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Investigation of the major human hepatic cytochrome P450 involved in 4-hydroxylation and *N*-dechloroethylation of trofosfamide

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Abstract Trofosfamide and its congeners ifosfamide and cyclophosphamide are cell-cycle-nonspecific alkylating agents that undergo bioactivation catalyzed by liver cytochrome P450 (CYP) enzymes. Two NADPH-dependent metabolic routes for the anticancer drug trofosfamide, i.e., 4-hydroxylation and *N*-dechloroethylation, were studied in human liver microsomes and in seven recombinant human CYP isoforms (i.e., CYP1A1, 1A2, 2A6, 2B6, 2D6, 2E1, and 3A4-OR) to identify the CYP enzymes involved. Recombinant human CYP3A4 and CYP2B6 exhibited catalytic activity with respect to both pathways of trofosfamide. Enzyme kinetic analyses revealed the dominant role of human CYP3A4 in 4-hydroxylation and *N*-dechloroethylation of trofosfamide. This was confirmed by the observation that only the CYP3A4 contents of five samples of human liver microsomes correlated with both pathways of trofosfamide. Furthermore, ketoconazole, a selective inhibitor of CYP3A4, substantially inhibited microsomal trofosfamide 4-hydroxylation and *N*-dechloroethylation (50% inhibitory concentration $< 1\mu M$ for both reactions). The present study indicates that human liver microsomal CYP3A4 preferentially catalyzes the two NADPH-dependent metabolic routes of trofosfamide, which emphasizes the necessity for awareness of potential interactions with any coadministered drugs that are CYP3A4 substrates.

Key words Trofosfamide · Ifosfamide · Cyclophosphamide · Metabolism · Cytochrome P450

Abbreviations *TRO* Trofosfamide · *IFO* Ifosfamide · *CYC* Cyclophosphamide · *7OH-CHIN* 7-Hydroxyquinoline · *4-KET-IFO* 4-Ketoifosfamide · *KTZ* Ketoconazole · *HPLC* High-performance liquid chromatography

Introduction

The oxazaphosphorine trofosfamide (TRO), a DNA-alkylating agent, has been commercially available since 1973. In comparison with its congeners cyclophosphamide (CYC) and ifosfamide (IFO), TRO has gained little acceptance in clinical application, presumably because it is marketed only in a formulation for oral use and most cancer treatment protocols have focused on the optimization of intravenous ifosfamide application. Moreover, as TRO is mainly employed in palliative situations, little is known about this agent.

Oxazaphosphorines are prodrugs that require biotransformation to become cytotoxic [32]. This occurs mainly in the liver by the action of a mixed-function oxidase, producing the active metabolites by hydroxylation at ring position 4 [14]. The product is supposed to maintain a spontaneous equilibrium with its 4-aldo tautomer. Subsequently, the 4-aldo metabolite undergoes decomposition by β-elimination, resulting in the DNA-alkylating oxazaphosphorine mustard and acrolein.

Oxazaphosphorines can also be oxidatively metabolized at the chloroethyl side chains. Side-chain oxidation at the exo- or endocyclic nitrogen of IFO leads to the

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formation of the pharmacologically inactive 2- and 3-dechloroethyl ifosfamide (2- and 3-d-IFO) and the simultaneous release of chloroacetaldehyde [12]. Quantitatively, the *N*-dechloroethylation pathway plays a minor role for CYC but is of major importance for IFO [26]. The different metabolic patterns of CYC and IFO, especially the formation of side-chain metabolites, are the subject of intense discussion against the background of an increasing incidence of renal and central nervous system side effects after application of ifosfamide [10, 23, 27].

According to previously reported in vitro investigations, the side-chain oxidation of TRO resulted in the formation of IFO and CYC [8]. Studies in rats and mice showed IFO to be the predominant metabolite of this pathway, showing a 5- to 6-fold excess as compared with CYC. In distinction to CYC and IFO, side-chain metabolism of TRO is consequently not a direct detoxification pathway. Figure 1 summarizes these potential pathways of TRO metabolism.

Although high-dose intravenous treatment, especially with IFO, has long dominated therapy regimens, there is an increasing need for reappraisal of oral treatment strategies on an outpatient basis. Clinical trials of oral IFO have been associated with considerable neurotoxicity [20, 21]. Following therapy with CYC, cardiotoxic side effects have been reported [3].

TRO, by contrast, can be given orally over long periods without producing any significant side effect [2, 36]. Our research interest therefore focused on the in vitro investigation of TRO metabolism. There are some indications suggesting differences in the side-chain oxidation of TRO and IFO; TRO is preferentially oxidized at the exocyclic nitrogen, whereas IFO shows favored oxidation at the endocyclic nitrogen [6, 7, 16]. One possible cause for this phenomenon could be biotransformation by different cytochrome P450 isoenzymes.

Whereas the involvement of cytochrome P450 3A4 (CYP3A4) in *N*-dechloroethylation of IFO [34] and the participation of CYP3A4 and CYP2B6 in ring oxidation of IFO and CYC [11, 34] have been established, the identity of the important CYPs that are relevant to TRO metabolism remains to be addressed. The major goal of the present study was to use microsomal fractions prepared from a panel of human B-lymphoblastoid cell lines transformed with individual CYP complementary DNAs as well as human liver microsomes to determine their relevance to TRO metabolism.

