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Participation of CYP2C8 in Retinoic Acid 4-Hydroxylation in Human Hepatic Microsomes

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ABSTRACT. Cytochromes P450 (CYPs) catalyze the 4-hydroxylation of all-*trans*-retinoic acid (ATRA), an agent used in the treatment of certain malignancies. Literature studies have implicated several CYPs in this reaction, but the relative importance of individual CYPs is unclear. Human microsomal CYPs that contribute to the activity were evaluated by correlation with activities of hepatic drug-metabolizing CYPs, the capacity of cDNA-derived CYPs to catalyze the reaction, and inhibition of the microsomal activity by chemicals. 4-HydroxyATRA formation in microsomes varied 7-fold (8.7 to 61 pmol/mg protein/min) and correlated partially with activities mediated by CYPs 3A, 2C, and 1A ($\rho = 0.53$ to 0.66). cDNA-derived CYPs 2C8, 2C9, and 3A4, but not 1A1 or 1A2, catalyzed ATRA 4-hydroxylation (2.53, 4.68, and 1.29 pmol/pmol CYP/hr). The K_m for the reaction was 9 ± 3 μM in hepatic microsomes ($N = 3$) and 6 μM in microsomes containing cDNA-derived CYP2C8; by comparison, K_m values for the activity mediated by CYPs 2C9 and 3A4 were 100 and 74 μM, respectively. Inhibition of microsomal ATRA 4-hydroxylation was elicited by chemicals that interact with CYP2C8 (paclitaxel and diclofenac), but not those that interact with CYP2C9 (sulfaphenazole, tolbutamide, and torasemide). The CYP3A inhibitor troleandomycin and an anti-CYP3A IgG inhibited the activity slightly. Greater inhibition was produced by the less selective CYP3A inhibitors parathion, quinidine, and ketoconazole; CYP1A inhibitors were ineffective. These findings suggest that CYP2C8 is a major contributor to ATRA 4-hydroxylation in human liver and that 3A subfamily CYPs may be minor participants. Individual variation in CYP2C8 and 3A4 expression may influence ATRA pharmacokinetics and drug interactions during therapy. BIOCHEM PHARMACOL **58**;7:1201–1208, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. all-*trans*-retinoic acid; cytochrome P450; human hepatic microsomes; retinoid 4-hydroxylation; oxidative biotransformation

ATRA§ is physiologically important in normal vision, development, and cellular differentiation. These effects are mediated by a series of nuclear receptors that bind ATRA and activate target genes [1]. Recently, ATRA has also proven useful in cancer chemotherapy [2, 3]. Thus, during the initial phase of treatment, patients with acute promyelocytic leukemia respond well to ATRA therapy. More prolonged therapy with ATRA results in an enhanced rate of retinoid elimination and the inability to maintain therapeutic levels of the retinoid [4, 5]. These clinical observations are consistent with an increase in the rate of ATRA catabolism.

CYP-mediated 4-hydroxylation is the first step in the termination of ATRA action (Fig. 1) [6–8]. Previous reports have established that reconstituted systems incorporating purified human CYPs 2C, which appeared to be CYP2C8, were active in ATRA 4-hydroxylation [9]. Recombinant human CYPs 1A2, 2B6, 2D6, 2E1, and 3A4 have also been reported to catalyze ATRA 4-hydroxylation *in vitro* [10]. Thus, it is unclear whether CYPs have a uniform capacity to support ATRA biotransformation, or whether specific CYPs are dominant *in vivo*. In the present study, significant, but incomplete, correlations were found between hepatic microsomal ATRA 4-hydroxylation and activities of CYP3A, CYP2C, and CYP1A. cDNA-derived CYPs 2C8, 2C9, and 3A4, but not CYPs 1A1 or 1A2, were active in ATRA 4-hydroxylation. Potent inhibition of ATRA 4-hydroxylation in human liver was effected by inhibitors and substrates of CYP2C8 and, to a lesser extent, chemicals that interact preferentially with CYP3A4. However, an anti-rat CYP3A IgG elicited only minimal inhibition of the activity, and modulators of CYP1A were essentially non-inhibitory. These findings suggest that CYP2C8 may be the major participant in the biotransformation of ATRA in human liver and that there may be a minor role for CYP3A4 in some individuals.

MATERIALS AND METHODS

Chemicals

[11,13-^{3}H]ATRA (50–60 Ci/mmol) was obtained from Amrad Pharmacia Biotech Australia. [4-^{14}C]Testosterone

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§ *Abbreviations*: ATRA, all-*trans*-retinoic acid; and CYP, cytochrome P450.

