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Inhibition of retinoic acid metabolism by imidazole antimycotics in F9 embryonal carcinoma cells*

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Retinoic acid is a metabolite of retinol [1–3] that promotes growth, maintains epithelia *in vivo* [4], and is more active than retinol in the induction of differentiation of most cultured cells and tissues [5]. Inhibitors of retinoic metabolism would be useful to help assess the structure/activity relationships of retinoids, since extensive metabolism occurs simultaneously with retinoid-induced differentiation. The F9 embryonal carcinoma cell line, for example, is induced to differentiate into parietal endoderm by retinoic acid [6, 7], as it converts retinoic acid into polar metabolites [8], and is, therefore, one model to relate retinoid metabolism with function.

This report compares inhibition of retinoic acid metabolism in F9 cells by four inhibitors of cytochromes P-450. The imidazole antimycotics, ketoconazole, clotrimazole, and miconazole, inhibit: the metabolism of xenobiotics in microsomes [9] and vitamin D_3 in LLC-PK_1 cells [10, 11]; sterol biosynthesis [12, 13]; and oxidative metabolism of steroids [14, 15]. Metyrapone is a relatively nonspecific inhibitor of cytochromes P-450 [16].

Methods

Materials. [11, 12-^{3}H]Retinoic acid (23–32 Ci/mmol) was a gift of Hoffmann-La Roche, Inc. Ketoconazole was obtained from Janssen Pharm., Inc. Clotrimazole, miconazole, and metyrapone were purchased from Sigma.

Cell culture. F9 cells were grown in Dulbecco's modified Eagle medium with 10% heat-inactivated fetal bovine serum. The cells were seeded in 35 mm dishes (8×10^5 cells/plate). After 12 hr, the medium was replaced, and an inhibitor in ethanol (2 μl) or ethanol alone was added. Thirty minutes after the additions, 100 pmol of all-*trans*-[^{3}H]retinoic acid, purified to 98% by HPLC, was added in ethanol.

Quantification of retinoic acid in F9 cultures treated with inhibitors (Table 1). Each incubation (cells plus medium) was quenched with 0.2 N HCl in methanol (2 ml). Radioinert retinoic acid (5 μg) was added, and each sample was extracted with hexane [2]. The hexane was evaporated and the residues were dissolved in methanol (100 μl) and analyzed by HPLC (see below). The radioinert retinoic acid recovered (80%) was determined from a standard curve relating peak height to mass. [^{3}H]Retinoic acid was measured by liquid scintillation counting.

Analysis of retinoic acid metabolites (Figures). After the incubations, medium was removed from cells. Cells were harvested with 0.02% EDTA in phosphate-buffered saline (PBS) and were washed twice with PBS. The washes were combined with the medium. The medium and cells were dehydrated separately by azeotropic distillation with ethanol. Internal standards were added, and the residues were extracted with three portions of methanol (1–3 ml) containing butylated hydroxytoluene (BHT) (5 μg/ml). Solvents were concentrated and samples were analyzed with a reverse-phase column (radially-compressed C-18, 0.8 × 10 cm) eluted at a flow rate of 2 ml/min with a linear gradient of 10 mM ammonium acetate in methanol/water (45/55) to 10 mM ammonium acetate in methanol/water (75/25) over 30 min. Retinoids were detected at 340 nm. Liquiscint (5 ml) was added to each fraction (1 ml), and the [^{3}H]retinoids were measured with an LKB scintillation counter. Detection limits were 10 fmol in cell and 500 fmol in medium extracts.

Results and discussion

The four inhibitors of cytochromes P-450 were tested at three concentrations (Table 1). Ketoconazole and clotrimazole were about equipotent. Miconazole had no more than 10% of the activity of clotrimazole, i.e. 100 μM miconazole inhibited retinoic and metabolism by 56%, compared to 10 μM clotrimazole inhibiting 62%. Metyrapone had about 1% of the activity of clotrimazole—100 μM metyrapone inhibited 26% compared to 1 μM clotrimazole inhibiting 22%.

Incubation for more than 8 hr with 100 μM ketoconazole or clotrimazole resulted in cell death. The cytotoxic effects were not observed at inhibitor concentrations of 10 μM during 24-hr incubations. The presence of 10 μM ketoconazole does not prevent F9 cell proliferation or retinoic acid-induced differentiation [8]. Consequently, careful use

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Table 1. Inhibition of retinoic acid metabolism in F9 embryonal carcinoma cells by inhibitors of cytochrome P-450-mediated metabolism

Inhibitor	% Inhibition		
	Concentration (μM)		
	1	10	100
Ketoconazole	42	84	100
Clotrimazole	22	62	84
Miconazole	8	12	56
Metyrapone	0	2	26

F9 cells were incubated with [^{3}H]retinoic acid (50 nM, 23 Ci/mmol) and inhibitors for 4 hr. The data are the average of duplicates; variation was less than 6%. Retinoic acid in the control, i.e. incubated in medium in the absence of cells, was stable for the duration of the experiment.

of these agents inhibits retinoic acid metabolism without compromising cell viability or the capacity to differentiate.

The antimycotics selectively inhibit specific cytochromes P-450 [15]. For example, miconazole is a potent inhibitor of human placental microsomal aromatase but is a poor inhibitor of the human placental mitochondrial cholesterol side-chain cleavage complex. Clotrimazole is a weak inhibitor of aromatase, but a potent inhibitor of cholesterol side-chain cleavage. Thus, it seemed appropriate to determine whether ketoconazole and clotrimazole, the two most potent inhibitors of retinoic acid metabolism, were selective in F9 cells and inhibit at different sites of retinoid metabolism.

