Molecular Pharmacology Fast Forward. Published on August 31, 2009 as doi:10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

MOL #59808

tert-Butylphenylacetylene is a potent mechanism-based inactivator of cytochrome P450 2B4: Inhibition of cytochrome P450 catalysis by steric hindrance

Haoming Zhang, Hsia-lien Lin, Vyvyca J. Walker, Djemel Hamdane, and Paul F. Hollenberg

Department of Pharmacology, The University of Michigan, Ann Arbor, Michigan (HZ, HLL, VJW, PFH) and Laboratoire d'Enzymologie et Biochimie Structurales UPR 3082 CNRS, Gif-sur-Yvette Cedex, France (DH)

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Inactivation of P450 2B4 by tBPA

Corresponding author: Paul F. Hollenberg, PhD

Department of Pharmacology, The University of Michigan

1150 West Medical Center Drive

2301 MSRB III

Ann Arbor, MI 48109-5632

Telephone: 734-764-8166; Fax: 734-763-5378

E-mail: phollen@umich.edu.

Text pages: 35

Figures: 7

References: 29

Words in Abstract: 250

Words in Introduction: 549

Words in Discussion: 1236

Nonstandard abbreviations: 7-EFC, 7-ethoxy-4-trifluoromethylcoumarin; 7-HFC, 7-hydroxy-4-trifluoromethylcoumarin; P450, cytochrome P450; CPR, NADPH-dependent cytochrome P450 reductase; DLPC, dilauroylphosphatidylcholine; ESI-LC/MS, electro-spray ionization liquid chromatography mass spectrometry; tBPA, *tert*-butylphenylacetylene.

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

We have demonstrated that 4-(*tert*-butyl)-phenylacetylene (tBPA) is a potent mechanism-based inactivator for cytochrome P450 2B4 (P450 2B4) in the reconstituted system. It inactivates P450 2B4 in a NADPH- and time-dependent manner with a K_I of 0.44 μM and k_{inact} of 0.12 min^{-1}. The partition ratio was approximately zero, indicating that inactivation occurs without the reactive intermediate leaving the active site. LC-MS analyses revealed that tBPA forms a protein adduct with a 1:1 stoichiometry. Peptide mapping of the tBPA-modified protein provides evidence that tBPA is covalently bound to Thr302. This is consistent with results of molecular modeling that show the terminal carbon of the acetylenic group is only 3.65 Å away from Thr302. To characterize the effect of covalent modification of Thr302, tBPA-modified P450 2B4 was purified to homogeneity from the reconstituted system. The Soret band of tBPA-modified protein is red-shifted by 5 nm to 422 nm compared to unmodified protein. Benzphetamine Binding to the modified P450 2B4 does not cause any spin shift, indicating that substrate binding and/or the heme environment has been altered by covalently bound tBPA. Cytochrome P450 reductase (CPR) reduces the unmodified and tBPA-modified P450s at approximately the same rate. However, addition of benzphetamine stimulates the rate of reduction of unmodified P450 2B4 by ~20-fold, but only marginally stimulates reduction of the tBPA-modified protein. This large discrepancy in the stimulation of the first electron transfer by benzphetamine strongly suggests that the impairment of P450 catalysis is due to inhibition of benzphetamine binding to the tBPA-modified P450 2B4.

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

The cytochromes P450 (P450s) are a superfamily of hemoproteins that catalyze oxidative biotransformation of numerous xenobiotics including drugs, carcinogens, and organic solvents. Mechanism-based inactivation of P450s often occurs when reactive intermediates generated by the P450-catalyzed reactions covalently modify the heme or critical amino acid residues. Inactivation of P450 catalysis can have significant ramifications on the pharmacokinetics of pharmaceutical drugs and other xenobiotics as it is often associated with adverse drug-drug interactions and toxicity (Hollenberg et al., 2008). It is therefore important to understand the mechanism of P450 catalysis in order to minimize the adverse effect.

All P450s contain a protoporphyrin IX prosthetic cofactor ligated to a cysteinyl residue. Through sequential addition of two electrons from cytochrome P450 reductase (CPR), the P450s activate the dioxygen molecule, split the O-O bond and subsequently insert one oxygen atom into their substrates (for details, see Denisov et al., 2005; Hamdane et al., 2008). The putative oxygenating intermediate has been postulated to be an oxyferryl intermediate referred to as Compound I in analogy to the reactive Compound I of heme peroxidases. Due to the oxidative nature of P450 reactions, P450s can be victims of their own reactions. Classic examples of mechanism-based inactivators of P450s are acetylenic compounds. Many aliphatic and arylacetylenes have been reported by our group and others to be mechanism-based inactivators of various isoforms of P450s (Hollenberg et al., 2008; Komives and Ortiz de Montellano, 1987; Ortiz de Montellano and Kunze, 1980; Wright et al., 2009). For example, 17α-

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

ethynylestradiol, a synthetic contraceptive, has been reported to inactivate P450 2B1 and 2B6 by protein alkylation of the peptides ^{347}PYTDAVIHEI^{376} and ^{347}PYTEAV^{365}, respectively (Kent et al., 2006). Both *tert*-butylacetylene (tBA) and *tert*-butyl-1-methyl-2-propynl ether (tBMP) inactivate P450 2E1 predominantly through the formation of heme adducts. A novel reversible formation of a tBA-heme adduct was observed for the T303A variant of P450 2E1 (Blobaum et al., 2005). Earlier work reported that phenylacetylenes inactivate P450 2B1 through heme alkylation whereas 1-ethynylpyrene inactivated P450 1A1 by protein alkylation (Chan et al., 1993; Gan et al., 1984; Komives and Ortiz de Montellano, 1987; Ortiz de Montellano and Komives, 1985). Other acetylenic mechanism-based inactivators of P450s include 5-phenyl-1-pentyne (Roberts et al., 1998), 2-ethynylnaphthalene (Roberts et al., 1994), 9-ethynylphenathrene (Roberts et al., 1995), 7-ethynylcoumarin (Kent et al., 2001), to name a few. Even though many acetylenic compounds have been identified as mechanism-based inactivators of P450s, the effects of protein alkylation by these acetylenic compounds on the catalytic mechanism have not yet been characterized in detail. This is in part due to low k_{inact} and high partition ratio for many mechanism-based inactivators. These factors contribute to the difficulty in obtaining homogenously modified P450s for further mechanistic studies.

