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Effect of P450 Isozyme-selective Inhibitors on In-vitro Metabolism of Retinoic Acid by Rat Hepatic Microsomes

MOBASHAR AHMAD, PAUL J. NICHOLLS, H. JOHN SMITH AND MASOUD AHMADI

Welsh School of Pharmacy, Cardiff University, Cathays Park, Cardiff, CF1 3XF, UK

Abstract

Cytochrome P450-mediated 4-hydroxylation of retinoic acid is an important pathway in the termination of its biological action and the activity of certain P450 isozymes has been studied in non-induced male rat hepatic microsomes using isozyme-selective inhibitors.

The importance of the activity of the isozyme to retinoic acid metabolism was, 2A6 (diethyl dithiocarbamate as selective inhibitor) $>$ 1A1/1A2 (7,8-benzoflavone) $\gg$ 1A1 (ellipticine) $>$ 3A4 (naringenin, ketoconazole) as shown by the respective apparent IC50 values of 0·12, 0·34, 2·7, 9·25 and 13·5 μM with 2C8-10, 2D6 and 2E1 having little effect on metabolism.

It is concluded that although the P450 3A family normally constitutes half the total rat hepatic P450 activity, other hepatic isozymes (1A1, 1A2 and 2A6) are also involved in retinoic acid metabolism. This suggests that the horizons for the design of potential anti-cancer agents acting through inhibition of retinoic acid metabolism may be widened to include structures which do not resemble the established hetereocyclic base P450 3A4 inhibitors.

Retinoic acid is the active metabolite of retinol (vitamin A) that executes vitamin A-dependent functions (except vision) including maintenance of the integrity of epithelial cells and regulation of cellular differentiation. Known pathways for clearance of physiological amounts of retinoic acid include C_4-hydroxylation, followed by dehydrogenation, isomerization (to 13-*cis*-4-oxo retinoic acid) and glucuronylation of retinoic acid (Fiorella & Napoli 1994). The pathway initiated by C_4-hydroxylation has received the most attention as a first event, committing retinoic acid to degradation in several retinoid target tissues (Roberts et al 1980; Frolik 1981; Van Wauwe et al 1994), and is catalysed by hepatic microsomal cytochrome P450 oxidase (Roberts et al 1979b).

The identity of the hepatic P450s that participate in retinoic acid 4-hydroxylation in-vivo remain to be established. P450 2B (rat, rabbit), P450 1A2 (rabbit), P450 2C7 (rat), and the P450 2C subfamily (human) have been shown to possess activity (Leo et al 1984, 1989; Roberts et al 1992). Continuous administration of retinoic acid in the treatment of cancer results in loss of effectiveness due to a decrease in plasma concentration. The causes of this situation in man are not clear but Sprague-Dawley rat studies have shown that retinoic acid does not induce an increase in hepatic P450 content (Howell et al 1998). However, in other species a different picture emerges of induction by retinoic acid of its own metabolism. A novel P450, CYP 26 (P450 RAI) which is cloned from zebra fish, human and mouse tissues, is induced by retinoic acid and is specific for the metabolism of retinoic acid to 4-hydroxy retinoic acid and other products (White et al 1997). This enzyme is considered to play a key role in retinoic acid metabolism, controlling retinoic acid levels in an autoregulatory manner.

This study has examined the effects of a range of established P450 isoform-selective inhibitors (Nedelcheva & Grut 1994) on the metabolism of [11,12-^{3}H]retinoic acid by male rat hepatic microsomes to provide a preliminary characterization of the relevant P450s. Although these inhibitors have only been established as selective for human P450s,

Correspondence: H. J. Smith, Welsh School of Pharmacy, Cardiff University, Cathays Park, Cardiff, CF1 3XF, UK.

their application to the rat forms here was considered useful in providing a preliminary characterization of the P450 isoform(s) involved in hepatic retinoic acid metabolism in this species.

