Additionally, this peak is not observed in the absence of NADPH.

Inactivation of P450 2B6 and P450 2B6.4 by 8-hydroxyefavirenz. The ability of 8-hydroxyefavirenz, the primary metabolite of efavirenz, to inactivate the wild-type and variant enzymes was also investigated. Interestingly, inactivation by 8-hydroxyefavirenz was markedly different from the inactivation by the parent. Incubation of both enzymes with 8-hydroxyefavirenz led to inactivation in time-, concentration-, and NADPH-dependent manners. The approximate K_I value for the inactivation of 2B6 by 8-hydroxyefavirenz was 6.4 μM, with a $t_{1/2}$ of 11 min, and a rate of inactivation of 0.06 min^{-1}, as measured by 7-EFC *O*-deethylation activity remaining Figure 3.5. In contrast to what had been observed with efavirenz, the P450 2B6 variant was also inactivated by 8-hydroxyefavirenz although the estimated K_I was approximately 10-fold higher than for the wild-type enzyme (75 μM) and the $t_{1/2}$ was 17 min with a rate of inactivation of 0.04

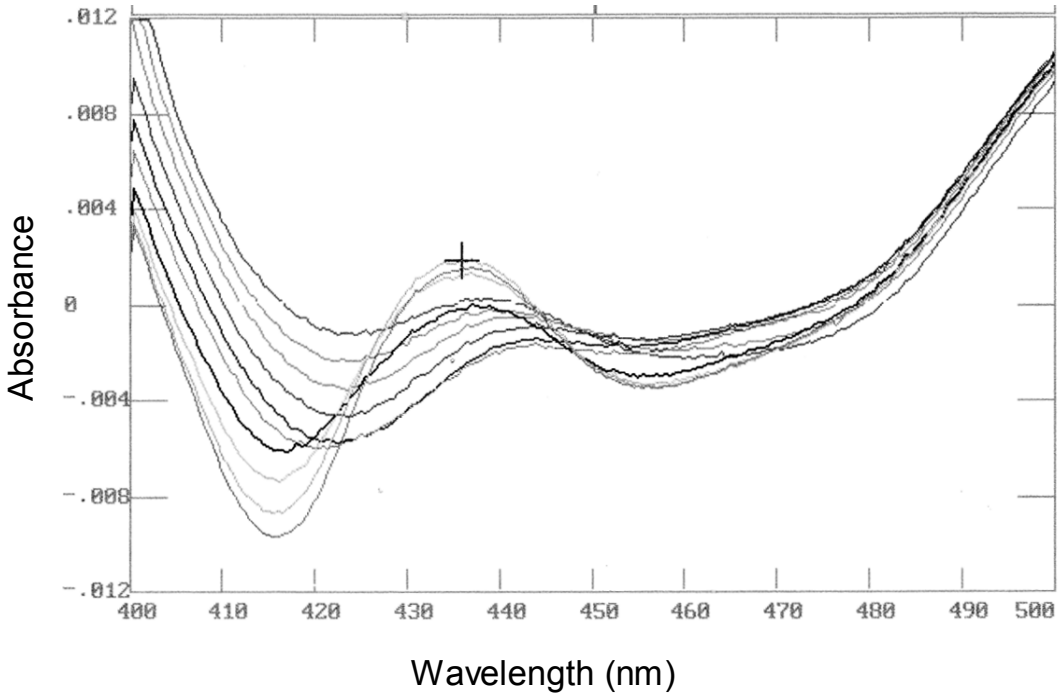


Figure 3.4 - Formation of a spectral intermediate during the inactivation of P450 2B6 by efavirenz. Reconstituted P450 2B6 was incubated with efavirenz and difference scans were recorded between 350 and 700 nm for 20 minutes (the traces obtained between 400-500 nm are shown here). After a baseline scan at 20 minutes, NADPH was added to the sample cuvette and water was added to the reference cuvette. Spectra were taken every 2 min. The absorbance at 400 nm was maximal at 0 time and decreased with successive scans. The star indicates peak maximum absorption observed at 435 nm.

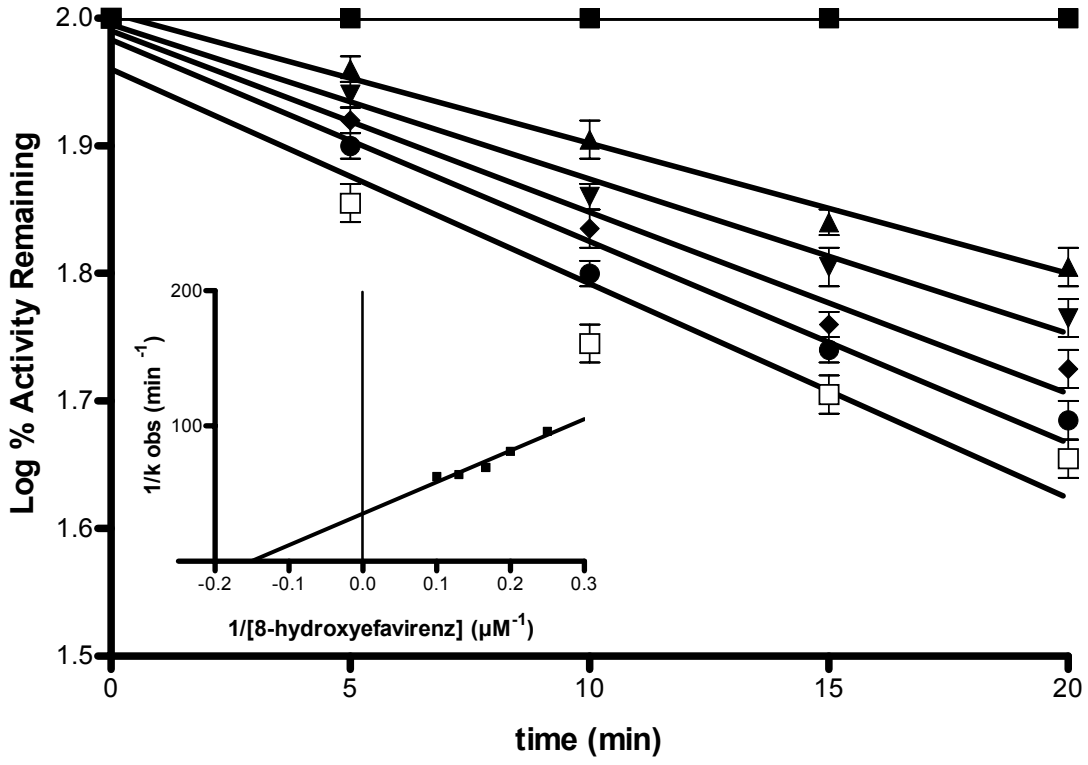


Figure 3.5 - Inactivation of P450 2B6 by 8-hydroxyefavirenz. P450 2B6 was inactivated by 8-hydroxyefavirenz and the activity remaining was measured using the 7-EFC *O*-deethylation assay. After the addition of NADPH, aliquots were removed from the primary reaction mixture at 0, 5, 10, 15, and 20 min. The concentrations of 8-hydroxyefavirenz were (■) 0 μM, (▲) 4 μM, (▼) 5 μM, (◆) 6 μM, (●) 8 μM, and (□) 10 μM. The data show the means and standard deviations from 3 separate experiments done in duplicate. The inset shows the double reciprocal plot of the rates of inactivation as a function of the 8-hydroxyefavirenz concentrations.

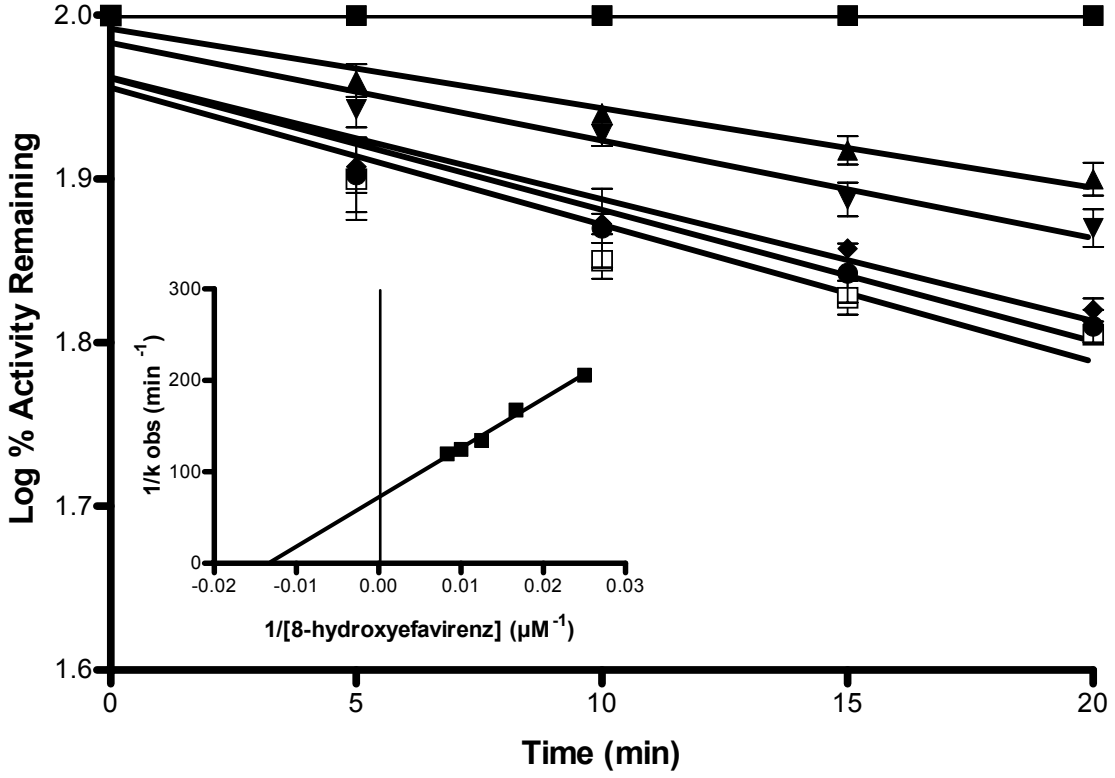


Figure 3.6 - Inactivation of P450 2B6.4 by 8-hydroxyefavirenz. Samples were reconstituted and incubated with 8-hydroxyefavirenz in the presence or absence of NADPH as described in Methods. Inactivation was measured by determining 7-EFC *O*-deethylation activity remaining. The concentrations of 8-hydroxyefavirenz were (■) 0 μM, (▲) 40 μM, (▼) 60 μM, (◆) 80 μM, (●) 100 μM, and (□) 120 μM. The data show the means and standard deviations from 3 separate experiments done in duplicate. The inset shows the double reciprocal plot of the rates of inactivation as a function of the 8-hydroxyefavirenz concentrations.

Table 3.4 - Effect of incubation with 8-hydroxyefavirenz on the bupropion and cyclophosphamide hydroxylation activities of P450 2B6 and P450 2B6.4. Assay conditions were described under Methods. Data are representative of the mean and standard deviations of 3 separate experiments done in duplicate. Primary reaction mixtures contained 8-hydroxyefavirenz at a concentration of 20 μM.

Primary Reaction Conditions	Percentage of Control Activity Remaining	
	Bupropion	Cyclophosphamide
2B6 -NADPH + 8-hydroxyefavirenz	100	100
2B6 +NADPH + 8-hydroxyefavirenz	32 ± 1	39 ± 2
2B6.4 -NADPH + 8-hydroxyefavirenz	100	100
2B6.4+NADPH + 8-hydroxyefavirenz	81 ± 5	84 ± 2

Table 3.5 - Irreversibility of 2B6 inactivation by 8-hydroxyefavirenz. Assay conditions were as described as under Experimental Procedures. Data are representative of the means and standard deviations of 3 separate experiments done in duplicate. P450 2B6 was incubated with 20 μM 8-hydroxyefavirenz for 15 min. P450 2B6.4 was incubated with 80 μM 8-hydroxyefavirenz for 15 min.

Primary Reaction Conditions	Percentage of Control		
	Activity Remaining	P450 Remaining	HPLC Heme
P450 2B6			
+8-OHefavirenz + NADPH (B.D.)	37 ± 3	41 ± 5	35 ± 2
+8-OHefavirenz + NADPH (A.D.)	46 ± 3	53 ± 4	43 ± 2
P450 2B6.4			
+8-OHefavirenz + NADPH (B.D.)	47 ± 6	53 ± 5	44±5
+8-OHefavirenz + NADPH (A.D.)	51 ± 4	55 ± 3	47 ± 4

B.D., before dialysis

A.D., after dialysis

min^{-1} Figure 3.6. Because the K_I for inactivation of the wild-type enzyme by efavirenz (30 μM) was greater than the K_I for inactivation by 8-hydroxyefavirenz (6.4 μM), it suggested that the 8-hydroxy metabolite was a more potent inactivator than the parent compound. Pre-incubation of the wild type and the variant enzyme with 8-hydroxyefavirenz in the presence of NADPH also resulted in marked decreases in the bupropion and cyclophosphamide hydroxylation activities of both enzymes Table 3.4.

The decrease in the enzymatic activity of the P450 2B6 that had been pre-incubated with 8-hydroxyefavirenz when measured by its ability to hydroxylate bupropion or cyclosphosphamide (CPA) was reduced to 32% and 39% activity remaining, respectively. The decrease in the catalytic activity of the variant enzyme that had been pre-incubated with 20 μM 8-hydroxyefavirenz and NADPH when measured by its ability to hydroxylate bupropion was 81% and for CPA it was 84% as compared to untreated controls. The losses in bupropion and CPA hydroxylation activities were similar to the activity decreases observed using the 7EFC assay.

Irreversibility of the inactivation of P450 2B6 and P450 2B6.4 by 8-hydroxyefavirenz. The changes in enzymatic activity and the losses in the CO spectra and heme were measured before and after overnight dialysis in samples inactivated by 8-hydroxyefavirenz. In complete contrast to what had been observed with efavirenz, the inactivation of both enzymes by 8-hydroxyefavirenz was irreversible (Table 3.5). The percentage of activity remaining after dialysis and after incubation with fresh reductase increased only slightly for both enzymes suggesting that although there may be a small

portion of the population of enzyme that is reversibly inactivated by 8-hydroxyefavirenz, the inactivation is essentially irreversible.

