Metabolism of N,N′,N″-Triethylenetriphosphoramid by CYP2B1 and CYP2B6 Results in the Inactivation of Both Isoforms by Two Distinct Mechanisms

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ABSTRACT

The anticancer drug \(N,N′,N″\)-triethylenetriphosphoramid (tTEPA) inactivated CYP2B6 and CYP2B1 in the reconstituted system in a time-, concentration-, and NADPH-dependent manner indicative of mechanism-based inactivation. The \(K_I\) value for the inactivation of CYP2B1 was 38 \(\mu\)M, the \(k_{inact}\) was 0.3 min\(^{-1}\), and the \(t_{1/2}\) value was 2.5 min. Spectral carbon monoxide (CO) binding and high-performance liquid chromatography heme studies of the tTEPA-inactivated CYP2B1 suggest that the loss in the enzymatic activity was primarily due to the binding of a reactive tTEPA intermediate to the 2B1 apoprotein. Inactivation by tTEPA in the presence of 7-ethoxycoumarin, an alternate substrate, reduced the rate of inactivation of CYP2B1. Incubations with tTEPA and NADPH resulted in greater than 90% loss in the 7-ethoxycoumarin O-deethylation and testosterone hydroxylation activity of CYP2B1. In contrast, benzphetamine metabolism was significantly less inhibited (47%). CYP2B6 was inactivated by tTEPA with a \(K_I\) value of 50 \(\mu\)M, a \(k_{inact}\) value of 0.1 min\(^{-1}\), and a \(t_{1/2}\) value of 14 min. However, unlike CYP2B1, the tTEPA-inactivated human isoform showed losses in the cytochrome P450 (P450) CO spectrum, the pyridine heme chromophore spectrum, and in the amount of native heme that were comparable with the loss in the 7-EFC and benzphetamine activity, suggesting that activity loss was brought about by a tTEPA-reactive intermediate damaging the CYP2B6 heme. CYP2B6 could only be protected from the tTEPA-dependent inactivation by the 2B6-specific substrate bupropion but not by other substrates of CYP2B6 such as benzphetamine, testosterone, or 7-ethoxycoumarin. The data indicate that tTEPA metabolism by these two 2B isoforms results in inactivation of the P450s by two distinct mechanisms.

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ABBREVIATIONS: P450, cytochrome P450; tTEPA, \(N,N′,N″\)-triethylenetriphosphoramid molecule; GSH, glutathione; 7-EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; DLPC, 1,2-dilaurylphosphatidylcholine; HPLC, high-performance liquid chromatography; 7-EC, 7-ethoxycoumarin.
Further complications arise from the fact that many drugs may be metabolized by more than one P450 isoform and therefore they may affect the clearance and metabolism of drugs administered at the same time, even though they may be metabolized by different P450s.

N,N,N’-Triethylenetriphosphoramid (tTEPA) is a non-specific antineoplastic agent that is used in the treatment of ovarian, breast, and bladder cancers (van Maanen et al., 2000). This drug was developed in the 1950s and has experienced a renewed interest due to its efficiency in treating cancer in high-dose regimens. Earlier studies by Ng and Waxman (1990) indicated that incubations of tTEPA with liver microsomes from phenobarbital-treated rats or purified rat 2B enzyme in the presence of NADPH resulted in a decrease in the testosterone 16β-hydroxylation activity in these preparations. The data were strongly indicative of a mechanism-based type of inactivation of CYP2B1 by tTEPA. However, it was not clear from these studies whether the loss in activity was irreversible (an absolute requirement for mechanism-based inactivation) and whether inactivation by tTEPA resulted in modification of the CYP2B1 protein or heme alkylated. It has been reported previously that tTEPA inhibited the S-mephenytoin N-demethylation activity of CYP2B6 in human liver microsomes, as well as in supersomes containing the expressed enzyme (Rae et al., 2002). No tTEPA-dependent loss in enzymatic activity was observed for other P450s that were tested (3A4, 2E1, 2C8, 2C9, 2C19, and 2D6). Interestingly, tTEPA was also reported to inhibit the conversion of cyclophosphamide to 4-hydroxycyclophosphamide, which is part of the metabolic pathway required for the activation of cyclophosphamide to a cytotoxic metabolite (Rae et al., 2002). Cyclophosphamide is often used in combination with other drugs for cancer chemotherapy. The highly toxic nature of cyclophosphamide, together with its narrow therapeutic window complicates its administration. If the metabolism of cyclophosphamide is interrupted by tTEPA, cyclophosphamide could accumulate to toxic levels in the plasma of patients and thereby lead to potentially serious clinical ramifications. Because mechanism-based inactivation of CYP2B6 by tTEPA could lead to adverse drug-drug interactions, it may require that combination therapies that include tTEPA together with other drugs metabolized by CYP2B6 be reevaluated. Therefore, understanding the mechanism(s) of the effects of tTEPA on CYP2B enzymes is essential due to the potential biomedical implications. In this report, we investigate the effects of tTEPA on the rat and human CYP2B isoforms. We present novel data showing that these two highly conserved isoforms of 2B metabolize tTEPA to reactive intermediates that inactivate the two P450s by different mechanisms. Our observations further suggest that P450 inhibitor data derived from rat enzyme preparations may not necessarily be valid in the human system.

