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tert-Butylphenylacetylene Is a Potent Mechanism-Based Inactivator of Cytochrome P450 2B4: Inhibition of Cytochrome P450 Catalysis by Steric Hindrance

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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. Liquid chromatography-mass spectrometry 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 to 422 nm compared with unmodified protein. Benzphetamine binding to the modified P450 2B4 causes no spin shift, indicating that substrate binding and/or the heme environment has been altered by covalently bound tBPA. Cytochrome P450 reductase 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.

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 in that 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 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. Because of 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 var-

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ABBREVIATIONS: P450, cytochrome P450; CPR, NADPH-dependent cytochrome P450 reductase; tBPA, *tert*-butylphenylacetylene; DLPC, dilauroylphosphatidylcholine; TFA, trifluoroacetic acid; 7-EFC, 7-ethoxy-4-trifluoromethylcoumarin; cyt b5, cytochrome b_5; ESI, electrospray ionization; LC, liquid chromatography; MSMS, mass spectrometry; MS/MS, tandem mass spectrometry.

ious isoforms of P450s (Ortiz de Montellano and Kunze, 1980; Komives and Ortiz de Montellano, 1987; Hollenberg et al., 2008; Wright et al., 2009). For example, 17α-ethynylestradiol, a synthetic contraceptive, has been reported to inactivate P450s 2B1 and 2B6 by protein alkylation of the peptides ^{347}PYTDAVIHEI^{376} and ^{347}PYTEAV^{365}, respectively (Kent et al., 2006). Both *tert*-butylacetylene and *tert*-butyl-1-methyl-2-propynyl ether inactivate P450 2E1 predominantly through the formation of heme adducts. A novel reversible formation of a *tert*-butylacetylene-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 (Gan et al., 1984; Ortiz de Montellano and Komives, 1985; Komives and Ortiz de Montellano, 1987; Chan et al., 1993). 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), and 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 due in part to low k_{inact} values 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 *tert*-butylphenylacetylene (tBPA) is a potent mechanism-based inactivator of P450 2B4 with a partition ratio near 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 monoalkylated 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.

Materials and Methods

Chemicals. All chemicals used are American Chemical Society reagent grade unless otherwise specified. Benzphetamine, 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 (Carlsbad, CA). 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).

Overexpression and Purification of P450 2B4, NADPH-Dependent CPR, and Cytochrome b_5. The plasmid for over-expression of P450 2B4 (pKK2B4dH) was a generous gift from Dr. James Halpert (University of California at San Diego, La Jolla, CA). P450 2B4 was expressed as a truncated form in which 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 overexpressed in *Escherichia coli* C41(DE3) cells and purified with a nickel-affinity column followed by a CM-Sepharose cation exchange column as described by Scott et al. (2001). NADPH-dependent CPR and cytochrome b_5 (cyt b5) were overexpressed and purified as described previously (Zhang et al., 2009).

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°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 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 in the preceding section was incubated as described previously except that incubation of the primary reaction mixture was allowed to proceed for 30 min at 30°C until the mechanism-based inactivation was complete. The activity remaining after the inactivation of P450 2B4 was analyzed using the secondary reaction mixture as described above. The partition ratio was then determined as described previously (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 an LCQ ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) as described previously (Zhang et al., 2009). After incubation with tBPA and NADPH for the times indicated in Fig. 3, an aliquot (50 μl) of the primary reaction solution was applied to a reversed-phase C3 column (2 × 150 mm, 5 μm) (Agilent Technologies, Santa Clara, 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 Bioworks software (Thermo Fisher Scientific).

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°C, the reaction was initiated by the addition of 1 mM NADPH and formation of the tBPA-protein adduct was monitored by LC-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 nickel-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°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 pmol) 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 h. The digested sample was centrifuged at 16,000*g* for 5 min, and a 50-μl aliquot of the supernatant was loaded onto a reversed-phase C18 column (2.0 × 100 mm, Jupiter; Phenomenex, 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 on an LCQ Deca XP mass spectrometer (Thermo Fisher Scientific). The instrument parameters were as follow: sheath gas flow, 60 (arbitrary units); auxiliary gas flow, 10 (arbitrary units); spray voltage, 4.5 kV; capillary voltage, 30 V; capillary temperature, 220°C; and collision-induced dissociation 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 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°C using a Hi-Tech SF61DX2 stopped-flow spectrophotometer (Hi-Tech, Wiltshire, UK) as described previously (Zhang et al., 2008). To preform an active P450-CPR complex, equimolar concentrations of P450 and CPR (3 μM each) and 0.15 mg/ml 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 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).

