Thr302 Is the Site for the Covalent Modification of Human Cytochrome P450 2B6 Leading to Mechanism-Based Inactivation by *tert*-Butylphenylacetylene

Hsia-lien Lin, Haoming Zhang, Matthew J. Pratt-Hyatt, and Paul F. Hollenberg

Department of Pharmacology, University of Michigan, Ann Arbor, Michigan

Received August 4, 2011; accepted September 19, 2011

ABSTRACT:

The mechanism-based inactivation of human CYP2B6 by *tert*-butylphenylacetylene (BPA) in the reconstituted system was investigated. The inactivation of CYP2B6 by BPA is time-, concentration-, and NADPH-dependent and exhibits a K_i of 2.8 μM , a k_{inact} of 0.7 min^{-1} , and a $t_{1/2}$ of 1 min. The partition ratio is ~ 5 . Unlike CYP2B1 and CYP2B4, in addition to the formation of an apoprotein adduct and a glutathione conjugate, a small heme adduct was observed when CYP2B6 was incubated with BPA. The mass increase of the adducted apoprotein and GSH conjugate is 174 Da, equivalent to the mass of one molecule of BPA plus one oxygen atom. To identify the adducted residue, BPA-inactivated CYP2B6 was digested with trypsin, and the digest was then analyzed by liquid chromatography-tandem mass spectrometry. A mass shift of 174 Da was used

for the SEQUEST database search, and the identity of the modified residue was confirmed by MS/MS fragmentation of the modified peptide. Two residues, Lys274 and Thr302, were identified as having been modified. Further mutagenesis studies have demonstrated that the residue that is modified to result in inactivation is Thr302, not Lys274. Docking studies show that in the enzyme-substrate complex, Thr302 is in close contact with the triple bond of BPA with a distance of 3.8 Å between the terminal carbon of BPA and the oxygen in the hydroxyl group of Thr302. In conclusion, Thr302 of CYP2B6 is covalently modified by a reactive metabolite of BPA, and this modification is responsible for the mechanism-based inactivation.

Introduction

The mammalian cytochrome P450 (P450) enzymes catalyze the metabolism of a wide variety of endogenous and exogenous substrates including clinically used drugs, chemical carcinogens, steroids, and environmental pollutants. For decades, the P450 2B enzymes have been the primary models for investigating structure-function relationships of mammalian P450s (Zhao and Halpert, 2007). In the past 5 years, the crystal structures of ligand-free, 4-(4-chlorophenyl)imidazole-bound, and bifenazole-bound rabbit cytochrome P450 2B4 have revealed that conformational changes in several regions of CYP2B4 can dramatically reshape the active site of the enzyme to fit the size and shape of the bound ligand (Scott et al., 2003, 2004; Zhao et al., 2006). By combining the results of studies using mechanism-based inactivators, mutagenesis studies, and homology modeling and substrate docking studies, the role of residues in the B' region, F-helix, I-helix, and K-helix of rat cytochrome P450 2B1 has been characterized (He et al., 1995; Lin et al., 2004, 2009; Honma et al., 2005; Kent et al., 2006). Phencyclidine and 17 α -ethynylestradiol are mechanism-based inactivators of CYP2B6, and covalent modification of the

apoprotein is believed to contribute to the mechanism of inactivation in both CYP2B1 and human cytochrome 2B6 (Jushchysyn et al., 2003; Kent et al., 2006). By using electrospray ionization-liquid chromatography-mass spectrometry (ESI-LC-MS) analysis, our laboratory has determined that the CYP2B1 apoprotein was covalently modified by phencyclidine and 17 α -ethynylestradiol, but the demonstration of protein adduct formation of these two inactivators with CYP2B6 has not been successful. The difficulty may be due to poor ionization of CYP2B6 with LC-MS or to the low efficiency of these mechanism-based inactivators in labeling the protein. In recent studies, a potent mechanism-based inactivator, 4-*tert*-butylphenylacetylene (BPA), for the inactivation of CYP2B1 and CYP2B4 has been characterized. We have had success in analyzing the BPA-inactivated apoprotein by LC-MS analysis (Lin et al., 2009, 2010; Zhang et al., 2009). Here, we used BPA as a probe to test whether it can also act as a mechanism-based inactivator of CYP2B6 and, most importantly, whether we can identify the adducted apoprotein and the residue that is covalently modified by BPA using LC-MS/MS analysis.

A variety of widely used drugs including bupropion, efavirenz, methadone, ifosfamide, and cyclophosphamide are preferentially metabolized or stereoselectively metabolized by CYP2B6 (Faucette et al., 2000; Huang et al., 2000; Ward et al., 2003; Gerber et al., 2004). Moreover, CYP2B6 is expressed in human liver, brain, kidney, and lung and exhibits significant genetic polymorphisms (Gervot et al.,

This work was supported in part by the National Institutes of Health National Cancer Institute [Grant CA16954] (to P.F.H.).

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

doi:10.1124/dmd.111.042176.

ABBREVIATIONS: P450, cytochrome P450; ESI, electrospray ionization; LC, liquid chromatography; MS, mass spectrometry; BPA, 4-*tert*-butylphenylacetylene; MS/MS, tandem mass spectrometry; EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; WT, wild-type.

1999; Lang et al., 2001). In the past few years, because of the growing list of substrates and the potential pharmacological/clinical significance of CYP2B6, the interest in research on CYP2B6 has increased significantly (Turpeinen et al., 2006; Wang and Tompkins, 2008).

By using CYP2B6 and reductase in the reconstituted system, we report here the following: 1) the kinetic values for inactivation were determined using 7-ethoxy-4-fluoromethylcoumarin (EFC) O-deethylation as the measure of catalytic activity; 2) the mechanism of inactivation due to heme loss and covalent modification of the apoprotein were characterized by high-pressure liquid chromatography (HPLC) and ESI-LC-MS, respectively; 3) the formation of BPA-glutathione conjugates was determined by LC-MS/MS; and 4) after digestion of the BPA-adducted apoprotein by trypsin, the amino acid residue that was covalently modified by BPA was analyzed by LC-MS/MS.

