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ACTIVATION OF THE ANTI-CANCER DRUG IFOSPHAMIDE BY RAT LIVER MICROSOMAL P450 ENZYMES

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Abstract—The NADPH-dependent metabolism of ifosphamide catalyzed by rat liver microsomes was investigated in order to identify individual P450 enzymes that activate this anti-cancer drug and to ascertain their relationship to the P450 enzymes that activate the isomeric drug cyclophosphamide. Pretreatment of rats with phenobarbital or clofibrate increased by up to 8-fold the activation of both ifosphamide and cyclophosphamide catalyzed by isolated liver microsomes. Studies using P450 form-selective inhibitory antibodies demonstrated that constitutively expressed P450s belonging to subfamily 2C (forms 2C11/2C6) make significant contributions to the activation of both oxazaphosphorines in uninduced male rat liver microsomes, while the phenobarbital-inducible P450 2B1 was shown to be a major catalyst of these activations in phenobarbital-induced microsomes. Pretreatment of rats with dexamethasone increased liver microsomal activation of ifosphamide ~6-fold without a corresponding effect on cyclophosphamide activation rates. Ifosphamide activation catalyzed by dexamethasone-induced liver microsomes was minimally inhibited by anti-P450 2B or anti-P450 2C antibodies, but was selectively inhibited by anti-P450 3A antibodies. Selective inhibition of liver microsomal ifosphamide activation was also effected by the macrolide antibiotic triacetyloleandomycin, an inhibitor of several dexamethasone-inducible 3A P450s. These studies establish that a dexamethasone-inducible family 3A P450 can make an important contribution to rat liver microsomal ifosphamide activation, and suggest that dexamethasone pretreatment might provide a useful approach for modulation of ifosphamide metabolism in order to improve its therapeutic efficacy in cancer patients.

Ifosphamide and cyclophosphamide are isomeric prodrug alkylating agents that undergo metabolic activation catalyzed by the hepatic cytochrome P450 (P450)†-linked monooxygenase system. P450-catalyzed hydroxylation at C4 initiates the metabolic pathway, which ultimately results in fragmentation of the oxazaphosphorines to yield 2-propenal (acrolein) and an electrophilic phosphoramide mustard metabolite (Fig. 1). While acrolein can exhibit cytotoxic effects by binding covalently to proteins, including cytochrome P450 [1], and can contribute to the hemorrhagic cystitis associated with oxazaphosphorine therapy [2], the phosphoramide mustards possess significant DNA alkylating activity and are believed to be the therapeutically significant, cytotoxic metabolites of these drugs [3]. Other pathways for metabolism of ifosphamide and cyclophosphamide include aldehyde dehydrogenase-catalyzed oxidation of the primary P450-generated 4-hydroxy/aldophosphamide metabolite, which can confer drug resistance [2, 4], and N-dechloroethylation of the parent drugs to yield inactive, monofunctional (non-DNA cross-linking) metabolites that are therapeutically inactive. The N-dechloroethylation pathway is quantitatively minor for cyclophosphamide, but is major in the case of ifosphamide, where it can consume an estimated 50% of the administered dose, and is associated with production of the neurotoxic metabolite chloroacetaldehyde [5].

Although cyclophosphamide and ifosphamide are closely related in structure and in their overall pathways of metabolism, ifosphamide is finding increased use as an active agent with its own characteristic pharmacological and toxicological properties [e.g. Ref. 6]. Ifosphamide produces less myelosuppression than cyclophosphamide and also exhibits little cross-resistance [7]. While some of these distinctive properties of ifosphamide may relate to the longer DNA cross-links that it induces (7 atom cross-links vs 5 atom cross-links for cyclophosphamide), the distinct pharmacokinetic properties of the two drugs are also a contributing factor. In the case of cyclophosphamide, hepatic activation is not limiting over a wide range of drug doses and activation rates, as demonstrated in rodent model studies and in the clinic [2, 8, 9]. In contrast, the activation of ifosphamide is dose-dependent and saturable at doses that are readily achieved clinically [10]. This not only contributes to the higher fractional metabolism via the non-productive N-dechloroethylation pathway, but also to the metabolism-limiting nature of ifosphamide that has been observed at standard clinical doses, and which may be of even greater importance at the substantially higher drug doses used in conjunction with autologous bone marrow transplantation protocols. Whereas modulation of the overall rate of liver cyclo-

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† Abbreviations: P450, cytochrome P450; and TAO, triacetyloleandomycin.

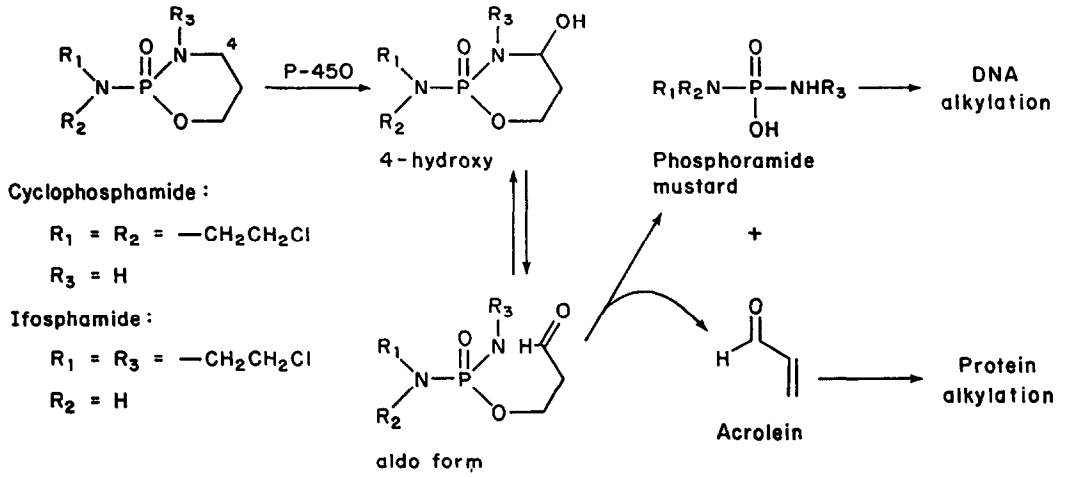

Fig. 1. P450-catalyzed metabolism of ifosphamide and cyclophosphamide via the 4-hydroxylation pathway.

