Enhanced Cyclophosphamide and Ifosfamide Activation in Primary Human Hepatocyte Cultures: Response to Cytochrome P-450 Inducers and Autoinduction by Oxazaphosphorines

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ABSTRACT

The anticancer oxazaphosphorine prodrugs cyclophosphamide and ifosfamide are activated in human liver by a 4-hydroxylation reaction catalyzed by multiple cytochrome P450 (CYP) enzymes. In the present study, we used a cultured human hepatocyte model to identify possible inducers of the CYP-catalyzed activation of these two anticancer prodrugs. Treatment of primary cultures of human hepatocytes with phenobarbital, dexamethasone, or rifampin elevated hepatocyte microsomal oxazaphosphorine 4-hydroxylation by up to 200-400% of control for both drug substrates. These inductions were associated with corresponding increases in immunoreactive CYP2B6, CYP2C8, CYP2C9, and CYP3A4, all previously shown to catalyze oxazaphosphorine activation. Rifampin (1 μM, 96-h exposure) was a particularly potent inducer of ifosfamide and cyclophosphamide 4-hydroxylation, as well as of CYP3A protein levels and CYP3A-dependent testosterone 6β-hydroxylation. CYP3A4, CYP2C8, and CYP2C9 protein levels were also increased by exposure of the hepatocytes to cyclophosphamide or ifosfamide (50 μM), which thereby enhanced their own rates of 4-hydroxylation in the cultured hepatocytes. In one human hepatocyte culture that contained the polymorphically expressed CYP3A5 in addition to the more widely expressed CYP3A4, only CYP3A4 was induced by cyclophosphamide, ifosfamide, and rifampin. These studies: (a) demonstrate an underlying metabolic basis for the clinically important oxazaphosphorine autoinduction pharmacokinetics seen with these drugs in cancer patients; and (b) identify rifampin and other CYP inducers as potentially useful for increasing the rates of cyclophosphamide 4-hydroxylation and ifosfamide 4-hydroxylation in human liver in a manner that could favorably impact the clinical pharmacokinetics of these anticancer prodrugs.

INTRODUCTION

Cyclophosphamide and its isomeric analogue, ifosfamide, are widely used in the clinical management of a variety of human malignancies (1, 2). Cyclophosphamide also exhibits immunosuppressive properties and is used in organ transplantation protocols and various autoimmune disorders (1). Cyclophosphamide and ifosfamide are oxazaphosphorine prodrugs that require bioactivation by P-450 enzymes to form a 4-hydroxy metabolite that equilibrates with the ring-opened aldophosphamide. This intermediate can undergo spontaneous β-elimination to yield acrolein and a mustard derivative (phosphoramid mustard or isophosphoramid mustard) or, alternatively, it can be inactivated by aldehyde dehydrogenase-catalyzed conversion to carboxyphosphamide (3). Multiple human P-450 enzymes are capable of activating oxazaphosphorines in vitro, including CYP2A6, CYP2B6, CYP3A4, CYP3A5, and all four individual CYP2C enzymes (6). In the case of ifosfamide, a clinically significant bio-transformation pathway is side-chain N-dechloroethylation (7–9), which is catalyzed by CYP2B and CYP3A enzymes in vitro (5, 10) and generates therapeutically inactive but neurotoxic metabolite(s) (11, 12).

Large interpatient differences are found in the clinical pharmacokinetics and biotransformation of cyclophosphamide (13–15) and ifosfamide (16–18). Genetic factors may account for some of this variability as suggested by in vitro experiments demonstrating that allelic variants of CYP2C9 can catalyze cyclophosphamide 4-hydroxylation and ifosfamide 4-hydroxylation with significantly different apparent Km values; moreover, the polymorphically expressed (S)-mephentoin 4′-hydroxylase CYP2C19 is a relatively low Km catalyst of the activation of these prodrugs (6). In addition to genetic factors, concurrently administered drugs may also contribute to the observed interindividual variability in oxazaphosphorine metabolism. For example, phenobarbital (19) and prednisone (20) can decrease the elimination half-life of cyclophosphamide in patients, whereas dexamethasone may increase the total body clearance of this alkylating agent (15). The underlying basis for these alterations in the pharmacokinetics of cyclophosphamide is uncertain, however, but could involve pharmacodynamic factors in addition to P-450-catalyzed drug metabolism. It is important to understand the basis for the large inter-patient variability in the pharmacokinetics and biotransformation of cyclophosphamide and ifosfamide because of the potential relationship between systemic exposure (as measured by AUC) and the toxicity of these agents (18, 21).

Repeated administration or continuous infusion of cyclophosphamide (13, 22–24) or ifosfamide (16, 25, 26) to cancer patients over a period of several consecutive days results in a decreased elimination half-life of these drugs. This is accompanied by an increase in total body clearance (16, 26–28) but no alteration in the volume of distribution (16, 23, 29) or renal clearance (16, 26, 28), suggesting that these oxazaphosphorines may enhance their own hepatic clearance. Although cyclophosphamide (30–33) and ifosfamide (31) can modulate P450 levels in the rat liver model, this modulation leads to a decrease rather than an increase in capacity for oxazaphosphorine activation (30, 31). Consequently, it is important to investigate what effects these cancer chemotherapeutic drugs have on human liver P-450 expression. Because cancer patients are often prescribed combination chemotherapy, such information would allow clinicians to optimize drug therapy by designing rational drug scheduling and avoiding drug combinations that might compromise therapeutic efficacy or exacerbate systemic toxicity.