In this study, we report that tBPA is a potent mechanism-based inactivator of P450 2B4 with a partition ratio of close to zero. tBPA inactivates P450 2B4 by forming a single covalent adduct with the Thr302 with a stoichiometry for inactivation of 1:1 without modification or loss of the heme. Furthermore, the

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

mono-alkylated protein can be purified to homogeneity from the reaction mixture for further characterization. The results from substrate binding studies, steady-state activity measurements, and stopped-flow experiments suggest that tBPA inactivates the catalytic activity of P450 2B4 by causing steric hindrance which prevents binding of the substrate to the active site of the enzyme.

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Chemicals - All chemicals used are ACS reagent grade unless otherwise specified. Benzphetamine, *tert*-butylphenylacetylene (tBPA), and dilauroylphosphatidylcholine (DLPC), catalase, and NADPH were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Trifluoroacetic acid (TFA) was purchased from Pierce Chemicals (Rockford, IL). 7-Ethoxy-4-trifluoromethylcoumarin (7-EFC) was purchased from Invitrogen Molecular Probes (Eugene, OR). The nonionic detergent Cymal-5 was purchased from Anatrace (Maumee, OH). Sequencing grade trypsin was purchased from Promega (Madison, WI). Carbon monoxide with purity > 99.5% was purchased from Cryogenic Gas (Detroit, MI).

Over-expression and purification of P450 2B4, NADPH-dependent cytochrome P450 reductase, and cytochrome b5. The plasmid for over-expression of P450 2B4 (pKK2B4dH) was a generous gift from Dr. James Halpert (University of California at San Diego, CA). P450 2B4 was expressed as a truncated form where the hydrophobic membrane-spanning domain had been removed (Δ3-21) and several positively charged residues introduced into the N-terminus to increase the expression yield. This truncated P450 2B4 was over-expressed in Escherichia coli C41(DE3) cells and purified with a Ni-affinity followed by a CM-sepharose cation exchange column as described by Halpert and co-workers (Scott et al., 2001). NADPH-dependent cytochrome P450 reductase (CPR) and cytochrome b5 (cyt b5) were over-expressed and purified as previously described (Zhang et al., 2009).

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

Kinetics for the mechanism-based inactivation of P450 2B4 by tert-butylphenylacetylene. The kinetics for the inactivation of P450 2B4 by tBPA were determined at 30 ^{o}C in 50 mM potassium phosphate buffer (pH 7.4). The primary reaction mixtures contained equimolar concentrations of P450 2B4 and CPR (1 μM each), 0.3 mg/mL DLPC, and varying concentrations of tBPA (0-10 μM). The reactions were initiated by addition of NADPH to a final concentration of 1 mM. At designated times, aliquots (10 μL) of the primary reaction mixture were transferred to a secondary reaction mixture that contained 0.1 mM 7-EFC and 0.3 mM NADPH in 50 mM potassium phosphate (pH 7.4). The secondary reactions were terminated after incubation for 10 min by the addition of 50 μL of ice-cold acetonitrile. The fluorescent intensity of the 7-hydroxy-4-trifluoromethylcoumarin (7-HFC) product was measured at 520 nm with excitation at 410 nm and used to calculate the activity remaining of the P450 2B4. The results were expressed as percentage of the control sample from which tBPA was omitted.

Partition ratio for the mechanism-based inactivation of P450 2B4 by tert-butylphenyl acetylene. To determine the partition ratio, the primary reaction mixture containing P450 2B4, CPR, cyt b5 (when present), catalase, and various concentrations of tBPA as indicated was incubated as previously described except that incubation of the primary reaction mixture was allowed to proceed for 30 min at 30 ^{o}C until the mechanism-based inactivation was complete. The activity remaining after the inactivation of P450 2B4 was analyzed using the

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

secondary reaction mixture as described above. The partition ratio was then determined as previously described (Kent et al., 1997).

Analyses of the tBPA-modified P450 2B4 protein by ESI-LC/MS. The tBPA-modified P450 protein was analyzed by ESI-LC/MS using a LCQ ion-trap mass spectrometer (ThermoScientific, Waltham, MA) as previously described (Zhang et al., 2009). Following incubation with tBPA and NADPH for the times indicated, an aliquot (50 μL) of the primary reaction solution was applied to a reverse-phase C3 column (2 × 150 mm, 5 μm) (Agilent Technologies, CA). P450 2B4 was separated from the other reaction components with a binary solvent system consisting of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) using the following gradient: 30% B for 5 min, linearly increased to 90% B in 20 min, and held at 90% B for 30 min. The flow rate was 0.25 mL/min. The molecular masses of the unmodified and tBPA-modified P450 2B4 were determined by deconvolution of the apoprotein charge envelopes using the Bio-works software (Thermo Scientific, Waltham, MA).

Preparations of tBPA-modified P450 2B4 under turnover conditions. In a typical reaction mixture labeling P450 2B4 with tBPA, P450 2B4, CPR, and cyt b5 were reconstituted in the presence of DLPC for 60 min on ice and diluted with 50 mM potassium phosphate (pH 7.4) to give final concentrations of 4, 2, 12 and 240 μM for the P450 2B4, CPR, cyt b5, and DLPC, respectively. tBPA (20 μM) and catalase (300 units/mL) were then added into the reaction mixture. After further incubation for 5 min at 30 ^{o}C, the reaction was initiated by the addition of 1 mM NADPH and formation of the tBPA-protein adduct was monitored by LC-

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

MS analyses of aliquots of the reaction mixture. The reaction was terminated by the addition of 0.5 M NaCl and 4.8 mM Cymal-5 once complete formation of the tBPA-protein adduct had been confirmed by LC-MS. The tBPA-modified P450 2B4 was then purified to homogeneity from the reaction mixture using a Ni-affinity column followed by cation-exchange chromatography as described previously (Scott et al., 2001). The purified tBPA-modified P450 2B4 was then stored at -80 ^{o}C until used for subsequent studies.

Identification of the modified amino acid residue in the tBPA-modified P450 2B4 using LC-MS/MS. To identify the covalently modified amino acid residue, we used LC-MS/MS to map tryptic digests of tBPA-modified P450 2B4. The purified tBPA-modified P450 2B4 (~500 pmoles) was digested with 2 μg of trypsin in 0.1 mL of 50 mM ammonium bicarbonate buffer (pH 8.0) at room temperature for 18 hours. The digested sample was centrifuged at 16,000×g for 5 min and a 50 μL aliquot of the supernatant was loaded onto a reverse-phase C18 column (2.0×100 mm, Jupiter, Phenomemnex, Torrance, CA). The tryptic peptides were then separated with a gradient of Solvent A and Solvent B at a flow rate of 0.3 mL/min. The gradient was linearly increased from 10% B to 25% B over 5 min, to 65% B over 35 min, and to 95% B over 10 min. The column eluents were introduced into the ion source through a silica capillary tube, ionized, and fragmented through collision-induced dissociation (CID) on a LCQ Deca XP mass spectrometer (Thermo Scientific, Waltham, MA). The instrument parameters were: sheath gas flow, 60 (arbitrary units); auxiliary gas flow, 10

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

(arbitrary units); spray voltage, 4.5 kV; capillary voltage, 30 V; capillary temperature, 220 ^{o}C; and CID energy, 37%.

