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Human cytochrome P450s involved in the metabolism of 9-*cis*- and 13-*cis*-retinoic acids☆

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Abstract

The purpose of this work was to identify the principal human cytochrome P450s (CYPs) involved in the metabolism of the retinoic acid (RA) isomers, 9-*cis*- and 13-*cis*-RA, by using a combination of techniques including human liver microsomes (correlation of activity and inhibition), and lymphoblast microsomes expressing a single CYP. Concerning the 9-*cis*-RA, 4-OH- and 4-oxo-9-*cis*-RA were formed with human liver microsomes, and their formation correlated with activities linked to CYPs 3A4/5, 2B6, 2C8, 2A6, and 2C9. The use of lymphoblast microsomes expressing a single human CYP identified CYPs 2C9 > 2C8 > 3A7 as the most active in the formation of 4-OH-9-*cis*-RA. With regard to 13-*cis*-RA, specific P450 activities linked to CYPs 2B6, 2C8, 3A4/5, and 2A6 were correlated with the formation of 4-OH- and 4-oxo-13-*cis*-RA. Microsomes expressing a single CYP identified CYPs 3A7, 2C8, 4A11, 1B1, 2B6, 2C9, 2C19, 3A4 (decreasing activity) in the formation of 4-OH-13-*cis*-RA. The use of CYP-specific inhibitors in human liver microsomes disclosed that the formation of the 4-OH-9-*cis*-RA was best inhibited by sulfaphenazole (72%) and quercetin (66%), whereas ketoconazole and troleandomycin inhibited its formation by 55 and 38%, respectively; the formation of 4-OH-13-*cis*-RA was best inhibited by troleandomycin (54%) and ketoconazole (46%), whereas quercetin was a weak inhibitor (14%). In conclusion, adult human CYPs 2C9, 2C8, 3A4 have been identified as active in the 9-*cis*-RA metabolism, whereas CYPs 3A4 and 2C8 were active in 13-*cis*-RA metabolism. The fetal form CYP3A7 was also identified as very active in either 9-*cis*- or 13-*cis*-RA metabolism. The role of these human CYPs in the biological response or resistance to RA isomers remains to be determined.

Keywords: 9-*Cis*-retinoic acid; 13-*Cis*-retinoic acid; Retinoids; Metabolism; Cytochrome P450s; Retinoic acid 4-oxidation; CYP; CYP2C9; CYP2C8; CYP3A7; CYP3A4

1. Introduction

In addition to the importance of retinoids (Vitamin A and its derivatives) in embryogenesis, vertebrate development, differentiation, and homeostasis [1], these compounds are under investigation in the prevention and treatment of a variety of cancers (reviewed in [2,3]). Retinoids are obtained in the diet either as preformed retinoids or as carotenoids (provitamin A). After several metabolic reactions in the intestines, retinol (Vitamin A) becomes the major retinoid absorbed and is stored in esterified form in the liver. After ester hydrolysis, retinol is transported by a plasma protein to the tissues. The conversion of retinol to retinal by retinol dehydrogenases and several CYPs is considered to be rate-limiting in the biosynthesis of RA [4]. The metabolism of retinals to retinoic acids is mediated by human CYPs 1A1, 1A2, 1B1 and 3A4 for the formation of *all-trans*-retinoic acid (atRA), and CYP1A2 for the formation of 9-*cis*-RA [5].

RA crosses the plasma membrane passively and is translocated by cellular retinoic acid binding proteins (CRABP I–II) to the nucleus where it can bind to nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each composed of three subtypes

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Abbreviations: RA, retinoic acid; atRA, *all-trans*-retinoic acid; 9-*cis*-RA, 9-*cis*-retinoic acid; 4-OH-9-*cis*-RA, 4-hydroxy-9-*cis*-retinoic acid; 4-oxo-9-*cis*-RA, 4-oxo-9-*cis*-retinoic acid; 13-*cis*-RA, 13-*cis*-retinoic acid; 4-OH-13-*cis*-RA, 4-hydroxy-13-*cis*-retinoic acid; 4-oxo-13-*cis*-RA, 4-oxo-13-*cis*-retinoic acid; HPLC, high-performance liquid chromatography; RAR, retinoic acid receptor; RXR, retinoid X receptor; Rt, retention time.

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(α, β, γ). The RARs are activated by both atRA and 9-*cis*-RA and function as ligand-inducible transcriptional regulators when heterodimerized with RXR [6]. The RXRs can also homodimerize, and act as transcriptional regulators under certain conditions, and are only activated by 9-*cis*-RA [6,7]. Biological responses to retinoids are therefore, 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 the retinoid to specific proteins, may also play a role in the cell response to atRA [8].

The metabolism of atRA is not only a simple catabolic process, as some oxidized metabolites display biological activity in the modulation of genes expressed in apoptosis, cellular growth and differentiation, embryonic development, and in the growth inhibition of several normal and neoplastic cells *in vitro* [9–15]. It has also been proposed that the metabolism of atRA may be linked to its growth inhibitory effects, as the most sensitive cell lines are intriguingly those that can metabolize atRA the most efficiently [16,17].

Although atRA metabolism appears to play a central role in its molecular mechanism of action, either in terms of sensitivity or resistance, the human enzymes involved in its metabolism have only recently been identified. In humans, in addition to the already known CYP2C8 [18,19] and CYP26 [20,21] several other CYPs have been identified in the metabolism of atRA, i.e., CYPs 3A7, 3A5, 2C18, 3A4, 2C9 and 1A1 [22–24].

The atRA can isomerize *in vitro* and *in vivo* to its stereoisomers 9-*cis*- and 13-*cis*-RA, which possess different nuclear receptor binding properties [7] and pharmacological activities. The clinical pharmacology of RA isomers is also markedly different, e.g., the 13-*cis*-RA pharmacokinetics are stable over time with a half-life in the range of 13–22 hr [25], whereas atRA and 9-*cis*-RA pharmacokinetics are variable over time with a decrease in plasma concentrations after repeated dosage [26,27].