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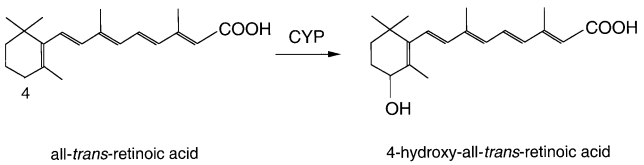

FIG. 1. CYP-mediated 4-hydroxylation of all-*trans*-retinoic acid. The 4-position is indicated.

(56 mCi/mmol) and [1-^{14}C]lauric acid (56 mCi/mmol) were from Amersham. Torasemide was a gift from Boehringer Mannheim, ketoconazole was from Janssen Pharmaceutica, parathion was from Rhone-Poulenc, and cyclophosphamide was from Farmitalia Carlo Erba; diclofenac and sulfaphenazole were from Ciba-Geigy, and tolbutamide, chlorpropamide, and hydroxytolbutamide were provided by Hoechst Pharmaceuticals. Unlabeled ATRA, other CYP substrates and inhibitors and biochemicals were obtained from Sigma-Aldrich. Analytical grade reagents were from Ajax Chemicals, and solvents for HPLC were from Rhone-Poulenc.

Preparation of Human Hepatic Microsomes

Ethical approval for the experimental use of human tissue was obtained from the Human Ethics Committee of the Western Sydney Area Health Service. Unwanted sections of donor human livers used for transplantation were obtained from the Queensland Liver Transplant Service (at Princess Alexandra Hospital) and the Australian Liver Transplant Center (at Royal Prince Alfred Hospital). Consent for the use of tissue in this project was also provided by the donors' relatives. Livers were perfused with cold Viaspan 228 solution (NEN-DuPont) and were stored in this solution for transport. Upon arrival, samples were snap-frozen in liquid nitrogen and stored at −70° until used for microsomal preparations.

Tissue was thawed and minced in ice-cold potassium phosphate buffer (10 mM, pH 7.4) containing 0.25 M sucrose and EDTA (1 mM), homogenized with a Kinematica polytron, and filtered through 250 μm nylon gauze (Allied Screen Fabrics). Hepatic microsomes were prepared by differential ultracentrifugation as described previously [11]. Microsomal protein was estimated by the method of Lowry *et al.* [12].

Microsomes (10 mg protein/mL) from human lymphoblastoid cells (AHH-1 TK+/−) containing cDNA-derived CYPs (1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 4A11) or vector alone were from the Gentest Corp. Inhibition and kinetic experiments were conducted in microsomes from baculovirus-infected insect cells (BTI-TN-5B1–4) containing overexpressed cDNA-derived CYP2C8, CYP2C9, CYP3A4, or vector (Gentest); protein concentrations were ~5 mg/mL, and CYP contents were measured by Gentest.

ATRA 4-Hydroxylation in Microsomal Fractions

Incubations (37°, final volume 1.0 mL, 45 min) contained hepatic microsomal protein (0.25 mg), [^{3}H]ATRA (7.5 μM, 1 × 10^6 dpm/incubation, except in kinetic analyses where the range of substrate concentrations was 5–100 μM), NADPH (1 mM), and potassium phosphate buffer (0.1 M, pH 7.4, containing 1 mM EDTA) [8]. Inhibitors were added in 5 μL of an appropriate solvent (ethanol, dimethylformamide, or dimethylsulfoxide), and solvent alone was added to control incubations; preliminary experiments confirmed that solvents did not influence ATRA 4-hydroxylation. Reactions were terminated by the addition of 0.5 mL of cold ethanol (containing 0.25 mg/mL of ascorbic acid and 0.25 mg/mL of EDTA). Substrate and metabolites were extracted into 2 mL of ethyl acetate (containing 1 mg/mL of butylated hydroxyanisole), and the organic phase was removed and layered onto a bed of anhydrous Na_2SO_4. After drying, the organic phase was removed under N_2.

In immunoinhibition experiments, microsomal protein and various amounts of anti-CYP3A IgG were incubated for 30 min at room temperature prior to the addition of NADPH to initiate the reactions; preimmune IgG was included in parallel control incubations.

ATRA 4-hydroxylation in microsomes from lymphoblastoid insect cells was measured similarly. Protein was increased to 2.5 mg/mL, and incubation time was increased to 60 min. CYPs 2C9 and 2A6 were incubated in Tris–HCl buffer (0.1 M, pH 7.5) in place of 0.1 M potassium phosphate buffer, according to the manufacturer's instructions. An NADPH-generating system (1.3 mM NADP, 3.3 mM glucose-6-phosphate, 0.4 U/mL of glucose-6-phosphate dehydrogenase, and 3.3 mM $MgCl_2$) was used to initiate reactions. Other procedures were identical to those used for human liver microsomes.