LC-MS/MS analysis of trypsin-digested CYP2B6 followed by a SEQUEST search (BioWorks; Thermo Fisher Scientific, Waltham, MA) suggested that Lys274 and Thr302 were the amino acids adducted by BPA, leading to inactivation. False-positive results can be observed in large-scale SEQUEST data searches, and, in addition, not all the covalent modification of an apoprotein is necessarily involved in mechanism-based inactivation. Thus, mutagenesis studies of Lys274 and Thr302 to Val were conducted to conclusively identify the residue whose modification was responsible for the inactivation.

To test the experimental results, BPA was docked into the active site of the crystal structure of a CYP2B6 genetic variant (Gay et al., 2010). The active site residues within 4 Å of the reversibly bound BPA, and the distance between the heme iron and the two adducted residues suggested by the SEQUEST search were examined. The docking studies are in agreement with the mutagenesis results, which have revealed that Thr302, not Lys274, is the critical residue modified by BPA and the one that contributes to the mechanism-based inactivation. To our knowledge, this is the first time that the highly conserved Thr302 residue in CYP2B6 has been identified and further confirmed by mutagenesis to be covalently modified by a mechanism-based inactivator. In addition, the functional role of Thr302 in CYP2B6 catalysis has not previously been characterized.

Materials and Methods

Chemicals. NADPH, GSH, catalase, and BPA were purchased from Sigma-Aldrich (St. Louis, MO). EFC was from Invitrogen (Carlsbad, CA). All other chemicals and solvents were of the highest purity available from commercial sources.

Site-Directed Mutagenesis. The human CYP2B6 plasmid was used as a template to construct two mutant proteins at positions Lys274 and Thr302. The mutations were performed using an *in vitro* QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The primer 5'-CCTACCTGCTCCACATGGAAGTAGAGAAATCCAACGCACAC-3' was used for the Lys274 to Val conversion (K274V mutant). The primer 5'-GCTGGCACTGAGGT-CACCAGCACCACCTC-3' was used for the Thr302 to Val conversion (T302V mutant). The mutations were confirmed by DNA sequencing performed out at the University of Michigan Biomedical Core Facility (Ann Arbor, MI).

Enzyme Assays and Inactivation of the P450s. CYP2B6, the two CYP2B6 variants, and reductase were expressed in *Escherichia coli* and purified according to procedures published previously (Scott et al., 2001; Lin et al., 2005). To assess catalytic activity, the purified CYP2B6 and reductase were reconstituted at 22°C for 30 min as described previously (Lin et al., 2009). The primary reaction mixture contained 500 pmol of P450, 500 pmol of reductase, 2 mM GSH, 100 units of catalase, and BPA (0.3–25 µM) in 500 µl of 100 mM potassium phosphate buffer (pH 7.7). After incubation of the primary reaction in the absence (as the control sample) or in the presence of 1 mM NADPH at 22°C with BPA for the time indicated (0.5–3 min), a 5-µl aliquot was removed and added to 250 µl of a secondary reaction mixture containing 50 µM EFC and 200 µM NADPH for 10 min. The formation of the

7-hydroxy-(trifluoromethyl)coumarin product was determined on a fluorescence plate reader (excitation 410 nm; emission, 510 nm) using a Wallac Victor II 1420-042 multilabel counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). The calculations of the kinetic values for mechanism-based inactivation were performed as described previously (Lin et al., 2005).

Determination of the Partition Ratio. BPA at concentrations ranging from 0.3 to 100 µM was added to the primary reaction mixture containing 1 µM CYP2B6. The reactions were initiated by the addition of 1 mM NADPH and incubated at 22°C for 20 min to allow the inactivation to go to completion (Silverman, 1996). Aliquots were removed and assayed for residual EFC deethylation activity as described above.

HPLC Analysis of the Heme. An HPLC system with a Waters 600E system controller was used to investigate the loss of native heme and the formation of heme adducts. Aliquots containing 100 pmol of control (–NADPH) and inactivated (+NADPH) P450, incubated with 10 µM BPA for 10 min as described above for the inactivation, were then analyzed using a C4 reverse-phase column (5 µm, 4.6 × 250 mm, 300 Å; Phenomenex, Torrance, CA). The solvent system consisted of solvent A (0.1% TFA in water) and solvent B (0.05% TFA in acetonitrile). The column was eluted with a linear gradient from 30 to 80% B over 30 min at a flow rate of 1 ml/min, and the column eluent was monitored at 400 nm using a model 996 photodiode array detector (Millipore Corporation, Billerica, MA).

ESI-LC-MS Analysis of the Apoprotein. Control and inactivated samples incubated with 10 µM BPA for 2 min were prepared. Aliquots of 50 pmol of P450 were analyzed on a C3 reverse-phase column (Zorbax 300SB-C3, 3.5 µm, 3.0 × 150 mm; Agilent Technologies, Santa Clara, CA) equilibrated with 40% acetonitrile and 0.1% TFA at a flow rate 0.3 ml/min. After 5 min, the column effluent was directed into the mass analyzer of a LCQ mass spectrometer (Thermo Fisher Scientific) as described previously (Kent et al., 2006). The acetonitrile concentration was increased linearly to 90% over the next 25 min to separate the components of the reconstitution mixture, and the mass spectra were recorded. The spectra corresponding to the protein envelopes for the P450s were deconvoluted to give the masses associated with each protein envelope using the BioWorks software package. The ESI source conditions were the following: sheath gas, 90 arbitrary units; auxiliary gas, 30 arbitrary units; spray voltage, 4.2 kV; and capillary temperature, 230°C.