phosphamide activation has little impact on therapeutic efficacy [2, 8], the above studies indicate that increased hepatic metabolism of ifosphamide may be a therapeutically beneficial objective.

The major goal of the present study was to use a rat model to determine the roles played by individual liver microsomal P450 enzymes in ifosphamide activation and to identify ways by which the expression of these P450s, and consequently hepatic ifosphamide activation rates, can be increased. Our findings lead us to conclude that ifosphamide activation in rat liver microsomes is catalyzed by P450 forms‡ 2B1, 2C6/2C11, as well as by a dexamethasone-inducible subfamily 3A P450 that does not contribute significantly to microsomal cyclophosphamide activation.

MATERIALS AND METHODS

Materials. Ifosphamide was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). 4-Hydroperoxy-ifosphamide was a gift from Asta-Werke, Inc. (Bielefeld, Germany). Cyclophosphamide, NADPH, bovine serum albumin (Sigma), 3-aminophenol (recrystallized before use by dissolving in distilled water, cooling, filtration, and drying over phosphorous pentoxide at 50°), hydroxylamine hydrochloride, semicarbazide hydrochloride (Aldrich), zinc sulfate, barium hydroxide (Mallinckrodt Chemical Works), Hydrofluor (National Diagnostics), and triacetyloleandomycin (TAO) (Pfizer, Inc.) were obtained from the sources indicated. Buffers were filtered through 0.45 μm microfilters and stored at 4°. All other solutions were prepared fresh daily. Fluorescence was measured using a Perkin–Elmer model MPF-4 spectrofluorometer.

‡ Individual liver cytochrome P450 (CYP) forms are designated according to the systematic nomenclature of Nebert *et al.* [11].

Liver microsomes and P450 enzymes. Adult male and female Fischer 344 rats, 8–10 weeks of age (Taconic Farms; Germantown, NY), were untreated, or were treated with various drugs or chemicals to induce the expression of individual hepatic P450 enzymes [12]. Rat liver microsomes were prepared by calcium precipitation as summarized elsewhere [13, 14]. Protein concentrations were determined with the Bio-Rad protein assay reagent using bovine serum albumin as a standard.

Purified rat liver P450 form 2B1 (P450 PB-4; [15]), 10 pmol hemeprotein, was reconstituted with purified rabbit liver NADPH cytochrome P450 reductase (0.7 U) and 2 μg of freshly sonicated dilauroyl phosphatidylcholine for 15 min at 20° to 22° [15]. Analysis of NADPH-dependent metabolism of ifosphamide and cyclophosphamide was assayed as described below.

Fluorescence assay for acrolein formation. Previous studies of cyclophosphamide activation reported by this laboratory employed ^{14}C-labeled drug and monitored covalent binding of the resultant [^{14}C]-acrolein to bovine serum albumin as an index of drug activation [12]. Since ^{14}C-labeled ifosphamide is not available commercially, activation of ifosphamide and cyclophosphamide was determined in the present study by using a fluorimetric assay that measures acrolein after derivatization with 3-aminophenol according to Alarcon [16] as modified [17]. These assays were carried out at 2 mM oxazaphosphorine substrate in order to increase the sensitivity of metabolite detection. In preliminary experiments, microsomal acrolein formation measured by this assay was shown to be both NADPH- and O_2-dependent (90% inhibition in reactions carried out under the atmosphere of N_2), consistent with the cytochrome P450-dependent nature of the reaction.

Standard assay mixtures included 2 mM ifosphamide or 2 mM cyclophosphamide in a final volume of 0.2 mL containing 0.1 M potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, 20% (v/v)

glycerol, 5 mM semicarbazide hydrochloride, and 0.5 mg microsomal protein/mL. Somewhat lower background values could be obtained by eliminating glycerol from the assay mix. Semicarbazide was included to stabilize the initial 4-hydroxy metabolite as a semicarbazone prior to chemical liberation and derivatization of acrolein. Samples were preincubated at 37° for 4 min and the monooxygenase reactions then started by addition of NADPH (final concentration, 1 mM). Incubations were continued at 37° for an additional 60–90 min with continuous shaking to provide aeration. These assay conditions were found to be within the linear range with respect to protein concentration and incubation time. Reactions were stopped by precipitation of the protein with 80 μL of 5.5% (w/v) zinc sulfate, 80 μL of saturated barium hydroxide and 40 μL of 0.01 M hydrochloric acid. The precipitate was removed by centrifugation, and 300 μL of the supernatant was added to 160 μL of a solution containing 6 mg of 3-aminophenol and 6 mg of hydroxylamine hydrochloride dissolved in 1 mL of 1 M hydrochloric acid. The samples were heated at 100° for 20 min and then allowed to cool to room temperature in the dark before reading the fluorescence (excitation at 350 nm, emission at 515 nm). Standard curves for acrolein were generated by incubating 4-hydroxyperoxy-ifosphamide (0 to 0.4 mM) with liver microsomal protein under the same assay conditions but in the absence of NADPH. The acrolein precursor 4-hydroxyperoxy-ifosphamide was used for the standard curve in place of acrolein itself, as recommended by Sladek *et al.* [18].