Materials and methods

Materials

TRO, the internal standard 4-ketoifosfamide (4-KET-IFO), and all metabolites were kindly supplied by Dr. J. Pohl, Asta Medica AG (Frankfurt, Germany). Spectrograde solvents such as isopropanol, dichloromethane, acetic acid, and acetonitrile, used in extraction or analytical procedures, were purchased from Baker-Mallinckrodt (Groß-Gerau, Germany). NADPH, hydrochloric acid, hydroxylamine hydrochloride, and 3-aminophenol were obtained from Merck (Darmstadt, Germany), and acrolein and *trans*-2-hexenal were supplied by Sigma (Deisenhofen, Germany). Inorganic reagents not listed above were of analytical grade. The cDNA-expressing human lymphoblast cell lines were derivatives of the AHH-1 $TK^{+/-}$ cell line [14], which also served as the control cell line for these studies. The cell microsomes containing human CYPs were purchased from Gentest Corporation (Woburn, USA), and human liver microsomes were obtained from the human liver bank at the Margarete Fischer-Bosch-Institut für Klinische Pharmakologie in Stuttgart.

Microsomal incubation

The incubations were carried out at 37 °C for specified times (30 min as a rule) in a shaking water bath. Incubations were initiated by the addition of cold microsomes to prewarmed substrate/cofactors/buffer (preincubation for 5 min at 37 °C). The incubation mixtures contained TRO at various concentrations, magnesium chloride (4 m*M*), and NADPH (1 m*M*) at a final volume of 1 ml. The system was buffered with 0.1 *M* potassium phosphate (pH 7.4). Reactions were stopped by placement of the tubes on ice. All incubations were done in duplicate unless stated otherwise.

Assay for determination of activated metabolites

4-Hydroxylation of TRO was measured as the fluorescence of 7-hydroxyquinoline (7-OH-CHIN) produced by the reaction of liberated acrolein with 3-aminophenol as described by Alarcon [1]. The incubation medium (100 µl) was transferred into new centrifuge tubes; 100 µl of an internal standard solution of 1 m*M trans*-2-hexenal and 400 µl of the reaction mixture of 3 *N* hydrochloric acid, 0.5% 3-aminophenol, and 0.6% hydroxylamine hydrochloride were added and the solution was vortexed for 15 s. The solution was heated at 100 °C for 6 min and cooled on ice, and 300 µl of HPLC-grade water was added. The mixture was then vortexed and centrifuged at 2000 *g* for 5 min; 100 µl of the supernatant was injected for analysis by HPLC.

Equipment

The HPLC system consisted of a double-piston pump (Pharmacia LKB model 2150), a low-pressure mixer for the eluation gradients, an HPLC controller (Pharmacia LKB model 2252), a fluorescence detector (Shimadzu RF 530), and the integration system Nelson Analytical Software 5.1. Analytes were separated on a Nucleosil C_{18} (5 µm, 12.5 cm × 4 mm inside diameter, ID) column (Macherey-Nagel, Düren, Germany). A 30-mm precolumn of the same packing material was used. Concentrations were quantified by the internal standard method.

Chromatography

The mobile phase consisted of acetonitrile/acetic acid/water (12.5:4:83.5, by vol.). The flowrate was 1 ml/min. The retention times of the internal standard and 7-OH-CHIN were 8.5 and 2.6 min, respectively. Fluorescence was measured at 505 nm using an excitation wavelength of 358 nm.

Calibration

The calibration curve (0.32–1.7 µ*M* acrolein) was obtained from the quotients of peak areas (7-OH-CHIN/internal standard). The mean correlation coefficient recorded for 7-OH-CHIN from five assays conducted on different days (0.9987 ± 0.00065 SD) exceeded 0.998. The limit of quantification was 0.32 µ*M*.

Assay for determination of side-chain oxidation metabolites

To the remaining 900 µl of the incubation assay, 20 µl of 364 µ*M* 4-KET-IFO was added as the internal standard, and protein was

