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Identification of Human Cytochrome P450 Isoforms that Contribute to All-*trans*-Retinoic Acid 4-Hydroxylation

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ABSTRACT. The role of specific human cytochrome P450 (CYP) isoforms in the oxidative metabolism of all-*trans*-retinoic acid was investigated by studies in human liver microsomes using isoform-specific chemical inhibitors and inhibitory antibodies. Studies using individual isoforms expressed in lymphoblastoid cells and correlation analysis using different microsome preparations were also performed. With expressed isoforms, evidence for a role for CYP2C8, CYP3A4, CYP2C9, and CYP1A1 in 4-hydroxylation was obtained, with the highest catalytic efficiency being observed for CYP2C8. Using inhibition studies and correlation analysis, we also concluded that CYP2C8 was the major all-*trans*-retinoic acid 4-hydroxylating cytochrome P450 in human liver microsomes, though CYP3A4 and, to a lesser extent CYP2C9, also made a contribution. In addition, we compared the rate of retinoic acid degredation in HepG2 cells when cultured in the absence and presence of 3-methylcholanthrene or all-*trans*-retinoic acid. Culture in the presence of all-*trans*-retinoic acid decreased the half-life twofold and resulted in an increased sensitivity of retinoic acid degredation to ketoconazole. Since no induction of either CYP1A1, CYP2C8, CYP2C9, or CYP3A4 was detected using immunoblotting and as mRNA encoding another cytochrome P450 enzyme, CYP26, has been previously demonstrated to be induced by retinoic acid treatment of HepG2 cells and to be highly sensitive to ketoconazole, this enzyme in addition to CYP2C8, CYP2C9 and CYP3A4 likely plays a role in all-*trans*-retinoic acid oxidation in the liver at high retinoic acid levels. BIOCHEM PHARMACOL **60**;4:517–526, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cytochrome P450; all-*trans*-retinoic acid; CYP2C8; CYP3A4; induction

RA† is a vitamin A derivative with a well-established role in cell differentiation and signalling. Several natural isomers occur, but the most biologically significant are all-*trans*-RA and 9-*cis*-RA. RA appears to exert its effects through binding to retinoic acid receptors, which are members of the steroid hormone superfamily (for review see [1]). All-*trans*-RA binds with highest affinity to RAR (retinoic acid receptor) receptors, whereas 9-*cis*-RA is the major ligand bound by the RXR (retinoid X receptor) receptors [1]. RAR is a transcriptional regulator of a variety of retinoic acid-responsive genes, but RXR is a coregulator of RAR together with other members of the nuclear hormone receptor superfamily, including thyroid hormone receptors, vitamin D receptor, and the recently identified pregnane receptor (PXR), which has a role in the regulation of metabolism of some drugs [1, 2]. RA in the body is mainly synthesised from β-carotene and retinol (vitamin A), but various naturally occurring isomers and synthetic derivatives are also used therapeutically, especially in the treatment of certain skin diseases and cancer. Both the biosynthesis and catabolism of RA is still relatively poorly understood [3]. It is believed that formation of the 4-hydroxy metabolite is an important catabolic step in all-*trans*-RA metabolism, although other oxidised metabolites are also formed, as is the acyl glucuronide [4]. There is clear evidence from animal studies that cytochromes P450 catalyse the 4-hydroxylation reaction [5, 6], and specific inhibition of all-*trans*-RA elimination in the rat by the P450 inhibitor ketoconazole has been shown [7]. Studies in humans have been limited, but it has been demonstrated that purified CYP2C8 can hydroxylate both retinoic acid and retinol at the 4-position [8]. However, there is also evidence from animal studies that members of the CYP3A family can carry out this reaction [9, 10], and there have been no systematic studies using a range of cytochromes P450 and human liver microsomes to determine the precise contribution of various isoforms to 4-hydroxylation. More recently, the cytochrome P450 CYP26 has been demonstrated to be a specific RA hydroxylase, though its precise contribution to RA oxidation in different cell types has not yet been clearly established [11].

Use of retinoic acid derivatives in cancer chemotherapy is a promising approach in the treatment of a number of

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† *Abbreviations:* RA, retinoic acid; CYP, cytochrome P450; 3-MC, 3-methylcholanthrene; and HLM, human liver microsomes.

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tumour types, but there is a problem with relapse due to development of resistance [12]. Determining which cytochromes P450 hydroxylate RA is particularly relevant to this problem of all-*trans*-RA resistance, since it appears to be due to increased levels of 4-hydroxylation resulting from enzyme induction [12, 13].

We describe a study of all-*trans*-RA 4-hydroxylation by human liver microsomes and recombinant cytochromes P450 and show that the major 4-hydroxylating enzyme is CYP2C8, but that there is also a significant role for CYP3A4. We also describe some studies on metabolism in HepG2 cells that suggest that CYP26 may have a more significant role in all-*trans*-RA metabolism following pretreatment with retinoic acid.

MATERIAL AND METHODS

Materials

All-*trans*-RA was purchased from Sigma. [11,12-^{3}H(N)] All-*trans*-RA (1 mCi/mL) was purchased from DuPont NEN, all-*trans*-4-oxo-RA was a gift from Dr Michael Klaus, Hoffman LaRoche, 4′-hydroxydiclofenac was a gift from Dr Degen, Novartis, and 1′-hydroxybufuralol was supplied by Hoffmann LaRoche. Lymphoblastoid cell microsomes expressing specific P450 isoforms were purchased from Gentest Corporation. Human livers were obtained from Vitron Inc. and human liver microsomes from the International Institute for the Advancement of Medicine. Anti-CYP3A, anti-CYP2C13, and anti-CYP2C antibodies were gifts from Dr J. Hardwick, Rootstown, Ohio, Dr C. Crespi, Gentest Corporation, and Professor P. Beaune, Paris, respectively. Furafylline and sulphaphenazole were from Ultrafine Chemicals.

