Contribution of CYP3A5 to hepatic and renal ifosfamide N-dechloroethylation

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Abstract

Ifosfamide nephrotoxicity is attributed to the formation of a toxic metabolite – chloroacetaldehyde via N-dechloroethylation, a reaction that is purportedly catalyzed by CYP3A and CYP2B6. Because allelic variants of CYP3A5 are associated with polymorphic expression of microsomal CYP3A5 in human liver and kidneys, we hypothesized that ifosfamide N-dechloroethylation depends on CYP3A5 genotype.

Methods: We compared ifosfamide N-dechloroethylation activity in cDNA-expressed CYP3A4 and CYP3A5. Ifosfamide N-dechloroethylation was also assessed in liver (N=20) and kidney (N=21) microsomes from human donors with different CYP3A5 genotypes. Results: Ifosfamide N-dechloroethylation was catalyzed by recombinant CYP3A5 at a rate comparable to recombinant CYP3A4. In human liver microsomes matched for CYP3A4 protein content, N-dechloroethylation was more than 2-fold higher in those from donors carrying CYP3A5*1 allele that express CYP3A5 relative to those from donors homozygous for the mutant CYP3A5*3. Correlation analysis revealed that ifosfamide N-dechloroethylation was significantly associated with CYP3A4 and CYP3A5 protein concentration, but not with age, sex or CYP2B6 protein concentration. In hepatic microsomes not expressing CYP3A5 protein, ifosfamide N-dechloroethylation was inhibited 53-61% and 0-3% by monoclonal antibodies specific for CYP3A4/5 or CYP2B6, respectively. Ifosfamide N-dechloroethylation was not detected in renal microsomes obtained from CYP3A5*3/*3 donors. In contrast, it was readily measurable in microsomes isolated from four kidneys of CYP3A5*1 carriers, which was almost completely inhibited by the CYP3A inhibitor—ketoconazole. CYP2B6 protein could not be detected in this panel of human renal microsomes. Conclusions: CYP3A5*1 genotype is associated with higher rates of ifosfamide N-dechloroethylation in human liver and kidneys.
Introduction

The alkylating agent ifosfamide causes moderate to severe nephrotoxicity in 18–28% of patients. (Loebstein et al., 1999; Skinner et al., 2000; McCune et al., 2004) Identifying patient-specific risk factors are of paramount importance to anticipate the potential for this long-term complication, as a chemoprotectant does not exist to prevent this adverse drug reaction. Cumulative ifosfamide dose greater than 60 g/m² is the most commonly identified risk factor, but no safe dose limit exists. (Loebstein et al., 1999; Skinner et al., 2000)

Ifosfamide itself is not cytotoxic in cultured renal epithelial cells or isolated perfused rat kidneys exposed to ifosfamide concentrations that have been observed in cancer patients receiving ifosfamide (i.e., 160 – 650 µM). (Kurowski and Wagner, 1993; Mohrmann et al., 1993; Zamlauski-Tucker et al., 1994; Springate et al., 1999; Boddy and Yule, 2000) This raises the possibility that ifosfamide metabolites are nephrotoxic. Ifosfamide is bioactivated via 4-hydroxylation to several reactive products including acrolein. In parallel, ifosfamide is also metabolized by N-dechloroethylation to 2-dechloroethylifosfamide (2DCEI), 3-dechloroethylifosfamide (3DCEI), and an equivalent molar amount of chloroacetaldehyde. Both acrolein and chloroacetaldehyde cause toxicity in LLC-PK1 cells; (Mohrmann et al., 1992; Mohrmann et al., 1993) however, acrolein does not impair the function of isolated perfused rat kidneys or after long term exposure in animal models. (Parent et al., 1992; Zamlauski-Tucker et al., 1994) Chloroacetaldehyde has consistently shown a concentration-dependent cytotoxic effect in several in vitro models (i.e., porcine or rabbit cultured renal tubules; isolated perfused rat kidneys) with a minimum toxic concentration that ranges from 12.5 to 64 µM. (Mohrmann et al., 1993; Springate, 1997; Springate et al., 1999) In cultured primary human proximal tubules cells, the minimum chloroacetaldehyde concentration for toxicity was reported to be 500 µM, which is well above the observed peak plasma concentrations (i.e., 0.5 – 109 µM) in patients receiving standard doses of ifosfamide. (Goren et al., 1986; Kurowski and Wagner, 1993; Dubourg et al., 2001) Hence, circulating concentrations of chloroacetaldehyde is not sufficient to explain nephrotoxicity. It has recently been
demonstrated that renal microsomes from pigs and humans catalyze ifosfamide N-dechlo-roethylation. (Woodland et al., 2000; Aleska et al., 2001) Intrarenal ifosfamide N-dechlo-roethylation, with the release of proximate toxin chloroacetaldehyde, has been postulated to be the primary cause of renal toxicity. (Aleska et al., 2004)

In human liver microsomes, N-dechlo-roethylation of ifosfamide correlates well with the catalytic activities of CYP3A and CYP2B6. (Walker et al., 1994; Roy et al., 1999; Huang et al., 2000) An earlier study by Gervot suggested renal expression of CYP2B6 protein, although the investigators cautioned that the observed immunoreactive protein may be CYP2B7. (Gervot et al., 1999) CYP3A4 and CYP3A5 are related isozymes that display 88% similarity in amino acid sequence and great overlap in their substrate specificity. (Wrighton and Thummel, 2000) Expression of CYP3A4 is largely confined to the liver and intestinal mucosa; it has a negligible presence in the kidneys. (Haehner et al., 1996; Koch et al., 2002; Givens et al., 2003) Tissue expression of CYP3A5 is more widespread, and is a major CYP enzyme in the kidneys. (Haehner et al., 1996; Wrighton and Thummel, 2000; Givens et al., 2003) However, it has been suggested that CYP3A5 mediated formation of chloroacetaldehyde from N-dechet roethylation of ifosfamide occurs at a much lower rate than CYP3A4. (Huang et al., 2000) This comparison was confounded by using two different expression systems of recombinant P450 (Supersomes™): CYP3A4 co-expressed with cytochrome b₅ versus CYP3A5 supplemented with an equimolar amount of exogenous cytochrome b₅. It is known that catalytic activity of CYP3A4 is higher when co-expressed with cytochrome b₅, in comparison to equimolar amounts of exogenous cytochrome b₅. (Yamazaki et al., 1999) Perhaps the previously reported lower ifosfamide N-dechlo-roethylation by CYP3A5 simply reflected the difference in expression systems. Thus, the differences in rates of ifosfamide N-dechlo-roethylation between CYP3A4 and CYP3A5 should be verified in comparable expression systems.

