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ORIGINAL ARTICLE

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Cytochrome P450 isozymes 3A4 and 2B6 are involved in the in vitro human metabolism of thiotepa to TEPA

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Abstract *Purpose*: To establish the cytochrome P450 (CYP) isozymes involved in the metabolism of the alkylating agent, thiotepa, to the pharmacologically active metabolite, TEPA. *Methods*: In vitro chemical inhibition studies were conducted by incubating thiotepa and pooled human hepatic microsomes in the presence of known inhibitors to CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. Studies were also performed with cloned, expressed CYP3A4, CYP2A6, CYP2E1 and CYP2B6 microsomes, and anti-CYP2B6 monoclonal antibody. *Results*: Known CYP3A4 inhibitors reduced TEPA production. Inhibition with CYP2E1 inhibitors was inconsistent. All other inhibitors produced little or no change in TEPA formation. Cloned, expressed CYP2B6 and CYP3A4 microsomes catalyzed TEPA formation, whereas CYP2A6 and CYP2E1 did not. Incubation of thiotepa with anti-CYP2B6 antibody and cloned, expressed CYP2B6 microsomes resulted in reductions in the formation of TEPA, but no change in TEPA formation occurred in human liver microsomes. *Conclusions*: Thiotepa is metabolized in human liver microsomes by CYP3A4 (major) and CYP2B6 (minor). There is a potential for CYP-mediated drug interactions with thiotepa. Pharmacokinetic variability of thiotepa may be related to expression of hepatic CYP isozymes.

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Introduction

Thiotepa, *N*,*N*′,*N*″-triethylene thiophosphoramide, is an alkylating agent that has been used in the treatment of many tumor types for nearly five decades. Thiotepa is associated with toxicity, especially bone marrow suppression. Its major use in the past 10 years has been in the setting of high-dose chemotherapy with stem cell transplant rescue [2, 3, 19, 20, 21, 26, 37, 38, 44]. Unfortunately, there are few metabolism, pharmacokinetic, or pharmacodynamic data in this setting. Thiotepa doses used in stem cell transplantation are up to 100 times greater than those used in conventional therapy, and hence there is a high potential for severe and possibly fatal drug toxicity [23, 25]. Up to 20% of patients die from a complication directly attributed to high-dose chemotherapy. Therefore, understanding chemotherapy metabolism is fundamental to designing chemotherapy regimens that minimize serious toxicity and improve survival.

Thiotepa undergoes oxidative desulfuration to *N*,*N*′,*N*″-triethylene phosphoramide (TEPA), a pharmacologically active compound, by the action of the cytochrome P450 enzyme system (Fig. 1) [30]. In rat liver microsomes, 50–80% of thiotepa is metabolized to TEPA [29]. TEPA has a longer plasma half-life than thiotepa, and is presumed to contribute substantially to its antineoplastic activity [10, 31]. In conventional and high-dose settings, TEPA excretion in the urine accounts for approximately 10% or less of the administered dose [9, 17, 43]. TEPA is further metabolized to *N*,*N*′-diethylene *N*″-2-chloroethylphosphoramide (monochloro-TEPA) which has alkylating activity significantly lower than that of TEPA [40]. Thiotepa also undergoes conjugation with glutathione catalyzed by glutathione S-transferase isozymes A1-1 and P1-1 to ultimately form thiotepa-mercapturate. Thiotepa-mercapturate excretion in the urine accounts for $<13\%$ of the administered