Preparation of Retinoid Stocks

All manipulations were performed under yellow light since retinoids are light-sensitive. RA and metabolites were dissolved in methanol. All-*trans*-4-hydroxy-RA was prepared by the reduction on ice of all-*trans*-4-oxo-RA with sodium borohydride (5 mg/mL in methanol) by the method of Aig *et al.* [14].

Analysis of RA Oxidation by Microsome Preparations

The standard assay mixture contained, in a total volume of 250 μL, 0.4 mM NADPH, 4 mM $MgCl_2$, 0.2 mg microsomal protein in 0.1 M sodium phosphate buffer, pH 7.4, and 10 μM unlabelled all-*trans*-RA containing 0.1 μCi ^{3}H all-*trans*-RA. Reactions, performed in duplicate, were initiated by the addition of NADPH in the case of human liver microsomes and by adding thawed ice-cold microsomes to prewarmed reaction mix in the case of lymphoblastoid cells, and terminated by the addition of 3 volumes ice-cold methanol containing butylated hydroxytoluene (0.05%) as antioxidant [15]. Non-radiolabelled retinoid standards (all-*trans*-RA, all-*trans*-4-oxo-RA, and all-*trans*-4-hydroxy-RA) were added, each at 10 μM. After extraction, samples were evaporated to dryness under vacuum in a GyroVap (Howe) and dissolved in 50 μL methanol. HPLC analysis was carried out using a Spherisorb ODS (octadecylsilyl)-2 column (5 μm, 125 mm × 4 mm, Hewlett Packard) attached to a Hewlett Packard HPLC Chemstation 1050 system. The initial mobile phase (adapted from [14]) was 58% methanol in 10 mM sodium acetate, pH 7.4 at a flow rate of 1 mL/min. After 6.5 min, the methanol concentration was increased linearly to 82% over a 2.5-min time period. Chromatography was continued at 82% methanol for a further 5 min. The detection wavelength was set at 343 nm. Each sample (30 μL) was injected onto the column using the autosampler, and the eluate was collected into scintillation vials at 0.5-min intervals, 4.5 mL scintillant (Optiphase Hisafe) added, and fractions counted for radioactivity in a Packard Tri-Carb 4530 scintillation counter programmed to calculate sample dpm.

Preparation of Human Liver Microsomes

Microsomes were prepared from frozen human liver samples stored at −80°. The liver material was thawed, placed in 3 volumes of ice-cold buffer (0.25 M sucrose, 2 mM HEPES, 1 mM EGTA, pH 7.2), and homogenised using a Polytron (Kinematica, GMBH). Microsomes were isolated by initial centrifugation at 20,000 *g* at 4° for 20 min followed by centrifugation of the supernatant at 100,000 *g* (Sorvall Ultracentrifuge OTD 55B, DuPont) for 60 min at 4° and resuspension of the microsomal pellet in a small volume of isolation buffer (0.25 M sucrose, 2 mM HEPES, 1 mM EGTA, pH 7.2) to give a final protein concentration of approximately 10 mg/mL. Microsomal protein content was estimated by the Bio-Rad assay according to the supplier's recommendations using immunoglobulin G (IgG) as a standard.

Determination of Kinetic Constants

In standard incubations, 0.2 mg microsomal protein was incubated with all-*trans*-RA for 30 min, which was within the linear range from velocity against time experiments. For the determination of values of K_m and V_{max} for RA oxidation, a range of RA concentrations from 0.1 to 5 μM was used. The data were fitted directly to the Michaelis–Menten equation using the program Ultrafit (Biosoft) to obtain estimates of kinetic constants.

Chemical Inhibition Studies of Microsomal RA Metabolism

The chemical inhibitors used in inhibition studies and their concentrations were as follows: ketoconazole (1 and 20 μM), paclitaxel (50 μM), troleandomycin (50 μM), sulphaphenazole (5 μM), furafylline (50 μM), 8-methoxypsoralen (50 μM), orphenadrine (100 μM), quinidine (10

μM), and chlorzoxazone (50 μM). Microsomal incubations were performed as described above with a concentration of 10 μM all-*trans*-RA, with inhibitors added to the reaction mix in a volume of 5 μL vehicle (water or methanol depending on solubility) and the equivalent volume of vehicle added to control incubations without inhibitors. When the mechanism-based inhibitors troleandomycin or furafylline were used, they were preincubated with microsomes and NADPH in phosphate buffer for 10 min prior to addition of all-*trans*-RA. The percentage inhibition of all-*trans*-RA oxidation by each inhibitor was determined by comparison with control incubations performed under identical conditions to which vehicle without inhibitor had been added.

Immunoinhibition Studies on Microsomal RA Metabolism

The effect of specific antisera on RA metabolism in human liver microsomes was studied by carrying out reactions as described above except that microsomes and antisera were preincubated together in 0.1 M phosphate buffer pH 7.4 for 10 min and the reaction then initiated by the addition of NADPH and all-*trans*-RA (10 μM). Percentage inhibition of all-*trans*-RA oxidation was determined by comparison with control incubations to which preimmune rabbit serum was added.