Materials and Methods

All *trans*-retinoic acid, NADPH, butylated hydroxy anisole, ketoconazole, 7,8-benzoflavone, ellipticine, diethyl dithiocarbamate, naringenin, sulphaphenazole, cimetidine, quinidine, phenformin, aminoacetonitrile, 4-methylpyrazole, isoniazid and protein standards were purchased from Sigma Chemical Company (Dorset). [11,12-^{3}H]All *trans*-retinoic acid was from Dupont (UK) Ltd (Stevenage, Herts). Formic acid, ammonium acetate and Hisafe III scintillation fluid (Optiphase III) were obtained from Fisons Ltd (Leicestershire). All solvents used for chromatography were of HPLC grade and were obtained from Rathburn Chemicals Ltd (Walkburn, UK). All other laboratory reagents were of analytical grade and obtained from British Drug House (Poole, Dorset).

Preparation of rat liver microsomes

Rat liver microsomes were prepared by the method previously described (Ahmad et al 1999) and stored at -80°C.

Determination of retinoic acid-metabolising activity of rat liver microsomes

The assay followed that described previously based on the general procedure of Roberts et al (1979a, 1992) and Van Wauwe et al (1992) and was briefly as follows. Hepatic microsomes (10 μL, 0·12 mg protein mL^{-1} final concentration) were incubated, in triplicate, with [11,12-^{3}H]retinoic acid (3 μM, 10 μL), NADPH (2 mM, 50 μL) and phosphate buffer (50 mM, pH 7·4, 320 μL) for 15 min at 37°C and then the reaction was terminated by adding formic acid (1% v/v, 0·1 mL). [11,12-^{3}H]Retinoic acid and its (oxidative) metabolites were extracted into ethyl acetate containing 0·05% (v/v) butylated hydroxyanisole (2 $\times$ 2 mL). The extract was taken to dryness in-vacuo at room temperature and the residue dissolved in acetonitrile–water–formic acid (75 : 25 : 0·05 v/v) containing ammonium acetate (10 mM) and the [^{3}H]metabolites separated and quantitatively determined by HPLC on a C_{18} μBondapak (3·9 $\times$ 300 mm, Millipore) with a model 970 detector (Reeve). The percentage metabolism was calculated from the areas under the curves: 100 [(metabolites)/(metabolites + retinoic acid)].

Determination of IC50 values for P450 isozyme-selective inhibitors of retinoic acid metabolism

The assay was conducted in the presence of P450 isozyme-selective inhibitors of 1A1 (7,8-benzoflavone, ellipticine), 1A2 (7,8-benzoflavone), 2A6 (diethyl dithiocarbamate), 3A4 (naringenin, ketoconazole), 2C8,9,10 (sulphaphenazole, cimetidine), 2D6 (quinidine, phenformin) and 2E1 (aminoacetonitrile, 4-melthylpyrazole, isoniazid) at 100 μM final concentration in DMSO (10 μL). A control incubation was conducted with DMSO (10 μL) alone. The percentage inhibition was calculated from the areas under the curves: 100 [(metabolites (control) $-$ metabolites (inhibitor))/metabolites (control)]. The IC50 values for the inhibitors were determined, using a range of suitable inhibitor concentrations, from a plot of percentage inhibition versus log inhibitor concentration using Cricket Graph 1.3.

Table 1. Inhibition of in-vitro hepatic microsomal metabolism of [11,12-^{3}H]retinoic acid by various P450 isozyme selective inhibitors.

