Metabolism of *N,N',N''*-Triethylenethiophosphoramidate by CYP2B1 and CYP2B6 Results in the Inactivation of Both Isoforms by Two Distinct Mechanisms

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ABBREVIATIONS: *N,N',N''*-triethylenethiophosphoramidate, tTEPA; dilauroyl-L- α -phosphatidylcholine, 7-ethoxy-4-(trifluoromethyl)coumarin, 7-EFC; cytochrome P450, CYP; cytochrome P450 2B1, CYP2B1; cytochrome P450 2B6, CYP2B6; NADPH-cytochrome P450 reductase, reductase; L- α -dilaurylphosphatidylcholine, DLPC; trifluoroacetic acid, TFA; glutathione, GSH; high performance liquid chromatography, HPLC; electrospray ionization mass spectrometry, ESI-MS.

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ABSTRACT

The anti-cancer drug *N,N'',N''*-triethylenethiophosphoramidate (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 for the inactivation of CYP2B1 was 38 μM , the k_{inact} was 0.3 min^{-1} , and the $t_{1/2}$ was 2.5 minutes. Spectral CO binding- and HPLC 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 (7-EC), an alternate substrate, reduced the rate of inactivation of CYP2B1. Incubations with tTEPA and NADPH resulted in greater than 90% loss in the 7-ethoxy-4-(trifluoromethyl)coumarin (7-EFC) *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 of 50 μM , a k_{inact} of 0.1 min^{-1} , and a $t_{1/2}$ of 14 min. However, unlike CYP2B1, the tTEPA-inactivated human isoform showed losses in the P450 CO spectrum, the pyridine hemochrome spectrum, and in the amount of native heme that were comparable to 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 CYP2B such as benzphetamine, testosterone, or 7-ethoxycoumarin. The data indicate that tTEPA metabolism by these two 2B isoforms results in inactivation of the CYPs by two distinct mechanisms.

Cytochromes P450 (CYP) catalyze the metabolism of both endogenous substrates such as steroids, fatty acids and fat-soluble vitamins, as well as exogenous compounds such as drugs and pesticides. CYP2B1, the major phenobarbital-inducible isoform found in rat (Gonzalez, 1989) and CYP2B6, the human isoform of CYP2B1, share greater than 70% sequence homology.

Inhibition of CYP enzymes by certain drugs such as raloxifene, mifepristone, and ketoconazole, or compounds found in food such as bergamottin and silybin can pose a problem in the clinic when these enzymes are required for the metabolism of co-administered drugs (Guengerich, 1995; Chen et al., 2002; He et al., 1998; Schmiedlin-Ren et al., 1997, Sridar et al., 2004). In contrast to mechanism-based inactivation of an enzyme, simple competitive inhibition can generally be overcome by washing out the inhibitor or by replacement with a higher affinity substrate. Mechanism-based inactivation is characterized by the enzymatic conversion of a substrate to a reactive intermediate that covalently binds to a moiety in the enzyme's active site resulting in an irreversible loss in enzymatic activity (Kent et al., 2001). As a consequence, drugs that function as mechanism-based inactivators can therefore exert substantial effects on the activity of CYP enzymes at concentrations well below their respective K_I values. Because new protein synthesis is required for the active CYP to be regenerated, mechanism-based inactivation can lead to a rapid build up of certain drugs with potentially toxic effects. Further complications arise from the fact that many drugs may be metabolized by more than one CYP 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 CYPs.

tTEPA is a nonspecific anti-neoplastic 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 7-ethoxycoumarin *O*-deethylation- as well as the testosterone 16- β -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 if inactivation by tTEPA resulted in modification of the CYP2B1 protein or heme alkylation. It has been previously reported 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 (CYPs 3A4, 2E1, 2C8, 2C9, 2C19, and 2D6). Interestingly, tTEPA was also reported to inhibit the conversion of cyclophosphamide to 4-OHCP, 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 CYP 2B enzymes is essential due to the potential biomedical implications. In this report we investigate the effects of tTEPA on the rat and human CYP 2B isoforms. We present novel data showing that these two highly conserved isoforms of 2B metabolize tTEPA to reactive intermediates that inactivate the two CYPs by different mechanisms. Our observations further suggest that CYP inhibitor data derived from rat enzyme preparations may not necessarily be valid in the human system.

