Relationship between Binding Affinities to Cellular Retinoic Acid-binding Protein and in Vivo and in Vitro Properties for 18 Retinoids


ABSTRACT

A new rapid assay has been developed for measurement of the binding of [3H]retinoic acid to cellular retinoic acid-binding protein. The assay, which uses activated charcoal for the separation of bound from unbound retinoic acid, was used to determine the concentration required to inhibit the binding of [3H]retinoic acid to cellular retinoic acid-binding protein by 50% for 18 retinoids with free carboxylic acid groups. Partially purified cellular retinoic acid-binding proteins isolated from rat testes and carcinogen-induced rat mammary tumors were used for these determinations. The following parameters were also determined for some or all of the retinoids: hypervitaminosis A doses; activity against carcinogen-induced mouse skin papillomas; inhibition of growth of a rat chondrosarcoma; inhibition of growth of 3T6 cells; and differentiation of the embryonal carcinoma cell line PCC4.azalR. While all retinoids that are potent in these biological test systems bind tightly to cellular retinoic acid-binding protein, the converse is not true. The lack of a consistent quantitative correlation between 50% inhibitory concentration and biological activity is probably due to insufficient concentrations of the retinoid in the target tissue or cell, which is a consequence of factors such as absorbability, metabolism, tissue distribution, and pharmacokinetics.

INTRODUCTION

The natural retinoids, retinol and all-trans-retinoic acid, are essential for the normal differentiation of epithelial tissues (44) where they may act in a manner analogous to the steroid hormones, which control differentiation of target organs (31, 46, 56). Both the natural and the synthetic retinoids also influence the changes in cellular differentiation that occur following exposure to carcinogens. Thus, retinoids prevent or delay the development of benign and malignant, chemically induced tumors in vivo (3, 5–9, 14, 16, 17, 21, 42, 43, 53, 55, 65, 66) and prevent or reverse carcinogen or vitamin A deficiency-induced changes of differentiation in the mouse prostate (10–12, 32, 33), chick embryo metatarsal skin (76), and hamster trachea (64) in organ culture. In tissue culture, retinoids prevent oncogenic transformation of mouse fibroblasts by chemical carcinogens (40) or X-irradiation (24) and inhibit several transformed and tumor cell lines (35, 36). Recently, retinoids have been shown to cause the differentiation of embryonal carcinoma cells that are normally nullipotent or differentiate very infrequently (28, 67).

The mechanism by which retinoids mediate these diverse effects is unknown. However, retinoids have been shown to be potent antipromoting agents, particularly in their ability to antagonize the effects of tumor-promoting phorbol esters on mouse skin (70, 71). In addition, since certain retinoids can stimulate immune responses (18, 30, 34, 41), some of the anticarcinogenesis effects in vivo may be mediated via stimulation of host immunity. However, in vitro, other mechanisms must be operative, and there is growing evidence that retinoids mediate their biological functions via specific cellular binding proteins. Two such proteins have been identified and recently purified to homogeneity from various sources: cellular retinol binding protein (1, 2, 50, 54, 74), which is specific for retinoids with a free alcohol function (48); and cellular retinoic acid-binding protein (47, 51, 54, 57, 59, 60, 75), which is specific for retinoids with a free carboxylic acid function (59, 60). A role of the cellular retinoic acid-binding protein in the mediation of retinoid biological activity is suggested by correlations between binding affinities and biological properties (15, 26, 27, 60, 61). However, thus far, binding to cellular retinoic acid-binding protein has been measured using the original sucrose density gradient assay for this protein (47) and only at 100-fold or 200-fold molar excess of the test retinoids. While such measurements can answer the question of whether retinoids bind to cellular retinoic acid-binding protein or not, they are not capable of quantitation of the binding affinities. In addition, since the separation of bound retinoic acid from unbound retinoic acid is achieved via sucrose density gradient centrifugation and is thus not rapid, measurements of binding constants by this methodology may be subject to error. In this paper, we describe a rapid, simple method for measurement of binding of retinoic acid to cellular retinoic acid-binding protein and its application to the determination of IC50’s2 for 18 retinoids.