ATRA and 4-hydroxyATRA were separated by reverse-phase HPLC [13]. Samples were dissolved in 100% methanol (50 μL) and injected onto an Ultrasphere C18 column (5 μm, 250 × 4.6 mm, Beckman Instruments Inc.). The mobile phase was methanol:acetonitrile:0.1 M ammonium acetate, pH 6.8 (73:12:15). The flow rate was 1 mL/min, and fractions were collected at 1-min intervals. Scintillant (ACS II, Amersham) was added, and the samples were subjected to β-counting. Elution times for 4-hydroxyATRA and ATRA were 3.40 and 10.01 min, respectively.

Other Assays

Testosterone 6β-hydroxylation was determined by established methods [11]. Briefly, [^{14}C]testosterone (50 μM, 0.18 μCi) was incubated (37°) with microsomal protein (0.15 mg) and NADPH (1 mM) in potassium phosphate buffer, (0.1 M, pH 7.4, 1 mM EDTA, final volume 0.4 mL). Reactions were terminated after 2.5 min with chloroform (5 mL), and products were extracted and separated by TLC.

Metabolite formation was determined by scintillation spectrometry after localization of radioactive zones on TLC plates by autoradiography.

7-Ethoxyresorufin *O*-deethylation (0.5 mg protein/2 mL incubation, substrate concentration 2.5 μM) was assayed in Tris–HCl buffer (0.1 M, pH 7.8) according to Prough *et al.* [14]. Product (resorufin) formation was determined in a Shimadzu RF-1501 spectrofluorometer.

N-Nitrosodimethylamine *N*-demethylation (2.5 mg protein/1 mL incubation, substrate concentration 4 mM) was determined in potassium phosphate buffer (0.1 M, pH 7.4, 1 mM EDTA). Reactions (20 min, 37°) were initiated with NADPH (1 mM), terminated by the addition of 10% trichloroacetic acid, and product (formaldehyde) was detected colorimetrically [15].

Tolbutamide methyl hydroxylation (0.3 mg protein/0.4 mL incubation, substrate concentration 300 μM) was assayed in potassium phosphate buffer (0.065 mM, pH 7.4, 1 mM EDTA) as described by Knodell *et al.* [16]. Reactions were initiated with NADPH (1 mM) and terminated after 15 min with HCl (7.5 M, 20 μL). Tolbutamide metabolism in insect cell microsomes was determined in a similar manner, but protein concentration and incubation time were 0.6 mg/0.4 mL and 60 min, respectively. Products were extracted into diethyl ether, evaporated to dryness, dissolved in acetonitrile, and subjected to HPLC (Ultrasphere C18 column, 5 μm). The mobile phase was 0.05% phosphoric acid:acetonitrile (3:2), and the flow rate was 1 mL/min. Retention times were: hydroxymethyltolbutamide, 4.14 min; chlorpropamide (internal standard), 9.25 min; and tolbutamide, 11.53 min.

Microsomal 7-ethoxycoumarin *O*-deethylation (0.4 mg protein/2 mL incubation, substrate concentration 400 μM) was measured in potassium phosphate buffer (0.1 M, pH 7.4, 1 mM EDTA) in a Shimadzu RF-1501 spectrofluorometer according to Prough *et al.* [14]. The activity in cDNA-derived microsomes was measured in analogous fashion except that 0.1 mg microsomal protein (CYPs 1A1, 2A6, 2B6, and 2E1) or 0.2 mg protein (CYPs 1A2, 2C8, 2C9, 2C19, 2D6, and 3A4) was used. After incubation at 37° for 30 min, reactions were terminated by the addition of HCl (2 M, 50 μL). 7-Hydroxycoumarin was extracted into chloroform, back-extracted into sodium borate (0.03 M, pH 9.2), and quantified by spectrofluorometry.

Hepatic microsomal lauric acid ω- and ω-1 hydroxylations were assayed (0.5 mg protein/0.4 mL incubation, [^{14}C]lauric acid concentration 20 μM; 0.2 μCi) in potassium phosphate buffer (0.1 M, pH 7.4, 1 mM EDTA) essentially as described [17, 18]. Incubations (37°, 5 min) were initiated with NADPH (1 mM) and terminated with H_2SO_4 (10%, 0.1 mL). Substrate and metabolites were extracted into ethyl acetate (5 mL), and solvent was evaporated under N_2. Metabolite formation was detected by HPLC (Ultrasphere C18 column, 5 μm). Isocratic elution with solvent A (1% acetic acid) and solvent B (100% acetonitrile) (1:2) for 16 min (flow rate, 1.5 mL/min) was followed by elution with a linear gradient over 1 min in which solvent B increased to 100%; isocratic elution continued until 25 min. Fractions were collected at 30-sec intervals from 12 min and subjected to β-counting. Elution times were: 11-hydroxylauric acid, 13.5 min; 12-hydroxylauric acid, 16.0 min; and lauric acid, 24.0 min.