Immunohistochemical studies have revealed localization of CYP3A5 in the epithelium of the proximal tubule, the thin loop of Henle, cortical collecting ducts, and the pelvis. (Leggat et al., 1994) Renal CYP3A5 protein expression is polymorphic, resulting in profound differences (>100-fold) in
CYP3A-dependent catalytic activity between individual donors. (Haehner et al., 1996; Koch et al., 2002; Givens et al., 2003) There is strong evidence to suggest that inherited mutations in the CYP3A5 gene contribute to polymorphic protein expression/function. A single base substitution within intron-3 of CYP3A5 (6986A>G) creates an alternative acceptor splice site that results in the production of aberrant mRNA and a truncated non-functional protein. (Kuehl et al., 2001) This mutation (CYP3A5*3) is the major cause of polymorphic CYP3A5 expression in the liver, small intestine, and kidneys. (Lin et al., 2002; Givens et al., 2003) Thus, we hypothesize that rates of ifosfamide N-dechlo-roethylation in human liver and kidney is dependent on CYP3A5 genotype.

The specific aims of our studies were: 1) to compare the rate of ifosfamide N-dechlo-roethylation by cDNA expressed CYP3A4 and CYPA5 with equivalent cytochrome b5 supplementation or expression; 2) to assess ifosfamide N-dechlo-roethylation activity in a panel of 20 liver microsomes matched for CYP3A4 content and in 21 kidney microsomes obtained from human donors with known CYP3A5 genotype; 3) to assess the relative contribution of CYP3A5 and CYP2B6 in ifosfamide N-dechlo-roethylation by microsomes isolated from human livers and kidneys.

**Methods**

**Materials.** NADPH, ketoconazole, thiopeta and troleandomycin (TAO) were obtained from Sigma Chemical Company (St. Louis, MO). Ifosfamide was provided by the National Cancer Institute, Drug Synthesis & Chemistry Branch. 2-Dechloroethylifosfamide and 3-dechloroethylifosfamide were kindly provided by Dr. Uif Niemeyer at Asta Medica (Frankfurt, Germany). Dipropylycyclophosphamide was synthesized as described by Kalhorn et al. (Kalhorn et al., 1999) Supersomes™ containing cDNA-expressed CYP3A4 + P450 reductase (lot #18, catalog no. 456207), CYP3A5 + P450 reductase (lot #18, catalog no. 456235), CYP3A4 + P450 reductase + cytochrome b5 (lot #55, catalog no. 456202), CYP3A5 + P450 reductase + cytochrome b5 (lot #1, catalog no. 456256), and CYP2B6 + P450 reductase + cytochrome b5 (lot #13513, catalog no. 456255) were purchased from BD Gentest (Woburn, MA).
Cytochrome b<sub>5</sub> (lot #23525) was purchased from Panvera (Carlsbad, CA). Monoclonal antibodies directed towards CYP3A4/5 (catalog no. 458334) and towards CYP2B6 (catalog no. 458326) were also purchased from BD Gentest (Woburn, MA); mouse IgG (whole molecule) (catalog no. 015-000-003) were purchased by Jackson Immunologics (West Grove, PA) to serve as a control. Anti-human polyclonal antibody (catalog no. 458226) for Western blot of CYP2B6 within renal tissue was purchased from BD Gentest (Woburn, MA).

**Tissue collection and preparation.** Human livers were obtained from the University of Washington School of Pharmacy Tissue Bank (Seattle, WA). Liver microsomes were prepared by differential centrifugation and stored at -80°C as previously described.(Lin et al., 2002) Twenty one adult human kidneys were obtained from the National Disease Research Interchange (Philadelphia, PA); 17 of the donors were Caucasian, one was Hispanic and three were of unknown ethnicity.(Givens et al., 2003) Upon receiving the kidneys, microsomes were prepared by differential centrifugation and stored at -80°C as previously described.(Dai et al., 2004) The final pellets were suspended in 100 mM potassium phosphate, 0.25 M sucrose, and 100 mM EDTA. Microsomal CYP3A5 specific content was measured by Western blot as previously described for liver (Lin et al., 2002) and renal microsomes (Dai et al., 2004), using a CYP3A5-specific antibody.

**CYP3A5 genotyping and midazolam 1’ hydroxylation.** All procedures for CYP3A5 genotyping followed methods described previously for human liver.(Lin et al., 2002) Single nucleotide polymorphisms (SNPs) in CYP3A5 corresponding to CYP3A5*3 (6986A>G), CYP3A5*6 (14690G>A) and CYP3A5*7 (27131-32, insertion T) allelic variants were evaluated. Midazolam 1’-hydroxylation activity of liver and renal microsomes used in this study has previously been reported.(Lin et al., 2002; Givens et al., 2003)

**CYP2B6 protein concentrations.** CYP2B6 protein content of microsomal preparations from human kidneys was analyzed according to the method of Ekins et al.(Ekins et al., 1997) Microsomes (5-
10 µg) were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE); after SDS-PAGE, proteins were transferred electrophoretically from the polyacrylamide gels to Immobilon P (Millipore) for immunoblotting using polyclonal antibodies produced against human CYP2B6-specific peptides.