Discussion

P450 2B6 is expressed in a number of organs including the liver, heart and brain, and has been shown to have widely variable expression levels [13-15]. P450 2B6 plays an important role in the metabolism a growing list of clinically important substrates which include bupropion, an anti-depressant and smoking cessation aid [16, 17] and cyclosphosphamide, an important chemotherapeutic agent [18]. Bupropion has been used previously as a tool to study the inactivation and inhibition of P450 2B6 [19]. It has recently been shown that a P450 2B6/reductase fusion protein catalyzed the metabolic activation of the pro-drug cyclophosphamide and markedly increased the cyclophosphamide-dependent cytotoxicity [20]. A number of SNPs have been found in the *P450 2B6* gene [21] and some of these have been shown to have effects on the catalytic activity of the enzyme. *P450 2B6*4*, which corresponds to a K262R mutation of the protein, has recently been shown to have close to a 50% mutation frequency in Ghanians and close to 30% in African-Americans and Caucasians [13]. We have previously shown that the K262R mutant of P450 2B6 has significant effects on the metabolism of the P450 2B6 specific substrate bupropion [6]. In this study we have compared the abilities of purified P450 2B6 and its K262R mutant, 2B6.4, to metabolize efavirenz to 8-hydroxyefavirenz in the reconstituted system. Our data indicate that P450 2B6.4 hydroxylates efavirenz to 8-hydroxyefavirenz at a rate almost two-fold greater than that of the wild-type enzyme. The K_m of the wild-type enzyme for efavirenz in the

present study was similar to that reported previously [2]. However, because of the marked variability in the expression levels of P450 2B6 in the human population, it is not possible to directly draw clinical conclusions based upon our current data. In patients, P450 2B6 polymorphisms have been shown to have an effect on plasma levels of efavirenz as patients homozygous for *2B6*6* (Q172H, K262R) had higher mean plasma concentrations of the parent drug [4]. Two other groups have recently reported similar findings in patients homozygous for the 516G>T mutation (Q172H) [22, 23].

Because many drugs, including efavirenz, are prescribed as part of a combination therapy, it is important to determine which substrates may have inhibitory effects on the enzyme. Adverse drug reactions are a major source of hospitalizations and even mortality [24-26], and are defined as "an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medical product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product" [27]. Polymorphisms appear to play an important role in adverse drug reactions since many of the drugs that are frequently cited in these studies are metabolized by at least one polymorphic enzyme [26, 28]. We have previously shown that the K262R mutation protected the enzyme against inactivation by 17-α-ethynylestradiol, which readily inactivates the wild-type enzyme [6, 29]. In the present study pre-incubation with efavirenz decreased the ability of P450 2B6 to catalyze the hydroxylation of bupropion and cyclophosphamide as well as decreasing 7-EFC *O*-deethylation activity. In contrast, no effect on the above mentioned activities was observed with the P450 2B6.4 mutant. The reason for the inability of the mutant to become inactivated by efavirenz is not clear but does not appear to involve the absence of

reversible binding and metabolism of efavirenz because the primary metabolite 8-hydroxyefavirenz was generated by the mutant. We are currently investigating this functional difference between the wild-type and the K262R mutant enzyme, and the affect that this mutation has on the ability of P450 2B6 to form a reactive intermediate from ethynyl-containing compounds capable of inactivating the wild-type enzyme.

Another interesting finding was that the inactivation of P450 2B6 by efavirenz was completely reversible after 24 hours of dialysis. A similar recovery of enzymatic activity has been described with purified rat P450 2B1 where the initial loss in activity was due to the formation of a metabolic intermediate (MI) complex [12]. MI complex formation results in the appearance of a characteristic maximum absorbance peak at 455 nm in the difference spectrum [30]. A second mechanism for reversible inactivation has recently been described for the inactivation of P450 2E1 T303A by *tert*-butyl acetylene. In this case, the inactivation was accompanied by the appearance of a spectral intermediate at 485 nm [31]. Difference spectra of efavirenz-inactivated samples versus controls exhibited a new peak with a maximum absorbance at 435 nm. This spectral intermediate formed during the inactivation by efavirenz is different than the intermediates reported previously. The reasons for this difference are under investigation.

Surprisingly, when 8-hydroxyefavirenz, the major metabolite of efavirenz, was used instead of efavirenz, both P450s were inactivated in a mechanism-based manner and the inactivation was irreversible. With either enzyme and 8-hydroxyefavirenz, we were unable to observe a spectral intermediate similar to that seen during the inactivation of the wild type enzyme by efavirenz (data not shown). These data suggest that the inactivation of P450 2B6 and the variant by efavirenz and 8-hydroxyefavirenz occurs

through two distinctly different mechanisms. Even though 8-hydroxyefavirenz is formed during efavirenz metabolism by both enzymes and the K_I value for the hydroxylated product with the wild-type enzyme is approximately 4-fold lower than the K_I value for efavirenz, the concentration of 8-hydroxyefavirenz required to achieve irreversible inactivation does not appear to have been achieved during the incubations. Therefore, we believe that during the metabolism of efavirenz in the reconstituted system, the concentrations of 8-hydroxyefavirenz produced contribute only negligibly to the inactivation. Further, the spectral intermediate that is formed during the reversible inactivation of P450 2B6 may be formed prior to the production of significant amounts of 8-hydroxyefavirenz. In contrast, incubations of 2B6 with 8-hydroxyefavirenz alone may lead to the formation of a reactive intermediate that is not produced during incubations of 2B6 with efavirenz alone. Our data suggest that efavirenz is initially bound in the P450 active site in an orientation that facilitates oxidation at or near the 8-hydroxy position. Once the 8 position is hydroxylated, the preferred orientation of the substrate in the active site may bring the ethynyl moiety into closer proximity to the heme iron with the activated oxygen. This could then result in the generation of the reactive intermediate which could be responsible for the irreversible inactivation. Studies are currently underway to attempt to trap the reactive intermediate formed during the metabolism of 8-hydroxyefavirenz and to obtain structural information of this intermediate.

We also observed a difference in inactivation of P450 2B6 by 8-hydroxyefavirenz when compared to P450 2B6.4. The approximate K_I for the wild-type enzyme was 6.4 μM, whereas it was 75 μM for the mutant. The 12-fold greater K_I for the mutant once again suggests that this mutation has a significant effect on the catalytic properties of the

enzyme. This effect was not substrate dependent as we have reported previously for inactivation of the mutant enzyme [6]. Similar levels of inactivation were seen with 7-EFC, cyclophosphamide, and bupropion.

These studies demonstrate that the K262R mutant of P450 2B6 catalyzes the metabolism of efavirenz to 8-hydroxyefavirenz at a significantly greater rate than the wild-type enzyme. P450 2B6 in the reconstituted system was inactivated by efavirenz, while P450 2B6.4 was not inactivated. Interestingly, the efavirenz-mediated inactivation of the wild-type enzyme was completely reversible after dialysis. The primary metabolite of efavirenz, 8-hydroxyefavirenz, inactivated both enzymes and the inactivation was irreversible. Since the inactivation by 8-hydroxyefavirenz was irreversible whereas the inactivation by efavirenz was reversible, these two closely related compounds inactivated the enzymes through mechanisms that are completely different from each other. This study has further shown a difference in the catalytic properties of the wild-type enzyme and K262R mutant. Efavirenz and 8-hydroxyefavirenz may prove to be useful tools for probing the structure of the P450 2B6 active site.

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CHAPTER 4

INVESTIGATION OF THE MECHANISMS UNDERLYING THE DIFFERENTIAL EFFECTS OF THE K262R MUTATION OF P450 2B6 ON CATALYTIC ACTIVITY

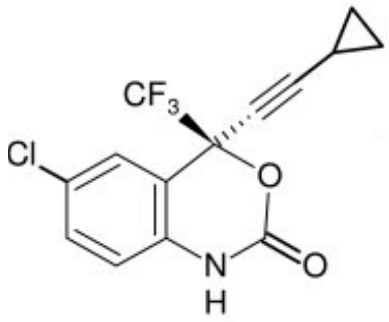
Introduction

The cytochromes P450 (P450) are a superfamily of heme-containing monooxygenases that catalyze the oxidative metabolism of a number of endogenous and exogenous compounds, including clinically relevant drugs, pesticides and carcinogens. The P450 catalytic cycle consists of a number of steps including: substrate binding to ferric P450; reduction, as a result of the transfer of an electron from NADPH via NADPH-cytochrome P450 reductase (reductase); binding of molecular oxygen to ferrous P450, leading to the formation of oxyferrous P450; transfer of a second electron to oxyferrous P450 from NADPH via reductase, or in some instances cytochrome b_5; formation of the oxygenating species; and subsequent oxidation of the substrate followed by product release. In addition, hydrogen peroxide can be formed via the decomposition of the oxyferrous complex or by autooxidation of the two-electron reduced P450 [1]. This phenomenon is referred to as "uncoupling".

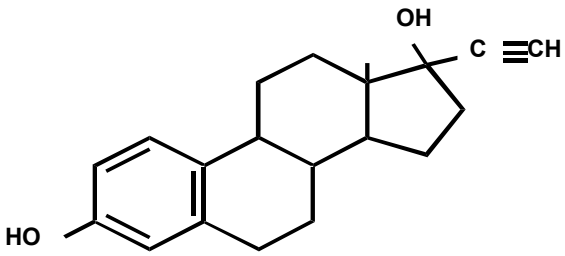
Human P450 2B6 plays a major role in the metabolism of a growing list of compounds including bupropion, an anti-depressant and smoking cessation aid;

efavirenz, a non-nucleoside HIV-1 reverse transcriptase inhibitor; and cyclophosphamide, a chemotherapeutic prodrug that requires metabolic activativation [2-5]. Certain substrates of P450 2B6, such as efavirenz, are also mechanism-based inactivators of the enzyme. Mechanism-based inactivation occurs when a substrate, in the process of metabolism, is converted to a reactive intermediate that binds covalently to the active site of the P450, rendering it inactive [6]. A number of single nucleotide polymorphisms have been found in the *P450 2B6* gene [7]. Recent studies in patients have demonstrated that some of these mutations can have significant effects on clinical outcomes [8, 9]. However, studies investigating the mechanisms underlying these effects are lacking. In order to address this issue, we have used recombinant P450 2B6 and a mutant P450 2B6 K262R (2B6.4), which corresponds to the *P450 2B6*4* variant allele observed in humans (785A>G, exon 5), to investigate the functional consequences of this amino acid mutation.

Previously, we demonstrated that efavirenz and 17EE (Figure 4.1), which both inactivate P450 2B6, do not inactivate P450 2B6.4 in the reconstituted system [10, 11]. Further, in those studies the mutant enzyme was not able to metabolize 17EE, a substrate readily metabolized by the wild-type enzyme. In this study, we have systematically investigated some of the aspects of P450 catalytic function that could potentially be altered by the K262R mutation. Therefore, we conducted studies to elucidate whether differences in the catalytic activities of P450 2B6 and P450 2B6.4 are related to: 1) active site topology; 2) substrate binding; 3) interaction with reductase; 4) reaction coupling. Our approach included the use of phenyldiazene to probe the active site of the P450, and the use of an alternate oxidant to support catalytic activity in the absence of reducing



Efavirenz



17EE

Figure 4.1 - Chemical structures of efavirenz and 17EE.

equivalents from NADPH. The results presented here suggest that there may be some differences in the active site topologies of the two enzymes, although the binding constants derived from spectral binding studies were similar. Interestingly, reaction stoichiometry experiments revealed that the reactions catalyzed by the mutant were more uncoupled than the reactions catalyzed by the wild-type enzyme. The addition of cytochrome b_5 improved the coupling of P450 2B6.4 and facilitated inactivation of the enzyme by both compounds.

Experimental Procedures

Materials. Benzphetamine, BSA, 17EE, catalase and NADPH were purchased from Sigma (St. Louis, MO). Efavirenz was purchased from Toronto Research Chemicals (Ontario, Canada). 7-Ethoxy-4-(trifluoromethyl)coumarin (7-EFC) was obtained from Molecular Probes (Eugene, OR). Phenyldiazene was purchased from Research Organics (Cleveland, OH). The P450 2B6 plasmid was a generous gift from Dr. James Halpert, University of Texas Medical Branch, Galveston, Texas. All other chemicals were of the highest grade commercially available.

Site-Directed mutagenesis, expression and purification of P450s and Reductase. Construction of the P450 2B6.4 mutant was performed as described by Bumpus et al. [11]. P450 2B6, P450 2B6.4, and NADPH-P450 reductase were expressed in *E. coli* Topp 3 cells and purified according to published protocols [12-14]. Cytochrome b_5 was purified from liver microsomes of phenobarbital-treated Long-Evans rats.

***N*-phenylprotoporphyrin IX regioisomer formation.** The procedures used in these studies was adapted from published protocols [15, 16]. The phenyldiazene stock used in these experiments was prepared by adding 2.5 µl of neat phenyldiazene to 200 µL of 1N KOH. For the myoglobin experiments, 5 nmol of myoglobin in 100 mM KPi, pH 7.4, was placed into a 1 ml cuvette and the absorbance spectrum from 400 to 500 nm was recorded. Then, 3 µl of the phenyldiazene stock was added to the cuvette and the absorbance spectrum was once again determined. A peak was observed at 430 nm, which is characteristic of a myoglobin phenyl-iron complex. Once the peak reached a maximum (approximately 10 min), the protein was denatured by adding the contents of the cuvette to 5 ml of 5% sulfuric acid (aq). After sitting for 2 hours the sample was extracted twice with an equal volume of methylene chloride. The extract was then dried down under a stream of nitrogen. For the P450 experiments, 2 nmol of P450 in 100 mM KPi, pH 7.4, was placed into a 1 ml cuvette and the absorbance from 400-500 nm was measured. Then, 1.5 µl of the phenyldiazene stock solution was added to the cuvette and peak formation at 478 nm was monitored. After the peak formation reached a maximum (approximately 10 min) 3 µl of potassium ferricyanide [50 µM] was added to the cuvette and the contents of the cuvette were mixed and allowed to sit for 3 minutes. This was repeated twice to induce migration of the phenyl group from the iron to the porphyrin nitrogens. The sample was then denatured and extracted with methylene chloride as described above for myoglobin. After being dried under nitrogen, the *N*-phenylprotoporphyrins were reconstituted in 150 µl of solvent A (40% water, 59.5% methanol, .5% acetic acid). The samples were analyzed by HPLC LC-MS using a Phenomenex phenyl-hexyl column under isocratic conditions with 70% A and 30% B

(99.5% methanol, .5% acetic acid). The area under the curve was determined for each of the four resulting regioisomers. These data were then expressed as a percentage of the total sum of the areas under the curve of all four peaks.

