

# Identification of Human Cytochrome P450s Involved in the Formation of All-*trans*-Retinoic Acid Principal Metabolites

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## ABSTRACT

Cytochrome P450 (P450)-dependent metabolism of all-*trans*-retinoic acid (atRA) is important for the expression of its biological activity. Because the human P450s involved in the formation of the principal atRA metabolites have been only partially identified, the purpose of this study was to identify the human P450s involved in atRA metabolism. The use of phenotyped human liver microsomes ( $n = 16$ ) allowed the identification of the following P450s: 2B6, 2C8, 3A4/5, and 2A6 were involved in the formation of 4-OH-RA and 4-oxo-RA; 2B6, 2C8, and 2A6 correlated with the formation of 18-OH-RA; and 2A6, 2B6, and 3A4/5 activities correlated with 5,6-epoxy-RA formation (30-min incubation, 10  $\mu$ M atRA, HPLC separation, UV detection 340 nm). The use of 15 cDNA-expressed human P450s from lymphoblast microsomes, showed the formation of 4-OH-RA by CYP3A7 > CYP3A5 > CYP2C18 > CYP2C8 > CYP3A4 > CYP2C9, whereas the 18-OH-RA formation involved CYPs 4A11 > 3A7 > 1A1 > 2C9 > 2C8 > 3A5 > 3A4 > 2C18. Kinetic studies identified 3A7 as the most active P450 in the formation of three of the metabolites: for 4-OH-retinoic

acid, 3A7 showed a  $V_{\max}/K_m$  of 127.7, followed by 3A5 ( $V_{\max}/K_m = 25.6$ ), 2C8 ( $V_{\max}/K_m = 24.5$ ), 2C18 ( $V_{\max}/K_m = 15.8$ ), 3A4 ( $V_{\max}/K_m = 5.7$ ), 1A1 ( $V_{\max}/K_m = 5.0$ ), and 4A11 ( $V_{\max}/K_m = 1.9$ ); for 4-oxo-RA, 3A7 showed a  $V_{\max}/K_m$  of 13.4, followed by a 10-fold lower activity for both 2C18 and 4A11 ( $V_{\max}/K_m = 1.2$ ); and for 18-OH-RA, 3A7 showed a  $V_{\max}/K_m$  of 10.5 compared with a  $V_{\max}/K_m$  of 2.1 for 4A11 and 2.0 for 2C8. 5,6-Epoxy-RA was only detected at high substrate concentrations in this system ( $>10 \mu$ M), and P450s 2C8, 2C9, and 1A1 were the most active in its formation. The use of embryonic kidney cells (293) stably transfected with human P450 cDNA confirmed the major involvement of P450s 3A7, 1A1, and 2C8 in the oxidation of atRA, and to a lesser extent, 1A2, 2C9, and 3A4. In conclusion, several human P450s involved in atRA metabolism have been identified, the expression of which was shown to direct atRA metabolism toward the formation of specific metabolites. The role of these human P450s in the biological and anticancer effects of atRA remains to be elucidated.

Retinoids (vitamin A and its derivatives, which include atRA) play a central role in embryogenesis, vertebrate development, differentiation, and homeostasis (Gudas et al., 1994), and have been used in the prevention and the treatment of certain types of cancer (Hong and Itri, 1994). In humans, retinoids are obtained in the diet in the form of carotenoids (provitamin A), or preformed retinoids. Retinol (vitamin A) is the major retinoid absorbed after complex metabolic reactions in the intestines, and is stored in esterified form in the liver. After ester hydrolysis, retinol is then transported by a plasma protein (retinol binding protein) to the tissues where it can exert its activity. A small fraction of

the plasma retinoids is available in the form of atRA bound to albumin, which is rapidly taken up by tissues (Blaner and Olson, 1994). The conversion of retinol to retinal by the P450s is considered to be the rate-limiting step for the biosynthesis of atRA, and, in addition to retinol dehydrogenases, P450s 1A1, 1A2, 1B1, and 3A4 have recently been shown to be the major human P450s that catalyze this reaction (Chen et al., 2000).

At the cellular level, atRA passively crosses the plasma membrane and is translocated by cellular retinoic acid binding protein type I or II to the nucleus where it can bind to nuclear receptors. The nuclear receptors are of two types: the RARs and the RXRs, each comprising three subtypes ( $\alpha$ ,  $\beta$ ,  $\gamma$ ). The RARs, which function as ligand-inducible transcriptional regulators, heterodimerize with RXR (Mangelsdorf and Evans, 1995) and are activated by both atRA and 9-*cis*-RA. The RXRs can also homodimerize, and act as transcriptional

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**ABBREVIATIONS:** atRA, all-*trans*-retinoic acid; P450, cytochrome P450; RAR, retinoic acid receptor; RXR, retinoid X receptor; 9-*cis*-RA, 9-*cis*-retinoic acid; 4-OH-RA, 4-hydroxy-retinoic acid; 4-oxo-RA, 4-oxo-retinoic acid; 18-OH-RA, 18-hydroxy-retinoic acid; 5,6-epoxy-RA, 5,6-epoxy-retinoic acid; 13-*cis*-RA, 13-*cis*-retinoic acid.

regulators under certain conditions, and are only activated by 9-*cis*-RA (Heyman et al., 1992; Mangelsdorf and Evans, 1995). Biological responses to retinoids may therefore be modulated by the availability of a specific ligand, and also by the type of nuclear receptors available. In addition to the nuclear receptor-mediated responses to retinoids, it has also been suggested that retinoylation, or covalent binding of retinoids to specific proteins, may also play a role in cellular response to atRA (Takahashi and Breitman, 1990).