Ifosfamide N-dechloroethylation by cDNA-expressed CYP3A4 and CYP3A5. First, the saturation kinetics of ifosfamide N-dechloroethylation was evaluated using CYP3A4 + P450 reductase Supersomes and CYP3A5 + P450 reductase Supersomes; their respective cytochrome c reductase activity was 370 and 2200 n mole/(min x mg protein). Ifosfamide at concentration ranging from 0.25 to 17.5 mM was preincubated with 10 pmol of recombinant enzyme and 30 pmol of cytochrome b₅ (a 1:3 molar ratio) in a pH 7.4, 0.1 M potassium phosphate buffer containing 1 mM EDTA at 37°C for 5-min before addition of NADPH (1 mM final concentration). The incubation volume was 500 µL. After 15-min, the reactions were stopped with the addition of 4-mL of an ice-cold dichloromethane and isopropyl alcohol mixture (95%:5%). All incubations were performed in triplicate in silanized glass tubes to prevent adsorptive loss of 2-DCEI and 3-DCEI.

To evaluate the effect of cytochrome b₅ supplementation, ifosfamide N-dechloroethylation by the two aforementioned Supersomes was measured with and without the addition of cytochrome b₅ at an ifosfamide concentration of 0.25 mM. We also compared ifosfamide N-dechloroethylation between CYP3A4 + P450 reductase + cytochrome b₅ Supersomes and the recently available CYP3A5 + P450 reductase + cytochrome b₅ Supersomes, which are well matched in their activity of cytochrome c reductase (i.e., 5100 versus 3900 n mole/min/mg protein, respectively) and cytochrome b₅ content (i.e., 1400 and 1600 pmol/mg protein, respectively).

Microsomal ifosfamide N-dechloroethylation. Microsomal N-dechloroethylation activity was determined at a fixed ifosfamide concentration of 0.25 mM, which is well below the reported Km and within the plasma concentration range observed in patients.(Kurowski and Wagner, 1993; Boddy and
Initially, ifosfamide N-dechloroethylation was evaluated in a panel of 20 human liver microsomes, ten of them from CYP3A5*3/*3 donors and the remaining ten from donors carrying a CYP3A5*I allele. Each of the ten pairs of microsomes were matched within 15% of CYP3A4 protein content. The levels of CYP2B6 in these human liver microsomes were determined by Western blot with polyclonal antibodies produced against human CYP2B6-specific peptides. Mean CYP2B6 protein concentrations did not differ between the two groups (p=0.67). Ifosfamide at a final concentration of 0.25 mM was pre-incubated with 100 µg of human liver microsomal protein in 0.5 ml volume of phosphate buffer containing 0.1 mM EDTA. Incubations with liver microsomes were conducted in triplicate. After a 5-minute preincubation, the reaction was initiated with the addition of NADPH at a final concentration of 1 mM. The incubations were stopped after 30 min by the addition of 4-mL of an ice-cold dichloromethane and isopropyl alcohol mixture (95%:5%).

Next, we evaluated ifosfamide N-dechloroethylation in 21 human kidney microsomes, four of which came from heterozygotes for the CYP3A5*I allele and the rest were from CYP3A*3/*3. Incubation conditions for the renal microsomes were identical to that for human liver microsomes, with the exception that the incubation duration was 120 min. Prior to initiating the human renal microsome experiments, the chemical stability of ifosfamide, 2-DCEI and 3-DCEI over 120 min of incubation with boiled human liver microsomes was confirmed. We also assessed possible complication from secondary metabolism of 2-DCEI and 3-DCEI during a prolonged renal microsomal incubation. To conserve the limited amount of human kidney microsomes, we used a dilute preparation of human liver microsomes as controls. Based on the previously determined rates of midazolam 1'-hydroxylation in the human kidney and liver microsomes, we scaled down the protein content of one human liver preparation to simulate the expected activity of the human kidney microsomes. The ifosfamide N-dechloroethylation rate of this dilute liver microsomal preparation was later shown to be 500-fold higher than the average rate in CYP3A5*I/*3 human kidney microsome preparations. Using this dilute liver microsomal preparation, we confirmed that 2-DCEI and 3-DCEI concentrations were stable over 120 min, i.e., no product loss due to secondary
metabolism. Control incubations with dilute human liver microsomes were included in each run: with ifosfamide and NADPH; NADPH alone (i.e., no ifosfamide), and ifosfamide alone (i.e., no NADPH). These controls provided a check for background, non-enzymatic formation of DCEIs.

**Inhibition studies.** The quantitative contribution of CYP3A and CYP2B6 towards ifosfamide N-dechloroethylation in human liver microsomes was assessed by CYP-specific chemical inhibitor and immuno-inhibition studies. Similarly, chemical inhibition studies were conducted to assess the relative contribution of CYP3A5 and CYP2B6 in human renal microsomes. Specificity of the chemical inhibitors was checked using cDNA-expressed CYPs – specifically CYP2B6 + P450 reductase + cytochrome b5 with ketoconazole and TAO and CYP3A4 + P450 reductase + cytochrome b5 with thiotepa. The condition of immuno-inhibition experiments with each monoclonal antibody were optimized using human liver microsomes based on manufacturer specifications. Incubation conditions were identical to that described above, with the notable exceptions that the immuno-inhibition experiments were conducted in plastic tubes and with 1 pmole of CYP3A4 + P450 reductase + cytochrome b5.