Holo-CYP and NADPH-cytochrome P450 reductase activity in human liver were determined spectrophotometrically [19, 20]. Relative levels of CYP2C and CYP3A immunoreactive protein were determined by immunoblotting, following electrophoretic separation of microsomal proteins on 7.5% sodium dodecyl sulfate-polyacrylamide gels and transfer to nitrocellulose filters [21]. The IgG to human CYP2C was provided by Professor P. H. Beaune, Inserm U75, Paris, France, and the anti-CYP3A IgG was raised against purified rat CYP3A in this laboratory as described for other anti-CYP IgG [22].

Statistics

All data are presented as means ± SEM of estimates in four individual microsomal fractions unless otherwise stated. Differences between means of control and test groups were detected using either nonparametric analysis of variance and the Kruskal-Wallis test (3 or more groups), or the Mann-Whitney test (2 groups). Correlations between CYP functions were determined by simple linear regression and Spearman's nonparametric procedure.

RESULTS

Interindividual Variation in ATRA Metabolism in Human Liver

Microsomal fractions were derived from excess tissue that was available after the transplantation of cut-down adult livers into pediatric recipients, or the normal margin adjacent to tumor tissue in patients undergoing hepatic resection. ATRA 4-hydroxylation in these livers varied over an approximate 7-fold range (8.7 to 61 pmol 4-hydroxyATRA formed/mg protein/min; Table 1). The recent drug histories of the donors are also shown in Table 1; some were reported in previous studies [23, 24]. Five of the donors were cigarette smokers, six had histories of alcohol intake, and some had received drugs with the potential to influence CYP activity, including enalapril, simvastatin, dexamethasone, and phenytoin. There was no apparent relationship between ATRA 4-hydroxylation activity and smoking, alcohol, or drug histories except that HL33, which contained the highest ATRA 4-hydroxylation activity, was obtained from a subject who had received phenytoin (Table 1).

The kinetics of ATRA 4-hydroxylation in human hepatic microsomes (N = 3) were determined (Fig. 2A). From Hanes-Woolf plots of [S]/V versus [S], K_m values of 6–15 μM were determined for the three individual livers (mean ± SEM: 9.0 ± 3.4 μM). V_{max} values ranged from 69 to 128 pmol/mg protein/min.

TABLE 1. Liver donor drug, smoking, and alcohol histories and microsomal CYP contents and ATRA 4-hydroxylation activities

Donor	Medications	Smoking history	Alcohol history	CYP content (nmol/mg protein)	ATRA 4-hydroxylation (pmol/mg protein/min)
HL1	Ranitidine	—	—	0.42	11
HL2	Oxazepam, α-methyldopa	—	—	0.33	8.7
HL4	None	—	—	0.37	18
HL8	None	—	—	0.59	21
HL10	Dopamine, desmopressin	—	—	0.42	39
HL16	Dopamine, desmopressin	—	—	0.42	29
HL17	Colloidal bismuth subnitrate, sucralfate	Y*	—	0.50	33
HL22	Unknown	?	?	ND†	51
HL24	Flucloxacillin, ceftriaxone	—	—	ND	45
HL25	Enalapril	—	Y	0.40	25
HL26	Dopamine, imipenem	—	Y	0.40	25
HL27	Dopamine, desmopressin	Y	—	0.43	21
HL28	Unknown	?	?	0.39	36
HL29	Simvastatin	—	—	0.37	34
HL30	Adrenaline, ranitidine, penicillin	—	Y	0.25	20
HL31	Dexamethasone, dopamine, desmopressin	Y	Y	0.12	25
HL32	Prazocin	—	—	0.44	13
HL33	Phenytoin, dopamine, desmopressin	Y	Y	ND	61
HL34	Alprazolam, dexamethasone	Y	Y	0.26	35

ATRA 4-hydroxylation data are means of duplicate determinations that varied by < 10%. Because of limited material, CYP values are individual estimations.
*Smoking history (≥ 3 cigarettes per day) or alcohol history (≥ 10 g/day); Y indicates positive history, and ? indicates an unknown history.
†ND, not determined because of limited availability of material.