Immunoblotting

Fifty micrograms of protein from human liver microsomes was fractionated by electrophoresis on a 10% SDS–polyacrylamide gel [16] and electrophoretically transferred onto a nitrocellulose membrane (Hybond-C, Amersham). Detection of CYP2C and CYP3A was achieved using rabbit anti-rat primary antibodies followed by incubation with a peroxidase-conjugated goat anti-rabbit secondary antibody and visualisation using an ECL™ (Enhanced Chemiluminescence) kit (Amersham) according to the manufacturer's instructions. To ensure separation of specific P450 isoforms (CYP3A4, CYP3A5, CYP2C8, and CYP2C9), electrophoresis was carried out for an extended period (20 hr at 50 V) and bands corresponding to specific isoforms were identified on the basis of their mobility compared with bands from lymphoblastoid cell lines overexpressing particular isoforms. Cytochrome P450 isoform levels were quantitated by scanning densitometry of photographs of immunoblots using a Camag TLC Scanner II with a tungsten lamp in absorbance mode connected to a Camag SP4290 TLC integrator. The concentration of cytochrome P450 isoform was determined by comparison with standards of lymphoblastoid microsomal protein of known P450 isoform content using several concentrations as recommended in the ECL kit instructions.

Enzyme Assays

Total cytochrome P450 content of human liver microsomes was determined by the method of Omura and Sato [17] using a Perkin Elmer Lambda 3 spectrophotometer. The microsomes were also assayed for four specific isoform marker substrate activities (coumarin 7-hydroxylase [CYP2A6], diclofenac 4′-hydroxylase [CYP2C9], ethoxyresorufin *O*-deethylase [CYP1], and bufuralol 1′-hydroxylase [CYP2D6] using incubation times and protein concentrations shown to be within the linear range in velocity against time experiments. Coumarin 7-hydroxylase activity was analysed by incubation of human liver microsomes with 50 μM coumarin in 0.1 M phosphate buffer, pH 7.4, containing 1 mM NADPH and 2.5 mM $MgCl_2$ in a final volume of 250 μL. The reaction was initiated by the addition of NADPH after a 3-min preincubation at 37° of microsomes with coumarin and was carried out for 20 min at 37° with shaking. Reactions were terminated with 250 μL ice-cold 90% (v/v) ethanol. Tubes were centrifuged for 5 min at 13,000 *g* and 420 μL supernatant removed and evaporated to dryness. 7-Hydroxycoumarin was measured by thin layer chromatography using the method of Cholerton *et al.* [18]. Chromatograms were visualised and coumarin and 7-hydroxycoumarin were quantitated by fluorescence densitometry under a mercury lamp at 313 nm in the reflection mode with a K400 secondary filter using a Camag TLC Scanner II connected to a Camag SP4290 TLC integrator. Diclofenac 4′-hydroxylase was measured using the method described by Leeman *et al.* [19], ethoxyresorufin *O*-deethylase by the method of Burke *et al.* [20], and bufuralol 1′-hydroxylase by the method of Kronbach [21].

All-trans-RA Metabolism Studies in HepG2 Cells

The liver hepatoma cell line HepG2 was maintained in minimum essential medium (MEM) supplemented with 10% foetal bovine serum, 1% L-glutamine, and non-essential amino acids, and the cells were cultured in a humidified 5% CO_2 atmosphere at 37°. For studies on the conversion of all-*trans*-RA to polar metabolites, 1 mL medium containing 10^5 cells was added to 1.5-cm diameter wells in 24-well plates and cells were allowed to adhere overnight (18 hr). At zero time, the medium was removed and replaced with fresh medium containing [11,12-^{3}H]all-*trans*-RA(20 nM, 1 μCi/mL) to initiate metabolism studies with individual wells used for each time point. At 0, 6, 12, and 24 hr, 800 μL medium was removed from specific wells and RA and its metabolites extracted by solid-phase extraction using Sep-Pak C18 columns (Waters) by the method of Garrabrant and End [22]. Medium from each well was added to 3.5 mL acetonitrile and centrifuged at 1000 *g* for 15 min to precipitate protein. The supernatant was added to 4 mL 40 mM acetic acid and passed through a preconditioned SepPak column. The eluate was collected and combined with eluate produced from washing the column with 1.7 mL 40% acetonitrile. This fraction contained polar retinoic

acid metabolites. The column was then washed with 3 mL methanol and the eluate, which contained unconverted retinoic acid, collected separately. Levels of radioactivity in both fractions were determined by liquid scintillation counting and the retinoid content of the two fractions was confirmed by HPLC analysis.

Studies on induction were performed using 0.1 μM all-*trans*-RA and 1 μM 3-MC. All induction was for 72 hr. For all-*trans*-RA induction, the normal medium was removed 24 hr after passaging and replaced with fresh medium containing retinoic acid in methanol (less than 0.5% vol.). Every 24 hr during the induction period, medium was removed and replaced with fresh medium containing 0.1 μM all-*trans*-RA. After 72 hr, the medium was removed and the cells were washed 3 times with PBS and twice with medium and plated into the wells of a 24-well plate for metabolism studies with sampling of individual wells at 0, 6, 12, and 24 hr.

The significance of differences in the rate of retinoic acid metabolism following treatment with various potential inducers or inhibitors was investigated by comparison of the data generated using a series of inhibitors or inducers plotting ln (natural log, percentage retinoic acid remaining) against time by analysis of variance using the program SYSTAT general linear model option. The dependent variable was ln (percentage retinoic acid remaining), and time together with inducer or inhibitor were independent variables. If the overall *P* value for the treatment was less than 0.05, the effect of various individual treatments was assessed.

