TRIETHYLENETHIOPHOSPHORAMIDE IS A SPECIFIC INHIBITOR OF CYTOCHROME P450 2B6: IMPLICATIONS FOR CYCLOPHOSPHAMIDE METABOLISM

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ABSTRACT:

Cytochrome P450 2B6 is a genetically polymorphic enzyme that is important in the metabolism of a number of clinically used drugs. This enzyme is not as well studied as other cytochrome P450 (P450) isoforms because of the lack of specific antibodies, probe drugs, and inhibitors. Although recent progress has been made toward specific antibodies and probe drugs, a specific enzyme inhibitor is still lacking. Studies suggest that CYP2B6 plays an important role in the 4-hydroxylation of cyclophosphamide and that this reaction can be inhibited by triethylenethiophosphoramide (thioTEPA). We therefore wished to test the hypothesis that thioTEPA is an inhibitor of CYP2B6. Using human liver microsomes (HLMs) and recombinant P450 enzymes, we demonstrated that thioTEPA is a potent and specific inhibitor of CYP2B6. Enzyme activity was reduced 76.1 ± 0.2% by 50 μM thioTEPA when CYP2B6 activity was measured by following the metabolism of 200 μM S-mephenytoin to nirvanol. thioTEPA did not significantly inhibit (<20% at 100 μM) the other isoforms tested (CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4). thioTEPA seems to be a potent noncompetitive inhibitor of CYP2B6, with Ki values of 4.8 ± 0.3 and 6.2 ± 0.7 μM for HLMs and recombinant CYP2B6, respectively, values that are within the plasma concentration range of thioTEPA at therapeutic doses (1.1–18.6 μM). We conclude that thioTEPA is a potent and specific inhibitor of CYP2B6 and that this is the likely mechanism by which thioTEPA inhibits the activation of cyclophosphamide. Furthermore, thioTEPA may prove to be a valuable new tool for the study of this important drug-metabolizing enzyme.

Recent studies into the role that cytochrome P450 2B6 plays in drug elimination have shown that this enzyme is important for the metabolism of a number of clinically used drugs. The growing list of compounds identified as substrates of CYP2B6 include nevirapine (Erickson et al., 1999), S-mephenobarbital (Kobayashi et al., 1999), artemisinin (Svensson and Ashton, 1999), bupropion (Faucette et al., 2000) and propofol (Court et al., 2001), ifosfamide (Huang et al., 2000), ketamine (Yanagihara et al., 2001), selegiline (Hidestrand et al., 2001), and methadone (Gerber and Rhodes, 2000). Despite this growing list, this enzyme remains one of the least studied P4503 isoforms. This is probably due to the lack of suitable in vitro and in vivo tools available to study this enzyme (Ekins et al., 1997). Although recent progress has been made with respect to a specific substrate probe (Faucette et al., 2000) and immunological inhibitors of CYP2B6 (Yang et al., 1998), a specific chemical inhibitor is still lacking.

The level of expression of CYP2B6 protein in the human liver has been controversial until recently, with some earlier articles showing that only a small proportion of livers (<25%) (Mimura et al., 1993; Edwards et al., 1998) contain protein, whereas other studies have shown a more extensive level of expression (up to 90%) (Gervot et al., 1999; Hanna et al., 2000). The differences in antibodies used for each study may well contribute to the different results reported. More recent studies used antibodies prepared against human protein and have shown that nearly all of the liver samples have detectable levels of CYP2B6 (Gervot et al., 1999). However, more than a 20-fold difference in the level of protein (ranging from 0.4 to 8 pmol/mg of protein) was observed (Gervot et al., 1999). This variability may be caused by differences in exposure to environmental factors that can induce or inhibit the expression of CYP2B6 or by genetic polymorphisms that alter the expression or catalytic activity of the enzyme.

CYP2B6 has been shown to catalyze the 4-hydroxylation of cyclophosphamide at a high rate in vitro (Roy et al., 1999); however, its contribution to this reaction in vivo remains unclear. Cyclophosphamide is the most widely used antitumor alkylating agent and is often used in combination chemotherapy regimens for the treatment of many malignancies (Teicher, 1997). It is a prodrug that requires metabolic activation by the P450 system to 4-hydroxycyclophosphamide (4-OHCP) before it exerts cytotoxicity (Colvin et al., 1973). This primary metabolite exists in equilibrium with its open-ring tautomer aldophosphamide, which enters cells and undergoes chemical decom-
position to form phosphoramidate mustard, a bifunctional DNA alkylator and the ultimate cytotoxic metabolite, and acrolein. The P450-mediated activation pathway may be diminished by genetic polymorphisms of the enzymes involved or by concurrent administration of inhibitor drugs, which may lead to reduced 4-OHCP formation and decreased anticancer efficacy. Indeed, clinical studies suggest that triethylenthiophosphoramide (thioTEPA) inhibits the conversion of cyclophosphamide to 4-OHCP, and it has been recommended that these two agents should not be given together (Huijtena et al., 2000). It follows that thioTEPA may inhibit one or more of the P450 isozymes implicated in cyclophosphamide activation.