Relationship between ATRA Metabolism and Other CYP-Mediated Hydroxylations

To assess the role of CYPs in ATRA 4-hydroxylation, a series of correlations with rates of well-defined CYP-catalyzed reactions were derived. Because these activities were not normally distributed, the nonparametric approach of Spearman based on rank correlations was used. Significant correlations were detected between 4-hydroxyATRA formation and CYP3A-mediated testosterone 6β-hydroxylation ($\rho = 0.664$, $P < 0.01$; N = 17; Table 2), CYP2C-mediated tolbutamide methylhydroxylation ($\rho = 0.532$, $P < 0.05$, N = 15), and CYP1A-mediated 7-ethoxyresorufin *O*-deethylation ($\rho = 0.539$, $P < 0.05$, N = 17) as well as relative CYP2C immunoreactive protein ($r = 0.525$, $P = 0.05$, N = 14) and relative CYP3A immunoreactive protein ($r = 0.681$, $P < 0.01$, N = 14). In contrast, correlations were not observed between ATRA 4-hydroxylation and CYP2E1-catalyzed *N*-nitrosodimethylamine *N*-demethylation, CYP4A11-mediated lauric acid ω- or ω-1 hydroxylation, or 7-ethoxycoumarin *O*-deethylation, which is catalyzed by several CYPs. Although statistically significant, the correlations with CYP1A, 2C, and 3A activities were incomplete, and the possible functional roles of these CYPs in ATRA 4-hydroxylation were evaluated further.

Metabolism of ATRA by cDNA-Derived Human CYPs

The capacities of individual CYPs to catalyze ATRA 4-hydroxylation were assessed in microsomes prepared from lymphoblastoid cells that had been transfected with CYP cDNAs. Of the eleven CYPs examined, 2C8, 2C9, and 3A4

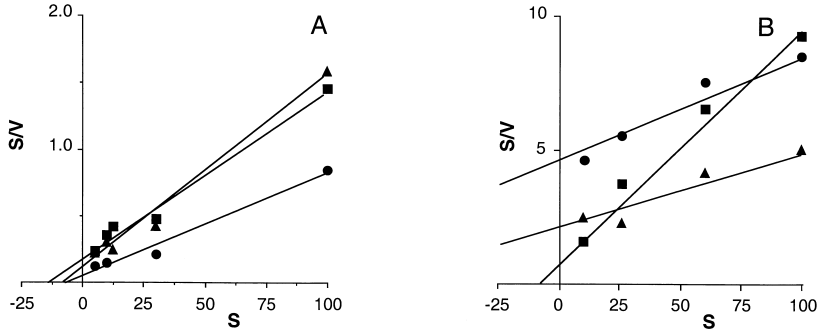

FIG. 2. Hanes-Woolf plots of ATRA 4-hydroxylation activity in: (A) microsomal fractions from three individual human livers (■, ●, and ▲), and (B) microsomal fractions from insect cells containing cDNA-derived CYP2C9 (●), CYP2C8 (■), and CYP3A4 (▲). Units: S, μM; and V, pmol/mg protein/min (liver microsomes) or pmol/pmol CYP/hr (cell microsomes). Data are means of two separate estimates that varied by < 10%.

TABLE 2. Rank correlation coefficients for the relationships between ATRA 4-hydroxylation and other CYP activities in human liver microsomes

	TEST	EROD	ECOD	NDMA	TOLB	LAω	LAω-1
ATRA	0.664*	0.539†	0.315	0.179	0.532†	0.221	0.409
TEST		0.146	0.223	−0.226	0.276	−0.078	0.206
EROD			0.645*	−0.176	0.321	0.270	0.203
ECOD				−0.399	0.325	0.029	0.053
NDMA					−0.027	−0.076	0.022
TOLB						0.571	−0.688*
LAω							0.868‡
LAω-1							

Correlation analysis was performed using data derived from 15 to 19 individual livers. Abbreviations: ATRA, ATRA 4-hydroxylation; TEST, tertosterone 6β-hydroxylation; EROD, 7-ethoxyresorufin *O*-deethylation; ECOD, 7-ethoxycoumarin *O*-deethylation; NDMA, *N*-nitrosodimethylamine *N*-demethylation; TOLB, tolbutamide methyl hydroxylation; LAω and LAω-1, lauric acid ω- and ω-1 hydroxylation, respectively.