Antibody inhibition of microsomal ifosphamide and cyclophosphamide activation. Complete reaction mixtures (minus NADPH) were preincubated with the indicated amounts of each antibody for 30 min at room temperature. NADPH was then added to initiate P450-dependent microsomal metabolism, followed by a determination of acrolein formation as described above. Mouse monoclonal anti-P450 antibodies of defined P450 form-specificity [19] were those used previously [12] and were provided by Dr. H. V. Gelboin, National Cancer Institute. Monoclonal antibody B3 (clone 4-7-1) is inhibitory toward rat P450s 2B1, 2B2 and 2A1; similar results were obtained with monoclonal antibody B4 (clone 4-29-5), which is selectively inhibitory toward rat P450 2B1 and to the closely related P450 2B2. Monoclonal antibody D1 (clone 1-68-11) is inhibitory toward P450s 2C6 and 2C11. Polyclonal rabbit anti-rat P450 antibodies were prepared and their specificities characterized as outlined elsewhere [20]. Purified monoclonal and polyclonal Ig fractions were prepared according to the procedures described in Ref. 21 for use in the P450 inhibition experiments. Non-specific monoclonal antibody IgG (anti-lysozome; clone HyHel-9) or non-specific rabbit IgG was used in control incubations carried out in parallel.

TAO and metyrapone inhibition experiments. TAO dissolved in dimethyl sulfoxide (5 μL) was added to complete assay mixtures *minus* ifosphamide and cyclophosphamide to give the final concentrations indicated in the text. Samples were incubated at 37° for 30 min to allow for TAO metabolism, following which ifosphamide or cyclophosphamide was added to a 2 mM concentration and acrolein formation determined as described above. The presence of dimethyl sulfoxide at 2.5% (v/v) decreased microsomal acrolein formation activity by ~30%, while the 30-min preincubation in the presence of this solvent reduced activity a further ~20% relative to non-incubated controls. Metyrapone inhibition experiments were carried out by including metyrapone in standard incubations to give final concentrations as indicated in the text.

Enzyme kinetics. Kinetic constants for microsomal enzymes were calculated by least squares analysis of Lineweaver–Burk and Eadie–Hoffstee plots and are based on the acrolein formation assay described above. Substrate concentrations were varied from 0.5 to 4 mM for rat liver microsomes and from 0.05 to 1 mM for purified cytochrome P450 2B1. K_m and V_{max} values were calculated for each individual experiment and the results presented as means ± range or SD for two to four independent experiments. Given the non-enzymatic nature of the conversion of 4-hydroxy-cyclophosphamide to acrolein [2, 3], the kinetic values obtained by these methods are indicative of the initial 4-hydroxylation reaction.

RESULTS

Influence of P450 inducers on liver microsomal activation of ifosphamide. Adult male and female rats were treated with drugs and chemicals known to elevate liver microsomal levels of P450 enzymes belonging to five different classes: β-naphthoflavone (inducer of P450 subfamily 1A), phenobarbital (inducer of P450 subfamily 2B), isoniazid (P450 2E inducer), dexamethasone (P450 3A inducer), and clofibrate (P450 4A and P450 2B inducer [12]). The effects of these P450-inducing agents on hepatic microsomal activation of ifosphamide were then assessed. β-Naphthoflavone and isoniazid had little or no effect on the rates of microsomal activation of ifosphamide or cyclophosphamide (Fig. 2), indicating that the β-naphthoflavone-inducible P450 1A and the isoniazid-inducible P450 2E activity are unlikely to make significant contributions to the activation of either oxazaphosphorine. In contrast, *in vivo* pretreatment with either phenobarbital or clofibrate, which both induce P450 2B1, led to a significant increase (up to 8-fold) in the activation of ifosphamide catalyzed by both male (Fig. 2A) and female (data not shown) rat liver microsomes. Microsomal cyclophosphamide activation, which proceeded at a higher rate than microsomal ifosphamide activation (Fig. 2B), was also induced by phenobarbital and clofibrate. In contrast, dexamethasone markedly increased hepatic microsomal ifosphamide activation, but not cyclophosphamide activation, in both male and female rats (5- to 6-fold increase) (Fig. 2 and data not shown). This suggests that one or more dexamethasone-inducible P450s, e.g. P450 3A1 [22], may contribute significantly to liver microsomal activation of ifosphamide but not cyclophosphamide.

Steady-state kinetic analysis. Apparent K_m and V_{max} values were determined for activation of the

