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IDENTIFICATION OF THE MAJOR HUMAN HEPATIC CYTOCHROME P450 INVOLVED IN ACTIVATION AND N-DECHLOROETHYLATION OF IFOSFAMIDE

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Abstract—Two NADPH-dependent metabolic routes for the anticancer drug ifosfamide, 4-hydroxylation (activation) and N-dechloroethylation (a detoxication pathway), were studied in human liver microsomes to identify the cytochrome P450 enzymes involved. Naringenin, a grapefruit aglycone and an inhibitor of cytochrome P450 3A4 (CYP3A4)-catalysed reactions, was found to inhibit ifosfamide activation and N-dechloroethylation by human liver microsomes. IC_{50} for both reactions was of the order of 70 μM. The CYP3A4-specific inhibitor triacetyloleandomycin inhibited ifosfamide N-dechloroethylation by human liver microsomes with an IC_{50} of approximately 10 μM. Furthermore, anti-human CYP3A4 antiserum inhibited by about 80% N-dechloroethylation of ifosfamide by human liver microsomes. The relative levels of cytochromes P450 1A, 2C, 2E and 3A4 in 12 human livers were determined by western blotting analysis. A strong correlation ($P < 0.001$) was observed between CYP3A4 expression and both activation and N-dechloroethylation of ifosfamide. A role for human CYP3A4 in both pathways of ifosfamide metabolism was thus demonstrated. This was substantiated by the observation that the nifedipine oxidase activities of the 12 samples of human liver microsomes correlated with ifosfamide activation ($P < 0.009$) and N-dechloroethylation ($P < 0.001$). These findings have important clinical implications. The involvement of the same key cytochrome P450 enzyme in both reactions prohibits selective inhibition of the N-dechloroethylation pathway, as might be desirable to reduce toxic side effects. They also demonstrate the need to consider interaction with co-administered drugs that are CYP3A4 substrates.

Key words: cytochrome P450 3A4; inhibition; naringenin; triacetyloleandomycin; western blotting; correlation

Progress in cancer care might best be achieved by improved prescribing of existing effective drugs. The oxazaphosphorine ifosfamide, a DNA alkylating agent, is used against a range of paediatric and adult malignancies [1, 2]. Therapeutic outcome, in terms of both tumour response and patient toxicity, is variable and unpredictable. The drug is inert in terms of both anti-tumour efficacy and toxicity until metabolically activated and an understanding of its metabolism will facilitate the development of techniques to identify at diagnosis responsive tumours in drug-tolerant patients.

The principal metabolic pathways of ifosfamide are well established [3] and are illustrated in Fig. 1. Isophosphoramide mustard has been identified as the ultimate DNA cross-linking species. The identity of the enzymes responsible for some of the metabolic interactions, however, remains unclear. Specifically, the human cytochromes P450 responsible for 4-hydroxylation, the activating step, and N-dechloroethylation, a probable detoxication pathway, remain to be identified. In the rat, hepatic cytochromes P450 2B1 and 2C6/11 have been shown to have a role in 4-hydroxylation of the oxazaphosphorines [4]. Consistent with this is that, when expressed in yeast, cytochrome P450 2B1 (CYP2B1) increased the frequency of mutation induced by cyclophosphamide exposure [5]. Recently, a role for cytochrome P450 3A in dexamethasone-treated rats has also been demonstrated [6]. Whereas cytochrome P450 involvement in N-dechloroethylation has been established [7], the identity of the important P450s remains to be addressed.

Relevant in terms of therapy is the question as to whether the cytochromes P450 involved in the two reactions are distinct or overlapping. Evidence indicates that N-dechloroethylation might be responsible for the major toxic side effects of oxazaphosphorine-induced neurotoxicity [8] and urotoxicity [9]. Selective inhibition of this reaction might, therefore, be desirable.

We demonstrate here that cytochrome P450 3A4 (CYP3A4) makes a major contribution to both 4-hydroxylation and N-dechloroethylation of ifosfamide.