*–‡Correlation significant: *$P < 0.01$, †$P < 0.05$, and ‡$P < 0.001$.

were quite active in ATRA hydroxylation (2.53, 4.68, and 1.29 pmol/pmol CYP/hr, respectively; Table 3); CYPs 2C19 and 4A11 also generated small amounts of product (0.62 and 0.31 pmol/pmol CYP/hr, respectively; Table 3). CYPs 1A1, 1A2, 2A6, 2B6, 2D6, and 2E1 did not produce 4-hydroxyATRA from ATRA. 7-Ethoxycoumarin *O*-deethylation was measured in most of these preparations as a general marker for CYP activity [25].

The kinetics of ATRA 4-hydroxylation were determined in insect cell microsomes containing cDNA-derived CYPs (Fig. 2B). From Hanes-Woolf plots of [S]/V versus [S], a value of 6.1 μM was determined for the K_m of CYP2C8-mediated ATRA 4-hydroxylation; the V_{max} was 10.8 pmol/pmol CYP/hr. In comparison, CYPs 2C9 and 3A4 exhibited, respectively, values of 100 and 74 μM for the reaction K_m and 22.8 and 33.6 pmol/pmol CYP/hr, respectively, for the reaction V_{max}. Thus, the ratio V_{max}/K_m, a measure of turnover, was 1.8, 0.23, and 0.45 for CYPs 2C8, 2C9, and 3A4, respectively.

TABLE 3. ATRA 4-hydroxylation activity by individual cDNA-derived human CYPs *in vitro*

CYP	ATRA 4-hydroxylation (pmol/pmol CYP/hr)	ECOD activity (pmol/pmol CYP/hr)	CYP content* (pmol/mg protein)
1A1	< 0.1	16	25
1A2	< 0.1	6.1	40
2A6	< 0.1	32	60
2B6	< 0.1	23	55
2C8	2.53	0.42	10
2C9	4.68	0.25	10
2C19	0.62	4.4	30
2D6	< 0.1	4.4	160
2E1	< 0.1	17	40
3A4	1.29	23	30
4A11	0.31	ND†	18

Data are means of duplicate determinations that varied by < 10%.

*CYP content was measured by the Gentest Corp.

†ND, not determined.

Modulation of ATRA 4-Hydroxylation by Substrates and Inhibitors of CYPs

A range of chemicals that interact with several CYPs were tested for their effects on ATRA 4-hydroxylation activity in human liver microsomes. Concentration-dependent inhibition of the activity was elicited by paclitaxel (IC_{50} = 46 μM) and diclofenac (IC_{50} = 120 μM), which are established substrates of CYPs 2C [26, 27] (Fig. 3A), but not by sulfaphenazole, tolbutamide, or torasemide, which interact with CYP2C9 [16, 28, 29] (Fig. 4). Consistent with other reports, both CYP2C8 and 2C9 supported tolbutamide methyl hydroxylation [30]. In the present study, diclofenac (250 μM) decreased cDNA-derived CYP2C8- and CYP2C9-mediated tolbutamide oxidation to 20 and 9.9% of the respective control (not shown). Thus, diclofenac elicited inhibition of both CYP2C enzymes.

Troleandomycin, a selective inhibitor of CYP3A, inhibited ATRA 4-hydroxylation in human hepatic microsomes

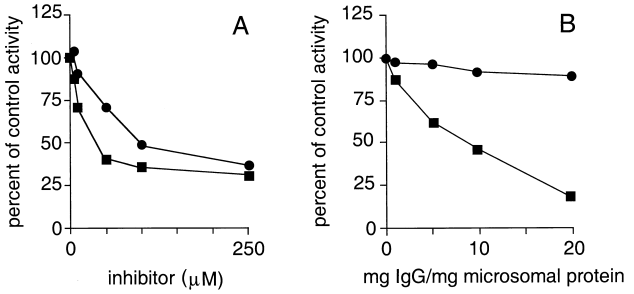

FIG. 3. (A) Inhibition of microsomal ATRA 4-hydroxylation in human liver by paclitaxel (■) and diclofenac (●). Data are means of measurements in four individual human liver fractions; uninhibited rates were 21, 29, 35, and 46 pmol/mg protein/min. (B) Effect of a rabbit anti-rat CYP3A IgG on testosterone 6β-hydroxylation (■) and ATRA 4-hydroxylation (●) activities in human liver microsomes. Data are means of estimates in two separate human liver microsomal fractions. Uninhibited rates were 4.0 and 8.0 nmol product/mg protein/min (testosterone) and 21 and 46 pmol/mg protein/min (ATRA). Replicates of percent inhibition data varied by less than 10%.