thioTEPA is a cell cycle-phase, nonspecific antineoplastic agent used in the treatment of breast, ovarian, and bladder carcinomas (Maenan et al., 2000). This drug was originally approved by the Food and Drug Administration (FDA) in 1959, but its dose-limiting toxicity (myelosuppression) limited its use until its use in preparative regimens before autologous bone-marrow and peripheral stem-cell transplantation was recognized. In this context, thioTEPA is frequently given in conjunction with cyclophosphamide in high-dose chemotherapy regimens (van der Wall et al., 1995).

In the present study, we used human liver microsomes (HLMs) and recombinant P450 enzymes to evaluate the inhibitory potency of thioTEPA on eight clinically relevant drug-metabolizing P450 enzymes in vitro. S-Mephenytoin N-demethylation to nirvanol was used as a substrate probe of CYP2B6 in this study, as we (Ko et al., 1998) and other authors (Heyn et al., 1996) have previously shown that this reaction is predominantly catalyzed by CYP2B6 at high substrate concentrations. Our goal was to identify which enzymes are inhibited by thioTEPA to test the hypothesis that thioTEPA is an inhibitor of CYP2B6. These studies may help identify the mechanism underlying the clinical interaction between thioTEPA and cyclophosphamide. In addition, thioTEPA may prove a valuable new tool for the study of this important drug-metabolizing enzyme.

Materials and Methods

Chemicals. thioTEPA was purchased from U.S. Pharmacopeia Convention (Rockville, MD). Tolbutamide, phenacetin, acetaminophen, midazolam, dextromethorphan, chlorzoxazone, G6P, G6PDH, NADP, and the disodium salt of EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). Nirvanol, S-mephenytoin, 4-hydroxy-S-mephenytoin, 6-hydroxychlorzoxazone, 4-hydroxymidazolam, and 4-methylhydroxytolbutamide were purchased from Ultrafine Chemicals (Manchester, UK). Dextromethorphan was purchased from F. Hoffman-La Roche, Inc. (Nutley, NJ). N-(4-Hydroxyphenyl)butabamide was kindly provided by John Strong (Division of Clinical Pharmacology, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Rockville, MD). Other reagents were of HPLC grade.

HLMs and Recombinant Human P450s. The HLMs used were prepared from human liver tissue that was medically unsuitable for liver transplantation and frozen at −80°C within 3 h of the cross-clamp time. The characteristics of liver donors, procedure for preparation of microsomal fractions, and their P450 contents have been described in detail elsewhere (Harris et al., 1994). The microsomal pellets were resuspended in a reaction buffer (0.1 M NaCl and KCl, 1.0 mM EDTA, and 5.0 mM MgCl2, pH 7.4) to a protein concentration of 10 mg/ml (stock) and were kept at −80°C until used. Protein concentrations were determined according to Pollard et al. (1978). Detailed protocols for the measurement of each P450 isoform activity using isoform-specific substrate reaction probes and their apparent kinetic parameters (Km and Vmax values) have been described in previous studies from our group (Desta et al., 2001). Baculovirus insect cell-expressed human CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2E1, and CYP3A4 were purchased from GEVENTE (Woburn, MA) and stored at −80°C. Protein concentrations and P450 contents were as supplied by the manufacturer.