AtRA is considered the active form of vitamin A and is involved in gene regulation, leading to differentiation and apoptosis of normal and cancer cells (Gudas et al., 1994). AtRA undergoes P450-mediated metabolism to oxidized compounds that include 4-OH-RA, 4-oxo-RA, 18-OH-RA, and 5,6-epoxy-RA (Napoli, 1999), which may also be glucuronidated by UGT2B7 (Samokyszyn et al., 2000). Metabolism appears to play a key role in the cellular resistance to atRA (Agadir et al., 1995; Kizaki et al., 1996) as well as in the pharmacokinetic resistance seen after several administrations due to an increase in systemic clearance via induced hepatic enzymes (Muindi et al., 1992; Lazzarino et al., 1996). Although the metabolism of atRA has been considered to be a catabolic process, some of its oxidized metabolites have been shown to display biological activity in the modulation of genes expressed in apoptosis, cellular growth and differentiation, embryonic development, and in the inhibition of proliferation of several normal and neoplastic cells in vitro (De Luca, 1991; Reynolds et al., 1993; Ramp et al., 1994; Carter et al., 1996; Braakhuis et al., 1997; Van heusden et al., 1998; Idres et al., 2000). It has also been proposed that atRA metabolism may be linked to its growth inhibitory effects because the most sensitive cell lines are those that metabolize atRA the most efficiently (Takatsuka et al., 1996; van der Leede et al., 1997).

Although atRA metabolism appears to play a central role in its molecular mechanism of action, both in terms of sensitivity and resistance, knowledge of the human enzymes involved in its metabolism is limited (Napoli, 1999). Studies using animal material have shown P450s to be important in the oxidation of atRA (Roberts et al., 1979a,b, 1980; Frolik et al., 1980), and several P450s have been identified in rabbits (Roberts et al., 1992; Raner et al., 1996). In humans, only a few P450s have been ascribed to atRA metabolism [e.g., CYP2C8 (Leo et al., 1989; Nadin and Murray, 1999) and CYP26 (Ray et al., 1997; White et al., 1997)].

The purpose of this study was to systematically determine the human P450s involved in atRA metabolism. Three methods were used: 1) correlation of atRA metabolite formation with P450-specific activity of human liver microsomes, 2) determination of the activity of cDNA expressed human P450s in lymphoblast microsomes, and 3) determination of the atRA metabolic activity of human embryonic kidney cells (293) stably transfected with a single human P450 cDNA. Our results show that, of the 15 human P450s investigated, several are significantly involved in atRA metabolism (CYP1A1, CYP2C8, CYP2C9, CYP2C18, CYP3A4, CYP3A5, CYP3A7, and CYP4A11), several of which were previously unidentified in atRA biotransformation (Nadin and Murray, 1999). In addition, the formation of 4-OH-RA and 18-OH-RA was found to be catalyzed more actively by CYP3A7 than by CYP2C8. Moreover, certain P450s were found to direct the metabolism of atRA toward the formation of specific metab-

olites, suggesting that P450-related metabolism plays an important role in retinoid signaling.

## Materials and Methods

**Chemicals.** atRA, 9-*cis*-RA, 13-*cis*-RA, and NADPH were purchased from Sigma-Aldrich (St-Quentin Fallavier, France). AtRA metabolites (4-OH-RA, 4-oxo-RA, 18-OH-RA, and 5,6-epoxy-RA) were kindly provided by Dr. Louise H. Foley (Hoffmann-La Roche, Inc., Nutley, NJ) and Dr. Michael Klaus (Hoffmann-La Roche, Ltd., Basel, Switzerland). Stock solutions of retinoids ( $10^{-2}$  M) were prepared in ethanol and stored at  $-20^{\circ}\text{C}$ . For microsomal incubations, atRA was further diluted in incubation buffer to  $10^{-4}$  M. The final ethanol concentration in incubation mixture was 0.1%. NADPH (12 mM) was freshly prepared before addition to the incubation buffer.

**AtRA Metabolism by Human Liver Microsomes.** Microsomal preparations from 16 human livers were phenotyped using the following P450-specific activities (XenoTech, Kansas City, KS): CYP1A2, 7-ethoxyresorufin *O*-dealkylation; CYP2A6, coumarin 7-hydroxylation; CYP2B6, 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylation, and *S*-mephenytoin *N*-demethylation; CYP2C8, paclitaxel 6 $\alpha$ -hydroxylation; CYP2C9, tolbutamide methyl-hydroxylation; CYP2C19, *S*-mephenytoin 4'-hydroxylation; CYP2D6, dextromethorphan *O*-demethylation; CYP2E1, chlorzoxazone 6-hydroxylation; CYP3A4/5, testosterone 6 $\beta$ -hydroxylation; and, CYP4A9/11, lauric acid 12-hydroxylation. The microsomal preparation was resuspended in potassium phosphate buffer (50 mM, pH 7.4) containing 1 mM EDTA and 3 mM  $\text{MgCl}_2$  at a final protein concentration of 0.25 mg/ml in a total volume of 0.5 ml. After a 3-min preincubation with 10  $\mu\text{M}$  atRA at  $37^{\circ}\text{C}$ , the reaction was initiated by the addition of NADPH at a final concentration of 1.2 mM. Reactions were carried out for 30 min in a shaking water bath, and stopped by the addition of 1 ml of ethyl acetate on ice. Tubes were vortexed for 2 min and centrifuged at 3500g for 15 min at  $4^{\circ}\text{C}$ . A second extraction procedure was carried out with 1 ml of ethyl acetate. The organic phases were pooled and evaporated under vacuum (Speed-Vac). The dry residue was kept at  $-80^{\circ}\text{C}$  until HPLC analysis. Under these conditions the atRA extraction was  $80 \pm 2\%$ . All procedures were carried out in the dark or in subdued lighting.

**AtRA Metabolism Using cDNA-Expressed Human P450s.** Microsomal preparations from human lymphoblasts transfected with the following human P450 cDNAs were used (Gentest Corp., Woburn, MA): 1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, and 4A11. Control microsomes were prepared from cells transfected with the expression vector without insert. The incubation and extraction conditions used were the same as described above for human liver microsomes.

**AtRA Metabolism by cDNA-Expressed Human P450s in 293 Cells.** Native 293 cells (American Type Culture Collection, Manassas, VA), also referred to as human embryonic kidney 293 or Ad293 cells), or 293 cells stably transfected with cDNA of human P450s 1A1, 1A2, 2C8, 2C9, 3A4, 3A5, or 3A7 inserted in the expression vector pMT2, and NADPH-cytochrome P450 reductase cDNA (Lacroix et al., 1997) were grown in Dulbecco's modified Eagle's medium with 25 mM glucose supplemented with 10% (v/v) fetal calf serum under 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . For the metabolism studies, cells were seeded at a concentration of  $6 \times 10^5$  cells/ml and incubated with 10  $\mu\text{M}$  atRA for 24 h. The culture medium was extracted twice with ethyl acetate and analyzed for the presence of atRA metabolites as described below. Total cell protein content was determined using the bicinchoninic acid assay (Smith et al., 1985) (Bio-Rad, Richmond, CA).