RESULTS

RA Metabolism in Human Liver Microsomes

In studies of all-*trans*-RA metabolism in human liver microsomes, it was found that it was possible to detect RA metabolism to more polar metabolites only when radiolabelled RA was used as substrate. The recovery of radioactivity was greater than 99%. Radiolabelled fractions corresponding to all-*trans*-RA eluted at 11.5 min, all-*trans*-4-hydroxy-RA at 4.0 min, and all-*trans*-4-oxo-RA at 3.5 min. Occasionally, 13-*cis*-RA was detected in small quantities (<3% of total radioactivity) eluting at 11.0 min. A typical profile is shown in Fig. 1. The appearance of polar metabolites was linear with respect to time (10 to 60 min) of incubation and protein amount (200 to 800 μg). In a typical incubation, only 5% of total all-*trans*-RA underwent conversion to the two polar metabolites. It has been previously demonstrated that conversion of 4-hydroxy-RA to 4-oxo-RA is not cytochrome P450-mediated and appears to involve a microsomal oxidoreductase active with either NAD or NADP as cofactor, though the precise enzyme involved in this conversion has not been identified [23]. The total radioactivity present in both 4-hydroxy and 4-oxo peaks therefore represented 4-hydroxylation activity within the microsomes. Estimates of K_m and V_{max} for the 4-hydroxylation of all-*trans*-RA by human liver microsomes prepared from 10 different subjects are shown in Table 1. Values of apparent K_m ranged from 0.37 μM (HLM7) to 0.95 μM (HLM1), with an overall mean value of 0.60 μM (standard deviation = 0.19). Similarly, a variation was seen in V_{max} values, which ranged from 5.90 pmol product/min/mg protein (HLM1) to 31.06 pmol product/min/mg protein (HLM2), while the ten HLM samples had an overall mean V_{max} of 14.66 pmol product/min/mg protein (standard deviation = 8.10). A typical Michaelis–Menten plot for all-*trans*-RA 4-hydroxylation is shown in Fig. 2.

The Effect of Chemical and Antibody Inhibitors of Cytochrome P450 Enzymes on All-trans-RA 4-Hydroxylation in Human Liver Microsomes

The effect of a range of chemical inhibitors specific for particular cytochrome P450 isoforms on all-*trans*-RA 4-hydroxylation was investigated for each of 10 human liver microsome preparations. Figure 3 shows the mean percentage inhibition achieved with the specific inhibitors. Highest inhibition (57.1%) was achieved with ketoconazole (20 μM), which indicates the participation of cytochromes P450 in RA metabolism, since it is a general P450 inhibitor at the concentration used [24]. When a lower ketoconazole concentration (1 μM) was used to more specifically detect CYP3A4 inhibition [24], the percentage inhibition decreased to 28.2%. Another specific CYP3A4 inhibitor, troleandomycin [25], gave a similar inhibition of 26.9%. Significant inhibition (46.2%) was also achieved with paclitaxel, which is a substrate for both CYP2C8 and CYP3A4 [26]. There was slight inhibition by both furafylline (50 μM) (specific for CYP1A2) and sulphaphenazole (5 μM) (specific for CYP2C9). Other P450 inhibitors used, namely quinidine (10 μM) (CYP2D6), methoxypsoralen (50 μM) (CYP2A6), chlorzoxazone (50 μM) (CYP2E1), and orphenadrine (100 μM) (CYP2B and other cytochromes P450), had no effect on all-*trans*-RA 4-hydroxylation.

Immunoinhibition studies were performed using antisera specific for particular cytochrome P450 enzymes in an attempt to confirm the results of the chemical inhibition studies. Due to the limited quantity of antisera available, studies were performed on sets of pooled microsomes. Inhibitions of approximately 40% and 30% were achieved with antisera specific for CYP2C8 and CYP3A, respectively (Table 2). When these antisera were added in combination, greater inhibition (approximately 50%) of all-*trans*-RA metabolism occurred compared to the effect of either antiserum used alone. Antisera specific for CYP2D6, CYP1A2, and CYP2E1 had no effect on all-*trans*-RA 4-hydroxylation.

Correlation of All-trans-RA 4-Hydroxylation with Isoform-Specific Activities and Protein Levels

The possibility of a correlation between RA-metabolising activity and several enzyme activities representative of