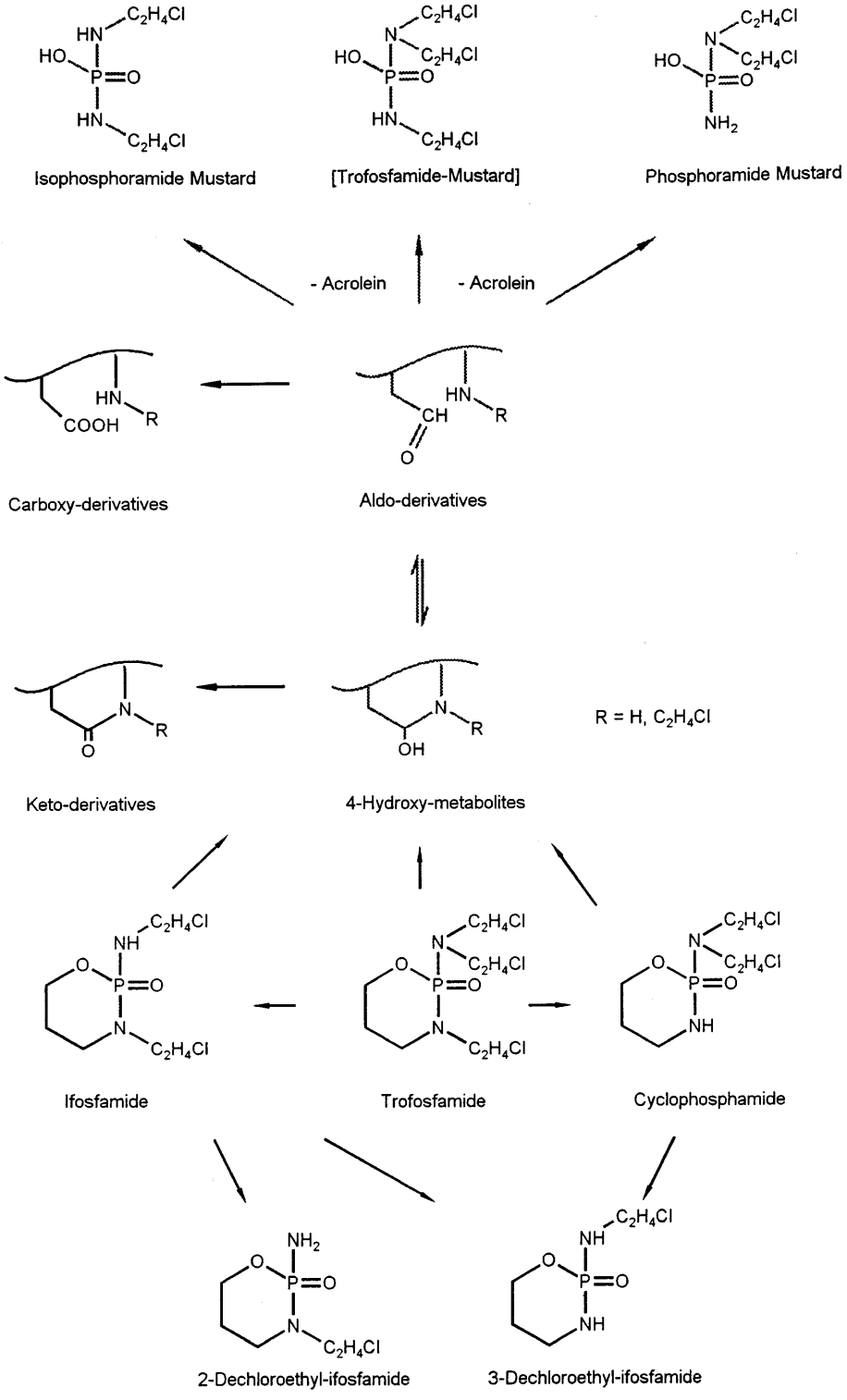

Fig. 1 Activation and inactivation pathways of trofosfamide metabolism

precipitated with an aliquot of ice-cold acetonitrile. After 10 s of vortex mixing the solution was placed on ice for 10 min. The precipitate was removed by centrifugation at 2000 *g* for 10 min in a Rotanta centrifuge. The supernatant liquid was transferred to clean centrifuge tubes, and TRO and metabolites were extracted from the incubation media by liquid-liquid separation using dichloromethane/isopropanol/acetic acid (85:15:1, by vol.) and subsequent evaporation as described elsewhere [8]; 200 µl of HPLC eluent was added to the residue. After vortex mixing the sample was subjected to HPLC analysis.

Equipment

The HPLC equipment was applied as described above using an LC-UV detector (Waters model 481). A Nucleosil C_{18} (5 µm,

25 cm × 4 mm ID) column (Macherey-Nagel, Düren, Germany) and a 30-mm precolumn of the same packing material were used for separation of TRO, IFO, and CYC.

Chromatography

A 50-min step gradient was used to separate the analytes and equilibrate the column. Mobile phase A consisted of 15% acetonitrile, 5% isopropanol, and 80% water. Mobile phase B comprised 40% acetonitrile, 5% isopropanol, and 55% water. The flow rate was 0.90 ml/min. The internal standard 4-KET-IFO, IFO, and CYC were eluted using eluent A. The proportion of mobile phase B was then increased from 0 to 70% over a 0.1-min interval, thereby increasing the acetonitrile concentration of the mixed mobile phase to 32.5%. After 16.4 min (during which TRO eluted), the proportion of mobile phase B was decreased to 0 over 0.1 min and the column was equilibrated with mobile phase A for 13.5 min before the next sample injection.

Calibration

Standard curves were constructed for IFO (9.6–766 μ*M*), CYC (8.9–534 μ*M*), and TRO (77–4635 μ*M*) using 4-KET-IFO (36.5 μ*M*) as the internal standard. The mean correlation coefficients recorded from five assays conducted on different days exceeded 0.998 for all substances. The limit of detection for IFO and CYC was 3.6 μ*M*.

Inhibition studies

Ketoconazole (KTZ) at concentrations of up to 100 μ*M* was added to the incubation medium before the reaction was initiated by the addition of microsomal protein.

Determination of the relative CYP monooxygenase levels in human liver microsomes by immunoblotting

Immunoblots were performed according to the method of Towbin et al. [33] as modified by Guengerich et al. [18]. The amounts of the different CYPs (CYP3A, CYP2B6, CYP2C9, CYP2D6, CYP2E1) were determined by densitometry of the spots.

Kinetic analyses

Estimates of apparent K_M and V_{max} values were obtained by graphical analysis of Lineweaver-Burk plots. Assay conditions were such that metabolite formation and parent-drug consumption were linear with respect to the time of incubation and the protein concentration.