**HPLC Analysis.** Dry residues from ethyl acetate extractions were resuspended in 200  $\mu\text{l}$  of methanol. The reaction products were quantitatively analyzed using an HPLC system consisting of a Varian 5000 liquid chromatograph, a reversed phase analytical column (Ultrasphere 5  $\mu\text{m}$ ,  $4.6 \times 250$  mm; Beckman Instruments, Columbia, MD), a Waters 484 tunable absorbance detector set at 340 nm, and a Varian 4400 integrator. Elution was performed using a linear gra-

dient with solvent A (1% ammonium acetate in water) and solvent B (methanol) as follows: after equilibration at 65% B and 35% A, %B was increased to 100% in 35 min, and then reequilibrated to initial conditions in 5 min at a flow rate of 1 ml/min. The atRA metabolites were identified by comparison to elution times and UV spectra of standard metabolites (Uvikon 923 spectrophotometer; Kontron, Zurich, Switzerland). Metabolites were quantified by comparison to a calibration curve of standards.

**Statistical Analyses.** Statistical significance ( $P \leq .05$ ) of the correlation coefficients between P450-specific activities and the formation of a given atRA metabolite was determined using a two-tailed Student's  $t_r$  test.  $V_{\max}$  and  $K_m$  values were estimated from the linear regression analysis of the Lineweaver-Burk transformation using the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

## Results

### Determination of Optimal Incubation Conditions.

For the *in vitro* formation of atRA metabolites, we first determined optimal incubation times, protein, and substrate concentrations using a pool of human liver microsomes. The production of metabolites was linear up to 45 min at protein concentrations up to 1 mg. By varying the atRA concentration it was possible to calculate a  $K_m$  value for the production of individual metabolites at approximately 15  $\mu\text{M}$ . To ensure a suitable yield of metabolite formation in further experiments, we routinely incubated 0.25 mg of protein/ml with 10  $\mu\text{M}$  atRA for 30 min. atRA was stable under these conditions, with minimal (2%) isomerization to 13-*cis*-RA and 9-*cis*-RA (data not shown).

**atRA Metabolite Separation and Identification.** Figure 1 shows the separation of atRA metabolites using reversed phase HPLC with gradient elution. Under these conditions, the metabolites were resolved and identified as 4-oxo-RA (9.9 min), 4-OH-RA (11.4 min), 18-OH-RA (13.5 min), and 5,6-epoxy-RA (16.5 min). The elution times for the retinoic acid isomers 13-*cis*-RA, 9-*cis*-RA, and atRA were 25, 26, and 27 min, respectively. Identity of the atRA metabolites was ascertained by comparing retention times and UV spectra of the material in individual peaks with those of authentic standards.

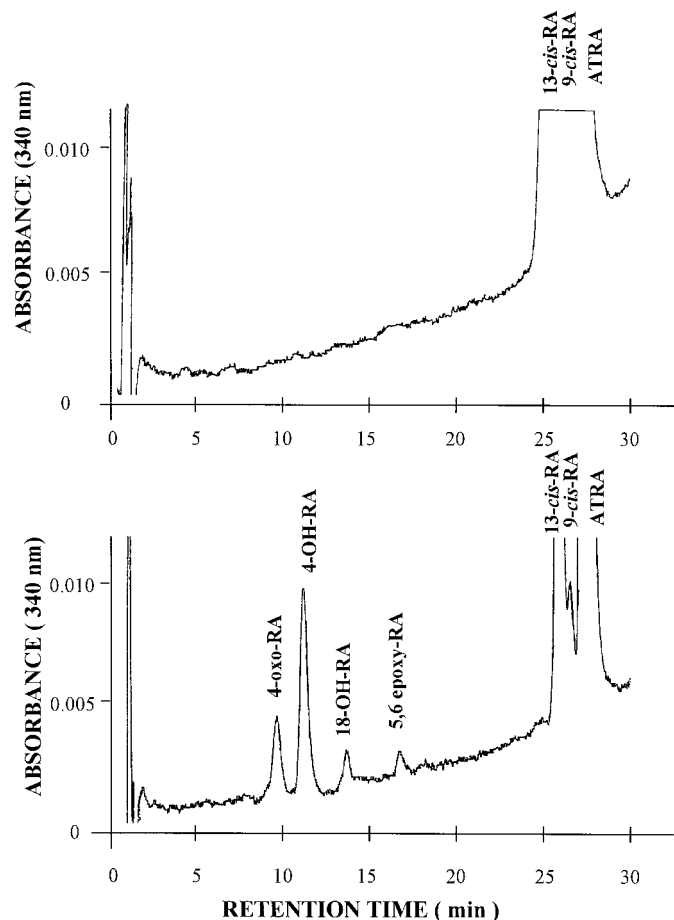
**Correlation of atRA Metabolite Formation with P450-Specific Activity.** To determine the human P450s involved in atRA metabolism, we used microsomal preparations from 16 different livers phenotyped for specific P450-dependent activities. The significant correlation coefficients between P450-dependent activity and formation of single atRA metabolites are presented in Table 1. Formation of 4-OH-RA and 4-oxo-RA was correlated with P450s 2B6 > 2C8 > 3A4/5 > 2A6. Hydroxylation at carbon 18 (18-OH-RA) was correlated with P450s 2C8, 2B6, and 2A6. Formation of 5,6-epoxy-RA was significantly correlated with P450s 2B6, 2A6, and 3A4/5. Figure 2 shows the most significant correlations observed between P450-dependent activity and formation of the 4 atRA metabolites.

**Correlation between atRA Metabolite Formation.** To assess whether the atRA metabolites were produced by the same P450s in liver microsomes, we carried out a linear regression analysis between each pair of metabolites. As expected, 4-OH-RA and 4-oxo-RA formation was highly correlated ( $r = 0.79$ ,  $P < .0001$ ); 4-OH-RA also correlated with 18-OH-RA formation ( $r = 0.55$ ,  $P < .001$ ). 4-oxo-RA corre-

lated also significantly with the formation of 18-OH-RA ( $r = 0.79$ ,  $P < .0001$ ), and 5,6-epoxy-RA ( $r = 0.37$ ,  $P = .03$ ). Finally, 18-OH-RA and 5,6-epoxy-RA formation were correlated ( $r = 0.43$ ,  $P < .01$ ).