In the present study, we used a cultured human hepatocyte model: (a) to examine whether hepatic cyclophosphamide and ifosfamide 4-hydroxylation reactions are subject to modulation by drug substrates that might compromise therapeutic efficacy or exacerbate systemic toxicity.

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Received 11/26/96; accepted 3/24/97.


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3 The abbreviations used are: P450 or CYP, cytochrome P-450; TCDD, 2,3,7,8-tetrachlorodibenzop-dioxin; AUC, area under the plasma concentration-time curve.

4 Individual cytochrome P-450 forms are designated according to systematic nomenclature (52).

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phorine autoinduction response seen in cancer patients reflects changes in liver P-450-dependent drug metabolism. Our findings establish that cyclophosphamide and ifosfamide activation can be enhanced in human hepatocytes by inducers of CYP2B, CYP2C, and CYP3A. Furthermore, cyclophosphamide and ifosfamide are shown to enhance their own activation by a mechanism that involves induction of liver P-450 enzymes.

MATERIALS AND METHODS

Materials. Cyclophosphamide and ifosfamide were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). Authentic 4-hydroperoxyifosfamide metabolite standard was kindly provided by Dr. J. Pohl (ASTA Pharma, Bielefeld, Germany). Collagenase, cell culture reagents, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO), FCS (Life Technologies, Inc., Paisley, Scotland), collagen-coated culture dishes (Corning, Iwaki Glass, Japan), and [4-14C] Testosterone (Amersham Corp., Arlington Heights, IL) were obtained from the sources indicated. cDNA-expressed human P-450s used as standards for Western blotting were obtained from Gentest Corp. (Woburn, MA).

Source of Human Liver Specimens. Approval for the scientific use of human liver tissues was obtained from the French National Ethics Committee. Liver specimens were obtained as discarded surgical material excised from patients undergoing hepatic lobectomy for medical reasons unrelated to the present study. The clinical characteristics of the liver tissue donors are given in Table 1.

Isolation and Primary Culture of Hepatocytes. Human hepatocytes were isolated and grown as primary cultures as described previously (34). Briefly, hepatic tissues were extensively washed with Eurocollins (2.05 g/liter KH2PO4, 7.4 g/liter K2HPO4, 1.12 g/liter KCl, 0.84 g/liter NaHCO3, and 35 g/liter glucose, pH 7.3) shortly after resection to eliminate erythrocytes and then transported to the laboratory in a sterile bag on ice. The tissue was then sequentially perfused with calcium-free HEPES, 0.5 mM EGTA, in the same buffer, and finally with 0.05% collagenase (Sigma; type IV) in HEPES buffer containing 7 mM calcium chloride at 37°C. After dissociation, hepatocytes were filtered through nylon gauze (250 μm), and cells were washed three times in culture medium by centrifugation at 50 × g. Cell viability, as assessed by trypan blue exclusion, was generally between 70 and 90%. The cells were plated into 60-mm plastic dishes precoated with collagen at 3 × 10⁶ cells/plate in a total volume of 3 ml of a hormonally and chemically defined medium consisting of a mixture of Williams’ E and Ham’s F-12 (1:1 in volume) and incubated at 37°C in a humidified atmosphere of air containing 5% CO2, 95% air, and 0.1 mM EDTA. Substrate oxidation was initiated by the addition of NADPH (final concentration, 1 mM) and stopped 20 min later with 1 ml of ethyl acetate. The supernatant was derivatized with a solution containing aminophenol (6 mg/ml) and hydroxylamine hydrochloride (6 mg/ml) in 1 M HCl (31). Authentic 4-hydroperoxyifosfamide was used as a standard for acrolein and measured fluorometrically (350-nm excitation wavelength, 515-nm emission wavelength).

Testosterone 6β-Hydroxylation Assay. Microsomal testosterone 6β-hydroxylation activity was determined by a TLC method (42). Each 200 μl of incubation mixture, containing 100 mM HEPES (pH 7.4), 0.1 mM EDTA, 20 μg of microsomal protein, and 50 μM [4-14C] Testosterone was preincubated at 37°C for 4 min. Reactions were initiated by the addition of NAPDH (final concentration, 1 mM) and stopped 20 min later with 1 ml of ethyl acetate. The incubation mixture was then extracted with ethyl acetate and chromatographed on silica gel TLC plates developed with dichloromethane:acetone:acetic acid (9:4:1) followed by chloroform:ethyl acetate:absolute ethanol (4:1:0.7). Metabolites were localized by autoradiography and quantitated by liquid scintillation counting.