Spectral binding. Spectral binding experiments were performed by titrating 1 μM of P450s 2B6 and 2B6.4 with either benzphetamine (dissolved in water) or efavirenz (dissolved in ethanol) at room temperature. Samples were brought to a total volume of 1 ml using 100 mM KPi, pH 7.4, and placed into a cuvette. The reference cuvette also contained 1 μM of the P450s in 100 mM KPi, pH 7.4. Vehicle solvent was added to the reference cuvette immediately following the titration of either benzphetamine or efavirenz into the sample cuvette. UV-visible spectra were recorded from 350-500 nm following the addition of each aliquot of the ligand to the sample cuvette and an equal volume of the vehicle solvent to the reference. The absorbance differences between the maximum and minimum absorbencies observed in the difference spectrum following each addition were recorded and plotted against the concentrations of benzphetamine or efavirenz added using GraphPad Prism (GraphPad software, San Diego, CA). Spectral binding studies to determine the apparent K_d of reductase binding to P450 were performed in a similar fashion by titrating 1 μM P450 with reductase (0–8 μM) as previously described by French et al., [17]. K_s of benzphetamine or efavirenz binding and the apparent K_d value of reductase binding values were approximated by plotting the inverse of the absorbance changes between 390 nm and 420 nm (type I) as a function of the concentration of either benzphetamine or reductase, and between 436 nm and 416 as a function of the efavirenz concentration.

Alternate oxidant studies. The alternate oxidant *tert*-butyl hydroperoxide (*t*BHP) was used to support P450 catalytic activity in place of NADPH, reductase and molecular oxygen. P450s 2B6 or 2B6.4 were placed in 50 mM KPi, pH 7.4, to a final volume of 200 µl. An aliquot (12 µl) of this primary mixture was transferred into 990 µl of assay mixture that contained 100 µM 7-EFC and 40 µg BSA/ml in 50 mM potassium phosphate buffer, pH 7.4. The assays were performed as previously described [18]. The concentration of *t*BHP (2.5 mM) used in experiments to test for inactivation was determined to be optimal by measuring 7-EFC *O*-deethylation activity at concentrations of *t*BHP ranging from 0 to 5 mM. The presence of 2.5 mM *t*BHP resulted in maximum formation of the 7-EFC *O*-deethylated product, with no measurable inhibition of enzyme activity.

Reaction stoichiometry. P450 2B6 or P450 2B6.4 (65 pmol) was incubated with reductase at a 1:2 molar ratio of P450:reductase for 45 min at 4ºC. In the experiments in the presence of cytochrome b_5, samples were reconstituted in a 1:2:1 molar ratio of P450:reductase:cytochrome b_5. P450 and reductase were incubated together on ice for 5 min prior to the addition of cytochrome b_5. The sample was brought to a total volume of 1 ml using 100 mM potassium phosphate buffer, pH 7.4 and placed into a cuvette. The sample was allowed to sit at room temperature for 3 min before the addition of NADPH to a final concentration of 200 µM. NADPH consumption was measured continuously, both in the presence and absence of substrate (10 µM 17EE or efavirenz), by monitoring the absorbance at 340 nm over 4 min. The concentration of NADPH was determined using an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ [19]. To measure product formation, 700 µl of the sample was removed and the reaction was quenched by the addition of 300 µl of

acetonitrile. Since the metabolism of both efavirenz and 17EE leads to the formation of multiple products, substrate depletion was used to quantify product formation. For this reason substrate concentrations were used where depletion could be readily determined. The samples were analyzed by HPLC as previously described [10, 20]. The remaining 300 μl of the sample was used to determine the amount of hydrogen peroxide formed using the ferrithiocyanate method [21].

Inactivation of P450 2B6.4 in the presence of cytochrome b_5. The purified P450 was reconstituted with reductase and cytochrome b_5 as described above for 45 minutes at 4 °C. The primary reaction mixtures contained 1 μM P450, 2 μM reductase, 1 μM cytochrome b_5, 110 U catalase and efavirenz (0-50 μM) or 17EE (0-160 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 the addition of NADPH to a final concentration of 1.2 mM. Following the initiation of the reaction by the addition of NADPH, 12 μL aliquots were removed from the primary reaction mixtures at the times indicated and transferred to 990 μL of the secondary reaction mixtures which contained 100 μM 7-EFC, 1 mM NADPH, and 40 μg BSA/mL in 50 mM potassium phosphate buffer, pH 7.4. The secondary reaction mixtures were incubated for 10 min at 30 °C, and then quenched by the addition of 334 μL of acetonitrile. The amount of 7-hydroxy-4-(trifluoromethyl) coumarin formed was measured at room temperature using an excitation wavelength of 410 nm and an emission wavelength of 510 nm on a RF-5310 Spectrofluorophotometer (Shimadzu Scientific Instruments, Inc., Wood Dale, IL).

17EE metabolism. P450 2B6.4 was reconstituted together with reductase and cytochrome b_5 as described above. The primary reaction mixture contained 1 μM P450, 2 μM reductase, 1 μM cytochrome b_5, 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 [20].

Results

P450 2B6 and P450 2B6.4 active site topology. Phenyldiazene forms a σ-bonded complex with the heme iron of the P450, resulting in the formation of a phenyl-iron complex. Oxidation facilitates the migration of the phenyl group to an available pyrrole nitrogen belonging to rings A, B, C or D. The ratio of formation of the resulting N-protoporphyrin IX regioisomers, denoted as N_A, N_B, N_C and N_D, allow for inferences to be made regarding the accessibility of each of the four pyrrole rings [15, 16, 22]. Phenyldiazene was added to P450s 2B6 and 2B6.4, and formation of the phenyl-iron complex was determined spectrally by monitoring the peak formation at 478 nm and a concomitant decrease at 418 nm (data not shown). Following oxidation using ferricyanide, the samples were analyzed by LC-MS and all four *N*-phenylprotoporphyrin IX regioisomers were observed. The elution times of the resulting regioisomers were compared to the standards produced from the incubation of phenyldiazene with myoglobin (data not shown). The major product formed by the wild-type enzyme was N_C, which accounted for 46 ± 2 % of the total regioisomer formation. N_A, N_B and N_D were also detected, and accounted for 10 ± .7 %, 7 ± .5 % and 37 ± 1 % respectively (Figure 4.2). Interestingly, there were some differences observed between the mutant and

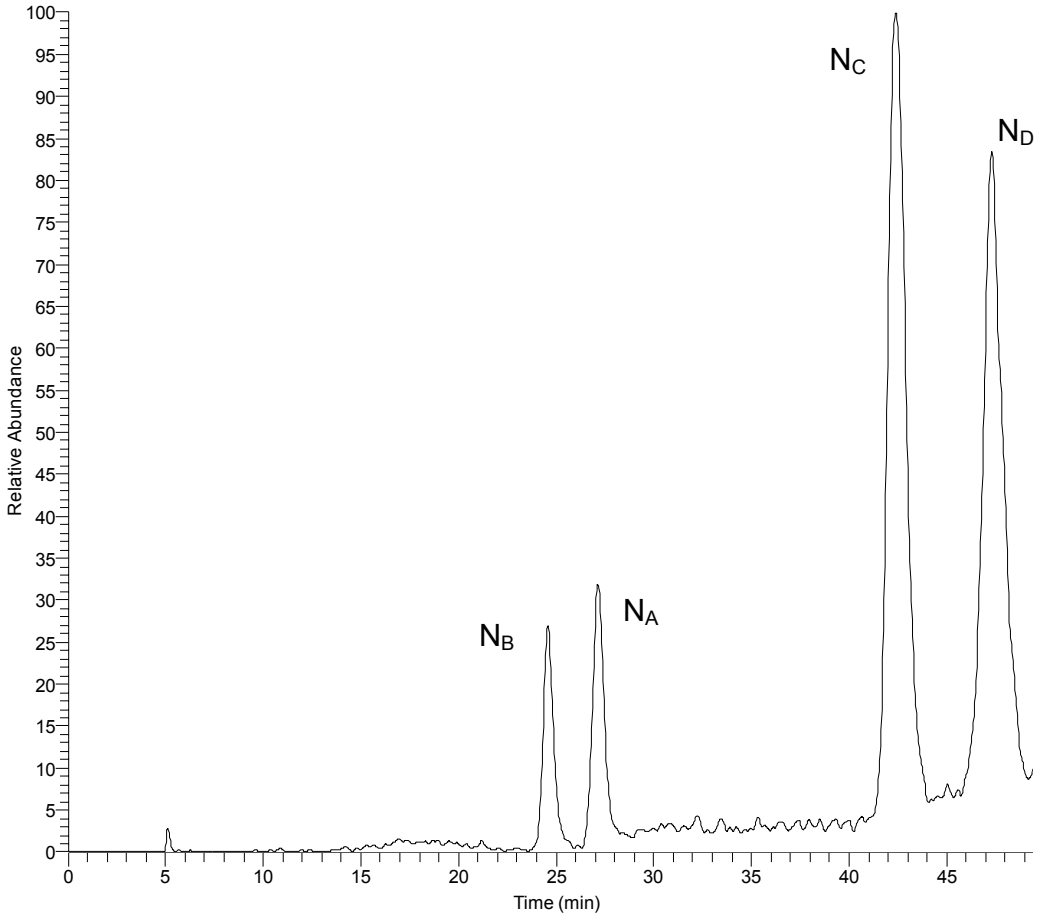


Figure 4.2 – P450 2B6 *N*-phenylprotoporphyrin IX regioisomers formation. P450 2B6 was incubated with phenyldiazene and phenyl-iron complex formation was observed spectrally as described under Experimental Procedures. Oxidation caused migration of the phenyl to the porphyrin nitrogens. The individual peaks represent migration of the phenyl to pyrrole rings A (N_A), B (N_B), C (N_C) or D (N_D). The chromatogram is representative of 3 separate experiments.

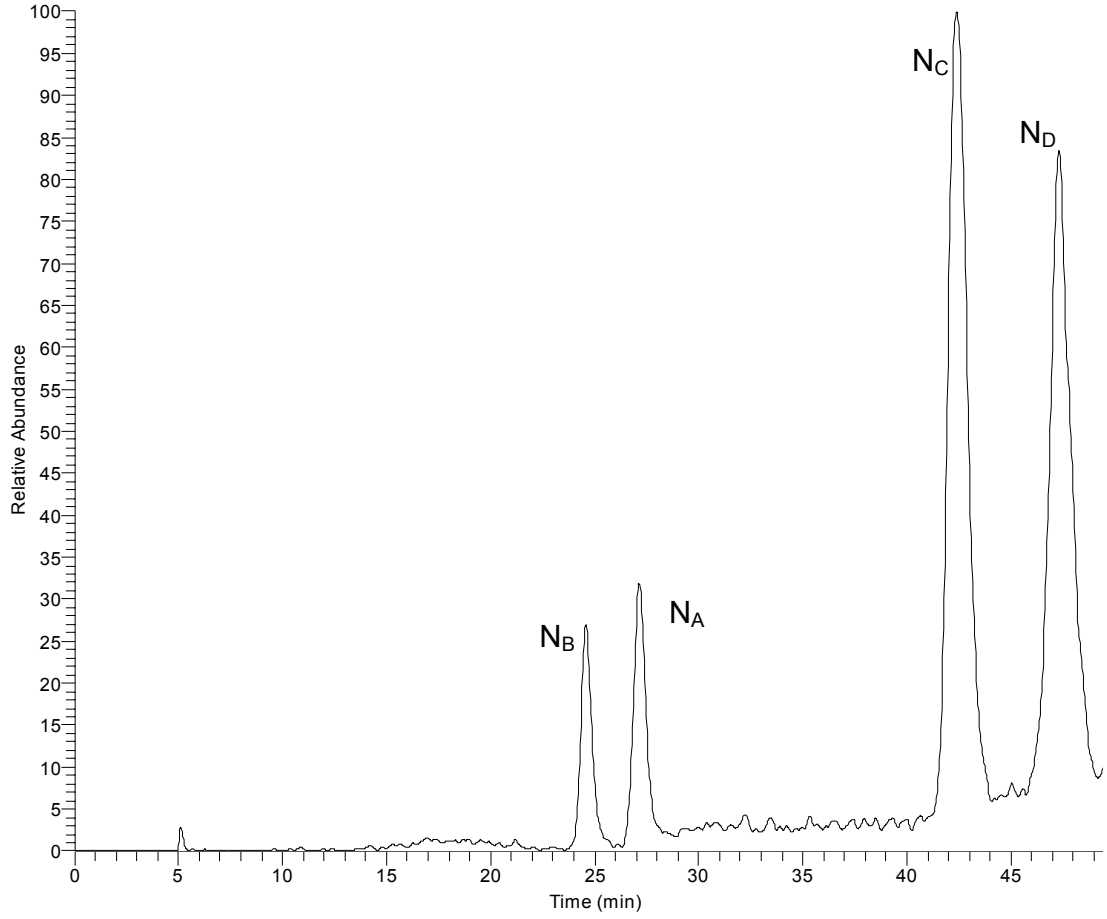


Figure 4.3 – P450 2B6.4 *N*-phenylprotoporphyrin IX regioisomers formation. P450 2B6.4 was incubated with phenyldiazene and phenyl-iron complex formation was observed spectrally as described under Experimental Procedures. Oxidation caused migration of the phenyl to the porphyrin nitrogens. The individual peaks represent migration of the phenyl to pyrrole rings A (N_A), B (N_B), C (N_C) or D (N_D). The chromatogram is representative of 3 separate experiments.

the wild-type enzyme. The reaction of phenyldiazene with P450 2B6.4 also resulted in the formation of all four *N*-phenylprotoporphyrin IX regioisomers (Figure 4.3). However, N_C only represented 37 ± 1 % of the overall formation, while N_A, N_B and N_D constituted 15 ± .5 %, 11 ± 3 % and 38 ± 4 %, respectively. These data suggest that there may be differences in the active site topologies of the two enzymes.