### atRA Metabolism by cDNA-Expressed Human P450s.

To investigate atRA metabolism by individual P450s, we used microsomes from lymphoblasts expressing single human P450s. Figure 3 shows the formation rate of 4-OH-RA and 18-OH-RA by microsomes from P450-transfected lymphoblasts. Control microsomes prepared from the parent cell line transfected with the empty vector did not show any detectable metabolite. Of the 15 different P450s tested, several were significantly involved in the formation of 4-OH-RA and/or 18-OH-RA. The most active P450s in the formation of 4-OH-RA (Fig. 3A) were 3A7 ( $1500 \pm 165$  pmol/min/nmol P450), followed by 3A5 ( $553 \pm 27$ ), 2C18 ( $317 \pm 18$ ), 2C8 ( $272 \pm 20$ ), 3A4 ( $112 \pm 57$ ), and 2C9 ( $105 \pm 26$ ). The most active P450s in 18-OH-RA formation (Fig. 3B) were 4A11 ( $64 \pm 13$ ), 3A7 ( $53 \pm 10$ ), and 1A1 ( $50 \pm 2$ ), followed by 2C9 ( $27 \pm 1$ ), 2C8 ( $24 \pm 4$ ), 3A5 ( $14 \pm 2$ ), 3A4 ( $10 \pm 10$ ), and 2C18 ( $7 \pm 0$ ); 2B6 and 2E1 also showed marginal activity in the



**Fig. 1.** HPLC separation of atRA metabolites. Representative chromatogram showing the separation of atRA metabolites from atRA and its stereoisomers 13-*cis*-RA and 9-*cis*-RA using reversed phase HPLC with gradient elution. AtRA (10  $\mu\text{M}$ ) was incubated in a phosphate buffer containing a human liver microsomal preparation (0.25 mg protein/ml) in the absence (top) or the presence (bottom) of 1.2 mM NADPH for 30 min at 37°C in a total volume of 0.5 ml. The reaction mixture was extracted twice with 1 ml of ethyl acetate. The organic phase was evaporated and the dry residue resuspended in 200  $\mu\text{l}$  of methanol and injected onto the HPLC system described under *Materials and Methods*.



TABLE 1

Correlation between P450-specific activity and the formation of atRA metabolites by human liver microsomes

atRA metabolites formed in vitro using human hepatic microsomes from 16 different livers incubated for 30 min at a substrate concentration of 10  $\mu$ M. Results are from three independent determinations.

Metabolite	Cytochrome P450 <sup>a</sup>	Correlation Coefficient ( <i>r</i> )	P <sup>b</sup>
4-OH-RA	CYP2B6 (S-MP)	0.95	<0.001
	CYP2B6 (EFC)	0.79	<0.001
	CYP2C8	0.75	<0.001
	CYP3A4/5	0.73	<0.01
	CYP2A6	0.63	<0.01
4-oxo-RA	CYP2B6 (S-MP)	0.90	<0.001
	CYP2C8	0.77	<0.001
	CYP2B6 (EFC)	0.66	<0.01
	CYP3A4/5	0.64	<0.01
	CYP2A6	0.63	<0.01
18-OH-RA	CYP2C8	0.74	<0.01
	CYP2B6 (S-MP)	0.68	<0.01
	CYP2B6 (EFC)	0.53	<0.05
	CYP2A6	0.51	<0.05
	(CYP3A4/5)	(0.45)	(<0.10) NS
	(CYP4A9/11)	(0.42)	(<0.20) NS
5,6-epoxy-RA	CYP2B6 (S-MP)	0.72	<0.01
	CYP2A6	0.58	<0.05
	CYP3A4/5	0.57	<0.05

NS, not significant at an  $\alpha$  level of .05.

<sup>a</sup> P450-specific activity was determined using the following substrates: CYP1A2, 7-ethoxyresorufin *O*-dealkylation; CYP2A6, coumarin 7-hydroxylation; CYP2B6 (EFC), 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylation; CYP2B6 (S-MP), *S*-mephenytoin *N*-demethylation; CYP2C8, paclitaxel 6 $\alpha$ -hydroxylation; CYP2C9, tolbutamide methyl-hydroxylation; CYP2C19, *S*-mephenytoin 4'-hydroxylation; CYP2D6, dextromethorphan *O*-demethylation; CYP2E1, chlorzoxazone 6-hydroxylation; CYP3A4/5, testosterone 6 $\beta$ -hydroxylation; and, CYP4A9/11, lauric acid 12-hydroxylation.

<sup>b</sup> *t*<sub>r</sub> test.

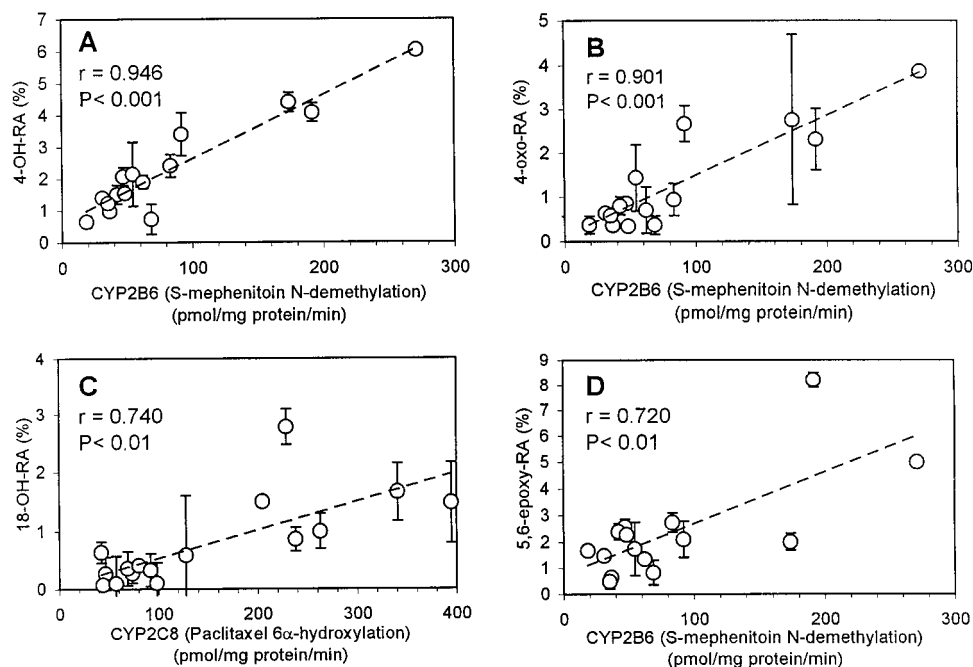
formation of this metabolite. It is noteworthy that almost the same P450s were involved in 4-OH-RA and 18-OH-RA formation, although two additional P450s, 1A1 and 4A11, were active only in the formation of 18-OH-RA. We observed that the formation rate of 4-OH-RA is approximately 10-fold higher than that of the 18-OH-RA for the P450s involved in the formation of both metabolites. The formation of 4-oxo-RA and 5,6-epoxy-RA was not observed at substrate concentra-