Preliminary experiments indicated that maximal inhibition of ifosfamide N-dechloroethylation was achieved in the presence of 10 µl monoclonal antibody against CYP3A4/5 and CYP2B6; hence, this volume of monoclonal antibody was used in all subsequent experiments. For immuno-inhibition, the monoclonal antibody and expressed enzymes or human liver microsomes were preincubated for 15 minutes on ice, followed by the addition of 37°C buffer. Ifosfamide was then added followed by an additional 5-min preincubation, and the reaction was started with the addition of NADPH. For both ketoconazole and thiotepa inhibition, ifosfamide and inhibitor were preincubated with expressed enzymes or human microsomes for 5-min, and reaction was started with the addition of NADPH. The final concentration of ketoconazole was 1 µM, which is 10 times its Ki for CYP3A5-mediated midazolam 1’-hydroxylation (109 ± 20 nM)(Gibbs et al., 1999) The final concentration of thiotepa was 50 µM, which is 10 times the Ki for CYP2B6-mediated S-mephenytoin N-demethylation in human liver microsomes (4.8 ± 0.3 µM)(Rae et al., 2002) TAO was preincubated with expressed enzyme or human liver
microsomes, NADPH and buffer for 30-minutes; the reaction was started with the addition of ifosfamide. The final TAO concentration was 10 µM, which has been reported to inhibit testosterone β–hydroxylation – CYP3A catalyzed reaction – by 85%, and did not inhibit bupropion hydroxylation – a CYP2B6 catalyzed reaction – in human liver microsomes.(Faucette et al., 2001). All inhibition data are expressed relative to ifosfamide N-dechloroethylation in the same expression system or human microsomes without inhibitor conducted on the same day under the same conditions.

The proportion of ifosfamide N-dechloroethylation mediated by CYP3A4 and CYP2B6 was assessed in three human liver microsomal preparations that did not express CYP3A5 (i.e., CYP3A5*3/*3 genotype). The effects of ketoconazole and thiotepa were evaluated in human renal microsomes that contained CYP3A5 protein or those that produced 2-DCEI and 3-DCEI at concentrations greater than four times the limit of quantitation. Ketoconazole and TAO were employed as CYP3A inhibitors.(Gibbs et al., 1999; Faucette et al., 2001) Their potential overlap in inhibition towards CYP2B6 was checked using CYP2B6 + P450 reductase + cytochrome b₅ Supersomes. Thiotepa was used as a specific CYP2B6 inhibitor.(Rae et al., 2002). The effect of thiotepa on CYP3A-mediated ifosfamide N-dechloroethylation was checked using CYP3A4 + P450 reductase + cytochrome b₅ Supersomes.

**Analysis of dechloroethylifosfamide metabolites.** Our early experience indicated that chloroacetaldehyde is inherently unstable in aqueous media, which poses tremendous difficulty in sample storage, handling and analysis. Instead, we developed a sensitive and robust LC-MS method for quantitating the parallel by-products of ifosfamide N-dechloroethylation, those being 2-DCEI and 3-DCEI, in microsomal incubates. The sum of 2-DCEI and 3-DCEI formation was used as an index of chloroacetaldehyde production; the two products are formed at an equi-molar ratio to chloroacetaldehyde.(Norpoth, 1976) After termination of reactions, the internal standard (dipropylcyclophosphamide, 40 ng) was added to all samples and control incubates. The tubes were then capped and shaken on a reciprocating shaker at room temperature, then centrifuged for 10 min. The aqueous layer was removed from the samples, and the organic layer was transferred to silanized 12x75
mm glass culture tubes. The organic layer was evaporated to dryness at 40°C under a stream of dry air. The residue was re-dissolved in 50 µl of mobile phase and gently vortexed for 5 seconds. The resulting solution was transferred to an autosampler vial with insert and capped.

Calibration standards were prepared in inactivated (boiled) human liver microsomes. Standard curves were prepared in the range of 0.025 to 10 ng for each analyte; the limit of quantitation was 0.025 ng in a 0.5 ml sample. Nominal chromatographic separation was achieved on a 3 µ Hypersil BDS-C18 3.0 mm x 125 mm column heated to 35°C, using a mobile phase of acetonitrile in 20 mM ammonium acetate buffer; the acetonitrile gradient was set at 15% from time 0 to 3 min; 15% to 75% from 3 to 7 min, maintained at 75% from 7 to 15 min, and then decreased to 15% from 15 to 16 minutes. Retention times of 2-DCEI, 3-DCEI and internal standard were 4.6, 5.4 and 10.5 min, respectively. The ionization mode was positive ion electrospray. Selective ion monitoring was set for m/e of 199 for 2-DCEI and 3-DCEI and 221.1 for the internal standard. Interday coefficient of variation for replicate analysis of quality control was 4.5% for 2-DCEI and 3.1% for 3-DCEI.

**Data analysis.** Ifosfamide N-dechloroethylation rate versus substrate concentration data were fitted to a Michaelis-Menten model using the numerical module of the general modeling program SAAM (SAAM Institute, Seattle, WA). Statistical analysis was performed by SAS (Version 9.1; Cary, NC). Wilcoxon rank-sum test was used for comparison of ifosfamide N-dechloroethylation by liver microsomes isolated from CYP3A5*3/*3 and *1/*3 donors. Multiple linear regression was used to estimate the association of ifosfamide N-dechloroethylation with the microsomal protein content of CYP3A4, CYP3A5, and CYP2B6 adjusted for the age and sex of the donor. The comparison of ifosfamide N-dechloroethylation rates in renal microsomes isolated from CYP3A5*3/*3 and *1/*3 donors was conducted using analysis of covariance. Detectable concentrations of 2-DCEI and 3-DCEI were observed in the 120-minute control incubations (i.e., non-enzymatic N-dechloroethylation). To adjust for this background reaction, analysis of covariance was conducted with the dependent variable being the log of ifosfamide N-dechloroethylation rate and the independent variables being the CYP3A5 genotype and...
ifosfamide-NADPH control values. To assess the effects of ketoconazole and thiotepa inhibition, an analysis of variance was carried out treating kidneys as blocks. Post-hoc comparisons of the ketoconazole and thiotepa groups with controls were carried out using Dunnett’s test.