LC-MS/MS Analysis of GSH Conjugates. The control and inactivated samples of CYP2B6 were prepared as described above. After the 10-min reaction, samples containing 1 nmol each of P450 were acidified with 60 µl of 10% TFA and then applied to a 1-ml AccuBond ODS-C18 solid-phase extraction cartridge (Agilent Technologies). The cartridges were previously washed with 2 ml of methanol followed by 2 ml of water. After the samples were loaded, the cartridges were washed with 2 ml of water and then eluted with 2 ml of methanol followed by 0.3 ml of acetonitrile. The eluted samples were dried under N₂ gas and resuspended in 80 µl of a 1:1 mixture of solvent A (0.1% acetic acid in H₂O) and solvent B (0.1% acetic acid in acetonitrile). The samples were analyzed on a C18 reverse-phase column (Luna, 3 µm, 4.6 × 100 mm; Phenomenex) using a gradient of 20 to 30% B over 5 min followed by a gradient to 40% B over 15 min and then increasing linearly to 90% B over 15 min at a flow rate of 0.3 ml/min. The column effluent was directed into the ESI source of a LCQ mass spectrometer (Thermo Fisher Scientific). The ESI conditions were the following: sheath gas flow rate, 90 arbitrary units; auxiliary gas, 30 arbitrary units; spray voltage, 4.5 kV; capillary temperature, 170°C; capillary voltage, 30 V; and tube lens offset, 25 V. Data were acquired in positive ion mode using Xcalibur software (Thermo Fisher Scientific) with one full scan followed by two data-dependent scans of the most intense and the second most intense ions.

LC-MS/MS Analysis of the BPA-Modified Peptide. BPA-inactivated CYP2B6 (500 pmol) was digested with 2 µg of trypsin in 100 µl of 50 mM ammonium bicarbonate buffer (pH 8.0) at 22°C for 18 h as described previously (Lin et al., 2010). The digested peptides were centrifuged at 16,000g, and the clear supernatant was subjected to analysis by mass spectrometry using a C18 reverse-phase column (Jupiter, 5 µm, 2.0 × 100 mm; Phenomenex). The initial gradient was 10 to 25% for 5 min followed by a gradient to 65% over 35 min and then increasing linearly to 95% over 10 min at a flow rate of 0.3 ml/min on an LC-10AD system (Shimadzu, Kyoto, Japan). The column effluent was directed into a LCQ Deca XP mass spectrometer (Thermo Fisher Scientific). The electrospray ionization conditions were the following: sheath

FIG. 1. Time- and concentration-dependent loss of EFC deethylation activity of CYP2B6 during inactivation by BPA. A, the reconstituted system was incubated with 0.625 μM (●), 1.25 μM (○), 2.5 μM (■), and 5.0 μM (□) BPA. Aliquots were removed at the times indicated and assayed for residual EFC deethylation activity as described under *Materials and Methods*. B, double reciprocal plots of the kinetic constants obtained from the initial plots. The K_I and k_{inact} values were determined from the double reciprocal plots. The data represent the average of two separate experiments performed in triplicate that did not differ by >10%.

gas flow rate, 60 arbitrary units; auxiliary gas, 10 arbitrary units; spray voltage, 4.5 kV; capillary voltage, 30 V; capillary temperature 220°C; and tube lens offset, 60 V. Data were acquired in positive ion mode using Xcalibur software in a data-dependent experiment in which MS/MS data were collected on the six most abundant ions in the survey scan.

Docking of BPA into the CYP2B6 Active Site. BPA was docked into the active site of CYP2B6 using the energy-based docking software AutoDock (version 4.0) (Morris et al., 1996). A homology model of WT CYP2B6 was constructed on the basis of a template of the crystal structure of a CYP2B6 genetic variant (Protein Data Bank code 3ibd) (Gay et al., 2010). The coordinates of BPA were built using ChemBioOffice 2008 (CambridgeSoft Corporation, Cambridge, MA), and the lowest energy conformations were obtained using the semiempirical quantum mechanics AM1 method. The flexible BPA ligands were docked to the rigid 2B6 using the Lamarckian Genetic Algorithm approach of AutoDock 4 with the following parameters: mutation rate = 0.02; crossover = 0.80; maximal number of generations = 2.7×10^7 ; and local search frequency = 0.06.

Results

Kinetics for Inactivation of CYP2B6 by BPA. The inactivation of the EFC deethylation activity of CYP2B6 by BPA was measured using various concentrations of BPA and various time points at each concentration. As shown in Fig. 1A, the inactivation of CYP2B6 was time- and concentration-dependent and required NADPH. Linear regression analysis of the time course data was used to estimate the initial rate constants (k_{obs}) for the inactivation of CYP2B6 by BPA. From the double reciprocal plots (Fig. 1B) of the values for k_{obs} and the concentration of BPA, the kinetic values for the K_I , k_{inact} , and $t_{1/2}$ were determined to be 2.8 μM , 0.7 min^{-1} , and 1 min, respectively.

Partition Ratio for the Inactivation. CYP2B6 was incubated with various concentrations of BPA for 20 min for the inactivation to reach completion. The percentage of activity remaining was plotted as a function of the molar ratio of inactivator to P450 (Fig. 2). The partition ratio was estimated from the intercept of the linear regression line obtained from the lower ratios of BPA to CYP2B6 with the straight line derived from higher ratios of BPA to CYP2B6. With this method, the partition ratio was estimated to be ~5.

Changes in the Native Heme after Inactivation as Determined by HPLC Analysis. The reaction mixtures incubated with 10 μM BPA in the absence (control sample) or presence (inactivated sample) of NADPH for 10 min were analyzed by HPLC to determine native heme remaining. The column eluates were monitored at 400 nm. There was a ~20% decrease in native heme of the inactivated CYP2B6 with the formation of a small amount of heme adduct that eluted at ~30 min (Fig. 3A). Using a diode array detector coupled to the HPLC system, the wavelengths for the maximal absorbances of

the native heme and heme adduct were 398 and 403 nm, respectively (Fig. 3B).

Characterization of the Adduct of BPA with the Apoprotein by ESI-LC-MS Analysis. The P450 reaction mixtures were incubated with BPA in the absence or presence of NADPH and then analyzed by ESI-LC-MS. The m/z spectrum of the CYP2B6 peak, shown in the insets of Fig. 4, was deconvoluted. The result shows that the control CYP2B6 apoprotein exhibited a mass of $54,558 \pm 6$ Da and the BPA-inactivated CYP2B6 apoprotein had a mass of $54,732 \pm 4$ Da. The difference in the masses of 174 ± 1 Da is consistent with the addition of the mass of one BPA molecule plus one oxygen atom.