The LC-MS/MS datasets for the tryptic digests of the tBPA-modified P450 2B4 were exported to the Bioworks program (ver. 3.3.1) and the modified amino acid residues were identified using the SEQUEST database search function of the Bioworks program. To reduce false hits, the cross-correlation score (XCorr) measuring the quality of the match between experimental data and the predicted fragment patterns was set at 2.0, 2.5, and 3.6 for singly, doubly and triply charged ions, respectively. Only those hits from the SEQUEST database search that had a possibility score less than 1×10^{-3} were considered.

Rates of reduction of unmodified and tBPA-modified P450 2B4 by P450 reductase. The rates of reduction of unmodified and tBPA-modified P450 2B4 by CPR were determined at 25 ^{o}C using a Hi-Tech SF61DX2 stopped-flow spectrophotometer as previously described (Zhang et al., 2008) (Hi-Tech, Wiltshire, UK). To pre-form an active P450-CPR complex, equimolar concentrations of P450 and CPR (3 μM each) and 0.15 mg/mL of DLPC were incubated in 100 mM potassium phosphate buffer (pH 7.4) on ice for 60 min. For those experiments in the presence of substrate, benzphetamine was added to a final concentration of 1 mM. After reconstitution for 60 min, the protein samples were gently bubbled with CO gas for ~5 min and then loaded into Syringe A of the stopped-flow spectrophotometer. The CO-saturated potassium phosphate buffer (0.1 M at pH 7.4) containing 0.1 mM NADPH and 1 mM benzphetamine (when present) was loaded into Syringe B. The kinetic traces at 450 nm were

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

recorded after rapid mixing of the contents of both syringes and the data were fit with double exponentials to obtain apparent rate constants using the KinetAsyst program (Hi-Tech, Wiltshire, UK).

Docking of *tert*-butylphenylacetylene into the active site of P450 2B4*. The energy-based docking software Autodock (ver. 4.0) was used to dock tBPA into the active site of P450 2B4 to examine the amino acid residues in the proximity of tBPA. The coordinates of P450 2B4 were obtained from the Protein Data Bank (PDB code, 1SUO). The coordinates of the water and other hetero atoms except for the heme were removed from the P450 2B4 prior to docking. The coordinates of the tBPA ligand were built using the ChemBioOffice 2008 software suite (CambridgeSoft, Cambridge, MA) and the geometry of tBPA was optimized using the semi-empirical quantum AM1 method included in the ChemBioOffice 2008 software suite.

Catalytic activity of the tBPA-modified P450 2B4 under steady-state conditions. The relative turnover rates for the metabolism of 7-EFC, benzphetamine and testosterone by the tBPA-modified P450 2B4 were determined under steady-state conditions as previously described (Zhang et al., 2009). To determine the effect of the covalently bound tBPA, the turnover rates for both the tBPA-modified and unmodified P450s were measured under identical conditions.

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Kinetics for the mechanism-based inactivation of P450 2B4 by tert-butylphenylacetylene. As shown in Fig. 1, inactivation of the 7-EFC O-deethylation activity of P450 2B4 by tBPA is NADPH-, concentration-, and time-dependent. The activity remaining for P450 2B4 in the absence of tBPA remains virtually unchanged with time, while in the presence of tBPA the remaining activity decreases significantly over time. In the absence of cyt b5, tBPA inactivates the 7-EFC O-deethylation activity with a K_I of 0.44 μM and a k_{inact} of 0.12 min^{-1}. In the presence of one equivalent of cyt b5, which is known to stimulate the activity of some P450s, both the K_I and k_{inact} are increased to 0.73 μM and 0.25 min^{-1} respectively. The overall inactivation efficiency (k_{inact}/K_I) is not significantly affected by cyt b5, but cyt *b5* seems to accelerate the rate of the mechanism-based inactivation by twofold. Based on this observation, cyt b5 was included in the primary reaction mixture to prepare the tBPA-modified P450 2B4.

Partition ratio for the mechanism-based inactivation of P450 2B4 by tert-butylphenylacetylene. The partition ratio for the mechanism-based inactivation of P450 2B4 by tBPA was determined in the absence and presence of cyt b5. As shown in Figure 2, the activity remaining for the inactivated P450 2B4 decreases with increasing molar ratios of tBPA to P450 as expected. The partition ratio was determined to be close to zero. The near zero partition ratio indicates that the reactive intermediate of tBPA inactivates P450 2B4 without leaving the active site (Silverman, 1995). The residual activity of the inactivated P450 2B4 is approximately 22% of the control sample in the absence of cyt b5.

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

Analysis of the tBPA-protein adduct of P450 2B4 by ESI-LC/MS. To investigate whether the mechanism-based inactivation of P450 2B4 by tBPA leads to formation of a tBPA-protein adduct, we determined the molecular mass of P450 2B4 that had catalyzed oxidation of tBPA under turnover conditions and been inactivated to varying extents. The results are shown in Figure 3. The P450 2B4 sample obtained after 5 min of incubation with NADPH yielded two mass peaks at 53948 and 54122 Da (Fig. 3A). The former mass represents that of the unmodified P450 2B4 (data not shown), whereas the latter exhibits a mass increase of 174 Da over the unmodified P450 2B4. This increase of 174 Da corresponds to the addition of one tBPA (158 Da) plus one oxygen atom to the unmodified protein. Thus, the mass peak at 54122 Da represents the tBPA-modified P450 2B4. The relative intensity of the tBPA-modified protein at 54122 Da increases with increasing incubation time at the expense of the unmodified protein. Following 15 min of the incubation, nearly all of the P450 2B4 was labeled with one tBPA molecule (Fig. 3C). These results demonstrate that the mechanism-based inactivation of P450 2B4 by tBPA leads to the formation of tBPA-protein adduct with a 1:1 stoichiometry. No loss in the heme content was observed by HPLC analyses of the tBPA-modified P450 2B4 (data not shown). The ability to make fully tBPA-labeled P450 2B4 provides a unique opportunity to study the mechanism(s) by which the covalently bound tBPA affects P450 catalysis.