Experimental Procedures

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

Purification of CYPs and Reductase. CYP2B1, CYP2B6 and NADPH reductase were expressed in *Escherichia coli* MV1304 cells and purified according to previously published procedures (Hanna et al., 2000, Hanna et al., 1998, Spatzenegger et al., 2003, Kent et al., 1999).

Enzyme Activity Assay and Inactivation of CYP2B1 and CYP2B6. Purified CYP2B1 and reductase were reconstituted with lipid for 45 minutes at 4°C. CYP2B6 was incubated with reductase for 45 minutes at 4°C. The primary reaction mixtures contained 1 μ M CYP2B1 or CYP2B6, 1 μ M reductase (or 2 μ M for the assays with CYP2B6), 60 μ g DLPC (or no DLPC for CYP2B6), 110 U catalase and tTEPA in water at concentrations between 0 μ M and 80 μ M (or 0 – 200 μ M 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, 11 and 20 min after the NADPH was added, 5 μ L (12 μ L for CYP2B6) aliquots were taken from the primary reaction mixture and transferred into a secondary reaction mixture containing 100 μ M 7-EFC, 1 mM NADPH, and 40 μ g BSA/mL in 50

mM potassium phosphate buffer (pH 7.4) (Buters et al., 1993). 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 μ L of cold acetonitrile. The activity remaining was determined spectrofluorometrically 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 CYPs. CYPs 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 μ M tTEPA (for CYP2B1) or 200 μ M tTEPA (for CYP2B6), and the third sample was inactivated with 80 μ M or 200 μ M 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 μ L of ice-cold quench buffer [40% glycerol and 0.6% Tergitol NP-10 in 50 mM potassium phosphate (pH 7.4)] and gently bubbled with CO for about 60 seconds. The spectra were recorded from 400 to 500 nm before and after the addition of solid dithionite on a DW2 UV/Vis 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 P450 samples (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 34.4 mM⁻¹ cm⁻¹ (Koop, 1990).

Effect of tTEPA Inactivation on the Heme of CYPs 2B1 and 2B6. HPLC analysis was performed in order to determine the effect of the inactivation by tTEPA on the CYP 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 4.9 mm x 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 – 95 % B over 35 min at a flow rate of 1 mL/min. The elution profile was monitored using a photodiode array detector (Waters, 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. P450s 2B1 and 2B6 were reconstituted with reductase for 45 min at 4°C as described above. Incubation mixtures contained 1 μM CYP2B1, 1 μM reductase, 1 μg DPLC/mL, and 110 U 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 μ M CYP2B6, 2 μ M reductase, and 110 U catalase/mL in 50 mM potassium phosphate buffer (pH 7.4). The primary reaction mixtures contained either 100 μ M tTEPA or 200 μ M 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 μ M 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 μ L) were dialyzed separately overnight at 4°C in Slide-A-Lyzer cassettes against 2 x 500 mL of 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. After dialysis the CYP samples were incubated with or without fresh reductase on ice for 15 min and then assessed for activity using the 7-EFC assay as described above.

Partition ratio. The partition ratios for the inactivation of CYPs 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 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 tTEPA ratios and the least squares straight line obtained at the higher ratios of tTEPA to CYP.

Substrate Protection from tTEPA-dependent Inactivation. Substrate protection from tTEPA-dependent inactivation of CYP2B1 was assayed 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 μ M tTEPA or 100 μ M tTEPA together with 1 mM 7-EC. Control reaction mixtures contained either solvent or 7-EC alone. Aliquots (10 μ L) were removed at 0, 1, 2, 4 and 8 minutes and added to a secondary 7-EFC reaction mixture and assayed for activity remaining as described above. CYP2B6 was reconstituted as describe above. Samples were incubated in the presence of either 100-200 μ M tTEPA alone, or 100-200 μ M tTEPA together with 1-2 mM 7-EC, 10 mM benzphetamine, 2 mM testosterone, or 2 mM bupropion. Aliquots (12 μ L) were removed at 0, 5, 10, 16 and 21 minutes and added to a secondary 7-EFC 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 μ M tTEPA (CYP2B1) or 200 μ M 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-EFC O-deethylation (5 pmol CYP2B1, 12 pmol CYP2B6), testosterone hydroxylation (25 pmol CYP2B1), or benzphetamine *N*-demethylation (25 pmol CYP2B1, 50 pmol CYP2B6) activity. Testosterone hydroxylation was 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 ethylacetate, 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 Corp.).