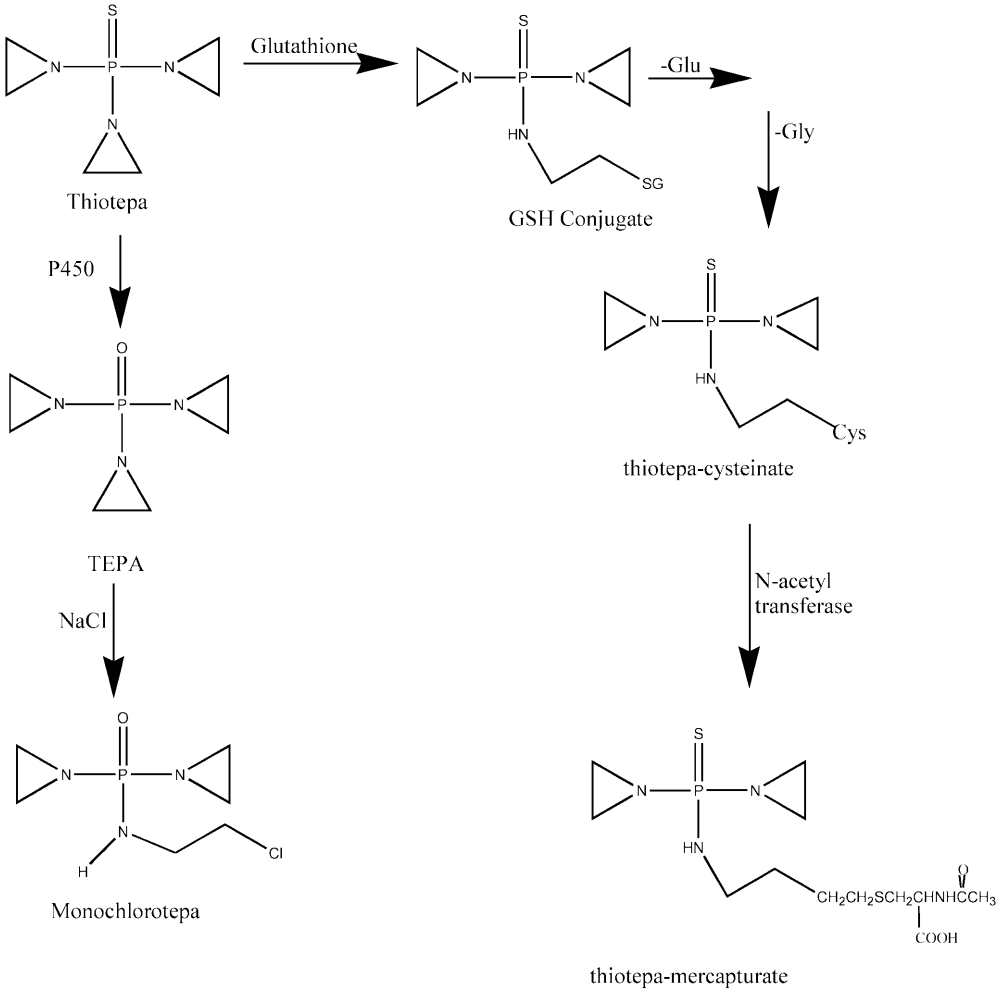

Fig. 1. Metabolism of thiotepa [41]

dose [40, 43]. This metabolite is believed to have minor alkylating activity [12, 40, 42]. The relative contribution of oxidative desulfuration and glutathione conjugation to human metabolism has not been well studied, especially in patients receiving high doses of thiotepa.

Metabolism studies in induced and uninduced rat liver microsomes have indicated that thiotepa is oxidized to TEPA by CYP isozymes 2B1, 2C11 and 2C6 [29, 30]. However, it is not known which human CYP isozymes are involved in its metabolism. The objective of this research was to identify the CYP isozyme(s) involved in the human metabolism of thiotepa to TEPA.

Materials and methods

Chemicals, cloned, expressed CYP microsomes, and antibody

Thiotepa was obtained from USP (Rockville, Md.). The following CYP isozyme inhibitors were obtained from Sigma Chemical Co (St. Louis, Mo.): furafylline (CYP1A2), coumarin (CYP2A6), omeprazole (CYP2C19), quinidine (CYP2D6), ketoconazole (CYP3A4), troleandomycin (CYP3A4), sulfaphenazole (CYP2C9), 4-methylpyrazole (CYP2E1), disulfiram (CYP2E1)], Tris acid, Tris base, β-NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and $MgCl_2$. The inhibitor paclitaxel (CYP2C8) was obtained from the Developmental Therapeutics Program, National Cancer Institute. Baclovirus-infected insect cells expressed CYP3A4 or CYP2E1 microsomes (Supersomes), human lymphoblast-expressed CYP2B6 microsomes, and anti-2B6 monoclonal antibody were obtained from Gentest (Woburn, Mass.). Cloned, expressed CYP2A6 insect cell microsomes were provided by Sharon Murphy, Ph.D., University of Minnesota. Diphenylamine was obtained from Acros Organics (Pittsburgh, Pa.). All chemicals and reagents were of analytical grade or higher. Control insect cell microsomes were purchased from Gentest.