LC-MS/MS Analysis of the Two GSH Conjugates of BPA Formed during Inactivation of CYP2B6. The extracted ion chromatograms for the two GSH conjugates with precursor ions at m/z 482 observed in the BPA-inactivated CYP2B6 reaction mixture are shown in Fig. 5A. The molecular masses of these GSH conjugates correspond to the sum of GSH plus BPA and one oxygen, suggesting that the mass of the reactive intermediate is 174 Da. The MS/MS spectra of the GSH adducts that eluted at 26.5 and 27.6 min are different and are displayed in Fig. 5, B and C, respectively. For both GSH conjugates, the major fragment ion at m/z 407 is from the loss of Gly (75 Da), the fragment ion at m/z 353 is from loss of Glu (129 Da), and further loss of water leads to formation of the ion at m/z 335 (Baillie and Davis, 1993). The ion at m/z 278 is from a combination of the loss of Glu and Gly, and the further loss of CO produces the fragment ion

FIG. 2. Determination of the partition ratio for the inactivation of 2B6 by BPA. The percentage of catalytic activity remaining was determined as a function of the molar ratio of BPA to CYP2B6 as described under *Materials and Methods*. The partition ratios were estimated from the intercept of the linear regression line for the lower ratios of BPA to 2B6 and the straight line obtained for the higher ratios of BPA to 2B6.

FIG. 3. Detection of a heme adduct by HPLC and analysis of the heme spectrum using a diode array detector. A, HPLC elution profiles monitored at 400 nm for the prosthetic heme in the -NADPH and +NADPH samples. Insets, magnified views of the samples eluting from 23 to 32 min. B, absorption spectra of the native heme and heme adduct that eluted at 26 and 30 min, respectively. The experimental procedures are described under *Materials and Methods*.

at m/z 250. All of the fragment ions indicate that GSH is a component of the BPA-GSH conjugates with m/z 482 in the extracted ion chromatogram. In Fig. 5B, the ion at m/z 232 is from the loss of a *tert*-butyl group of ion at m/z 278. In Fig. 5C, the ion at m/z 205 results from the cleavage of the C-S bond within the GSH moiety. However, whether the oxygen has been added to the internal or terminal carbon cannot be ascertained from the MS/MS spectrum. Ortiz de Montellano and colleagues (Ortiz de Montellano and Komives, 1985; Chan et al., 1993) reported previously that if the oxygen atom is added to the internal carbon, the heme will be alkylated and if the oxygen is added to the terminal carbon, the protein will be modified. The MS/MS spectrum shown in Fig. 5C for the GSH adduct formed by CYP2B6 is similar to that of the GSH conjugate formed by CYP2B1 (Lin et al.,

2009). Because a protein adduct was the major contributor to the inactivation of CYP2B1, the structure proposed for the GSH conjugate has the oxygen inserted into the terminal carbon of the acetylene as shown in the right panel of Fig. 5C. In a recent study, the BPA-adducted CYP2B4 crystal structure has shown that the ligation of the nucleophilic residue points toward the terminal carbon of the BPA triple bond (Gay et al., 2011). The mass increase of 174 Da for the BPA-GSH conjugate is equivalent to the mass of BPA plus one oxygen and is identical to the mass increase of the apoprotein adduct in the inactivated P450, indicating that the same reactive species that is trapped to form the GSH conjugate may also be reacting with the P450 apoprotein. Unlike our findings with CYP2B1, a small heme adduct was found with CYP2B6. Therefore, the MS/MS spectrum of

FIG. 4. Characterization of the BPA-adducted CYP2B6 apoprotein by ESI-LC-MS analysis. The incubation conditions, HPLC, and MS analysis conditions were as described under *Materials and Methods*. A, a representative deconvoluted mass spectrum of CYP2B6 incubated with BPA in the absence of NADPH. B, a representative deconvoluted mass spectrum of CYP2B6 incubated with BPA in the presence of NADPH. The insets show the m/z spectra of native CYP2B6 and the inactivated CYP2B6.

FIG. 5. LC-MS/MS analysis of GSH conjugates formed during the metabolism of BPA by 2B6. A reaction mixture containing 2B6 was incubated with BPA in the presence of NADPH, and the reactive intermediates were trapped with GSH. The GSH conjugates were analyzed as described under *Materials and Methods*. A, extracted ion chromatogram of the GSH conjugates with the MH^+ ion at m/z 482 eluting at 26.5 and 27.6 min. B, MS/MS spectrum of the GSH conjugate eluting at 26.5 min. C, MS/MS spectrum of the GSH conjugate eluting at 27.6 min. The proposed structures of the GSH conjugates are displayed in the right panels. The dashed lines indicate the sites of fragmentation. The MS/MS spectra were obtained in the positive ion mode and analyzed using Xcalibur software.

GSH conjugate shown in Fig. 5B may correspond to the structure for a GSH conjugate in which the oxygen has been inserted on the internal carbon of triple bond as shown in the right panel of Fig. 5B.

MS/MS Analysis of the BPA-Modified Peptide. Purified CYP2B6 was inactivated by incubation with BPA, the inactivated CYP2B6 was digested with trypsin, and then the tryptic peptides were analyzed as described under *Materials and Methods*. The modification of nucleophilic residues in the active site of CYP2B6 by the addition of a reactive metabolite formed by monooxygenation of BPA would be expected to result in a mass shift of 174 Da. On the basis of this mass shift, analysis of the MS/MS data using the SEQUEST search program suggested two modified peptides for the BPA-modified sample, whereas none were identified in digests from control samples. The MS/MS spectra of the modified peptides were further analyzed using Xcalibur software.