Because metabolism plays an important role in the response to retinoids, and also because little information is presently available concerning the identity of the human CYPs involved in the metabolism of the principal atRA isomers (9-*cis*- and 13-*cis*-RA), the purpose of this study was to identify the principal human CYPs involved in their metabolism, to identify the metabolites, and also to compare to the CYPs already identified in the metabolism of atRA.

2. Materials and methods

2.1. Chemicals

atRA, 9-, 13-*cis*-RA, quercetin, sulfaphenazole, troleandomycin, and NADPH were purchased from Sigma–Aldrich, and ketoconazole was purchased from ICN-Biochemicals. 4-Oxo-9- and 4-oxo-13-*cis*-RA, were kindly provided by Eva-Maria Gutknecht and Pierre Weber (Hoffmann-La Roche, Ltd., Basel, Switzerland). The 4-OH-13- and 4-OH-9-*cis*-RA were obtained by reduction of the corresponding 4-oxo standard metabolite using a molar excess of sodium borohydride (1 mg/mL). Stock solutions of retinoids (10^{-2} M) were prepared in methanol and stored at −20°. For microsomal incubations, the RA of interest was further diluted in incubation buffer to 10^{-4} M. The final methanol concentration in incubation mixture was 0.1%. NADPH (12 mM) was freshly prepared before addition to the incubation mixtures.

2.2. 9-Cis- and 13-cis-RA metabolism by human liver microsomes

Microsomal preparations from 16 human livers were phenotyped by the supplier (XenoTech) using the following CYP-specific activities: CYP1A2, 7-ethoxyresorufin *O*-dealkylation; CYP2A6, coumarin 7-hydroxylation; CYP2B6, 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylation (EFC), and *S*-mephenytoin *N*-demethylation (*S*-MP); CYP2C8, paclitaxel 6α-hydroxylation; CYP2C9, tolbutamide methyl-hydroxylation; CYP2C19, *S*-mephenytoin 4′-hydroxylation; CYP2D6, dextromethorphan *O*-demethylation; CYP2E1, chlorzoxazone 6-hydroxylation; CYP3A4/5, testosterone 6β-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 $MgCl_2$ at a final protein concentration of 0.25 mg/mL in a total volume of 0.5 mL. After a 3-min pre-incubation with 10 mM of the RA under study at 37°, the reaction was initiated by the addition of NADPH at a final concentration of 1 mM. The 10 μM concentration was chosen based on preliminary studies showing linearity of the reaction up to 20 μM of the retinoid, on the detectability and quantification of the metabolites formed, and also because this is in the range of achievable pharmacological concentrations [28,29]. Reactions were carried out for 30 min for 9-*cis*-RA or 45 min for 13-*cis*-RA, in a shaking water bath. In these conditions, the assays were linear with respect to microsomal protein concentration, incubation time, and substrate concentration. After the incubation, the reaction was stopped by the addition of 1 mL ethyl acetate on ice. Tubes were vortexed for 2 min and centrifuged at 3500 *g* for 15 min at 4°. A second extraction procedure was carried out with 1 mL ethyl acetate. The organic phases were pooled and evaporated under vacuum (Speed-Vac). The dry residue was kept at −80° until high-performance liquid chromatography (HPLC) analysis. Under these conditions, the RA extraction was 80 ± 2%. All procedures were carried out in the dark or under subdued lighting.

2.3. 9-Cis- and 13-cis-RA metabolism by lymphoblast microsomes expressing a single human CYP

Microsomal preparations from human lymphoblasts stably transfected with the following human CYP cDNAs were used (Gentest Corp.): CYPs 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 were the same as for human liver microsomes.

2.4. HPLC analysis

Dry residues from ethyl acetate extractions were resuspended in 200 μL methanol. The reaction products were quantitatively analyzed using an HPLC system consisting of a Varian 5000 liquid chromatograph, a reversed-phase analytical column (Beckman, Ultrasphere 5 μm, 4.6 mm × 250 mm), a Beckman 168 diode array detector, and the 32-Karat software (Beckman–Coulter, Inc.). Elution was performed using a linear gradient with solvent A (1% ammonium acetate in water) and solvent B (100% methanol) as follows: after equilibration at 65% B and 35% A, percent B was increased to 100% in 35 min, then re-equilibrated to initial conditions in 5 min (flow rate, 1 mL/min). The RA metabolites were identified by comparison with elution times and UV spectra of standard metabolites. Identified metabolites were quantified using a calibration curve of standards.

2.5. Inhibition analysis

The following CYP-specific inhibitors were tested for their effect on retinoic acid isomers oxidation in pooled human liver microsomes: 10 μM ketoconazole [30], 40 μM sulfaphenazole [31], 50 μM troleandomycin [32], 20 μM quercetin [33,34]. The inhibitors were dissolved in methanol such that final concentration of solvent in the incubation mixture was <1%. The inhibitors were added to the incubation mixture containing NADPH 10 min before the addition of the retinoic acid substrates at 10 μM and compared with identical incubation mixtures without inhibitor.

2.6. Statistical analyses

Simple linear regression analyses and stepwise multiple regression analyses (forward and backward) were performed using the SigmaStat software (SPSS Science Software GmbH). Statistical significance ($P \leq 0.05$) of the simple linear regression analyses between CYP-specific activities and the formation of 9-*cis*- or 13-*cis*-RA metabolites was determined using an F test. Statistical significance for the multiple linear regression analyses was determined using t statistic. V_{max} and K_m values were determined by fitting the experimental data directly to the Michaelis–Menten equation using the software GraphPad Prism (version 3).