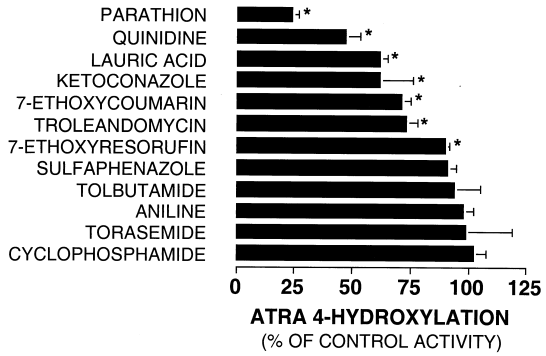

FIG. 4. Effects of putative inhibitory chemicals on ATRA 4-hydroxylation in human hepatic microsomes. The ATRA concentration was 7.5 μM and inhibitor concentrations were 250 μM, with the exception of ketoconazole (25 μM), lauric acid (100 μM), tolbutamide (1 mM), and 7-ethoxyresorufin (1 μM). Data are means ± SEM of separate estimates in four livers; unihibited rates, 13, 35, 36, and 39 pmol/mg protein/min. Asterisks indicate a significant difference from control ($P < 0.05$).

as did several other chemicals that also modulate CYPs 3A, although with lower selectivity (Fig. 4). Thus, parathion (250 μM), ketoconazole (5 μM), and quinidine (250 μM) decreased the rate of 4-hydroxyATRA formation to 24 ± 3, 62 ± 14, and 46 ± 6% of control activity, respectively ($N = 4$, $P < 0.05$, Fig. 4). However, the low affinity CYP3A substrate cyclophosphamide (250 μM) was not inhibitory (Fig. 4). Minimal inhibition (<25%) was produced by coumarin (250 μM; oxidized by CYP2A6), but other chemicals were non-inhibitory, including those that are substrates for CYP1A (7-ethoxyresorufin) and CYP2E1 (aniline) (Fig. 4). Similar findings were made with the CYP1A2 substrate phenacetin and the CYP2D6 substrate debrisoquine (not shown).

To clarify the involvement of CYPs 2C in human ATRA 4-hydroxylation, the CYP2C9 substrate tolbutamide (250 μM) was included in incubations containing cDNA-derived CYPs 2C8, 2C9, and 3A4. ATRA 4-hydroxylation by CYP2C9 was inhibited extensively by tolbutamide to 33% of control rates, whereas the CYP2C8- and CYP3A4-dependent activities were refractory to tolbutamide (data not shown). Because the activity in intact microsomal fractions was not inhibited by tolbutamide, these findings enhanced the association between CYP2C8 and ATRA 4-hydroxylation activity.

An anti-rat CYP3A IgG was examined for its effects on ATRA 4-hydroxylation in human hepatic microsomes. As shown in Fig. 3B, a ratio of 20 mg anti-CYP3A IgG/mg microsomal protein extensively inhibited testosterone 6β-hydroxylation (to 17% of preimmune control) but had only a minimal effect on ATRA 4-hydroxylation (to 89% of preimmune control). Thus, the contribution of CYP3A4 to human ATRA 4-hydroxylation appears to be quantitatively minor with respect to that of CYP2C8.

DISCUSSION

The results of the present investigation implicate a major role for CYP2C8 and a possible minor role for CYP3A4 in the biotransformation of ATRA in human liver. The Michaelis constants for ATRA 4-hydroxylation in three human hepatic microsomal preparations (9.0 ± 3.4 μM) were quite similar to that exhibited by cDNA-expressed CYP2C8 (6 μM) and different from those mediated by cDNA-expressed CYPs 2C9 and 3A4 (>70 μM). Thus, CYP2C8 supported ATRA 4-hydroxylation most efficiently, with CYP2C9 and CYP3A4 appearing to be catalysts of lower affinity and higher capacity, at least when evaluated in isolation. Paclitaxel, a selective substrate of CYP2C8 [26], and diclofenac, a substrate/inhibitor of CYP2C8/2C9 [27, and this study], potently inhibited ATRA 4-hydroxylation in human liver microsomes. On the other hand, substrates and inhibitors of CYP2C9 were not inhibitory toward ATRA 4-hydroxylation activity in human hepatic microsomes, a finding consistent with the low affinity of this enzyme for the retinoid. Troleandomycin, the selective chemical inhibitor of CYP3A4, inhibited ATRA 4-hydroxylation activity only weakly; any role for CYP3A therefore appears to be minor. Several other CYP3A4 inhibitors, including ketoconazole, quinidine, and parathion, were also inhibitory toward the activity, but these chemicals are somewhat less specific than troleandomycin for CYP3A4.