Formation of an adduct of BPA at Lys274 in the peptide sequence $^{263}\text{DLIDTYLLHMEKEK}^{276}$ with a MH^+ ion at m/z 1922.5 was suggested by the SEQUEST search. The b and y ions for the modified peptide were predicted using ProteinProspector software (Table 1). The identities of modified peptide and the residue adducted by BPA were confirmed by MS/MS fragmentation of the doubly charged precursor ion (m/z 961.8) shown in Fig. 6. Almost all of the singly charged fragment ions and doubly charged y10, y11, and y13 fragment ions for this modified peptide can be identified, suggesting that Lys274 is modified by a reactive metabolite of BPA.

Formation of an adduct of BPA at Thr302 in the peptide sequence $^{299}\text{GTETTSTTLR}^{308}$ with a MH^+ ion at m/z 1240.7 was also suggested by the SEQUEST search. The b and y ions for the modified peptide were predicted using ProteinProspector (Table 2). The modified peptide and the identity of residue modified by BPA were confirmed by MS/MS fragmentation of the doubly charged precursor ion (m/z 620.9) shown in Fig. 7. Almost all of the singly charged fragment ions and doubly charged ions y8 and $[\text{MH} + 2\text{H} - 2\text{H}_2\text{O}]^{+2}$ fragment ions for this modified peptide can be identified in Fig. 7, suggesting that Thr302 is modified by a reactive metabolite of BPA.

Mutagenesis Studies of Lys274 and Thr302. Because LC-MS/MS analysis indicated that two residues had been modified by

BPA but only one molecule of BPA was adducted per molecule of CYP2B6 apoprotein, the K274V mutant and the T302V mutant were constructed to elucidate the primary residue responsible for the mechanism-based inactivation. The EFC deethylation activity remaining after incubation of the WT and the two variant proteins with 5 and 20 μM BPA for 5 and 10 min was compared. As shown in Fig. 8, both the WT enzyme and K274V mutant were inactivated by BPA to very similar extents, but the T302V mutant was almost completely resistant to inactivation by BPA. These results strongly suggest that the site covalently modified by reaction with BPA and responsible for the mechanism-based inactivation is Thr302, not Lys274.

Discussion

The inactivation of CYP2B6 by BPA is concentration- and time-dependent. The inactivation requires the presence of NADPH. The apparent K_i is 2.8 μM , the k_{inact} is 0.7 min^{-1} , the $t_{1/2}$ is 1 min, and the partition ratio is ~ 5 . These data indicate that BPA is a potent mech-

TABLE 1
Predicted b and y ion series for the modified peptide sequence $^{263}\text{DLIDTYLLHMEKEK}^{276}$ with MH^+ at m/z 1922.5 for adduct formation by BPA at the Lys274^a

b Ions	m/z	Amino Acid Sequence	y Ions	m/z
b1		D	y14	
b2	229.1	L	y13	1806.9
b3	342.2	A	y12	1693.7
b4	457.2	I	y11	1580.7
b5	558.3	D	y10	1465.7
b6	721.3	T	y9	1364.6
b7	834.4	Y	y8	1201.6
b8	947.5	L	y7	1088.5
b9	1084.6	H	y6	975.4
b10	1215.6	M	y5	838.3
b11	1344.7	E	y4	707.3
b12	1646.8	K + BPA + oxygen	y3	578.3
b13	1775.8	E	y2	276.2
b14		K	y1	

^a The b and y ion series for the Lys274-modified peptide with an MH^+ ion at m/z 1922.5 were calculated from the theoretical b and y ions for the unmodified peptide with an increase of 174 Da using the ProteinProspector software.

Precursor ion: 961.8 [MH+2H]²⁺

FIG. 6. LC-MS/MS analysis of the peptide ²⁶³DLIDTYLLHMEKEK²⁷⁶ that has been modified by the reactive intermediate formed by BPA. The predicted fragment ion series for the singly charged ion with MH⁺ at *m/z* 1992.5 formed by covalent binding of BPA to Lys274 is indicated in Table 1. Data presented are from the MS/MS spectrum of the precursor ion with [M + 2H]²⁺ at *m/z* 961.8 obtained in the positive ion mode using Xcalibur software.

anism-based inactivator of CYP2B6. After incubation of CYP2B6 with 10 μM BPA for 10 min, the loss of catalytic activity is 80%, but native heme loss is ~20%. Unlike with CYP2B1 and CYP2B4, with CYP2B6 formation of a small amount of heme adduct was observed (Lin et al., 2009; Zhang et al., 2009). The LC-MS analysis demonstrated a mass increase of 174 Da, equivalent to the mass of BPA plus one oxygen atom, in the apoprotein inactivated by BPA. This result indicates that a reactive metabolite of BPA is covalently adducted to the inactivated CYP2B6 apoprotein. Therefore, the primary mechanism for the inactivation of 2B6 by BPA appears to involve labeling of the apoprotein. However, heme modification may be a secondary mechanism.

Both BPA-glutathione conjugates and the BPA-adducted apoprotein of CYP2B6 have indicated that the mass increase due to the adduct is 174 Da, equivalent to the mass of BPA plus one oxygen (Figs. 4 and 5) and similar to that obtained previously with 2B1 and 2B4 (Lin et al., 2009, 2010; Zhang et al., 2009). The BPA-inactivated CYP2B6 was digested by trypsin and analyzed by LC-MS/MS. When the mass shift of 174 Da was used for the SEQUEST data search, two peptides were identified. The identity of residues modified was then determined by MS/MS fragmentation. Lys274 and Thr302 were identified as the targets for covalent modification by the reactive metabolites of BPA. Solely on the basis of the LC-MS/MS results it is impossible to determine whether the covalent modification of Lys274

or Thr302 or both may be responsible for the inactivation. Therefore, K274V and T302V mutants of CYP2B6 were constructed and purified and used in the reconstituted system to characterize the possible roles of these residues in the inactivation by BPA. As shown in Fig. 8, the inactivation of the K274V mutant by BPA is similar to that of WT 2B6, whereas the inactivation of the T302V mutant by BPA is essentially abolished. These results suggest that covalent modification of Thr302 but not of Lys274 contributes to the mechanism-based inactivation of CYP2B6 by BPA. Because it is possible to observe false-positive results in large-scale SEQUEST data searches and not all covalent binding of reactive intermediate to a protein may be involved in the mechanism-based inactivation, mutagenesis studies provide a valuable tool for elucidating the residue(s) contributing to the inactivation. Here, Thr302 was confirmed to be the only residue covalently modified by BPA in CYP2B6 that is responsible for the inactivation.