Docking of *tert*-Butylphenylacetylene into the Active Site of P450 2B4. The energy-based docking software Autodock (ver. 4.0; http://autodock.scripps.edu/) 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 (code 1suo). The coordinates of the water and other hetero atoms except for the heme were removed from the P450 2B4 before 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 semiempirical 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 described previously (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.

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, whereas 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 2-fold. 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 Fig. 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 near zero, which 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.

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

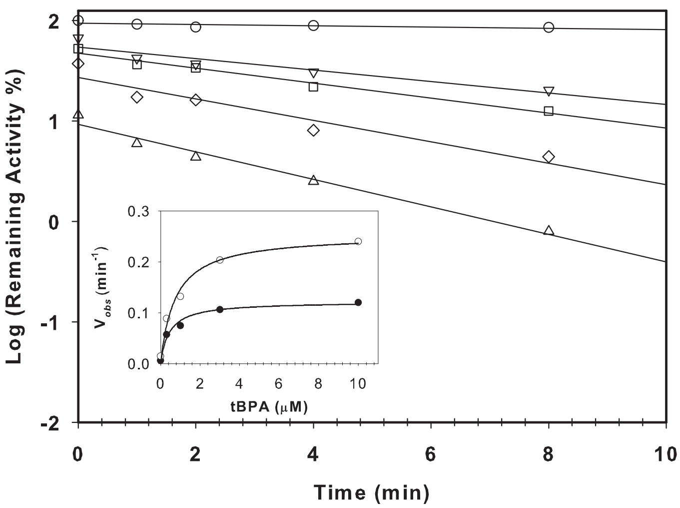


Fig. 1. Kinetics for the mechanism-based inactivation of P450 2B4 by *tert*-butylphenylacetylene at 30°C. The primary reaction mixture contained equal amounts of P450 and CPR (1 μM each), and varying concentrations of tBPA. An aliquot of 10 μl of the primary reaction mixture was transferred to the secondary reaction mixture at the designated times to determine the 7-EFC *O*-deethylation activity remaining as described under *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.

P450 2B4 that had catalyzed oxidation of tBPA under turnover conditions and been inactivated to varying extents. The results are shown in Fig. 3. The P450 2B4 sample obtained after 5 min of incubation with NADPH yielded two mass peaks at 53,948 and 54,122 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 54,122 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. After 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 Fig. 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 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. It is clear that benzphetamine binding to the unmodified P450 2B4 results in a typical substrate-induced low spin-to-high spin shift, or type I spectral change, as a result of 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 prox-

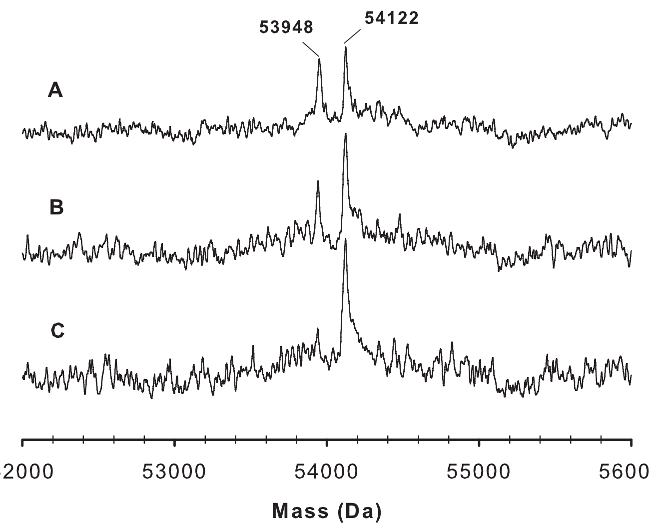


Fig. 3. Deconvoluted molecular masses of P450 2B4 and the modified P450 2B4 after reaction with *tert*-butylphenylacetylene under turnover conditions. Aliquots of the primary reaction mixture containing ~50 pmoles of P450 2B4 were loaded onto a RP C3 column and the P450 2B4 sample was separated from the rest of the components in the reconstitution mixture. The molecular mass of P450 2B4 was analyzed using ESI-LC/MS as described under *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.