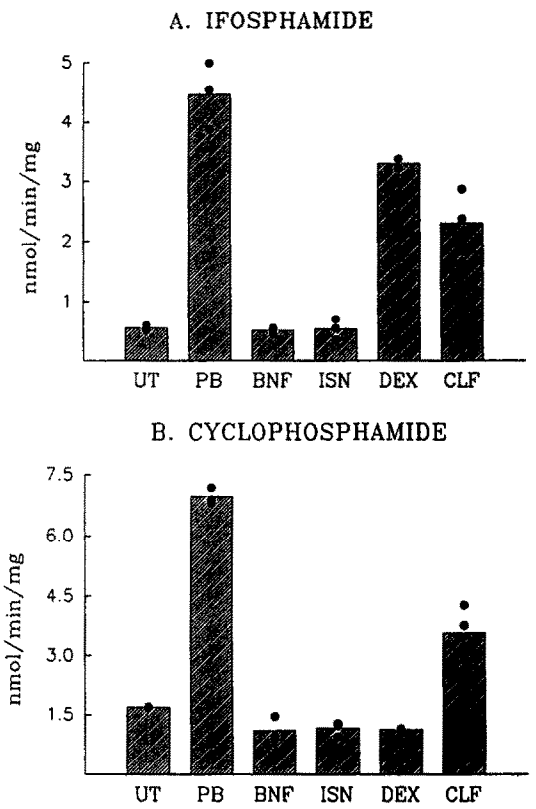

Fig. 2. Influence of monooxygenase inducers on ifosphamide (A) and cyclophosphamide (B) activation catalyzed by isolated rat liver microsomes. Adult male rats were untreated (UT) or were treated with phenobarbital (PB), β-naphthoflavone (BNF), isoniazid (ISN), dexamethasone (DEX) or clofibrate (CLF). Isolated liver microsomes were then assayed for metabolism of ifosphamide and cyclophosphamide as described under Materials and Methods. Activities determined for liver microsomes isolated from individual rats (N = 3/group) are shown as filled circles. Shaded bar = mean activity of the group. Comparable effects of these monooxygenase inducers were observed in experiments carried out in adult female rats (not shown).

two oxazaphosphorines by uninduced and drug-induced liver microsomes (Table 1). V_{max} values for cyclophosphamide activation were 1.5- to 2-fold higher than V_{max} values for ifosphamide activation in both uninduced and phenobarbital-induced male rat liver microsomes. In the dexamethasone-induced microsomes, however, ifosphamide was activated with a V_{max} approximately twice that of cyclophosphamide. Phenobarbital increased the V_{max} and decreased the K_m for both cyclophosphamide and ifosphamide (relative to the uninduced microsomes), while dexamethasone had these effects on ifosphamide but not on cyclophosphamide activation. Indeed, dexamethasone actually decreased the V_{max} for cyclophosphamide activation relative to that of the uninduced liver microsomes in male (Table 1) but not in female liver microsomes (data not shown). This may result from the associated suppression of the male-specific cyclophosphamide 4-hydroxylase P450 2C11 [12] in dexamethasone-pretreated microsomes (unpublished experiments). Ifosphamide and cyclophosphamide exhibited similar K_m values in each of the male rat liver microsomes studied. In uninduced female liver microsomes, however, K_m values were ~2-fold lower for ifosphamide as compared to cyclophosphamide. V_{max} values for both substrates were 2- to 3-fold lower for uninduced female liver microsomes as compared to uninduced male liver microsomes. This probably reflects the absence of the male-specific cyclophosphamide 4-hydroxylase P450 2C11 in the female liver microsomes.

Enzymatic analysis of purified, reconstituted P450 2B1 established that this liver P450 is able to activate ifosphamide as well as cyclophosphamide (Table 1). K_m values for ifosphamide were somewhat higher and the V_{max} values somewhat lower than those of cyclophosphamide, giving a 4-fold lower overall catalytic efficiency (V_{max}/K_m) for ifosphamide. Analogous differences in K_m and V_{max} values were observed when ifosphamide activation was compared to cyclophosphamide activation by phenobarbital-induced microsomes (Table 1), to which 2B1 makes a significant contribution (see antibody inhibition experiments, below).

Contributions of individual P450 forms to the microsomal activation of ifosphamide. Two approaches were taken to specify which individual P450 forms contribute to microsomal ifosphamide activation: use of inhibitory anti-P450 antibodies and application of diagnostic chemical inhibitors.

Figure 3 shows the influence of several inhibitory anti-P450 antibodies on ifosphamide and cyclophosphamide activation catalyzed by either uninduced liver microsomes (panel A) or liver microsomes isolated from rats pretreated with phenobarbital (panel B) or dexamethasone (panels C and D). In the uninduced male liver microsomes, 70% of microsomal cyclophosphamide activation, but only 35% of microsomal ifosphamide activation was inhibited by a monoclonal antibody reactive with P450 forms 2C6 and 2C11; monoclonal antibody reactive with P450 2B1 had no effect (Fig. 3A). The residual, uninhibited activity was similar for both substrates (approx. 0.8 to 1 nmol acrolein formed/min/mg). In contrast, in phenobarbital-induced male liver microsomes, cyclophosphamide activation was inhibited by 65% and ifosphamide activation by approximately 50% by a monoclonal antibody that is inhibitory toward the phenobarbital-inducible P450 form 2B1* (Fig. 3B). The residual activity in these phenobarbital-induced microsomes, however, was not inhibitable by the anti-2C6/2C11 antibody. This finding demonstrates that the constitutive P450s 2C11 and 2C6 together make a relatively small contribution to the activation of the oxazaphosphorines in phenobarbital-induced liver microsomes, and is consistent with the suppression of liver microsomal P450 2C11 by phenobarbital treatment

* These experiments do not distinguish between rat P450s 2B1 and P450 2B2, which are 97% similar in amino acid sequence, are fully cross-reactive with the available antibodies, and exhibit similar substrate specificity patterns.

Table 1. Kinetic data for the activation of ifosphamide and cyclophosphamide by rat liver microsomes

Liver microsomes	Ifosphamide			Cyclophosphamide		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
Untreated female	1.72 ± 0.22	1.61 ± 0.18	0.94	3.74 ± 0.37	1.78 ± 0.16	0.48
Untreated male	2.40 ± 0.23	3.04 ± 0.26	1.27	2.93 ± 0.32	6.01 ± 0.66	2.1
+ Phenobarbital	0.86 ± 0.19	8.1 ± 1.3	9.4	0.72 ± 0.11	12.3 ± 2.0	17.1
+ Dexamethasone	0.93 ± 0.15	5.55 ± 0.05	5.9	1.24 ± 0.11	2.46 ± 0.05	2.0
P450 2B1*	0.48 ± 0.02	17.8 ± 0.1	37	0.30 ± 0.03	39.1 ± 6.2	130

K_m values are in mM and V_{max} values in nmol acrolein formed/min/mg microsomal protein, except as noted. Substrate concentrations were varied between 0.5 and 4 mM for the liver microsomes and between 0.05 and 1 mM for purified P450 2B1. Values are means ± SD values for N = 3–4 independent determinations, except as noted.