Results

Role of individual cDNA-expressed human CYP enzymes in TRO 4-hydroxylation and *N*-dechloroethylation

To identify the CYP enzymes that are catalytically competent in metabolizing TRO, we examined a panel of human B-lymphoblastoid cell lines that were stably transformed with individual CYP cDNAs [13].

Only CYP2B6 and CYP3A4 showed catalytic activity, whereas CYP1A1, 1A2, 2A6, 2D6, and 2E1 did not yield any measurable product when TRO served as the substrate. There were different patterns of metabolism in the presence of recombinant human CYP2B6 and CYP3A4 (Fig. 2). When TRO (1.25 m*M*) was incubated in the presence of recombinant human CYP2B6, the side-chain oxidation metabolite CYC was formed in marginally higher concentrations as compared with IFO, which indicated higher metabolic activity at the endocyclic as opposed to the exocyclic nitrogen. By contrast, recombinant human CYP3A4 was 8-fold more active in metabolizing TRO at the exocyclic nitrogen, with IFO being the main side-chain metabolite. With respect to the ring oxidation pathway, recombinant CYP2B6 was somewhat more active than CYP3A4 in hydroxylating TRO at position 4.

Experiments performed to determine the apparent K_M and V_{max} involved in the formation of IFO, CYC, and 7-OH-CHIN were conducted using incubation periods of 30 min. V_{max} data were normalized with respect to the levels of CYP2B6 and CYP3A4, respectively (100 pmol), rather than those of total protein [29]. TRO metabolism in the presence of recombinant CYP2B6 and CYP3A4 was described by monophasic Michaelis-Menten kinetics over a substrate concentration range of 0.08–2.5 m*M* (Figs. 3–5). The C-4 hydroxylation of TRO was characterized by a V_{max} value of 34.1 nmol 30 min^{-1} 100 pmol CYP^{-1} and an apparent K_M of 0.062 m*M* in the presence of CYP3A4 and by a V_{max} value of 87.2 nmol 30 min^{-1} 100 pmol CYP^{-1} and an apparent K_M of 1.134 m*M* in the presence of CYP2B6 (Table 1). These data suggest that CYP3A4 has greater affinity than CYP2B6 with respect to 4-hydroxylation. The two recombinant enzymes also differed concerning the pathway of side-chain oxidation (formation of IFO – CYP2B6: V_{max} = 10.2 nmol 30 min^{-1} 100 pmol CYP^{-1}, K_M = 1.157 m*M*; CYP3A4: V_{max} = 50.1 nmol 30 min^{-1} 100 pmol CYP^{-1}, K_M = 0.252 m*M*; formation of CYC – CYP2B6: V_{max} = 36.7 nmol 30 min^{-1} 100 pmol CYP^{-1}, K_M = 0.492 m*M*; CYP3A4: V_{max} = 8.2 nmol 30 min^{-1} 100 pmol CYP^{-1}, K_M = 1.215 m*M*).

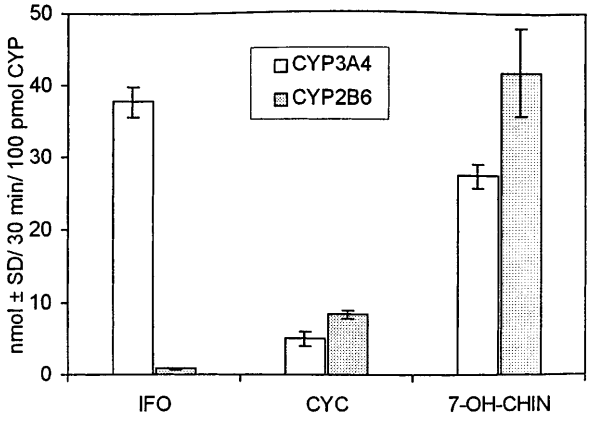

Fig. 2 Formation of IFO, CYC, and 4-OH metabolites after incubation with recombinant human CYP2B6 and CYP3A4. Data represent mean values ± SD for four separate incubations

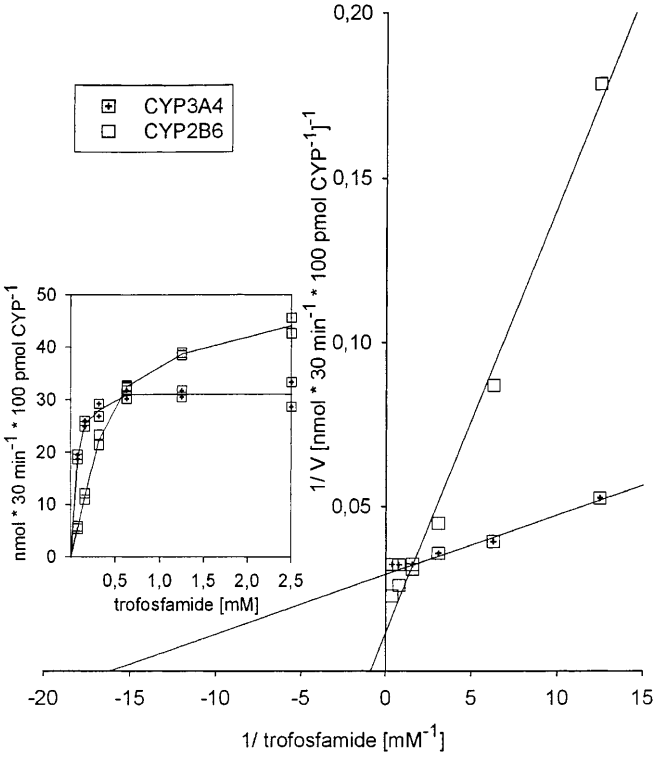

Fig. 3 Lineweaver-Burk and Michaelis-Menten plots of TRO metabolism to 4-OH metabolites in the presence of CYP2B6 and CYP3A4