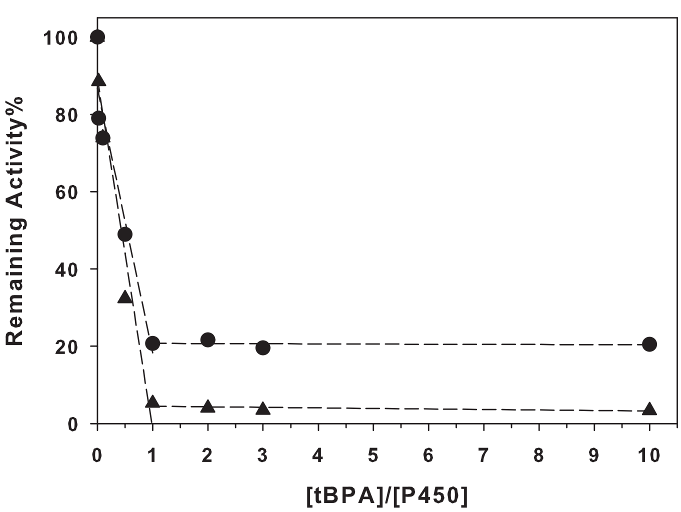


Fig. 2. Determination of the partition ratios for the mechanism-based inactivation of P450 2B4 by *tert*-butylphenylacetylene at 30°C. The primary reactions were incubated with tBPA and NADPH for 30 min at various molar ratios of tBPA to P450 as indicated; the concentration of P450 was 1 μM and the concentrations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

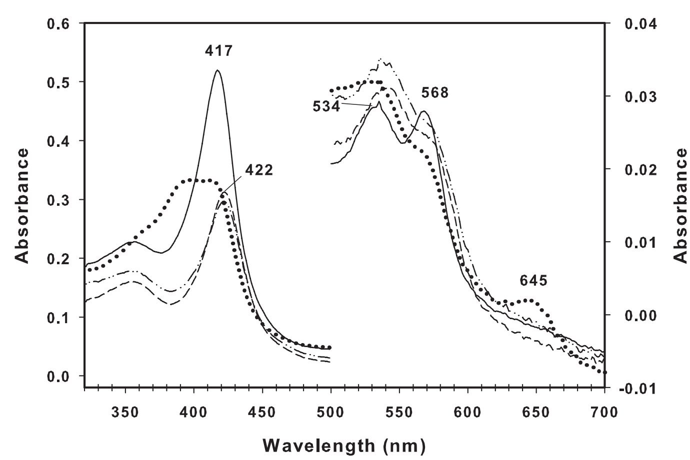


Fig. 4. UV-visible spectra of unmodified P450 2B4 and tBPA-modified P450 2B4 in the presence or absence of benzphetamine. The spectra were measured at 30°C in 0.1 M potassium phosphate buffer, pH 7.4, containing 15% glycerol and 0.2 mg/ml of DLPC. The concentrations of the unmodified and tBPA-modified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

imity 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 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 Fig. 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 best be fit with double exponential equations to give the apparent rate constants, k_{obs}, of 0.14 (12%) and 0.007 s^{-1} (88%). The numbers in parentheses 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 a 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). 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).