tions of 10  $\mu$ M using microsomes containing a single P450. This was likely due to the extremely low rate of formation of these metabolites in our incubation conditions and at our limits of detection.

**Kinetic Parameter Determination for the Formation of atRA Metabolites.** Using cDNA expressed human P450s, the kinetic parameters ( $K_m$  and  $V_{max}$ ) for the eight most active P450s involved in the formation of atRA metabolites were determined (Table 2). A general feature was the higher  $V_{max}$  values for most of the P450s involved in the hydroxylation at position 4, because the magnitude of this reaction largely exceeded the formation of either 4-oxo-RA, 18-OH-RA, or 5,6-epoxy-RA. This is in agreement with the above-mentioned data collected with human liver microsomes.

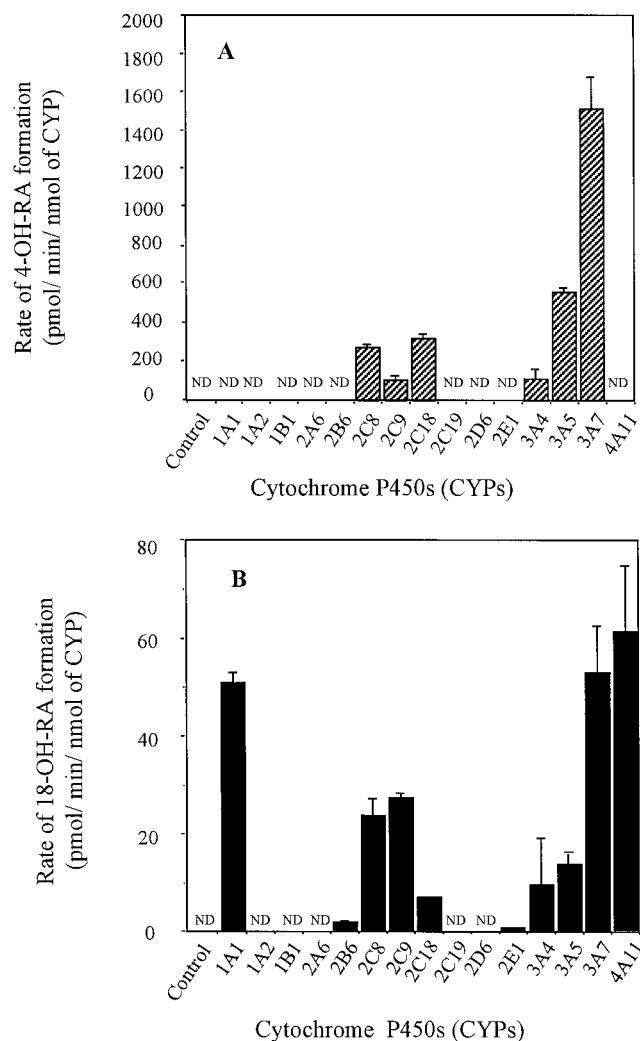
For the formation of 4-OH-RA, higher  $V_{max}$  values were observed with CYP3A7 (1869 pmol/min/nmol CYP), 2C8 (1211) (Fig. 4), and 3A5 (1124). AtRA appeared to have a better affinity for 3A7 ( $K_m$  = 15  $\mu$ M) and 2C18 ( $K_m$  = 16  $\mu$ M) than for 2C8 ( $K_m$  = 50  $\mu$ M). Using the ratio  $V_{max}/K_m$  to assess the P450s efficiency in atRA metabolism, CYP3A7 was found to be the most effective, with a  $V_{max}/K_m$  ratio of 127.7, followed by 3A5 ( $V_{max}/K_m$  = 25.6), 2C8 ( $V_{max}/K_m$  = 24.5), 2C18 ( $V_{max}/K_m$  = 15.8), 3A4 ( $V_{max}/K_m$  = 5.7), 1A1 ( $V_{max}/K_m$  = 5.0), and 4A11 ( $V_{max}/K_m$  = 1.9).

In this series of experiments, the formation of 4-oxo-RA was only observed at the highest substrate concentrations used (50 and 100  $\mu$ M) for most P450s, which did not allow the calculation of kinetic parameters for all the P450s studied. However, it was observed that the formation of this metabolite was preferentially catalyzed by CYP3A7 ( $V_{max}/K_m$  = 13.4), followed by a 10-fold lower activity with 2C18 and 4A11 ( $V_{max}/K_m$  = 1.2). 18-OH-RA was formed preferentially by CYP3A7 ( $V_{max}/K_m$  = 10.5), CYP4A11 ( $V_{max}/K_m$  = 2.1), and CYP2C8 ( $V_{max}/K_m$  = 2.0). In general, the 5,6-epoxidation required high atRA concentrations due to its elevated  $K_m$ , and the highest metabolic capacity ( $V_{max}/K_m$ ) was observed with P450s 2C8, 2C9, 1A1, and 3A5.