Previous studies of binding affinities were carried out with cellular retinoic acid-binding protein from chick embryo skin (56, 57), rat testes (15), or an embryonal carcinoma cell line (26). Cellular retinoic acid-binding protein has also been reported to be present in transplantable animal tumors (49, 58) and in human neoplastic tissue but not in the corresponding normal tissue from which the tumor arose (25, 52). However, when these initial observations were extended to larger numbers of tumors, it was found that only approximately 50% of human mammary tumors contained cellular retinoic acid-binding protein (25). We therefore examined NMU-induced rat mammary tumors induced in our laboratory and found cellular retinoic acid-binding protein to be present. After our studies were completed, Mehta and Moon (39) reported a similar

1 To whom requests for reprints should be addressed, at Department of Chemotherapy, Hoffman-La Roche Inc., Nutley, N. J. 07110.

Received June 21, 1979; accepted October 16, 1979.

2 The abbreviations used are: IC50, molar excess of retinoid required to inhibit 50% of the specific binding of [3H]retinoic acid under the assay conditions used; NMU, N-nitroso-N-methylurea; TMMP, trimethylmethoxyphenyl.
finding. The rat tumors were of mixed type, as described in "Materials and Methods," and undoubtedly also contained normal tissue. However, we felt that it would be of interest to compare the results of IC\textsubscript{50} determinations using rat testis cellular retinoic acid-binding protein with those obtained using cellular retinoic acid-binding protein from the NMU-induced tumors.

**MATERIALS AND METHODS**

**Materials**

[11,12-\textsuperscript{3}H]Retinoic acid (31 Ci/mmol) was synthesized by our Isotope Synthesis Group, Chemical Research Department, and was supplied in solution in toluene under nitrogen. The toluene was evaporated with a jet of nitrogen, and an 8 \muM solution of the [\textsuperscript{3}H]retinoic acid was prepared in dimethyl sulfoxide containing butylated hydroxyanisole (1 mg/ml) as antioxidant. All other retinoids used in this study were synthesized by members of our Chemical Research Department. Stock solutions (1.6 mm) for use in binding study assays were prepared in dimethyl sulfoxide containing butylated hydroxyanisole (1 mg/ml). All solutions of retinoids were stored in the dark at ~20°C or, in the case of [\textsuperscript{3}H]retinoic acid, in a liquid nitrogen freezer. Charcoal (Norit A; Fisher Scientific Co., Pittsburgh, Pa.) was heated in a chemical hood until it glowed red and then placed in a 70°C oven, where it was stored until use.

**Assay Procedure**

Partially purified cellular retinoic acid-binding protein (approximately 1 mg/ml protein; 100 \mul) was mixed with 5 \muL [\textsuperscript{3}H]retinoic acid stock solution (40 pmol; 1.24 \muCi) in the presence or absence of various amounts of retinoic acid or other retinoids and incubated at 4°C for 16 hr in the dark. Preliminary experiments had shown that maximum binding was achieved in 24 hr; the longer incubation period was used for convenience. Fifty \muL of a freshly made 1% suspension of activated charcoal (see "Materials") in 0.0025% Dextran T-40 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) —0.01 M Tris-HCl —0.25 M sucrose —1 mM EDTA, pH 7.5, were added and mixed. After 10 min, the charcoal was removed by pressure filtration through a filter made as follows. A 0.25-sq cm piece of glass fiber filter (934 AH; H. Reeve Angel & Co., Inc., Clifton, N. J.) was compressed, using a 2-mm-diameter rod, into the tip of an Eppendorf 100- to 1000-\muL pipet tip (Brinkmann Instruments, Inc., Westbury, N. Y.) such that a 3-mm column of glass fiber filter was generated. Using such filters, small volumes of suspensions may be filtered with minimal losses. Aliquots (50 \muL) of the filtrate were mixed with 15 ml Hydrofluor (National Diagnostics, Parsippany, N. J.), and their radioactivity was determined.