Materials and Methods

Materials. NADPH, catalase, dilauroyl-L-α-phosphatidylcholine (DLPC), bovine serum albumin, and reduced glutathione (GSH) were purchased from Sigma-Aldrich (St. Louis, MO). tTEPA was purchased from U.S. Pharmacopeia (Rockville, MD), and 7-ethoxy-4-(trifluoromethyl)coumarin (7-EFC) was from Molecular Probes (Eugene, OR). Trifluoroacetic acid and Slide-A-Lyzer cassettes were from Pierce Chemical (Rockford, IL). All other reagents were reagent grade and purchased from commercial sources.

Purification of P450s and Reductase. CYP2B1, CYP2B6, and NADPH reductase were expressed in Escherichia coli MV3104 cells and purified according to previously published procedures (Hanna et al., 1998, 2000; Kent et al., 1999; Spatzenegger et al., 2003).

Enzyme Activity Assay and Inactivation of CYP2B1 and CYP2B6. Purified CYP2B1 and reductase were reconstituted with lipid for 45 min at 4°C. CYP2B6 was incubated with reductase for 45 min at 4°C. The primary reaction mixtures contained 1 μM CYP2B1 or CYP2B6, 1 μM reductase (or 2 μM for the assays with CYP2B6), 60 μg of DLPC (or no DLPC for CYP2B6), 110 U of catalase, and tTEPA in water at concentrations between 0 and 80 μM (or 0–200 μM for CYP2B6) in 50 mM potassium phosphate buffer (pH 7.4) in a total volume of 1 ml. The primary reaction mixtures were incubated for 5 min at 30°C before initiating the reactions with 1.2 mM NADPH. At 0, 5, 10, and 20 min after the NADPH was added, 12 μl (12 μl for CYP2B6) aliquots were taken from the primary reaction mixture and transferred into a secondary reaction mixture containing 100 μM 7-EFC, 1 mM NADPH, and 40 μg of bovine serum reductase in 50 mM potassium phosphate buffer (pH 7.4) (Buters et al., 1983). The secondary reaction mixtures were incubated for 5 min (10 min for CYP2B6) at 30°C, and the reaction mixtures were then quenched with 334 μl of cold acetone. The activity remaining was determined spectrophotometrically by measuring the amount of 7-hydroxy-4-(trifluoromethyl) coumarin formed using an excitation of 410 nm and an emission of 510 nm on a RF-5301 spectrofluorophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD).

Spectrophotometric Quantitation of P450s. P450s and reductase were reconstituted with or without lipid and incubated for 45 min at 4°C as described above. The reconstitution mixture was divided into three aliquots and incubated at 30°C for 10 min. The control sample received water and 1.2 mM NADPH. The exposed sample received 80 μM tTEPA (for CYP2B1) or 200 μM tTEPA (for CYP2B6), and the third sample was inactivated with 80 or 200 μM tTEPA and 1.2 mM NADPH. Activity was measured at 0 and 10 min (15 min for CYP2B6) using 7-EFC as the substrate as described above. The remaining samples were added to 920 μl of ice-cold quench buffer [40% glycerol and 0.6% Tergitol Nonidet P-10 in 50 mM potassium phosphate (pH 7.4)] and gently bubbled with CO for about 60 s. The spectra were recorded from 400 to 500 nm before and after the addition of solid dithionite on a DW2 UVNis spectrophotometer (SLM Aminco, Urbana, IL) equipped with an OLIS spectroscopy operating system (On-Line Instrument Systems, Inc., Bogart, GA). The absorbance difference between 450 and 490 nm was used to assess the extent of CO binding to the P450s (Omura and Sato, 1964). The pyridine hemochrome spectrum for the control and tTEPA-inactivated samples was also analyzed as described by Koop using an extinction coefficient of 34.6 mM cm⁻¹ (Koop, 1990).

Effect of tTEPA Inactivation on the Heme of P450s 2B1 and 2B6. HPLC analysis was performed to determine the effect of the inactivation by tTEPA on the P450 heme. CYP2B1 or CYP2B6 and reductase were reconstituted as described above. Samples were either incubated only with 80 μM tTEPA (for CYP2B1) or 200 μM tTEPA (for CYP2B6), or inactivated in the presence of tTEPA and 1.2 mM NADPH for 10 or 15 min. The enzymatic activity remaining was determined at 0 and 10 (15) min using the 7-EFC assay as described above. The remaining samples were then analyzed for P450 remaining using the CO reduced spectral assay as described above and for the intact heme by HPLC on a 4.6 × 250-mm C₄ column (Phenomenex, Torrance, CA) equilibrated with 30% solvent B. The solvent system consisted of solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile). The sample components were resolved using a linear gradient from 30 to 95% B over 35 min at a flow rate of 1 ml/min. The elution profile was monitored using a photodiode array detector (Waters, Milford, MA). The native
heme eluted at 21.8 min and was quantified by integrating the area under the heme peak at 405 nm.