Benzphetamine *N*-demethylation was measured spectrofluorometrically (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 μ L of sample, 500 μ L of FluoralP 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 500F 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 nm and 450 nm. Samples contained 4 μ M CYP2B1, 4 μ M reductase, 400 μ g DLPC, 985 U of catalase, and 200 μ M 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 μ L aliquots (5 pmol 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 nm and 600 nm following the addition of increasing concentrations of benzphetamine or n-octylamine. Difference spectra were recorded for benzphetamine or testosterone concentrations between 0 – 200 μ M and n-octylamine concentrations between 0 - 8 μ M. K_s values were estimated by plotting the inverse of the absorbance changes between 385 nm and 419 nm (type I spectrum) versus the concentration of benzphetamine or testosterone, and between 392 nm and 425 nm (type II spectrum) as a function of the concentration of n-octylamine.

Identification of tTEPA metabolites by ESI-LC-MS/MS. tTEPA metabolites were analyzed by ESI-LC-MS/MS on a LCQ (Finnigan, Thermoquest) mass analyzer controlled by Excalibur software (Finnigan, Thermoquest) essentially as described previously with minor modifications (van Maanen and Beijnen, 1999). CYPs (1 μ M) were reconstituted as described above and samples containing 250 pmol CYP 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 μ l of a 100 μ g/mL solution) was added to each sample and the entire sample was immediately applied to a 500 μ L 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 x 100 mm, 5 micron, 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: Auxilliary gas 30 arbitrary units, sheath gas 90 arbitrary units, capillary temperature 170 °C, spray voltage 4.5 kV, capillary voltage 45 V.

Results

Inactivation of CYP2B1 and CYP2B6 by tTEPA. Initial studies were performed to test if 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 CYPs 2B1 and 2B6 are shown in Figures 2 and 3. Pseudo-first order kinetics were 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 CYPs 2B1 and 2B6 by tTEPA were determined from the double-reciprocal plots of rate^{-1} versus $[\text{tTEPA}]^{-1}$ (insets to Fig. 2 and 3). For CYP2B1 the maximal rate of inactivation at saturation (k_{inact}) was 0.3 min^{-1} , the concentration of tTEPA required for half maximal inactivation (K_I) was $38 \mu\text{M}$, and the time required for half of the enzyme molecules to be inactivated ($t_{1/2}$) was 2.5 minutes. These values are in good agreement with the data for CYP 2B1 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: $K_I = 50 \mu\text{M}$, $k_{\text{inact}} = 0.1 \text{ min}^{-1}$, and $t_{1/2} = 14$ minutes. Metabolic turnover of the inhibitor by the target enzyme is a prerequisite for mechanism-based inactivation. The data in Table 1 shows an absolute requirement for NADPH in the inactivation of CYPs 2B1 and 2B6 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 CYPs 2B1 and 2B6 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 following 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 CYP reduced CO spectrum, the pyridine hemochrome spectrum, and in the amount of HPLC-detectable intact heme that were comparable to 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-inactivated CYP2B6 samples (data not shown). This suggests that the tTEPA-reactive intermediate generated by CYP2B6 caused fragmentation of the heme and that the activity loss was due primarily to decomposition of the heme moiety.

Effect of exogenous nucleophiles on the rate of inactivation of CYPs 2B1 and 2B6 by tTEPA. Figure 4A shows the effect of the exogenous nucleophile glutathione (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 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 CYPs 2B1 and/or 2B6 and compete with tTEPA, thereby slowing the inactivation. The data suggests, 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 %) following 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 Figure 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 supports 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 CYP. 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 if the consequence of this inactivation was an inability of the tTEPA-modified CYP to bind substrate. The 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 of the tTEPA-inactivated sample when compared to 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 CYPs 2B1 and 2B6. Table 4 shows that the loss in the 7-EFC *O*-deethylation activity (93% loss) of the tTEPA-inactivated CYP2B1 samples was comparable to the decrease (92% loss) in the testosterone hydroxylation activity. The formation of all three metabolites (16 α -hydroxy testosterone, 16 β -hydroxy testosterone, and androstenedione) decreased to similar extents and no difference in the ratios of 16 α :16 β :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 when compared to control samples in the benzphetamine assay. Similar differences in activity losses measured by these two substrates were seen previously with *N*-benzyl-1-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 CYP 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 190) in the presence of NADPH resulted in the formation of TEPA (m/z 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 309) (Table 5). GSH-TEPA (but