Spectral binding of benzphetamine and efavirenz to P450 2B6 and P450 2B6.4. Spectrophotometric titrations were performed to investigate whether these two substrates of P450 2B6 and P450 2B6.4 showed differences in binding affinity to the two enzymes. The dissociation constants (K_s) were determined from the titration curves. Benzphetamine was chosen because it produces a prominent type I spectral change in P450 2B6. Efavirenz caused a type II spectral change, which is characteristic of a nitrogen atom coordinating to the heme. Both enzymes showed similar affinities for both of the substrates. The K_s values for benzphetamine binding to P450s 2B6 and 2B6.4 were 18 μM and 17 μM, respectively (Table 4.1). The efavirenz spectral dissociation constants were also similar between the two enzymes, with a value of 85 μM for the wild-type enzyme and 123 μM for the variant enzyme (Table 4.1). The K_s values for 17EE binding could not be determined since 17EE does not induce measurable spectral shifts. These data on the binding of benzphetamine and efavirenz suggest that P450 2B6 and P450 2B6.4 are able to bind substrates in a similar manner. Therefore, the differences in catalysis may not be directly related to substrate binding.

Table 4.1 - Spectral binding of benzphetamine and efavirenz to P450 2B6 and P450 2B6.4. P450s were titrated with benzphetamine or efavirenz until the maximum type I (benzphetamine) or type II (efavirenz) spectral shift was observed as described under Experimental Procedures. The k_s values were estimated by plotting the inverse of the absorbance changes (average of 3 separate experiments done in duplicate) associated with either type I or type II binding as a function of the concentration of benzphetamine or efavirenz. The data are representative of 3 separate experiments performed in duplicate.

	Substrate	K_s
2B6	Benzphetamine	18 μM
2B6.4	Benzphetamine	17 μM
2B6	Efavirenz	85 μM
2B6.4	Efavirenz	123 μM

Inactivation of P450s by 17EE and efavirenz using an alternate oxidant. We used *tert*-butyl hydroperoxide as an activated oxygen surrogate to investigate whether the wild-type and/or mutant enzymes could become inactivated by 17EE or efavirenz in a reductase and electron-free system. *t*BHP produces an active iron-oxygen species with the P450 that can support P450 catalytic activity in the absence of molecular oxygen and reducing equivalents from NADPH [23]. Both P450 2B6 and P450 2B6.4 were inactivated by 17EE and efavirenz when *t*BHP was used as an oxidant. P450 2B6 7-EFC *O*-deethylation activity remaining was 52 ± 2 % when the concentration of efavirenz was 50 μM and 32 ±1% when incubated with 80 μM efavirenz (Table 4.2). P450 2B6 was also inactivated by 17EE in the presence of tBHP in a concentration-dependent manner (Table 4.2). Interestingly, tBHP was also able to support the inactivation of P450 2B6.4 by both compounds (Table 4.2). Thus, in a system that is not dependent upon reductase, NADPH and oxygen, the variant enzyme behaved in a manner similar to the wild-type enzyme. These data suggest that the ability of P450 2B6.4 to interact with reductase may be compromised.

Determination of the apparent K_d of reductase binding to P450s 2B6 and 2B6.4. To determine whether the lack of inactivation of the mutant enzyme by 17EE and efavirenz in the reconstituted system was the result of impaired interaction with reductase, complex formation of the P450s with reductase was measured spectrophotometrically. The binding of reductase to P450s results in a low to high spin shift in the heme iron, characterized spectrally by a decrease in the absorbance at 418 nm and an increase in the absorbance at 385 nm [17]. The apparent K_d for the interaction of the reductase with P450 2B6.4 was

Table 4.2 - Inactivation of P450s 2B6 and 2B6.4 using *tert*-butylhydroperoxide to support the reaction. P450s were incubated with tBHP and the inactivators indicated as described in the Experimental Procedures section. Activity remaining was determined using the 7EFC *O*-deethylation assay. The data are presented as percent activity remaining as compared to control sample incubated with tBHP in the absence of efavirenz or 17EE.

		Percent activity remaining	
	Inactivator	50 μM inactivator	80 μM inactivator
2B6	Efavirenz	52 ± 2	32 ± 1
2B6.4	Efavirenz	66 ± 3	48 ± 1
2B6	17EE	33 ± 1	21 ± 2
2B6.4	17EE	81 ± 4	70 ± 2

Table 4.3 - Determination of the apparent K_d of reductase binding to P450s 2B6 and 2B6.4. P450 (1 μM) was added to 100 mM KPi (pH 7.4) to a final volume of 1 ml. Reductase (0 – 8 μM) was titrated and readings were taken following each addition. The apparent dissociation constant was determined by plotting the inverse of the absorbance changes (average of 3 separate experiments done in duplicate) between 390 nm and 420 nm (type I) as a function of the reductase concentration.

	Apparent K_d
2B6	240 nM
2B6.4	918 nM

almost 4-fold greater than the value obtained from experiments with the wild-type enzyme (Table 4.3). This difference does not seem to be marked enough to solely account for the differences in catalytic activity we observed between the two enzymes since we routinely used reductase concentrations in excess of P450. To gain a more complete understanding of the catalytic activities of the two enzymes, the reaction stoichiometry for the metabolism of 17EE and efavirenz by P450 2B6 and P450 2B6.4 was determined.

Reaction stoichiometry. As shown in Table 4.4, NADPH consumption was not increased by the presence of substrate when measured for either of the enzymes. During the metabolism of 17EE and efavirenz by P450 2B6, hydrogen peroxide was formed at a rate similar to the rate of product formation. In contrast, the majority of NADPH consumed by P450 2B6.4 resulted in the formation of hydrogen peroxide (Table 4.4). These data indicate that the metabolic reactions of P450 2B6.4 with 17EE and efavirenz are more uncoupled than the metabolism of these compounds by P450 2B6.

Several studies have demonstrated that cytochrome b_5 can increase the coupling of P450 catalyzed reactions, including those involving P450 2B enzymes [24, 25]. With this in mind, we measured NADPH consumption, hydrogen peroxide formation and product formation in the presence of cytochrome b_5. Reconstitution of P450 2B6.4 with cytochrome b_5 as well as reductase dramatically improved the coupling of both reactions (Table 4.5). Interestingly, cytochrome b_5 only had a minimal effect on the coupling of the wild-type reactions (Table 4.5). Further, P450 2B6.4-mediated 17EE product formation, as measured by substrate depletion, was observed when cytochrome b_5 was present in the reconstitution mixture (Table 4.5). In light of these findings, we went on to test whether

Table 4.4 - Stoichiometry for the metabolism of 17EE and efavirenz by P450s 2B6 and 2B6.4. P450s were reconstituted with reductase as described under Experimental Procedures. NADPH oxidation was measured spectrally by monitoring absorbance at 340 nm. Portions of the sample were then used to determine hydrogen peroxide and product formation. Product formation was measured by substrate depletion. All of the values are presented as nmol/nmol P450/min.

P450	Substrate	NADPH oxidation	H_2O_2 formed	Product formed
2B6	No substrate	15.7±.5	2.2±.1	0
2B6	Efavirenz	16.2±.9	6.3±.1	6.8±.2
2B6	17EE	15.1±.6	7.8±.3	7.3±.4
2B6.4	No substrate	14.7±.6	7.2±.3	0
2B6.4	Efavirenz	16.3±.5	10.1±.4	3.8±.1
2B6.4	17EE	16.9±.4	12.6±.6	.78±.2

Table 4.5 - Improvement of P450 2B6.4 reaction coupling upon reconstitution with cytochrome b_5. P450s were reconstituted with reductase and cytochrome b_5. The assay was performed as described under Experimental Procedures. NADPH oxidation was measured for 4 min then the sample was used to determine both hydrogen peroxide formation and product formation. All values are presented as nmol/nmol P450/min.

P450	Substrate	NADPH oxidation	H_2O_2 formed	Product formed
2B6	No substrate	14.3±.4	1.8±.2	0
2B6	Efavirenz	15.8±.7	5.6±.3	9.6±.6
2B6	17EE	15.0±.7	6.5±.1	8.2±.4
2B6.4	No substrate	13.1±.2	5.1±.1	0
2B6.4	Efavirenz	15.4±.5	5.2±.2	7.7±.2
2B6.4	17EE	15.0±.3	6.9±.1	5.3±.3

inactivation of P450 2B6.4 by efavirenz and 17EE could occur in the presence of cytochrome b_5.

Inactivation of P450 2B6.4 by 17EE and efavirenz in the presence of cytochrome b_5. We previously reported that P450 2B6.4 is not inactivated by 17EE and efavirenz when reconstituted with reductase alone [10, 11]. Inactivation of the mutant enzyme by 17EE and efavirenz in the presence of cytochrome b_5 was measured using the 7-EFC *O*-deethylation assay. P450 2B6.4 was inactivated by efavirenz (Figure 4.4) and 17EE (Figure 4.5) in a time- and concentration-dependent manner and the inactivation exhibited an absolute requirement for NADPH. The activity loss followed pseudo first order kinetics. Linear regression analysis was performed and the kinetic constants for the efavirenz- mediated inactivation of the mutant enzyme were determined from the inset of figures 4 and 5. The K_I values for inactivation of P450 2B6.4 by efavirenz and 17EE were 30 μM and 113 μM respectively.

Metabolism of 17EE by P450 2B6.4 requires cytochrome b_5. Studies were conducted to determine which 17EE metabolites were formed during metabolism by P450 2B6.4 in the presence of cytochrome b_5. 17EE was incubated with P450 2B6.4 that was reconstituted with reductase and cytochrome b_5 in the presence or absence of NADPH. The metabolites were analyzed using reverse phase HPLC as shown in Figure 4.6. P450 2B6.4 metabolized 17EE to give a number of major metabolites denoted as C, D and E as well as a minor metabolites, such as A and B. Metabolite B was not present in the profiles of the wild-type enzyme in our previous studies [11, 20].

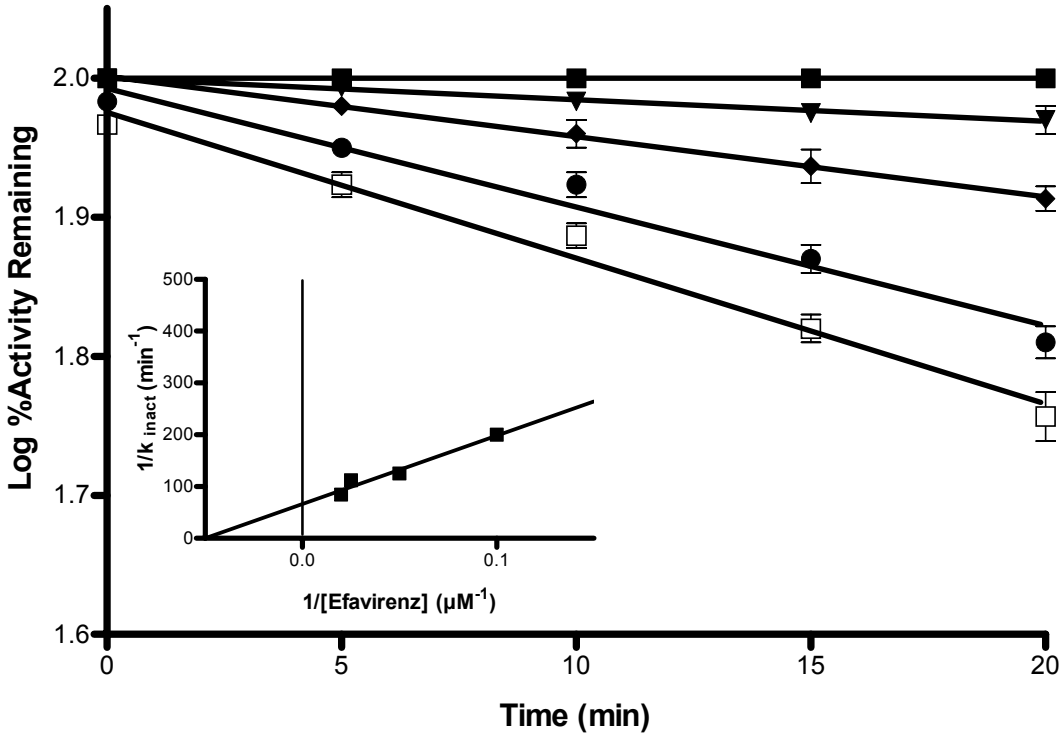


Figure 4.4 – Inactivation of P450 2B6.4 by efavirenz in the presence of cytochrome b_5. The time- and concentration-dependent inactivation of P450 2B6.4 by efavirenz in the presence of cytochrome b_5 was measured by determining the 7-EFC *O*-deethylation activity. After initiation of reaction by the addition of NADPH, aliquots were removed from the primary reaction mixture at 0, 5, 10, 16 and 21 minutes. The concentration of efavirenz were (■) 0 μM, (▼) 10 μM, (◆) 20 μM, (●) 40 μM, and (□) 50 μM. The data show the means and standard deviations from 4 separate experiments done in duplicate. In some cases the standard deviations were less than the size of the symbols. The inset shows the double reciprocal plot of the rates of inactivation as a function of the efavirenz concentrations.