MATERIALS AND METHODS

Materials. Ifosfamide was obtained as Mitoxana

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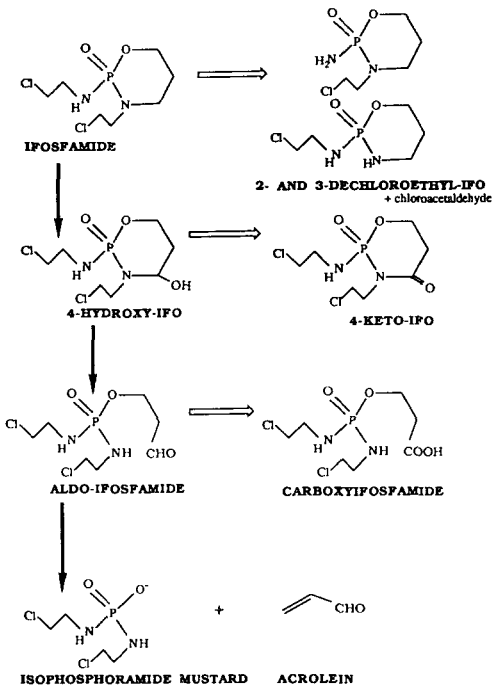

Fig. 1. Metabolic transformations of ifosfamide. Solid arrows indicate activation pathways and open arrows show detoxication pathways.

for intravenous use from Asta Medica (Cambridge, U.K.). 4-Hydroperoxy ifosfamide was a gift from Asta Medica AG (Frankfurt, Germany). Triacetyloleandomycin (Troleandomycin) was a gift from Pfizer Ltd (Sandwich, U.K.). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), purified from baker's yeast, β-NADP (monosodium salt), glucose-6-phosphate (monosodium salt), semicarbazide hydrochloride, hydroxylamine hydrochloride, nifedipine, chloroacetonitrile and 7-ethoxycoumarin were purchased from the Sigma Chemical Co. (Poole, U.K.). 3-Aminophenol and chloroacetaldehyde were from the Aldrich Chemical Co. (Poole, U.K.). Toluene was HPLC grade. Inorganic reagents not listed above were AnalaR grade. Human liver microsomes HHM1, HHM2, HHM9, HHM15, HHM19 and HHM25, additional to those used in the correlation studies, were purchased from the Keystone Skin Bank (Exton, PA, U.S.A.).

Preparation of human liver microsomes. Microsomes were prepared from 12 human liver samples collected and stored as described previously [10]. Cytochrome P450 was measured as described by Schoene *et al.* [11] and protein was assayed by the BCA method (Pierce) according to the supplier's recommendations and using BSA as standard.

Determination of ifosfamide 4-hydroxylation. 4-Hydroxylation of ifosfamide was measured as fluorescence of 7-hydroxyquinoline produced by reaction of liberated acrolein with 3-aminophenol as described by Masurel *et al.* [12]. Microsomes were incubated at 0.25–0.5 mg/mL protein in 0.4 mL vol. in 100 mM potassium phosphate, pH 7.4, 5 mM $MgCl_2$, 1 mM NADP, 5 mM glucose-6-phosphate, 1 EU/mL glucose-6-phosphate dehydrogenase, 5 mM semicarbazide HCl and 1 mM ifosfamide. Reactions were stopped by the addition of 160 μL 5.5% $ZnSO_4$, 160 μL saturated $Ba(OH)_2$ and 80 μL 0.01 M HCl at various times up to 45 min, throughout which the reaction rate was linear. The precipitate was removed by centrifugation at 13,000 rpm for 10 min in an Eppendorf centrifuge. Acrolein was liberated and derivatized by the addition to 0.6 mL of the reaction supernatant of 0.3 mL 6 mg/mL hydroxylamine HCl, 6 mg/mL 3-aminophenol in 1 M HCl. The mixture was heated to 100° for 20 min and cooled to room temperature in the dark. Fluorescence of 7-hydroxyquinoline was measured at 510 nm with excitation at 350 nm. Standard curves were constructed from 4-hydroperoxy ifosfamide added to incubation buffer and treated exactly as above. This compound liberates acrolein spontaneously under the conditions of the derivatization reaction.

Determination of ifosfamide N-dechloroethylation. N-Dechloroethylation of ifosfamide liberates chloroacetaldehyde in a molar ratio very close to 1:1 [13]. Reaction rate was determined as the rate of chloroacetaldehyde liberation. Microsome incubations were as described for 4-hydroxylation but with the omission of semicarbazide HCl. Fifty microlitres of 100 μM chloroacetonitrile were added as internal standard and protein was precipitated as described above. Two microlitres of the supernatant were injected onto a Porpak column attached to a Hewlett Packard 5890 series II gas chromatograph equipped with electron capture detection. Injector and detector temperatures were 200° and the oven temperature was 100°. Nitrogen flow rate was 60 mL/min. Chloroacetaldehyde eluted at 6.5 min and chloroacetonitrile at 16.5 min. Standard curves were constructed from chloroacetaldehyde diluted in incubation buffer and treated in the same way as the samples.