To further investigate whether the T302V mutant metabolizes BPA to form reactive intermediates, the formation of GSH conjugates in the reconstituted system in the presence of BPA and NADPH was investigated. LC-MS/MS analysis revealed that the T302V mutant formed two BPA conjugates (data not shown), which are similar to those formed by the WT. These results demonstrate that the T302V mutant is catalytically active and has the ability to form reactive intermediates of BPA but that there is no inactivation of the mutant because it does not have a nucleophilic residue available at position 302 for covalent binding. Thus, the inactivation by BPA is eliminated in the T302V mutant. Using the same conditions as described in the legend to Fig. 4, LC-MS analysis of the mutant proteins after incubation with BPA and NADPH revealed that a protein adduct with a mass increase of ~174 Da was observed for the K274V mutant, which exhibited the mechanism-based inactivation characteristic of WT 2B6, but no apoprotein adduct was observed for the T302V mutant, which was not inactivated by BPA. Our mutagenesis studies showing inactivation of the catalytic activity and apoprotein adduct formation further emphasize the possibility of obtaining false-positive results in SEQUEST data searches.

To better understand the experimental results, BPA was docked into the active site of the crystal structure of CYP2B6 (Gay et al., 2010). Figure 9A illustrates the geometric relationships of K274, T302, the F-, G-, H-, and I-helices, the heme, and the inactivator BPA docked in the active site before catalytic activation of the BPA. At this point,

TABLE 2

Predicted b and y ion series for the modified peptide sequence ²⁹⁹GTETTSTTLR³⁰⁸ with MH⁺ at *m/z* 1240.7 for adduct formation by BPA at the Thr302^a

b Ions	<i>m/z</i>	Amino Acid Sequence	y Ions	<i>m/z</i>
b1		G	y10	
b2	159.2	T	y9	1184.1
b3	288.3	E	y8	1082.9
b4	563.4	T + BPA + oxygen	y7	953.9
b5	664.5	T	y6	678.8
b6	751.6	S	y5	577.7
b7	852.7	T	y4	490.6
b8	953.8	T	y3	389.5
b9	1066.9	L	y2	288.4
b10		R	y1	175.2

^a The b and y ion series for the Thr302-modified peptide with an MH⁺ ion at *m/z* 1240.7 were calculated from the theoretical b and y ions for the unmodified peptide with the increase of 174 Da using the ProteinProspector software.

FIG. 7. LC-MS/MS analysis of the peptide $^{299}\text{GTETTSTTLR}^{308}$ that has been modified by the reactive intermediate of BPA. The predicted fragment ion series for the singly charged ion with MH^+ at m/z 1240.7 with the BPA reactive intermediate bound to Thr302 is indicated in Table 2. Data presented are from the MS/MS spectrum of the precursor ion with $[\text{M} + 2\text{H}]^{+2}$ at m/z 620.9 obtained in the positive ion mode using the Xcalibur software.

BPA is reversibly bound to the active site with the terminal carbon atom of the acetylenic group pointing to the heme iron with the distance between the two of 2.8 Å. Lys274, located in the H-helix, is 19 Å away from the heme iron and exposed to solvent. The distance between the terminal carbon of BPA and the hydroxyl group of Thr302, located in the I-helix, is 3.8 Å, making Thr302 a primary target for covalent modification by a reactive intermediate of BPA. Although this docking simulation is consistent with our experimental results suggesting that Thr302, not Lys274, is the target for covalent modification by BPA, resulting in the mechanism-based inactivation, it does not test them experimentally. Figure 9B shows the following residues in the active site within 4 Å of BPA: Ile101, Ile114, Phe115, Phe206, Ala298, Glu301, Thr302, and Val477. All of these residues fall within the putative substrate recognition sites proposed by Gotoh (1992). The six hydrophobic residues lead to relatively tight binding of BPA to the active site, resulting in the low K_i and high k_{inact} for CYP2B6. Thr302 in the CYP2Bs corresponds to Thr252 in CYP101 (Hasemann et al., 1995). Thr252 and Asp251 are part of the proton delivery network involved in activating O–O bond scission for the generation of compound I (Raag et al., 1991; Gerber and Sligar,

1994). Moreover, it was proposed that Thr252 accepts a hydrogen bond from the hydroperoxy intermediate and promotes the second protonation on the distal oxygen atom, leading to O–O bond cleavage (Nagano and Poulos, 2005). In CYP2B6, Thr302 participates in hydrogen bonding to Glu301. Therefore, disruption of the hydrogen bonding network in the active site due to chemical modification of Thr302 by covalent binding of BPA could dramatically decrease the catalysis.

A role for Thr302 in the mechanism-based inactivation of CYP2B4 by 2-ethynylnaphthalene has been characterized by mutagenesis studies (Roberts et al., 1996). The inactivation may possibly be through steric hindrance of optimal binding of the substrate, hydrophobic disruption of a proton relay pathway, or restriction of structural changes required for interaction with reductase. In a recent study, substrate binding studies, steady-state activity measurements, and stopped-flow experiments have suggested that BPA inactivates the catalytic activity of CYP2B4 by causing steric hindrance, which prevents the required binding of substrate to the active site of the enzyme in the proper orientation (Zhang et al., 2009). In this study, Thr302 was identified as the site covalently modified by BPA that was

FIG. 8. Loss of 7-EFC deethylation activity of the WT and mutant P450s after incubation in the reconstituted system with 5 or 20 μM BPA for 5 (□) or 10 min (■). The EFC deethylation activity at time 0 for each P450 was used as the 100% control. BPA at 5 and 20 μM was tested for each P450. The results presented here were from three experiments done in triplicate.