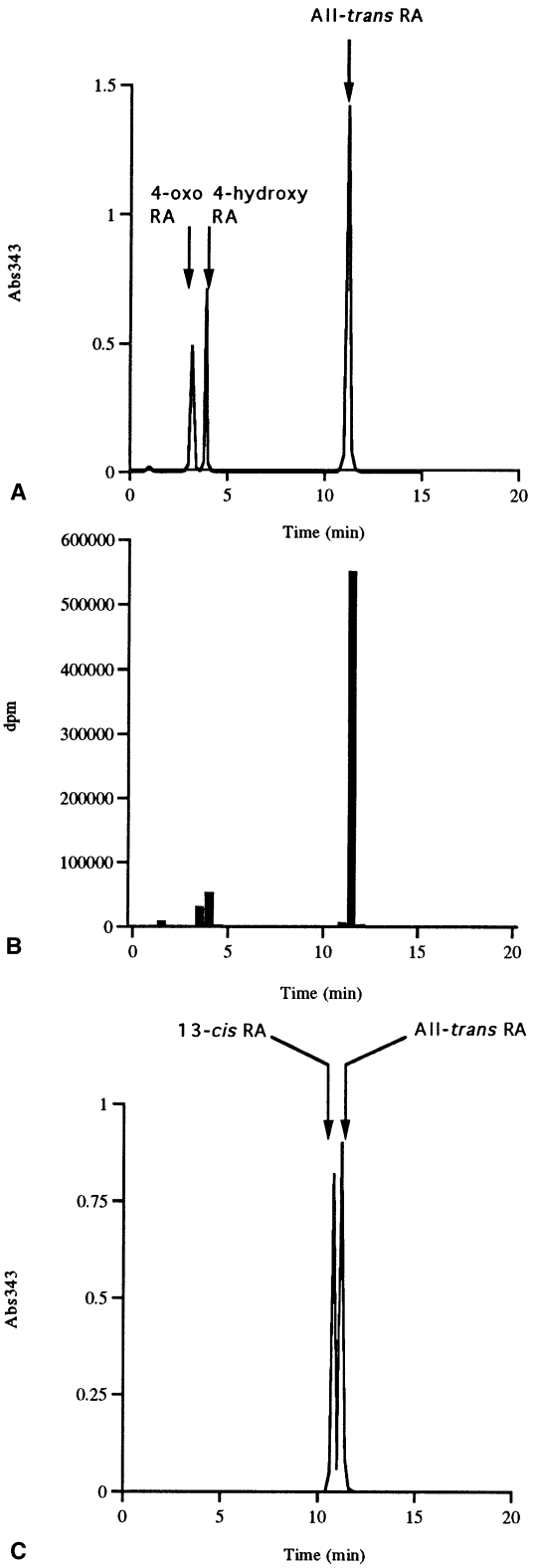

FIG. 1. Analysis of RA metabolites by HPLC. (A) UV absorbance trace (Abs) for unlabelled all-*trans*-RA, all-*trans*-4-hydroxy-RA, and all-trans-4-oxo-RA standards. (B) Typical radioactivity trace for incubation of human liver microsomes incubated with [^{3}H] all-*trans*-RA (5 μM) for 30 min. The dpm values are for 0.5-mL fractions. (C) UV absorbance trace (Abs) showing separation between unlabelled 13-*cis*- and all-*trans*-retinoic acid standards.

TABLE 1. 4-Hydroxylation of all-*trans*-RA by human liver microsomes

Human liver microsome preparation	K_m (μM)	V_{max} (pmol/min/mg protein)
1	0.95 ± 0.12	5.94 ± 0.34
2	0.72 ± 0.16	31.06 ± 2.92
3	0.68 ± 0.21	20.82 ± 2.68
4	0.46 ± 0.10	13.11 ± 1.10
5	0.47 ± 0.06	7.89 ± 0.36
6	0.58 ± 0.07	6.91 ± 0.32
7	0.37 ± 0.13	8.88 ± 1.13
8	0.38 ± 0.21	21.91 ± 4.26
9	0.82 ± 0.36	12.52 ± 2.44
10	0.55 ± 0.24	17.55 ± 2.97

Data were fitted to the Michaelis–Menten equation by non-linear regression. Values ± SEM are indicated.

specific P450 isoforms together with protein levels for CYP3A4 and CYP2C8 was assessed. Isoform-specific activities and protein levels together with total P450 levels for human liver microsomes from 10 different donors are shown in Table 3. When all-*trans*-RA 4-hydroxylase activities of the human liver microsomes were correlated with the activities of the microsomes with specific cytochrome P450 substrates, only very weak correlations were found with coumarin 7-hydroxylase (CYP2A6), diclofenac 4′-hydroxylase (CYP2C9), and bufuralol 1′-hydroxylase (CYP2D6). However, a weak to moderate correlation was seen with ethoxyresorufin O-deethylase activity, which in liver represents predominantly CYP1A2 (Table 4). In the case of CYP3A4 and CYP2C8, protein content was estimated by densitometric scanning of immunoblots of micro-

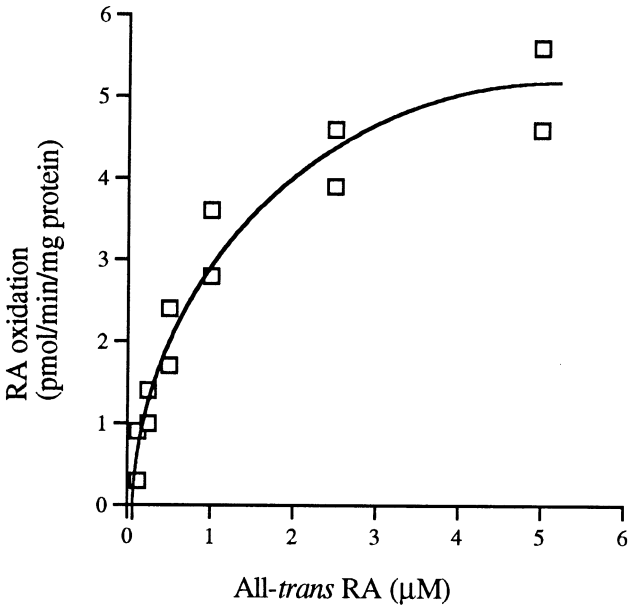

FIG. 2. Typical Michaelis–Menten plot of initial rate against substrate concentration for 4-hydroxylation of [^{3}H] all-*trans*-RA by human liver microsomes. The data were fitted to the Michaelis–Menten equation by non-linear regression.

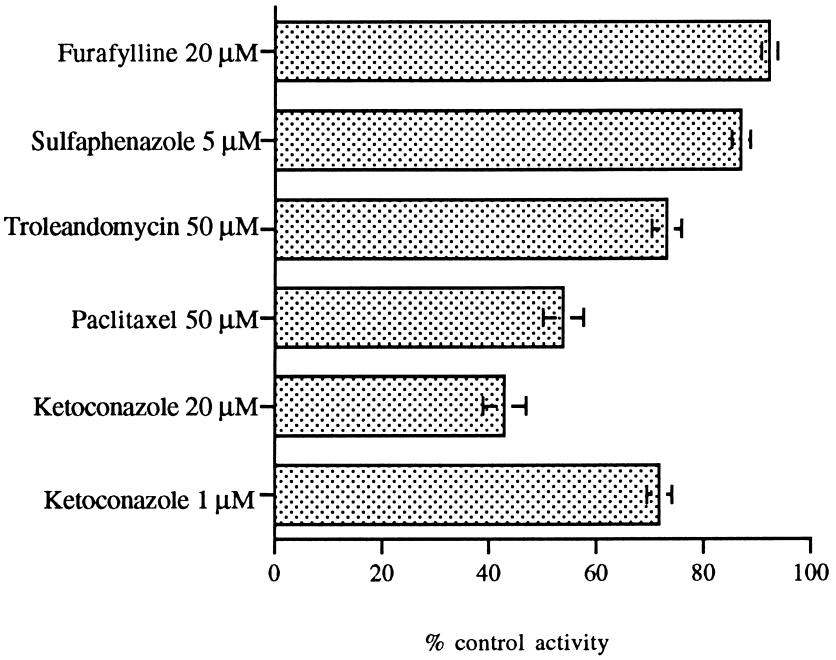

FIG. 3. Inhibition of all-*trans*-RA 4-hydroxylation in human liver microsomes. Inhibitors were used at the concentrations indicated. Microsomal protein (0.2 mg) was incubated with 10 μM all-*trans*-RA for 30 min. Control incubations were performed under identical conditions with an equal volume of the appropriate vehicle without inhibitor added. Inhibition values given are the means and standard deviations of results from 10 different microsome samples.