not tTEPA) adducts with a m/z of 484 were seen only in CYP2B1 samples incubated with NADPH, whereas GSH-tTEPA adducts with a 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 min versus 14 min, respectively. The K_I for both enzymes was in the μM range and well within concentrations observed previously for other mechanism-based inactivators of 2B enzymes. The estimated partition ratios of 166 and 96 for CYPs 2B1 and 2B6, respectively, which are the number of times each CYP 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 appeared to be biphasic (data not shown). Presumably this was due to substrate depletion since 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 re-incubated

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 CYP 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 CYP 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 CYPs was used to test if the heme was modified or if 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 CYP because no 405 nm peak was seen co-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 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 to 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 CYP, 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 heme complex, and in the recovery of native heme by HPLC. tTEPA appears 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, phencyclidine, 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, Jushchyshyn et al., 2003, Chun et al., 2000, Kent et al., 2002, and unpublished results). 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 if 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 co-incubated 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 and CYP2B6 have been shown to have rather large active sites and can easily accommodate molecules such as benzphetamine, steroids and phenanthrenes (Domanski et al., 1999, Kent et al., 2004, Roberts et al., 1995). 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 *N*-acetylcysteine 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 CYPs 2B1 and 2B6 was tested in order to determine if the CYPs were inactivated by the covalent binding of a reactive tTEPA intermediate to the CYP 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 CYPs 2B1 and 2B6 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 CYP 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-ethoxycoumarin (7-EC), was added to the reaction mixture to test if 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) ($K_m \approx 115 \mu\text{M}$, Ekins and Wrighton, 1999), a 50-fold excess of benzphetamine (10 mM) ($K_m \approx 93 \mu\text{M}$ Ekins and Wrighton, 1999), or a 2-fold excess of testosterone (2mM) ($K_m \approx 50 \mu\text{M}$, Ekins and Wrighton, 1999) over tTEPA ($K_I \approx 50 \mu\text{M}$ from this report, $K_I \approx 5\text{-}6 \mu\text{M}$, Rae et. al., 2002). In contrast, bupropion at a 10-fold molar excess (2 mM) with a reported K_m value of 85 – 156 μM for CYP2B6 was able to protect the human enzyme somewhat from tTEPA-dependent inactivation (Hesse et. al., 2000, Faucette et al., 2000). In light of the similar K_m values and the use of a 10 – 40-fold excess over the reported K_m 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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Legends to Figures

Fig. 1. Chemical Structure of *N,N',N''*-Thiotriethylenephosphoramidate (tTEPA).

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 in the Experimental Procedures. The primary reaction mixtures contained (□) 0, (▲) 5, (△) 10, (◆) 20, (◇) 40, and (●) 80 μM of 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.

Fig. 3. Time- and concentration-dependent inactivation of CYP2B6 7-EFC O-deethylation activity by tTEPA. Aliquots were removed from the primary reaction mixture at 0, 5, 10, 16 and 21 min after NADPH was added and assayed for activity using 7-EFC as described in the Experimental Procedures. The primary reaction mixtures contained (□) 0 μM, (▲) 5 μM, (△) 10 μM, (◆) 20 μM, (◇) 40 μM, (●) 80 μM, (○) 140 μM, 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.

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.

Aliquots were removed from the primary reaction mixtures at 0, 1, 2, 4, and 8 min and assayed as described in the Experimental Procedures. The data shown are the mean and standard deviation from three separate experiments. A) CYP2B1; B) CYP2B6; (■) Control, (□) Control with 10 mM GSH, (●) tTEPA-inactivated, (○) tTEPA-inactivated with 10 mM GSH.

