All-*trans*-retinoic acid 4-hydroxylation in human liver microsomes: *in vitro* modulation by therapeutic retinoids

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All-*trans* retinoic acid (ATRA) induces remission in patients with acute promyelocytic leukaemia. Other retinoids, including 9-*cis*- and 13-*cis*-retinoic acid (9-*cis*- and 13-*cis*-RA), are now being evaluated for their therapeutic potential. The elimination of ATRA is partially dependent on cytochrome P450 (P450)-mediated 4-hydroxylation, but the interaction of other retinoids with P450 has not yet been assessed. In the present study 9-*cis*- and 13-*cis*-RAs, as well as all-*trans*-retinol and three isomeric retinals were found to inhibit ATRA 4-hydroxylation in human hepatic microsomes, but the arotinoids acitretin and etretinate were not inhibitors. 9-*cis*- and 13-*cis*-RA were competitive inhibitors of ATRA 4-hydroxylation ($K_i:K_m$ ratios 3.5 ± 0.8 and 6.3 ± 0.5 , respectively) suggesting that these retinoids are alternate, but inferior, substrates for the P450 enzyme(s) that mediate the activity. The biotransformation of therapeutic retinoids containing the β -ionone ring system is likely to involve the microsomal ATRA 4-hydroxylase P450.

Keywords all-*trans* retinoic acid retinoids cytochrome P450

Introduction

All-*trans*-retinoic acid (ATRA) is valuable in the treatment of haematological malignancies, such as acute promyelocytic leukaemia (APL) [1]. Newer strategies that include the use of other retinoids, such as 9-*cis*- and 13-*cis*-retinoic acids (9-*cis*- and 13-*cis*-RAs) [2], or combination therapy with cytokines [3], may be implemented in future. Microsomal cytochromes P450 (P450) contribute to the elimination of ATRA by catalysing the 4-hydroxylation of the retinoid [4]. The specific P450(s) that participate in ATRA 4-hydroxylation in human liver have not been identified unequivocally, although it has been reported that 1A2, 2B6, 2C8, 2D6, 2E1 and 3A4 can catalyse the reaction [5, 6]. In the present study the ability of a series of retinoids, including 9-*cis*- and 13-*cis*-RA, to inhibit ATRA 4-hydroxylation in human liver microsomes was tested. The demonstration of competitive inhibition suggests that these compounds are substrates of the ATRA 4-hydroxylase.

Methods

Chemicals

[15-¹⁴C] ATRA (14 mCi mmol⁻¹) was obtained from Amersham Australia (North Ryde, NSW). 13-*cis*-RA, isomeric retinals, retinyl palmitate, and biochemicals were obtained from Sigma-Aldrich (Castle Hill, NSW), retinol was from Fluka Biochemika (Switzerland) and unlabelled all-*trans* RA was from Boehringer Mannheim (Sydney, NSW). 9-*cis*-RA, 4-hydroxy-ATRA, acitretin and etretinate were gifts from Hoffman-La Roche (Nutley, NJ, USA). Analytical reagents and h.p.l.c. solvents were from Ajax and Rhone Poulenc (Sydney, NSW), respectively.

Preparation of human liver microsomal fractions

The experimental protocols used in this study were approved by the Human Ethics Committee of the Western Sydney Area Health Service. Human micro-

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Figure 1 Kinetic analysis of the inhibition of human hepatic microsomal ATRA 4-hydroxylation activity by 13-*cis*-retinoic acid. (a) Lineweaver-Burk plots at (■) 0 μM , (◆) 5 μM , (▼) 10 μM , (▲) 20 μM , (●) 50 μM , and (X) 100 μM 13-*cis*-retinoic acid; (b) Dixon plots at (■) 5 μM , (◆) 10 μM , (▼) 20 μM , (▲) 50 μM , and (●) 100 μM ATRA; (c) replot of the slopes of the Lineweaver-Burk plots vs 13-*cis*-retinoic acid concentration, and (d) replot of the slopes of the Dixon plots versus reciprocal ATRA concentration. Units of substrate (S, ATRA) and inhibitor (I, 13-*cis*-retinoic acid) concentration are μM and rates of ATRA 4-hydroxylation (V) are in $\text{pmol min}^{-1} \text{mg}^{-1}$ protein.

somal fractions were prepared from excess biopsy tissue or unwanted fragments of donor livers used in hepatic transplantation (three individual livers were available for this study). Livers were placed in ice cold ViaSpan solution (Belzer University of Wisconsin solution; DuPont Pharmaceuticals, Wilmington, USA) and were then frozen in liquid nitrogen. For microsomal preparation, livers were homogenized in cold 10 mM potassium phosphate (pH 7.4) containing 1 mM EDTA and 0.25 M sucrose and subjected to differential ultracentrifugation [7]. Washed microsomes were stored at -70°C in 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 1 mM EDTA.

Assay of ATRA 4-hydroxylation activity and separation of products by h.p.l.c.