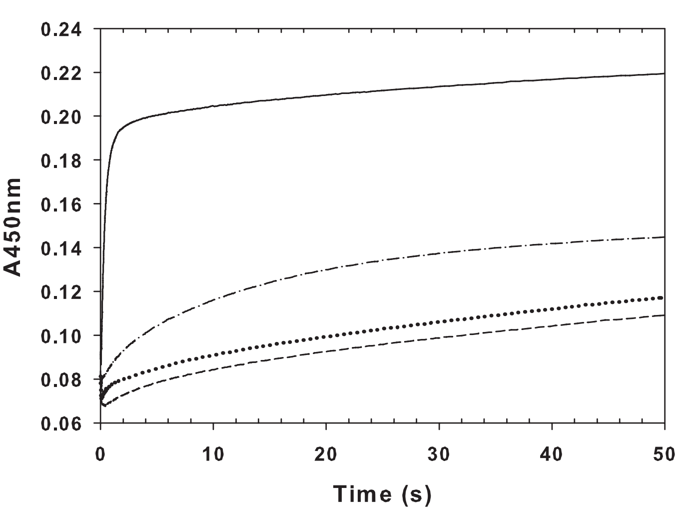


Fig. 5. Kinetics for the first electron transfer from P450 reductase to unmodified and tBPA-modified P450 2B4 determined by stopped-flow spectrophotometry at 25°C. The reconstituted P450 2B4 and CPR solution (3 μM each) were rapidly mixed with 0.1 mM NADPH solution in the stopped-flow spectrophotometer and the kinetics of the electron transfer were monitored at 450 nm as described under *Materials and Methods*. Both solutions were saturated with CO and contained 1 mM benzphetamine (when present) before being loaded into the stopped-flow spectrophotometer. - - -, unmodified P450 2B4; —, unmodified P450 2B4 + 1 mM benzphetamine; ···, modified P450 2B4; - · -, modified P450 2B4 + 1 mM benzphetamine.

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 modified by tBPA. As shown previously in Fig. 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.

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 Fig. 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 Fig. 7, tBPA is bound to the active site with the terminal carbon atom of the acetylenic group pointing toward 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 Ile101, Phe115, Phe297, Ala298, Glu301, Thr302, Ile363, Val367, and Val477. 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.

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 nea-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, because a single tBPA-protein adduct has been identified by LC-MS analysis (Fig. 3), whereas 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 Fig. 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 (Ortiz de Montellano and Kunze, 1980; Ortiz de Montellano and Komives, 1985; Chan et al., 1993). 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 (Komives and Ortiz de Montellano, 1987; Chan et al., 1993). Therefore, we propose that tBPA is covalently bound to Thr302 through an ester bond as shown in Scheme 1. 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 Fig. 7, tBPA is bound in the active site with the acetylenic moiety being tilted at approximately a ~45° angle