3. Results

3.1. 9-Cis- and 13-cis-RA metabolites separation and identification

Fig. 1 presents the separation of 9-*cis*-RA metabolites by reversed-phase HPLC. Two metabolite peaks were detected at retention times of 11.2 and 13.6 min which were identified as the 4-oxo-9- and the 4-OH-9-*cis*-RA, respectively, by co-elution with authentic standards and UV spectra. Fig. 2 presents the separation of the two major

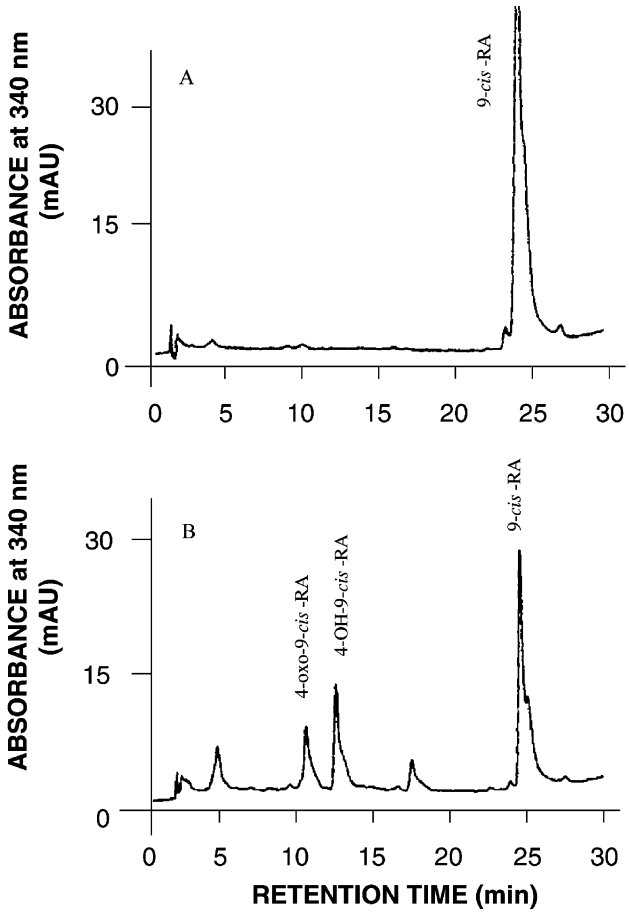

Fig. 1. HPLC separation of 9-*cis*-RA metabolites. Representative chromatogram showing the separation of 9-*cis*-RA metabolites using reversed-phase HPLC with gradient elution. 9-*Cis*-RA (10 μM) was incubated in phosphate buffer containing human liver microsomes (0.25 mg protein/mL) in absence (A) or presence (B) of 1 mM NADPH for 30 min at 37° in a total volume of 0.5 mL. The reaction was stopped and extracted twice with 1 mL ethyl acetate. The organic phase was evaporated and the dry residue resuspended in 200 μL methanol and injected onto the HPLC system described in Section 2.

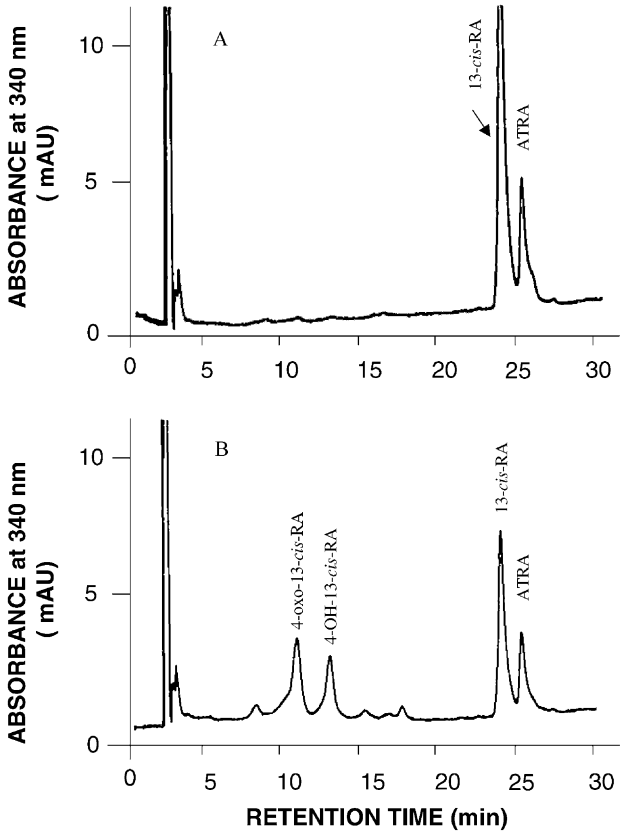

Fig. 2. HPLC separation of 13-*cis*-RA metabolites. Representative chromatogram showing the separation of 13-*cis*-RA metabolites from 13-*cis*-RA and atRA using reversed-phase HPLC with gradient elution. 13-*Cis*-RA (10 μM) was incubated in a phosphate buffer containing a human liver microsomal preparation (0.25 mg protein/mL) in the absence (A) or the presence (B) of 1 mM NADPH for 45 min at 37° in a total volume of 0.5 mL. The reaction was stopped and extracted twice by the addition of 1 mL ethyl acetate. The organic phase was evaporated and the dry residue resuspended in 200 μL methanol and injected onto the HPLC system described in Section 2.

metabolites of 13-*cis*-RA detected at retention times of 10.9 and 13 min that were identified as the 4-oxo- and the 4-OH-13-*cis*-RA, respectively, by co-elution with authentic standards and UV spectra. It can also be observed that within the 45-min incubation period, 13-*cis*-RA (Rt = 24.5 min) also underwent substantial isomerization to the *all-trans* isomer (atRA, Rt = 25 min).

3.2. Correlation of 9-cis- and 13-cis-RA metabolites formation with CYP-specific activities

Microsomal preparations from 16 human livers phenotyped for specific CYP-dependent activities were used to determine the CYPs most involved in 9-*cis*- or 13-*cis*-RA metabolism.