**Preparation of Partially Purified Cellular Retinoic Acid-binding Protein**

**Rat Testes.** Individually frozen rat testes (573 g; Pel-Freeze Biologicals, Inc., Rogers, Ark.) were thawed, and freed of fat and epididymis, and 200-\textsuperscript{3}g amounts were homogenized in 0.05 M Tris-HCl, pH 7.5 (2 ml/g tissue), with a Polytron homogenizer. The homogenates were centrifuged at 16,000 \times g for 15 min. The supernatant fluids were removed, combined, and justed to pH 5 with glacial acetic acid, and centrifuged at 16,000 \times g for 15 min. The supernatant fluid was again removed, adjusted to pH 7.2 with 5 N NaOH, and centrifuged at 100,000 \times g for 1 hr. The supernatant fluid was then removed and lyophilized.

The lyophilized extract was reconstituted in 30 ml of 0.05 M Tris-HCl, pH 7.5; centrifuged at 16,000 \times g for 15 min to remove a small amount of insoluble material; and then chromatographed on a 5-x 100-cm column of Ultrogel AcA54 (LKBI Instruments, Inc., Rockville, Md.) in 0.05 M Tris-HCl, —0.2 M NaCl, pH 7.5. Aliquots (100 \muL) of fractions (5 ml) from the column were tested for their ability to bind [\textsuperscript{3}H]retinoic acid using the assay procedure described above. Incubations were carried out in the absence and presence of a 200-fold molar excess of retinoic acid to locate material binding [\textsuperscript{3}H]retinoic acid specifically. Such fractions were pooled, and ammonium sulfate was added to reach 50% saturation. The precipitate was removed by centrifugation. The supernatant was subjected to another ammonium sulfate precipitation (75%), and the precipitate was removed and dissolved in water. The partially purified cellular retinoic acid-binding protein was exhaustively dialyzed against 0.05 M Tris-HCl, pH 7.5, and stored frozen in 1.5-ml aliquots at ~20°C until used.

**Rat Mammary Tumors.** Mammary tumors were induced in 50-day-old female Sprague-Dawley rats (CD-1; Charles River Breeding Laboratories, Inc., Wilmington, Mass.) by i.v. injection of NMU as described by Moon et al. (43). Two doses of NMU, either 50 or 25 mg/kg, were given 1 week apart. After 25 weeks, animals bearing tumors were sacrificed, and their tumors were removed and weighed. A total of 386.5 g of tumor tissue was obtained from 41 and 47 animals that had received NMU, 50 and 25 mg/kg, respectively. Subsequent histopathological evaluation of these tumors revealed them to be a mixture of adenocarcinomas, papillary carcinomas, fibroadenocarcinomas, and less than 5% fibroadenomas.

The tumor tissue was homogenized and prepared for Ultrogel chromatography as described above. The lyophilized solids (16.3 g) were dissolved in 0.05 M Tris-HCl, pH 7.5, and chromatographed as described above. Analysis of the column fractions for ability to bind [\textsuperscript{3}H]retinoic acid revealed that the cellular retinoic acid-binding protein was not completely separated from other substances that bind [\textsuperscript{3}H]retinoic acid nonspecifically. Fractions containing the cellular retinoic acid-binding protein were therefore pooled and, after concentration by ammonium sulfate precipitation, rerechromatographed on the same Ultrogel column. The cellular retinoic acid-binding protein was then well separated from other substances that bind [\textsuperscript{3}H]retinoic acid. The appropriate fractions were pooled; concentrated by ammonium sulfate precipitation; dialyzed against 0.05 M Tris-HCl, pH 7.5; and then stored frozen at ~20°C until used.

**Determination of IC\textsubscript{50}**

Stock solutions (1.6 mm) of retinoids were prepared in dimethyl sulfoxide containing butylated hydroxyanisole (1 mg/ml), and dilutions were made in the same solvent to give 533.3, 177.8, 59.3, 19.8, and 8 \muM. Aliquots (5 \muL) of each of these 6 solutions were added to the standard incubation mixture described above. The resulting mixtures contained 200, 66.7, 22.2, 7.4, 2.5, and 1 mol of retinoid per mol of [\textsuperscript{3}H]retinoic acid specifically. Fractions containing the cellular retinoic acid-binding protein were therefore pooled and, after concentration by ammonium sulfate precipitation, rerechromatographed on the same Ultrogel column. The cellular retinoic acid-binding protein was then well separated from other substances that bind [\textsuperscript{3}H]retinoic acid. The appropriate fractions were pooled; concentrated by ammonium sulfate precipitation; dialyzed against 0.05 M Tris-HCl, pH 7.5; and then stored frozen at ~20°C until used.