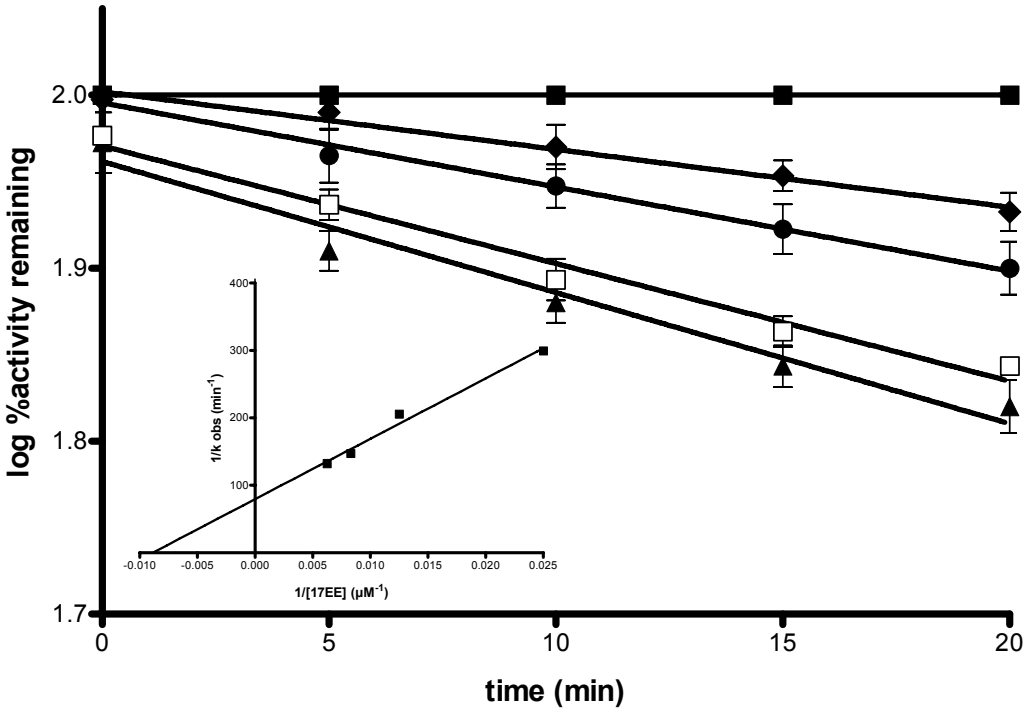


Figure 4.5 – Inactivation of P450 2B6.4 by 17EE in the presence of cytochrome b_5. The time- and concentration-dependent inactivation of P450 2B6.4 by 17EE was measured by determining the 7-EFC *O*-deethylation activity. After initiation of reaction by the addition of NADPH, aliquots were removed from the primary reaction mixture at 0, 5, 10, 16 and 21 minutes. The concentration of efavirenz were (■) 0 μM, (◆) 40 μM, (●) 80 μM, (□) 120 μM and (▲) 160 μM. The data show the means and standard deviations from 4 separate experiments done in duplicate. In some cases the standard deviations were less than the size of the symbols. The inset shows the double reciprocal plot of the rates of inactivation as a function of the 17EE concentrations.

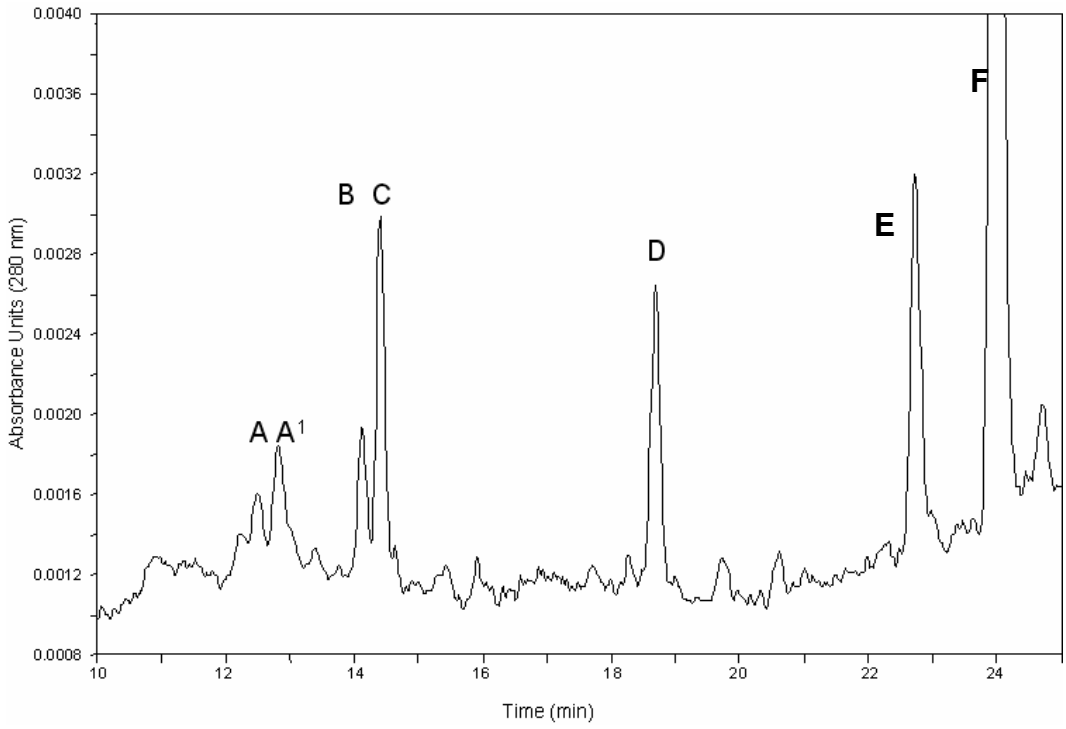


Figure 4.6 – Metabolism of 17EE by P450 2B6.4 in the presence of cytochrome b_5. Metabolites A, A1, and C are mono-hydroxylated metabolites, though the exact identities have not yet been determined. Metabolite D corresponds to 2-hydroxy-17EE, metabolite E corresponds to estrone and F corresponds to the substrate, 17EE. We previously showed that in the absence of cytochrome b_5 P450 2B6.4 does not readily metabolize 17EE [11].

Discussion

P450 2B6 is involved in the metabolism of a growing number of substrates including drugs, pesticides and procarcinogens [26, 27] and is expressed in a number of organs including the liver, kidney, skin, heart and brain [28-31]. Clinical studies have demonstrated a large degree of inter-individual variability in the metabolism of P450 2B6 specific substrates. Genetic polymorphisms appear to play a major role in this variability and a number of single nucleotide polymorphisms have been found in the *P450 2B6* gene [32]. Several studies have shown that these polymorphisms can have clinical consequences. The majority of studies regarding P450 polymorphisms have focused on correlating patient genotypes to a particular phenotype. However, there are very few *in vitro* studies investigating the mechanisms underlying effects of these mutations. The goal of our studies has been to understand how a particular naturally occurring genetic mutation can affect the structure and function of the P450.

We have previously demonstrated that the mutation of lysine 262 of the P450 2B6 protein to arginine can result in marked changes in catalytic activity [10, 11]. Our findings that P450 2B6.4 was not inactivated by efavirenz and 17EE, known inactivators of the wild-type enzyme, were the most intriguing [11]. This mutant, which corresponds to the *P450 2B6*4* variant allele of the *P450 2B6* gene, is of particular interest because it is present in at least 3 variant alleles and has a high mutation frequency. This mutation has been found across all ethnic groups genotyped for P450 2B6 polymorphisms and has been shown to have nearly a 50% mutation frequency in Ghanians and a frequency of nearly 30% in African-American, Caucasian, Japanese and Taiwanese populations [32].

Previous studies using a number of P450s have demonstrated that phenyldiazene is a useful tool for gaining information about the topology of the P450 active site [22, 33, 34]. Phenyldiazene forms a σ-bonded complex with the heme iron of the P450, resulting in the formation of a phenyl-iron complex. Oxidation results in the migration of the phenyl group to an available pyrrole nitrogen belonging to rings A, B, C or D. The ratios of formation of the resulting N-protoporphyrin IX regioisomers, denoted as N_A, N_B, N_C and N_D, allow for inferences to be made regarding the accessibility of, or open space above, each of the four pyrrole rings. In the case of bacterial P450 isoforms, the information gained using phenyldiazene has been consistent with X-ray crystallography data [35]. In the present study, we used phenyldiazene to investigate whether the K262R mutation leads to significant changes in active site topology. Although the overall profiles for the formation of the regioisomers were similar between the two enzymes, there was a difference in the migration of the phenyl group to the nitrogen of the pyrrole ring C. In the experiments with the wild-type enzyme, the N_C regioisomer accounted for 46% of the total formation. However, in the case of the mutant, N_C formation only accounted for 37% of the total. Though this difference is small, it suggests that the active site topologies of the two enzymes differ to some extent.

To further investigate whether there were differences in the active sites of the two enzymes, spectral binding studies were performed. Benzphetamine and efavirenz were chosen because these two substrates result in pronounced spectral changes. Other substrates were tested, such as 17EE, and spectral changes were not detected when these substrates were added to P450 2B6. The K_s values determined by measuring spectral the changes resulting from titration of P450s 2B6 and 2B6.4 with benzphetamine and

efavirenz were similar between the two enzymes, suggesting that the K262R mutation does not significantly affect binding. The ΔA values were similar in all cases.

According to the P450 2B4 crystal structure, the only structure of a P450 2B family member currently available, residue 262 is in the G/H loop [36, 37]. Although this region is not in close proximity to the active site, it could potentially play a role in the interaction with reductase. To test whether the K262R mutation alters the ability of the enzyme to interact with reductase, we performed experiments to measure the ability of each P450 to associate with reductase. Spectral studies to investigate the association of the P450s with reductase suggested that the mutant may have a somewhat lower affinity for reductase, however, the difference between the mutant and wild-type enzymes in reductase binding did not seem profound enough to account for the marked differences in catalytic activity. In addition, we used *t*BHP as an oxidant to determine if the mutant enzyme would be catalytically similar to the wild-type in the absence of the requirement to interact with reductase. Interestingly, in the presence of alternate oxidants the mutant was readily inactivated by 17EE and efavirenz suggesting that electron transfer to P450 2B6.4 may be compromised during these reactions when using the reconstituted system.

In order to gain a comprehensive understanding of a particular reaction it is necessary to determine the stoichiometry of the reaction. Investigation of the stoichiometry for metabolism of 17EE and efavirenz by the two enzymes indicated that the mutant appeared to be more uncoupled. Coupling can be defined as the fraction of electrons used towards the formation of monooxygenated metabolites. Therefore, uncoupling refers to a decrease in monoxygenated metabolite formation and a concomitant increase in non-productive metabolite product formation, namely the

autooxidation of the oxyferrous P450 to superoxide which is converted to hydrogen peroxide, as well as the release of hydrogen peroxide from the peroxo-iron intermediate. Reaction stoichiometry relates NADPH and oxygen consumption with hydrogen peroxide and metabolite formation. The mutant produced more hydrogen peroxide than the wild-type both in the presence and absence of substrate. In an attempt to improve the coupling of the P450 2B6.4 reactions, cytochrome b_5 was added to the reconstitution mixture. The presence of cytochrome b_5 improved the coupling of the mutant enzyme, and the hydrogen peroxide levels were closer to those observed for the reactions catalyzed by the wild-type enzyme. Further, the addition of cytochrome b_5 to the reconstitution mixture resulted in the inactivation of P450 2B6.4 by efavirenz and 17EE.

Cytochrome b_5 is a 17-kDa heme-containing protein that is located in the membrane of the endoplasmic reticulum [38], and it functions as an electron donor in a number of reactions, including cholesterol biosynthesis and certain P450-catalyzed reactions [39]. Depending upon the P450 isoform and the substrate being investigated, cytochrome b_5 has been shown to increase, inhibit, or have no effect on P450 activity [40]. Studies on the metabolism of methoxyflurane by P450 2B4, the rabbit isoform of P450 2B6, in the reconstituted system revealed that cytochrome b_5 was absolutely required [41, 42]. Interestingly, cytochrome b_5 had no effect on P450 2B4-mediated metabolism of benzphetamine [43]. There are two primary hypotheses to explain the stimulatory effect of cytochrome b_5 on some P450-mediated reactions. The first is that reduced cytochrome b_5 donates the second electron in the catalytic cycle to the P450 [44-46]. This is supported by studies showing electron transfer from cytochrome b_5 to P450 as well as the observation that "uncoupling" is decreased in the presence of the b_5 protein

[25]. In the latter instance, the presence of cytochrome b_5 results in a decrease in hydrogen peroxide formation and a subsequent increase in product formation, possibly by stabilizing the oxyferrous P450 complex, leading to a decrease in release of superoxide [25]. The second hypothesis is that cytochrome b_5 physically interacts with the P450 causing a conformational change that facilitates interaction with the substrate or reductase. This notion is supported by studies where the apo-cytochrome b_5, which cannot be reduced and donate the second electron, was able to stimulate P450-catalyzed reactions [47]. However, the exact role of cytochrome b_5 in P450-dependent hydroxylation and oxidations is unclear. In our studies, cytochrome b_5 improved the coupling of the mutant enzyme, facilitating metabolism of 17EE by the mutant and inactivation by both compounds. This suggests that for these particular reactions the former hypothesis may be true. We performed studies using both apo-cytochrome b_5 and Mn-cytochrome b_5, which cannot act as an electron donor, and did not see any improvement in the catalytic activity of P450 2B6.4 (data not shown). Studies performed by Zhang et al., under single turnover conditions demonstrate that cytochrome b_5 and reductase donate the second electron to the P450 (in this case P450 2B4) at a similar rate, however catalysis occurs faster in the presence of cytochrome b_5 [48]. These authors hypothesize that the conformation of the oxyferrous P450 may be different in the presence of cytochrome b_5 and reductase. This proposal is a potential explanation for the findings reported in the present study. However, it is also possible that the oxyferrous P450 2B6.4 and/or peroxo-iron intermediate in the presence of 17EE and efavirenz are less stable than the same complexes formed by the wild-type enzyme. Further studies are necessary to elucidate the precise mechanism by which cytochrome b_5 increases the

catalytic activity of P450 2B6.4. These studies could potentially include measuring the formation and stability of the oxyferrous complex of P450 2B6.4 versus P450 2B6.

In summary, we have investigated the effects of the K262R mutation on active site topology, substrate binding, interaction with reductase and reaction stoichiometry. The inactivation of P450 2B6.4 by efavirenz and 17EE showed an absolute requirement for cytochrome b_5. In the presence of cytochrome b_5, the reactions catalyzed by the mutant enzyme exhibited improved coupling. These studies provide evidence that the differences in the catalytic properties of P450 2B6 and P450 2B6.4 are related to uncoupling of P450 2B6.4 mediated metabolism.

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CHAPTER 5

CROSS-LINKING OF CYTOCHROME P450 2B6 TO NADPH-CYTOCHROME P450 REDUCTASE: IDENTIFICATION OF A POTENTIAL SITE OF INTERACTION

Introduction

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. P450s are involved in the oxidative, peroxidative, and reductive metabolism of a variety of structurally diverse compounds. All P450s share a common catalytic mechanism which involves the two-electron reduction of molecular oxygen resulting in the formation of a reactive oxygen intermediate and water [1]. Electrons are transferred from NADPH to the P450 via NADPH-P450-reductase (reductase), which leads to the reductive activation of molecular oxygen followed by the insertion of one oxygen atom into the substrate [1].