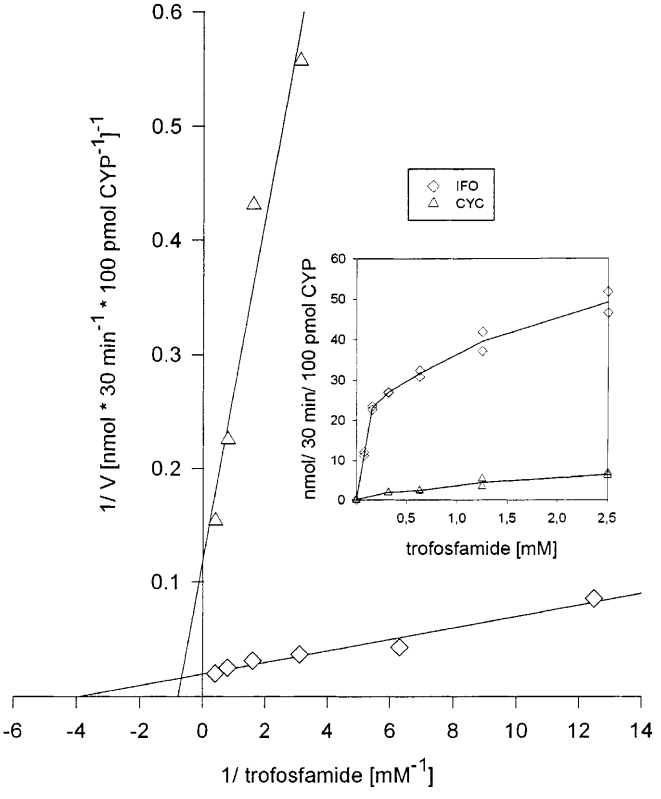

Fig. 4 Lineweaver-Burk and Michaelis-Menten plots of TRO metabolism to the metabolites CYC and IFO in the presence of CYP3A4

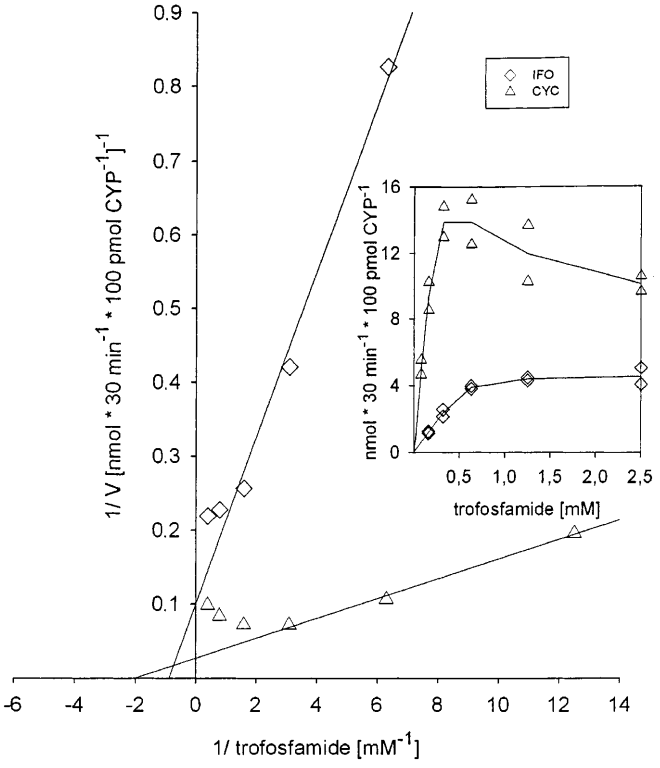

Fig. 5 Lineweaver-Burk and Michaelis-Menten plots of TRO metabolism to the metabolites CYC and IFO in the presence of CYP2B6

Inhibition by KTZ of TRO 4-hydroxylation and *N*-dechloroethylation in human liver microsomes

Inhibition studies with KTZ (0.1–100 μ*M*) indicated that this N^1-substituted imidazole effectively inhibited TRO metabolism. In the presence of human liver microsomes, CYC was formed in very small quantities. Hence, for purposes of comparison the *N*-dechloroethylation of TRO can be determined by IFO formation. The effect of inhibition was even more distinct, with a 50% inhibitory concentration (IC_{50}) of ≈0.07 μ*M* being recorded for the *N*-dechloroethylation (formation of IFO) pathway and an IC_{50} of ≈0.4 μ*M* being noted for

Table 1 Michaelis-Menten parameters obtained with TRO as the substrate. Formation of IFO, CYC, and 4-OH metabolites catalyzed by cDNA-expressed human P-450

Expressed cDNA	IFO	CYC	4-OH metabolites
CYP3A4:			
K_m [m*M*]	0.252	1.215	0.062
V_{max} [nmol 30 min^{-1} 100 pmol CYP^{-1}]	50.13	8.18	34.09
CYP2B6:			
K_m [m*M*]	1.157	0.492	1.134
V_{max} [nmol 30 min^{-1} 100 pmol CYP^{-1}]	10.17	36.72	87.24