RESULTS

Enhanced Cyclophosphamide 4-Hydroxylation and Ifosfamide 4-Hydroxylation following Treatment with Phenobarbital, Rifampin, or Dexamethasone. Primary cultures of human hepatocytes were treated with β-naphthoflavone (25 or 50 μM), TCDD (1 nm), phenobarbital (2 mM), rifampin (1–50 μM), dexamethasone (50 μM), or DMSO (0.1%, vehicle control) for 96–120 h, and hepatocyte microsomes were then prepared. As shown in Table 2, phenobarbital and rifampin elevated cyclophosphamide activation by at least 2–3-fold when measured at 2 mM substrate concentration. At 0.25 mM cyclophosphamide substrate, a modest induction (average of 30–35% for three to four independent cultures) was obtained. Similarly, phenobarbital and rifampin treatment increased ifosfamide 4-hydroxylation to a greater extent at 2 mM substrate concentration (~4-fold) than at 0.25 mM (50–60% average increase; Table 3). In addition, dexamethasone treatment increased these two drug activation reactions (2 mM substrate) by ~3-fold in culture HTL92 (data not shown). By contrast, the P-450 inducers β-naphthoflavone (Tables 2 and 3) and TCDD (data not shown) did not increase 4-hydroxylation of 10⁶ cells and by measuring the rate of de novo protein synthesis after labeling human hepatocytes with tritiated leucine. No sign of cytotoxicity was observed at the oxazaphosphorine concentrations tested (50–250 μM). Moreover, concentrations of cyclophosphamide and ifosfamide as high as 1 mM did not decrease the yield of isolated microsomal protein (data not shown).

Preparation of Hepatocyte Microsomes. At various times after drug treatment (as indicated in each table or figure legend), microsomes were isolated from the cultured hepatocytes by differential ultracentrifugation (35). Microsomal protein was measured by the Bradford method with BSA as standard.

Western Blotting Using Anti-P450 Antibodies. Polyclonal rabbit anti-rat CYP2B (36), anti-rat CYP2C (37), and anti-rat CYP3A antibodies (38) were prepared as detailed elsewhere. These antibody preparations have been shown to cross-react with human liver microsomal CYP2B, CYP2C, and CYP3A proteins (4), respectively. Polyclonal goat anti-rabbit CYP1A1 and goat anti-rabbit CYP1A2 antibodies were purified as described (35). These latter two antibodies have been shown to cross-react with the corresponding cDNA-expressed human P-450 (39). Microsomes isolated from cultured hepatocytes were analyzed for expression of individual microsomal human P-450 proteins by Western blot analysis (40). Each blot was probed with the P-450 subfamily-specific polyclonal antibodies indicated above.

Oxazaphosphorine Activation Assay. Microsomal cyclophosphamide 4-hydroxylation and ifosfamide 4-hydroxylation reactions were assayed by a fluorometric method (41) as described previously (4). Briefly, 100 μg of microsomal protein was preincubated with 0.25 or 2 mM cyclophosphamide or ifosfamide (as indicated in each figure legend) at 37°C for 4 min in a buffer containing 100 mM potassium phosphate (pH 7.4), 0.5 mM semicarbazide HCl, and 0.1 mM EDTA. Substrate oxidation was initiated by the addition of NADPH (final concentration, 1 mM) and stopped 60 min later by the addition of 80 μl of ice-cold 5.5% zinc sulfate solution, followed by 80 μl of saturated barium hydroxide and 40 μl of 0.01 M HCl. After centrifugation, 300 μl of the supernatant was derivatized with a solution containing aminophenol (6 mg/ml) and hydroxylamine hydrochloride (6 mg/ml) in 1 M HCl (31). Authentic 4-hydroperoxyifosfamide was used as a standard for acrolein and measured fluorometrically (350-nm excitation wavelength, 515-nm emission wavelength).

<table>
<thead>
<tr>
<th>Table 1 Liver tissue donors</th>
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<tr>
<td>Liver identification</td>
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<td>Age</td>
<td>Diagnosis</td>
</tr>
<tr>
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<td>F</td>
<td>60</td>
<td>Metastatic colon cancer</td>
</tr>
<tr>
<td>HTL92</td>
<td>M</td>
<td>73</td>
<td>Metastatic colon cancer</td>
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<td>FH105</td>
<td>M</td>
<td>57</td>
<td>Pancreas cancer</td>
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<tr>
<td>FTO8</td>
<td>F</td>
<td>29</td>
<td>Adenoma</td>
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of either cyclophosphamide or ifosfamide. These data demonstrate that the rate of activation of both oxazaphosphorines can be enhanced by select P-450 inducers, albeit to an extent that may vary between individual livers. Analysis of the data from nine individual human hepatocyte cultures (Table 1) suggests that the extent to which rifampin induces oxazaphosphorine 4-hydroxylation activity, expressed as a percentage of uninduced control, is inversely related to the basal activity (i.e., uninduced activity) of the culture (data not shown).