Optical absorption spectra of the tBPA-modified P450 2B4. The optical absorption spectrum of the tBPA-modified P450 2B4 is shown in Figure 4 in

comparison with that of the unmodified one. As expected, the unmodified ferric P450 2B4 has a Soret band at 417 nm and α and β bands at 568 and 534 nm respectively. These spectral features are characteristic of a hexacoordinated low spin heme. Binding of benzphetamine to the unmodified P450 2B4 leads to clear spectral changes; the most prominent changes are marked increases in the absorbances at 390 nm and 645 nm and the marked decrease in the Soret peak at 417 nm. These changes are characteristic for the conversion of a low-spin heme to a pentacoordinated high spin heme. Clearly, benzphetamine binding to the unmodified P450 2B4 results in a typical substrate-induced low spin-to-high spin shift, or type I spectral change, due to the dissociation of the axial water ligand at the sixth position. In the absence of benzphetamine, the overall optical absorption spectrum of the tBPA-modified P450 2B4 resembles that of the unmodified P450 2B4 except that its Soret band appears at 422 nm, a red-shift of 5 nm compared with that of the unmodified P450 2B4. This shift demonstrates that the local environment of the heme in the tBPA-modified P450 2B4 has been perturbed. Thus, it is reasonable to suggest that the site of covalent modification of the P450 by tBPA is located in proximity to the heme. In marked contrast to the results with the unmodified P450 2B4, the addition of benzphetamine to the tBPA-modified P450 2B4 does not result in any significant spectral changes (Fig. 4). The spectrum of the tBPA-modified P450 2B4 in the presence of 1 mM benzphetamine is virtually identical to that observed in the absence of 1 mM benzphetamine. In this case, the lack of a type I spectral change suggests that

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

ligand binding to the active site of the modified P450 2B4 may have been altered by the presence of the covalently bound tBPA.

Effect of the covalently bound tBPA on the catalytic activity of P450 2B4. The impact of the covalently bound tBPA on the catalytic activity was evaluated by determining the relative turnover rates of the tBPA-modified protein for 7-EFC, benzphetamine, and testosterone under steady-state conditions. Like the unmodified P450 2B4, the tBPA-modified P450 2B4 metabolizes testosterone to give three major metabolites as reported by Hernandez et al. (2006). The amounts of all three metabolites were added together to calculate the relative overall turnover rate. The results show that the tBPA-modified P450 2B4 catalyzes the oxidation of 7-EFC, benzphetamine, and testosterone at 30, 21, and 9.6% of the rates for the unmodified P450 2B4, respectively. It is clear that the covalent modification by tBPA impairs the catalytic activity of P450 2B4, but the extent of the impaired activity varies with the nature of substrates.

Effect of the covalent modification of P450 2B4 by tBPA on the rate of electron transfer from P450 reductase to ferric P450 2B4. To investigate the mechanisms of the impaired catalytic activity, we measured the rate of electron transfer from CPR to the ferric P450 2B4, referred to as the first electron transfer. The kinetic traces for the first electron transfer to the unmodified P450 2B4 are shown in Figure 5, together with those for the tBPA-modified P450 2B4. In the absence of 1 mM benzphetamine, the kinetic trace for the unmodified P450 2B4 is biphasic and can be fit best with double exponentials to give the apparent rate constants, k_{obs}, of 0.14 (12%) and 0.007 s^{-1} (88%). The numbers in parentheses

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

are the relative amplitudes for respective kinetic phases. These rate constants and relative amplitudes are very similar to those of 0.33 (17%) and 0.008 s^{-1} (83%) for the tBPA-modified P450 2B4 in the absence of 1 mM benzphetamine. This similarity demonstrates that modification of the protein by tBPA does not result in a significant alteration in the rate of electron flow from CPR to the ferric P450 2B4. In contrast, the rate of the first electron transfer to the unmodified P450 2B4 is greatly enhanced in the presence of 1 mM benzphetamine and to much lesser extent for the tBPA-modified P450 2B4. Fitting of the kinetic trace for the reduction of the unmodified P450 2B4 in the presence of benzphetamine gives biphasic rate constants of 2.8 (86%) and 0.27 s^{-1} (14%). Thus, the k_{obs} for the fast phase is increased by ~20-fold in the presence of benzphetamine. A significant increase in the rate of the first electron transfer in the presence of benzphetamine (~ 10-fold) has previously been reported for the full-length P450 2B4 by other investigators (Imai et al., 1977; Vatsis et al., 1979). When compared with unmodified P450 2B4, the rate of the first electron transfer for tBPA-modified P450 2B4 is only marginally increased in the presence of 1 mM benzphetamine to give rate constants of 0.48 (22%) and 0.063 (78%). This marginal increase in the rate of electron transfer observed in the presence of benzphetamine is consistent with earlier conclusion that the binding of benzphetamine to the active site of P450 2B4 is impaired in tBPA-modified P450 2B4 (see Fig. 4).

Identification of the site for tBPA modification of P450 2B4 by LC-MS/MS. Peptide mapping was performed to identify the amino acid residue covalently

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

modified by tBPA. As previously shown in Figure 3, alkylation of the protein by tBPA leads to a mass increase of 174 Da. This mass increase of 174 Da was then used to search the dataset of the tryptic peptide fragments to determine the covalently modified peptide and amino acid residue. The SEQUEST search returned a doubly charged peptide with an m/z of 903.7 as the potential peptide modified by tBPA. This peptide corresponds to the amino acid sequence in P450 2B4 of ^{294}SLFFAGTETTSTTLR^{308}. The mass of this modified peptide was determined to be 1805.4 Da, which is 174 Da more than the theoretical mass for the unmodified peptide of 1631.8 Da. Thus, it is clear that tBPA is covalently bound to an amino acid residue in this peptide.

In order to determine the identity of the specific amino acid residue covalently modified by tBPA, the modified peptide was sequenced by LC-MS/MS and the result is shown in Figure 6. As shown, most of the expected fragment ions were detected. The y3 to y6 ions have the same masses as their theoretical masses at m/z 389.1, 490.2, 577.2 and 678.3, respectively. From the y7 ion forward, all the y ions observed (y7-y12) have a mass increase of 174 Da compared with the theoretical masses for the unmodified peptide, indicating that tBPA is covalently bound to the Thr302 (marked with an asterisk). This is also consistent with the observed b ions; the observed b9, b11, and b12 ions show a mass increase of 174 Da whereas the b3, b5, and b8 ions do not.

Molecular modeling of tBPA binding to P450 2B4. To better understand the mechanism of tBPA binding and inactivation at the atomic level, we used molecular modeling to investigate tBPA binding to P450 2B4. As shown in

Figure 7, tBPA is bound to the active site with the terminal carbon atom of the acetylenic group pointing towards the heme iron. The distance between the heme iron and the terminal carbon of tBPA is 2.78 Å. The residues within 4 Å of the bound tBPA in the active site include I101, F115, F297, A298, E301, T302, I363, V367, and V477. The distance between the terminal carbon of tBPA and the Oγ atom of Thr302 is 3.65 Å which puts them in van der Waals contact. The close proximity of Thr302 to tBPA makes it a primary target for covalent modification by the reactive intermediate of tBPA formed during catalysis. Therefore, molecular modeling provides evidence that helps to explain why Thr302 is the preferred site for covalent modification by tBPA.

