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SPECIFIC, COVALENT BINDING OF AN AZIDORETINOID TO CELLULAR RETINOIC ACID-BINDING PROTEIN

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Two C(5)-azido substituted aromatic retinoids were evaluated as photoaffinity probes for studying the mechanism of retinoid action. The secondary azide 1 and the tertiary azide 2 were equipotent with the parent C(5)-geminal-dimethyl substituted aromatic retinoid 3 in stimulating F9-cell differentiation. Both azides bound covalently to cellular retinoic acid-binding protein upon photolysis, but the secondary azide was twice as efficient, likely because of lesser steric hindrance. The covalent binding of azide 1 was specific, since it was inhibited by retinoic acid. Thus substitution of a photolabile group onto aromatic retinoids does not abolish biological activity and affinity for cellular retinoic acid-binding protein.

Naturally-occurring retinoids (vitamin A) are essential for the differentiation and maintenance of epithelia (1,2). Retinoic acid is a physiologically-occurring retinoid (3-5) that promotes the differentiation of a wide variety of cell types in culture (6). Retinoic acid is the most potent naturally-occurring substance that induces differentiation of the F9 embryonal carcinoma cell line (7,8). Therefore, F9 cells are a useful model for studying the mechanism of retinoid action and evaluating the biological activity of retinoids.

Altered gene expression is one mechanism of retinoic acid-induced differentiation (9). The specific uptake of retinoic acid by isolated nuclei

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Abbreviations used are: CRABP, cellular retinoic acid-binding protein; HPLC, high-performance liquid chromatography; HPSEC, high-performance size-exclusion chromatography; MOPS, 3-[N-morpholino]propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PHMB, p-hydroxymericuribenzoate; DTT, dithiothreitol; ELISA, enzyme-linked immunoadsorbent assay.

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is mediated by CRABP (10,11), which is occupied by retinoic acid *in vivo* (12). One approach to studying the relationship among CRABP, retinoic acid, and the nuclear binding of retinoids is to use photoaffinity-labelled retinoic acid analogs. Covalent labelling of binding sites with photolabile ligands is a powerful technique for probing the interactions between a ligand and its binding sites (13). We report the biological activity and covalent binding to CRABP of two photolabile aromatic retinoic acid analogs.

MATERIALS AND METHODS

Retinoids. All-trans-retinoic acid was purchased from Sigma. 11,12-[^{3}H]Retinoic acid (23 Ci/mmol), a gift from Hoffmann-LaRoche, Inc., Nutley, NJ, was purified by HPLC as described (14). 6-(5-Azido-5,6,7,8-tetrahydro-8,8-dimethyl-2-naphthalenyl)-2-naphthalenecarboxylic acid (1), 6-(5-azido-5,6,7,8-tetrahydro-5,8,8-trimethyl-2-naphthalenyl)-2-naphthalenecarboxylic acid (2), and 6-(5,6,7,8-tetrahydro-8,8-dimethyl-2-naphthalenyl)-2-naphthalenecarboxylic acid (4) were prepared by the method of Dawson *et al.* (15). 6-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-2-naphthalenecarboxylic acid (3) was prepared by the method of Dawson *et al.* (16). Retinoic acid and aromatic retinoids were handled under yellow light. Experiments with photolabile compounds were performed in darkness to the greatest feasible extent.

Assay of CRABP-Ligand Binding. Retinoid binding to CRABP was evaluated by HPSEC as described by Ranier *et al.* (17). Briefly, cytosol was prepared from a 1/1 (w/v) homogenate of decapsulated calf testes in 10 mM MOPS, 10 mM KCl, 2 mM 2-mercaptoethanol, 1 mM EDTA, and 0.5 mM PMSF, pH 7.5 (buffer A). The pH of the cytosol was adjusted to 5.0 with acetic acid. The supernatant was recovered after centrifugation for 10 min at 12,000 X g, and its pH was readusted to 7.5 with KOH. Protein in the purified cytosol was measured by the dye-binding method of Bradford (18).

Retinoids were added in 2 µl of ethanol to 2-4 mg of protein from the purified cytosol in a total volume of 0.2-0.4 ml of buffer A . After a 30-min incubation at 25 °C, the incubation mixture was placed on ice and unbound ligand was removed with dextran-coated charcoal (2.5% charcoal/0.25% dextran in buffer A). After 10 min, the charcoal was sedimented at 27,000 X g for 10 min. The supernatant was filtered through a 0.22-micron filter (Gelman). The sample was applied to a TSK 3000 SW column (1 X 30 cm, Varian Associates) and was eluted with buffer A containing 20 mM KCl (Waters Associates HPLC). Fractions (0.5-ml each) were counted for radioactivity in 5 ml of Liquiscint.