**Induction of Multiple P-450 Proteins by Phenobarbital, Dexamethasone, and Rifampin.** Phenobarbital, dexamethasone and rifampin have been shown to induce CYP3A mRNA (43-45), protein, and activity (46) in primary cultures of human hepatocytes. To confirm the effect of these drugs on CYP3A and to determine whether they also induce other P-450 forms (e.g., CYP2B6 and CYP2C) in our experiments, Western blot analysis was carried out using heterologous P-450 subfamily-specific polyclonal antibodies (4, 35). As shown in Fig. 1A, phenobarbital induced an immunoreactive CYP2B protein (Lane 6 versus Lane 4), which had an electrophoretic mobility corresponding to that of cDNA-expressed CYP2B6 (Lane 3). This CYP2B protein was also induced by dexamethasone and rifampin (Lanes 7 and 8 versus Lane 4). Phenobarbital, dexamethasone, and rifampin also induced two major CYP2C proteins (Fig. 1B, Lanes 6-8). The top band corresponds in electrophoretic mobility to CYP2C9, whereas the lower band has a mobility equivalent to cDNA-expressed CYP2C8 (Fig. 1B, Lane 1; Ref. 4). Whereas the same three inducers were each effective in inducing CYP3A protein(s) (Fig. 1C, Lanes 6-8), none of these compounds altered CYP1A or CYP1A2 protein content in these cells (data not shown). The CYP1A inducer TCDD (Lane 5) did not induce CYP2B, 2C, or 3A proteins in the human hepatocytes, although it did increase an unidentified CYP3A-immunoreactive protein (Fig. 1C, Lane 5) with a mobility similar to a CYP3A-immunoreactive protein that is expressed constitutively in the lymphoblast cell line cDNA expression system (Fig. 1C, band marked by asterisk, Lanes 1-3). In control experiments, TCDD was shown to induce CYP1A2 in these same cultures (39).

**Concentration and Time Dependence of Rifampin Induction Response.** To investigate the potency of rifampin with respect to induction of cyclophosphamide and ifosfamide 4-hydroxylation, primary cultures of human hepatocytes were treated with 1, 2, 5, 25, or 50 μM rifampin for 96 h. A near-maximal increase in cyclophosphamide 4-hydroxylation and ifosfamide 4-hydroxylation was achieved with 1 μM rifampin, both when the 4-hydroxylation assays were performed at 0.25 mM substrate (Fig. 2A) and at 2 mM substrate (Fig. 2B). The rifampin-induced increase in ifosfamide 4-hydroxylation was, however, greater than the increase in cyclophosphamide 4-hydroxylation at both substrate concentrations. A similar concentration dependence was observed for induction of immunoreactive CYP3A protein (Fig. 3A) and CYP3A-mediated testosterone 6β-hydroxylase activity (Fig. 3B). A time course study demonstrated that CYP3A protein was detectably elevated as early as 72 h and was sustained at an elevated level from 96-192 h (Fig. 3A, Lanes 5 and 7-11). The preferential induction by rifampin of ifosfamide 4-hydroxylation activity seen in this experiment (Fig. 2) was also observed in hepatocyte cultures from other individuals, albeit to different extents (e.g., Fig. 4; see below). This induction of ifosfamide 4-hydroxylation may in part reflect the preferential role of CYP3A enzymes in catalyzing this reaction (4).

**Autoinduction of Cyclophosphamide and Ifosfamide Activation in Human Hepatocytes.** Repeated treatment of cancer patients with cyclophosphamide (27, 28) or ifosfamide (16, 26, 47) results in a decrease in elimination half-life and an increase in total body clearance of these alkylating agents. To determine whether these oxazaphosphorines enhance their own activation, primary cultures of human hepatocytes were treated with dimethylsulfoxide (vehicle control, 0.1%), β-naphthoflavone (25 μM), phenobarbital (2 mM), or rifampin (50 μM) for 96 h, and microsomes were prepared. Microsomal cyclophosphamide 4-hydroxylation activity was determined as described in “Materials and Methods.” Results shown as relative activity (control = 100). Absolute activity values, expressed as pmol/min/mg microsomal protein, are shown in parentheses for the uninduced controls.

**Table 2 Effect of P-450 enzyme inducers on cyclophosphamide 4-hydroxylation in primary cultures of human hepatocytes**

<table>
<thead>
<tr>
<th>Culture no.</th>
<th>Control</th>
<th>BNF</th>
<th>PB</th>
<th>RIF</th>
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<tbody>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>FT43</td>
<td>(97)</td>
<td>45a</td>
<td>189</td>
<td>123</td>
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<td>(395)</td>
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<td>75</td>
<td>112</td>
</tr>
<tr>
<td>FT59</td>
<td>(494)</td>
<td>89</td>
<td>NA</td>
<td>106</td>
</tr>
<tr>
<td>FT60</td>
<td>(406)</td>
<td>147</td>
<td>142</td>
<td>172</td>
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<td>Mean</td>
<td>(348)</td>
<td>94</td>
<td>135</td>
<td>128</td>
</tr>
<tr>
<td>SD</td>
<td>(173)</td>
<td>51</td>
<td>57</td>
<td>30</td>
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</table>

* BNF, β-naphthoflavone; PB, phenobarbital; RIF, rifampin; NA, data not available.