Inhibition of P450 by thioTEPA. The inhibitory effects of thioTEPA on the activities of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were tested in recombinant human P450 isoforms and HLMs using isoform specific substrate probes, as described in our previous publications (Ko et al., 1998; Desta et al., 2001). The reaction probes used were as follows: phenacetin O-deethylation for CYP1A2, S-mephenytoin N-demethylation to nirvanol for CYP2B6, tolbutamide 4-methylhydroxylation for CYP2C8 and CYP2C9, S-mephenytoin 4-hydroxylation for CYP2C19, dextromethorphan O-demethylation for CYP2D6, chlorzoxazone 6-hydroxylation for CYP2E1, and midazolam 4-hydroxylation for CYP3A. Using incubation conditions specific to each isoform that were linear for time and substrate and protein concentrations, as described in our previous publications (Ko et al., 1998; Desta et al., 2001), isoform-specific substrate probes were incubated in duplicate at 37°C with HLMs or recombinant P450 isoforms and an NADPH-generating system in the absence (control) or presence of varying concentrations of thioTEPA (0–100 μM). Unless specified, a 5-min preincubation was carried out before the reaction was initiated by adding HLMs or recombinant P450. Preliminary experiments were carried out by incubating a single isoform-specific substrate concentration around its Km value with a single (50 μM) or range (0–100 μM) of thioTEPA concentrations. Incubation conditions and HPLC methods for measurement of each activity are validated and have been routinely used in our laboratory and details are described elsewhere (Ko et al., 1998; Desta et al., 2001). Concentrations of substrate probes were as follows: phenacetin (50 μM), tolbutamide (50 μM), S-mephenytoin (25 and 200 μM), dextromethorphan (25 μM), chlorzoxazone (25 μM), and midazolam (25 μM).

Computer simulation of the preliminary data thus obtained was then used to estimate the appropriate substrate and thioTEPA concentrations for Dixon plot analysis and the determination of exact inhibition constants (K). Since our data showed a potent effect of thioTEPA on CYP2B6 activity alone, Dixon plots were constructed for this isoform only. S-Mephenytoin (50, 75, and 150 μM) was incubated for 90 min at 37°C with a range of thioTEPA concentrations (0–15 μM) in HLMs and recombinant human CYP2B6 in the presence of an NADPH-generating system, as described above.

thioTEPA is a tri-azidine-phosphine that could undergo P450-mediated biotransformation to metabolites that may alkylate (inactivate) CYP2B6 and contribute to the overall inhibition. To test for the presence of mechanism-based inactivation of CYP2B6 by thioTEPA, HLMs and recombinant human CYP2B6 were preincubated with an NADPH-generating system with or without thioTEPA (5 μM) for 0, 5, 10, 20, and 30 min. The reaction was started by adding a substrate probe and then further incubated for 90 min. To avoid false-positive inhibitions that may result from loss of enzyme activity due to prolonged incubation, parallel control experiments were determined by preincubating HLMs and an NADP-generating system without thioTEPA before the reaction was initiated by the addition of S-mephenytoin and further incubated.

After termination of the incubation reactions with appropriate reagents, samples were centrifuged and injected into an HPLC system either directly or after extraction and reconstitution with the respective mobile phase. The concentrations of the metabolites and internal standards were measured by HPLC with UV or fluorescent detection specific for each assay. The rates of production of each metabolite from the substrate probes were quantified by using the ratio of the area under the curve (AUC) of the metabolite to the AUC of each internal standard using an appropriate standard curve. The rates of metabolite formation from substrate probes in the presence of thioTEPA were compared with controls in which the inhibitor was replaced with vehicle.

HPLC. Instruments used for HPLC were controlled by a Waters (Milford, MA) Millennium 2010 chromatography manager and included a Waters model 510 or 600 HPLC pump, Waters 710B or 717 Autosampler, Waters 490 or 484 UV detector, and Spectrovision FD-300 Dual Mono-Chromator Fluorescence Detector (Groton Technology Inc., Concord, MA). Full chromatographic conditions for each assay have been described elsewhere (Ko et al., 1998; Desta et al., 2001).

Enzyme Assays. In a previous study, we have shown that a relatively high concentration (200 μM) of S-mephenytoin can be used to probe for CYP2B6 activity (Ko et al., 1998). Therefore, CYP2B6-catalyzed S-mephenytoin N-demethylation was measured as described in our previous work (Ko et al., 1998). Assays for CYP2C19-catalyzed (S)-mephenytoin 4-hydroxylation, an NADPH-generating system in the absence (control) or presence of varying concentrations of thioTEPA (0–100 μM). Unless specified, a 5-min preincubation was carried out before the reaction was initiated by adding HLMs or recombinant P450. Preliminary experiments were carried out by incubating a single isoform-specific substrate concentration around its Km value with a single (50 μM) or range (0–100 μM) of thioTEPA concentrations. Incubation conditions and HPLC methods for measurement of each activity are validated and have been routinely used in our laboratory and details are described elsewhere (Ko et al., 1998; Desta et al., 2001).
CYP2C8 (recombinant)-mediated 4-methylhydroxylation of tolbutamide were describe in detail previously (Desta et al., 2001).