Effect of Exogenous Nucleophiles on the Rate of Inactivation of CYP2B1 and CYP2B6 by tTEPA. CYP2B1 and CYP2B6 were reconstituted with reductase for 45 min at 4°C as described above. Incubation mixtures contained 1 μM CYP2B1, 1 μM reductase, 1 μg of dilauroyl-L-α-phosphatidylcholine/ml, and 110 U of catalase/ml in 50 mM potassium phosphate buffer (pH 7.4). The primary CYP2B1 reaction mixtures contained either 80 μM tTEPA or 80 μM tTEPA together with 10 mM glutathione. Control samples received either solvent or glutathione. The reaction mixtures were initiated with NADPH, and the enzymatic activity was assayed using 7-EFC as described above. Similarly, CYP2B6 was reconstituted with reductase. Incubation mixtures contained 1 μM CYP2B1, 2 μM reductase, and 110 U of catalase/ml in 50 mM potassium phosphate buffer (pH 7.4). The primary reaction mixtures contained either 100 μM tTEPA or 200 μM tTEPA together with 10 mM glutathione.

Irreversibility of Inactivation of CYP2B1 and CYP2B6 by tTEPA. CYP2B1 or CYP2B6 was reconstituted and inactivated with 80 or 200 μM tTEPA as described above. Control samples were incubated with tTEPA in the absence of NADPH. Aliquots were removed at 0 and 10 min (15 min for CYP2B6) to assess the extent of inactivation using the 7-EFC assay as described above. Control and tTEPA-inactivated samples (300 μl) were dialyzed separately overnight at 4°C in Slide-A-Lyzer cassettes against 2 × 500 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. After dialysis, the P450 samples were incubated with or without fresh reductase on ice for 15 min and then assayed for activity using the 7-EFC assay as described above.

Partition Ratio. The partition ratios for the inactivation of P450s 2B1 and 2B6 by tTEPA were determined by incubating the reconstituted P450s with increasing concentrations of tTEPA in the presence of 1.2 mM NADPH. Aliquots were removed to determine the residual activity at 10 and 15 min for CYP2B1 or at 20 min for CYP2B6 after adding NADPH to ensure that the inactivation reaction had gone to completion. The percentage of activity remaining was plotted as a function of the ratio of the molar concentrations of tTEPA versus CYP2B1 or CYP2B6. The partition ratio was estimated from the intercept between the linear regression line derived from the lower CYP2B1 or CYP2B6. The partition ratio was estimated from the intercept between the linear regression line derived from the lower CYP2B1 or CYP2B6. The partition ratio was estimated from the intercept between the linear regression line derived from the lower CYP2B1 or CYP2B6.

Substrate Protection from tTEPA-Dependent Inactivation. Substrate protection from tTEPA-dependent inactivation of CYP2B1 was assessed using the alternate CYP2B1 substrate 7-ethoxycoumarin (7-EC). Samples were reconstituted as described above and were incubated with NADPH in the presence of 100 μM tTEPA or 100 μM CYP2B1 together with 1 mM 7-EC. Control reaction mixtures contained either solvent or 7-EC alone. Aliquots (10 μl) were removed at 0, 1, 2, 4, and 8 min and assayed on a secondary 7-EC reaction mixture and assayed for activity remaining as described above.

CYP2B6 was reconstituted as described above. Samples were incubated in the presence of either 100 to 200 μM tTEPA alone or 100 to 200 μM tTEPA together with 1 to 2 mM 7-EC, 10 mM benzphetamine, 2 mM testosterone, or 2 mM bupropion. Aliquots (12 μl) were removed at 0, 5, 10, 16, and 21 min and added to a secondary 7-EC reaction mixture and assayed for activity remaining as described above.

Other Assays. CYP2B1 and CYP2B6 were reconstituted with reductase and inactivated with tTEPA as described above. Samples were incubated with 100 μM tTEPA (CYP2B1) or 200 μM tTEPA (CYP2B6) at 30°C for 15 min in the presence or absence of 1 mM NADPH. Aliquots were removed at 0 and 15 min and assayed in duplicate for 7-EC O-deethylation (5 pmol of CYP2B1, 12 pmol of CYP2B6), testosterone hydroxylations (25 pmol of CYP2B1), or benzphetamine N-demethylation (25 pmol of CYP2B1, 50 pmol of CYP2B6) activity. Testosterone hydroxylations were measured using HPLC chromatography (Aoyama et al., 1989). The testosterone assay mixture contained 0.2 mM testosterone, 0.2 mM NADPH in 50 mM potassium phosphate buffer, pH 7.4. Metabolites were extracted twice with 1 ml of ethyl acetate, the solvent was evaporated under nitrogen, and the samples were chromatographed on a C18 reverse-phase column equilibrated with 65% methanol and detected at 254 nm using a photodiode array detector (Waters).

Benzphetamine N-demethylation was measured spectrophotometrically (deAndre et al., 1996). The reaction mixture contained 1.5 mM benzphetamine and 0.2 mM NADPH in 50 mM potassium phosphate, pH 7.4. Enzymatic activity was stopped by the addition of one-half volume of 60% trichloroacetic acid. All samples were cooled on ice for 10 min and centrifuged for 30 min at 12,000 rpm in an Eppendorf Microfuge at 4°C. To 500 μl of sample, 500 μl of FluorolP reagent was added, and the samples were incubated at 37°C for 20 min. Fluorescence was measured with excitation at 410 nm and emission at 510 nm on a Shimadzu 500FP spectrofluorometer (Shimadzu Scientific Instruments, Inc., Columbia, MD).