**Table 3 Effect of P-450 enzyme inducers on ifosfamide 4-hydroxylation in primary cultures of human hepatocytes**

<table>
<thead>
<tr>
<th>Culture no.</th>
<th>Control</th>
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<th>RIF</th>
</tr>
</thead>
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<td>NA</td>
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</tr>
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<tr>
<td>SD</td>
<td>(175)</td>
<td>30</td>
<td>121</td>
<td>45</td>
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* BNF, β-naphthoflavone; PB, phenobarbital; RIF, rifampin; NA, data not available.

* Culture treated with 50 μM BNF.

* Culture treated with 25 μM RIF.
hepatocytes were treated for 96 h with 50 μM cyclophosphamide or ifosfamide. This corresponds to a concentration of oxazaphosphorine that is clinically relevant and is without apparent toxicity to the cells (see "Materials and Methods"). As shown in Fig. 4, cyclophosphamide treatment of human hepatocytes increased microsomal cyclophosphamide 4-hydroxylase activity by as much as 140%. Ifosfamide (50 μM) increased its own 4-hydroxylation activity, as well as cyclophosphamide 4-hydroxylation activity, in a similar manner. Clear evidence for significant autoinduction of oxazaphosphorine metabolic activity was obtained in three of five human hepatocyte cultures examined (FT60, FH105, and FT108) but not in cultures FT59 or FT80 (Fig. 4 and data not shown), despite the responsiveness of the latter two cultures to rifampin. This variability in oxazaphosphorine inducibility is reminiscent of the interindividual differences in the autoinduction response seen in the clinic (13, 48) and is analogous to the variability in responsiveness of cultured human hepatocytes to classic P-450 inducers seen in Tables 2 and 3.

Effect of Cyclophosphamide and Ifosfamide Treatment on CYP2B, CYP2C, and CYP3A Protein Expression in Cultured Hepatocytes. CYP2B6, CYP2C8, CYP2C9, and CYP3A4 are each catalytically competent in activating cyclophosphamide and ifosfamide (4). Therefore, Western blot analysis was performed to determine whether the autoinduction of oxazaphosphorine activation in cultured hepatocytes seen in Fig. 4 is accompanied by induction of any of these enzymes. As is shown in Figs. 5 and 6, both oxazaphosphorines stimulated increases in CYP3A, CYP2C8, and CYP2C9 proteins that were detectable at 50 μM oxazaphosphorine inducer and were maximal at 250 μM. No increases or marginal increases were detected in the case of CYP2B6 (Fig. 6A). The enhanced CYP3A protein expression could also be monitored by the associated increase in CYP3A-catalyzed hepatocyte microsomal testosterone 6β-hydroxylase activity (Table 4). Interestingly, oxazaphosphorine induction of CYP3A protein and activity could be detected (albeit to a small extent) in hepatocyte culture FT59 (Fig. 6; Table 4), although in this case the increase did not lead to a major elevation of microsomal cyclophosphamide or ifosfamide 4-hydroxylase activity (Fig. 4A). Together, these findings demonstrate that cyclophosphamide and ifosfamide can enhance their own activation by inducing P-450 enzymes such as CYP2C8, CYP2C9, and CYP3A.

Differential Effect of Oxazaphosphorines on Hepatocyte Microsomal CYP3A4 and CYP3A5 Protein Levels. As shown in Fig. 5A, cyclophosphamide and ifosfamide induced an immunoreactive CYP3A protein that displayed an electrophoretic mobility indistinguishable from that of cDNA-expressed CYP3A4. In addition to CYP3A4, the polymorphically expressed CYP3A5 is found at significant levels in ~20–25% of adult human livers (49–51). We, therefore, investigated whether CYP3A5 is also subject to oxazaphosphorine induction. Fig. 6B shows that cyclophosphamide, ifosfamide, and rifampin each induced CYP3A4 (lower band of doublet in Lanes 4–8) but not CYP3A5 (upper band) in one culture of human hepatocytes (FT59) where both of these proteins were detected by immunoblot assay of uninduced cells.
Catalysts of these two drug activation reactions (4), whereas CYP2C using the same microsomal samples shown in A. Lanes 1—6. with the results expressed as to 192 h, as indicated. B, microsomal testosterone 6@3-hydroxylase activities determined hepatocyte microsomes probed with anti-rat CYP3A antibodies. Lane 1, vehicle control; hepatocytes (culture Ff69) were treated for 96 h with 0.1% DMSO (vehicle control) or I, rifampin. Human P450 enzymes CYP2B6, CYP2C8, CYP2C9, and CYP3A4. That these activity increases are associated with induction of the polymorphically expressed CYP3A5 may not respond to typical CYP3A inducers (Fig. 6; Ref. 44). The present study confirms previous findings that the antituberculosis drug rifampin elevates CYP3A protein and activity levels in primary cultures of human hepatocytes (39, 44, 46, 53). This is in agreement with the clinical observation that rifampin accelerates the elimination pharmacokinetics of drugs such as quinidine (54) and 17α-ethinylestradiol (55), which are metabolized by hepatic CYP3A (56, 57). Rifampin was presently found to be a relatively potent inducer of hepatocyte oxazaphosphorine 4-hydroxylase activity, as well as CYP3A protein and enzyme activity, with 1 μM rifampin stimulating near maximal increases in each of these activities (Figs. 2 and 3). Rifampin at 1 μM was also effective in enhancing CYP3A-mediated lidocaine N-deethylation activity (58) and CYP3A mRNA levels (59) in cultured human hepatocytes. Although the present studies identify rifampin as a particularly potent inducer of ifosfamide 4-hydroxylation and cyclophosphamide 4-hydroxylation in human liver cells, further studies, including a controlled clinical trial, will be needed to ascertain whether rifampin effects corresponding changes in the pharmacokinetics and pharmacodynamics of these alkylating agents and whether this translates into an enhanced therapeutic effect. The typical serum drug concentration achieved in patients after standard dosages of rifampin is 2—30 μM (60), which corresponds to the range of concentrations (1—50 μM) that enhance oxazaphosphorine activation in cultured human hepatocytes. In a previous study, prednisone was found to decrease the elimination half-life of cyclophosphamide in patients (20). This may be related to the fact that this corticosteroid increases CYP3A4 mRNA, protein, and activity levels (43).