Inhibition studies. Naringenin and 7-ethoxycoumarin, at concentrations up to 500 μM, were added to the incubation medium prior to the initiation of reaction by the addition of microsomal protein. Triacetyloleandomycin was added to complete assay mixtures minus ifosfamide and the samples were incubated at 37° for 30 min to allow for triacetyloleandomycin metabolism. The reaction was then initiated by the addition of ifosfamide.

Determination of nifedipine oxidation. Nifedipine oxidation was measured as the disappearance of the substrate over 30 min. Incubations were as described for ifosfamide 4-hydroxylation but semicarbazide was omitted and ifosfamide was replaced with nifedipine at an initial concentration of 100 μM. All operations were carried out under yellow light and the incubations were in amber glass vials due to the photosensitivity of nifedipine [14]. The reaction was stopped on ice-water prior to extraction of 0.4 mL into 1 mL toluene. Unmetabolized nifedipine was determined by electron capture gas chromatography using the method of Schmid *et al.* [14].

Measurement of 7-ethoxycoumarin deethylase activity. Microsomes were incubated in 0.4 mL vol. as described for determination of ifosfamide 4-hydroxylation above, but semicarbazide HCl was omitted and ifosfamide was replaced with 0.25 mM 7-ethoxycoumarin. Reactions were stopped after 15 or 45 min, over which the reaction rate remained linear, by the addition of 40 μL 20% trichloroacetic acid, and the fluorescent product, umbelliferone (7-hydroxycoumarin), was extracted into 400 μL of chloroform. Two-hundred microlitres of this were extracted with 800 μL 0.01 M NaOH and fluorescence was measured at 456 nm with excitation at 368 nm.

Anti-P450 antibody preparation. Anti-rat CYP1A1/2 was produced in rabbit against CYP1A1 purified from liver microsomes of β-naphthoflavone-treated rats. Anti-human CYP1A1/2 was produced in rabbit against human CYP1A2 expressed in bacteria. They do not cross-react with human CYP2C9, 2C18, 2E1, 3A4 and rat CYP2B1/2. Both antibodies recognised human liver microsomes as a single band and gave similar results in quantitative western blotting.

Anti-human CYP3A4 was produced in rabbit against pure human CYP3A4 expressed in bacteria or against purified human CYP3A4 [15]. Both recognised human liver microsomes as a single band and did not cross-react with CYP1A1, 1A2, 2C9, 2C18.

Anti human CYP2E1 was a generous gift of Prof. F. P. Guengerich (Center for Molecular Toxicology, Vanderbilt University, Nashville, TN, U.S.A.) or was produced in rabbit against pure human CYP2E1 expressed in bacteria. Both recognised one band in human liver microsomes.

Anti-CYP2C9 was produced in rabbits against purified CYP2C, a generous gift of Prof. F. P. Guengerich [16]. It recognised one main band in human liver microsomes and recognised pure CYP2C9 and CYP2C18 expressed in yeast. These two cytochromes P450 are clearly separated in western blots. They did not cross-react with human CYP1A1, 1A2, 3A4, 2E1 expressed in yeast or bacteria.

Determination of the relative P450 monooxygenase levels in human liver microsomes by western blotting. Microsomal protein was separated by SDS–PAGE according to Laemmli [17] and then electro-transferred onto nitrocellulose sheets. Cytochromes P450 were detected by primary antibodies and peroxidase-conjugated secondary antibodies. Staining was with 4-chloro-1-naphthol as described previously [18]. Cytochrome P450 was quantified by densitometry using a Hewlett Packard Scan Jet II and results were expressed as arbitrary units/mg microsomal protein. Linearity was checked using different loadings of the human liver microsomes.

Antibody inhibition studies. Microsomes were preincubated at room temperature at 0.25 mg/mL protein with anti-CYP3A4 anti-serum at 400, 800 or 1600 μL/mg (approximately 3–12 mg IgG/nmol cytochrome P450). The incubation medium contained 100 mM potassium phosphate, pH 7.4, 5 mM $MgCl_2$. Reaction was initiated by the addition of substrate to 1 mM plus NADPH generated from a 10× stock solution giving final concentrations of 1 mM NADP, 5 mM glucose-6-phosphate and 1 EU/mL glucose-6-phosphate dehydrogenase. Aliquots of 0.4 mL were processed as described above for measurement of N-dechloroethylation.