Binding Spectra. The ability of tTEPA-inactivated CYP2B1 to bind substrate was determined by exposing the control and tTEPA-inactivated CYP2B1 to increasing concentrations of benzphetamine or n-octylamine and measuring the change in absorbance between 350 and 450 nm. Samples contained 4 μM CYP2B1, 4 μM reductase, 400 μg of DLPC, 985 U of catalase, and 200 μM tTEPA in 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 4 ml. This incubation mixture was split into two samples. The control sample received water and the inactivated sample was incubated with 1.2 mM NADPH. At 0 and 10 min after adding NADPH, 5-μl aliquots (5 pmol of CYP2B1) were removed and assayed for 7-EFC activity as described above. Control and tTEPA-inactivated samples were dialyzed at 4°C overnight against 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol to remove free tTEPA and NADPH. The dialyzed control or tTEPA-inactivated samples were then analyzed by recording the absorbance spectra between 400 and 600 nm after the addition of increasing concentrations of benzphetamine or n-octylamine. Difference spectra were recorded for benzphetamine or testosterone concentrations between 0 and 200 μM and n-octylamine concentrations between 0 and 8 μM. Ks values were estimated by plotting the inverse of the absorbance changes between 385 and 419 nm (type I spectrum) versus the concentration of benzphetamine or testosterone, and between 392 and 425 nm (type II spectrum) as a function of the concentration of n-octylamine.

Identification of tTEPA Metabolites by Electrospray Ionization-Liquid Chromatography-Tandem Mass Spectrometry. tTEPA metabolism was analyzed by electrospray ionization-liquid chromatography-tandem mass spectrometry on a LCQ (Thermoquest; Thermo Finnigan, San Jose, CA) mass analyzer controlled by Excalibur software (Thermoquest; Thermo Finnigan) essentially as described previously with minor modifications (van Maanen and Beijen, 1999). P450s (1 μM) were reconstituted as described above and samples containing 250 pmol of P450 each were incubated with tTEPA in the presence or absence of NADPH for 20 or 60 min. Some samples also received either 10 mM GSH or 10 mM N-acetylcysteine during the incubation with tTEPA. Enzymatic activity was assessed at the beginning and after 20 (CYP2B1) or 60 min (CYP2B6) using 7-EFC as described above. At the end of the incubation time the internal standard sulfadiazine (10 μl of a 100 μg/ml solution) was added to each sample, and the entire sample was immediately applied to a 500-μl C18 solid-phase extraction cartridge. The cartridge was washed with water, and tTEPA and the metabolites were eluted with 60% methanol. The metabolite-containing fractions were combined, dried under nitrogen, dissolved in water, and applied to a C18 reverse-phase column (4.5 × 100 mm, 5 μm; Waters) equilibrated with 10 mM ammonium acetate, pH 4.8, and 17.5% acetonitrile. Samples were chromatographed at 0.2 ml/min under isocratic conditions and the effluent was directed into the mass analyzer. The analyzer conditions were auxiliary gas, 30 arbitrary units; sheath gas, 90 arbitrary units; capillary temperature, 170°C; spray voltage, 4.5 kV; and capillary voltage, 45 V.
Results

Inactivation of CYP2B1 and CYP2B6 by tTEPA. Initial studies were performed to test whether tTEPA (Fig. 1) was a mechanism-based inactivator of rat CYP2B1 or human CYP2B6 in the reconstituted system with 7-EFC as the substrate. The time- and concentration-dependent losses in the 7-EFC O-deethylation activity of CYP2B1 and CYP2B6 are shown in Figs. 2 and 3. Pseudo first order kinetics was observed for CYP2B1 between 5 and 80 μM and for CYP2B6 between 5 and 200 μM of tTEPA. The kinetic constants describing the inactivation of CYP2B1 and CYP2B6 by tTEPA were determined from the double-reciprocal plots of rate\(^{-1}\) versus [tTEPA]\(^{-1}\) (Figs. 2 and 3, insets). For CYP2B1, the maximal rate of inactivation at saturation (\(k_{\text{inact}}\)) was 0.3 min\(^{-1}\), the concentration of tTEPA required for half-maximal inactivation (\(K_I\)) was 38 μM, and the time required for half of the enzyme molecules to be inactivated (\(t_{1/2}\)) was 2.5 min. These values are in good agreement with the data for CYP2B1 purified from rat and reported previously using 7-EC or androstenedione as substrates (Ng and Waxman, 1990). The approximate kinetic constants describing the inactivation of CYP2B6 by tTEPA were \(K_I = 50 \mu M\), \(k_{\text{inact}} = 0.1\) min\(^{-1}\), and \(t_{1/2} = 14\) min. Metabolic turnover of the inhibitor by the target enzyme is a prerequisite for mechanism-based inactivation. The data in Table 1 show an absolute requirement for NADPH in the inactivation of CYP2B1 and CYP2B6 by tTEPA. Activity losses were seen only when the samples were incubated in the presence of both NADPH and tTEPA.

The partition ratios obtained for CYP2B1 and CYP2B6 with tTEPA were approximately 166 and 96, respectively (data not shown).