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

In this study we have demonstrated that tBPA is a potent mechanism-based inactivator of P450 2B4. tBPA inactivates P450 2B4 with a K_I of 0.44 μM and a k_{inact} of 0.12 min^{-1} (Fig. 1). One outstanding feature for the mechanism-based inactivation of P450 2B4 by tBPA is the very low partition ratio, which is a measure of the ratio of product release to inactivation (Silverman, 1995). The near zero partition ratio indicates that the reactive intermediate of tBPA inactivates P450 without leaving the active site. The loss in catalytic activity is due to protein alkylation and not heme alkylation since a single tBPA-protein adduct has been identified by LC-MS analysis (Fig. 3) while HPLC analysis shows no loss in the unmodified heme content (data not shown).

The results of peptide mapping and the lack of evidence for the formation of any modified heme indicate that Thr302 is the primary target for covalent modification and inactivation by tBPA. Our molecular modeling studies shown in Figure 7 support this conclusion and shed light on why Thr302 is the preferred target for modification. It is well documented that acetylenic compounds may inactivate P450s by protein alkylation or heme alkylation (Chan et al., 1993; Ortiz de Montellano and Kunze, 1980; Ortiz de Montellano and Komives, 1985). Oxygenation of the internal carbon of the acetylenic group results in N-alkylation of the prosthetic heme whereas oxygenation of the terminal carbon usually leads to protein alkylation via a ketene intermediate. Evidence supporting the formation of a ketene intermediate includes formation of acetic acid product resulting from hydrolysis of the ketene during inactivation of P450 by acetylenes

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

(Chan et al., 1993; Komives and Ortiz de Montellano, 1987) . Therefore, we propose that tBPA is covalently bound to Thr302 through an ester bond as shown in Scheme I. It is likely that the initial oxygenation of the terminal carbon leads to formation of a ketene intermediate and this is then followed by nucleophilic attack by the Oγ atom of Thr302 to link the tBPA to the Thr302 via the ester bond. The results from molecular modeling are consistent with oxygenation of the terminal carbon. As shown in Figure 7, tBPA is bound in the active site with the acetylenic moiety being tilted at approximately a ~45^{o} angle to the heme plane and the terminal carbon is only about 2.78 Å away from the heme iron. This orientation would strongly favor the activated oxygen adding to the terminal carbon, leading to formation of the ketene intermediate. The residues in the proximity of the bound tBPA are mostly hydrophobic including I101, F115, F297, I363, V367 and V477. This is consistent with the hydrophobic nature of tBPA. However two hydrophilic residues, E301 and T302 are also within 4 Å of the bound tBPA. In particular, the terminal carbon of the acetylene is only 3.65 Å away from the Oγ of Thr302, which makes Thr302 the preferred target for covalent modification by the ketene intermediate. This close proximity of the ketene to the Thr302 may also explain the very low partition ratio of tBPA.

Covalent modification of the Thr302 residue in P450 2B4 may have multiple effects on P450 catalysis. Thr302 is thought to be a critical residue for P450 catalysis since it is believed to be involved in proton relay leading to formation of the oxyferryl species (Davydov et al., 1999; Davydov et al., 2001). Mutation of the Thr302 of P450 2B4 to an alanine results in loss of ~85% of the

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

activity for benzphetamine metabolism due to disruption of the proton delivery pathway (unpublished data). It is unclear at this stage how the covalent modification of Thr302 affects the proton delivery pathway. However, it is clear that covalent modification of Thr302 impairs the catalytic activity of P450 2B4. The remaining activities of the tBPA-modified P450 2B4 for the metabolism of 7-EFC, benzphetamine, and testosterone are only 30, 21 and 9.6% of the comparable activities of the unmodified P450 2B4. The loss in activity seems to correlate with the volumes of the substrates. The volumes of 7-EFC, benzphetamine, and testosterone are 226.6, 289.1, and 313.9 $Å^3$, respectively as calculated by a semi-empirical quantum PM3 method (unpublished data). The tBPA adduct seems to have the largest impact on the catalytic activity of the bulky substrate testosterone. This is conceivable because tBPA may reduce the active site volume by 198.7 $Å^3$ (volume of tBPA) and hence prevents testosterone from effectively binding into the active site.

The notion that the tBPA adduct affects substrate binding gains support from the stopped-flow experiments. In the absence of benzphetamine, CPR transfers the first electron to the tBPA-modified P450 2B4 at approximately the same rate as to the unmodified one (see Fig. 5). The rate of the first electron transfer is governed primary by two factors: the redox potential of P450s and the interaction between P450s and CPR. The similarity in the rate of the first electron transfer in the absence of benzphetamine indicates that the covalent modification by tBPA may not alter these factors significantly. It is reasonable to assume that the interactions between P450s and CPR are not affected since the

effect of covalent modification of Thr302 is likely localized in the vicinity of the active site.

It is unclear whether the tBPA adduct has any influence on the redox potential of the heme. As tBPA is hydrophobic, adduction of tBPA to Thr302 may change the hydrophobicity of the active site and hence modulate the redox potential. However, the lack of any significant change in the rate of the first electron transfer observed in the absence of benzphetamine suggests that any modulation in the redox potential is relatively insignificant with respect to its effect on the electron transfer rate. The substantial increase in the rate of the electron transfer to the unmodified protein in the presence of 1 mM benzphetamine can be attributed to an increase in the redox potential of P450 2B4 as it has been reported that benzphetamine binding to P450 2B4 raises the redox potential by ~85 mV, leading to a ~10-fold increase in the rate of the electron transfer from CPR to the ferric P450 2B4 (Zhang et al., 2003). This substantial stimulation of the rate of electron transfer is absent for the tBPA-modified P450 2B4. Thus, it can be postulated that benzphetamine is not bound in the active site or if it is bound it is not in the proper orientation inside the active site to raise the redox potential because of the steric hindrance imposed by the tBPA adduct. The active site void of P450 2B4 may not be large enough to allow the binding of a second substrate, in addition to tBPA, so that it is properly positioned for optimal catalysis. It is intriguing that the tBPA-modified P450 2B4 retains partial activities for metabolism of 7-EFC, benzphetamine, and testosterone even though substrate binding to the active site is adversely

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

affected by the tBPA adduct. The residual activities may arise from the conformational flexibility of the P450 structure that allows transient access of the substrates to the active site since it is well documented that the secondary structure of P450 2B4 is very flexible (Muralidhara et al., 2006; Zhao et al., 2006). Further studies are under way to investigate the effect of the tBPA adduct on the formation and stability of the oxygenated intermediates, the proton delivery processes, and the conformational flexibility of the P450 active site.