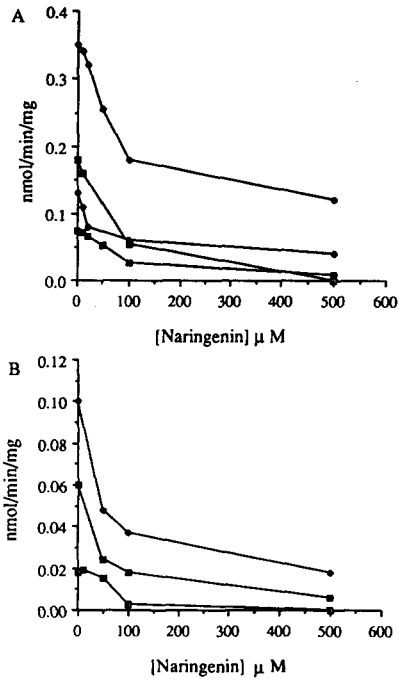

Fig. 2. Inhibition by naringenin of ifosfamide 4-hydroxylation (A) and N-dechloroethylation (B) in human liver microsomes. Reaction rates were determined at an initial ifosfamide concentration of 1 mM at the indicated concentrations of naringenin. Data for 4-hydroxylation are for microsomes from human liver samples HL63 (□), HHM25 (◆), HHM15 (◇) and HHM19 (■). Data for N-dechloroethylation are for human liver samples HL76 (□), HHM2 (■) and HHM9 (◆).

Statistical methods. A non-parametric measure of the degree of association between cytochrome P450 expression and enzyme activities was determined using Spearman's rank correlation.

RESULTS

Inhibition by naringenin of ifosfamide 4-hydroxylation and N-dechloroethylation in human liver microsomes

Naringenin inhibited, over a similar concentration range, ifosfamide 4-hydroxylation and N-dechloroethylation in human liver microsomes (Fig. 2). The concentration range over which inhibition occurs is similar to that observed for nifedipine and felodipine oxidation and for aflatoxin B1 activation [19]. These are all CYP3A4-catalysed reactions with the IC_{50} for naringenin inhibition in the order of 100 μM [19]. This is comparable to the value of around 70 μM determined here for both routes of ifosfamide metabolism. Recently, naringenin inhibition of caffeine metabolism by human liver microsomes has been demonstrated [20], indicating that the inhibitory

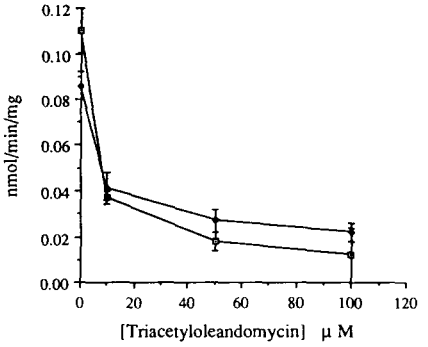

Fig. 3. Inhibition by triacetyloleandomycin of ifosfamide N-dechloroethylation in human liver microsomes. Microsomes from human liver samples HHM9 (□) or HHM1 (◆) were pre-incubated with triacetyloleandomycin at the indicated concentrations before initiating reaction by the addition of 1 mM ifosfamide. Results are shown as means ± SE of two separate incubations.

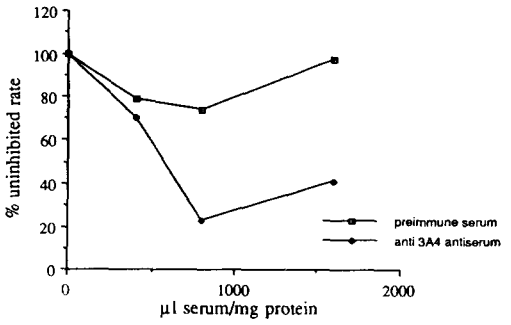

Fig. 4. Inhibition by anti-human CYP3A4 antiserum of ifosfamide N-dechloroethylation in human liver microsomes. Microsomes from human liver sample HL60 were pre-incubated with anti-3A4 antiserum or with pre-immune rabbit serum at the indicated concentrations before initiating reaction by the addition of 1 mM ifosfamide and NADPH.

properties of naringenin extend to CYP1A2 also. The inhibition data are, therefore, consistent with a role for human CYP3A4 in ifosfamide activation and N-dechloroethylation but alone are insufficient to conclude involvement of this isozyme.