The effects of the tTEPA-dependent inactivation on the CYP2B1 and CYP2B6 heme are shown in Table 1. Whereas CYP2B1 lost approximately 60% of its enzymatic activity, the amount of a spectrally detectable CO complex was not decreased after the inactivation by tTEPA. Similarly, the levels of pyridine hemochrome complex obtained with the control and the tTEPA-inactivated CYP2B1 samples were comparable. HPLC analysis of CYP2B1 samples incubated with tTEPA in the presence or absence of NADPH showed that approximately 91% of the area under the heme peak at 405 nm was retained by the tTEPA-inactivated sample. Together, the data suggest that the activity loss in CYP2B1 was not due to the destruction of the heme moiety during inactivation by tTEPA. In contrast, CYP2B6 showed losses in the P450-reduced CO spectrum, the pyridine hemochrome spectrum, and in the amount of HPLC-detectable intact heme that were comparable with the loss in enzymatic activity. No new peaks absorbing at 405 nm indicative of intact heme adducts were observed in the HPLC chromatograms of the tTEPA-inacti-

![Chemical structure of tTEPA](Image 110x72 to 230x164)

Fig. 1. Chemical structure of tTEPA.

![Concentration- and time-dependent inactivation of CYP2B1 7-EFC O-deethylation activity by tTEPA.](Image 308x291 to 560x467)

Fig. 2. Concentration- and time-dependent inactivation of CYP2B1 7-EFC O-deethylation activity by tTEPA. Aliquots were removed from the primary reaction mixtures at 0, 1, 2, 4, and 5 min and assayed for activity using 7-EFC as described under Materials and Methods. The primary reaction mixtures contained 0 (□), 5 (▲), 10 (●), 20 (●), 40 (○), and 80 (●) μM tTEPA. The data shown are the mean and standard deviation from six separate experiments. The inset shows the double-reciprocal plot of the rates of inactivation as a function of the tTEPA concentration.

![Time- and concentration-dependent inactivation of CYP2B1 and CYP2B6 by tTEPA.](Image 308x557 to 560x737)

Fig. 3. Time- and concentration-dependent inactivation of CYP2B6 7-EFC O-deethylation activity by tTEPA. Aliquots were removed from the primary reaction mixtures at 0, 1, 2, 4, and 5 min after NADPH was added and assayed for activity using 7-EFC as described under Materials and Methods. The primary reaction mixtures contained 0 (□), 5 (▲), 10 (●), 20 (●), 40 (○), 80 (●), 140 (○), and 200 (●) μM tTEPA. The data shown are the mean and standard deviation from three to five separate experiments. The inset shows the double-reciprocal plot of the rates of inactivation as a function of the tTEPA concentration.

![Effect of Exogenous Nucleophiles on the Rate of Inactivation of CYP2B1 and CYP2B6 by tTEPA.](Image 308x720 to 560x900)

Effect of Exogenous Nucleophiles on the Rate of Inactivation of CYP2B1 and CYP2B6 by tTEPA. Figure 4A shows the effect of the exogenous nucleophile GSH (10 mM) on the inactivation of CYP2B1 by tTEPA. Similar to previous observations, GSH did not greatly affect the rate of inactivation of CYP2B1 when 7-EFC was used as the substrate (Ng and Waxman, 1990). The rate of inactivation by tTEPA in the presence of GSH was 30% slower than the rate of inactivation in the absence of GSH. When CYP2B6 was incubated in the
Table 1
Effect of tTEPA on the 7-EFC O-deethylation activity, P450 content, heme, and pyridine hemochrome of CYP2B1 and CYP2B6

<table>
<thead>
<tr>
<th>Primary Reaction Conditions</th>
<th>Activity Remaining</th>
<th>P450 Remaining</th>
<th>HPLC Heme</th>
<th>Pyridine Hemochrome</th>
</tr>
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<tbody>
<tr>
<td>CYP2B1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- tTEPA + NADPH</td>
<td>102 ± 16</td>
<td>108 ± 13</td>
<td>100 ± 8</td>
<td>100</td>
</tr>
<tr>
<td>+ tTEPA - NADPH</td>
<td>108 ± 8</td>
<td>111 ± 18</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>+ tTEPA + NADPH</td>
<td>41 ± 6</td>
<td>126 ± 6</td>
<td>91 ± 5</td>
<td>90 ± 12</td>
</tr>
<tr>
<td>CYP2B6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- tTEPA + NADPH</td>
<td>90 ± 4</td>
<td>93 ± 1</td>
<td>94 ± 2</td>
<td>100</td>
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<tr>
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<td>96 ± 2</td>
<td>84 ± 3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>+ tTEPA + NADPH</td>
<td>63 ± 3</td>
<td>72 ± 5</td>
<td>60 ± 4</td>
<td>60 ± 12</td>
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</table>

ND, not determined.

The presence of tTEPA and 10 mM GSH, a 30% reduction in the rate of inactivation was again seen (Fig. 4B). In the absence of GSH the inactivation of CYP2B6 by tTEPA was slowed at later time points, whereas more linear kinetics were observed in the presence of 10 mM GSH. The reduction in the rates of inactivation in the presence of GSH may indicate that some of the reactive tTEPA intermediate leaves the active site to disrupt enzymatic activity by either binding somewhere else on the P450 molecule or by affecting reductase activity or binding. Alternatively, GSH may have access to the active sites of P450s 2B1 and/or 2B6 and compete with tTEPA, thereby slowing the inactivation. The data suggest, however, that the majority of the reactive tTEPA intermediate does not leave the enzyme active site.