The significant linear regression correlation coefficients between CYP-dependent activity and the formation of 9-*cis*-RA metabolites are presented in Table 1. The formation of the 4-OH-9-*cis*-RA was best correlated with the activities supported by CYPs 3A4/5, 2B6, 2C8, and 2A6. Although not significant at the 0.05 level, CYP2C9 was near significance with a *P* value of 0.053. The 4-oxo-9-*cis*-RA formation was best correlated with activities linked to CYPs 2B6, 2A6, 3A4/5, 2C8, and 2C9. Examples of the best correlations observed between 4-oxo- or 4-OH-9-*cis*-RA formation and activities related to CYPs 2A6, 3A4/5, and 2C8, are presented in Fig. 3. Using the correlation method, it thus, appear that CYPs 3A4/5, 2B6, 2A6, 2C8 and 2C9 are the principal CYPs involved in 9-*cis*-RA metabolism. Stepwise forward and backward linear regression analyses for the formation of 9-*cis*-RA metabolites disclosed additional information on the potential joint contribution of several CYPs to their formation. For the 4-OH-9-*cis*-RA formation, the contribution of CYPs 3A4/5

Table 1
Significant linear correlation between P450-linked activity and the formation of 9-*cis*-RA metabolites by human liver microsomes[a]

9-*Cis*-RA metabolites	CYP-related activities[b]	*r*[c]	*P*[d]
4-OH-9-*cis*-RA (13.6 min)	CYP3A4/5	0.783	<0.001
	CYP2B6 (*S*-MP)	0.749	<0.001
	CYP2B6 (EFC)	0.737	<0.001
	CYP2C8	0.729	<0.002
	CYP2A6	0.665	<0.005
	(CYP2C9)	(0.491)	(0.053)
4-Oxo-9-*cis*-RA (11.2 min)	CYP2B6 (*S*-MP)	0.755	<0.001
	CYP2B6 (EFC)	0.737	<0.002
	CYP3A4/5	0.743	<0.001
	CYP2A6	0.742	<0.001
	CYP2C8	0.733	<0.002
	CYP2C9	0.562	<0.05

[a] 9-*Cis*-RA metabolites formed *in vitro* using microsomes from 16 different human livers. Incubations were performed as detailed in Section 2. Results from three independent determinations.

[b] The CYP-specific activity was determined using the substrates listed in Section 2. EFC, 7-ethoxy-4-trifluoromethylcoumarin; *S*-MP, *S*-mephenytoin.

[c] Correlation coefficient determined by linear regression analysis between a CYP-specific activity and the production of a given metabolite.

[d] Statistical significance of the linear regression analysis between CYP-specific activities and the formation of the indicated metabolite was determined using an *F* test.

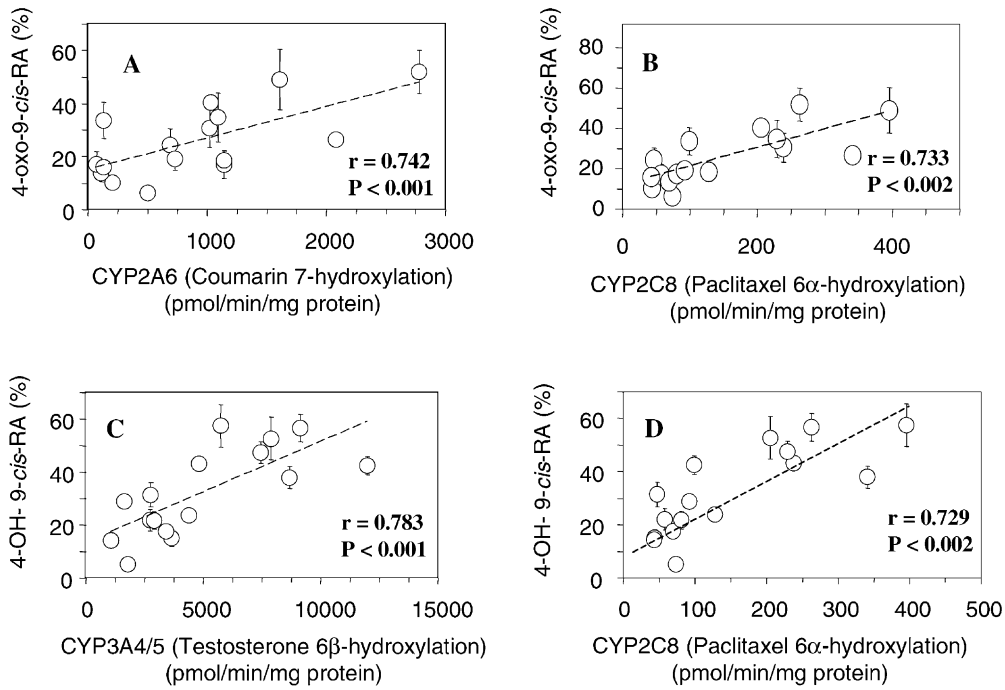

Fig. 3. Correlation between CYP-specific activity and 9-*cis*-RA metabolites formation. 9-*Cis*-RA metabolites formation was determined by HPLC, and correlated with the activity of phenotyped microsomal preparations using the CYP-specific substrates listed in Section 2. Representative significant correlations observed for the 4-oxo-9-*cis*-RA formation with CYPs 2A6 and 2C8 linked activities are shown in panels A and B, respectively; panels C and D show the correlation between the 4-OH-9-*cis*-RA formation and CYP3A4/5 and CYP2C8 linked activities, respectively. The *y*-axis is expressed as percent of metabolite formation relative to the total (retinoic acid isomer plus the metabolite). The indicated *P* values refer to the statistical significance of the linear regression analysis between CYP-specific activities and the formation of the indicated metabolite determined using an *F* test. Each data point is the mean of three independent determinations; error bars, SEM.

and 2B6 (*S*-MP) yielded a combined *r* value of 0.864 which was greater than either activity alone (Table 2). Likewise, the combined contribution of CYPs 3A4/5 and 2A6 to the formation of 4-oxo-9-*cis*-RA was also better than either activity alone (Table 2).