**Retinoic Binding and Biological Activity**

Retinoic binding and biological activity
acid. All incubation mixtures were run in duplicate. Each assay contained a titration of unlabeled retinoic acid and control tubes to which no unlabeled retinoid was added. Reaction mixtures were incubated and processed as described above. Specific binding of [3H]retinoic acid in the presence of the various concentrations of the retinoids was calculated by subtracting the dpm not displaceable by a 200-fold molar excess of retinoic acid from the dpm bound at each concentration of competing retinoid. The IC50 of each retinoid was obtained from plots of percentage of inhibition of specific binding versus the logarithm of the molar excess.

Sucrose Density Gradient Centrifugation

Samples to be analyzed by sucrose density gradient centrifugation were treated with charcoal as described above. Aliquots of the filtrate were layered onto 5 to 20% sucrose gradients in 0.05 M Tris-HCl, pH 7.5, and centrifuged at 175,000 × g in an SW41 rotor at 4°C for 48 hr. The gradients were fractionated into twenty 0.6-ml fractions by the method of Tan (68). The fractions were mixed with 15 ml Hydrofluor, and the radioactivity was determined.

Determination of in Vitro Properties of Retinoids

Stimulation of differentiation of PCC4.aza1R cells by retinoids was determined as described previously (28). Cell aggregates were treated with the desired retinoid and, after 3 days of incubation, the percentage of aggregates with differentiating outgrowth was determined by the fibrin-agar overlay technique (4). Inhibition of growth of 3T6 cells by retinoids was determined as described previously (27).

Determination of in Vivo Properties of Retinoids

The hypervitaminosis dose for each retinoid was determined as described by Bollag (8). The hypervitaminosis A symptoms, which are manifested as weight loss, redness and scaling of the skin, hair loss, changes in the mucosa of the nose and mouth, and bone fractures, were evaluated (8). The hypervitaminosis dose is defined as the lowest daily dose for which 2 of 3 mice tested have one or more symptoms of hypervitaminosis A.

The effects of various retinoids on dimethyl-1,2-benz(a)anthracene-induced skin papillomas in mice was determined as described by Pawson et al. (53). Briefly, groups of 8 mice were treated i.p. with aqueous suspensions of the retinoids 10 times in a 14-day period. The change in papilloma diameter for the group, determined from photographs taken at the beginning and the end of the treatment period, was expressed as a percentage of the total papilloma diameter for the group at the beginning of the experiment. The papillomas of vehicle-treated control mice either increased in diameter or showed no net change for the group. Statistical significance levels were determined using a 2-tailed t test on the net changes in papilloma diameter per animal. When the probability that the differences between the means of these net changes for control and treated groups could have arisen by chance was <0.01, the retinoid was considered active. Such statistical significance was usually associated with ≥25% decrease in papilloma diameter for the treated group. Most retinoids were tested at several doses. However, where possible, in order to facilitate comparisons among the compounds, only those results obtained at a constant fraction (two-fifths) of the hypervitaminosis dose are presented in "Results and Discussion."

Inhibition of the growth of a transplantable chondrosarcoma in rats was determined as described by Trown et al. (69). Groups of 8 chondrosarcoma-bearing rats were treated i.p. with aqueous suspensions of the retinoids 5 days/week for 4 weeks. Tumors were excised and weighed. The inhibition of growth was calculated as

\[
\frac{C - T}{C} \times 100\%
\]

where C and T are the mean tumor weights for control and treated groups, respectively. An inhibition of ≥50% was considered evidence of antitumor activity. Again, retinoids were usually tested at several doses where possible, but only those results obtained at a constant fraction (one-fifth) of the hypervitaminosis dose are presented in order to facilitate comparisons between compounds.