Irreversibility of CYP2B1 and CYP2B6 Inactivation by tTEPA. Inactivation of CYP2B1 by tTEPA was irreversible with overnight dialysis (Table 2). Two separate experiments where the CYP2B1 enzyme was inactivated to different extents are shown in Table 2. The percentage of activity remaining in each case increased only slightly (approximately 10%) after overnight dialysis. Upon reconstitution with fresh reductase, an additional small increase in the CYP2B1 activity was observed. The data suggest that with CYP2B1, there is a small population of enzyme (10–15%) that is reversibly inhibited by tTEPA or a product of tTEPA metabolism. With CYP2B6, there was no recovery of activity after overnight dialysis and the addition of fresh reductase also did not restore activity to the samples (Table 2). These data support the observations recorded in Table 2 concerning the CYP2B6 heme. If heme destruction is the primary cause of activity loss, no recovery in the enzymatic activity would be expected.

Substrate Protection. The effect of an alternate substrate on the inactivation of CYP2B1 by tTEPA is shown in Fig. 5A. The alternate CYP2B1 substrate 7-EC, at a 10-fold molar excess over tTEPA, was able to afford CYP2B1 some protection from inactivation. Inclusion of 7-EC alone had no effect on the 7-EFC assay used to measure inactivation. The data support the notion that tTEPA metabolism by CYP2B1 leads to the formation of a reactive intermediate that inactivates the enzyme by binding to an amino acid residue within the active site of the P450. Substrate protection from tTEPA-dependent CYP2B6 inactivation was attempted using the 2B substrates 7-EC, benzphetamine, or testosterone in 5- to 50-fold molar excess over tTEPA. None of these 2B substrates were able to compete for inactivation of CYP2B6 by tTEPA. If, however, bupropion was used in a 10-fold molar excess over tTEPA, some substrate protection was seen with CYP2B6 as well (Fig. 5B).

Effect of tTEPA Inactivation on the Spectral Binding of Benzphetamine, n-Octylamine, and Testosterone. The inactivation of CYP2B1 by tTEPA presumably was brought about by the binding of a reactive tTEPA intermediate to the apoprotein. Spectral studies were performed to test whether the consequence of this inactivation was an inability of the tTEPA-modified P450 to bind substrate.

Fig. 4. Time- and concentration-dependent inactivation of the CYP2B1 and CYP2B6 7-EFC O-deethylation activity by tTEPA in the presence or absence of glutathione. All quotients were removed from the primary reaction mixtures at 0, 1, 2, 4, and 8 min and assayed as described under Materials and Methods. The data shown are the mean and standard deviation from three separate experiments. CYP2B1 (A) and CYP2B6 (B): control (■), control with 10 mM GSH (□), tTEPA-inactivated (●) and tTEPA-inactivated with 10 mM GSH (○).
ability of tTEPA-inactivated CYP2B1 to bind substrate was examined using three 2B substrates. Addition of benzphetamine or testosterone to the control and the tTEPA-inactivated CYP2B1 brought about a spectral change characteristic of a type I binding spectrum with a trough at 385 nm and a peak at 419 nm (data not shown). For benzphetamine or testosterone, the inactivation (62% of control) resulted in an approximately 1.5-fold increase in the $K_s$ value of the tTEPA-inactivated sample compared with the control. With $n$-octylamine, a typical type II spectral binding spectrum with a peak at 425 nm and a trough at 392 nm was observed (data not shown). Virtually no difference in the extrapolated spectral binding constant was seen for $n$-octylamine (Table 3).

**Effect of tTEPA Inactivation on the Metabolism of Testosterone and Benzphetamine by CYP2B1 and CYP2B6.** Table 4 shows that the loss in the 7-EFC O-deethylation activity (93% loss) of the tTEPA-inactivated CYP2B1 samples was comparable with the decrease (92% loss) in the testosterone hydroxylation activity. The formation of all three metabolites (16$\alpha$-hydroxy testosterone, 16$\beta$-hydroxy testosterone, and androstenedione) decreased to similar extents, and no difference in the ratios of 16$\alpha$/16$\beta$/androstenedione was seen between the tTEPA-exposed and the tTEPA-inactivated CYP2B1. In contrast, the same tTEPA-inactivated CYP2B1 samples exhibited only a 41% loss in formaldehyde formed compared with control samples in the benzphetamine assay. Similar differences in activity losses measured by these two substrates were seen previously with $N$-benzyl-l-aminobenzotriazole, another mechanism-based inactivator of CYP2B1 that inactivated the enzyme by forming a protein adduct (Kent et al., 2004). As would be expected for P450 samples where the loss in activity was due to destruction of the heme moiety, the CYP2B6 samples inactivated by tTEPA showed comparable losses in both the 7-EFC and benzphetamine activities.

**Metabolism of tTEPA.** Incubations of CYP2B1 and CYP2B6 with tTEPA ($m/z$ of 190) in the presence of NADPH resulted in the formation of TEPA ($m/z$ of 174) as the primary product. The levels of TEPA produced by CYP2B6 were similar when the incubations were carried out either in the absence or presence of GSH or $N$-acetylcysteine (Table 5). In contrast, approximately twice as much TEPA was formed in the CYP2B1 incubation mixtures that contained 10 mM GSH ($m/z$ of 309) (Table 5). GSH-TEPA (but not tTEPA) adducts with an $m/z$ of 484 were seen only in CYP2B1 samples incubated with NADPH, whereas GSH-tTEPA adducts with an $m/z$ of 497 were observed only in the CYP2B6 preparations (data not shown). The higher amounts of TEPA observed in the CYP2B6 incubations are a reflection of the slower rates of inactivation that were observed with the human enzyme. No other hydroxylated tTEPA or TEPA metabolites were observed under these analysis conditions.