FIG. 9. Results of docking BPA into the CYP2B6 crystal structure. A, a ribbon-and-stick depiction of BPA docking into the active site. K274 in the H-helix and T302 in the I-helix are shown in sticks. B, residues within 4.0 Å of BPA shown here are Ile101, Ile114, Phe115, Phe206, Ala298, Glu301, Thr302, and Val477. T302 participates in hydrogen bonding to E301. Shown in color are BPA (green) and the heme (red).

contributing to the mechanism-based inactivation in CYP2B6. The results suggest that Thr302 is not required for catalytic activity or substrate binding when tested with EFC and BPA, but it is required for the binding of the reactive intermediate of BPA for the inactivation in CYP2B6. Although the LC-MS results showed that almost 100% of the CYP2B6 protein was adducted by BPA (Fig. 4), ~20% of the catalytic activity still remained (Figs. 2 and 8). The determination of the crystal structure of BPA-bound CYP2B4 demonstrating the existence of both open and closed conformations could explain the partial catalytic activity observed here even though all of the protein is modified (Gay et al., 2011). Docking experiments using the open structure show that BPA is able to rotate upward to give substrates some access to the heme. Moreover, the crystal structure of the BPA-inactivated CYP2B4 has shown the formation of an ester bond between the oxygenated BPA and the hydroxyl group of Thr302 with the oxygen inserted onto the terminal carbon of the carbon-carbon triple bond. As displayed in Fig. 9B, our docking studies on the CYP2B6 active site structure show that Thr302 is in close contact with the acetylene moiety of BPA. Therefore, our docking model of unmetabolized BPA reversibly bound to CYP2B6 is in good agreement with the BPA-bound CYP2B4 crystal structure. In both P450s, covalent modification of the apoprotein by BPA is the predominant mechanism contributing to the inactivation. However, a noticeable difference is that a small amount of heme adduct was observed with CYP2B6, but not with CYP2B4, during the inactivation by BPA. Thus far, except for BPA, detection of both the apoprotein adduct and the identity of the covalently modified residue(s) formed by mechanism-based inactivators such as 17 α -ethinylestradiol or bergamottin with CYP2B6 has not been successful (Lin et al., 2005; Kent et al., 2006, 2008).

Acknowledgments

We thank Dr. Richard Neubig, Department of Pharmacology, University of Michigan (Ann Arbor, MI) for the use of the Wallac Victor II plate reader.

Authorship Contributions

Participated in research design: Lin, Zhang, and Hollenberg.

Conducted experiments: Lin, Zhang, and Pratt-Hyatt.

Contributed new reagents or analytic tools: Lin and Pratt-Hyatt.

Performed data analysis: Lin, Zhang, and Hollenberg.

Wrote or contributed to the writing of the manuscript: Lin, Zhang, and Hollenberg.

References

- Baillie TA and Davis MR (1993) Mass spectrometry in the analysis of glutathione conjugates. *Biol Mass Spectrom* **22**:319–325.
- Chan WK, Sui Z, and Ortiz de Montellano PR (1993) Determinants of protein modification versus heme alkylation: inactivation of cytochrome P450 1A1 by 1-ethynylpyrene and phenylacetylene. *Chem Res Toxicol* **6**:38–45.
- Faucette SR, Hawke RL, Lecluyse EL, Shord SS, Yan B, Laethem RM, and Lindley CM (2000) Validation of bupropion hydroxylation as a selective marker of human cytochrome P450 2B6 catalytic activity. *Drug Metab Dispos* **28**:1222–1230.
- Gay SC, Shah MB, Talakad JC, Maekawa K, Roberts AG, Wilderman PR, Sun L, Yang JY, Huelga SC, Hong WX, et al. (2010) Crystal structure of a cytochrome P450 2B6 genetic variant in complex with the inhibitor 4-(4-chlorophenyl)imidazole at 2.0-Å resolution. *Mol Pharmacol* **77**:529–538.
- Gay SC, Zhang H, Wilderman PR, Roberts AG, Liu T, Li S, Lin HL, Zhang Q, Woods VL Jr, Stout CD, et al. (2011) Structural analysis of mammalian cytochrome P450 2B4 covalently bound to the mechanism-based inactivator *tert*-butylphenylacetylene: insight into partial enzymatic activity. *Biochemistry* **50**:4903–4911.
- Gerber JG, Rhodes RJ, and Gal J (2004) Stereoselective metabolism of methadone *N*-demethylation by cytochrome P4502B6 and 2C19. *Chirality* **16**:36–44.
- Gerber NC and Sligar SG (1994) A role for Asp-251 in cytochrome P-450cam oxygen activation. *J Biol Chem* **269**:4260–4266.
- Gervot L, Rochat B, Gautier JC, Bohnenstengel F, Kroemer H, de Berardinis V, Martin H, Beaune P, and de Waziers I (1999) Human CYP2B6: expression, inducibility and catalytic activities. *Pharmacogenetics* **9**:295–306.
- Gotoh O (1992) Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. *J Biol Chem* **267**:83–90.
- Hasemann CA, Kurumbail RG, Boddupalli SS, Peterson JA, and Deisenhofer J (1995) Structure and function of cytochromes P450: a comparative analysis of three crystal structures. *Structure* **3**:41–62.
- He YQ, He YA, and Halpert JR (1995) *Escherichia coli* expression of site-directed mutants of cytochrome P450 2B1 from six substrate recognition sites: substrate specificity and inhibitor selectivity studies. *Chem Res Toxicol* **8**:574–579.
- Honma W, Li W, Liu H, Scott EE, and Halpert JR (2005) Functional role of residues in the helix B' region of cytochrome P450 2B1. *Arch Biochem Biophys* **435**:157–165.
- Huang Z, Roy P, and Waxman DJ (2000) Role of human liver microsomal CYP3A4 and CYP2B6 in catalyzing *N*-dechloroethylation of cyclophosphamide and ifosfamide. *Biochem Pharmacol* **59**:961–972.
- Jushchyshyn MI, Kent UM, and Hollenberg PF (2003) The mechanism-based inactivation of human cytochrome P450 2B6 by phenylclidine. *Drug Metab Dispos* **31**:46–52.
- Kent UM, Lin HL, Mills DE, Regal KA, and Hollenberg PF (2006) Identification of 17- α -ethinylestradiol-modified active site peptides and glutathione conjugates formed during metabolism and inactivation of P450s 2B1 and 2B6. *Chem Res Toxicol* **19**:279–287.
- Kent UM, Sridar C, Spahlinger G, and Hollenberg PF (2008) Modification of serine 360 by a reactive intermediate of 17- α -ethinylestradiol results in mechanism-based inactivation of cytochrome P450s 2B1 and 2B6. *Chem Res Toxicol* **21**:1956–1963.
- Lang T, Klein K, Fischer J, Nüssler AK, Neuhaus P, Hofmann U, Eichelbaum M, Schwab M, and Zanger UM. Schwab M, and Zanger UM (2001) Extensive genetic polymorphism in the human CYP2B6 gene with impact on expression and function in human liver. *Pharmacogenetics* **11**:399–415.
- Lin HL, Kent UM, and Hollenberg PF (2005) The grapefruit juice effect is not limited to cytochrome P450 (P450) 3A4: evidence for bergamottin-dependent inactivation, heme destruction, and covalent binding to protein in P450s 2B6 and 3A5. *J Pharmacol Exp Ther* **313**:154–164.