* V_{max} values are expressed as nmol acrolein/min/nmol purified P450 2B1. Data for P450 2B1 are expressed as mean ± range for N = 2 determinations.

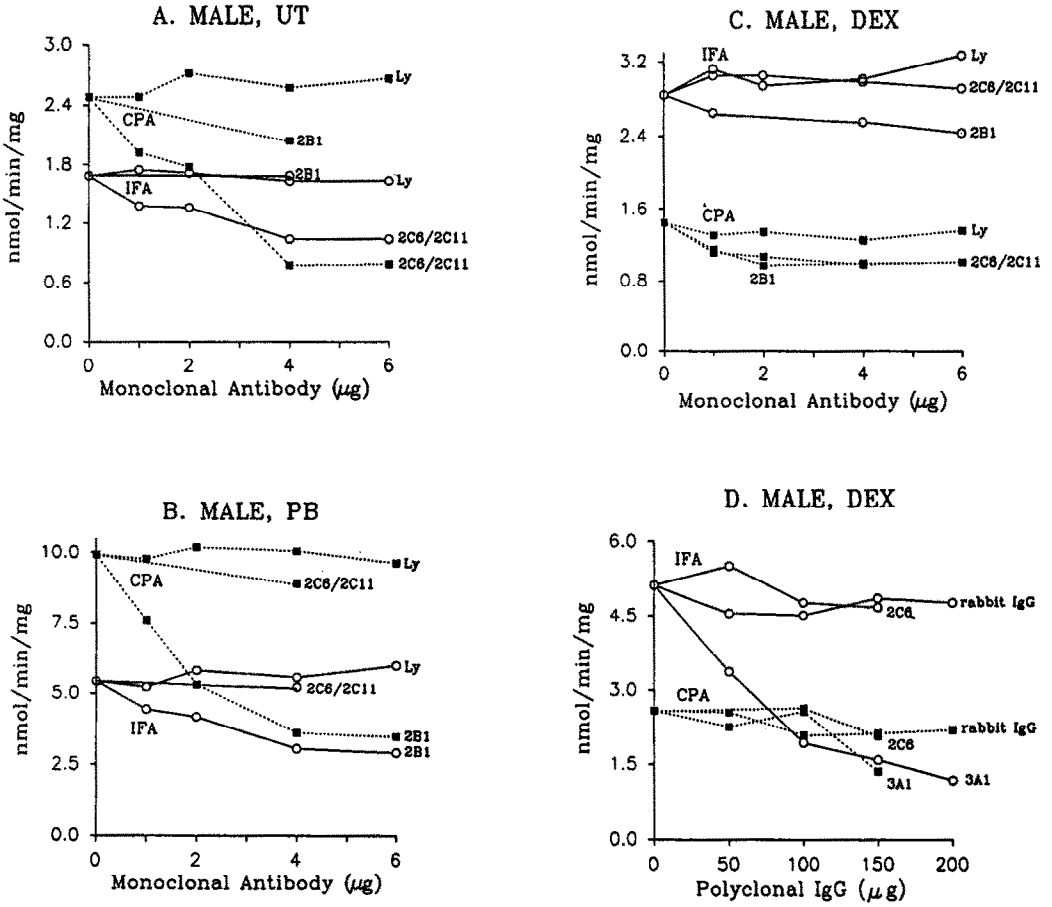

Fig. 3. Influence of anti-rat P450 antibodies on liver microsomal metabolism of ifosphamide (IFA) (solid lines, open circles) and cyclophosphamide (CPA) (dashed lines, closed squares). The antibody titration experiments shown were carried out as described under Materials and Methods using adult male liver microsomes isolated from uninduced rats (A), or from rats induced with phenobarbital (B) or dexamethasone (C and D). Purified monoclonal antibodies reactive with P450s 2B1 (MAb B3) and P450s 2C6 + 2C11 (MAb D1) were used at the indicated amounts per 50 μg microsomes/0.2 mL assay in the experiments shown in panels A–C. Ly, anti-lysozyme antibody, was used as a control for the effects of non-specific immunoglobulin. Polyclonal antibodies to P450s 2C6 and 3A1 (purified IgGs at the indicated amounts of IgG per 50 μg microsomes/0.2-mL assay) were used for the titrations shown in panel D.

[20]. Since the antibodies used in the above experiments are inherently inhibitory towards microsomal P450 activities by at least 85–90% [19], the residual, uninhibited activities are probably catalyzed by immunochemically distinct microsomal P450 enzymes.