P450 Isoform	Selective Inhibitor	% Inhibition[a] (100 μM)	IC50[a] (μM)
1A1	7,8-benzoflavone	85·8 ± 0·4	0·34 ± 0·01
	ellipticine	86·7 ± 0·2	2·7 ± 0·14
1A2	7,8-benzoflavone	85·8 ± 0·4	0·34 ± 0·01
2A6	diethyl dithiocarbamate	85·6 ± 1·3	0·34 ± 0·01
3A4	naringenin	80·8 ± 3·0	9·25 ± 0·07
	ketoconazole	87·5 ± 0·4	13·5 ± 1·3
2C8,9,10	sulphaphenazole	4·8 ± 0·1	ND
	cimetidine	0·0	ND
2D6	quinidine	3·9 ± 0·1	ND
	phenformin	12·0 ± 0·1	ND
2E1	aminoacetonitrile	0·0	ND
	4-methylpyrazole	0·0	ND
	isoniazid	0·0	ND

Values are the means of three determinations ± s.d. [a]Retinoic acid concentrations = 3 μM. ND, not determined.

Results and Discussion

Inhibition of in-vitro rat hepatic microsomal metabolism of [^{3}H]retinoic acid by various P450 isozyme selective inhibitors at 100 μM concentration (Table 1) indicates that those inhibitors selective for the P450 families 1, 2 and 3 and sub family A were most effective in reducing retinoic acid metabolism viz 1A1 (85·8, 86·7% inhibition), 1A2 (85·8%), 2A6 (85·6%), 3A4 (80·8, 87·5%). The IC50 values for the inhibitors (Table 1) were 0·34 μM for 7,8-benzoflavone (1A and 1A2), 2·7 μM for ellipticine (1A1), 0·12 μM for diethyl dithiocarbamate (2A6), 9·25 μM for naringenin (3A4) and 13·5 μM for ketoconazole (3A4). These results suggest that in rat liver microsomes the 3A4 isozyme is less engaged in retinoic acid metabolism than the 2A6, 1A1 and 1A2 isoforms.

4-Hydroxylation of retinoic acid (and further metabolism to 4-keto retinoic acid) is a major pathway mediated by P450(s) in its metabolism (Williams & Napoli 1985), as demonstrated by administration to rats of ketoconazole and liarozole (known P450 inhibitors) when retinoic acid serum levels were elevated (Van Wauwe et al 1990, 1992) and in-vitro studies where P450 2B, P450 1A2 and P450 2C7 forms have been involved (Leo et al 1984, 1989; Roberts et al 1992). However, the identity of the P450(s) that participate in 4-hydroxylation of retinoic acid in-vivo remains to be established.

The present study was undertaken to characterise the P450s present in non-induced rat hepatic microsomes responsible for the 4-hydroxylation of retinoic acid by using a range of established P450 isoform-selective inhibitors. Although these have only been used in human P450 studies, their application in this work might provide a preliminary characterization of the P450 isoforms involved in hepatic retinoic acid metabolism in this species.

The results obtained (Table 1) with the inhibitors at concentrations of 100 μM show that P450s in families 1, 2 and 3, with sub family A, are most effective in reducing metabolism of retinoic acid. The apparent lower IC50 values for diethyl dithiocarbamate (0·12 μM; 2A6 inhibitor), 7,8-benzoflavone (0·34 μM; 1A1 and 1A2) than naringenin and ketoconazole (9·25 and 13·5 μM, respectively; 3A4) support a ranking order for metabolic activity of $2A6 > 1A1/1A2 \gg 1A1 > 3A4$. However, it must be borne in mind that these values are weighted means since several isoforms may be inhibited due to some non-selectivity of the inhibitor and that the potency (i.e. K_i) of the inhibitor for a specific isoform is also involved, i.e. $IC50 = K_i\ (1 + S/K_m)$.

Martini & Murray (1993) reported a potential role for P450 3A in the metabolism of retinoic acid. However, their studies were limited in that the effects of 1A1, 1A2, 2A6, 2C8, 9, 10 and 2D6 inhibitors were not included. Here the isoforms 2A6, 1A1 and 1A2 have been shown to have a more important role than 3A4. This conclusion would suggest that the design of potential inhibitors of retinoic acid metabolism could extend beyond the current heterocyclic base P450 3A4 inhibitors to include new structural types.

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