CYP2B6 is the only CYP2B protein that has been isolated and purified from human liver (61, 62). The present study provides the first demonstration that an immunoreactive CYP2B protein, likely CYP2B6, is inducible by several xenobiotics, such as phenobarbital, rifampin, and dexamethasone in cultured human hepatocytes. That CYP2B6 is inducible in human cells is consistent with studies in animal models where one or more liver CYP2B proteins are subject to barbiturate induction (63). In the case of human hepatocytes, phenobarbital increased not only CYP2B6 but also CYP2C8, CYP2C9, and CYP3A. The previous finding that phenobarbital decreases the elimination half-life of cyclophosphamide in human subjects (19) may now be explained by the phenobarbital induction of both the high capacity (high Vmax) CYP2B6 (4) and the lower capacity CYP2C enzymes (6) that contribute to hepatic activation of this anticancer drug. From the present study, it appears likely that phenobarbital will also impact on the clinical pharmacokinetics of ifosfamide.

Treatment of primary human hepatocyte cultures with rifampin or phenobarbital was previously shown to increase CYP2C mRNA and protein levels (53). Using SDS-PAGE conditions that can resolve CYP2C8 from CYP2C9 and using polyclonal anti-rat CYP2C antibodies that cross-react with both of these human CYP2C forms, we presently demonstrate that rifampin, phenobarbital, and dexamethasone elevate both CYP2C8 and CYP2C9 in human hepatocytes. That CYP2C9 is inducible in these liver cells is consistent with the clinical finding that rifampin enhances the total body clearance of tolbutamide.

DISCUSSION

Clinical pharmacokinetic studies have suggested that hepatic clearance of cyclophosphamide in cancer patients can be increased by concurrent administration of drugs such as phenobarbital (19), prednisone (20), and dexamethasone (15), which are inducers of human P-450 (46). The present study shows that cyclophosphamide 4-hydroxylation and ifosfamide 4-hydroxylation are both increased in cultured human hepatocytes treated with select P450 inducers, and that these activity increases are associated with induction of the human P-450 enzymes CYP2B6, CYP2C8, CYP2C9, and CYP3A4. Phenobarbital and rifampin, which induced all four P-450 proteins in these cells, increased cyclophosphamide and ifosfamide activation to a greater extent at 2 μM than at 0.25 μM substrate concentration. This is consistent with our finding that CYP2B6 and CYP3A4 are high Km catalysts of these two drug activation reactions (4), whereas CYP2C forms such as the wild-type CYP2C9-11e359 allele are lower Km oxazaphosphorine 4-hydroxylases (6). We have observed previously that in human liver, CYP2B6 preferentially contributes to cyclophosphamide 4-hydroxylation, whereas CYP3A4 is a major catalyst of ifosfamide 4-hydroxylation (4). In several hepatocyte cultures, phenobarbital and rifampin enhanced ifosfamide 4-hydroxylation to a greater extent than cyclophosphamide 4-hydroxylation (Tables 2 and 3; Fig. 4). This suggests that although CYP2B6 and CYP3A are increased by both P-450 inducers, the CYP3A induction may dominate the metabolic profile with these anticancer drug substrates. In contrast to phenobarbital and rifampin, the CYP1A inducers β-naphthoflavone and TCDD did not affect cyclophosphamide 4-hydroxylation or ifosfamide 4-hydroxylation activities in cultured human hepatocytes. This is in accord with the finding that cDNA-expressed CYP1A1 and CYP1A2 do not catalyze these reactions (4). Therefore, tobacco smoke, which induces CYP1A, is unlikely to influence cyclophosphamide and ifosfamide activation in humans.