Inhibition by triacetyloleandomycin of ifosfamide N-dechloroethylation in human liver microsomes

The macrolide antibiotic triacetyloleandomycin is a selective inhibitor of human CYP3A4-catalysed reactions and is metabolized by CYP3A4 to yield the inhibitory product(s) [21]. After pre-incubation with human hepatic microsomes to allow for its metabolic activation, triacetyloleandomycin inhibited ifosfamide N-dechloroethylation with an IC_{50} of approximately 10 μM (Fig. 3), indicating strongly a role for CYP3A4 in ifosfamide N-dechloroethylation. Pre-incubation without triacetyloleandomycin resulted in no detectable loss of ifosfamide N-dechloroethylating activity, thus any selective denaturation of cytochromes P450 had no effect on the total inhibition observed. Interference by triacetyloleandomycin with the fluorescence assay prevented investigation of its effect on ifosfamide 4-hydroxylation.

Inhibition by anti-CYP3A4 antibody of ifosfamide N-dechloroethylation in human liver microsomes

Anti-human CYP3A4 antiserum from rabbit was found to inhibit by 80% the N-dechloroethylation of ifosfamide by human liver microsomes at a ratio of approximately 5 mg IgG/nmol cytochrome P450 (Fig. 4). Corresponding reduction in activity observed on the addition of equivalent amounts of pre-immune rabbit serum was very low. A major role for human CYP3A4 in ifosfamide metabolism is thus further implicated.

Correlations between rates of ifosfamide metabolism and cytochrome P450 expression

A correlation ($P < 0.0001$) between the 4-hydroxylation and N-dechloroethylation activities of microsomes prepared from 12 human livers indicates the involvement of the same cytochrome P450 isozyme(s) in both pathways, responsible for most or all of the activity measured (Fig. 5, Table 1). Both activities correlate ($P < 0.001$) with CYP3A4 expression as determined by western blot analysis (Fig. 6, Table 1) but not with the expression of 1A, 2C, 2E or total cytochrome P450 (Table 1). A major role for CYP3A4 in ifosfamide 4-hydroxylation and N-dechloroethylation by human liver is thus demonstrated.

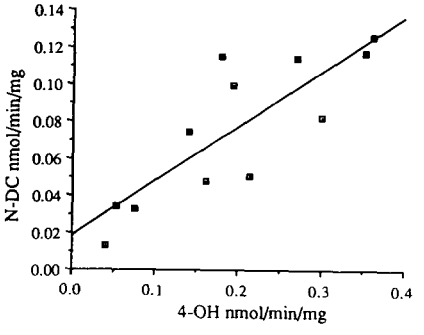

Fig. 5. Ifosfamide N-dechloroethylating (N-DC) *vs* 4-hydroxylating (4-OH) activity for microsomes prepared from 12 human livers. Rates of ifosfamide metabolism were determined at 1 mM ifosfamide. A significant Spearman rank coefficient ($P < 0.0001$) was calculated for the correlation.

Correlations between rates of ifosfamide and nifedipine metabolism

Ifosfamide 4-hydroxylation and N-dechloroethylation were found to correlate with the nifedipine oxidase activities of the 12 livers ($P < 0.009$ for 4-hydroxylation; $P < 0.001$ for N-dechloroethylation) when measured as rate of nifedipine disappearance

Table 1. Associations between ifosfamide 4-hydroxylation (4-OH) and N-dechloroethylation (N-DC), nifedipine oxidation and cytochrome P450 expression in microsomes from 12 human livers

	Correlation coefficient	Significance (P<)
4-OH vs:		
N-DC	0.853	0.0001
CYP3A	0.825	0.001
CYP1A	−0.377	0.2
CYP2C	−0.615	0.03
CYP2E	0.189	0.5
Total P450	0.140	0.6
Nifedipine oxidation	0.720	0.009
N-DC vs:		
4-OH	0.853	0.0001
CYP3A	0.832	0.001
CYP1A	−0.084	0.5
CYP2C	−0.288	0.3
CYP2E	0.276	0.8
Total P450	−0.255	0.4
Nifedipine oxidation	0.831	0.001

Spearman's rank correlation was used to determine the degree of correlation between the various parameters.