**Results**

We compared ifosfamide N-dechloroethylation by CYP3A4 + P450 reductase Supersomes and CYP3A5 + P450 reductase Supersomes supplemented with cytochrome b₅ at a 1:3 molar ratio, and by Supersomes containing CYP3A4 co-expressed with P450 reductase and cytochrome b₅ that was used in the earlier study by Huang et al. (Huang et al., 2000) The designated molar ratio of CYP-to-cytochrome b₅ was set based on the study by Yamazaki et al. (Yamazaki et al., 1999), which observed maximal CYP3A4 activity at a 3-fold cytochrome b₅ supplementation. As shown in Table 1, supplementation with cytochrome b₅ at a 3-fold excess did lead to a modest increase in the rate of ifosfamide N-dechloroethylation for CYP3A5, but not for CYP3A4. CYP3A5 + P450 reductase Supersomes catalyzed ifosfamide N-dechloroethylation at a higher rate than CYP3A4 + P450 reductase Supersomes, either with or without cytochrome b₅ supplementation. It appears that the previously observed higher catalytic rate of CYP3A4 co-expressed with cytochrome b₅ relative to CYP3A5 with supplementation of cytochrome b₅ reflected the difference between co-expression and supplementation of cytochrome b₅ rather than an intrinsic difference in catalytic efficiency between CYP3A4 and CYP3A5 (Table 1). Our ifosfamide N-dechloroethylation rates were similar to chloroacetaldehyde formation rates reported by Huang et al. (Huang et al., 2000) in CYP3A4 + P450 reductase + cytochrome b₅ Supersomes (4.1 versus 5.43 pmol/min/pmol P450, respectively) and CYP3A5 + P450 reductase Supersomes supplemented with cytochrome b₅ at a 1 to 1.5 ratio (0.330 versus 0.541 pmol/min/pmol P450, respectively). It should be noted that the two aforementioned Supersomes are not matched in terms of co-expressed P450 reductase activity; i.e., cytochrome c reductase activity of 2200 nmol/(min x mg protein) for CYP3A5 Supersomes compared to 370 nmol/(min x mg protein) for CYP3A4 Supersomes. Hence, the higher CYP3A5 Supersomes activity may be related to its higher co-expressed level of P450 reductase. An additional
study with the recently available CYP3A5 + P450 reductase + cytochrome b₅ Supersomes showed that ifosfamide N-dechloroethylation activity was 50% lower in CYP3A5 as compared to CYP3A4 (see Table 1). These Supersomes were well matched; co-expressed cytochrome c reductase activity at 3900 and 5100 n mole/(min X mg protein), and cytochrome b₅ content was 1600 and 1400 pmol/mg protein for CYP3A5 and CYP3A4, respectively.

Data from the saturation experiments with CYP3A4 and CYP3A5, both supplemented with 1:3 molar ratio of cytochrome b₅, are presented in Figure 1. The Km and Vmax estimates for CYP3A4 were 2.63 mM and 3.11 pmol/min/pmol P450, respectively; intrinsic clearance (Vmax/Km) was 1.18 µL/min/nmol P450 (see Eadie-Hofstee plot in Figure 2). For CYP3A5, the Km and Vmax were 1.55 mM and 5.48 pmol/min/pmol P450, respectively; intrinsic clearance was 3.54 µL/min/nmol P450. Thus, recombinant CYP3A5 has a slightly higher affinity for ifosfamide and is roughly 3-fold more efficient in mediating N-dechloroethylation of ifosfamide. The enzymes formed 2-DCEI and 3-DCEI at comparable amounts, i.e., similar regioselectivity.

As shown in Figure 3 and Table 2, rate of ifosfamide N-dechloroethylation was more than 2-fold higher in liver microsomes from donors carrying the CYP3A5*1 allele that expressed CYP3A4 and CYP3A5 relative to microsomes from homozygotes of CYP3A5*3/*3 that expressed CYP3A4 only (p=0.0001). Multiple regression analysis indicated that ifosfamide N-dechloroethylation was dependent upon CYP3A4 and CYP3A5 protein concentration (P=0.0007 and 0.0230, respectively), but not upon age, sex or CYP2B6 protein concentration (p>0.05). For every 10-unit increase in CYP3A5 content (pmol/mg protein), ifosfamide N-dechloroethylation would increase by 33 pmol/min/mg-protein, while the rate would be increased by only 8 pmol/min/mg-protein for every 10-unit increase in CYP3A4 content (pmol/mg protein). These data suggest that CYP3A5 is the major microsomal N-dechloroethylase of ifosfamide in individuals carrying the CYP3A5*1 allele.

The contribution of CYP3A4 and CYP2B6 was quantified by isoform-specific inhibitor studies.
with three human liver microsomal preparations that did not express CYP3A5 (i.e., CYP3A5*3/*3 genotype). The three microsomes were chosen from approximately 30 of the available human liver microsomes at the University of Washington Tissue Bank to represent varying proportions of the two enzymes: low CYP3A4 protein + high CYP2B6 protein, moderate expression of both enzymes, and high CYP3A4 protein + low CYP2B6 protein. Addition of ketoconazole (1 µM) – a recognized CYP3A inhibitor led to a similar degree of inhibition (i.e., 69-81%) regardless of the CYP3A4 protein concentration; a similar trend was noted with TAO (10 µM) which inhibited ifosfamide N-dechloroethylation within 5% of that of ketoconazole (Table 3). The CYP3A4/5 monoclonal antibody inhibited microsomal ifosfamide N-dechloroethylation to a lesser degree (53-61%), suggesting that ketoconazole and TAO might not be specific for CYP3A4. Indeed, ketoconazole and TAO inhibited ifosfamide N-dechloroethylation in cDNA-expressed CYP2B6 by 40% and 60%, respectively. The degree of inhibition by thiotepa (50 µM) – a CYP2B6 inhibitor did increase with an increasing amount of CYP2B6 protein (Table 3). However, this concentration of thiotepa inhibited ifosfamide N-dechloroethylation by 63% in CYP3A4 + P450 reductase + cytochrome b5 Supersomes. In contrast, CYP2B6 monoclonal antibody experiments demonstrated a negligible (0-3%) inhibition of ifosfamide N-dechloroethylation in human liver microsomes.