somal protein. As summarised in Table 4, when values of V_{max} for oxidation of all-*trans*-RA by HLM were compared with corresponding CYP2C8 protein levels, a strong correlation was found, with an r value of 0.81 suggesting a high likelihood of CYP2C8 involvement in the 4-hydroxylation of all-*trans*-RA. In addition, CYP3A4 content showed a strong correlation ($r = 0.76$) with all-*trans*-RA 4-hydroxylating activity, but a correlation was also detected between levels of CYP2C8 and CYP3A4 ($r = 0.79$). Multiple regression analysis of all the data suggested that the best fit was obtained by a model including both the CYP2C8 levels and the CYP1A2 activity levels ($P = 0.007$).

All-trans-RA Metabolism Studies in Lymphoblastoid Cell Microsomes

The ability of specific P450 isoforms to 4-hydroxylate all-*trans*-retinoic acid was examined by incubation with microsomes from human lymphoblastoid cells that overexpressed particular cytochrome P450 isoforms, namely CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4, together with control lymphoblastoid cell microsomes. CYP1A1, CYP2C8, CYP2C9, and CYP3A4 were found to metabolise all-*trans*-RA, and the radiolabelled metabolite fraction had retention times corresponding to all-*trans*-4-hydroxy-RA and all-*trans*-4-oxo-RA. Values of kinetic constants with their standard errors are given in Table 5. The CYP2C8 isoform exhibited the greatest activity for the oxidation of all-*trans*-RA, with a V_{max} value of 2.08 pmol/min/pmol P450 and K_m equal to 1.5 μM. Lower activities were found with the CYP3A4, CYP1A1, and CYP2C9 isoforms (V_{max} 0.04, 0.01, and 0.02 pmol/min/pmol P450, respectively) with corresponding K_m values of 2.6, 15.9, and 1.1 μM, respectively. CYP2C8 had the highest V_{max}/K_m value (1.41), followed by CYP3A4 and CYP2C9 (0.020). No metabolism of all-*trans*-RA was detected with microsomes from cells overexpressing the other cytochrome P450 isoforms studied or with control lymphoblastoid cell microsomes.

TABLE 2. The effect of specific cytochrome P450 antibodies on all-*trans*-RA 4-hydroxylation

	% Inhibition	
Inhibitor	Set 1 Range (N = 2)	Set 2 Range (N = 2)
Control (anti-CYP2D6)	0	0
Anti-CYP2C13	38.8–41.6	36.7–39.3
Anti-CYP3A4	25.4–28.8	27.5–32.3
Anti-CYP2C13 + anti-CYP3A4	49.0–52.6	49.9–53.3

Immunoinhibition studies were performed on 2 sets of pooled microsomes, 4 microsome samples per set equalised for protein content. Set 1 consisted of microsomes HLM1, HLM2, HLM4, and HLM9 and set 2 microsomes HLM3, HLM5, HLM7, and HLM10.

Studies in All-trans-RA Metabolism in HepG2 Cells

Incubation of HepG2 cells with radiolabelled all-*trans*-RA resulted in metabolism of all-*trans*-RA to more polar compounds over the time points studied. The rate of disappearance of all-*trans*-RA was 10.2 ± 0.6 pmol/10^5 cells/24 hr, with the half-life 23.1 ± 1.5 hr in cells which had not been treated with inducers or inhibitors.

The effect of no pretreatment and pretreatment of cells with all-*trans*-RA and the CYP1A inducer 3-MC on the half-life of all-*trans*-RA was examined. The results with the rate of RA disappearance expressed as half-life are shown in

TABLE 3. Characterisation of cytochrome P450 isoform activities and levels in human liver microsomes

Human liver microsome number	P450 (nmol/mg/ protein)	CYP1A2 activity (pmol/min/mg)	CYP2A6 activity (nmol/min/mg)	CYP2C9 activity (nmol/min/mg)	CYP2D6 activity (nmol/min/mg)	CYP3A4 levels (pmol/mg protein)	CYP2C8 levels (pmol/mg protein)
1	0.21	15.1	0.12	4.52	0.35	39.92	4.84
2	0.58	56.8	0.77	4.68	0.26	152.38	14.54
3	0.47	50.1	0.14	7.53	0.24	86.86	8.13
4	0.64	44.8	0.60	10.15	0.13	44.11	2.57
5	0.43	38.3	0.86	7.03	0.09	105.3	4.95
6	0.35	30.5	0.48	4.58	0.68	49.54	2.39
7	0.72	58.7	1.58	3.10	0.44	82.53	3.35
8	0.70	41.1	1.40	4.45	0.38	130.5	18.74
9	0.30	27.0	0.61	3.22	0.79	93.35	4.78
10	0.33	24.5	1.05	9.40	0.23	82.42	8.74

CYP1A2 activity is the rate of ethoxyresorufin O-deethylation, CYP2A6 activity is the rate of coumarin 7-hydroxylation, CYP2D6 activity is the rate of bufuralol 1-hydroxylation, and CYP2C9 activity is the rate of diclofenac 4-hydroxylation, while CYP3A4 and CYP2C8 were determined by immunoblotting. The values given are the means of duplicate determinations.