Concerning 13-*cis*-RA, the significant linear correlation coefficients observed between CYP-dependent activity and the formation of 13-*cis*-RA metabolites are presented in Table 3. The formation of the two major metabolites, i.e., 4-oxo- and 4-OH-13-*cis*-RA, was significantly correlated with CYP2C8 activity, and the activities supported by CYPs 2B6, 3A4/5, and 2A6. Examples of the best correlations observed between 4-oxo- or 4-OH-13-*cis*-RA formation and CYPs 2C8 or 2B6 activities, are presented in Fig. 4. Using the correlation method, it thus, appears that CYPs 2B6, 2C8, 3A4/5, and 2A6 are the principal CYPs involved in 13-*cis*-RA metabolism. Forward and backward stepwise linear regression analyses for the formation of 13-*cis*-RA metabolites uncovered the contribution of some CYPs not identified above by simple linear regression analysis (Table 4). The formation of 4-OH-13-*cis*-RA was contributed significantly by CYPs 1A2 and 2C19 in addition to the already identified 2B6 and 2C8 with a notable increase in *r* value (0.960) over the individual *r* values. Likewise the formation of 4-oxo-13-*cis*-RA was best predicted by a linear combination of CYPs 2C8, 1A2 and 4A9/11 activities.

3.3. 9-Cis- and 13-cis-RA metabolism by lymphoblast microsomes expressing a single human CYP

To ascertain the results obtained with the above correlation method, we then used microsomes expressing a single human CYP. For the 9-*cis*-RA, the single metabolite formed was identified as the 4-OH-9-*cis*-RA. Fig. 5A shows the formation rate of this metabolite as a function

Table 2
Stepwise linear regression analysis for the formation of 9-*cis*-RA metabolites

9-*Cis*-RA metabolites	Equation	*r*	*P*
4-OH-9-*cis*-RA	10.953 + (0.00303 × 3A4/5) + (0.0549 × 2B6 (*S*-MP))	0.864	<0.001
4-Oxo-9-*cis*-RA	6.492 + (0.00167 × 3A4/5) + (0.00690 × 2A6)	0.870	<0.001

Stepwise multiple regression analyses were accomplished using the SigmaStat software with the data generated from the microsomal incubation from 16 different human livers. Incubations were performed as detailed in Section 2. Statistical significance was determined using *t* statistic.

Table 3
Significant linear correlation between P450-specific activity and the formation of 13-*cis*-RA metabolites by human liver microsomes[a]

13-*Cis*-RA metabolites	CYP-related activities[b]	*r*[c]	*P*[d]
4-OH-13-*cis*-RA (13.0 min)	CYP2B6 (*S*-MP)	0.846	<0.001
	CYP2B6 (EFC)	0.843	<0.001
	CYP2C8	0.773	<0.001
	CYP3A4/5	0.653	<0.01
	CYP2A6	0.648	<0.01
4-Oxo-13-*cis*-RA (10.9 min)	CYP2C8	0.779	<0.001
	CYP2B6 (*S*-MP)	0.687	<0.01
	CYP2B6 (EFC)	0.691	<0.01
	CYP3A4/5	0.618	<0.02
	CYP2A6	0.614	<0.02

[a] 13-*Cis*-RA metabolites formed *in vitro* using microsomes from 16 different human livers. Incubations were performed as described in Section 2. Results from three independent determinations.
[b] The CYP-specific activity was determined using the substrates listed in Table 1.
[c] Correlation coefficient determined by linear regression analysis between a CYP-specific activity and the production of a given metabolite.
[d] Statistical significance of the linear regression analysis between CYP-specific activities and the formation of the indicated metabolite was determined using an *F* test.

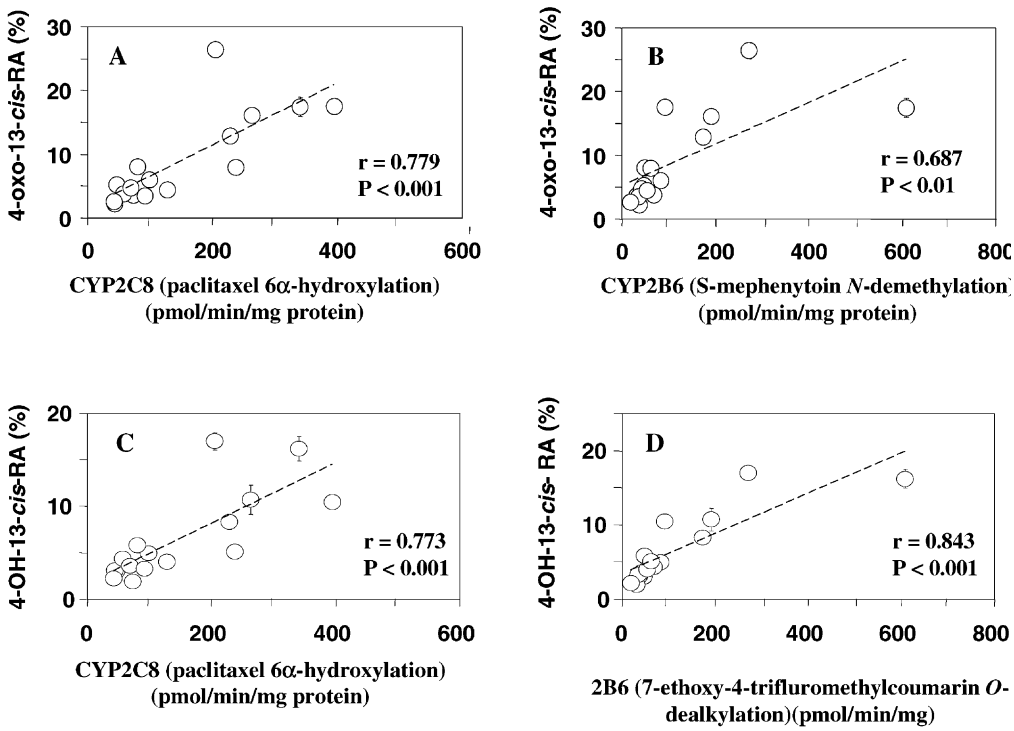

Fig. 4. Correlation between CYP-specific activity and 13-*cis*-RA metabolites formation. 13-*Cis*-RA metabolite formation was determined by HPLC, and correlated with the activity of phenotyped microsomal preparations using the CYP-specific substrates listed in Section 2. Correlations observed for the two principal metabolites are shown: A and B, 4-oxo-13-*cis*-RA formation with CYPs 2C8 and 2B6 linked activities, respectively; C and D, 4-OH-13-*cis*-RA formation with CYPs 2C8 and 2B6, respectively. The *y*-axis is expressed as percent of metabolite formation relative to the total (retinoic acid isomer plus the metabolite). The indicated *P* values refer to the statistical significance of the linear regression analysis between CYP-specific activities and the formation of the indicated metabolite determined using an *F* test. Results are the mean of three independent determinations (error bars, SEM).