**Fig. 2.** Correlation between P450-specific activity and atRA metabolite formation. atRA metabolite formation was determined by HPLC, and correlated with the activity of phenotyped microsomal preparations using the P450-specific substrates listed under *Materials and Methods*. The best correlations observed for the four atRA metabolites formation are shown: 4-OH-RA formation with *S*-mephenytoin-*N*-demethylation (CYP2B6) (A); 4-oxo-RA formation with *S*-mephenytoin-*N*-demethylation (CYP2B6) (B); 18-OH-RA formation with paclitaxel 6 $\alpha$ -hydroxylation (CYP2C8) (C); and 5,6-epoxy-RA formation with *S*-mephenytoin-*N*-demethylation (CYP2B6) (D). Results are the mean of three independent determinations (error bars, S.E.M.).

**atRA Metabolism Using Individual P450s Expressed in 293 Cells.** To examine the profile and intensity of formation of the atRA metabolites in a more complete biological



**Fig. 3.** Rate of formation of atRA metabolites by cDNA expressed human P450s. A, rate of formation of 4-OH-RA. B, rate of formation of 18-OH-RA. Control microsomes were from human lymphoblasts transfected with the empty vector. Results are the mean of three independent determinations (error bars, S.E.M.). ND, not detected: the limit of detection was 5 pmol for 4-OH-RA and 2 pmol for 18-OH-RA.

**TABLE 2**

Determination of kinetic parameters for the formation of atRA metabolites by human lymphoblast-expressed P450s

Microsomes (0.25 mg/ml) from cDNA expressed human P450s in lymphoblasts were incubated at 37°C in phosphate buffer with atRA at concentrations of 0, 5, 10, 20, 50, and 100  $\mu$ M (total volume, 0.5 ml). The reaction was started by the addition of 1 mM NADPH and stopped 30 min later by the addition of 1 ml of ethyl acetate on ice. Retinoids were extracted and analyzed by HPLC as described under *Materials and Methods*.

Cytochrome P450	4-OH-RA			4-oxo-RA			18-OH-RA			5,6-epoxy-RA		
	$V_{max}$	$K_m$	$V_{max}/K_m$	$V_{max}$	$K_m$	$V_{max}/K_m$	$V_{max}$	$K_m$	$V_{max}/K_m$	$V_{max}$	$K_m$	$V_{max}/K_m$
	pmol/min/nmol P450	$\mu$ M		pmol/min/nmol P450	$\mu$ M		pmol/min/nmol P450	$\mu$ M		pmol/min/nmol P450	$\mu$ M	
CYP1A1	204	41	5.0	nd	nd		28	43	0.7	840	439	1.9
CYP2C8	1211	50	24.5	nd	nd		33	17	2.0	450	130	3.5
CYP2C9	199	81	2.5	nd	nd		38	86	0.5	293	107	2.7
CYP2C18	251	16	15.8	19	16	1.2	9	16	0.5	71	50	1.4
CYP3A4	195	34	5.7	nd	nd		nd	nd		153	117	1.3
CYP3A5	1124	44	25.6	nd	nd		20	39	0.5	36	22	1.6
CYP3A7	1869	15	127.7	52	4	13.4	52	5	10.5	49	80	0.6
CYP4A11	278	145	1.9	36	32	1.2	57	27	2.1	28	58	0.5

nd, not determined because formation of this metabolite was only at the highest atRA concentrations (50 and 100  $\mu$ M).

system, we used human embryonic kidney cells (293) stably transfected with human cDNA of P450s 1A1, 1A2, 2C8, 2C9, 3A4, 3A5, and 3A7 (Fig. 5). As in the microsomes, P450s 3A7, 1A1, and 2C8 were again found to be strongly implicated in the formation of the three metabolites detected in this system, 4-OH-RA, 4-oxo-RA, and 18-OH-RA. The major metabolite was 4-OH-RA with a maximum formation rate 3-fold higher than that of 4-oxo-RA, and 40-fold higher than that of 18-OH-RA. P450s 2C9, 1A2, and 3A4 were, to a lesser extent, also involved in 4-OH-RA formation. Rates of formation of 4-oxo-RA were moderate by CYP1A2.

## Discussion

Despite the importance of metabolism in the mechanism of action of atRA, little information is presently available concerning the identity of the human P450s involved in atRA biotransformation, with the exception of CYP2C8 (Leo et al., 1989; Nadin and Murray, 1999) and the recently characterized CYP26 (Ray et al., 1997; White et al., 1997). The purpose of this study was therefore to characterize the principal atRA metabolites formed in vitro using human preparations, and to identify the human P450s responsible for these reactions.

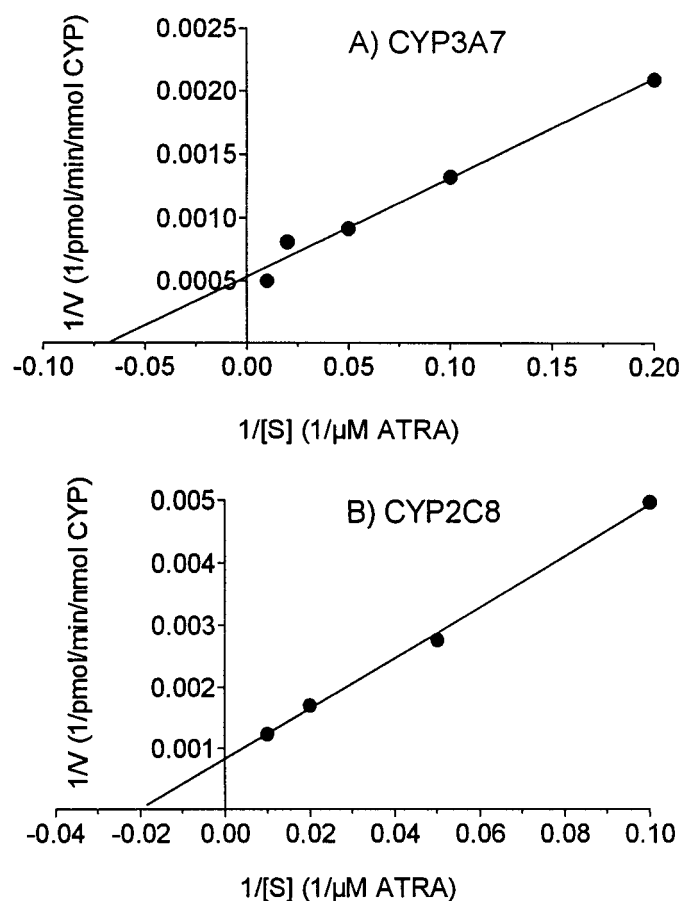
The use of a reversed phase HPLC method with gradient elution allowed baseline separation of four metabolites (4-OH-RA, 4-oxo-RA, 18-OH-RA, and 5,6-epoxy-RA) and quantitation of individual metabolites formed during in vitro incubation. The results obtained with the 3 complementary methods used are summarized in Table 3. The major metabolite generated during in vitro incubation of liver microsomes with 10  $\mu$ M atRA was 4-OH-RA, followed by 4-oxo-RA, 18-OH-RA and 5,6-epoxy-RA. This indicates a preferential P450 activity at position 4 of the atRA molecule. The formation of 4-OH-RA was found to be significantly correlated with that of 4-oxo-RA and 18-OH-RA, indicating that the same P450s are involved in their formation. This is not the case for atRA conversion to 5,6-epoxy-RA, which suggests that different P450s are responsible for these reactions.