In contrast to the above findings, anti-2C6/2C11 and anti-2B1 antibodies had only a small effect on ifosphamide and cyclophosphamide activation catalyzed by dexamethasone-induced liver microsomes (≤15–25% inhibition) (Fig. 3C). These data, together with the experiments reported in Fig. 1, indicate that ifosphamide activation can be catalyzed by P450 enzymes that are dexamethasone-inducible and are distinct from those that metabolize this drug in uninduced and phenobarbital-induced liver microsomes. The possible role of dexamethasone-inducible 3A P450s was therefore probed using a polyclonal antibody against P450 3A1, which in our earlier experiments has been shown to be inhibitory toward microsomal steroid 6β-hydroxylation reactions catalyzed by several family 3A P450s [23]. Anti-P450 3A IgG inhibited in a concentration-dependent manner the activation of ifosphamide catalyzed by the dexamethasone-induced microsomes, but did not inhibit microsomal activation of cyclophosphamide (Fig. 3D). This indicates that a dexamethasone-inducible subfamily 3A P450, perhaps P450 3A1, makes an important contribution to the dexamethasone-inducible ifosphamide activation activity.

The macrolide antibiotic TAO is a selective inhibitor of rat and human 3A P450s, and can be activated via P450 3A-catalyzed metabolism to yield more potent inhibitory products [23–25]. TAO was therefore employed as a probe for P450 3A-catalyzed microsomal metabolism of ifosphamide and cyclophosphamide. TAO was first incubated with microsomes in the presence of NADPH for 30 min at 37° to allow for its metabolic activation. Subsequently, ifosphamide was added to give a final concentration of 2 mM and the incubation was continued for an additional 90 min. Figure 4 shows that TAO selectively inhibited ifosphamide activation catalyzed by both male and female dexamethasone-induced rat liver microsomes. No inhibition of ifosphamide activation was observed in uninduced, phenobarbital-induced or clofibrate-induced microsomes (Fig. 4 and data not shown). Moreover, TAO did not inhibit cyclophosphamide activation in any of the microsomes examined, including those induced with dexamethasone. Thus, TAO-inhibitable subfamily 3A P450s make an important contribution to ifosphamide activation catalyzed by dexamethasone-inducible liver microsomes, in agreement with the antibody inhibition experiments shown in Fig. 3D.

Metyrapone is a dipyridyl heme ligand that at low concentrations (≤ 10 μM) preferentially (although not exclusively) inhibits P450 2B1, while at higher concentrations (≥ 250 μM) can, in some instances, stimulate P450 2C6-catalyzed enzyme activities [26]. We therefore examined the effects of metyrapone on microsomal ifosphamide activation (Fig. 5). Low concentrations of metyrapone (10 μM) substantially inhibited both ifosphamide and cyclophosphamide metabolism catalyzed by phenobarbital-induced liver microsomes, consistent with the role of the phenobarbital-inducible P450 2B1 indicated by the antibody inhibition experiments. In contrast, metyrapone was a much poorer inhibitor of oxazaphosphorine activation catalyzed by uninduced liver microsomes (Fig. 5). No evidence for a metyrapone-dependent stimulation of P450 2C6-associated metabolism [cf. Ref. 26] was observed in these microsomes, even at concentrations of 1 mM metyrapone (data not shown).

Impact of cisplatin treatment on microsomal ifosphamide activation. Treatment of rats with cisplatin leads to a significant alteration in the profile of P450 enzymes expressed in liver tissue. This can consequently reduce the capacity for liver cyclophosphamide metabolism, owing to the associated loss of the cyclophosphamide 4-hydroxylase P450 2C11 [13, 27]. The impact of cisplatin treatment *in vivo* on the capacity of isolated liver microsomes to activate ifosphamide was assessed in the present study. Cisplatin was administered to adult male rats as a single i.v. injection (0.72 mg/100 g body wt) and its effects on microsomal drug metabolism were assessed in animals killed up to 28 days later (Fig. 6). Cisplatin was found to depress liver microsomal ifosphamide metabolism, although the effects (~55% decrease 7 days after cisplatin treatment) were somewhat less severe than the loss of cyclophosphamide activation (~75% decrease). Moreover, whereas microsomal cyclophosphamide activity was only partially recovered by 28 days, the initial loss of ifosphamide activity was fully reversed by 21–28 days (Fig. 6). This finding suggests that P450 2C11, which is still suppressed 28 days after cisplatin treatment [13], makes a more prominent contribution to liver microsomal cyclophosphamide metabolism than to ifosphamide metabolism, in agreement with the antibody inhibition studies in Fig. 3A.

DISCUSSION

Several alkylating agent anti-cancer drugs, including cyclophosphamide, ifosphamide, procarbazine and *N,N′,N″*-triethylenethiophosphoramide (thio-TEPA), are metabolized by hepatic cytochrome P450 enzymes to reactive metabolites that contribute to the anti-tumor activity, and probably also account for some of the host toxicity of the parent drug [2, 28, 29]. Our earlier studies on P450-catalyzed cyclophosphamide metabolism identified two constitutive P450s (2C6 and 2C11) and one phenobarbital-inducible P450 enzyme (2B1) that activate the drug in rat liver [12]. Those studies were key to the subsequent elucidation of the mechanisms by which cyclophosphamide treatment alters the rate of hepatic cyclophosphamide activation in the rat model [30]. The present studies establish that the same three rat P450 enzymes also catalyze ifosphamide activation, as judged from P450 induction studies and antibody and chemical inhibition experiments. This conclusion is supported by the ability of ifosphamide to serve as a competitive inhibitor of cyclophosphamide activation catalyzed by microsomes prepared from mouse liver [31] or by microsomes isolated from uninduced, phenobarbital-induced or dexamethasone-induced rat liver (data