Fig. 5. Substrate protection of CYP2B1 and CYP2B6 from inactivation by tTEPA. Aliquots were removed from the primary reaction mixtures at 0, 1, 2, 4, and 8 min and assayed for residual activity as described in Experimental Procedures. A) The primary CYP2B1 reaction mixtures contained (□) solvent and methanol, (○) solvent together with 1 mM 7EC, (■) 100 μ M tTEPA, and (●) 100 μ M tTEPA together with 1 mM 7EC. B) The CYP2B6 reaction mixtures contained (□) solvent, (○) solvent and 2 mM bupropion, (■) 200 μ M tTEPA, and (●) 200 μ M tTEPA together with 2 mM bupropion. The data shown are the mean and standard deviation from three separate experiments.

TABLE 1

Effect of tTEPA on the 7-EFC O-deethylation activity, P450 content, heme, and pyridine hemochrome of CYP2B1 and CYP 2B6.

Assay conditions were as described in Experimental Procedures. The data shown represent the means and standard deviations from 3-6 experiments obtained for CYP2B1 after 10 min and for CYP2B6 after 15 min of incubation in the presence or absence of NADPH and are shown as a percentage of the control samples at 0 min incubated in the absence of tTEPA. The HPLC data are the average from two separate experiments. The data from one experiment are shown for the pyridine hemochrome assay.

		Percentage of Control		
Primary Reaction	Activity Remaining	P450 Remaining	HPLC	Pyridine
Conditions			Heme	Hemochrome
<hr/>				
CYP2B1				
- tTEPA + NADPH	102 ± 16	108 ± 13	100 ± 8	100
+ tTEPA - NADPH	108 ± 8	111 ± 18	ND	ND
+ tTEPA + NADPH	41 ± 6	126 ± 6	91 ± 5	90 ± 12
<hr/>				
CYP2B6				
- tTEPA + NADPH	90 ± 4	93 ± 1	94 ± 2	100
+ tTEPA - NADPH	96 ± 2	84 ± 3	ND	ND
+ tTEPA + NADPH	63 ± 3	72 ± 5	60 ± 4	60 ± 12
<hr/>				
ND, not determined				

TABLE 2

Irreversibility of CYP2B1 and CYP2B6 Inactivation by tTEPA.

Assay conditions were as described in Experimental Procedures. Data from two separate experiments are shown.

Samples	% of Control 2B1 Activity		% of Control 2B6 Activity	
	Sample 1	Sample 2	Sample 1	Sample 2
Before dialysis	23	32	46	37
After dialysis	33	47	42	33
After dialysis + fresh reductase	37	59	47	34

TABLE 3

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

Assay conditions were as described in Experimental Procedures. With the exception of the testosterone experiment, the data shown represent the means and standard deviations from at least 3 experiments.

Incubation Condition	K _s (μM)		
	Benzphetamine	Testosterone	n-Octylamine
+ tTEPA - NADPH	40 ± 7	79	0.76 ± 0.02
+ tTEPA + NADPH	72 ± 10	109	0.68 ± 0.01

TABLE 4

Effect of tTEPA inactivation on the ability of CYPs 2B1 and 2B6 to metabolize 7-EFC, benzphetamine, and testosterone.

Assay conditions were as described in Experimental Procedure. The data shown represent the means and standard deviations from three experiments.

% of Control Activity of					
2B1 after 15 min incubation			2B6 after 20 min incubation ^a		
Sample	7-EFC	Benzphetamine	Testosterone	7-EFC	Benzphetamine
- NADPH	92 ± 10	90 ± 4	84 ± 14	89 ± 8	91 ± 1
+ NADPH	7 ± 3	53 ± 3	8 ± 2	61 ± 1	63 ± 3

^athe levels of testosterone hydroxylation by CYP2B6 were too low to evaluate the effect of tTEPA on activity.

TABLE 5

Metabolism of tTEPA to TEPA by CYP2B1 and CYP2B6 in the presence of NADPH.

Assay and analysis conditions were as described in Experimental Procedures. The data shown represent the means and standard deviations from three samples for each condition tested.

Incubation Conditions	nmols TEPA formed/nmol CYP	
	CYP2B1 ^a	CYP2B6 ^b
CYP + tTEPA	12.2 ± 1.8	144 ± 4.2
CYP + tTEPA + GSH	26.0 ± 3.8	152 ± 6.6
CYP + tTEPA + NacCys ^c	14.8 ± 2.0	170 ± 4.2

^aCYP2B1 samples were incubated for 20 min and ^bCYP2B6 samples were incubated for 60 min at 30 °C with tTEPA.

^cN-acetylcysteine