**Data Analysis.** The reaction velocity of each substrate probe in the presence of thioTEPA was expressed as the percentage of the control velocity with no thioTEPA present. Approximate $K_i$ values were calculated from experiments that were conducted using single substrate and multiple thioTEPA concentrations with use of the following equation assuming competitive inhibition:

$$\% \text{ Inhibition} = \frac{100 \times [I]}{[I] + K_i \times \left(1 + \frac{[S]}{K_m}\right)}$$

where $I$ is the thioTEPA concentration, $K_i$ is the inhibitory constant, $S$ is the substrate concentration, and $K_m$ is the substrate concentration at half of the maximum velocity ($V_{max}$) of the reaction.

Estimates for kinetic parameters from this analysis were used to generate computer-simulated optimal concentrations of substrate and thioTEPA for the determination of Dixon plots. The inhibition data from Dixon plots were fitted to appropriate nonlinear regression models of enzyme inhibition, and accurate $K_i$ values were calculated (WinNonlin Version 3.1; Pharsight Corporation, Mountain View, CA). The $K_i$ values obtained from visual inspection of the Dixon plots served as initial estimates for this determination. An appropriate model and mechanism of inhibition were decided graphically and from parameters of the model using the dispersion of residuals and standard errors of the parameter estimates.

**Results**

thioTEPA has been shown to inhibit the P450-mediated metabolism of cyclophosphamide to 4-OHCP in the clinical setting and in vitro using HLMs. These studies however, did not determine which cytochrome P450 enzymes were inhibited by this agent. Therefore, we tested the inhibitory effects of thioTEPA on the activity of eight clinically relevant P450 isoforms. In our initial experiments, we used HLMs to determine the effects of thioTEPA on the activity of P450s, as assessed by isoform-specific substrate reaction probes at their approximate $K_m$ value. thioTEPA (50 µM) inhibited CYP2B6 activity by 78.1 ± 0.2%, as determined by following the N-demethylation of S-mephenytoin (200 µM) to nirvanol in HLMs, but it had little effect (≤20%) on the microsomal activity of CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A (Fig. 1A; shown as the mean of duplicates ± S.E. of the mean). Because some of the substrate probes might also be metabolized by other P450 isoforms,
we tested the ability of thioTEPA to inhibit the activity of recombinant human P450s. These results were consistent with those shown in Fig. 1A in that thioTEPA (50 μM) inhibited the activity of recombinant human CYP2B6 by 42.5 ± 2.4% while having no major effect (≤15%) on the activity of recombinant human CYP2C8, CYP2C9, CYP2C19, CYP2E1, or CYP3A4 (Fig. 1B; shown as the mean ± S.D. of four determinations).

A range of thioTEPA concentrations (0–100 μM) was used to generate inhibitory dose response curves in HLMs and in recombinant human CYP2C8 and CYP2C9 preparations. The recombinant proteins were used since tolbutamide 4-methylhydroxylation is not specific for the individual isoforms. The results in Fig. 2 (shown as the mean of duplicates) show that thioTEPA is a potent and specific inhibitor of CYP2B6, with 5 μM drug inhibiting the activity of this enzyme by 51%. The effects seem to plateau around 50 μM, with 50 and 100 μM inhibiting the activity of CYP2B6 by 78 and 83% respectively. In addition, this figure shows that thioTEPA has little effect on the other cytochrome P450 isoforms tested at concentrations up to 100 μM.

The inhibitory data on CYP2B6 were used to approximate the range of S-mephenytoin and thioTEPA concentrations needed to construct Dixon plots for the inhibition of CYP2B6 by thioTEPA to allow, in turn, the calculation of inhibition constants (Kₐ values). Dixon plots were generated in both HLMs and recombinant CYP2B6, and the results were compared. The representative plots in Fig. 3 show that very similar results were obtained. The mean Kₐ values derived from two different human liver microsomal preparations (4.8 ± 0.3 μM) were very similar to those obtained using recombinant CYP2B6 (6.2 ± 0.7 μM). This type of inhibition, based on visual inspection of the Dixon plots and analysis of the data by nonlinear regression analysis using WinNonlin, was consistent with noncompetitive inhibition.