Using phenotyped human liver microsomes, it was found that the catalytic activity of P450s 2B6, 2C8, 3A4/5, and 2A6 correlated with the production of 4-OH-RA and 4-oxo-RA; 2C8, 2B6, and 2A6 correlated with the production of 18-OH-RA; and 2B6, 2A6, and 3A4/5 correlated with 5,6-epoxidation. Correlation studies between P450-specific activities showed trends that indicated that particular P450s might be in-

involved in the formation of a given atRA metabolite. However, a major limitation of the correlation approach is the lack of specificity of the substrates used to characterize P450-dependent reactions.

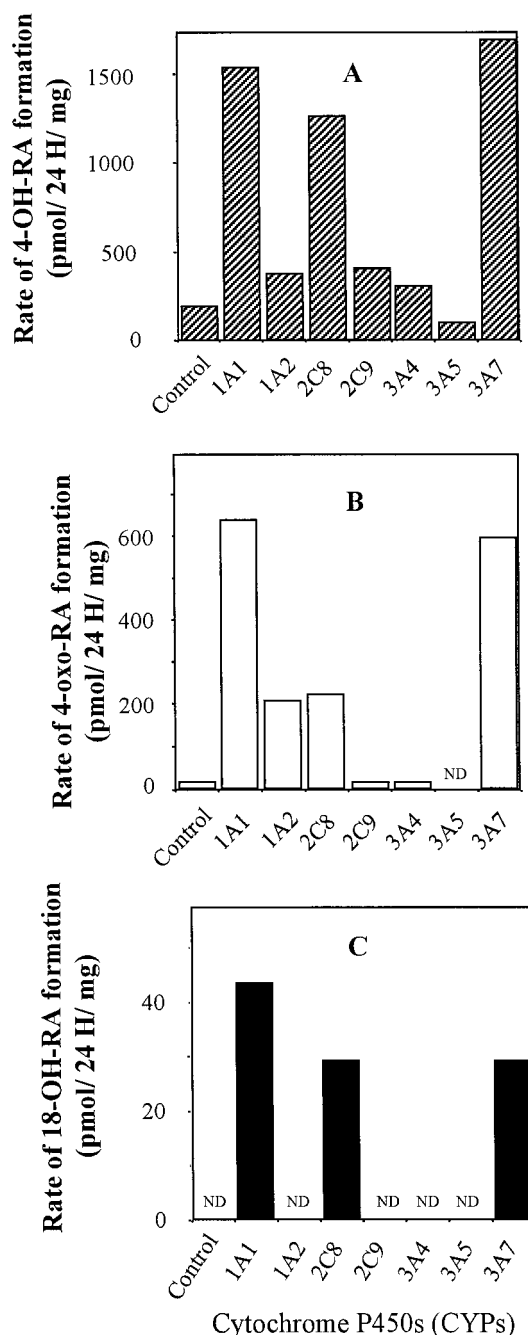
We then used single P450-expressing microsomes for the 15 most abundant human P450s. The combination of the two methods highlighted the role of P450s 2C8 and 3A4 in the overall atRA metabolism. P450s 2A6 and 2B6, which displayed a positive correlation between reference activities (respectively, coumarin hydroxylation and ethoxytrifluoromethyl coumarin dealkylation) and the formation of atRA metabolites by human liver microsomes, were found to be inefficient in atRA metabolism in lymphoblast microsomes. This may be due to a lack of specificity of the "reference substrates" for a given P450 because recent studies have indicated the involvement of several other P450s [i.e., 1A, 2B6, 2C, and 2E1 in the ethoxytrifluoromethyl coumarin dealkylation reaction (Buters et al., 1993; Code et al., 1997)]. In addition to P450s 2C8 and 3A4, the lymphoblast-expressed human P450s allowed the identification of several other P450s significantly involved in the formation of 4-OH-RA (i.e., 3A7, 3A5, 2C9, and 2C18). P450s 4A11, 3A7, and 1A1 were important in the formation of 18-OH-RA, followed by 2C9 and 2C8.

Interestingly, the participation of the P450s involved in atRA metabolism varies with the metabolite considered. For



**Fig. 4.** Lineweaver-Burk plot of the rate of formation of 4-OH-RA by cDNA-expressed human P450s. A, CYP3A7 or B, CYP2C8. Substrate concentrations were 5, 10, 20, 50, and 100  $\mu$ M (30-min incubation time). The metabolite formation was determined by HPLC as described under *Materials and Methods*.

example, 4-OH-RA, the major metabolite formed in vitro, is preferentially generated by P450s 3A7, 3A5, 2C18, 2C8, 3A4, and 2C9, whereas 18-OH-RA is generated by P450s 4A11, 3A7, 1A1, 2C9, 2C8, 3A5, 3A4, and 2C18. The rate of 18-OH-RA formation, however, is 10- to 20-fold lower than that of the 4-OH-RA for the same P450. This indicates that 4-OH-RA is the preferred route of atRA metabolism, which is in agreement with the liver microsome data, even though  $K_m$



**Fig. 5.** Rate of formation of atRA metabolites by cDNA-expressed human P450s expressed in human embryonic kidney cells (293). Cells were seeded at a concentration of  $6 \times 10^5$  cells/ml, and cultured in the presence of 10  $\mu$ M atRA for 24 h. The culture medium was extracted twice with ethyl acetate and analyzed for the presence of atRA metabolites using reversed phase HPLC with gradient elution as described under *Materials and Methods*. Results are the mean of two independent determinations giving similar results. ND, not detected; the limit of detection was 5 pmol for 4-OH-RA and 2 pmol for both 4-oxo-RA and 18-OH-RA.

values of 4- and 18-hydroxylation by individual P450s were in the same range.