Human P450 2B6 has received increased attention in recent years in part due to its expression in a number of extrahepatic tissues, as well as its role in the oxidative metabolism of a growing list of xenobiotics. However, as the crystal structure of P450 2B6 is yet to be solved, structural information regarding this enzyme is lacking. Many of the inferences that have been made relating to P450 2B6 structure and function have

been drawn from knowledge of the rabbit isoform P450 2B4. Crystal structures of both the open and closed forms of P450 2B4 are available and these data have helped to confirm mutagenesis data that identified residues of the P450 reported to be responsible for the interaction with reductase [2, 3]. The interaction between P450 and reductase is electrostatic, involving basic residues on the P450 and acidic residues on the surface of reductase [4, 5]. Mutation of 25 amino acids on the surface of P450 2B4 to alanines by Bridges et al., revealed that residues in the C helix, as well as R422 near the β bulge and R443 in the L helix play a role in the interaction of the P450 with reductase [6]. Unfortunately, similar studies have not been performed for P450 2B6. Therefore, we aimed to identify the P450 2B6 residues that may be involved in the interaction with reductase. To approach this, we used the water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as a crosslinker. EDC has been shown to be a useful tool in the identification of sites of interaction between proteins in a number of protein-protein complexes, including reductase-cytochrome c and P450 2E1-b_5 [7, 8]. However, a previous attempt to cross-link P450 2B4 to reductase using EDC was reported to be unsuccessful [9]. In the present study, we were able to cross-link P450 2B6 to reductase and to structurally characterize this complex. EDC covalently links lysine residues to either aspartic or glutamic acid residues, making this an appropriate cross-linker to use in these studies since the interactions between the P450 and reductase are believed to be electrostatic.

In order to identify P450 2B6-reductase cross-linked peptides we used ^{18}O-water to isotopically label cross-linked peptides. Proteolysis by an enzyme such as trypsin, results in the ability of the tryptic peptides to specifically exchange oxygen atoms from

water with the two oxygen atoms of the carboxyl terminus [10]. By comparing the masses of proteolytic peptides after exchange with either ^{18}O-water or ^{16}O-water, cross-linked peptides can be identified based on the magnitude of the mass shift due to the incorporation of two ^{18}O atoms. The incorporation of two ^{18}O atoms into the carboxyl terminus of a peptide will result in a 4 Da mass shift. Therefore, if two peptides are cross-linked, the observed mass shift would be 8 Da, since the complex should consist of two linked peptides, each with a C-terminus capable of incorporating two of the ^{18}O atoms. Also, incomplete incorporation can occur where cross-linked peptides may only incorporate three ^{18}O atoms. In this case a 6 Da mass shift would be observed. This method has been used previously to identify cross-linked peptides [7, 11, 12]. The studies reported here detail the use of this method to identify a P450 2B6-reductase cross-linked peptide.

Experimental Procedures

Materials. EDC was purchased from Pierce (Rockford, IL). Efavirenz was purchased from Toronto Research Chemicals (Toronto, Canada).

Expression and purification of P450 2B6 and reductase. P450 2B6 and NADPH-P450 reductase were expressed in *E. coli* Topp 3 cells and purified according to published protocols [13-15].

Cross-linking reactions. P450 2B6, reductase and DLPC were reconstituted by incubating all three components at 4ºC for 45 minutes using a molar ratio of 1:1:600,

respectively. The components were then allowed to sit at room temperature for 10 minutes. EDC was added to a final concentration of 10 mM from a 100 mM stock made fresh just prior to the experiment. After 2 hours, the reaction was quenched by dialysis against 100 mM ammonium bicarbonate buffer, pH 8.5 at 4ºC.

Activity assay. In order to isolate the cross-linked complex, the free P450 as well as free reductase had to be removed from the sample. To do this, we used affinity purification techniques. The P450 2B6 used in these studies was his-tagged; therefore, we applied the cross-linked sample to a nickel column. Only P450 2B6 or the P450 2B6-crosslinked complex should bind to the column. Therefore, once the column was eluted and the contents collected, free reductase was absent from the sample. In order to remove the free P450 2B6, the sample was then bound to an ADP-sepharose column which binds NADPH binding domains. Following the use of both of these columns, only the cross-linked complex was present. The efavirenz metabolism assay was then carried out according to Bumpus et al., 2006 [16].

Proteolytic digestions. The in-gel digestions as well as analyses using MALDI TOF/TOF were performed by the Michigan Proteome Consortium (Ann Arbor, Michigan) according to the protocol posted on their website (www.proteomeconsortium.org). For the in-solution digests and isotopic labeleling, the cross-linked sample was dialyzed against 100 mM ammonium bicarbonate, pH 8.5, twice for four hours. Trypsin was then added such that the amount of protein from the EDC reaction was in 50-fold molar excess. The digestion was allowed to proceed for 24 hours

at 37ºC. Subsequently, the samples were divided into two portions and dried using a SpeedVac. The resulting peptides were then reconstituted using either ^{16}O-water or ^{18}O-water and analyzed using a LTQ mass spectrometer (ThermoElectron Finnigan) in the University of Michigan Biomedical Mass Spectrometry Facility.

Data analysis. Data were analyzed using the Pro-CrossLink Version 1 suite of software tools developed by Gao et al., at the University of Washington (Seattle, Washington) [17].

Results

P450 2B6 and reductase complex formation. Cross-linking of P450 2B6 to reductase was analyzed using SDS-PAGE. Upon incubation with EDC, reductase and P450 form a 1:1 complex as illustrated by a band at approximately 130 kDa in the sample containing both enzymes (Figure 5.1, circled). This complex was absent in the control samples consisting of P450 or reductase (Figure 5.1) alone incubated with EDC. Interestingly, P450 2B6-P450 2B6 complexes were also observed as well as reductase-reductase complexes (Figure 5.1). However, in the absence of EDC none of the abovementioned complexes were formed (data not shown). The presence of both P450 2B6 and reductase in the 130 kDa complex was confirmed unequivocally following digestion of the excised band. Polypeptide chains from both P450 2B6 and reductase were identified in the digest by searching the MASCOT database (Matrix Science, London, UK).

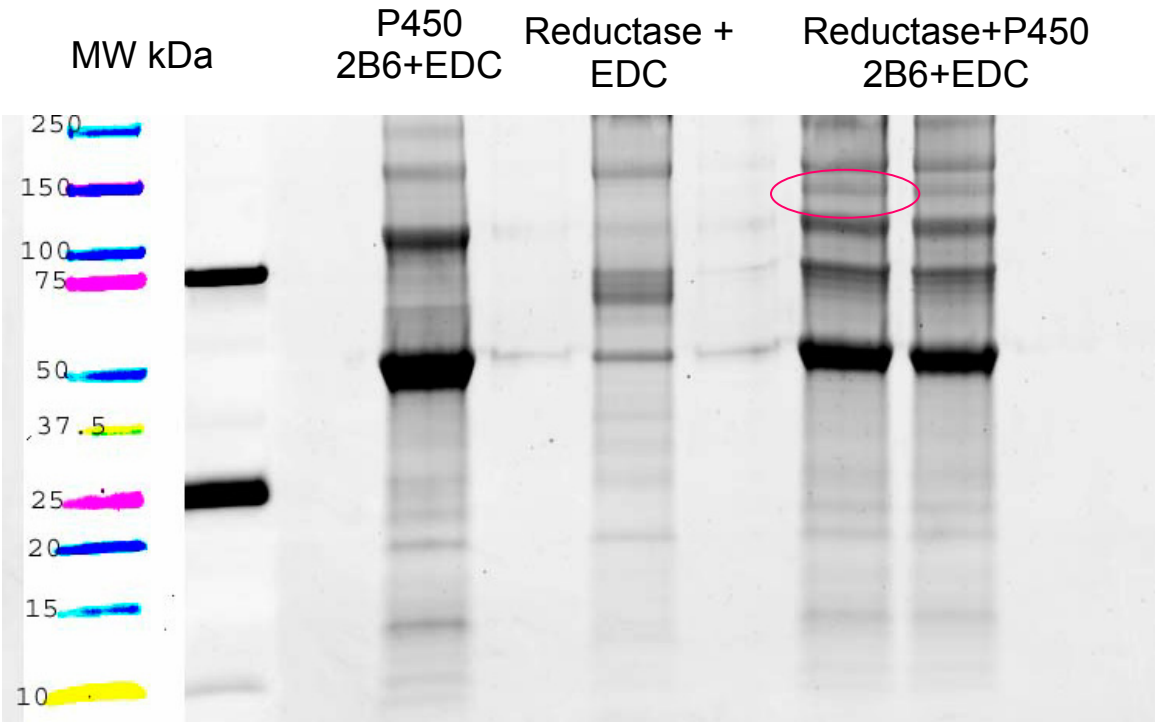

Figure 5.1 – SDS-PAGE analysis of cross-linked complexes. P450 2B6 (54 kDa), reductase (77 kDa) and P450 2B6 reconstituted with reductase in the presence of lipid (this sample was divided in half) were all incubated separately with EDC as described under Experimental Procedures. The gel was stained with Sypro Ruby. The circled band represents the cross-linked P450 2B6-reductase complex at approximately 130 kDa. This band was excised, digested and analyzed by mass spectrometry, which confirmed the presence of both P450 2B6 and reductase based upon a database search using MASCOT.

Determination of the cross-linked P450 2B6-reductase complex as functionally active. In order to determine whether the cross-linked P450 2B6-reductase complex observed exists in a functional conformation, the complex was separated from the non-cross-linked proteins by column chromatography and activity assays were performed. SDS-PAGE followed by coomassie staining showed that the cross-linked complex was the only protein remaining in the sample following the separation procedures (data not shown). As shown in Table 5.1, the isolated cross-linked complex was able to metabolize the P450 2B6 substrate efavirenz. The data obtained were normalized to total protein in the sample using a BCA protein assay (Pierce). However, the metabolite formation by the isolated cross-linked complex was approximately 30% less than that of reconstituted P450 2B6 and reductase that were not incubated with EDC. The 8-hydroxyefavirenz formed by the non-crosslinked sample was approximately 3.7 pmol 8-hydroxyefavirenz/mg protein, while product formation by the cross-linked sample was approximately 2.6 pmol 8-hydroxyefavirenz/mg protein.

Mass spectrometric analysis of peptides. The identification of the cross-linked peptides from the P450 2B6-reductase was approached in two ways. First, peptides resulting from the in-gel digests of P450 2B6 alone, reductase alone and P450 2B6-reductase all incubated with EDC were analyzed using a MALDI TOF/TOF mass spectrometer. Since inter- and intra-molecular cross-links between P450 and reductase are possible, the peptide masses found in the P450 2B6 and reductase alone samples were subtracted from the peptide masses present in the P450 2B6-reductase cross-linked

Table 5.1 – Determination of cross-linked P450 2B6 and reductase activity. P450 2B6 and reductase were reconstituted in the presence of lipid as described under Experimental Procedures. 8-hydroxyefavirenz formation was measured and normalized to total protein as determined by performing a BCA protein assay.

	pmol 8-hydroxyefavirenz/mg protein
2B6 + reductase - EDC	3.7 ± 0.5
2B6 + reductase +EDC	2.6 ± 0.3

sample. The tandem mass spectra (MS/MS) of the peptides present in the P450 2B6-reductase sample and not in the controls, were input into the Pro-CrossLink software which subsequently identified a peptide with a mass of 3809.4 Da as a cross-linked peptide (data not shown). To confirm this identification, we took a second approach where we isotopically labeled the peptides with ^{18}O water following proteolytic digestion, as described under Experimental Procedures. The samples were then analyzed using an LTQ equipped with a photodiode array detector. Figures 5.2 and Figure 5.3 show the respresentative UV spectra (280 nm) and total ion chromatograms, respectively, of peptides reconstituted in either ^{16}O-water or ^{18}O-water. Under electrospray ionization conditions, peptide ions can exist in multiple charge states. In this case, the expected mass shift of the precursor ion is dependent upon the charge state of the ion. For instance, a singly charged ion upon the incorporation of four ^{18}O atoms would have a mass shift of 8 Da. However, a mass shift of 4 Da would be observed for a doubly charged ion. Through the use of the Pro-CrossLink software, as well as manually searching through the mass spectra, a cross-linked peptide candidate was identified with its quintuply charged ion at *m/z* 762.6 in the ^{16}O-water samples and at *m/z* 763.8 in the ^{18}O-water samples; an ion at *m/z* 763.8 was not observed in the ^{16}O-water sample. Figure 5.4 shows the co-elution of these ions in the total ion chromatograms. This mass shift of 1.2 for the quintuply charged ion indicates the incorporation of three ^{18}O atoms at the C-terminus of this peptide. Since incomplete incorporation and/or back exchange can occur, peptides with a mass shift commensurate with the incorporation of three or four ^{18}O atoms were considered cross-linked peptide candidates. The measured peptide mass matched the mass of the peptide previously identified from the in-gel digest using MALDI TOF/TOF.