The data described above (Figs. 1–3) suggest that thioTEPA is a potent inhibitor of CYP2B6, with little or no effect on the activities of other P450s tested. To further understand the mechanism by which thioTEPA inhibits CYP2B6 activity, thioTEPA was preincubated in the presence of an NADPH-generating system and microsomes from human liver or recombinant human CYP2B6 before initiating the reaction by the addition of S-mephenytoin. Figure 4 demonstrates that indeed preincubation of thioTEPA (5 μM) with HLMs before the addition of S-mephenytoin (200 μM) slightly increased the degree of CYP2B6 inhibition. thioTEPA inhibited CYP2B6 activity by ~43% at a 30- versus 0-min preincubation and by ~21% when corrected for the control incubation (without thioTEPA) at a 30-min incubation. The effect of preincubation on the ability of thioTEPA to inhibit CYP2B6 was minimal when recombinant enzymes were used. The small decrease in the activity of CYP2B6 versus the duration of preincubation (Fig. 4B) was not unique, as it also happens in the control (without thioTEPA).

Discussion
The metabolism of cyclophosphamide is complex and shows a great deal of interpatient variability (Moore et al., 1994), thus confounding the desire of prescribers to provide predictable consistent treatment. This variability in metabolism may account for some of the differences seen in response and toxicity, and it is therefore possible that drug interactions that alter metabolism may change cyclophosphamide effects. The data that we present here provide a possible mechanism for the clinical drug interaction between cyclophosphamide and thioTEPA and show that thioTEPA is a potent and specific inhibitor of CYP2B6.

Anderson et al. (1996) investigated a possible drug-drug interaction between thioTEPA and cyclophosphamide by measuring the pharmacokinetics of cyclophosphamide and 4-OHCP in patients before and during coadministration of thioTEPA. Their results showed that the...
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vivo, our study did include the principal cytochrome P450 enzymes known to be responsible for cyclophosphamide hydroxylation, and it would seem unlikely that other enzymes play a major role.

It is interesting to note that the type of inhibition of CYP2B6 by thioTEPA is noncompetitive. thioTEPA is tris-aziridino-phosphine and could be metabolized by cytochrome P450 enzymes to an alkylating agent that could alkylate P450s. This phenomenon in turn may account for the noncompetitive nature we observed but would also result in time-dependent inhibition. Although the specific P450 isoform involved in the metabolism of thioTEPA in humans is not yet clear, evidence from animal studies suggest that thioTEPA is metabolized by P450s, notably CYP2B1 and CYP2C11 (Chang et al., 1995). There is also evidence that thioTEPA is a suicide inhibitor of certain rat P450s (Ng and Waxman, 1990). We have noted that the ability of thioTEPA to inhibit CYP2B6 was increased with the duration of preincubation of thioTEPA with an NADPH-generating system and HLMs before the addition of S-mephenytoin. Although, the effect was modest, it does suggest that inhibition of CYP2B6 by thioTEPA is time-dependent. The possibility that thioTEPA alkylates CYP2B6 and thus may contribute to the noncompetitive inhibition observed was not directly tested and cannot be ruled out.

Besides cyclophosphamide, recent articles have suggested that CYP2B6 plays an important role in the metabolism of a number of other clinically used drugs. The variability in the pharmacokinetics of these agents may be related to the variability in the level of CYP2B6 expression. CYP2B6 is highly inducible by drugs, such as phenobarbital, and this may explain differences in its level of expression, and recent advances in the underlying biochemical mechanisms of induction have elucidated this (Honkakoski et al., 1998). These advances may help identify other environmental factors that affect CYP2B6 expression. In addition, genetic polymorphisms in CYP2B6 may affect its activity. One such polymorphism was described in a Japanese population (Ariyoshi et al., 2001). This polymorphism has an allelic frequency of 20% and is the result of a G to T nucleotide change at position 516. The variant allele exhibits increased catalytic activity for O-deethylation of 7-ethoxycoumarin in vitro, whereas its in vivo effect has yet to be determined. Another study by Lang et al. (2001), found nine polymorphisms in the CYP2B6 gene, five of which resulted in amino acid substitutions. The authors showed that a polymorphism in exon 9 was associated with significantly reduced CYP2B6 protein expression and S-mephenytoin N-demethylase activity in human liver specimens. Taken together, these studies suggest an underlying genetic component to the variability in CYP2B6 activity.

Our data demonstrate for the first time that thioTEPA is a potent and specific inhibitor of CYP2B6. These findings have important implications. First, the specificity of thioTEPA can be used as a tool to study the activity of CYP2B6 in vitro so that we may be able to further characterize the role of this enzyme in human drug metabolism. Second, the clinical interaction of cyclophosphamide and thioTEPA documented in the literature seems to be mediated by the ability of thioTEPA to inhibit CYP2B6 and underlines the role of CYP2B6 in cyclophosphamide activation in vivo. Finally, thioTEPA is likely to inhibit the metabolism of agents beyond cyclophosphamide and caution should be used during coadministration with other CYP2B6 substrate drugs.

References