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

ACKNOWLEDGEMENTS

We would like to thank Drs. Mike Tarasev and David Ballou for their help with stopped-flow experiments.

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES

Blobaum AL, Harris, DL and Hollenberg PF (2005) P450 active site architecture and reversibility: Inactivation of cytochrome P450 2B4 and 2B4 T302A by tert-butyl acetylenes. *Biochemistry* **44**:3831-3844.

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.

Davydov R, Macdonald ID, Makris TM, Sligar S and Hoffman BM (1999) EPR and ENDOR of catalytic intermediates in cryoreduced native and mutant oxy-cytochromes P450cam: Mutation-induced changes in the proton delivery system. *J Am Chem Soc* **121**:10654-10655.

Davydov R, Makris TM, Kofman V, Werst DE, Sligar SG and Hoffman BM (2001) Hydroxylation of camphor by reduced oxy-cytochrome P450cam: mechanistic implications of EPR and ENDOR studies of catalytic intermediates in native and mutant enzymes. *J Am Chem Soc* **123**:1403-1415.

Denisov IG, Makris TM, Sligar SG and Schlichting I (2005) Structure and chemistry of cytochrome P450. *Chem Rev* **105**:2253-2277.

Gan LS, Acebo AL and Alworth WL (1984) 1-Ethynylpyrene, a suicide inhibitor of cytochrome P-450 dependent benzo[α]pyrene hydroxylase activity in liver microsomes. *Biochemistry* **23**:3827-3836.

Hamdane D, Zhang H and Hollenberg P (2008) Oxygen activation by cytochrome P450 monooxygenase. *Photosynth Res* **98**:657-666.

Hernandez CE, Kumar S, Liu H and Halpert JR (2006) Investigation of the role of cytochrome P450 2B4 active site residues in substrate metabolism based on crystal structures of the ligand-bound enzyme. *Arch Biochem Biophys* **455**:61-67.

Hollenberg PF, Kent UM and Bumpus NN (2008) Mechanism-based inactivation of human cytochromes p450s: experimental characterization, reactive intermediates, and clinical implications. *Chem Res Toxicol* **21**:189-205.

Imai Y, Sato R and Iyanagi T (1977) Rate-limiting step in the reconstituted microsomal drug hydroxylase system. *Journal of Biochemistry* **82**:1237-1246.

Kent UM, Bend JR, Chamberlin BA, Gage DA and Hollenberg PF (1997) Mechanism-based inactivation of cytochrome P450 2B1 by N-benzyl-1-aminobenzotriazole. *Chem Res Toxicol* **10**:600-608.

Kent UM, Juschyshyn MI and Hollenberg PF (2001) Mechanism-based inactivators as probes of cytochrome P450 structure and function. *Curr Drug Metab* **2**:215-243.

Kent UM, Lin HL, Mills DE, Regal KA and Hollenberg PF (2006) Identification of 17-alpha-ethynylestradiol-modified active site peptides and glutathione conjugates formed during metabolism and inactivation of P450s 2B1 and 2B6. *Chem Res Toxicol* **19**:279-287.

Komives EA and Ortiz de Montellano PR (1987) Mechanism of oxidation of pi bonds by cytochrome P-450. Electronic requirements of the transition state in the turnover of phenylacetylenes. *J Biol Chem* **262**:9793-9802.

Muralidhara BK, Negi S, Chin CC, Braun W and Halpert JR (2006) Conformational flexibility of mammalian cytochrome P450 2B4 in binding imidazole inhibitors with different ring chemistry and side chains. Solution thermodynamics and molecular modeling. *J Biol Chem* **281**:8051-8061.

Ortiz de Montellano P and Kunze KL (1980) Occurrence of a 1,2 shift during enzymatic and chemical oxidation of a terminal acetylene. *J Am Chem Soc* **102**:7373-7375.

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.

Paul KG, Theorell H and Akeson A (1953) The molar light absorption of pyridine ferroprotoporphyrin (Pyridine haemochromogen). *Acta Chem Sacnd* **7**:1284-1287.

Roberts ES, Alworth WL and Hollenberg PF (1998) Mechanism-based inactivation of cytochromes P450 2E1 and 2B1 by 5-phenyl-1-pentyne. *Arch Biochem Biophys* **354**:295-302.

Roberts ES, Hopkins NE, Zaluzec EJ, Gage DA, Alworth WL and Hollenberg PF (1994) Identification of active-site peptides from 3H-labeled 2-ethynylnaphthalene-inactivated P450 2B1 and 2B4 using amino acid sequencing and mass spectrometry. *Biochemistry* **33**:3766-3771.

Roberts ES, Hopkins NE, Zaluzec EJ, Gage DA, Alworth WL and Hollenberg PF (1995) Mechanism-based inactivation of cytochrome P450 2B1 by 9-ethynylphenanthrene. *Arch Biochem Biophys* **323**:295-302.

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.

Silverman RB (1995) Mechanism-based enzyme inactivators. *Methods Enzymol* **249**:240-283.

Vatsis KP, Oprian DD and Coon MJ (1979) Kinetics of reduction of purified liver microsomal cytochrome P-450 in the reconstituted enzyme system studied by stopped flow spectrophotometry. *Acta Biol Med Ger* **38**:459-473.

Wright AT, Song JD and Cravatt BF (2009) A Suite of Activity-Based Probes for Human Cytochrome P450 Enzymes. *J Am Chem Soc*., in press.

Zhang H, Gruenke L, Arscott D, Harris DL, Glavanovich M, Johnson R and Waskell L (2003) Determination of the rate of reduction of oxyferrous cytochrome P450 2B4 by 5-deazaFAD T491V cytochrome P450 reductase. *Biochemistry* **42**:11594-11603.

Zhang H, Hamdane D, Im SC and Waskell L (2008) Cytochrome b5 inhibits electron transfer from NADPH-cytochrome P450 reductase to ferric cytochrome P450 2B4. *J Biol Chem* **283**:5217-5225.

Zhang H, Kenaan C, Hamdane D, Hui Bon Hoa G and Hollenberg P (2009) Effect of conformational dynamics on substrate recognition and specificity as probed by the introduction of a de novo disulfide bond into cytochrome P450 2B1. *J. Biol. Chem.,* in press.