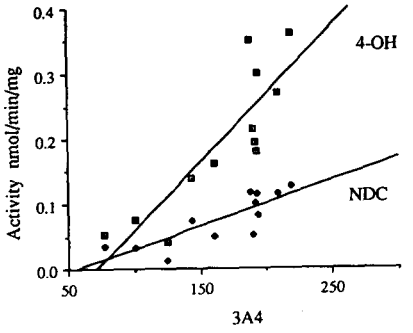

Fig. 6. Rates of ifosfamide 4-hydroxylation and N-dechloroethylation *vs* expression of CYP3A4, expressed in arbitrary units, in microsomes prepared from 12 human livers. Rates of metabolism were determined at 1 mM ifosfamide. Determination of CYP3A4 content was by western blotting. Significant Spearman rank coefficients ($P < 0.001$) were calculated for both correlations.

(Fig. 7, Table 1). The predominant involvement of CYP3A4 in ifosfamide metabolism is thus further demonstrated.

Absence of in vitro *metabolic interactions between ifosfamide and 7-ethoxycoumarin*

7-Ethoxycoumarin at concentrations up to 500 μM had no inhibitory effect on N-dechloroethylation of ifosfamide by human liver microsomes (liver sample HL553). Deethylation of 500 μM 7-ethoxycoumarin by human liver microsomes (liver sample HL1004) was unaffected by 500 μM ifosfamide. The absence of any reciprocal metabolic effect of these two

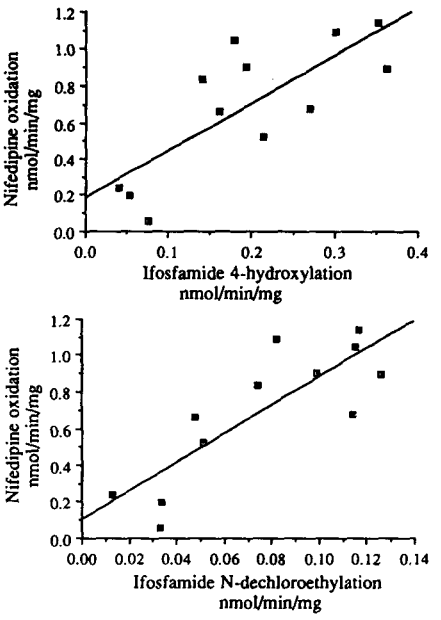

Fig. 7. Rates of ifosfamide 4-hydroxylation and N-dechloroethylation *vs* the measured rate of nifedipine oxidation in microsomes prepared from 12 human livers. Rates of metabolism were determined at 1 mM ifosfamide or 100 μM nifedipine. Significant Spearman rank coefficients ($P < 0.009$ for 4-hydroxylation and $P < 0.001$ for N-dechloroethylation) were calculated for the correlations.

cytochrome P450 substrates indicates the strong likelihood of distinct isozymes in their metabolism.

DISCUSSION

CYP3A4 has been identified as the major cytochrome P450 responsible for both ifosfamide 4-hydroxylation and N-dechloroethylation in the human liver. Correlations between rates of ifosfamide metabolism and hepatic cytochrome P450 levels determined by western blotting and also between ifosfamide and nifedipine metabolism are the major criteria in this study for the assignment of CYP3A4 as the major hepatic enzyme responsible for ifosfamide 4-hydroxylation and N-dechloroethylation. The validity of this approach might be challenged in respect of the argument that levels of particular cytochromes P450 might correlate with each other or with total cytochrome P450 content. This was indeed demonstrated in one study on a panel of 12 human livers [22]. In the present study no such correlations were evident, and chemical and antibody inhibition data further substantiate the conclusion. Whilst studies using purified cytochromes P450 or cell lines over-expressing single forms can demonstrate unequivocally that a given compound is a substrate for a particular cytochrome P450, they in no way alone reflect the actual contribution of this single enzyme form to the total hepatic metabolism of the compound. Levels of hepatic expression of cytochromes P450 are required also for such studies to be informative in this respect.

The present study is unable to exclude the minor involvement of cytochromes P450 other than CYP3A4 in ifosfamide metabolism. In fact, correlation between activity of the N-dechloroethylation pathway in the 12 human liver microsome preparations and nifedipine oxidase activity is better than for the 4-hydroxylation pathway, suggesting a bigger contribution from other cytochromes P450 to the latter. Identification of candidate enzymes using purified forms or single expression systems will guide further investigations into their contribution.