TEPA synthesis

TEPA was synthesized by published procedures [11, 39]. The purity and identity of TEPA dissolved in methanol was determined by liquid chromatography on a Finnigan MAT LCQ Deca system with a Waters 600 mass spectrophotometer (>98% pure). The identity was confirmed by nuclear magnetic resonance spectroscopy. The white solid was stored in glass at –80°C under nitrogen. A working stock solution of TEPA (1 mg/ml) was prepared in ethanol as needed and stored at –80°C. At this concentration and temperature, TEPA was found to be stable for at least 7 days.

Preparation of human liver microsomes

Transplant grade human livers from adult male and female donors were obtained from Human Biologics (Phoenix, Ariz.), Anatomical Gift Foundation (Pittsburgh, Pa.) and LifeSource (Minneapolis, Minn.). Microsomes were processed by standard procedures and stored at –80°C. Equal microsomal aliquots from 12 livers were combined to form pooled microsomes. The total protein content of the pooled microsome solution was determined with a bicinchoninic acid protein assay kit (Pierce Chemical Company, Rockford, Ill.).

TEPA assay

TEPA production in liver microsomes was quantified by gas chromatography on a Shimadzu GC-14 system equipped with a nitrogen-phosphorus detector by previously published methods [29, 39]. TEPA, thiotepa and diphenylamine (internal standard) were separated on a J & W DB-17 fused silica capillary column (30 m×0.25 mm). The column oven and injection port were set at 155°C and 250°C, respectively. Helium at a flow rate of 9 ml/min was used as the carrier gas. The nitrogen-phosphorus detector was maintained at 275°C with a hydrogen flow rate of 3.5 ml/min and an air flow rate of 15 ml/min. A TEPA standard curve was prepared in human liver microsomes at concentrations of 0.05, 0.1, 0.25, 0.5, 1, 5 and 10 μg/ml. Quality control samples were prepared in human liver microsomes that contained 0.075, 0.75 and 7.5 μg/ml TEPA. Quality control samples were within 10% of the nominal values. TEPA concentrations were determined by comparing the peak area ratios of the TEPA to internal standard with those of authentic standards prepared in parallel. Intraday and interday coefficients of variation (CVs) were 6.5–21.3% and 6.9–22.6%, respectively. The highest CVs were observed at the lowest concentration (0.05 μg/ml).

Sample preparation

Microsome standards (100 μl) containing TEPA or incubated microsomal samples (100 μl) were extracted with 5 ml ethyl acetate after the addition of 10 μl internal standard solution (100 μg/ml solution of diphenylamine dissolved in ethanol, prepared fresh daily). The mixture was vortexed for 30 s and placed in a refrigerator (3°C) for 5 min. The samples were immediately centrifuged for 10 min at 2500 rpm, and the aqueous layer discarded. The solvent layer was transferred to a 13×100 mm glass test tube and dried under nitrogen at 30°C to a volume of approximately 750 μl. Samples were refrigerated for approximately 5 min, and aliquots transferred to autosampler vials containing 350 μl flat bottomed glass inserts. The vials were placed in a water-cooled autoinjector during analysis. Cooling techniques were used during the extraction and analysis process to avoid potential decomposition of TEPA. The samples were injected onto the column in 2-μl aliquots.