Retinoids in F9 cells and their medium were analyzed by HPLC (Fig. 1) after incubation of 50 nM [^{3}H]retinoic acid (32 Ci/mmol). After 12 hr, 83% of the substrate had been converted into polar metabolites, and 99% of the retinoids were in the medium. Three clusters of polar metabolites were detected migrating at 3–11, 25–32, and 45–58 ml. The most rapidly eluting group of metabolites comprised 52 and 50% of the retinoids in the medium and cells respectively. Retinoic acids (all-*trans* and 13-*cis*) represented 13 and 30% of the retinoids in the medium and the cells respectively. Relative to the quantities of the fastest eluting cluster and retinoid acids, a lesser contribution of the metabolites migrating in 25–32 and 45–58 was observed in the cells than in the medium. A minor peak co-eluted with 4-hydroxyretinoic acid. In the controls, in which 50 nM [^{3}H]all-*trans*-retinoic acid was incubated in medium alone, greater than 95% of the substrate was recovered as unchanged retinoic acids. At least 15% of the retinoic acid was present as 13-*cis*-retinoic acid, which demonstrates that the mere detection of 13-*cis*-retinoic acid does not imply enzymatic synthesis, nor original presence in the biological material.

These results were compared to those obtained with 10 μM ketoconazole (data not shown) or 10 μM clotrimazole (Fig. 2) under the same conditions. No gross differences were noted in the nature of the metabolites produced during ketoconazole or clotrimazole treatment, which suggests that these inhibitors do not selectively inhibit different enzymes of retinoid catabolism. Both markedly reduced the quantity of the most rapidly eluting metabolites (3–11 ml) and nearly completely inhibited the accumulation of metabolites eluting at 25–32 ml. The amounts of metabolites eluting at 45–58 ml were increased 3- and 2-fold by ketoconazole and clotrimazole, respectively, possibly as a result of increased substrate. A 4-fold increase in the *total* retinoic acids (cells and medium) was produced by both inhibitors. The *cellular* retinoic acids increased 15-fold with ketoconazole and 11-fold with clotrimazole.

Ketoconazole and clotrimazole are potent inhibitors of retinoic acid metabolism, inhibiting at sites beyond 4-hydroxylation of retinoic acid. This suggests roles for cytochrome P-450-dependent enzymes in a broad spectrum of retinoid metabolism. These inhibitors should be useful for further delineation of the relationship between the metabolism and function of retinoids. Perhaps co-dosing these antimycotics could enhance the clinical effectiveness of retinoids in dermatology and oncology [17] by helping to sustain tissue levels of the active retinoids.

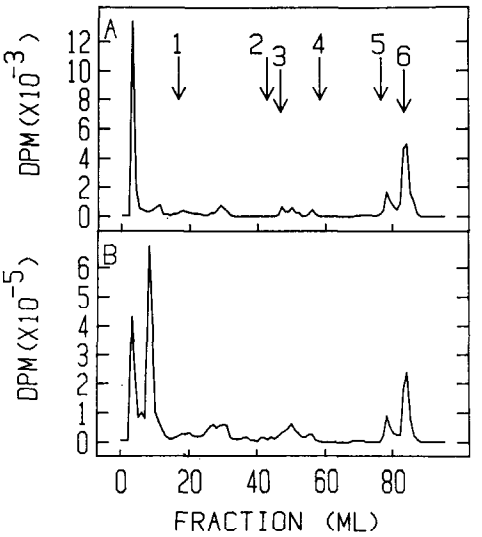

Fig. 1. Analysis of the [^{3}H]retinoic acid metabolites produced by F9 cells. The cells (panel A) and their medium (panel B) were analyzed by HPLC. Internal standards used were: (1) 4-oxo-16-hydroxyretinoic acid; (2) 4-oxoretinoic acid; (3) 4-hydroxyretinoic acid; (4) 5,6-epoxyretinoic acid; (5) 13-*cis*-retinoic acid; and (6) all-*trans*-retinoic acid. Fractions were 1 ml.

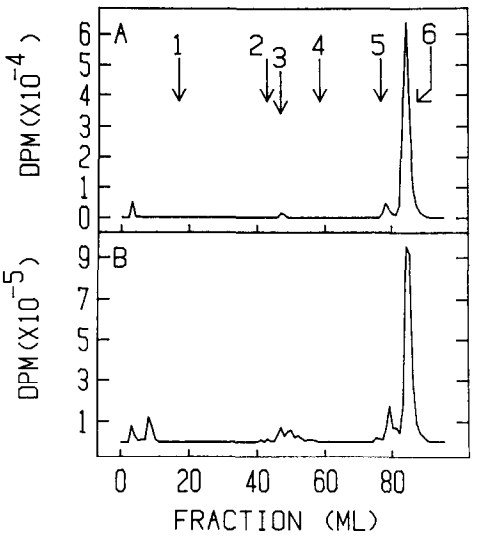

Fig. 2. Analysis of the [^{3}H]retinoic acid metabolites produced by F9 cells in the presence of 10 μM clotrimazole. Cells (A) and their medium (B) were analyzed as described in the legend to Fig. 1.

Department of Biochemistry JOHN B. WILLIAMS*
SUNY-Buffalo JOSEPH L. NAPOLI†
Baffalo, NY 14214, U.S.A.

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* This work was done in partial fulfillment of the Ph.D. degree, University of Texas, Health Science Center, Dallas.

† To whom correspondence should be addressed.

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