Table 6. Comparison of the rate of disappearance of RA by analysis of variance showed that there were significant differences between the various treatments (P value 0.00004). For the individual treatments, all-*trans*-RA treatment gave a significant difference when compared with untreated cells, but 3-MC did not.

The effect of ketoconazole, at concentrations of both 2 and 20 μM, on the metabolism of all-*trans*-RA was also examined in both uninduced HepG2 cells and following treatment with inducers. The results are summarised in Table 7. We found that ketoconazole at either concentration was not a strong inhibitor of RA metabolism in untreated cells (13.3% inhibition at 2 μM and 30.0% at 20 μM); however, following treatment with all-*trans*-RA, there was a 2.1-fold increase in the rate of metabolism, with this metabolism being highly sensitive to ketoconazole at both 2 and 20 μM (42.6% and 51.5% inhibition, respectively). A similar effect was not seen in 3-MC-treated cells, where the ketoconazole inhibition pattern was broadly similar to that observed for untreated cells. Treatment of HepG2 cells with 3-MC gave an approx. 10-fold induction of CYP1A1 levels by immunoblotting, but no induction of CYP1A1, CYP2C8, CYP2C9, or CYP3A4 was detected in all-*trans*-RA-treated cells.

DISCUSSION

Using the three complementary approaches of inhibition studies with chemical inhibitors and antibodies, correlation analysis on human liver microsomes characterised for specific P450 isoforms, and kinetic studies with cDNAs expressed in lymphoblastoid cells, we have obtained evidence that two cytochrome P450 isoforms, CYP2C8 and CYP3A4, make an important contribution to the 4-hydroxylation of all-*trans*-RA in human liver. Although the turnover number for CYP2C8 was found to be 50-fold higher than that for CYP3A4, immunoblotting studies from the present study together with data from other reports suggest that CYP2C8 represents only approx. 2% of total P450 in human liver, whereas CYP3A4 represents approx. 20 to 30% [27]. The 10-fold difference in abundance means that CYP3A4 makes a contribution to all-*trans*-RA metab-

TABLE 4. Correlation between V_{max} values for 4-hydroxylation of all-*trans*-RA and activity or content of specific cytochrome P450 isoforms in 10 human liver microsome preparations

	V_{max} all-*trans*-RA	CYP1A2	CYP2A6	CYP2C9	CYP2D6	CYP2C8	CYP3A4
V_{max} all-*trans*-RA	$r = 1$						
CYP1A2	$r = 0.492$	$r = 1$	—	—	—	—	—
CYP2A6	$r = 0.134$	$r = 0.402$	$r = 1$	—	—	—	—
CYP2C9	$r = 0.107$	$r = -0.039$	$r = -0.197$	$r = 1$	—	—	—
CYP2D6	$r = -0.255$	$r = -0.287$	$r = -0.024$	$r = -0.710$†			
CYP2C8	$r = 0.812$*	$r = 0.240$	$r = 0.335$	$r = -0.115$	$r = -0.181$	$r = 1$	—
CYP3A4	$r = 0.761$†	$r = 0.494$	$r = 0.453$	$r = -0.256$	$r = -0.124$	$r = 0.796$*	$r = 1$

Pearson correlations (r) were performed between mean values in human liver microsomes of V_{max} for retinoic acid (RA) 4-hydroxylation and mean cytochrome P450 activity or content for cytochromes P450 CYP1A2, CYP2A6, CYP2C9, CYP2D6, CYP2C8, and CYP3A4 for 10 human liver microsome preparations.

*Correlation is significant at the 0.01 level; †Correlation is significant at the 0.05 level.

TABLE 5. Kinetic constants for all-*trans*-RA 4-hydroxylation by microsomes from lymphoblastoid cells expressing specific cytochrome P450 isoforms

P450 isoform	K_m (μM)	V_{max} (pmol product/min/pmol P450)	V_{max}/K_m (μL/min/pmol)
CYP1A1	15.9 ± 10.2	0.01 ± 0.004	0.0008 ± 0.0003
CYP2C8	1.4 ± 0.3	2.08 ± 0.20	1.41 ± 0.39
CYP2C9	1.1 ± 0.7	0.02 ± 0.01	0.02 ± 0.006
CYP3A4	2.6 ± 1.0	0.04 ± 0.01	0.02 ± 0.008

Data were fitted to the Michaelis–Menten equation by non-linear regression. Values ± SEM are indicated.

olism, as is confirmed by the inhibition studies with troleandomycin, ketoconazole, and anti-CYP3A antibodies. Together, CYP2C8 and CYP3A4 appear to account for 50 to 60% of all 4-hydroxylation of all-*trans*-RA in human liver. The next most important enzyme appears to be CYP2C9, but the contribution by this enzyme appears small, probably not more than 5 to 10% on the basis of the sulphaphenazole inhibition studies, but this would be consistent with the relatively small amount of this isoform present in most human livers [27]. Some evidence for a minor role for CYP1A2 was suggested by the correlation and furafylline inhibition studies, but this was not confirmed when lymphoblastoid cell microsomes overexpressing CYP1A2 were analysed. Our findings are broadly in line with those reported in a very recent study indicating that CYP2C8 is the major isoform catalysing all-*trans*-RA 4-hydroxylation, with some contributions by CYP3A4 and CYP2C9 as well [28].