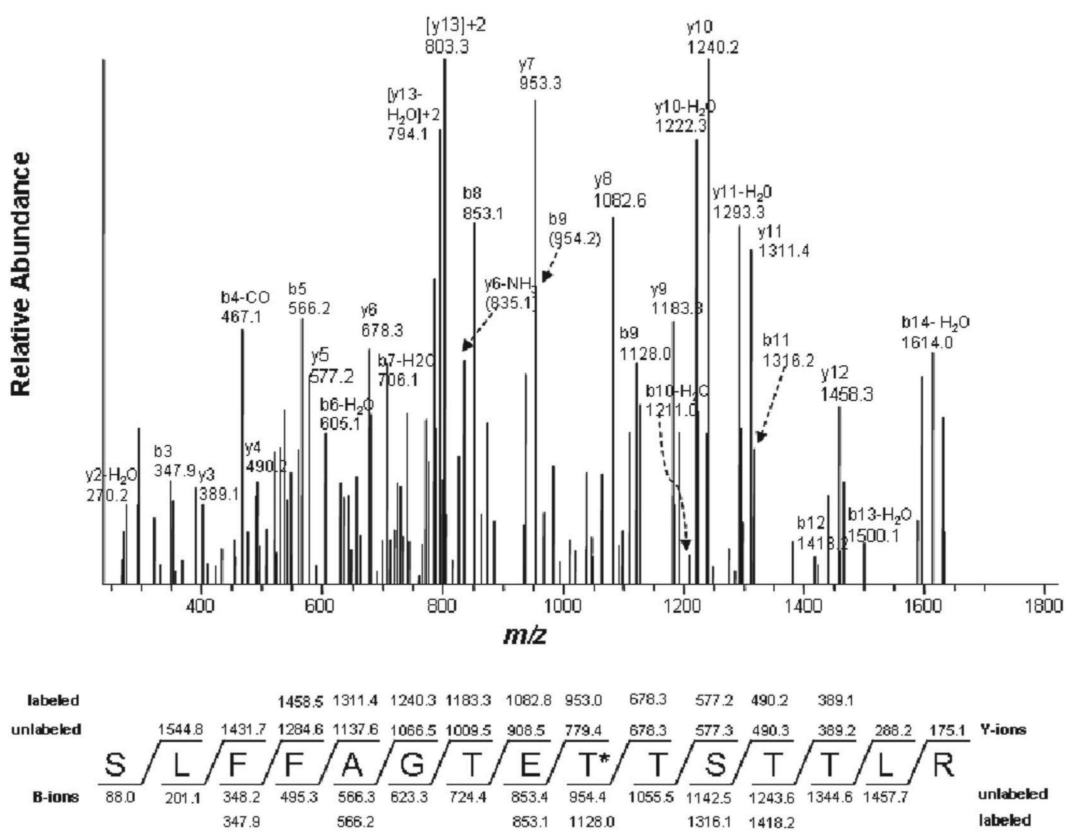


Fig. 6. LC-MS/MS analysis of the tBPA-modified peptide [294]SLFFAGTETTSTTLR[308] showing that Thr302 is the tBPA-modified residue. Thr302 is marked with an asterisk. The predicted fragment ion series (b and y ions) for the singly charged ion at *m/z* 1805.4 are denoted as unlabeled and the fragment ions 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 under *Materials and Methods*.

to the heme plane, and the terminal carbon is only approximately 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 Ile101, Phe115, Phe297, Ile363, Val367, and Val477. This is consistent with the hydrophobic nature of tBPA. However two hydrophilic residues, Glu301 and Thr302 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 because it is believed to be involved in proton relay leading to formation of the oxyferryl species (Davydov et al., 1999, 2001). Mutation of the Thr302 of P450 2B4 to an alanine results in loss of ~85% of the activity for benzphetamine metabolism as a result of disruption of the proton delivery pathway (H. Zhang and P. Hollenberg, 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 semiempirical quantum PM3 method (H. Zhang and P. Hollenberg, 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.

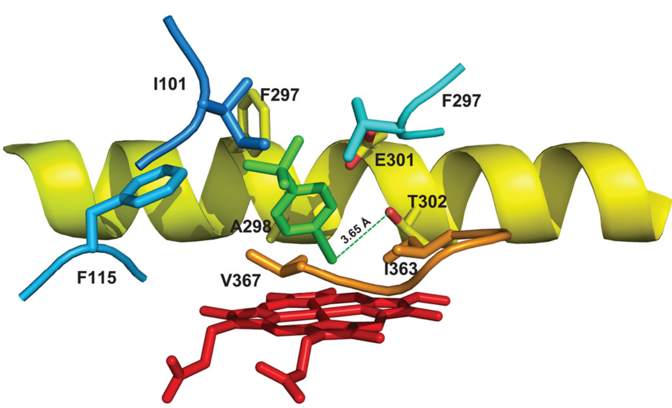
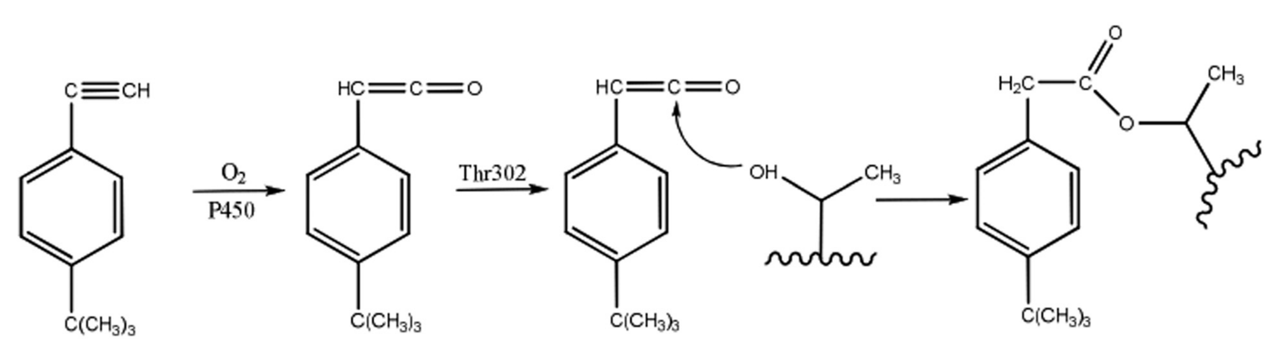


Fig. 7. Molecular modeling showing the binding of tBPA in the active site of P450 2B4. tBPA was docked to the active site of P450 2B4 using Autodock software (ver. 4.0) as described under *Materials and Methods*. The I-helix and the heme are shown in yellow ribbon and red stick, respectively. Only the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

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, because the effect of covalent modification of Thr302 is probably 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. Because 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, given that 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



Scheme 1. Proposed mechanism for the formation of the tBPA-protein adduct.

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 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 because 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.

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