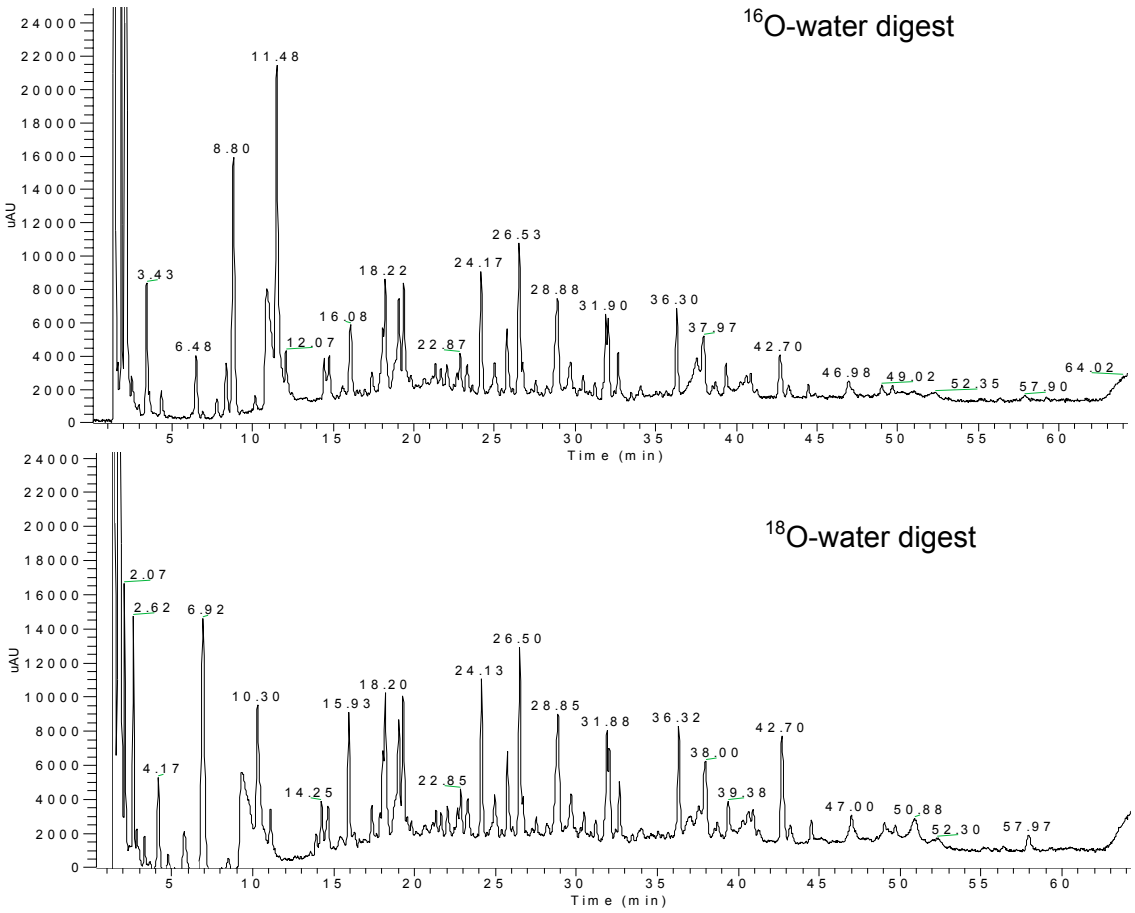

Figure 5.2 – UV spectra of cross-linked P450 2B6-reductase peptides reconstituted in ^{16}O-water or ^{18}O-water. P450 2B6 and reductase were reconstituted, then incubated with EDC as described under Experimental Procedures. In-solution digests were then performed and the samples were dried down using a SpeedVac. The dried peptides were there reconstituted in either ^{16}O-water or ^{18}O-water. The figure shows the peptides monitored at 280 nm.

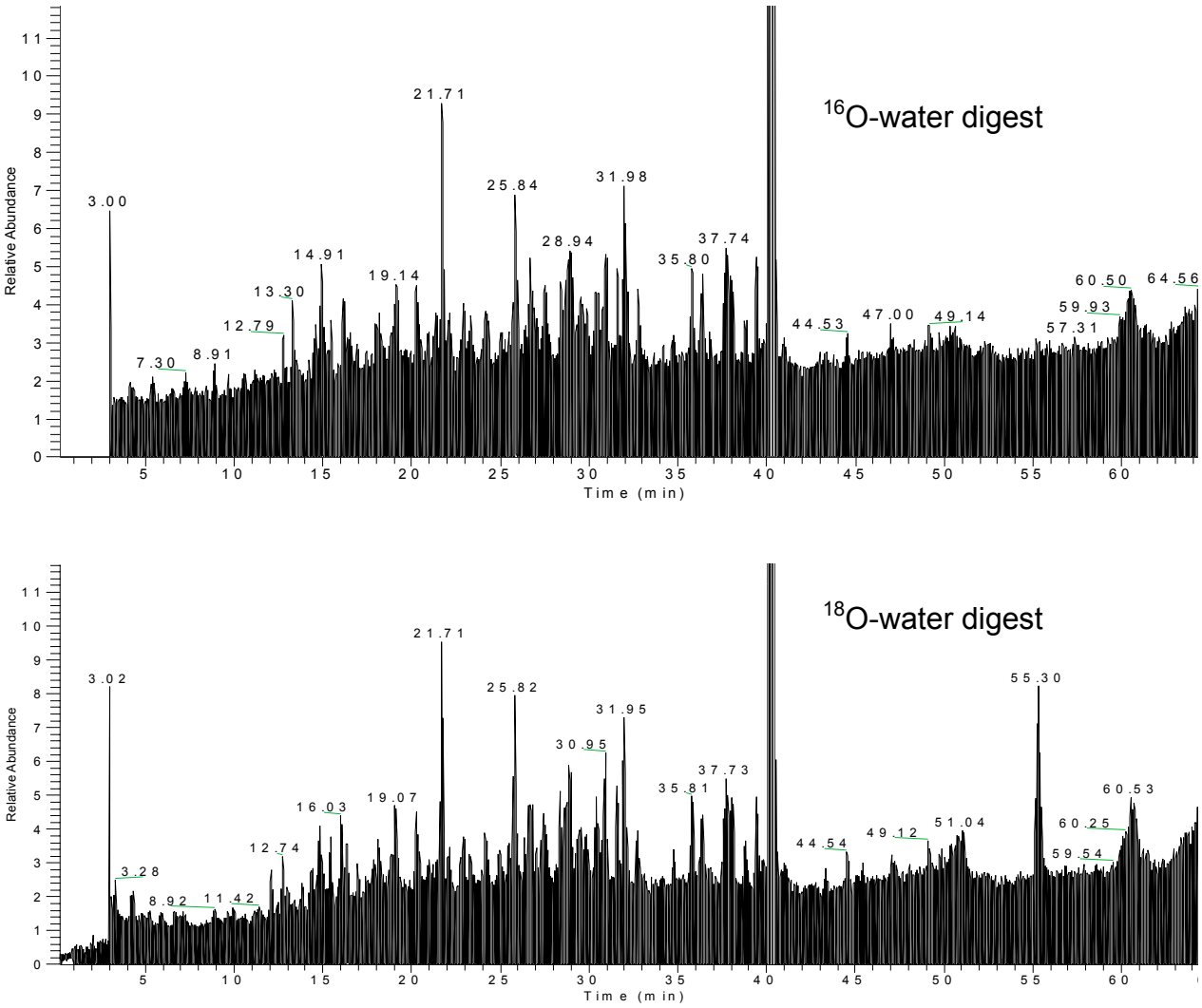

Figure 5.3 – Total ion chromatograms of digested cross-linked P450 and reductase. P450 2B6 and reductase were cross-linked as described under Experimental Procedures. In-solution digests were then performed and the samples were dried down using a SpeedVac. The dried peptides were there reconstituted in either ^{16}O-water or ^{18}O-water. The figure shows co-elution of the ions present in the sample analyzed on a LTQ mass spectrometer.

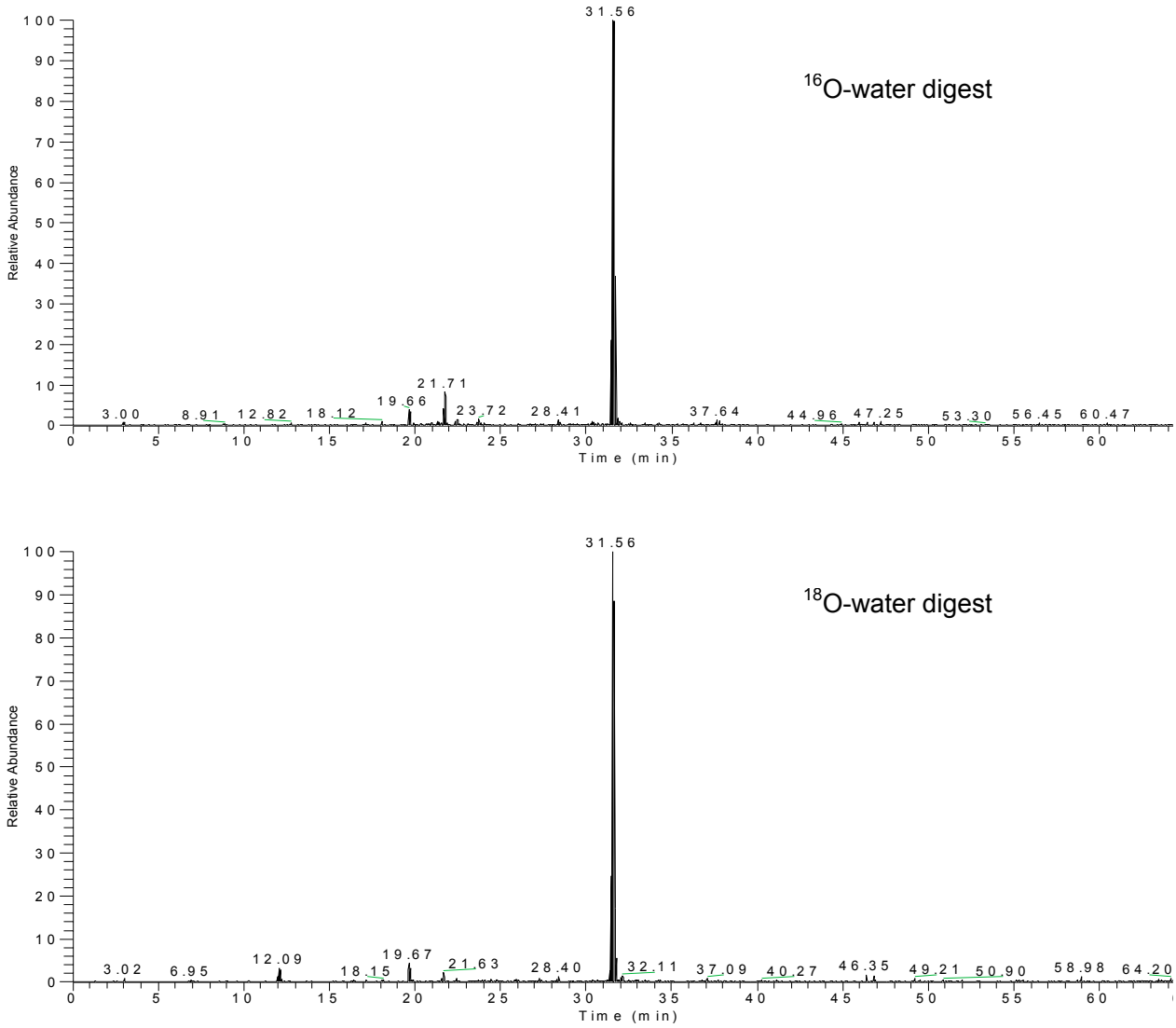

Figure 5.4 – Co-elution of the quintuply charged ion at *m/z* 762.6 and *m/z* 763.8. Mass spectra of cross-linked peptides were analyzed manually and using the Pro-CrossLink software to determine mass shifts between the ^{16}O-water and ^{18}O-water reconstituted samples. The mass shift of 1.2 Da for the quintuply charged ion indicates the incorporation of three ^{18}O atoms at the C-terminus of this peptide. The ion at *m/z* 763.8 was not present in the ^{18}O-water sample.

Using the Pro-CrossLink software, the cross-linked peptide (P450 2B6: DFGMGKR)-(reductase: RHILAILQDCPSLRPPIDHLCELLPR) was identified based upon the mass and charge state of the monoisotopic peak at *m/z* 762.6, as well as the MS/MS spectrum of this ion. De novo sequencing of the peptide using the MS/MS spectrum suggests that this may be the identity of the peptide (Figure 5.5).

Discussion

Structural information pertaining to P450 2B6 is seriously lacking, particularly with regard to interactions with redox partners. To address this, we have cross-linked P450 2B6 to reductase and have structurally characterized the resulting complex. Cross-linking coupled with mass spectrometry is a powerful tool for elucidating sites of interaction between two proteins. The use of isotopic labeling during this process assists in unambiguous identification of cross-linked peptides. To facilitate the identification of as many peptide candidates as possible, conditions for ^{18}O incorporation were optimized. During preliminary experiments it was determined that in-solution proteolysis resulted in more complete incorporation of ^{18}O atoms compared to in-gel digestion. Further, instead of using the isotopic label during proteolysis, we performed the proteolysis in ^{16}O water, dried the sample down using a SpeedVac and reconstituted the sample in ^{18}O water [10]. This resulted in increased incorporation of ^{18}O atoms, and potentially less back exchange.

We have used two different approaches to identify the potential sites of interaction between P450 2B6 and reductase. Since EDC covalently links basic and acidic residues that come into very close proximity, it is an appropriate cross-linker to use

P450 2B6: D**FGM**GK**R** Reductase: RHI**L**AI**L**QD**C**P**S**LR**P**PID**H**LC**E**LLPR

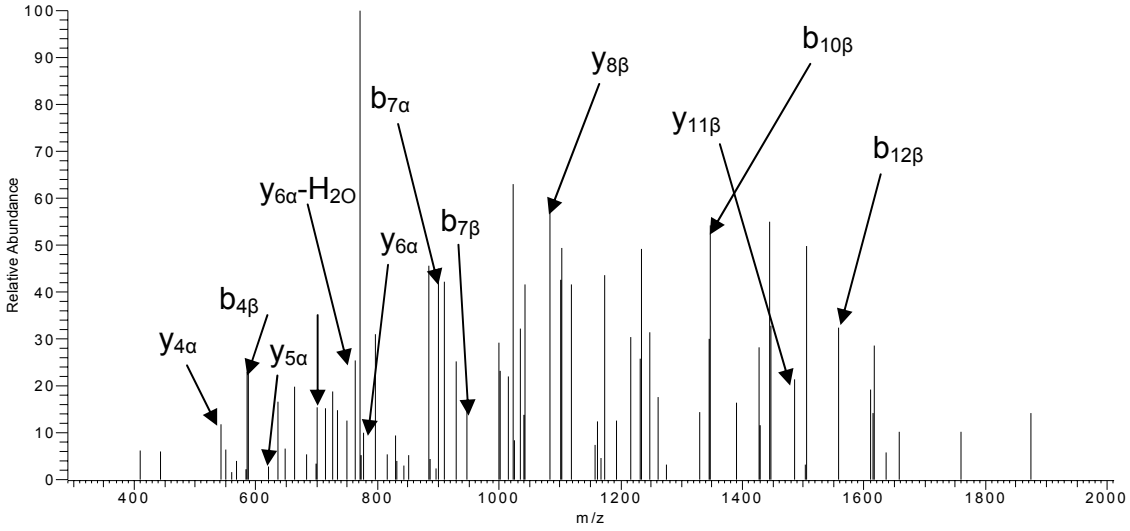

Figure 5.5 – MS/MS spectrum of the precursor ion $[M+5]^{5+}$ = 762.0. The cross-linked candidate ion was fragmented for MS/MS using an LTQ mass spectrometer. De novo sequencing was used to determine the potential amino acid sequence of the peptide as shown above. Y and b ions identified in the spectrum are shown above. The ions denoted as α refer to P450 from P450 2B6 peptide D^{134}-R^{140}. The ions denoted as β are derived from the reductase peptide R^{428}-R^{453}. Residues underlined and in bold above the figure represent amino acids identified in the spectrum.

for the identification of amino acids involved in an electrostatic interaction. Further, we performed the cross-linking reaction in the presence of lipid using both full-length P450 2B6 and reductase in order to simulate the actual physiological interaction as closely as possible. However, it is possible to generate false-positive cross-linked peptides which may not actually represent the binding orientations of the two proteins. For this reason, we attempted to isolate the complex and perform an activity assay to determine whether or not this complex was functional. Oxidative metabolism of efavirenz was observed, however, we cannot be certain that the cross-linked population is homogenous with respect to the sites of cross-linking and that the product formation can actually be attributed to the complex that was bound through the regions found in our studies.