**Discussion**

Three novel observations were made in this study: 1) In the reconstituted system with reductase, CYP2B1 and CYP2B6 were inactivated by tTEPA in a mechanism-based manner; 2) the two enzymes were inactivated by different mechanisms; and 3) the active site of CYP2B1 may have different binding sites for different substrates analogous to CYP3A4. Mechanism-based inactivation was demonstrated by a loss in activity that was time-, concentration-, and NADPH-dependent. Comparisons of the kinetic constants indicated that CYP2B1 was more rapidly inactivated than CYP2B6 with a $t_{1/2}$ of 2.5 versus 14 min, respectively. The $K_s$ value for both enzymes was in the micromolar range and well within concentrations observed previously for other mechanism-based inactivators of 2B enzymes. The estimated partition ratios of 166 and 96 for CYP2B1 and CYP2B6, respectively, which are the number of times each P450 metabolizes tTEPA before a reactive intermediate inactivates the enzyme, were also similar.

It was necessary to determine the kinetics of inactivation at early time points where the rates of inactivation were still linear. At later time points the reaction mechanism seemed to be biphasic (data not shown). Presumably this was due to
that heme alkylation was not the cause for the loss in activity and that tTEPA inactivates CYP2B1 by binding to the apoprotein. Direct analysis of the CYP2B1 apoprotein to test for a tTEPA protein adduct was attempted using LC-MS analysis as previously described (Regal et al., 2000). Although mass spectra for the control protein were obtained without difficulty, the tTEPA-inactivated sample was resistant to this procedure. In all instances, a loss comparable with loss in activity was observed in the total ion chromatogram of the tTEPA-inactivated CYP2B1. Presumably this suggests that the inactivated CYP2B1 could not be ionized or vaporized. It is also possible that the labile nature of tTEPA and its ability to form cross-links may have contributed to the selective loss of the adducted P450, presumably due to precipitation or aggregation of the protein (van Maanen et al., 2000). In contrast to what was seen with CYP2B1, the loss in activity of the tTEPA-inactivated CYP2B6 correlated with a loss in the ability of the human isoform to bind CO, to form a pyridine hemochrome complex, and in the recovery of native heme by HPLC. tTEPA seems to be the first mechanism-based inactivator where a difference in the type of inactivation was seen between the human and the rat 2B isoforms. In previous reports where 2B enzymes were compared using n-propylxanthate, phenyclidine, 2-phenyl-2-piperidino propane, 17-α-ethynylestradiol, tert-butylacetylene, and 7-ethynylcoumarin, the same type of inactivation, either adduct formation to the protein or the heme moiety, was observed (Kent et al., 1999; Chun et al., 2000; Kent et al., 2002; Jushchyshyn et al., 2003; our unpublished data). In all instances, CYP2B6 was inactivated by the same compounds and by the same mechanism as CYP2B1 but at a considerably slower rate.

Mechanism-based inactivation requires that a substrate is metabolized to a reactive intermediate that binds at the active site of the enzyme and does not leave the active site to interact with any part of the protein external to the active site or with other proteins in the incubation mixture causing loss in enzymatic activity via a secondary process. GSH was used as a trapping agent to test whether a tTEPA-reactive intermediate left the active site of CYP2B1 or CYP2B6. GSH affected the rates of inactivation of both enzymes. The decrease in the rate of inactivation in the presence of GSH may be due to some of the metabolites leaving the active site and becoming trapped by GSH. Whereas the same amount of TEPA was formed by CYP2B6 in the absence or presence of GSH or N-acetylcysteine after 60 min, CYP2B1 produced twice as much TEPA when coincubated with GSH. Presumably, these differences are due to the slower rate of inactivation of CYP2B6 at longer times in the absence of GSH. The extent of inactivation of the two CYP2B6 preparations was similar after 60 min. Alternatively, GSH may have more access to the active site of CYP2B1 than CYP2B6. CYP2B1

substrate depletion because addition of more tTEPA at a later time restored the linear kinetics of inactivation (data not shown). This observation was not unexpected because the turnover numbers indicated the inactivation process was not extremely efficient and may lead to substrate depletion in the reaction mixture. In contrast to CYP2B1, where essentially 100% inactivation could be observed, 36% residual activity was still present even at the highest tTEPA concentrations tested with the expressed CYP2B6. When these samples were dialyzed and then reincubated with a second aliquot of fresh tTEPA and NADPH, only a minor additional loss in activity (4–6%) was observed (data not shown), suggesting that the residual activity was due to a fraction of the population of CYP2B6 that was resistant to inactivation.