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 bifonazole: insight into P450 conformational plasticity and membrane interaction. *J Biol Chem* **281**:5973-5981.

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

FOOTNOTES

This work was supported in part by a grant from the National Cancer Institute and National Institute of Health [CA16954].

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

LEGENDS FOR FIGURES

Scheme I. Proposed mechanism for the formation of the tBPA-protein adduct.

Figure 1. Kinetics for the mechanism-based inactivation of P450 2B4 by *tert*-butylphenylacetylene at 30 ^{o}C. The primary reaction mixture contained equal amounts of P450 and CPR (1 μM each), and varying concentrations of tBPA. An aliquot of 10 μL of the primary reaction mixture was transferred to the secondary reaction mixture at the designated times to determine the 7-EFC O-deethylation activity remianing as described in Materials and Methods. The concentrations of tBPA varied from 0 (○), 0.3 (▽), 1 (□), 3 (◇) to 10 μM (△). The inset is a plot of the concentration dependence of the observed rates, V_{obs}, in the absence (●) and presence (○) of one equivalent of cyt b5.

Figure 2. Determination of the partition ratios for the mechanism-based inactivation of P450 2B4 by *tert*-butylphenylacetylene at 30 ^{o}C. The primary reactions were incubated with tBPA and NADPH for 30 min at various molar ratios of tBPA to P450 as indicated; the concentration of P450 was 1 μM and the concentrations of tBPA varied from 0 to 10 μM. The remaining activity for the inactivated P450 2B4 was assayed in the secondary reaction as described in Materials and Methods. (●) indicates the dataset obtained in the absence of cyt b5 in the primary reaction; (▲) indicates the dataset obtained in the presence of 1 μM cyt b5 in the primary reaction.

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3. Deconvoluted molecular masses of P450 2B4 and the modified P450 2B4 following reaction with *tert*-butylphenylacetylene under turnover conditions. Aliquots of the primary reaction mixture containing ~ 50 pmoles of P450 2B4 were loaded onto a RP C3 column and the P450 2B4 sample was separated from the rest of the components in the reconstitution mixture. The molecular mass of P450 2B4 was analyzed using ESI-LC/MS as described in Materials and Methods. The three traces represent the samples obtained at 5 (A), 10 (B), and 15 min (C) after addition of NADPH to the primary reaction mixture.

Figure 4. UV-visible spectra of unmodified P450 2B4 and tBPA-modified P450 2B4 in the presence or absence of benzphetamine. The spectra were measured at 30 ^{o}C in 0.1 M potassium phosphate buffer (pH 7.4) containing 15% glycerol and 0.2 mg/mL of DLPC. The concentrations of the unmodified and tBPA-modified P450 2B4 were 4.3 and 3.0 μM respectively as determined by the haemochromagen method (Paul et al., 1953). (—) indicates the unmodified ferric P450 2B4; (●●●) indicates the unmodified ferric P450 2B4 + 1 mM benzphetamine; (---) indicates the modified ferric P450 2B4; (-●●-) indicates the modified ferric P450 2B4 + 1 mM benzphetamine.

Figure 5. Kinetics for the first electron transfer from P450 reductase to unmodified and tBPA-modified P450 2B4 determined by stopped-flow spectrophotometry at 25 ^{o}C. The reconstituted P450 2B4 and CPR solution (3

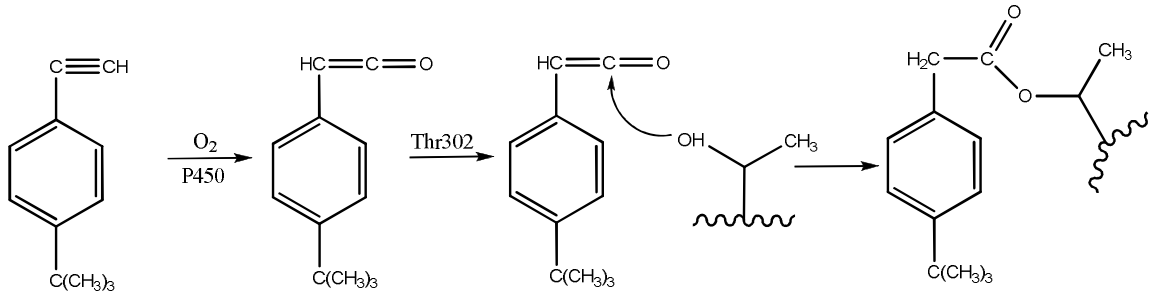
Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.

μM each) were rapidly mixed with 0.1 mM NADPH solution in the stopped-flow spectrophotometer and the kinetics of the electron transfer were monitored at 450 nm as described in Materials and Methods. Both solutions were saturated with CO and contained 1 mM benzphetamine (when present) prior to being loaded into the stopped-flow spectrophotometer. (---) indicates the unmodified P450 2B4; (—) indicates the unmodified P450 2B4 + 1 mM benzphetamine; (•••) indicates the modified P450 2B4; (-•-) indicates the modified P450 2B4 + 1 mM benzphetamine.

Figure 6. LC-MS/MS analysis of the tBPA-modified peptide ^{294}SLFFAGTETTSTTLR^{308} showing that Thr302 is the tBPA-modified residue. Thr302 is marked with an asterisk. The predicted fragment ion series (b and y ions) for the singly charged ion at *m/z* 1805.4 are denoted as unlabeled and the fragment ions observed for the modified peptide are indicated as labeled. The observed fragment ions are MS/MS spectra of the doubly charged precursor ion at *m/z* 903.7 obtained in positive mode using the Xcalibur software as described in Materials and Methods.

Figure 7. Molecular modeling showing the binding of tBPA in the active site of P450 2B4. tBPA was docked to the active site of P450 2B4 using Autodock software (ver. 4.0) as described in Materials and Methods. The I-helix and the heme are shown in yellow ribbon and red stick, respectively. Only the amino acid residues within 4 Å of the tBPA bound in the active site are shown.

Molecular Pharmacology Fast Forward. Published on August 31, 2009 as DOI: 10.1124/mol.109.059808
This article has not been copyedited and formatted. The final version may differ from this version.



Scheme I

Figure 1.