We also obtained evidence that CYP1A1 can 4-hydroxylate all-*trans*-RA, though the K_m was approximately 10-fold higher than that for the other three enzymes. This finding is of interest in view of a report by Vecchini *et al.* [29] of the presence of a retinoic acid responsive element in the CYP1A1 promoter. CYP1A1 is not expressed at significant levels in the majority of human livers, though it is a major isoform in some extrahepatic tissues including skin, where RA may contribute to normal development and tissue maintenance. In the case of HepG2 cells, culturing in the presence of polycyclic aromatic hydrocarbons such as 3-MC increases levels of CYP1A1 [30, 31]. However, we found no evidence that treatment with 3-MC resulted in an increased rate of all-*trans*-RA metabolism in HepG2 cells,

TABLE 6. Effect of inducers on all-*trans*-RA metabolism in HepG2 cells

Inducer	Half-life of all-*trans*-RA (hr)	Fold induction
None	23.0 ± 1.5	(1)
All-*trans*-RA	10.8 ± 0.3*	2.12
3-MC	22.3 ± 2.2	1.03

Half-lives were determined by linear regression and values ± SEM are indicated.
*Significantly different from uninduced level by multiple regression analysis ($P = 0.001$).

TABLE 7. Effect of ketoconazole on all-*trans*-RA metabolism by HepG2 cells

Inducer	% Inhibition by ketoconazole 2 μM	% Inhibition by ketoconazole 20 μM
None	13.3% ± 1.3%	30.0% ± 3.4%
All-*trans*-RA	40.6% ± 3.3%*	51.5% ± 2.9%*
3-Methylcholanthrene	16.7% ± 2.32%	26.7% ± 3.8%

*Significantly different from extent of inhibition in uninduced cells.

possibly because the K_m for all-*trans* 4-hydroxylation is higher for CYP1A1 than for the other P450 isoforms showing activity, and we also failed to detect significant induction of CYP1A1 by all-*trans*-RA in HepG2 cells or other cultured cells.* Whether CYP1A1 makes a biologically significant contribution to RA oxidation in cells other than HepG2 remains unclear.

The precise contribution by the apparently specific RA-metabolising enzyme CYP26 is more difficult to assess, as it has not yet been fully characterised with regard to its inhibitor specificity. However, a recent study of CYP26 in human keratinocytes showed that the IC_{50} for CYP26 inhibition by ketoconazole was approx. 1 μM [32], a value only slightly higher than that of 0.2 μM previously reported for CYP3A4 [24]. In the present study, the extent of inhibition by 1 μM ketoconazole, troleandomycin, and anti-CYP3A of all-*trans*-RA 4-hydroxylation in human liver appeared similar, suggesting that all the activity inhibited by 1 μM ketoconazole is due to CYP3A rather than CYP26. This would be consistent with several studies of CYP26 that suggest that levels are normally very low in a number of tissues and cell types including rat liver and HepG2 cells, but that treatment with RA induces enzyme expression [11, 33]. We have investigated the possible involvement of CYP26 indirectly by use of HepG2 cells and found a twofold autoinduction of all-*trans*-RA metabolism in these cells that was highly sensitive to inhibition by ketoconazole, but we found no evidence for CYP3A induction by immunoblotting, making it possible that the ketoconazole-sensitive enzyme induced is CYP26. In general, all-*trans*-RA metabolism in HepG2 cells that had not been pretreated with all-*trans*-RA was less sensitive to ketoconazole inhibition than liver microsomes, but this may be due to lower levels of cytochrome P450 expression in HepG2 cells than in human liver.

Under normal physiological conditions, most retinoic acid will be formed intracellularly from retinol or β-carotene with plasma levels of all-*trans*-retinoic acid in the range 1 to 10 nM [34]. Intracellular levels are unclear but may be higher than plasma levels, with the majority bound to cellular retinoic acid binding protein (CRABP) in many cell types [3]. In hepatocytes, most all-*trans*-RA oxidation may be catalysed by CYP2C8 and CYP3A4, since levels of each are probably higher than uninduced CYP26, and RA

* McSorley LC and Daly AK, unpublished observations.

levels are unlikely to be sufficiently high for CYP26 induction. It has been reported that the approximate K_m for all-*trans*-RA oxidation by CYP26 is 1 μM, which is comparable to that obtained for CYP2C8 in the present study; the enzymes may thus be kinetically quite similar, though complete information on the catalytic efficiency of CYP26 is not yet available [11]. Levels of CYP2C8 and CYP3A4 will be lower in extrahepatic cells, but may still be sufficient to make a contribution to the metabolism of locally formed all-*trans*-retinoic acid. When high doses of all-*trans*-RA are used therapeutically, it is likely that CYP26 or other RA-inducible P450s will make an important contribution to metabolism because of their induction, though metabolism by the other P450s may also continue to occur. Plasma levels of all-*trans*-RA may be as high as 10 μM during therapeutic use [35] and levels within the liver may exceed this, especially during first-pass metabolism. It is therefore possible that coadministration of all-*trans*-RA with other drugs metabolised by CYP3A4 or CYP2C8 may result in drug interactions. The number of commonly prescribed drugs known to be metabolised by CYP2C8 is limited but includes paclitaxel and verapamil [26, 36]. A recent study has shown that all-*trans*-RA is a potent inhibitor of paclitaxel metabolism by both human liver microsomes and expressed CYP2C8 [37]. CYP3A4 is the major isoform for the oxidation of a wide variety of commonly prescribed drugs [38] and therefore drug interactions involving all-*trans*-RA and a large number of drugs may be possible.

Resistance at the target site due to increased oxidative metabolism both locally and in the liver is an important problem when all-*trans*-RA is used clinically. Strategies involving coadministration of metabolic inhibitors such as ketoconazole have been described [35] and the present study indicates that this may be an effective approach for the inhibition of 4-hydroxylation, since all the major isoforms contributing to this reaction are ketoconazole-sensitive though varying in their precise sensitivity. Use of other RA isomers such as 9-*cis* may also be advantageous, as they have been reported to undergo less oxidative metabolism than the all-*trans*-isomer, possibly because of differences in their transport intracellularly due to low affinity for CRABP, the cellular retinoic acid binding protein [3].

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