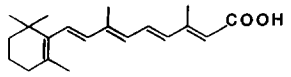
Photolysis. Incubation mixtures were photolyzed for 5 min at ambient temperature with a Mineralight UVSL-25 (Scientific Products, maximum intensity at 254 nm) at a distance of 2.5 cm. The samples were then incubated sequentially with: 5 mM PHMB for 1 hr at 4 °C; 25 mM DTT for 1 hr at 4 °C; 28 nM [^{3}H]retinoic acid for 30 min at 25 °C.

Assay of Biological Activity. The ability of the retinoids to stimulate F9 embryonal carcinoma cell differentiation was determined by measuring laminin production with an ELISA as described by Williams and Napoli (14). Three dishes of cells were used for each concentration; each dish was assayed in triplicate. The data were analyzed with the Allfit program (19), a statistical method for fitting sigmoidal curves, adapted for use with an IBM PC.

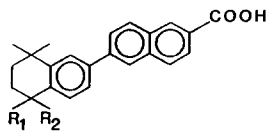
RESULTS AND DISCUSSION

An ELISA for the basement membrane glycoprotein laminin, a marker of F9 cells differentiated into parietal endoderm, was used to compare the activity of the four retinoids shown in Fig. 1 to that of retinoic acid. Dose-response curves were generated for each compound in the range of 0.01 nM to 1000 nM. Retinoids 1 to 4 promoted differentiation of F9 cells but were less active than retinoic acid (Table 1). The groups at C(5) of the tetrahydronaphthalenyl ring were clearly important to higher activity, because the C(5)-dihydro analog 4 was 6-fold less potent than the C(5)-derivatized compounds. Most important, substituting an azide for a methyl group at C(5) did not diminish the ability of the substituted compounds to induce F9-cell differentiation. Also assessed was the effect of azide substitution on binding to CRABP. The 5-azido retinoid 1, the 5-methyl-5-azido retinoid 2, and radioinert retinoic acid, at 200-fold molar excess concentrations, were compared for their ability to compete with 55 nM [^{3}H]retinoic acid for binding to calf testes cytosolic CRABP. The 5-azido analog 1 displaced 78% of the label, relative to 88% displacement by radioinert retinoic acid (Fig. 2). The 5-azido-5-methyl analog 2 displaced 49% of the [^{3}H]retinoic acid (data not shown).

To assess the degree of covalent binding of the azido analogs to CRABP upon photolysis, 55 nM [^{3}H]retinoic acid (control) or 4.7 μM azidoretinoid 1



all-trans-retinoic acid



aromatic retinoid	R_1	R_2
1	H	N_3
2	CH_3	N_3
3	CH_3	CH_3
4	H	H

Fig. 1: Structures of retinoids.

Table 1: Biological Activity of Aromatic Retinoids 1 to 4 Relative to Retinoic Acid in Stimulating Differentiation of F9 Cells

Retinoid	[Half-Maximum Response]	[Maximum Response]	Relative Potency
	nM	nM	%
retinoic acid	1	10-100	100
1	31	1000	3
2	31	1000	3
3	31	1000	3
4	184	1000	0.5

or 2 was allowed to bind to CRABP. After incubation, the free ligands were removed and the CRABP-retinoid complexes were photolyzed. The photolyzed CRABP-retinoid complexes were then incubated with PHMB, to release non-covalently bound ligand (20). CRABP was then reduced with DTT to restore its retinoic acid-binding integrity (20). The CRABP regenerated from each sample was incubated with 55 nM [^{3}H]retinoic acid, separated from free ligand, and analyzed by HPSEC to determine available binding sites.

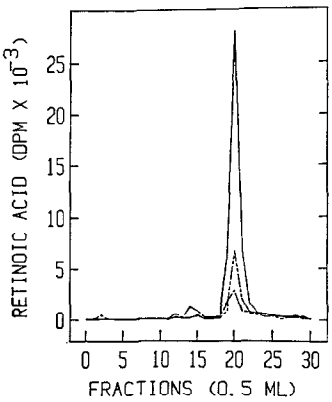


Fig. 2: Competition between aromatic retinoid 1 and retinoic acid for CRABP. Protein (2 mg/tube) from the purified calf testes cytosol was incubated with 55 nM [^{3}H]retinoic acid alone (——), or in the presence of 200-fold molar excesses of radioinert retinoic acid (-·-·-), or aromatic retinoid 1 (-·-·-). After 30 min, unbound ligand was removed with dextran-coated charcoal. The sample was analyzed for CRABP by HPSEC.