RESULTS AND DISCUSSION

Partial Purification of Cellular Retinoic Acid-binding Protein

The primary purpose of partially purifying cellular retinoic acid-binding protein was to remove interfering substances that bind [3H]retinoic acid nonspecifically so that a rapid assay based on charcoal absorption of unbound ligand could be developed. Chromatography on Ultrogel AcA54 achieved this purpose; Chart 1 shows the elution profile of a typical column to which an extract of rat testes was applied. Material binding [3H]retinoic acid specifically was eluted in Fractions 190 to 240 and was clearly separated from material (presumably serum albumin) that bound [3H]retinoic acid nonspecifically (Fractions 80 to 185). When an extract of rat mammary tumors was applied to the same column, the absorbance pattern differed, but again 2 peaks containing material that bound [3H]retinoic acid were obtained. The second peak eluted at the same position (Fractions 190 to 240) as that obtained from rat testes. The material binding [3H]retinoic acid specifically in Fractions 190 to 240 appears to consist mainly of cellular retinoic acid-binding protein as shown by sucrose density gradient analysis (Chart 2). Specifically bound [3H]retinoic acid sediments with a coefficient of 2S as does cellular retinoic acid-binding protein (Chart 2). It should be noted that no [3H]retinoic acid sedimented to the position of serum albumin (Fractions 17 and 18).

Properties of the Charcoal Assay for Cellular Retinoic Acid-binding Protein

The amount of [3H]retinoic acid bound specifically to the partially purified cellular retinoic acid-binding protein was linearly related to the protein concentration over the range 80 µg/ml to 10 mg/ml. At lower protein concentrations, results were erratic, presumably because of adsorption of the cellular retinoic acid-binding protein to the plastic ware and/or charcoal used in the assay. Specifically bound [3H]retinoic acid ranged from 68 to 84% of total [3H]retinoic acid bound for testes cellular retinoic acid-binding protein and from 68 to 77% of total [3H]retinoic acid bound for mammary tumor cellular retinoic acid-binding protein. Specific activities of the cellular retinoic acid-binding protein preparations were 56 to 64 and 12 to 13 pmol retinoic acid bound per mg protein for testes...
Retinoid Binding and Biological Activity

Chart 1. Chromatography of rat testis extract on Ultrogel AcA54. Aliquots (100 µl) of column fractions were tested for their ability to bind [3H]retinoic acid in the presence (C) and in the absence (O) of a 200-fold molar excess of unlabeled retinoic acid as described in "Materials and Methods."

Chart 2. Sucrose density gradient analysis of partially purified cellular retinoic acid-binding protein from rat testis after incubation with [3H]retinoic acid in the absence (O) and presence (X) of a 200-fold molar excess of unlabeled retinoic acid.

Chart 3. Inhibition of binding of [3H]retinoic acid to rat testis cellular retinoic acid-binding protein by selected retinoids. O, unlabeled retinoic acid; ▲, 4-ketoretinoic acid (Ro 12-4824); ■, 6-fluoro-TMMP analog (Ro 21-0600); △, dimethoxytetramethyltetrahydroxynaphthalenyl analog (Ro 22-2180).

and mammary tumor cellular retinoic acid-binding protein, respectively.

The reproducibility of the assay was determined by measuring total and specific binding of [3H]retinoic acid in 10 replicate samples on 3 separate occasions. The standard deviations ranged from 8 to 28% of the means for each day. The repeatability of the assay, as measured by the [3H]retinoic acid specifically bound to a given preparation of testes cellular retinoic acid-binding protein under standard assay conditions in 9 separate assays, was ±18% (S.D.).

Relationship of IC50's to in Vivo Properties of Retinoids

IC50's for each retinoid were determined from plots of percentage of inhibition of specific binding versus the logarithm of the molar excess; typical examples are shown in Chart 3. The repeatability of the IC50 determinations was determined for only one retinoid, retinoic acid; it was found to be 1.12 ± 0.50 (mean ± S.D. of 10 determinations).

The IC50's for cellular retinoic acid-binding protein derived from rat testes and mammary tumors of 20 retinoids of various structural types are presented in Table 1 together with the structures of the retinoids, their hypervitaminosis doses, and their effects on mouse skin papillomas and rat chondrosarcoma growth.