At least two P-450s belonging to the CYP3A subfamily may be expressed in adult human liver (52). Whereas CYP3A4 is inducible, the polymorphically expressed CYP3A5 may not respond to typical CYP3A inducers (Fig. 6; Ref. 44). The present study confirms previous findings that the antituberculosis drug rifampin elevates CYP3A protein and activity levels in primary cultures of human hepatocytes (39, 44, 46, 53). This is in agreement with the clinical observation that rifampin accelerates the elimination pharmacokinetics of drugs such as quinidine (54) and 17α-ethinylestradiol (55), which are metabolized by hepatic CYP3A (56, 57). Rifampin was presently found to be a relatively potent inducer of hepatocyte oxazaphosphorine 4-hydroxylase activity, as well as CYP3A protein and enzyme activity, with 1 μM rifampin stimulating near maximal increases in each of these activities (Figs. 2 and 3). Rifampin at 1 μM was also effective in enhancing CYP3A-mediated lidocaine N-deethylation activity (58) and CYP3A mRNA levels (59) in cultured human hepatocytes. Although the present studies identify rifampin as a particularly potent inducer of ifosfamide 4-hydroxylation and cyclophosphamide 4-hydroxylation in human liver cells, further studies, including a controlled clinical trial, will be needed to ascertain whether rifampin effects corresponding changes in the pharmacokinetics and pharmacodynamics of these alkylating agents and whether this translates into an enhanced therapeutic effect. The typical serum drug concentration achieved in patients after standard dosages of rifampin is 2—30 μM (60), which corresponds to the range of concentrations (1—50 μM) that enhance oxazaphosphorine activation in cultured human hepatocytes. In a previous study, prednisone was found to decrease the elimination half-life of cyclophosphamide in patients (20). This may be related to the fact that this corticosteroid increases CYP3A4 mRNA, protein, and activity levels (43).

CYP2B6 is the only CYP2B protein that has been isolated and purified from human liver (61, 62). The present study provides the first demonstration that an immunoreactive CYP2B protein, likely CYP2B6, is inducible by several xenobiotics, such as phenobarbital, rifampin, and dexamethasone in cultured human hepatocytes. That CYP2B6 is inducible in human cells is consistent with studies in animal models where one or more liver CYP2B proteins are subject to barbiturate induction (63). In the case of human hepatocytes, phenobarbital increased not only CYP2B6 but also CYP2C8, CYP2C9, and CYP3A. The previous finding that phenobarbital decreases the elimination half-life of cyclophosphamide in human subjects (19) may now be explained by the phenobarbital induction of both the high capacity (high Vmax) CYP2B6 (4) and the lower capacity CYP2C enzymes (6) that contribute to hepatic activation of this anticancer drug. From the present study, it appears likely that phenobarbital will also impact on the clinical pharmacokinetics of ifosfamide.

Treatment of primary human hepatocyte cultures with rifampin or phenobarbital was previously shown to increase CYP2C mRNA and protein levels (53). Using SDS-PAGE conditions that can resolve CYP2C8 from CYP2C9 and using polyclonal anti-rat CYP2C antibodies that cross-react with both of these human CYP2C forms, we presently demonstrate that rifampin, phenobarbital, and dexamethasone elevate both CYP2C8 and CYP2C9 in human hepatocytes. That CYP2C9 is inducible in these liver cells is consistent with the clinical finding that rifampin enhances the total body clearance of tolbutamide.
INDUCTION OF OXAZAPHOSPHORINE ACTIVATION

Fig. 4. Enhanced cyclophosphamide and ifosfamide activation in human hepatocytes treated with cyclophosphamide or ifosfamide. Four independent human hepatocyte cultures were treated for 96 h with 0.1% DMSO (vehicle control), cyclophosphamide (CPA; 50 μM, A, C, and D; 250 μM, B), ifosfamide (IFA; 50 μM, A, C, and D; 250 μM, B), or rifampin (RIF; 25 μM, A and B; 1 μM, C and D). Microsomal cyclophosphamide 4-hydroxylation and ifosfamide 4-hydroxylation activities were determined at 2 mM substrate as described in "Materials and Methods." Results are expressed as the percentage of increase in enzyme activity above the DMSO control (means; bars, one-half the range for duplicate determinations). Control (uninduced) enzyme activities were 0.60, 0.49, 1.40, and 0.31 nmol 4-hydroxycyclophosphamide/min/mg and 0.56, 0.47, 1.36, and 0.46 nmol 4-hydroxyifosfamide/min/mg for hepatocyte cultures FT59, FT60, FH105, and FT108, respectively.

(64) and warfarin (65), both of which are metabolized to a large extent by CYP2C9 (66–68).