Table 4
Stepwise linear regression analysis for the formation of 13-*cis*-RA metabolites

13-*Cis*-RA metabolites	Equation	*r*	*P*
4-OH-13-*cis*-RA	$-0.00053 + (0.000208 \times 2B6\text{-MP}) + (0.00206 \times 2C8) + (0.000527 \times 1A2) - (0.000164 \times 2C19)$	0.960	<0.001
4-Oxo-13-*cis*-RA	$0.0386 + (0.000666 \times 2C8) + (0.000903 \times 1A2) - (0.0000839 \times 4A9/11)$	0.919	<0.001

Stepwise multiple regression analyses were accomplished using the SigmaStat software with the data generated from the microsomal incubation from 16 different human livers. Incubations were performed as detailed in Section 2. Statistical significance was determined using *t* statistic.

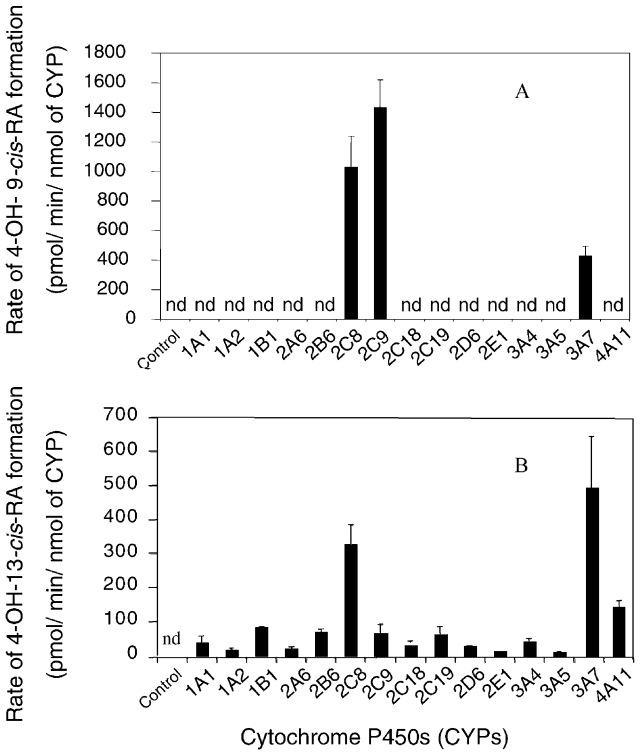

Fig. 5. Rate of formation of the 4-hydroxy metabolite of 9-*cis*-RA (panel A) or of 13-*cis*-RA (panel B) by lymphoblast microsomes expressing a single human CYP. The substrate was incubated with microsomes expressing a single human CYP and the metabolite formation was determined by HPLC as described in Section 2. Control microsomes were from human lymphoblasts transfected with the empty vector. Results are the mean of three independent determinations (error bars, SEM). ND, not detected.

of the single CYP preparation used. CYP2C9 was the most active (1432 ± 187 pmol/min/nmol of CYP), followed by CYPs 2C8 (1029 ± 208), and 3A7 (428 ± 64). Concerning the 13-*cis*-RA, the major metabolite formed was the 4-OH-13-*cis*-RA which was mostly formed by CYPs 3A7 (495 ± 153 pmol/min/nmol of CYP), 2C8 (328 ± 56), 4A11 (143 ± 19), 1B1 (82 ± 3), 2B6 (68 ± 10), 2C9 (66 ± 25), 2C19 (63 ± 22), and 3A4 (41 ± 6) (Fig. 5B).

3.4. Kinetic parameters determination for the formation of 4-OH-9- and 4-OH-13-cis-RA using lymphoblast expressed human CYPs

The kinetic parameters (apparent K_m and V_{max}) for the three most active CYPs involved in the formation of 4-OH-9-*cis*-RA were determined at concentrations ranging from 0 to 100 μM (0, 5, 10, 20, 50, and 100 μM) and the results are presented in Table 5. For CYP2C9, apparent K_m and V_{max} were 5 μM and 2163 pmol/min/nmol of CYP ($V_{max}/K_m = 433$), respectively; CYP2C8, $K_m = 7$ μM and $V_{max} = 948$ pmol/min/nmol of CYP ($V_{max}/K_m = 135$); CYP3A7, $K_m = 80$ μM and $V_{max} = 3665$ pmol/min/nmol of CYP ($V_{max}/K_m = 45$). These data clearly identified CYP2C9 as the most active CYP in the metabolism of 9-*cis*-RA. The kinetic parameters for the 4-OH-13-*cis*-RA was determined for the CYP most active in its formation (CYP3A7), and an apparent K_m of 49 μM and a V_{max} of 1587 pmol/min/nmol CYP were found ($V_{max}/K_m = 32$). Concerning CYPs 2C8 and 4A11, the kinetic values could not be calculated because a plateau phase was not reached at 300 μM.

3.5. Inhibition of 9-cis- and 13-cis-RA metabolites formation by CYP-specific inhibitors

In a pool of human liver microsomes, the formation of the 4-OH-9-*cis*-RA was best inhibited by sulfaphenazole (72%) and quercetin (66%), whereas ketoconazole and troleandomycin inhibited the formation of this metabolite by 55 and 38%, respectively (Fig. 6A). As expected, in microsomes expressing a single CYP, the 4-OH-9-*cis*-RA formation was $93 \pm 1\%$ inhibited by sulfaphenazole (40 μM) for CYP2C9 microsomes, $78 \pm 4\%$ inhibited by troleandomycin (50 μM) for CYP3A7 microsomes, and $87 \pm 2\%$ inhibited by quercetin (20 μM) for CYP2C8 microsomes (data not shown).