Human liver microsome incubations

Microsomes from six individual human livers were used for assay validation and to determine optimal protein and thiotepa concentration, and incubation conditions. Thiotepa (20–100 μg/ml, 105.8–529 μ*M*) was incubated in six different liver samples (protein concentrations 0.25–0.5 mg/ml) to determine the concentration of thiotepa required to achieve measurable turnover to TEPA. Observed plasma concentrations in humans (Cp_{max}) range from 1 to 22 μg/ml [1, 23, 33]. However, at 20 μg/ml thiotepa, TEPA production was not detectable with our analytical method. Therefore, a thiotepa concentration of 100 μg/ml was selected for the inhibition studies. Incubations of human hepatic microsomes were performed at 37°C in an Eberbach shaking water bath in 1.5 ml polypropylene Eppendorf microcentrifuge tubes (Westbury, N.Y.). The final incubation volume was 0.5 ml, and consisted of human liver microsomes (0.5 mg protein/ml), thiotepa (100 μg/ml) prepared fresh daily in Tris buffer, inhibitor when appropriate, and Tris buffer (50 m*M*, pH 7.4, at 37°C). After a 2-min preincubation at 37°C, the reaction was initiated by the addition of an NADPH-generating system consisting of 100 μl cofactor solution (containing 1 m*M* β-NADP, 5 m*M* $MgCl_2$ and 5 m*M* glucose-6-phosphate) and 100 μl glucose-6-phospate dehydrogenase (1 IU/ml). The mechanism-based inhibitors, furafylline and troleandomycin, were preincubated for 15 min with the microsomes and the NADPH-generating system, and the reaction was initiated by the addition of thiotepa [28]. Control tubes were initiated using 200 μl Tris buffer in place of the NADPH-generating system. The reaction was allowed to proceed for 30 min and was terminated by flash freezing in an isopropanol dry-ice bath. The samples were stored at –80°C until analyzed for TEPA production.

Inhibition experiments

Pooled human liver microsomes (0.5 mg protein/ml) were incubated with each CYP isozyme inhibitor and thiotepa (100 μg/ml). The final inhibitor concentrations selected were approximately 25–30 times the reported K_i values. The concentrations selected for the inhibitors were 20 μ*M* for furafylline, 50 μ*M* for troleandomycin, 35 μ*M* for paclitaxel, 50 μ*M* for disulfiram, 10 μ*M* for sulfaphenazole, 30 μ*M* for omeprazole, 48.7 μ*M* for 4-methylpyrazole, 5 μ*M* for quinidine, 1, 10 and 50 μ*M* for ketoconazole and 20 μ*M* for coumarin [4, 15, 24, 28, 32, 34, 35, 36]. The inhibitors were dissolved in methanol, except where noted below, and then further diluted 10 or 100 times with Tris buffer to obtain the final concentrations. To enable solubility and minimize the solvent effects on CYP isozymes [5, 7], the solvent concentrations were less than 0.1% (v/v) for all inhibitors except omeprazole which contained 1% methanol, paclitaxel which contained 1% ethanol, and disulfiram which contained 0.2% acetonitrile. 4-Methylpyrazole was dissolved in Tris buffer. Microsomal incubations containing inhibitors, solvents and thiotepa were compared to control incubations containing identical concentrations of solvents and thiotepa, but without inhibitors. The incubations were conducted as described in the previous section.

Cloned, expressed CYP microsome incubations

Incubations containing thiotepa (50–100 μg/ml) and cloned, expressed CYP3A4 and CYP2E1 microsomes (50 pmol CYP450), or expressed CYP2A6 microsomes (50 pmol CYP450), or expressed CYP2B6 microsomes (0.5 mg protein/ml) were conducted in a manner identical to those of the human liver microsomes. The CYP3A4 and CYP2E1 microsomal preparations also contained P450 reductase and coexpressed cytochrome b_5. Control incubations, with insect cell microsomes that contained no expressed isozymes, were performed.

Anti-CYP2B6 monoclonal antibody incubations

To further study metabolism by CYP2B6, thiotepa (100 μg/ml) was incubated with lymphoblast-expressed CYP2B6 microsomes (77.5 pmol CYP) and pooled human liver microsomes (0.5 mg protein/ml). Each sample was incubated with and without anti-CYP2B6 monoclonal antibody (0.1 mg protein). The anti-CYP2B6 antibody was added to a solution of CYP2B6 microsomes and Tris buffer, and placed on ice for 15 min. The incubations were then carried out in an identical manner to the human liver microsome incubations. Control incubations with insect cell microsomes were performed.