Table 1A includes data on all-trans-retinoic acid (Ro 1-5488), its 13-cis-isomer (Ro 4-3780), 4-ketoretinoic acid (Ro 12-4824), 4-hydroxy-retinoic acid (Ro 10-2655), and 5,6-dihydroxyretinoic acid (Ro 22-5112). 4-Ketoretinoic acid has been shown to be a fecal metabolite of retinoic acid in the rat (22), and 4-hydroxyretinoic acid is its possible precursor. 5,6-Dihydroxyretinoic acid is the formal hydration product of another
Table 1

Structures, IC₅₀'s, and in vivo biological data

<table>
<thead>
<tr>
<th>Ro no.</th>
<th>Structure</th>
<th>IC₅₀'s of cellular retinoic acid-binding protein</th>
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<td>% of change</td>
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<td>(mg/kg/day)</td>
<td>(mg/kg/day)</td>
<td>(mg/kg/day)</td>
<td>% change</td>
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metabolite of retinoic acid, 5,6-epoxyretinoic acid (29, 38). While the latter compound has been shown to be converted to 5,6-epoxyretinoic acid in vivo (45), it could also be converted to 5,6-dihydroxyretinoic acid, although this latter compound has not yet been isolated as a metabolite of retinoic acid.

13-cis-Retinoic acid, 4-ketoretinoic acid, and 4-hydroxyretinoic acid all competed very well with retinoic acid for binding to cellular retinoic acid-binding protein from either rat testes or rat mammary tumors with IC_{50}’s close to those of retinoic acid. 5,6-Dihydroxyretinoic acid was a less effective competitor for binding to cellular retinoic acid-binding protein from rat testes; its IC_{50} was more than 10-fold that of retinoic acid. This compound was not tested for binding to cellular retinoic acid-binding protein from mammary tumors. These patterns of binding to cellular retinoic acid-binding protein were not consistently reflected in the in vivo properties of this group of retinoids, however. For example, 13-cis-retinoic acid was inactive at the dose tested (80 mg/kg/day, i.e., two-fifths of the hypervitaminosis dose) against established mouse skin papillomas, whereas retinoic acid and the other retinoids tested in this group were active at the same fraction of the hypervitaminosis dose. 13-cis-Retinoic acid has been reported by Verma et al. (72) to be almost as active against mouse skin papillomas as retinoic acid is. However, in the latter studies, the retinoids were applied topically during the promotion phase of papilloma induction, whereas, in the present study, all retinoids were administered systemically after papillomas had been induced. The results from the 2 studies may not, therefore, be comparable. All retinoids in this group inhibited the growth of the rat chondrosarcoma; retinoic acid and 13-cis-retinoic acid showed the weakest activity when dose titrations were carried out (data not shown).

Table 1B includes data on 6 retinoids in which the trimethylcyclohexene ring of retinoic acid is replaced by variously substituted aromatic rings. The compounds are the phenyl analog (Ro 8-8717), a phenol hydroxydecylether analog (Ro 22-1342), a dichlorotrimethoxyphenyl analog (Ro 22-2131), a methylmethoxyquinolyl analog (Ro 22-1260), a trimethylpyrimidinyl analog (Ro 22-1401), and a dimethoxytetramethyltetrahydrodronaphthalenyl analog (Ro 22-2180). Four of these 6 retinoids bound less tightly to cellular retinoic acid-binding protein than did retinoic acid with IC_{50}’s 1 to 2 orders of magnitude greater than that for retinoic acid; the pyrimidine analog and Ro 22-2180 did not appear to bind to cellular retinoic acid-binding protein under the conditions used. All 6 of these compounds were relatively nontoxic, with hypervitaminosis doses of >200 mg/kg/day, and they were inactive or weakly active against mouse skin papillomas. Only 2 compounds were tested against rat chondrosarcoma; Ro 22-2131 was inactive, and Ro 22-1760 was weakly active. Thus, for this group of compounds, there appeared to be a correlation between weak binding to cellular retinoic acid-binding protein and low in vivo activity. However, it should be remembered that 5,6-dihydroxyretinoic acid had an IC_{50} of 12.5 (Table 1A), which is comparable with those of Ro 22-1342 and Ro 22-2131 (8.2 and 14, respectively), but it was active against skin papillomas and chondrosarcoma. The IC_{50} for the phenyl analog was 200; at 100-fold molar excess, there was 40% inhibition of retinoic acid binding (data not shown), in good agreement with the 30% inhibition of retinoic acid binding to chick