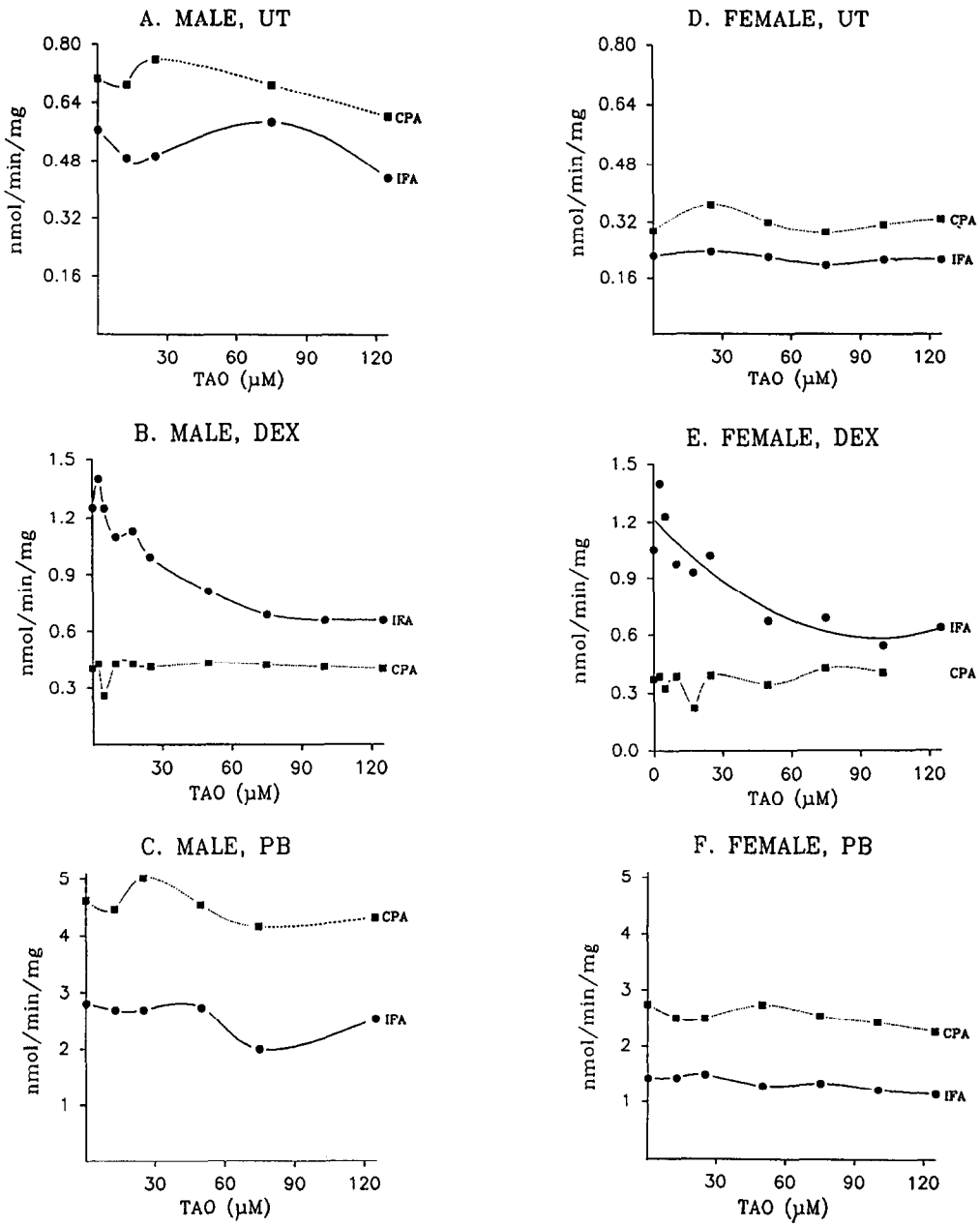

Fig. 4. Inhibition of liver microsomal metabolism of ifosphamide (IFA) and cyclophosphamide (CPA) by the P450 3A-selective inhibitor TAO. Liver microsomes isolated from adult male or female rats that were untreated (UT) or induced with dexamethasone (DEX) or phenobarbital (PB) were preincubated for 30 min at 37° with the indicated concentrations of TAO, and then the residual, uninhibited oxazaphosphorine metabolism activity (acrolein formation) was determined as described under Materials and Methods. Basal activities (i.e. in the absence of TAO) were lower than those shown in Figs. 2 and 3 due to the inhibitory effects of the dimethyl sulfoxide vehicle used to deliver TAO (see Materials and Methods). TAO was seen to inhibit selectively ifosphamide activation catalyzed by the dexamethasone-induced liver microsomes.

not shown). The present studies also point to the involvement of additional P450 enzymes, particularly in the case of dexamethasone-treated rats, where a dexamethasone-inducible subfamily 3A P450 was found to contribute to the metabolic activation of ifosphamide but not cyclophosphamide. Several dexamethasone-inducible P450 3A subfamily members have been identified in rat liver [22, 25, 32] and in human liver [33, 34], and the antibody and chemical inhibition studies described in the present

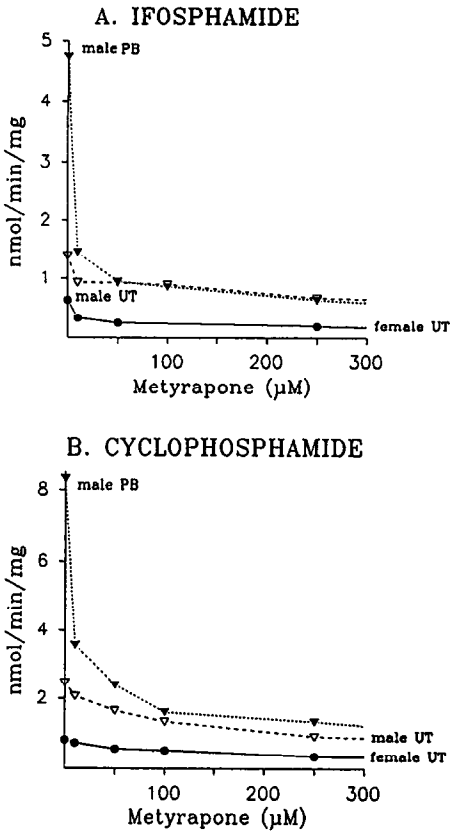

Fig. 5. Metyrapone inhibition of oxazaphosphorine metabolism catalyzed by uninduced male and female (UT) and by phenobarbital (PB)-induced male rat liver microsomes. Microsomal incubations were carried out in the presence of metyrapone at the indicated final concentrations. Liver microsomes used in these experiments were isolated from uninduced male (dashed line, open triangle), uninduced female (solid line, closed circle) or phenobarbital-induced rat liver (dashed line, closed triangle).