Kinetic analysis

Incubations identical to those described above, containing thiotepa (20–4000 μg/ml, 105.8 μ*M* to 21 m*M*), were conducted in both pooled human liver microsomes and cloned, expressed CYP3A4 and CYP2B6 to determine the K_m and V_{max} for thiotepa metabolism. Saturation was not achieved at these thiotepa concentrations and therefore K_m and V_{max} were not obtained.

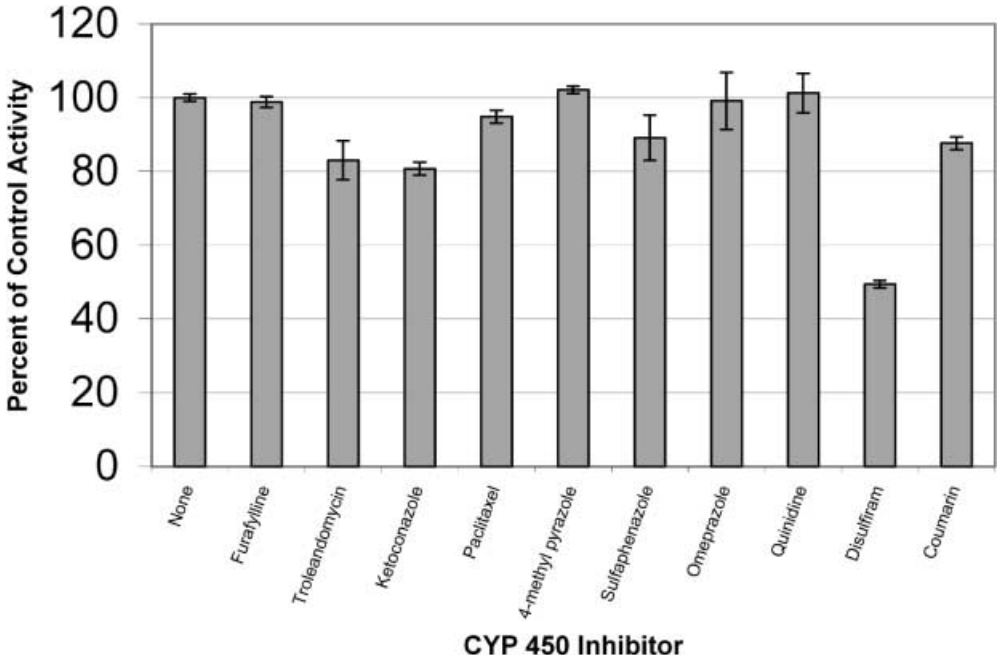

Fig. 2. Inhibition of TEPA formation in pooled human liver microsomes in the presence of CYP inhibitors. Each bar represents the mean ± SD of triplicate experiments

Data analysis

All experiments were conducted in triplicate. The percent inhibition in relation to control activity was the ratio of the mean TEPA concentration in the presence of inhibitor to the mean TEPA concentration in the control with no inhibitor. A 20% reduction in the formation of TEPA was considered significant.

Results

Preliminary experiments

Experiments were performed to determine the linearity of TEPA production with respect to human liver microsome protein concentration and incubation time, and efficiency of turnover of thiotepa to TEPA. TEPA formation from incubations of human liver microsomes (0.1, 0.25, 0.5 and 1 mg protein/ml) with thiotepa (20–100 μg/ml) was linear (results not shown). Incubation periods of 0, 5, 15, 30, 45 and 60 min were evaluated with thiotepa at 50 and 189 μg/ml. TEPA formation was linear to approximately 45 min (data not shown). A protein concentration of 0.5 mg/ml and a 30-min incubation time was therefore selected for all further experiments. The mean TEPA formation rate under these conditions was 2.2 μg/h per mg protein.

Inhibition of TEPA formation

The results of the inhibitor studies are presented in Fig. 2. In the presence of troleandomycin and ketoconazole (1 μ*M*) (CYP3A4 inhibitors) and coumarin (CYP2A6 inhibitor) the formation of TEPA was reduced by ≥18% compared to thiotepa without inhibitor [35].