Ifosfamide N-dechloroethylation could not be detected in human renal microsomes which were homozygotes for CYP3A5*3/*3. Significant but variable rates of N-dechloroethylation were observed in heterozygotes of CYP3A5*1, suggesting that CYP3A5 is a critical determinant of chloroacetaldehyde formation in the human kidneys (Figure 4). The median ifosfamide N-dechloroethylation rate in the four kidneys obtained from CYP3A5*1 carriers was 0.26 pmol/min/mg-protein, which is comparable to that reported by Woodland et al. (Woodland et al., 2000) in renal microsomes from one human subject (i.e., 0.19 pmol/min/mg-protein). Ketoconazole (1 µM) inhibited ifosfamide N-dechloroethylation by 93 – 100% in these four kidneys (P<0.05), relative to control incubations without ketoconazole (Figure 4). Thiotepa (50 µM) inhibited ifosfamide N-dechloroethylation by 12-31% in these four kidneys (P>0.05).
relative to control incubations without thiotepa (Figure 4). We also checked for CYP2B6 protein expression in human kidney microsomes, which was below detection limit (1 pmol/mg protein) in seven human kidney microsomes.

**Discussion**

CYP3A5 catalyzed ifosfamide $N$-dechloroethylation is comparable or more efficient than CYP3A4 when the measurements were conducted using the same cytochrome P450 expression systems, at the same level of cytochrome b$_5$ supplementation or co-expression (Table 1). In the presence of fortified cytochrome b$_5$, the intrinsic clearance of ifosfamide $N$-dechloroethylation by CYP3A5 was 3-fold greater than that for CYP3A4 (i.e., 3.54 vs. 1.18 µl/min/nmol P450). We also observed that cytochrome b$_5$ supplementation increased the velocity of the $N$-dechloroethylation reactions by CYP3A5 but not CYP3A4; Yamazaki et al and Dai et al previously noted this trend with several other CYP3A substrates.(Yamazaki et al., 1999; Dai et al., 2004) Moreover, we were also able to reproduce the apparent difference in ifosfamide $N$-dechloroethylation rate between the CYP3A4 and cytochrome b$_5$ co-expression system and CYP3A5 with a 1:1.5 molar ratio cytochrome b$_5$ supplementation as reported by Huang et al.(Huang et al., 2000) It now seems likely that the previously reported higher rate of ifosfamide $N$-dechloroethylation in CYP3A4 compared to CYP3A5 by these investigators was confounded by differences in the two expression systems, i.e., co-expression versus supplementation of cytochrome b$_5$. It should, however, be noted that the content of P450 reductase does differ between the Supersome™ preparations of CYP3A4 and CYP3A5, i.e., 370 versus 2200 nmole/(min x mg protein), respectively. Hence, we cannot rule out that the higher CYP3A5 activity could in part be due to the higher reductase expression. Subsequent experiments with the recently available Supersomes CYP3A5 co-expressed with P450 reductase and cytochrome b$_5$ showed that this enzyme catalyzes ifosfamide $N$-dechloroethylation at approximately half the rate of CYP3A4 in a similar expression system which was well matched for cytochrome c and cytochrome b$_5$ (Table 1).
We also showed in a panel of human liver microsomes that ifosfamide \(N\)-dechloroethylation is highly correlated with CYP3A4 and CYP3A5 protein content—but not with CYP2B6 protein content, age and gender of the donor. In pairs of human liver microsomes matched for their CYP3A4 protein content, \(N\)-dechloroethylation of ifosfamide was approximately 2-fold higher in liver microsomes obtained from donors carrying a \(CYP3A5^{*1}\) allele than those from \(CYP3A5^{*3/*3}\) donors (Figure 3). Our present comparative activity data between CYP3A5 genotypes for ifosfamide \(N\)-dechloroethylation agree with other CYP3A-dependent reactions reported earlier with the same matched set of microsomes (e.g., midazolam 1’ hydroxylation, erythromycin \(N\)-demethylation). (Lin et al., 2002; Huang et al., 2004) Thus, ifosfamide appears to be another addition to the growing list of drugs for which hepatic CYP3A5 contribute significantly to their oxidative metabolism in individuals carrying \(CYP3A5^{*1}\). (Huang et al., 2004)