The peptide that we have identified in the present study includes P450 2B6 residues believed to be in the C-helix (based upon the P450 2B4 crystal structure) and reductase residues that lie in the connecting domain between the FAD and FMN domains. The same residues in the C-helix of P450 2B4 have been demonstrated by Bridges et al., to be involved in the interaction between the P450 and reductase [6]. When these authors mutated lysine 139 to alanine, the apparent K_d for reductase binding increased 23-fold. Since EDC cross-linked lysines to glutamic or aspartic acid residues, we propose that K139 is the P450 residue that is cross-linked residue in our system. Several reductase residues have been proposed to interact with P450s based upon mutagenesis data; however, all of these residues are located within the FMN domain [4, 18]. During reductase-mediated reduction of P450, the electrons are believed to be transferred via the FMN domain to the P450. Other proteins that are reduced by reductase, such as heme oxygenase, interact with regions of reductase other than the FMN domain [19]. With this

in mind, it is plausible that the specificity for the site of interaction of the reductase with the P450 may be more related to the structure of the P450 than that of reductase. The reductase amino acids (428-453) identified by our cross-linking studies are within the connecting domain. Although the connecting domain has not been shown to interact with P450, the residues do appear to be on the surface of the protein based upon the reductase crystal structure and, thus, would be logical candidates for interaction [20]. However, it is possible that this cross-linked peptide was simply more abundant, or perhaps ionized more readily, than other existing cross-linked peptides.

During our analysis, two other precursor ions were identified as potential cross-linked peptides as a result of their mass shifts in the ^{18}O sample; unfortunately, we were not able to generate MS/MS data regarding these peptides. However, since the peptide data for the P450 cross-linking site coincides precisely with the mutagenesis data, it is possible that the complex characterized is biologically relevant. Further, multiple binding orientations may have the ability to facilitate electron transfer. Subsequent studies, such as mutagenesis of lysine 139 in P450 2B6, are necessary to determine whether the identified residues actually play a role in the interaction between the two proteins. Ultimately, co-crystallization of P450 with reductase may provide the most useful insights into the sites of interaction.

In summary, we have identified a P450 2B6-reductase cross-linked peptide using mass spectrometry coupled with isotopic labeling. This study provides the first direct information regarding potential sites of interaction between P450 2B6 and reductase.

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CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

Summary

In the clinical setting, P450 inhibition can result in elevated plasma levels of other drugs that are primarily metabolized by the particular P450 being inhibited. As a result, inhibition of P450s has the potential to cause severe adverse events, particularly if the co-administered drug has a narrow therapeutic index. Since mechanism-based inactivation of P450s is irreversible, in order to regain catalytic activity, the inactivated P450 has to be replaced by newly synthesized P450. The mechanism-based inactivation has a much greater potential to lead to drug-drug interactions than reversible inhibition [1]. A number of clinically relevant drugs and dietary components have been shown to be mechanism-based inactivators *in vitro*. Clinical studies have confirmed that some of these mechanism-based inactivators affect the pharmacokinetics of co-administered drugs. Certain mechanism-based inactivators of P450 2B6 have been shown to cause drug-drug interactions including, bergamottin and 17EE. The effect of grapefruit juice on the pharmacokinetics of prescription drugs has received significant attention, since patients who ingest grapefruit juice as part of their diets exhibit significantly greater mean oral bioavailability of drugs belonging to several different classes, including lipid-lowering

drugs, calcium channel blockers and immunosuppressive agents [2, 3]. An effect of grapefruit juice has been reported on both the pharmacokinetics and metabolism profiles of more than 40 drugs [4]. These effects have been attributed to mechanism-based inactivation of P450s, including P450 2B6, by bergamottin a furanocoumarin present in grapefruit juice. The P450 2B6 inactivator 17EE has been reported to interact with a variety of co-administered drugs; however, the most striking interaction described thus far comes from a study with selegiline [5]. Selegiline is a selective, irreversible inhibitor of monoamine oxidase-B used in the treatment of Parkinson's disease, and is extensively metabolized by P450s 2B6 and 2C19 [6]. Healthy female volunteers taking 17EE and selegiline concomitantly exhibited a 20-fold increase in the selegiline AUC and a decrease in the formation of metabolites [5]. Although, a two-fold or greater increase in drug plasma concentration significantly increases the risk of adverse events [7], less dramatic changes in plasma concentrations may still be clinically relevant if the co-administered compound has a narrow therapeutic index. Further, the outcomes of drug-diet and drug-drug interactions may vary depending on the age of the patient, gender, pathological conditions and genetic polymorphisms. With this in mind, the effects of a prominent P450 2B6 polymorphism (K262R; P450 2B6.4) on mechanism-based inactivation were studied.

Initial studies to investigate the effects of the K262R mutation were performed using bupropion as a probe substrate, since there was clinical evidence that this mutation caused an increase in plasma concentrations of hydroxybupropion, the pharmacologically active metabolite of bupropion [8]. Our studies, summarized in Chapter 2 were thus commensurate with the clinical data in demonstrating that the purified enzyme in the

reconstituted system could potentially mimic catalytic activity observed in humans [9]. To further probe the effects of this mutation, several structurally unrelated mechanism-based inactivators were used. To our knowledge, the studies in this thesis were the first to examine the effects of a P450 genetic polymorphism on mechanism-based inactivation either *in vitro* or *in vivo*. The mutant enzyme was inactivated by tTEPA and bergamottin with K_I values that were very similar to those observed for the wild-type enzyme. Interestingly, P450 2B6.4 was not inactivated by 17-α-ethynylestradiol, which readily inactivates wild-type P450 2B6 [10]. Subsequent metabolism studies revealed that the mutant enzyme did not metabolize 17EE. This finding led us to question whether the lack of inactivation of the mutant enzyme by 17EE was specific for 17EE, or whether there were other inactivators that would produce similar results.

Efavirenz, which has been shown to be a P450 2B6 specific substrate, was shown to inhibit P450 2B6 activity in human liver microsomes in studies using only one concentration and one time point [11]. Therefore, we investigated efavirenz as a mechanism-based inactivator of P450 2B6 and/or P450 2B6.4. As described in Chapter 3, efavirenz inactivated P450 2B6 in a time-, concentration-, and NADPH-dependent manner and the losses in activity exhibited pseudo-first order kinetics [12]. However, one of the generally accepted hallmarks of mechanism-based inactivation is that it is irreversible and the efavirenz-dependent inactivation observed in the present studies was reversible after 24 hours of dialysis. P450 2B6.4 was not inactivated by efavirenz, although the mutant enzyme metabolized efavirenz to its primary metabolite, 8-hydroxyefavirenz. These data suggested that P450 2B6.4 was not able to produce the metabolite or "inactivating intermediate" responsible for the inactivation of the wild-type

enzyme. A number of experiments were performed in attempts to identify the reactive metabolite responsible for inactivation including analyzing metabolites formed during inactivation using an Orbitrap mass spectrometer, which provides exact mass information and is a powerful tool for metabolite identification. Using this instrument, a di-hydroxylated metabolite was detected; however, the tandem mass spectrometry data was inconclusive, leading to the use of liquid chromatography NMR in an attempt to obtain precise identification of the reactive intermediate. Unfortunately, enough metabolite could not be collected to permit unequivocal identification of the metabolite. Since the metabolite of efavirenz, 8-hydroxyefavirenz was available in the lab, we also investigated whether the metabolite itself could inactivate the P450s. Similar to the parent compound, 8-hydroxyefavirenz inactivated the wild-type enzyme in a time-, concentration-, and NADPH-dependent matter. However, in this instance the inactivation was irreversible. This finding was significant, since it demonstrates for the first time that a metabolite can inactivate a P450 through a different mechanism than the parent compound. In addition, the mutant enzyme was inactivated by 8-hydroxyefavirenz, suggesting that the inactivation occurs by a mechanism distinct from that by which the parent compound caused inactivation.

Since P450 2B6.4 exhibited marked differences in catalytic activity from the wild-type P450 2B6, the experiments in Chapter 4 were designed to try to elucidate the mechanism(s) underlying the observed differences. We systematically investigated the individual steps in the P450 catalytic cycle in order to determine which one(s) might be affected during catalysis by the mutant enzyme. Since the data from our studies with 17EE and efavirenz were the most intriguing, we focused our mechanistic studies on

these two compounds. The first step was to determine whether the effects of the mutation could be related to changes in the active site structure. Studies using phenyldiazene to label the heme and spectral binding studies, respectively, indicated that both the active site topology and substrate binding to the active site were similar between the two enzymes. Analysis of the crystal structure of P450 2B4, the rabbit isoform of P450 2B6, suggests that the K262R mutation may lie in the G/H loop [13, 14]. It has been postulated that the G/H loop may be involved in the interaction between the P450 and its redox partners, suggesting that this mutation could affect the binding of reductase to the P450. However, spectral studies measuring the low to high spin shift that results from reductase binding indicated that P450 2B6.4 associates with reductase in a manner similar to P450 2B6 and exhibits a similar binding affinity. In order to gain a more complete understanding of the similarities and differences between the two enzymes, we determined the reaction stoichiometries for the metabolism of efavirenz and 17EE. These data revealed that the mutant enzyme was more uncoupled than the wild-type. Coupling is generally defined as those electrons from NADPH that are used by the enzyme to produce monooxygenated metabolites. Conversely, uncoupling refers to those electrons from NADPH that are consumed and do not lead to the formation of monooxygenated metabolites but result in the formation of non-monoxygenated metabolite products, such as superoxide, hydrogen peroxide and water. The primary product produced during the metabolism of 17EE and efavirenz by the mutant was hydrogen peroxide. The hydrogen peroxide can either originate from the decomposition of the oxyferrous P450 or from hydrogen peroxide shunting from the peroxo-iron intermediate. Interestingly, the addition of cytochrome b_5 resulted in increased coupling of the P450 2B6.4-catalyzed reactions,

with little effect on the coupling of the P450 2B6 wild-type-catalyzed reactions. Alhough the mechanism by which cytochrome b_5 enhances reaction coupling is unclear, studies performed by Zhang et al. under single turnover conditions demonstrate that cytochrome b_5 and reductase donate the second electron to the P450 (in this case P450 2B4) at a similar rate, however catalysis occurs faster in the presence of cytochrome b_5 [15]. These authors hypothesize that the conformation of the oxyferrous P450 may be different in the presence of cytochrome b_5 and reductase thereby favoring the formation of monoxygenated metabolites as opposed to the products associated with uncoupling. This hypothesis could potentially explain the findings in the present study. Taken together, these data demonstrate that P450 2B6.4 exhibits differences in catalytic activity from the wild-type and that these differences are substrate dependent. Further, the results presented here demonstrate for the first time the effect of a genetic polymorphism on individual steps in the P450 catalytic cycle.

Although it is believed that proximal surface residues of P450s are involved in interactions with the reductase, studies have not been performed to determine the specific site(s) of interaction between P450 2B6 and reductase [16, 17]. The studies described in Chapter 5 were designed to investigate the site(s) of interaction between P450 2B6 and reductase using the cross-linker EDC. The resulting cross-linked complex was structurally characterized using mass spectrometry. With this approach, we have identified a cross-linked peptide that appears to contain residues that lie within the C-helix of P450 2B6 and residues from the reductase connecting domain. The spatial locations of these residues in the proteins are based upon the crystal structures of P450 2B4 and reductase [13, 14, 18]. Although the reductase connecting domain has not

previously been implicated in its interaction with P450s, the P450 residues identified coincide with amino acids that have been previously shown to interact with reductase [16]. In order to determine whether the binding orientations of P450 2B6 and reductase in the cross-linked complex were physiologically relevant, we performed the cross-linking in the presence of lipid and isolated the cross-linked complex to assay for activity. The cross-linked P450 2B6 and reductase readily metabolized efavirenz to 8-hydroxyefavirenz, indicating that P450 2B6-reductase cross-linked species that we identified exists in a functional conformation. These studies provide the first information about the potential site(s) of interaction between P450 2B6 and reductase.

Future directions

The work in this thesis has raised a number of interesting questions that should be addressed in the future. Of particular interest will be determining whether the amino acids in the peptide identified through the cross-linking studies are actually involved in the interaction with reductase. These studies could include mutagenesis and competition studies using synthetic peptides. Also, we believe that there are potentially other cross-linked complexes yet to be identified. To address this, studies should be performed to optimize cross-linking efficiency. The use of higher concentrations of P450 2B6 and reductase could also increase the abundance of other cross-linked complexes. It would also be interesting to repeat these studies using cytochrome b_5 to determine whether the binding sites to both the P450 and reductase overlap.

The mechanistic studies suggested that the formation of the oxyferrous and/or peroxo-iron intermediates may be different between the K262R mutant and the wild-type

P450 2B6. Stopped flow spectroscopy studies investigating the rates of decomposition of the oxyferrous intermediate may help to answer this question. Ultimately, more detailed structural characterization of the two proteins using various techniques such as crystallization, hydrogen/deuterium exchange and in-solution NMR will be necessary to provide more conclusive insights into the differences between the two enzymes.

Finally, since very little information is currently available regarding the P450 2B6 active site, the identification of the site of adduct formation and the reactive intermediate responsible for the inactivation of P450 2B6 could be very useful. Since efavirenz and 8-hydroxefavirenz appear to inactivate the enzyme through distinct pathways, more detailed information regarding the amino acid modified and mechanisms underlying the inactivation by these two compounds could lead to the generation of improved P450 2B6 active site models.

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