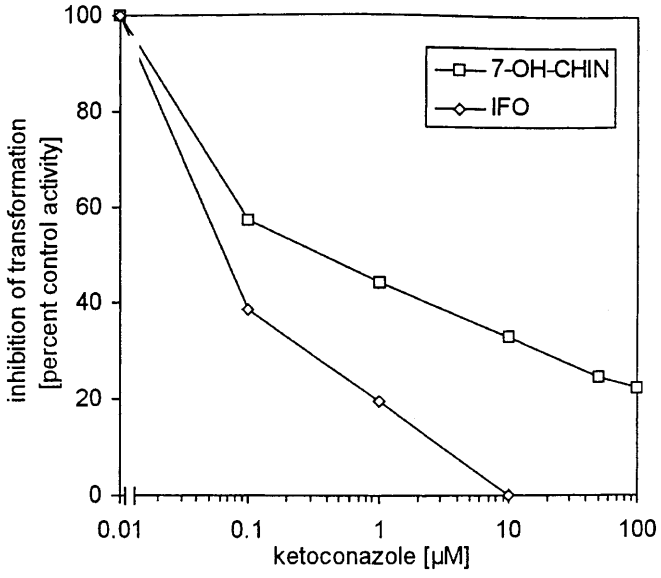

Fig. 6 Inhibitory effect of ketoconazole on the metabolism of trofosfamide after incubation with human liver microsomes

the ring oxidation (Fig. 6). KTZ is known to be a selective inhibitor of CYP3A4 at this concentration (1 μ*M*) [4].

Correlation between rates of TRO metabolism and CYP expression

The formation of 4-OH metabolites ($P < 0.05$, $r = 0.9001$) and IFO ($P < 0.05$, $r = 0.9960$) after incubation of 1.25 m*M* TRO with microsomes prepared from five human livers was found to correlate with the expression of CYP3A as determined by immunoblotting, but not with the expression of CYP2C9, CYP2D6, or CYP2E1 (Table 2). This finding indicates a major role for CYP3A4 in TRO 4-hydroxylation and *N*-dechloroethylation by human liver. A correlation study examining rates of TRO metabolism and CYP2B6 expression will require a larger number of human liver samples. The formation of IFO and 4-OH metabolites followed monophasic Michaelis-Menten kinetics (Table 2).

Discussion

The present investigation shows that the *N*-dechloroethylation and 4-hydroxylation of TRO is mainly mediated by CYP3A4 in the human liver; this is apparent in the catalytic activity of cDNA-expressed CYP3A4 and in the significant inhibition of human microsomal activity by KTZ. The correlation observed between the rates of TRO metabolism and the hepatic CYP levels determined by Western blotting further substantiates this conclusion.

When TRO served as the substrate, cDNA-expressed CYP3A4 as well as CYP2B6 were catalytically active in 4-hydroxylation and *N*-dechloroethylation. The following observation should be considered in the interpretation of this result: the relative levels of CYP3A4 and CYP2B6 in human liver microsomes differ considerably. Specifically, CYP3A4 and CYP2B6 contribute >25% and ≤2%, respectively, of the total CYP pool in human liver microsomes [22, 30, 31]. However, the specific content of the cDNA-expressed proteins in B-lymphoblastoid cell microsomes is similar (50–100 pmol/mg). Consequently, minor forms of CYP such as CYP2B6 will contribute significantly to metabolism only if this is mediated with relatively high affinity. The apparently high K_m values observed after incubation with CYP2B6 suggest that *N*-dechloroethylation and 4-hydroxylation of TRO are catalyzed only with low affinity, whereas CYP3A4 can catalyze the activation pathway of TRO with high affinity (low K_m). As the 4-hydroxylation of TRO is quantified through the liberation of acrolein, this might also include the 4-hydroxylation of IFO and CYC formed by side-chain oxidation of TRO. Any 4-hydroxylation of IFO/CYC, however, plays a minor role at most, as the K_m of CYP3A4-mediated 4-hydroxylation is 4 times lower than the generation of IFO and CYC. Moreover, it is the sum of the 4-hydroxy metabolites as active metabolites that is pharmacologically relevant.

Table 2 Correlation of the content of various P450 enzymes (reported as arbitrary units per milligram of protein, from densitometric evaluation of immunoblots) versus the rate of formation ($V_{1.25mM}$) of IFO and 4-OH metabolites from TRO in the microsomal fraction of 5 different human livers

Liver number	P450 content (units/mg protein)					V (1.25 m*M* TRO) [pmol h^{-1} μg $protein^{-1}$]		K_m [m*M*]		V_{max} [pmol h^{-1} μg $protein^{-1}$]	
	CYP3A	CYP2B6	CYP2C9	CYP2D6	CYP2E1	IFO	7OH-CHIN	IFO	7OH-CHIN	IFO	7OH-CHIN
10	33.3	17.2	38.4	0.5	0.2	36.15	36.93	0.13	0.14	37.1	39.5
14	32.1	28.6	84.8	1.2	0.3	28.55	32.88	–	0.17	–	0.19
16	25.8	35.3	87.3	1.7	0.2	27.74	48.91	–	–	–	–
19	88.2	37.6	104.5	1.1	0.2	89.43	73.89	0.43	0.15	112.7	78.8
22	68.3	36.7	43.6	–	–	69.80	61.59	–	–	–	–
^{r}(IFO)	0.9960	0.5482	0.1760	−0.1543	−0.3816						
^{r}(7-OH-CHIN)	0.9001 ($P = 0.05$)	0.7558	0.3278	0.2076	−0.5516						

Nonetheless, further studies using refined analytical methodology are warranted to quantify the levels of the active 4-hydroxy derivatives of TRO, IFO, and CYC. A quantitative comparison of the active 4-hydroxy derivatives of TRO and of IFO would be of particular interest, as clinical trials of oral IFO have shown unexpected neurologic toxicity at higher doses [9].