Mechanism-based inactivation has been shown to involve either one or a combination of the three following events: 1) The reactive intermediate alkylates the P450 heme moiety leading to heme modification and/or destruction, 2) the reactive intermediate covalently binds to an amino acid residue that is critical in substrate binding or catalysis, or 3) the reactive intermediate cross-links the heme to the P450 apoprotein rendering the enzyme incapable of catalysis (Osawa and Pohl, 1989). CYP2B1 and CYP2B6 were inactivated by two different mechanisms. Analysis of the CO spectra indicated that the tTEPA-inactivated CYP2B1 was capable of forming a CO complex to nearly the same extent as the control sample, suggesting that the heme moiety was intact. HPLC analysis of the P450s was used to test whether the heme was modified or whether cross-linking of the heme to the apoprotein had occurred after incubating the samples with tTEPA in the presence of NADPH. The HPLC profile at 405 nm of the control and the tTEPA-inactivated CYP2B1 sample showed a peak for the heme with the same retention time (21 min) and of nearly the same intensity in both samples. This observation indicated that the CYP2B1 heme was not destroyed upon inactivation with tTEPA, and that intact heme did not become cross-linked to the P450 because no 405 nm peak was seen eluting at a later time in the chromatogram together with CYP2B1 (data not shown). Furthermore, the diode array spectra of the heme peak obtained from the control and the inactivated sample were identical suggesting

<table>
<thead>
<tr>
<th>Incubation Condition</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ tTEPA − NADPH</td>
<td>40 ± 7</td>
</tr>
<tr>
<td>+ tTEPA + NADPH</td>
<td>72 ± 10</td>
</tr>
</tbody>
</table>

**TABLE 3**

Apparent binding constants obtained for expressed for control and tTEPA-inactivated CYP2B1 in the reconstituted system

Assay conditions were as described under Materials and Methods. With the exception of the testosterone experiment, the data shown represent the means and standard deviations from at least three experiments.

<table>
<thead>
<tr>
<th>Percentage of Control Activity of 2B1 after 15-min Incubation</th>
<th>Percentage of Control Activity of 2B6 after 20-min Incubation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-EFC</td>
<td>Benzphetamine</td>
</tr>
<tr>
<td>NADPH</td>
<td>92 ± 10</td>
</tr>
<tr>
<td>+ NADPH</td>
<td>7 ± 3</td>
</tr>
</tbody>
</table>

* Levels of testosterone hydroxylation by CYP2B6 were too low to evaluate the effect of tTEPA on activity.

**TABLE 4**

Effect of tTEPA inactivation on the ability of CYP2B1 and CYP2B6 to metabolize 7-EFC, benzphetamine, and testosterone

Assay conditions were as described under Materials and Methods. The data shown represent the means and standard deviations from three experiments.
and CYP2B6 have been shown to have rather large active sites and can easily accommodate molecules such as benzphetamine, steroids, and phenanthrenes (Roberts et al., 1995; Domanski et al., 1999; Kent et al., 2004). Because CYP2B1 and CYP2B6 generated different GSH-adducts, it is also possible that CYP2B1 may have produced a different reactive tTEPA intermediate than CYP2B6. With the CYP2B1 samples, a difference in the amount of TEPA generated in the presence of GSH or NaOAc was seen. The reason for this is not clear at this time and could be due to a difference in the reactivity of these two scavengers for the reactive intermediate(s) formed in the CYP2B1 or CYP2B6 samples.

The reversibility of the inactivation of CYP2B1 and CYP2B6 was tested to determine whether the P450s were inactivated by the covalent binding of a reactive tTEPA intermediate to the P450 and not just inhibited by tTEPA. The enzymatic activity of CYP2B6 did not return after dialysis, and only a slight increase was observed for CYP2B1, suggesting that CYP2B1 and CYP2B6 were inactivated in an irreversible mechanism-based manner by tTEPA. The dialyzed, inactivated samples were divided, and fresh reductase was added to one of the samples. There was only a slight recovery of activity by CYP2B1 in the presence of fresh reductase that suggested that the major loss in activity was primarily due to the inactivation of the P450 and not due to an effect on the reductase.

To further confirm that the inactivation occurred at the active site, an alternate CYP2B1 substrate, 7-EC, was added to the reaction mixture to test whether this substrate could compete with tTEPA and protect CYP2B1 from inactivation. A 10-fold molar excess of 7-EC over tTEPA partially protected CYP2B1 from inactivation. Concentrations of 7-EC could not be increased to completely protect CYP2B1 from inactivation because of solubility limitations of 7-EC in aqueous solutions. Surprisingly, CYP2B6 inactivation by tTEPA could not be protected against with a 20-fold molar excess of 7-ethoxycoumarin (2 mM) (Km of ~115 μM; Ekins and Wrighton, 1999), a 50-fold excess of benzphetamine (10 mM) (Km of ~93 μM; Ekins and Wrighton, 1999), or a 2-fold excess of testosterone (2 mM) (Km of ~50 μM; Ekins and Wrighton, 1999) over tTEPA (Kd of ~50 μM from this report, Kd of ~5–6 μM; Rae et al., 2002). In contrast, bupropion at a 10-fold molar excess (2 mM) with a reported Kd value of 85–156 μM for CYP2B6 was able to protect the human enzyme somewhat from tTEPA-dependent inactivation (Faucette et al., 2000; Hesse et al., 2000). In light of the similar Kd values and the use of a 10- to 40-fold excess over the reported Kd values for these substrates, it was surprising that only bupropion was able to compete with tTEPA. One possible interpretation of this result is that analogous to CYP3A4, CYP2B6 may have distinct substrate binding site pockets within its active site.

The studies described in this report establish that tTEPA is an efficient mechanism-based inactivator of CYP2B1 and CYP2B6 and advance our understanding of the different mechanisms by which the activity of these two isoforms is inhibited. These results could also imply potentially detrimental consequences if tTEPA is administered in high-dose combination regimes with other drugs that are metabolized by CYP2B6.

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References


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