These findings are in accord with the work of Leo *et al.* [9], who described the ability of a purified CYP2C protein, probably CYP2C8, to catalyze ATRA 4-hydroxylation in a reconstituted system. However, these workers suggested that other CYPs may function in ATRA 4-hydroxylation in liver. Similarly, it has been proposed that CYP3A4 mediates ATRA oxidation [10], which suggests that this quantitatively important enzyme may have a functional role in human liver. Five other cDNA-expressed human CYPs also catalyzed ATRA 4-hydroxylation at similar rates [10]. In the present study, several human CYPs—1A1, 1A2, 2A6, 2B6, 2D6, and 2E1—did not oxidize ATRA at perceptible rates. The reason for this discrepancy between the studies is unclear, but could be related to differences in substrate concentration (0.16 to 5 μM in that study) and shorter incubation times, which would have necessitated the use of substrate of very high specific activity and could have complicated the elution of radioactivity during chromatography. Notwithstanding these possibilities, the literature indicates that several CYPs may support ATRA 4-hydroxylation under particular incubation conditions. The uncertainty over the relative contributions of different CYPs to the activity in microsomes has been clarified by the findings of the present study using kinetic analysis and cDNA-expressed CYPs that support a major role for CYP2C8 and a minor role for CYP3A4.

Previous findings from this laboratory and others have demonstrated the partial involvement of CYP3A enzymes in microsomal ATRA 4-hydroxylation in rat liver [11, 31].

Thus, several lines of investigation, including enzyme induction, the use of inhibitory antibodies and chemicals, and developmental patterns of activity, were used to strengthen the association between CYP3A function and ATRA 4-hydroxylation. Because only ~40% of the microsomal activity was inhibited by an anti-CYP3A IgG (20 mg IgG/mg protein) [11], it was clear from these studies that other CYPs may contribute to the microsomal reaction.

The recent cloning of CYP26 and the demonstration that the cDNA-expressed CYP26 catalyzes ATRA 4-hydroxylation suggest that other CYPs may contribute to ATRA biotransformation, especially in cells and tissues that do not express CYP2C8 or CYP3A4 [32–34]. In these studies, CYP26 was found to be expressed constitutively in some tumor cells, was inducible in others, and was absent from other cell types. Thus, CYP26 appears to be a strong candidate as an ATRA-inducible CYP, although its role in hepatic ATRA 4-hydroxylation is unclear. Indeed, the quantitative significance of CYP26 in human liver, especially in relation to major multifunctional CYPs like CYP3A and CYP2C, remains to be established. Clarification of the role of CYP26 is also dependent upon further information on the substrate and inhibitor specificity of this enzyme.

Different CYPs may contribute to ATRA 4-hydroxylation activity in a tissue-specific manner. Dermal ATRA 4-hydroxylation was not inhibited by 9-*cis*- and 13-*cis*-retinoic acids, which suggests that these isomers may not be alternate substrates for the CYP ATRA 4-hydroxylase(s) in this tissue [35]. In contrast, retinoic acid isomers, as well as a number of oxidized retinoids, inhibited ATRA 4-hydroxylation in breast cancer cells [36]. Furthermore, in human liver microsomes ATRA 4-hydroxylation was inhibited by all isomers of retinoic acid, retinol, and retinal that were tested [8]. Thus, tissue-specific CYP expression is an important factor that determines the nature of ATRA 4-hydroxylation. The present findings complement other literature evidence that implicates several CYPs with the potential to mediate ATRA 4-hydroxylation in tissues.

Like most CYPs, those from the 2C and 3A subfamilies exhibit substantial variation in expression between subjects [37]. The use of ATRA as a drug in cancer chemotherapy, therefore, is likely to be complex, as suggested by the present finding of a 7-fold variation in 4-hydroxylation activity in liver and by previous pharmacokinetic analyses [38]. The potential for interaction between drugs is enhanced by the findings that CYP2C8 and, to a lesser extent, CYP3A4 are involved in ATRA biotransformation. A range of other anti-cancer drugs, including taxol [26] and tamoxifen [39], are also oxidized by these enzymes. Thus, inhibitory effects of such agents may impair ATRA elimination, leading to accumulation of the drug in serum and possible precipitation of retinoid resistance, such as retinoic acid syndrome [4]. Further complications of therapy with ATRA include the apparent enhanced rates of retinoid elimination that occur with prolonged therapy. This situation is characterized by the inability to maintain adequate serum levels in patients, the failure of retinoid therapy and unresponsiveness toward subsequent rounds of therapy with ATRA. Concurrent administration of agents such as liarozole has been shown to maintain ATRA levels in serum and in tumors, most likely by inhibiting ATRA catabolism [40]. It will be of interest to determine the specificity of such drugs against CYPs that mediate ATRA 4-hydroxylation.

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