Our observation of the dominant role of CYP3A4 in the 4-hydroxylation and *N*-dechloroethylation of TRO is in agreement with an inhibition study on KTZ, a selective CYP3A4 inhibitor [4], which caused substantial inhibition of TRO 4-hydroxylation and *N*-dechloroethylation. Since a CYP2B6-specific chemical inhibitor has not yet been found [28], the selective inhibition of CYP2B6 remains to be addressed. Furthermore, a significant ($P < 0.05$) correlation was found between the rates of TRO metabolism and the human hepatic CYP3A4 levels determined by Western blotting, whereas no significant correlation was observed with CYP2B6.

Earlier pharmacokinetics investigations in humans showed that a high amount of the drug was metabolized through hydroxylation in position 4 and that dechloroethylation of TRO led mainly to the formation of IFO, which could be further activated [19]. Thus, the clinical monitoring of oral TRO therapy shows a metabolite pattern resembling in vitro incubations with CYP3A4, where IFO is the predominant metabolite rather than CYP2B6 (where CYC is the predominant dechloroethylated metabolite).

These findings are also consistent with those of Walker et al. [34], indicating that the 4-hydroxylation and *N*-dechloroethylation of the related oxazaphosphorine IFO is mainly mediated by CYP3A4. Bohnenstengel et al. [5] demonstrated that CYP3A4 was the major enzyme involved in side-chain oxidation of CYC, and Ren et al. [28] reported that CYP2C9 and CYP3A4/5 seemed to be the major P450 isoforms involved in 4-hydroxy CYC formation and that CYP3A4/5 seemed to be the major CYP isoform involved in dechloroethyl CYC formation at high concentrations of CYC (0.7 and 5 m*M*). The kinetic parameters reported for 4-hydroxy CYC and dechloroethyl CYC formation in human liver microsomes [28] were similar to our results.

Recent studies have shown that considerable amounts of CYP3A4 are expressed in the duodenum [15]. Since TRO is exclusively given p.o., one may assume that its metabolic degradation, which produces IFO and CYC, starts in the duodenum. Thus, the side-chain oxidation of TRO, as opposed to CYC, which is also occasionally given by the oral route, does not produce inactive metabolites at the first step of metabolic degradation (Fig. 1). 4-Hydroxylation, i.e., the activation pathway, may thus occur in the liver.

It can be predicted that other drugs that are metabolized by CYP3A4 are likely candidates for interactions with TRO (e.g., dexamethasone [24], erythromycin [35]; for a complete list of CYP3A4 substrates, see the publication by Guengerich [17]). The therapeutic consequences of drug interactions involving enzyme-mediated processes depend on the relative affinity of the interacting drugs for the catalyzing enzyme. For the minimization of possible interactions the individual affinity constants for the respective isoenzymes should therefore always be considered during the selection of drugs for cytotoxic therapy.

Finally, CYP3A has been found in different kinds of tumors [25, 26], and the significance of the activation of TRO in tumors as compared with that in the duodenum or in the liver is an issue deserving of further study. In the future the expression of CYP3A4 in tumors might even be used as a marker predicting the response to TRO therapy.

References

1. Alarcon RA (1968) Fluorometric determination of acrolein and related compounds with *m*-aminophenol. Anal Chem 40: 1704
2. Albrecht M, Kleinkauf-Houken A, Trams G, Thomsen K (1984) 5jährige Erfahrungen mit adjuvanter Chemotherapie beim primären Mammakarzinom. Geburtshilfe Frauenheilkd 44: 550
3. Ayash LJ, Wright JE, Tretyakov O, Gonin R, Elias A, Wheeler C, Eder JP, Rosowsky A, Antman K, Frei E III (1992) Cyclophosphamide pharmacokinetics: correlation with cardiac toxicity and tumor response. J Clin Oncol 10: 995
4. Baldwin SJ, Bloomer JC, Smith GJ, Ayrton AD, Clarke SE, Chenery RJ (1995) Ketoconazole and sulphaphenazole as the respective selective inhibitors of P4503A and 2C9. Xenobiotica 25: 261
5. Bohnenstengel F, Hofmann U, Eichelbaum M, Kroemer HK (1996) Characterization of the cytochrome P450 involved in side-chain oxidation of cyclophosphamide in humans. Eur J Clin Pharmacol 51: 297
6. Boos J, Welslau U, Ritter J, Blaschke G, Schellong G (1991) Urinary excretion of the enantiomers of ifosfamide and its inactive metabolites in children. Cancer Chemother Pharmacol 28: 455
7. Boos J, Göring M, Küpker F, Jürgens H, Blaschke G (1993) In-vitro studies on the formation of active and inactive metabolites of ifosfamide by different organ systems. Pharm Pharmacol Lett 3: 119
8. Boos J, Küpker F, Blaschke G, Jürgens H (1993) Trofosfamide metabolism in different species – ifosfamide is the predominant metabolite. Cancer Chemother Pharmacol 33: 71
9. Brade WP, Herdrich K, Kachel-Fischer U, Araujo CE (1991) Dosing and side effects of ifosfamide plus mesna. J Cancer Res Clin Oncol 117: 164
10. Cerny T, Küpfer A (1994) The enigma of ifosfamide encephalopathy. Ann Oncol 3: 679
11. Chang TKH, Weber GF, Crespi CL, Waxman DJ (1993) Differential activation of cyclophosphamide and ifosphamide by cytochromes P-450 2B and 3A in human liver microsomes. Cancer Res 53: 5629
12. Connors TA, Cox PJ, Farmer PB, Foster AB, Jarman M (1974) Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. Biochem Pharmacol 23: 115
13. Crespi CL (1991) Expression of cytochrome P450 cDNAs in human B lymphoblastoid cells: applications to toxicology and metabolite analysis. Methods Enzymol 206: 123
14. Crespi CL, Thilly WG (1984) Assay for gene mutation in a human lymphoblast line, AHH-1, competent for xenobiotic metabolism. Mutat Res 128: 221
15. Fromm MF, Busse D, Kroemer HK, Eichelbaum M (1996) Differential induction of prehepatic and hepatic metabolism of verapamil by rifampin. Hepatology 24: 797