<table>
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<th>Ro no.</th>
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<th>IC_{50}'s of cellular retinoic acid-binding protein</th>
<th>Hypervitaminosis A dose (mg/kg/day)</th>
<th>Papilloma Dose (mg/kg/day) % of changea</th>
<th>Chondrosarcoma Dose (mg/kg/day) % of inhibitionb</th>
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a The mean percentage change in papilloma diameter for the treated group during 2 weeks of treatment with the retinoid.

b The percentage of inhibition of tumor growth for the treated group relative to controls treated with vehicle only.

c --- not done.

d Results obtained with methyl or ethyl ester.

e Effective dose required to cause a 50% decrease in papilloma diameter, determined from plot of percentage change vs. log dose; at least four 2-fold serial doses used.
skin cellular retinoic acid-binding protein reported for this compound at 100-fold molar excess by Sani et al. (61).

Of the 7 retinoids included in Table 1C, 5 can be considered to be derivatives of the TMMP analog that, as its ethyl ester or ethyl amide, is one of the most extensively studied synthetic retinoids (13, 19, 20, 23, 37, 62, 63, 69, 73). The compounds are the TMMP analog itself (Ro 10-1670), the trimethylphenol analog (Ro 12-7310), the analogs in which fluorine is substituted in the side chain of TMMP at the 4- and 6-positions (Ro 22-0599 and Ro 22-0600, respectively), and the analog of TMMP in which the methoxy group is replaced by a bis-(2-chloroethyl)carbamoyl ether (Ro 22-1196). The remaining 2 compounds are analogs in which the trimethylcyclhexenene ring of retinoic acid has been replaced either by a trimethylthiophene (Ro 21-6668) or a dimethylacetylcyclopentene (Ro 8-7699).

The group of retinoids in Table 1C competed well with retinoic acid for binding to cellular retinoic acid-binding protein from testes but not, when measured, with the cellular retinoic acid-binding protein from mammary tumors. This difference in binding affinities may indicate that the 2 binding proteins are not identical. However, further purification of both cellular retinoic acid-binding proteins would be needed to clarify this point. Despite similar IC50's, these 7 retinoids had widely different toxicities, with hypervitaminosis doses ranging from 12.5 to >200 mg/kg/day. There was also a large variation in efficacy against mouse skin papillomas and rat chondrosarcoma. The retinoids, Ro 10-1670, Ro 22-0599, Ro 22-0600, and Ro 8-7699, were very active against papillomas and rat chondrosarcoma, whereas the remaining 3 compounds were less active or inactive. Thus, for this group of compounds, there does not seem to be a relationship between IC50's and in vivo properties.

Overall, for the 18 compounds listed in Table 1, the correlation between IC50's for the cellular retinoic acid-binding protein from rat testes or mammary tumors and in vivo properties is not consistent. Retinoids that do not bind to cellular retinoic acid-binding protein or that bind weakly compared to retinoic acid are generally of low toxicity and low activity against papillomas and chondrosarcoma. However, a high degree of binding to cellular retinoic acid-binding protein does not necessarily mean that the compound will have a low hypervitaminosis dose or be very active in the papilloma or chondrosarcoma systems. This is not, perhaps, too surprising since many factors such as absorbability, metabolism, tissue distribution, and pharmacokinetics determine the concentration of a substance in a target tissue. Thus, even though a retinoid may be tightly bound to a cellular retinoic acid-binding protein in a target tissue, it may never reach that tissue in sufficient amounts to cause any biological effects therein.