The relative contribution of CYP3A4 and CYP2B6 in ifosfamide \(N\)-dechloroethylation was examined by selective inhibitors in three human liver microsomes with varying CYP3A4 and CYP2B6 content (Table 3). The microsomes used in these experiments were from \(CYP3A5^{*3/*3}\) donors to minimize any confounding effects of CYP3A5 because an inhibitor completely specific for CYP3A4 has not yet been reported. Ketoconazole and thiotepa were initially evaluated; however, recent data from Turpeinen et al. (Turpeinen et al., 2004) demonstrated that ketoconazole inhibits CYP2B6-catalyzed bupropion hydroxylation with an IC\(_{50}\) of 3.5 \(\mu\text{M}\), which is slightly above the 1 \(\mu\text{M}\) concentration used in these experiments. It has been reported that TAO (10 \(\mu\text{M}\)) does not inhibit buproprion hydroxylation – a CYP2B6 catalyzed reaction – in pooled human liver microsomes. (Faucette et al., 2001) However, in this study both ketoconazole and TAO inhibited ifosfamide \(N\)-dechloroethylation by cDNA-expressed CYP2B6 + P450 reductase + cytochrome b\(_5\) to the extent of 40% and 60%, respectively. This explains the higher percentage of inhibition of \(N\)-dechloroethylation in human liver microsomes by both ketoconazole and TAO (65-81%) compared to anti-CYP3A4/5 monoclonal antibody (53-61%). Inhibition of \(N\)-dechloroethylation by thiotepa also lacked specificity; thiotepa inhibited ifosfamide \(N\)-
dechloroethylation by 63% in CYP3A4 + P450 reductase + cytochrome b₅ Supersomes. Our result stands in contrast to the report of Rae et al. (Rae et al., 2002), who observed that thiotepa had minimal effect upon CYP3A activity (i.e., midazolam 4’ hydroxylation). CYP2B6 immuno-inhibition experiments demonstrated barely measurable (<3%) inhibition of ifosfamide N-dechloroethylation in human liver microsomes. Thus, these data suggest that CYP3A4 plays a predominant role in hepatic ifosfamide N-dechloroethylation, whereas CYP2B6 has a negligible role, even in those hepatic microsomes with a high expression of CYP2B6 protein (i.e., HLM 142, Table 3). Our data contradict those reported by Huang et al, who reported that CYP2B6 monoclonal antibody inhibited ifosfamide N-dechloroethylation by 40% in human liver microsomes. In another study by Granvil et al., CYP2B6 activity (i.e., S-mephenytoin N-demethylation) was highly correlated ($R^2>0.95$) with the formation rate of S-3-dechloroethylifosfamide and S-2-dechloroethylifosfamide from (R)-ifosfamide and (S)-ifosfamide, respectively; however, the relative contribution of CYP3A4/5 and CYP2B6 toward N-dechloroethylation of each ifosfamide enantiomer was not evaluated. (Granvil et al., 1999) We have no obvious explanation for the discrepancy between our data and the previous study by Huang et al. Further studies are needed regarding the relative importance of CYP3A4/5 and CYP2B6 in hepatic ifosfamide N-dechloroethylation.

Until recently, it had been assumed that hepatic ifosfamide N-dechloroethylation leads to the release of chloroacetaldehyde into the systemic circulation and would inflict nephrotoxicity upon reaching the kidneys. However, this scenario is questionable because peak plasma chloroacetaldehyde concentrations range from 0.5 – 109 µM in patients receiving standard doses of ifosfamide, which are appreciably lower than its reported minimum nephrotoxic concentration. (Goren et al., 1986; Kurowski and Wagner, 1993; Dubourg et al., 2001) In freshly isolated human renal proximal tubules, the minimal chloroacetaldehyde concentration leading to toxicity (e.g., lactate dehydrogenase release, impaired lactate metabolism) was 500 µM. (Dubourg et al., 2001) This has led to the alternative hypothesis that ifosfamide N-dechloroethylation is mediated by CYP3A within renal proximal tubule; i.e., chloroacetaldehyde is produced locally at the site of toxicity. (Woodland et al., 2000; Aleska et al., 2001; Aleksa et al., 2004)
Ifosfamide N-dechloroethylation could not be detected in human renal microsomes from CYP3A5*3*3 donors, i.e., when CYP3A5 expression is negligible. In the renal microsomes obtained from CYP3A5*1 carriers, ifosfamide N-dechloroethylation ranged from 0.06 – 0.45 pmol/min/mg-protein (Table 2 and Figure 4). This catalytic activity was effectively inhibited by ketoconazole, but not by thiotepa (Figure 4). Although the presence of CYP2B6 in human renal tubules has been suggested, (Gervot et al., 1999; Aleksa et al., 2004) we could not detect CYP2B6 protein in our panel of human renal microsomes. Thus, current data suggests that CYP2B6 does not appear to be important in renal ifosfamide N-dechloroethylation.

These data predict that cancer patients who carry CYP3A5*1 would catalyze ifosfamide N-dechloroethylation at a greater rate, leading to higher chloroacetaldehyde concentrations and a higher risk of ifosfamide induced nephrotoxicity. The effects of renal ifosfamide N-dechloroethylation are expected to be the primary contributor to nephrotoxicity because chloroacetaldehyde degrades rapidly in human blood. (Kurowski and Wagner, 1993) Interestingly, there is increasing recognition for the importance of extrahepatic CYP3A5 in the pathophysiology of hypertension and prostate cancer risk. (Givens et al., 2003) Although mesna and amifostine are capable of preventing chloroacetaldehyde induced renal toxicity in-vitro, they have not proven to be clinically effective. (de Kraker et al., 2000; Zaki et al., 2003) Therefore, efforts to minimize ifosfamide nephrotoxicity have focused upon identifying patient-specific risk factors. (Loebstein et al., 1999; Skinner et al., 2000; McCune et al., 2004) Further studies are now needed to demonstrate the association of CYP3A5*1 with ifosfamide nephrotoxicity in cancer survivors.
References


Footnotes

Supported by University of Washington Royalty Research Fund Award (#2578)
Legends for Figures

Figure 1: Saturation kinetics of ifosfamide N-dechloroethylation

Figure 2: Eadie-Hofstee plot of ifosfamide N-dechloroethylation by CYP3A4 and CYP3A5

Figure 3: Variation in ifosfamide N-dechloroethylation rate in human liver microsomes as a function of CYP3A5 genotype.