16. Goren MP (1991) Determination of urinary 2- and 3-dechloroethylated metabolites of ifosfamide by high-performance liquid chromatography. J Chromatogr 570: 351
17. Guengerich FP (1991) Mechanisms of catalysis and substrate specificity of human cytochrome P450 enzymes. Falk Symp 57: 85
18. Guengerich FP, Dannan G, Wright ST (1982) Purification and characterization of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. Biochemistry 21: 6019
19. Hempel G, Krümpelmann S, May-Manke A, Hohenlöchter B, Blaschke G, Jürgens H, Boos J (1997) Pharmacokinetics and metabolism of trofosfamide and its dechloroethylated metabolites. Cancer Chemother Pharmacol 40: 45
20. Kurowski V, Cerny T, Küpfer A, Wagner T (1991) Metabolism and pharmacokinetics of oral and intravenous ifosfamide. J Cancer Res Clin Oncol 117 [Suppl IV]: 148
21. Lind MJ, Margison JM, Cerny T, Thatcher N, Wilkinson PM (1989) Comparative pharmacokinetics and alkylating activity of fractionated intravenous and oral ifosfamide in patients with bronchogenic carcinoma. Cancer Res 49: 753
22. Mimura M, Baba T, Yamazaki H, Ohmori S, Inui Y, Gonzalez FJ, Guengerich FP, Shimada T (1993) Characterization of cytochrome P-450 2B6 in human liver microsomes. Drug Metab Dispos 21: 1048
23. Mohrmann M, Ansorge S, Schmich U, Schönfeld B, Brandis M (1994) Toxicity of ifosfamide, cyclophosphamide and their metabolites in renal tubular cells in culture. Pediatr Nephrol 8: 157
24. Molowa DT, Schuetz EG, Wrighton SA, Watkins PB, Kremers P, Mendez-Picon G, Parker GA, Guzelian PS (1986) Complete cDNA sequence of a cytochrome P450 inducible by glucocorticoids in human liver. Proc Natl Acad Sci USA 83: 5311
25. Murray GI, Paterson PJ, Weaver RJ (1993) The expression of cytochrome P-450, epoxide hydrolase, and glutathione S-transferase in hepatocellular carcinoma. Cancer 71: 36
26. Murray GI, Weaver RJ, Paterson PJ (1993) Expression of xenobiotic metabolizing enzymes in breast cancer. J Pathol 169: 347
27. Patterson WP, Khojasteh A (1989) Ifosfamide-induced renal tubular defects. Cancer 63: 649
28. Ren S, Yang JS, Kalhorn TF, Slattery JT (1997) Oxidation of cyclophosphamide to 4-hydroxycyclophosphamide and deschloroethylcyclophosphamide in human liver microsomes. Cancer Res 57: 4229
29. Rodrigues AD (1994) Use of in vitro human metabolism studies in drug development – an industrial perspective. Biochem Pharmacol 48: 2147
30. Shimada T, Iwasaki M, Martin MV, Guengerich FP (1989) Human liver microsomal cytochrome P-450 enzymes involved in the bioactivation of procarcinogens detected by *umu* gene response in *Salmonella typhimurium* TA 1535/pSK1002. Cancer Res 49: 3218
31. Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. J Pharmacol Exp Ther 270: 414
32. Sladek NE (1988) Metabolism of oxazaphosphorines. Pharmacol Ther 37: 301
33. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350
34. Walker D, Flinois J-P, Monkman SC, Beloc C, Boddy AV, Cholerton S, Daly AK, Lind MJ, Pearson ADJ, Beaune PH, Idle JR (1994) Identification of the major human hepatic cytochrome P450 involved in activation and *n*-dechloroethylation of ifosfamide. Biochem Pharmacol 47: 1157
35. Watkins PB, Wrighton SA, Maurel P, Schuetz EG, Mendez-Picon G, Parker GA, Guzelian PS (1985) Identification of an inducible form of cytochrome P450 in human liver. Proc Natl Acad Sci USA 82: 6310
36. Wist E, Risberg T (1991) Trofosfamide in non-Hodgkin's lymphoma – a phase II study. Acta Oncol 30: 819