ATRA 4-hydroxylation was assayed in amber borosilicate tubes as described previously [4]. Briefly, [$^{15}\text{-}^{14}\text{C}$] ATRA ($3 \times 10^5 \text{ d min}^{-1}$; 20 μM , except in kinetic experiments) was incubated with 0.25 mg microsomal protein [8] in 0.1 M potassium phosphate buffer containing 1 mM EDTA (pH 7.4). Reactions were initiated by the addition of NADPH (1 mM) and were terminated after 45 min with 0.5 ml ethanol containing EDTA (0.125 mg) and ascorbic acid (0.125 mg). Ethyl acetate (2 ml) was added, the samples were vortexed and centrifuged and

anhydrous sodium sulphate was added to the organic phase. The organic phase was evaporated to dryness under a stream of N₂.

Microsomal extracts were dissolved in 50 µl methanol and applied to an Ultrasphere C₁₈ column (5 µm, 250 × 4.6 mm; Beckman, San Ramon, CA, USA) [4]. The mobile phase was methanol: acetonitrile: 0.1 M ammonium acetate, pH 6.8 (73:12:15) and the flow rate was 1 ml min⁻¹. Fractions (collected over 1 min intervals) were subjected to β-counting (ACS II, Amersham). ATRA and 4-hydroxy-ATRA eluted at 9.8 and 3.3 min, respectively.

In preliminary experiments [15-¹⁴C] 4-hydroxy-ATRA was generated from [15-¹⁴C] ATRA in NADPH-supplemented microsomes and was collected as the eluate following h.p.l.c. as described. The radioactive metabolite (10 000 d min⁻¹) was added in place of [15-¹⁴C] ATRA into a microsomal incubation and was then subjected to the extraction procedure; the radioactivity was recovered quantitatively. The inter-assay reproducibility of microsomal ATRA 4-hydroxylation activities was determined in the microsomal fractions on several separate occasions (coefficients of variation <8%).

Kinetic analysis of the inhibition of microsomal ATRA hydroxylation

The kinetics of the effects of 9-*cis*- and 13-*cis*-RAs (0–100 µM) on microsomal ATRA 4-hydroxylation (5–100 µM) were examined. Data were obtained from single complete experiments from each of three livers and were subjected to graphical analysis by the method

of Lineweaver-Burk (1/*V* vs 1/*S*) to determine the *K_m* (Michaelis constant) and *V_{max}* (maximal reaction velocity) of 4-hydroxylation. Dixon plots (1/*V* vs *I*) and replots (slopes of Dixon plots vs 1/*S*) were used to determine *K_i* (dissociation constant of the enzyme-inhibitor complex) and the nature of inhibition [9].

Statistics

All data are presented as mean ± s.e. mean. Statistical analyses were performed using the Student's *t*-test to detect differences between means.

Results

Effect of other retinoids on ATRA 4-hydroxylase activity

From the data in Table 1 it is evident that ATRA 4-hydroxylation was inhibited by the isomeric RAs, retinol and the isomeric retinals (28–53% of activity remaining at the test concentrations of 100 µM). Retinyl palmitate and the arotinoids, acitretin and etretinate, were not inhibitory. The apparent *K_m* for ATRA 4-hydroxylation in these livers was 10 ± 1 µM and the *V_{max}* was 85 ± 3 pmol min⁻¹ mg⁻¹ protein. *K_i* values of 34 ± 13 and 62 ± 11 µM, were obtained for 9-*cis*- and 13-*cis*-RA, respectively (Figure 1). Thus, respective *K_i*: *K_m* ratios of 3.4 ± 0.8 and 6.3 ± 0.5 were obtained for these RA derivatives. Competitive inhibition is indicated from the appearance of the Dixon plot (Figure 1b) and

Table 1 Effects of retinoids on ATRA 4-hydroxylation activity in human hepatic microsomes

<p>4</p> <p>9</p> <p>13</p> <p>Ring A (β-ionone system)</p>	<p>CH₃O</p> <p>Ring B (aromatic system)</p>							
<div> <div>ATRA 4-hydroxylase activity</div> <div>(% remaining activity)</div> </div>								
Retinoid	Ring	R	isomer					Mean $\pm$ s.e. mean
			9—	13—	HL1	HL2	HL3	
9- <i>cis</i> -retinoic acid	A	COOH	<i>cis</i>	<i>trans</i>	36	28	23	29 $\pm$ 4 ^a
13- <i>cis</i> -retinoic acid	A	COOH	<i>trans</i>	<i>cis</i>	67	54	38	53 $\pm$ 8 ^c
all- <i>trans</i> -retinol	A	CH ₂ OH	<i>trans</i>	<i>trans</i>	43	38	38	40 $\pm$ 2 ^a
all- <i>trans</i> -retinal	A	CHO	<i>trans</i>	<i>trans</i>	49	41	28	39 $\pm$ 6 ^b
9- <i>cis</i> -retinal	A	CHO	<i>cis</i>	<i>trans</i>	57	37	31	42 $\pm$ 8 ^c
13- <i>cis</i> -retinal	A	CHO	<i>trans</i>	<i>cis</i>	28	33	ND	28, 33
retinyl palmitate	A	CH ₂ OCOC ₁₅ H ₃₁	<i>trans</i>	<i>trans</i>	92	90	ND	92, 90
acitretin	B	COOH	<i>trans</i>	<i>trans</i>	106	92	64	87 $\pm$ 12
etretinate	B	CO ₂ C ₂ H ₅	<i>trans</i>	<i>trans</i>	113	101	113	110 $\pm$ 4