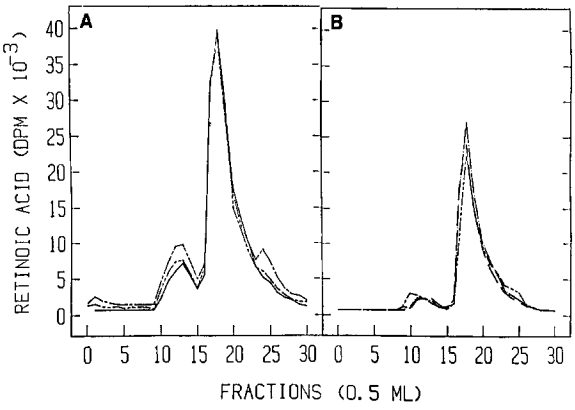


Fig. 3: Covalent binding of aromatic retinoid 1 to CRABP. CRABP (4 mg protein/tube) was incubated in triplicate with either 55 nM retinoic acid (A) or 4.7 μM aromatic retinoid 1 (B) for 30 min. Free ligands were removed with dextran-coated charcoal, and the supernatants were photolyzed for 5 min at ambient temperature. Each solution was then treated sequentially with PHMB, DTT, and 28 nM [^{3}H]retinoic acid. Unbound retinoic acid was removed and CRABP-retinoic acid complexes were analyzed by HPSEC.

In the first experiment (Fig. 3), 1.9 ± 0.1 pmol of [^{3}H]retinoic acid/tube rebound to the irradiated CRABP, which had been photolyzed in the presence of [^{3}H]retinoic acid. In contrast, 1.2 ± 0.2 pmol/tube (mean ± SD, n = 3, p less than 0.025) rebound to the CRABP, which had been photolyzed in the presence of the secondary azide 1 (37% covalent binding). In the second experiment, the average of duplicate determinations was 3.2, 2.0, and 2.6 pmol of [^{3}H]retinoic acid/tube rebound for the photolyzed CRABP-retinoic acid, secondary azide 1, and tertiary azide 2 complexes, respectively (36 and 17% covalent binding by 1 and 2, respectively). The less efficient covalent binding of the photospecies generated from the tertiary azide, compared to that generated from the secondary azide, probably reflects either the steric hindrance afforded by the C(5) methyl group on the tetrahydronaphthalenyl ring or the increased lability of the tertiary azido group.

To determine the specificity of binding by the secondary azide 1, CRABP was incubated with 250 nM retinoic acid for 30 min; 4.7 μM azide 1 was added to three of the six incubation mixtures. After 5 min, all six mixtures were photolyzed. All of the mixtures were then treated with PHMB/DTT, incubated with 28 nM [^{3}H]retinoic acid, and measured for pmol of bound retinoic acid

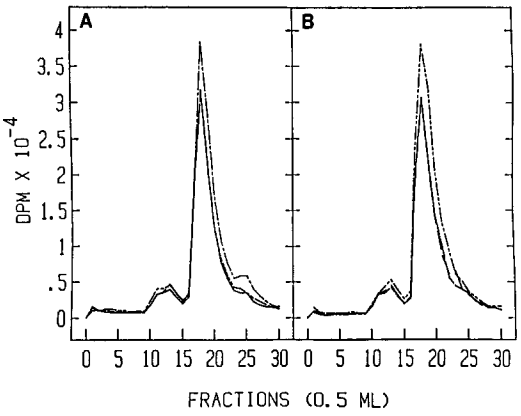


Fig. 4: Specificity of covalent binding of aromatic retinoid 1 to CRABP. CRABP (4 mg protein/tube) was incubated with 250 nM retinoic acid for 30 min, followed by incubation with 4.7 μM aromatic retinoid 1 (A), or vehicle alone (B). After 5 min, the solutions were photolyzed, free ligands were removed, and each solution was then treated sequentially with PHMB, DTT, and 28 nM [^{3}H]retinoic acid. Unbound retinoic acid was removed and CRABP-retinoic acid complexes were analyzed by HPSEC.

(Fig. 4). The CRABP photolyzed in the presence of retinoic acid alone rebound 1.5 ± 0.2 pmol of [^{3}H]retinoic acid/tube, and the CRABP photolyzed in the presence of both retinoic acid and the azide 1 rebound 1.6 ± 0.2 pmol of retinoic acid/tube (mean ± SD, n = 3). Thus, retinoic acid protected CRABP from covalent modification by the azido aromatic retinoid.

This report has demonstrated the efficacy of new synthetic retinoids as the first photoaffinity labels for CRABP. Incorporation of a photolabile group onto aromatic retinoids did not abolish biological activity and affinity for CRABP. The use of photoaffinity labelled retinoids is likely to provide new insights into the mechanisms of retinoid action, particularly when applied to probe interactions among retinoids, CRABP, and putative nuclear acceptor sites.

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