This is the first definitive study on human hepatic metabolism of ifosfamide to be reported and comparison with studies on ifosfamide metabolism in the rat [4–6] highlights important interspecies differences. CYP2B1 and CYP2C6/11 are the principal catalysts of ifosfamide activation in the rat, with a detectable contribution from CYP3A being apparent only following dexamethasone induction [6]. In the present study, the lack of any correlation between ifosfamide metabolism and CYP2C expression leads us to reject CYP2C9 and CYP2C18 as having a major contribution to ifosfamide metabolism in human liver. Failure of ifosfamide to inhibit 7-ethoxycoumarin deethylation by human liver microsomes and of 7-ethoxycoumarin to inhibit ifosfamide N-dechloroethylation suggest that CYP2B6, the human enzyme orthologous to rat CYP2B1 and a catalyst of 7-ethoxycoumarin deethylation, has no role in hepatic ifosfamide metabolism. This result might indeed be predicted in light of the low levels of hepatic expression of CYP2B6, and it leaves open the possibility that extrahepatic CYP2B, for instance in the tumour, kidney or lung, might contribute to ifosfamide metabolism. These differences between rat and human hepatic metabolism of ifosfamide exemplify the danger of extrapolating between species with regard to P450 substrate specificity and demonstrate with respect to ifosfamide in particular the limitations of using animal models for human drug metabolism. Other examples of differences between rats and humans with respect to the complement of cytochromes P450 responsible for the metabolism of a specific compound can be cited. A case showing a similar pattern to ifosfamide is 7-pentoxyresorufin, a specific substrate for CYP2B1 and CYP2B2 in rats and for CYP3A4 in humans [23].

One possible clinical application following from identification of the human hepatic cytochromes P450 involved in the two major routes of ifosfamide metabolism was anticipated to be selective inhibition of the N-dechloroethylation pathway to limit toxicity and improve efficacy. Demonstration of the involvement of the same principal cytochrome P450 in both pathways abrogates this possibility. Nevertheless, knowledge of specific cytochrome P450 involvement in ifosfamide metabolism is clinically important. Patient metabolism of CYP3A4-metabolized marker drugs, such as erythromycin, might, for example, prove a useful predictor of therapeutic response and toxicity.

Certainly of importance in the clinical use of ifosfamide is its interaction with co-administered drugs. Substrates and/or inducers of CYP3A4 such as dexamethasone [24], rifampicin [25], prednisolone [26], erythromycin [27], and the benzodiazapines midazolam and triazolam [28] are now identified as being of particular importance in this respect. Indeed clinical data concerning co-metabolism of cyclophosphamide, a structural isomer of ifosfamide, and CYP3A4 substrates/inducers are consistent with the involvement of CYP3A4 in cyclophosphamide clearance. In one such study, prednisolone initially inhibited the biotransformation of cyclophosphamide, but prolonged pretreatment with prednisone resulted in an increased rate of cyclophosphamide metabolism, consistent with induction of cyclophosphamide-metabolizing cytochromes P450. These data were explained on the basis of a single prednisone-inducible cytochrome P450 responsible for the metabolism of both drugs [29]. A second study suggested an associated increase in cyclophosphamide and dexamethasone clearance in patients receiving high dose cyclophosphamide to be through induction of common hepatic metabolizing enzymes [30].

It is interesting to note a parallel between the metabolism of ifosfamide and of the antihistamine drug, terfenadine. The latter undergoes a C-hydroxylation and an N-dealkylation and CYP3A4 plays a major role in both reactions in human liver [31].

To establish the contribution of CYP3A4 variability to therapeutic outcome and toxicity a number of questions need be addressed. Most pertinent is whether or not hepatic and/or tumour activation of the drug is important, and whether or not the site of N-dechlororethylation responsible for specific toxic effects is local: in the kidney for nephrotoxicity and in the brain for encephalopathy. The suggestion that tumour response might indeed be dependent on intratumoural, rather than hepatic, drug metabolism is appealing in view of the lack of toxicity of the ultimate DNA cross-linking metabolite, phosphoramide mustard, when generated extracellularly [32], coupled with the highly reactive nature and short half-life of the activated 4-hydroxylated metabolite of cyclophosphamide [33]. Tumour-expression of CYP3A4 and its contribution to ifosfamide activation requires investigation and might ultimately prove to be a marker for the prediction of tumour response to ifosfamide therapy.

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