Repeated administration of cyclophosphamide (27, 28) and ifosfamide (16, 26) can shorten the plasma half-life and increase the total body clearance of the parent oxazaphosphorine. Consequently, it has been suggested that these alkylating agents undergo autoinduction (17, 29, 47, 69). In principle, autoinduction of cyclophosphamide and ifosfamide biotransformation could occur via enhanced 4-hydroxylation (activation) and/or N-dechloroethylation (inactivation). Clinical pharmacokinetic studies have shown that fractionated ifosfamide therapy is associated with increased formation of the N-dechloroethylated metabolites (17, 70, 71). In patients administered cyclophosphamide by i.v. infusion on each of two consecutive days, the AUC for the parent drug is lower on the second day than on the first day, but the AUCs for the metabolites 4-hydroxycyclophosphamide and phosphoramide mustard are not altered (13). A potential explanation for the lack of a change in the AUC of the activated metabolites is that repeated oxazaphosphorine administration induces not only the microsomal P-450s that catalyze the 4-hydroxylation reaction but also the cytosolic aldehyde dehydrogenase enzymes that oxidize aldophosphamide to the carboxy metabolite and thereby inactivate it. Consistent with this proposal, repeated administration of ifosfamide is associated with elevated urinary levels of carboxyifosfamide (70, 71). In the present study, we used microsomes isolated from primary human hepatocytes treated with cyclophosphamide or ifosfamide to demonstrate directly that autoinduction of oxazaphosphorine biotransformation involves enhanced 4-hydroxylation. It has been suggested that induction of P-450 enzymes may not impact on the therapeutic efficacy of cyclophosphamide because of the unaltered AUCs for 4-hydroxycyclophosphamide and phosphoramid mustard (13), which is thought to be the therapeutically active metabolite (3). However, induction of P450 enzymes such as CYP3A could potentially impact...

Fig. 5. Effect of cyclophosphamide and ifosfamide on immunoreactive CYP2C8, CYP3A, and CYP3A protein levels in microsomes from cultured human hepatocytes. Primary cultures of human hepatocytes (culture FT108; Lanes 2–9) were treated for 120 h with 0.1% DMSO (vehicle control), cyclophosphamide (CPA), or ifosfamide (IFA) at 50, 250, or 1000 μM, as indicated above each lane. Rifampin treatment (20 μM for 96 h) was included as a positive control in Lane 6. Shown is an immunoblot of isolated hepatocyte microsomes probed with anti-rat CYP3A antibodies (A) and anti-rat CYP2C antibodies (B). Lane 1, human liver microsomes HLS9. Lane 2, vehicle control. Lanes 3–9, induction with CPA, IFA, or RIF at the concentrations indicated. Lanes 10–12, cDNA-expressed CYP3A4, CYP2B6, and CYP2C9-Ile™ standards.

A. 
HL9 UT CPA 50 CPA 250 CPA 1000 RIF 20 IFA 50 IFA 250 IFA 1000 CYP 2A4 CYP 2B6 CYP 2C8
CYP 3A

B. 
CYP 2C9
CYP 2C8 2C6 3A 4A 5A 6A 7A 8A 9A 10A 11A 12A

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on the host toxicity of ifosfamide because this compound is extensively metabolized by N-dechloroethylation (7–9), which is at least in part catalyzed by CYP3A enzymes in human liver (5) and leads to the production of neurotoxic metabolite(s) (11, 12). Recent studies indicate, however, that the subset of P-450 enzymes that catalyzes oxazaphosphorine N-dechloroethylation is distinct from that which catalyzes drug activation via 4-hydroxylation, suggesting possible strategies for improving the balance between these two competing metabolic pathways based on the use of P-450 inducers in combination with P-450 form-selective inhibitors (10). The present identification of several effective inducers of oxazaphosphorine activation in human liver cells is an important first step leading to testing of the therapeutic utility of such strategies.

The present study establishes that cyclophosphamide and ifosfamide are modulators of human P-450 expression and have the potential to induce P-450 enzymes such as CYP2C8, CYP2C9, and CYP3A4. That cyclophosphamide induces CYP3A4 in primary cultures of human hepatocytes is consistent with the finding in bone marrow transplant patients that this drug increases total body clearance of dexamethasone (27), which is metabolized by CYP3A in human liver (72). Although cyclophosphamide and ifosfamide were both effective in inducing CYP3A4 protein in cultured human hepatocytes, the present study showed that these drugs are ineffective in modulating the protein levels of the polymorphically expressed CYP3A5. However, these alkylating anticancer agents might affect CYP3A5 mRNA expression because in the case of another CYP3A, rifampin increases the levels of CYP3A7 mRNA but not the corresponding protein in cultured human hepatocytes (73). The lack of an effect of the oxazaphosphorines on CYP3A5 protein levels is consistent with previous findings that the expression of this P-450 is not subject to modulation by known CYP3A4 inducers in cultured human hepatocytes (44) or in vivo (51). Our observation that oxazaphosphorine anticancer drugs are inducers of human P-450 in cultured liver cells suggests the potential for a pharmacokinetic drug interaction in patients given combination cancer chemotherapy regimens that include other drugs subject to CYP2C8, CYP2C9, or CYP3A metabolism. Future clinical studies will be required to investigate this possibility.

In summary, the treatment of primary cultures of human hepatocytes with rifampin, phenobarbital, or dexamethasone, which induced CYP2B, CYP2C, and CYP3A, increased cyclophosphamide 4-hydroxylation and ifosfamide 4-hydroxylation. These two oxazaphosphorines enhanced their own activation by inducing P-450 enzymes such as CYP2C8, CYP2C9, and CYP3A4.

**ACKNOWLEDGMENTS**

We thank Dr. J. Pohl (ASTA Pharma, Bielefeld, Germany) for kindly providing authentic 4-hydroxyifosfamide metabolite standard and Dr. Lydiane Pichard for providing some of the primary hepatocyte microsomes used in this study.

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