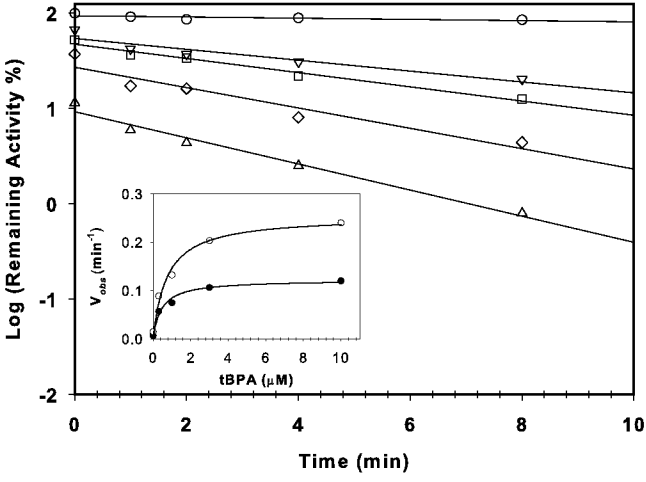

Log (Remaining Activity %)
2
1
0
-1
-2
0
2
4
6
8
10
Time (min)
v_{obs} (min^{-1})
0.3
0.2
0.1
0.0
0
2
4
6
8
10
tBPA (μM)

Figure 2.

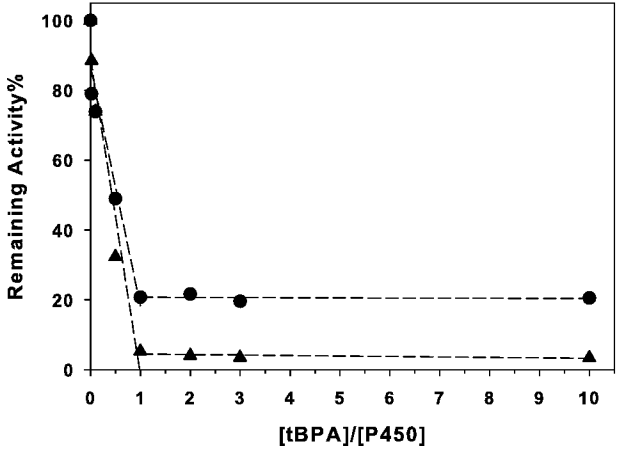


Figure 3.

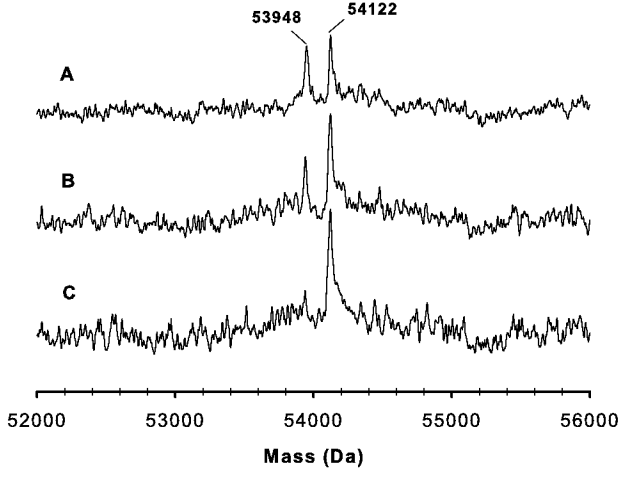


Figure 4

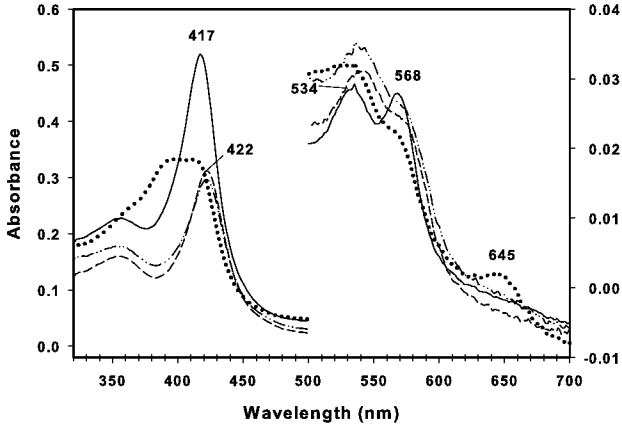

417
422
534
568
645
Absorbance
Wavelength (nm)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.04
0.03
0.02
0.01
0.00
-0.01
350
400
450
500
550
600
650
700

Figure 5

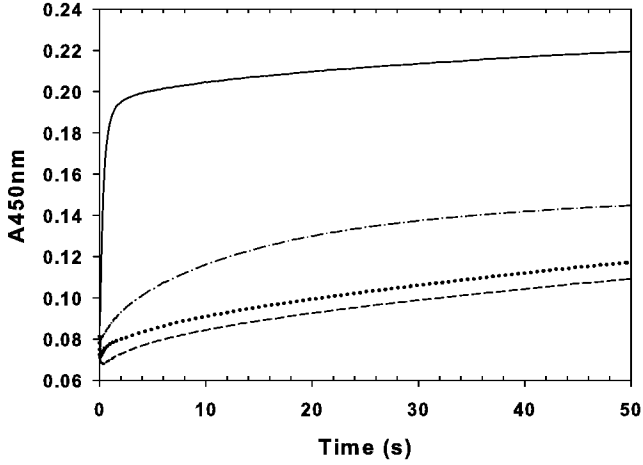

0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
A450nm
0
10
20
30
40
50
Time (s)

Figure 6

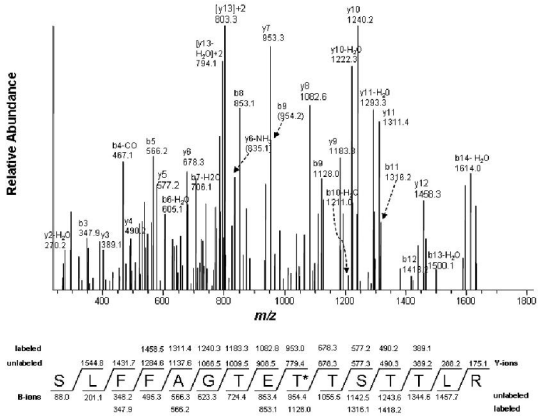

Relative Abundance
m/z
400
600
800
1000
1200
1400
1600
1800
[y13]+2 803.3
y10 1240.2
y7 953.3
[y13-H2O]+2 794.1
y10-H2O 1222.2
y8 1082.6
y8 (954.2)
y11-H2O 1293.3
y11 1311.4
b8 853.1
y6-NH3 (835.1)
b4-CO 467.1
b5 566.2
y9 678.3
y9 1183.3
b14- H2O 1614.0
y5 577.2
b7-H2O 706.1
b9 1128.0
b11 1316.2
y12 1468.3
b6-H2O 605.1
b10-H2O 1211.0
y2-H2O 220.2
b3 347.9
y4 490.2
y3 389.1
b12 1413.1
b13-H2O 1500.1
labeled
unlabeled
B-ions
Y-ions
S L F F A G T E T* T S T T L R
unlabeled
labeled

Figure 7