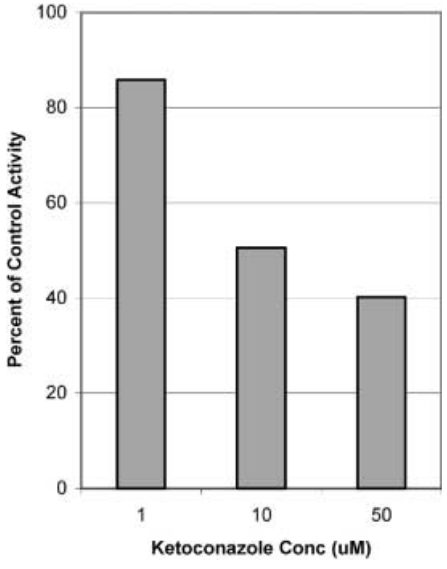

Fig. 3. Mean inhibition of TEPA formation by ketoconazole

Disulfiram (CYP2E1 inhibitor) resulted in a 49% reduction in TEPA formation, whereas 4-methylpyrazole (CYP2E1 inhibitor) had no effect. All other inhibitors produced minimal or no reduction in TEPA. Various concentrations of ketoconazole were studied. As expected, at higher concentrations of ketoconazole greater inhibition of TEPA production was observed (Fig. 3).

Thiotepa metabolism by cloned, expressed microsomes

Due to the lack of a known specific CYP2B6 inhibitor, thiotepa was incubated with cloned, expressed CYP2B6

microsomes, and turnover to TEPA occurred (Fig. 4). To verify the results of the inhibitor experiments, thiotepa was incubated with cloned, expressed human CYP3A4, CYP2E1 and CYP2A6 microsomes. Cloned, expressed CYP3A4 microsomes catalyzed TEPA production, whereas CYP2E1 and CYP2A6 microsomes did not, suggesting that CYP2E1 and CYP2A6 are not involved in thiotepa metabolism (Fig. 4).

Inhibition of metabolism by anti-CYP2B6 monoclonal antibody

The presence of anti-2B6 monoclonal antibody in incubations of thiotepa and human liver microsomes resulted in no reduction in TEPA formation compared to control incubations containing no antibody. However, the presence of anti-2B6 monoclonal antibody in incubations of thiotepa and cloned, expressed CYP2B6 microsomes resulted in a 69% reduction in TEPA formation compared to control incubations (data not shown).

Discussion

This is the first study to identify the specific human CYP isozymes involved in the oxidative metabolism of thiotepa to the active metabolite, TEPA. The isozymes were identified by chemical inhibitors, cloned and expressed CYP microsomes and monoclonal antibody methods. CYP3A4 is the major enzyme, with CYP2B6 making a minor contribution.

Ten chemical inhibitors commonly used in CYP identification studies were incubated with pooled human hepatic microsomes and thiotepa. With the exception of disulfiram, the selected inhibitors have specificity at the selected concentrations for individual isozymes. In the presence of ketoconazole (CYP3A4), troleandomycin (CYP3A4), and coumarin (CYP2A6), TEPA formation was reduced compared to control incubations. Disulfiram (CYP2E1) resulted in 49% reduction in TEPA production, but 4-methylpyrazole (CYP2E1, CYP2D6) resulted in no inhibition. Disulfiram has been shown to be a nonselective inhibitor, so reductions in TEPA formation in its presence were most likely due to the other active isozymes or mechanisms [6, 14]. In addition, in further experiments, no TEPA was formed from thiotepa in incubations with cloned, expressed CYP2E1 microsomes, and thus CYP2E1 is unlikely to be involved. The involvement of CYP3A4 and CYP2A6 was further investigated with cloned, expressed microsomal CYP enzymes. Thiotepa incubated with cloned, expressed CYP3A4 showed good turnover to TEPA, whereas incubations with cloned, expressed CYP2A6 showed no TEPA production.