To compare the metabolic activity of the eight most active P450s involved in atRA metabolism, the kinetic parameters were determined, and it was found that, using the ratio  $V_{\max}/K_m$  as an efficiency criterion for the formation of 4-OH-RA, CYP3A7 was 5-fold more efficient than either 3A5 or 2C8. To our knowledge, it is the first time that P450s 3A7 and 3A5 have been found to be more active than CYP2C8 in atRA metabolism. We have not compared the efficiency of these human P450s with that of the recently identified CYP26 (Ray et al., 1997; White et al., 1997) because there is presently no model substrate or inhibitor identified, and there is no commercially available preparation for this P450.

Despite their formation in the incubations performed with liver microsomes, no 4-oxo-RA or 5,6-epoxy-RA was detected with the lymphoblast microsomes expressing a single P450 (at 10  $\mu$ M atRA). Concerning the formation of 4-oxo-RA, this may indicate that a multistep process involving several P450s would be needed to generate this metabolite, as has been reported with other substrates (Guengerich, 1999). Although the 5,6-epoxidation of atRA has been suggested to be P450-independent (Roos et al., 1998), the formation of this metabolite in our incubation conditions was seen only in the presence of NADPH, thus suggesting P450 participation.

The metabolism of atRA was also evaluated in a more biologically relevant system (i.e., in 293 cells stably transfected with P450s active in atRA biotransformation). This system confirmed the participation of P450s 1A1, 2C8, and 3A7 in the production of 18-OH-RA and of P450s 3A7, 2C8, 3A4, and 3A5 in the formation of 4-OH-RA, as observed using lymphoblast microsomes expressing single P450s. In this cellular system, 4-OH-RA is also formed by P450s 1A1 and 1A2. The greater efficiency of transfected 293 cells in the generation of 4-OH-RA could be due to the presence of an intrinsic intracellular atRA transporter, such as the retinoic acid binding protein (cellular retinoic acid binding protein). This may increase the concentration, and thus atRA metabolism by P450s in the cell (Fiorella and Napoli, 1994). 4-oxo-RA was formed in the cell system by the same P450s as 4-OH-RA; this would be expected because it appears that 4-oxo-RA is derived from 4-OH-RA (Roberts et al., 1979b). 5,6-epoxy-RA has not been detected in this cell system perhaps due to the formation of adducts with cellular nucleophiles, and/or to the hydration by epoxide hydrolase to its vicinal diol.

These data clearly emphasize the role of several human P450s in the biotransformation of atRA. The information relative to the catalytic efficiency of a given P450 to drive a specific reaction should however be kept in perspective with regard to the relative concentration of individual P450s

found in human tissues. The two P450s highly implicated in atRA metabolism (i.e., 3A7 and 1A1) are not, or only marginally, expressed in nonpathological adult human tissues, particularly in the liver. CYP3A7 is, however, highly expressed in the fetal liver and is replaced by CYP3A4 within a few weeks after birth (Lacroix et al., 1997; Cresteil, 1998; Hakkola et al., 1998b). The present novel finding that CYP3A7 is the most efficient in atRA metabolism, compared with the other P450s tested, would indicate that this enzyme could regulate atRA levels and metabolite formation in the fetus for normal developmental and differentiation purposes. It is also possible that the teratogenic effects of high levels of retinoids in the fetus may be prevented by the high expression of CYP3A7 before birth. Because CYP3A7 has also been reported to be expressed at low levels in the adult liver (Hakkola et al., 1994a; Schuetz et al., 1994), the expression of CYP3A7 could also alter atRA therapy in adults. The emergence of CYP1A1 as a major contributor to atRA metabolism is also an interesting observation. In agreement with our results, it was recently reported that atRA is a potent inhibitor of CYP1A1-dependent oxidation (Yamazaki and Shimada, 1999). Because a major function of CYP1A1 is to activate precarcinogens, it is conceivable that the cancer prevention properties of retinoids could be due to their competitive inhibition of precarcinogen activation.

Because some atRA metabolites have been reported to be active in cell growth inhibition (Takatsuka et al., 1996; van der Leede et al., 1997), organogenesis (Pijnappel et al., 1993), or differentiation (Ramp et al., 1994; Idres et al., 2000), it is possible that the expression of a given set of P450s in a given tissue could orient the formation of atRA metabolites that possess selective binding to nuclear receptors, thereby triggering the expression of specific sets of genes. For example, it has been reported that 4-oxo-RA activates RAR $\beta$  more efficiently than atRA (Pijnappel et al., 1993).

In conclusion, human P450s 3A7, 1A1, 2C8, 2C18, 2C9, 3A4, 3A5, and 4A11 have been identified as major contributors to atRA metabolism. This indicates that fine regulation of the effects of retinoids could be possible via modulation of the concentration of these enzymes in relation to the physiological status of human tissues. The biological importance of the expression of these human P450s in the cellular response to atRA remains to be elucidated.

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TABLE 3

Summary of the three methods used to determine the human P450s involved in atRA metabolism

Method	P450s Involved			
	4-OH-RA	4-oxo-RA	18-OH-RA	5,6-epoxy-RA
Correlation of activity	2B6, 2C8, 3A4/5, 2A6	2B6, 2C8, 3A4/5, 2A6	2C8, 2B6, 2A6	2B6, 2A6, 3A4/5
Lymphoblast-expressed P450s	3A7, 3A5, 2C18, 2C8, 3A4, 2C9	nd	4A11, 3A7, 1A1, 2C9, 2C8, 3A5, 3A4, 2C18	nd
293 Cells transfected with a single P450	3A7, 1A1, 2C8, 2C9, 1A2, 3A4	1A1, 3A7, 2C8, 1A2	3A7, 1A1, 2C8	nd

nd, not detected. In our HPLC conditions, the limit of detection was 2 pmol for 4-oxo-RA.



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