Most of these considerations do not apply to the experiments in tissue culture, the results of which are summarized in Table 2. Again, however, the correlation between IC50 and biological activity was not consistent. The 2 compounds that bound very poorly or not at all, Ro 8-8717 and Ro 22-1401, did not inhibit the growth of 3T6 cells or cause differentiation of PCC4.azaR cells to a significant degree. In addition, several retinoids with IC50's close to those of retinoic acid (Ro 4-3780, Ro 10-1670, Ro 22-0600, Ro 22-0599, Ro 12-4824, Ro 10-2655, and Ro 8-7699) inhibited the growth of 3T6 cells to approximately the same extent at 10-8 M and also caused differentiation of 100% of the PCC4.azaR cell aggregates at 10-7 M. However, other retinoids (Ro 21-6668 and Ro 12-7310) also had low IC50's and caused similar inhibitions of 3T6 cell growth to the above compounds but were much less active in causing differentiation of PCC4.azaR cell aggregates, being completely inactive at 10-7 M. Since, in these experiments, the retinoids were in direct contact with the cells, only lack of cellular uptake, metabolism by the cells, or chemical instability could be invoked as reasons for inactivity. Alternatively, cellular retinoic acid-binding proteins from different sources may not be identical; thus, differences in binding affinities, as shown for testis and mammary tumor cellular retinoic acid-binding proteins, may account for the different results obtained in the various systems.

It may therefore be concluded from this study that, while the retinoids that have low hypervitaminosis dosage levels and are highly active in the mouse skin papilloma, rat chondrosarcoma, 3T6 cell growth inhibition, or PCC4.azaR differentiation systems all bind tightly to cellular retinoic acid-binding protein from rat testis, the converse is not true. Many retinoids with low IC50's are weakly active or inactive in one or more of these biological test systems. This does not prove that cellular retinoic acid-binding protein is not involved in the mediation of the

### Table 2

<table>
<thead>
<tr>
<th>Ro No.</th>
<th>Testis</th>
<th>Mammary adenocarcinoma</th>
<th>Inhibition of growth of 3T6 cells (%)</th>
<th>% of PCC4.azaR cell aggregates differentiating</th>
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</thead>
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<tr>
<td>01-5488</td>
<td>1.1</td>
<td>1</td>
<td>66</td>
<td>100</td>
</tr>
<tr>
<td>04-3780</td>
<td>1</td>
<td>1.7</td>
<td>65</td>
<td>100</td>
</tr>
<tr>
<td>10-1670</td>
<td>8.1</td>
<td>ND</td>
<td>61</td>
<td>100</td>
</tr>
<tr>
<td>22-0600</td>
<td>9.0</td>
<td>100</td>
<td>52</td>
<td>100</td>
</tr>
<tr>
<td>22-0599</td>
<td>3.2</td>
<td>80</td>
<td>55</td>
<td>100</td>
</tr>
<tr>
<td>21-6668</td>
<td>6.8</td>
<td>ND</td>
<td>62</td>
<td>100</td>
</tr>
<tr>
<td>06-8717</td>
<td>200</td>
<td>ND</td>
<td>17</td>
<td>100</td>
</tr>
<tr>
<td>12-7310</td>
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<td>ND</td>
<td>43</td>
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</tr>
<tr>
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<td>1.2</td>
<td>4.5</td>
<td>65</td>
<td>100</td>
</tr>
<tr>
<td>10-2655</td>
<td>0.8</td>
<td>2.2</td>
<td>65</td>
<td>100</td>
</tr>
<tr>
<td>06-7699</td>
<td>0.7</td>
<td>ND</td>
<td>63</td>
<td>100</td>
</tr>
<tr>
<td>22-1401</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

* For structures, see Table 1.
* a Retinoids were used at 10^-5 M.
* ND, not done.
biological activity of retinoids. On the contrary, the many positive correlations observed in this study, and by others, indicate that it is involved. The lack of quantitative correlation between ICGO and biological activity is more likely to be a consequence of other factors, already discussed, which result in low intra-cellular concentrations of the retinoid in the target tissue.

ACKNOWLEDGMENTS

We are most grateful to the Isotope Synthesis Group, who synthesized the [3H]retinoids used in this study, and to the following people who synthesized the retinoids: E. Aig, K.-K. Chan, R.-J. Han, F. Humec, M. Klaus, A. Lovey, Y. Pan, B. A. Pawson, B. Pecherer, M. Rosenberger, A. Specian, and J. Swistok.

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Relationship between Binding Affinities to Cellular Retinoic Acid-binding Protein and *in Vivo* and *in Vitro* Properties for 18 Retinoids

Patrick W. Trown, Alicia V. Palleroni, Oksana Bohoslawec, et al.


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