- Lin HL, Kent UM, Zhang H, Waskell L, and Hollenberg PF (2004) The functional role of threonine-205 in the mechanism-based inactivation of P450 2B1 by two ethynyl substrates: the importance of the F helix in catalysis. *J Pharmacol Exp Ther* **311**:855–863.
- Lin HL, Zhang H, Jushchyshyn M, and Hollenberg PF (2010) Covalent modification of Thr302 in cytochrome P450 2B1 by the mechanism-based inactivator 4-*tert*-butylphenylacetylene. *J Pharmacol Exp Ther* **333**:663–669.
- Lin HL, Zhang H, Noon KR, and Hollenberg PF (2009) Mechanism-based inactivation of CYP2B1 and its F-helix mutant by two *tert*-butyl acetylenic compounds: covalent modification of prosthetic heme versus apoprotein. *J Pharmacol Exp Ther* **331**:392–403.
- Morris GM, Goodsell DS, Huey R, and Olson AJ (1996) Distributed automated docking of flexible ligands to proteins: parallel applications of AutoDock 2.4. *J Comput Aided Mol Des* **10**:293–304.
- Nagano S and Poulos TL (2005) Crystallographic study on the dioxygen complex of wild-type and mutant cytochrome P450cam. Implications for the dioxygen activation mechanism. *J Biol Chem* **280**:31659–31663.
- Ortiz de Montellano PR and Komives EA (1985) Branchpoint for heme alkylation and metabolite formation in the oxidation of arylacetylenes by cytochrome P-450. *J Biol Chem* **260**:3330–3336.
- Raag R, Martinis SA, Sligar SG, and Poulos TL (1991) Crystal structure of the cytochrome P-450CAM active site mutant Thr252Ala. *Biochemistry* **30**:11420–11429.
- Roberts ES, Pernecky SJ, Alworth WL, and Hollenberg PF (1996) A role for threonine 302 in the mechanism-based inactivation of P450 2B4 by 2-ethynylnaphthalene. *Arch Biochem Biophys* **331**:170–176.
- Scott EE, Spatzenegger M, and Halpert JR (2001) A truncation of 2B subfamily cytochromes P450 yields increased expression levels, increased solubility, and decreased aggregation while retaining function. *Arch Biochem Biophys* **395**:57–68.
- Scott EE, He YA, Wester MR, White MA, Chin CC, Halpert JR, Johnson EF, and Stout CD (2003) An open conformation of mammalian cytochrome P450 2B4 at 1.6-Å resolution. *Proc Natl Acad Sci USA* **100**:13196–13201.
- Scott EE, White MA, He YA, Johnson EF, Stout CD, and Halpert JR (2004) Structure of mammalian cytochrome P450 2B4 complexed with 4-(4-chlorophenyl)imidazole at 1.9-Å resolution: insight into the range of P450 conformations and the coordination of redox partner binding. *J Biol Chem* **279**:27294–27301.
- Silverman RB (1996) Mechanism-based enzyme inactivation, in *Contemporary Enzyme Kinetics and Mechanisms* (Purich DL ed) pp 291–335, Academic Press, San Diego, CA.
- Turpeinen M, Raunio H, and Pelkonen O (2006) The functional role of CYP2B6 in human drug metabolism: substrates and inhibitors *in vitro*, *in vivo* and *in silico*. *Curr Drug Metab* **7**:705–914.
- Wang H and Tompkins LM (2008) CYP2B6: new insights into a historically overlooked cytochrome P450 isozyme. *Curr Drug Metab* **9**:598–610.
- Ward BA, Gorski JC, Jones DR, Hall SD, Flockhart DA, and Desta Z (2003) The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary metabolism: implication for HIV/AIDS therapy and utility of efavirenz as a substrate marker of CYP2B6 catalytic activity. *J Pharmacol Exp Ther* **306**:287–300.
- Zhang H, Lin HL, Walker VJ, Hamdane D, and Hollenberg PF (2009) *tert*-Butylphenylacetylene is a potent mechanism-based inactivator of cytochrome P450 2B4: inhibition of cytochrome P450 catalysis by steric hindrance. *Mol Pharmacol* **76**:1011–1018.
- Zhao Y and Halpert JR (2007) Structure-function analysis of cytochromes P450 2B. *Biochim Biophys Acta* **1770**:402–412.
- Zhao Y, White MA, Muralidhara BK, Sun L, Halpert JR, and Stout CD (2006) Structure of microsomal cytochrome P450 2B4 complexed with the antifungal drug bifenazole: insight into P450 conformational plasticity and membrane interaction. *J Biol Chem* **281**:5973–5981.

Address correspondence to: Dr. Paul F. Hollenberg, Department of Pharmacology, 2301 MSRB III, 1150 W. Medical Center Dr., Ann Arbor, MI 48109-5632. E-mail: phollen@umich.edu