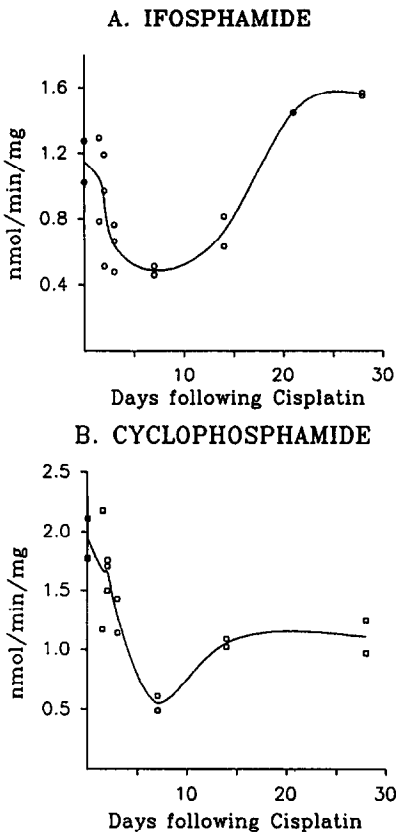

Fig. 6. Influence of *in vivo* cisplatin treatment on oxazaphosphorine metabolism catalyzed by isolated rat liver microsomes. Cisplatin (0.72 mg/100 g body wt) was administered on day 0, and individual rats were killed 1.5, 2, 3, 7, 14, 21 and 28 days later (N = 2 or 3 rats per time point). Isolated liver microsomes were assayed for metabolism of ifosphamide (A) and cyclophosphamide (B). Each data point represents the microsomal activities for an individual rat. Some heterogeneity in the responses of individual rats was seen over the first 2 days of the experiment.

report do not permit a precise determination of which specific rat 3A P450 activates ifosphamide in liver microsomes. Possible candidates include P450 3A1 and a second dexamethaxone-inducible 3A P450, form 6β-2, which has not yet been characterized at the cDNA level [32]. P450 form 3A2 is unlikely to be the catalyst of the dexamethasone-inducible microsomal ifosphamide activation, since P450 3A2 expression in adult rats is restricted to males [35], whereas both male and female rat liver microsomes catalyze a dexamethasone-inducible ifosphamide activation (Fig. 2 and data not shown). If hepatic ifosphamide activation can also be induced in humans by pretreatment with dexamethasone or other known P450 3A-inducing drugs (e.g. erythromycin, rifampicin, TAO), this could lead to an increase in therapeutic efficacy. In contrast to cyclophosphamide, ifosphamide activation is both obligatory and rate-limiting, in part due to its more extensive inactivation by N-dechloroethylation as compared to cyclophosphamide [2, 5]. While cyclophosphamide activation is required for anti-tumor activity, it is generally not a rate-limiting process [2, 8, 9]; hence the failure of dexamethasone to increase cyclophosphamide activation in the rat model should not be of major consequence.

Several anticancer drugs, including cisplatin [13, 27] and cyclophosphamide [28, 36], can modulate the expression and the activity of individual hepatic P450 enzymes. Since most chemotherapeutic regimens, including those containing ifosphamide, utilize combinations of two or more active agents, drug–drug interactions at the level of the hepatic P450 monooxygenase system could potentially compromise the effects of drugs that undergo P450 metabolism and are included in the combination. The cisplatin suppression of rat liver ifosphamide activation described in the present study, if it occurs in humans as well, would probably alter the pharmacokinetics of ifosphamide and its metabolites,

and could also have an adverse impact on drug therapy, unless drug scheduling takes such an interaction into account.

While liver activation is believed to be the major route for oxazaphosphorine metabolism, the possibility that extrahepatic and perhaps even intratumoral activation of ifosphamide and cyclophosphamide might occur has not been fully explored. The major P450 catalysts of ifosphamide and cyclophosphamide activation described in the present study are primarily found in the liver, although some extrahepatic expression has also been observed, particularly for P450 2B1 [37, 38]. Recent studies suggest, however, that in lung tissue other enzyme systems, such as prostaglandin synthase/cyclooxygenase, can also activate cyclophosphamide [39]. This raises the possibility that while hepatic metabolism may contribute to systemic drug activation, extrahepatic sites might be more important for reason of their proximity to sites relevant to therapeutic activity. If, indeed, intratumoral activation of these oxazaphosphorines contributes significantly to their anti-tumor effect, then a useful modulation strategy could involve selective inhibition of hepatic activation, such as might be achieved by the application of P450 inhibitors like metyrapone or TAO, in order to decrease systemic exposure to toxic circulating metabolites. Further investigation will be required to fully assess the potential usefulness of modulating the multiple pathways of cytochrome P450-dependent and cytochrome P450-independent [39] oxazaphosphorine metabolism with the goal of achieving decreased toxicity and improved therapeutic effects.

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