Concerning the inhibition of 13-*cis*-RA metabolism by CYP-specific inhibitors in human liver microsomes, the formation of the 4-OH-13-*cis*-RA was best inhibited by troleandomycin (54%) and ketoconazole (46%), whereas quercetin was a weak inhibitor (14%) (Fig. 6B). Using

Table 5
Determination of kinetic parameters for the formation of the 4-hydroxy-metabolites of 9-*cis*- and 13-*cis*-RA by human lymphoblast-expressed P450s

Cytochrome P450	4-OH-9-*cis*-RA			4-OH-13-*cis*-RA		
	V_{max} (pmol/min/nmol)	K_m (μM)	V_{max}/K_m	V_{max} (pmol/min/nmol)	K_m (μM)	V_{max}/K_m
CYP2C8	948	7	135	ND[a]	ND[a]	ND[a]
CYP2C9	2163	5	433	–	–	–
CYP3A7	3665	80	46	1587	49	32
CYP4A11				ND[a]	ND[a]	ND[a]

Microsomes (0.25 mg/mL) from cDNA expressed human P450s in lymphoblasts were incubated at 37° in a phosphate buffer with 9-*cis*- or 13-*cis*-RA at concentrations of 0, 5, 10, 20, 50 and 100 μM (total volume, 0.5 mL). The reaction was started by the addition of 1 mM NADPH and stopped by the addition of 1 mL of ethyl acetate on ice. Retinoids were extracted and analyzed by HPLC as described under Section 2.

[a] ND: not determined because the plateau phase was not reached at concentrations up to 300 μM.

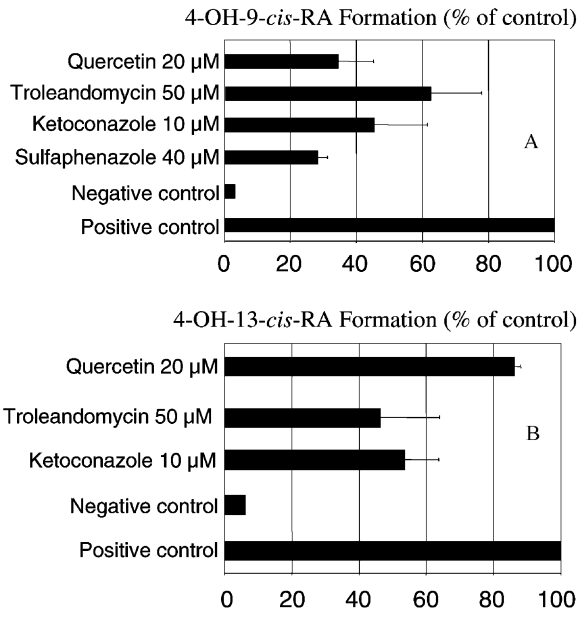

Fig. 6. Effect of various CYP inhibitors on the formation of 4-OH-9-*cis*-RA (panel A), or 4-OH-13-*cis*-RA (panel B) by human liver microsomes. The substrate (9-*cis*- or 13-*cis*-RA at 10 µM) was incubated with a pool of human liver microsomes in presence or absence of the indicated inhibitors (quercetin, 2C8; troleandomycin and ketoconazole, 3A; and sulfaphenazole, 2C9). The metabolite formation was determined by HPLC. Negative control, without NADPH; positive control, with NADPH and without inhibitor. Mean of three experiments ± SEM.

microsomes expressing a single human CYP, the 4-OH-13-*cis*-RA formation was inhibited by 89 ± 3% by quercetin (20 µM) for CYP2C8, and by 79 ± 2% by troleandomycin (50 µM) for CYP3A7 (data not shown).

4. Discussion

The aims of this study were to identify the human CYPs involved in the metabolism of the retinoic acid isomers 9-*cis*- and 13-*cis*-RA, to determine their major metabolites, and to compare the identified CYPs with those already known to be involved in the metabolism of atRA [18–24].

In the first part of this work, phenotyped human liver microsomes were used to identify the CYPs most likely to be involved in the metabolism of 9-*cis*- and 13-*cis*-RA. It was found that catalytic activities linked to CYPs 3A4/5, 2B6, 2C8, and 2A6 correlated best with metabolite formation of either isomer. In addition, CYP2C9 was also identified as being involved in the metabolism of 9-*cis*-RA. The use of stepwise regression analyses to identify possible cooperation between CYPs disclosed that 3A4/5 was cooperating with either 2B6 or 2A6 for the formation of 4-OH- or 4-oxo-9-*cis*-RA. Likewise, for the formation of 4-OH-13-*cis*-RA, cooperation was observed between several CYP-related activities including 2B6, 2C8, 1A2, and 2C9; for the 4-oxo-13-*cis*-RA, cooperation was found between CYPs 2C8, 1A2, and 4A9/11. These data suggest that in the liver, several CYPs are probably cooperating to metabolize these retinoic acid isomers.

Although the formation of 4-oxo-RA correlated with the same CYPs as the ones identified for the 4-OH-RA formation, this oxidation could be due to a non-P450 reaction, since some studies have shown this reaction is non-P450 dependent in cultured human keratinocytes and fibroblasts (reviewed in [35]). In the present study, the fact that the formation of 4-oxo-metabolites correlated with the same P450 activities as the ones identified for the 4-OH-metabolites is therefore, understandable since the 4-oxo-metabolites formation require the initial conversion to the 4-hydroxy-metabolites.