Data obtained in individual human hepatic microsomal fractions are presented. Significant difference from control: ^a*P* < 0.0001, ^b*P* < 0.001, ^c*P* < 0.01. ND, not determined. The general structures differ in the nature of the ring systems (ring A = β-ionone, ring B = substituted aromatic). Carbons 9 and 13 in the side chain and the 4-position of the β-ionone system are indicated.

the closeness of the intersection of the line in the Dixon replot (Figure 1d) to the origin [9].

Discussion

Retinoids have important biological functions, but are also of major importance in cancer chemotherapy. The elimination of ATRA is partially dependent on 4-hydroxylation, although ATRA biotransformation also includes glucuronidation and epoxidation pathways [10]. It has been demonstrated that several P450s, including 1A2, 2B6, 2C8, 2D6, 2E1 and 3A4, can catalyse this pathway but the identity of the P450(s) that mediate the reaction in the liver are presently undefined [5, 6]. The principal finding to emerge from the present study is that 9-*cis*- and 13-*cis*-RAs are competitive inhibitors of the P450(s) responsible for ATRA 4-hydroxylation, which suggests that 9-*cis*-, 13-*cis*-RAs and ATRA are hydroxylated by a common P450 enzyme. However, in view of the high K_i values relative to the K_m for ATRA 4-hydroxylation, the *cis*-RAs probably have a lower affinity than ATRA for the enzyme.

Serum concentrations of ATRA and 13-*cis*-RA are somewhat variable after oral dosage but range up to $\approx 10 \mu\text{M}$ [11, 12]. Because the K_m and K_i values determined in the present study are similar to these serum levels, the P450 oxidation pathway contributes significantly to the elimination of ATRA and probably other *cis*-RAs of therapeutic importance.

The β -ionone ring (ring system A in the general structure at the top of Table 1) seems necessary for inhibition of ATRA 4-hydroxylation. This is noteworthy because 4-hydroxylation occurs at an allylic carbon, where oxidation is favoured. The arotinoids do not possess an allylic carbon, which may be why they appear not to interact with the ATRA 4-hydroxylase P450.

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References

- 1 Castaigne S, Chomienne C, Daniel MT, *et al.* All-*trans* retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical Results. *Blood*. 1990; **76**: 1704–1709.
- 2 Miller WH, Jr, Jakubowski A, Tong WP, *et al.* 9-*cis*-Retinoic acid induces complete remission but does not reverse clinically acquired retinoid resistance in acute promyelocytic leukemia. *Blood* 1995; **85**: 3021–3027.
- 3 Moore DM, Kalvakolanu DV, Lippman SM, *et al.* Retinoic acid and interferon in human cancer: Mechanistic and clinical studies. *Semin Hematol* 1994; **31** (Suppl): 31–37.
- 4 Martini R, Murray M. Participation of P450 3A enzymes in rat hepatic microsomal retinoic acid 4-hydroxylation. *Arch Biochem Biophys* 1993; **303**: 57–66.
- 5 Leo MA, Lasker JM, Raucy JL, *et al.* Metabolism of retinol and retinoic acid by human liver cytochrome P450 IIC8. *Arch Biochem Biophys* 1989; **269**: 305–312.
- 6 Muindi JF, Young CW. Lipid hydroperoxides greatly increase the rate of oxidative catabolism of all-*trans*-retinoic acid by human cell culture microsomes genetically enriched in specific cytochrome P-450 isoforms. *Cancer Res* 1993; **53**: 1226–1229.
- 7 Murray M, Wilkinson CF, Dube CE. Effects of dihydrosafrole on cytochrome P-450 and drug oxidation in hepatic microsomes from control and induced rats. *Toxicol Appl Pharmacol* 1983; **68**: 66–76.
- 8 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265–270.
- 9 I.H. Segel: *Enzyme kinetics. Behavior and analysis of rapid equilibrium and steady-state systems*. Wiley-Interscience (New York), 1975.
- 10 Cullum ME, Zile MH. Metabolism of all-*trans*-retinoic acid and all-*trans*-retinyl acetate. Demonstration of common physiological metabolites in rat small intestinal mucosa and circulation. *J Biol Chem* 1985; **260**: 10590–10596.
- 11 Lefebvre P, Thomas G, Gourmel B, *et al.* Pharmacokinetics of oral all-*trans*-retinoic acid in patients with acute promyelocytic leukemia. *Leukemia* 1991; **5**: 1054–1058.
- 12 Villablanca JG, Khan AA, Avramis VI, *et al.* Phase I trial of 13-*cis*-retinoic acid in children with neuroblastoma following bone marrow transplantation. *J Clin Oncol* 1995; **13**: 894–901.

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