Figure 4: Effect of thiotepa and ketoconazole upon ifosfamide N-dechloroethylation in human kidney microsomes from CYP3A5*1 carriers
Table 1. Effect of cytochrome b₅ upon ifosfamide N-dechloroethylation\textsuperscript{a} by cDNA-expressed CYP3A4 and CYP3A5

<table>
<thead>
<tr>
<th>CYP</th>
<th>3A4</th>
<th>3A5</th>
<th>3A4</th>
<th>3A5</th>
<th>3A4</th>
<th>3A5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome b₅</td>
<td>None</td>
<td>Added (1:3 ratio)\textsuperscript{b}</td>
<td>Co-expressed\textsuperscript{c}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-DCEI formation</td>
<td>0.059</td>
<td>0.046</td>
<td>0.024</td>
<td>0.077</td>
<td>0.378</td>
<td>0.068</td>
</tr>
<tr>
<td>3-DCEI formation</td>
<td>0.283</td>
<td>0.676</td>
<td>0.296</td>
<td>0.938</td>
<td>5.05</td>
<td>2.25</td>
</tr>
<tr>
<td>2- &amp; 3-DCEI formation</td>
<td>0.342</td>
<td>0.722</td>
<td>0.320</td>
<td>1.02</td>
<td>5.43</td>
<td>2.32</td>
</tr>
</tbody>
</table>

\textsuperscript{a}pmol of 2-DCEI and 3-DCEI formed/min/pmol P450; \textsuperscript{b}molar ratio of CYP to cytochrome b₅; cytochrome c reductase activity (nmole/(min x mg protein)) of 370 and 2200 for CYP3A4 and CYP3A5, respectively; \textsuperscript{c}co-expressed cytochrome c reductase activity (nmole/(min x mg protein)) at 5100 and 3900, and cytochrome b₅ (pmol/mg protein) content at 1400 and 1600 for CYP3A4 and CYP3A5, respectively.
Table 2. Ifosfamide N-dechloroethylation\textsuperscript{a} in liver and renal microsomes

<table>
<thead>
<tr>
<th></th>
<th>All Genotypes</th>
<th>CYP3A5*3/*3</th>
<th>CYP3A5*1/*3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver microsomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-DCEI formation</td>
<td>5.89 (1.26 – 29.2)</td>
<td>4.35 (1.26 – 7.42)</td>
<td>10.7 (1.76 – 29.2)</td>
</tr>
<tr>
<td>3-DCEI formation</td>
<td>51.1 (12.5 – 178)</td>
<td>32.6 (12.5 – 55.4)</td>
<td>78.4 (23.1 – 178)</td>
</tr>
<tr>
<td>2-DCEI + 3-DCEI formation</td>
<td>57.5 (12.8 – 206)</td>
<td>37.4 (12.8 – 64.3)</td>
<td>92.2 (30.8 – 206)</td>
</tr>
<tr>
<td><strong>Kidney microsomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-DCEI formation</td>
<td>0\textsuperscript{b}</td>
<td>0\textsuperscript{b}</td>
<td>0\textsuperscript{b}</td>
</tr>
<tr>
<td>3-DCEI formation</td>
<td>0 (0 – 0.45)</td>
<td>0 (0 – 0.07)</td>
<td>0.26 (0.06 – 0.45)</td>
</tr>
<tr>
<td>2-DCEI + 3-DCEI formation</td>
<td>0 (0 – 0.45)</td>
<td>0 (0 – 0.07)</td>
<td>0.26 (0.06 – 0.45)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} median (range) as pmol/min/mg-protein; \textsuperscript{b} less than ifosfamide – NADPH control
Table 3. Effects of ketoconazole and thiotepa upon ifosfamide N-dechloroethylation in human liver microsomes isolated from CYP3A5*3/*3 donors

<table>
<thead>
<tr>
<th>Enzyme Content</th>
<th>CYP2B6</th>
<th>CYP3A4</th>
<th>3A4 MAB&lt;sup&gt;c&lt;/sup&gt;</th>
<th>TAO</th>
<th>Ketoconazole</th>
<th>2B6 MAB&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Thiotepa</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM 148</td>
<td>Low CYP2B6, High CYP3A4</td>
<td>12.2</td>
<td>132.9</td>
<td>61%</td>
<td>77%</td>
<td>81%</td>
<td>3.3%</td>
</tr>
<tr>
<td>HLM 120</td>
<td>Average CYP2B6, Average CYP3A4</td>
<td>15.5</td>
<td>58.3</td>
<td>53%</td>
<td>65%</td>
<td>69%</td>
<td>0%</td>
</tr>
<tr>
<td>HLM 142</td>
<td>High CYP2B6, Low CYP3A4</td>
<td>29.4</td>
<td>24.9</td>
<td>60%</td>
<td>80%</td>
<td>75%</td>
<td>0%</td>
</tr>
</tbody>
</table>

<sup>a</sup>all donors were women between 45 – 60 years of age; <sup>b</sup>% inhibition = 1 – [(Rate + inhibitor) / Rate – inhibitor] x 100; <sup>c</sup>MAB=monoclonal antibody; <sup>d</sup>&gt;80% inhibition with Supersomes™ CYP2B6 as positive control.
Figure 1.

[Graph showing two curves labeled CYP3A4 and CYP3A5, with the x-axis labeled Ifos (mM) and the y-axis labeled Ifosamide N-dechloroethylation (pmol/min/pmol P450)].
Figure 2.

CYP3A4:

\[ y = -2.64x + 3.56 \]
\[ R^2 = 0.989 \]

CYP3A5:

\[ y = -1.54x + 6.26 \]
\[ R^2 = 0.912 \]
Figure 3.

Hatch marks represent median of that group.
Figure 4.

Data shown as mean with standard deviations; \textsuperscript{b} No detectable \textit{N}-dechloroethylation of ifosfamide presence of ketoconazole