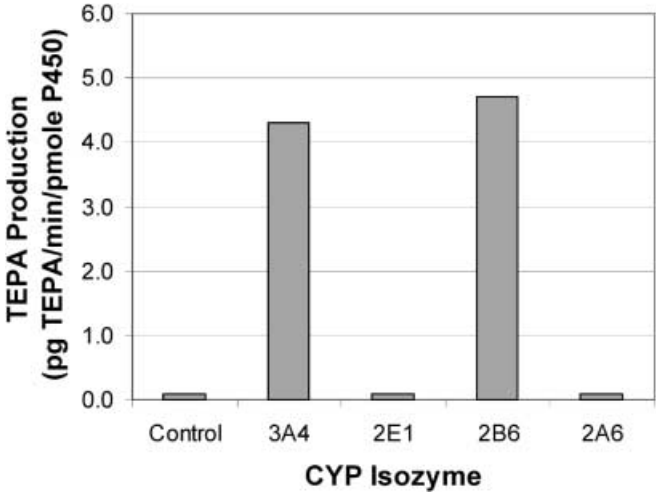

Fig. 4. Mean TEPA production with cloned, expressed microsomes

Because there is no selective inhibitor for CYP2B6, we investigated its role in metabolism with human liver microsomes, cloned, expressed CYP2B6 microsomes, and anti-CYP2B6 antibody. In human liver microsomes, CYP2B6 antibody did not reduce TEPA formation, but when thiotepa was incubated with cloned, expressed CYP2B6 microsomes and antibody, a 69% reduction in TEPA formation was observed. Therefore, CYP2B6 appears to be involved in CYP metabolism of thiotepa, but is most likely a minor contributor. This is consistent with CYP2B6 representing only a small proportion of total hepatic CYP ($<$ 1–5% of total P450 in human liver) [13, 18].

Ketoconazole is known to be a selective inhibitor of CYP3A4 at low concentrations. Ketoconazole inhibited TEPA production by only about 20% at low concentrations and by nearly 60% at higher nonspecific concentrations (Fig. 3). These results suggest that TEPA formation may occur through multiple isozymes.

Many agents administered with thiotepa are CYP2B6 or CYP3A4 substrates, inhibitors or inducers, and hence there is the potential for enhanced toxicity or reduction in efficacy. TEPA has a substantially longer half-life than thiotepa, and therefore provides more antitumor activity. In several clinical pharmacokinetic studies it has been found that proper sequencing of high-dose chemotherapy containing thiotepa is critical, presumably due to the changes in antineoplastic exposure due to CYP isozyme induction or inhibition [22, 23]. These findings suggest that regimens resulting in higher TEPA concentrations may be associated with greater toxicity. Induction of thiotepa metabolism has been suggested to be responsible for higher TEPA exposure. Others have found that thiotepa may inhibit in vivo activation of cyclophosphamide, possibly through competition for CYP isozymes (particularly CYP3A4).

Van Maanen et al. have recently reported the results of an in vitro inhibition study of thiotepa metabolism in human liver microsomes [41]. Thiotepa metabolism to TEPA was not inhibited by cyclophosphamide, carboplatin, ciprofloxacin, amphotericin B, itraconazole, fluconazole, ondansetron, granisetron, dexamethasone,

acyclovir, ranitidine, or lorazepam. These findings suggest that thiotepa metabolism may not be influenced by these agents, perhaps because thiotepa metabolism also occurs through other enzyme systems including glutathione conjugation through glutathione S-transferase [8, 12]. However, many common oncology agents have not been tested. Care must be exercised in the extrapolation of these findings to the clinical setting, since previous clinical pharmacokinetic studies have suggested that thiotepa disposition may be modified by concomitant therapy. In the case of thiotepa, which is converted to a highly active compound, induction of CYP3A4 and CYP2B6 activity may be highly relevant [16, 27]. To date, no human hepatocyte induction studies evaluating the effect of CYP induction on human thiotepa metabolism have been reported.

The therapeutic window is narrow in the setting of high-dose chemotherapy and transplantation. Small changes in metabolism may be the difference between aggressive ablative regimens and fatal toxicity. Whereas, in standard-dose regimens, induction or inhibition of CYP isozymes may result in insignificant changes in chemotherapy exposure and toxicity. Therefore, drug metabolism should be carefully considered when designing chemotherapy regimens.

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