The summary presented in Table 6 shows that the correlation method identified exactly the same CYPs for the two isomers as the ones already identified for atRA [23], with the exception of CYP2C9 for 9-*cis*-RA. Therefore, to identify more precisely the various CYPs involved in the metabolism of the two isomers, we then made use of lymphoblast microsomes expressing a single human CYP. This technique allowed a better discrimination between specific CYPs with regard to the metabolism of the different RA isomers and allowed a ranking of the CYPs based on activity. For the 9-*cis*-RA, this technique confirmed the correlation method concerning the implication of CYPs 2C9 and 2C8, but also allowed the identification of CYP3A7. Although the correlation method has not allowed the identification of CYP2C9 in the 13-*cis*-RA and atRA metabolism, the use of lymphoblast microsomes could detect not only the major contribution of CYP2C9 in the 9-*cis*-RA metabolism, but could also identify the minor implication of this CYP in the 13-*cis*-RA and atRA metabolism. In addition, this method firmly established the predominant role played by CYP2C9 in the metabolism of 9-*cis*-RA since CYP2C9 was more active than CYP2C8

Table 6
Summary of the human P450s significantly involved in the 4-oxidation of retinoic acid isomers

Retinoic acid isomer	CYPs determined by correlation of activity[a]	Lymphoblast-expressed CYPs[b]
9-*Cis*-retinoic acid[c]	3A4/5, 2B6, 2C8, 2A6, 2C9	2C9, 2C8, 3A7
13-*Cis*-retinoic acid[c]	2B6, 2C8, 3A4/5, 2A6	3A7, 2C8, 4A11, 1B1, 2B6, 2C9, 2C19, 3A4
All-trans-retinoic acid[d]	2B6, 2C8, 3A4/5, 2A6	3A7, 3A5, 2C18, 2C8, 3A4, 2C9

[a] Presented by decreasing order of *r* values.
[b] Presented by decreasing order of activity.
[c] Data from this article.
[d] Data from Marill *et al.* [23].

toward this substrate, with a V_m/K_m ratio of about four times higher for the former compared to the latter. For the 13-*cis*-RA, the lymphoblast-expressed CYPs corroborated the findings of the correlation method with regard to CYPs 2C8, 2B6, and 3A4, and also identified CYP3A7 as the most active CYP.

Concerning CYPs 2B6 and 2A6 that were identified with the correlation method, but not with the microsomes expressing a single CYP (except 2B6 for 13-*cis*-RA), this discrepancy may be due to the lack of specificity of several model substrates used to phenotype human microsomes. For example, several studies have indicated the involvement of several other CYPs, i.e., 1A, 2B6, 2C, and 2E1 in the ethoxytrifluoromethyl coumarin dealkylation reaction [36,37].

The use of CYP-specific inhibitors with hepatic microsomes confirmed the major involvement of CYPs 2C9, 2C8, and 3A4 in the adult liver metabolism of 9-*cis*-RA. For 13-*cis*-RA, the strong inhibition of metabolism by troleandomycin and ketoconazole indicated the importance of CYP3A4 in adult liver metabolism, whereas 2C8 was shown to play a minor role for this isomer. These inhibition studies underline the importance of these CYPs to the overall metabolism of retinoic acid isomers in adult liver, because CYP3A4 represents 20–30% of total P450, and CYPs 2C8 and 2C9 accounts for about 20% of total P450 [38,39].

The identification of CYP2C9 in the metabolism of 9-*cis*-RA, and to a lesser extent of 13-*cis*-RA, is of importance not only because this CYP is the most abundant of the CYP2C subfamily, but also because it can metabolize a large variety of commonly used drugs [40], and care should therefore, be taken in co-administration of 9-*cis*- or 13-*cis*-RA with CYP2C9 substrates. In addition, since CYP2C9 exhibits polymorphism, it is expected that a significant proportion (about 10%) of a Caucasian population may show impaired metabolism [41] of these RA isomers, that could impact significantly on their *in vivo* disposition.

The present finding that CYP3A7 is very active with regard to 9-*cis*- and 13-*cis*-RA metabolism, is in line with the reported major implication of this CYP in retinoic acids metabolism [22,23]. Since CYP3A7 is the primary CYP isoform expressed in human fetal liver, this CYP may therefore, detoxify retinoic acids to less toxic 4-hydroxylated metabolites, and thereby protect the fetus. It is also possible that the expression of CYP3A7 could play a role in adult therapy with RA, since this CYP is expressed at low levels in adult liver [42,43].

This study did not include an assessment of CYP26 contribution to the metabolism of retinoic acid isomers because this CYP is not yet commercially available. Duell *et al.* [44] reported that the three retinoic acid isomers could induce 4-hydrolylase activity (CYP26) in human skin, but this activity was apparently selective for the *all-trans*-isomer, suggesting that CYP26 appears to be isomer-selective, at least in human skin. Another report states that 13-*cis*- and 9-*cis*-RA can inhibit the 4-hydroxylation of atRA in human liver [45], which suggests that there is some cross-metabolism between several CYPs for the RA isomers. This last observation is in line with the findings of this study that show that several identical CYPs are involved in the metabolism of the various RA isomers, although with different efficacy. Another point that could be considered at least for whole cell metabolism, is that cytoplasmic binding proteins, e.g., CRABP, can play a major role at modulating the *in vivo* disposition of retinoic acids, as it has been suggested that the half-life of 13-*cis*-RA is considerably longer than that of the *all-trans* isomer probably due to the fact that it does not bind efficiently to CRABP (reviewed in [35]).

Because several oxidized metabolites of atRA have been shown to display biological activity in the modulation of several genes involved in the growth inhibition of normal and neoplastic cells *in vitro* [9–15], it is likewise possible that the metabolism of 9-*cis*- and 13-*cis*-RA by the identified human CYPs may play an important role in the local concentration of these retinoids.

In conclusion, considering the liver microsomal data (activity and inhibition), adult human CYPs 2C8, 2C9, and 3A4 are the major CYPs involved in 9-*cis*-RA, whereas CYPs 2C8 and 3A4 are the principal CYPs involved in 13-*cis*-RA metabolism. Additional data generated from lymphoblast microsomes expressing a single CYP allowed the identification of the fetal CYP3A7 as very active in 9-*cis*- or 13-*cis*-RA metabolism. When compared to atRA, it appears that each RA stereoisomer is preferentially metabolized by a specific set of human CYPs which could impact on the local concentrations of the isomer and also direct the formation of specific metabolites with different biological properties. The role of these human CYPs in the biological response or resistance to RA isomers remains to be determined.

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