Relationship between Binding Affinities to Cellular Retinoic Acid-binding Protein and Biological Potency of a New Series of Retinoids

Brahma P. Sani,™ Marcia I. Dawson,™ Peter D. Hobbs, Rebecca L.-S. Chan, and Leonard J. Schiff


ABSTRACT

Binding affinities of a new and unusual series of retinoic acid analogues to cellular retinoic acid-binding protein, a possible mediator of their biological function in the control of differentiation and tumorigenesis, and to serum albumin, their plasma transport protein, were determined. Also, biological activity of these retinoids in the reversal of keratinization in hamster tracheal organ cultures was assessed and compared with their binding affinities. Analogues that possessed high biological activity showed high binding efficiency to cellular retinoic acid-binding protein. Those that were biologically less active were poor binders to the binding protein. Three retinoids, 4657-57, 3920-59, and 4445-75, which showed 90 to 100% binding efficiency of that of retinoic acid for cellular retinoic acid-binding protein expressed high biological activity detectable in the range of 10^{-10} M as against 10^{-11} M for retinoid acid. The correlation noticed in these two activities not only enhances the confidence in the two assay procedures but also paves the way for design and development of potential chemopreventive agents. No apparent differences were observed in the binding affinities of the retinoids to binding proteins of a normal tissue or a tumor tissue. No correlation existed between the binding affinities of these retinoids to serum albumin and their biological activity. Structure-activity relationships of the retinoids in relation to their binding affinities and biological activities have been discussed.

INTRODUCTION

Retinol and its oxidized form, retinoic acid, are involved in the control of normal differentiation and general growth (1, 12). On account of the chemopreventive potential of retinoids against cancer, the emphasis of research in this area has attained a new dimension (11, 31). The prophylactic and tumor inhibiting action, and the effects on differentiation produced by retinoic acid are generally more pronounced than are those produced by any other retinoids (2, 29, 36). In some epithelia, such as those of trachea, bronchi, and skin, a potentially premalignant lesion (squamous metaplasia) with heavy keratinization occurs in the absence of retinoids (29, 36-38). Addition of retinoids to organ cultures of such tissues causes reversal of the process of keratinization and replacement of the abnormal squamous cells by columnar ciliated mucous epithelia (29, 36). The structure-activity relationships of 87 retinoids summarized by Newton et al. (17) reveal that all-trans- and 13-cis-retinoic acid possess the highest biological activity in this series of compounds in the reversal of keratinization in hamster tracheal organ culture systems. A similar pattern in the expression of biological potency of retinoids is observed in other organ culture systems as well (29, 36).

The action of retinoic acid in the control of epithelial differentiation and in the reversal of preneoplastic lesions may be mediated by CRABP. The demonstration of the existence of CRABP in the nuclei of certain cells and a CRABP-mediated transmigration of retinoic acid into the nuclei may be an important step towards understanding the molecular mechanism of retinoic acid action (15, 20, 24, 35). Likewise, a cellular retinol binding protein-dependent delivery of retinol to the nucleus with specific binding sites located on chromatin also has been illustrated (16). A retinoic acid-like structure with a ring, side chain, and a terminal free carboxyl group is essential for maximal binding of retinoids to CRABP (18, 25, 26). Various analogues of retinoic acid with modifications in the ring and side chain have been evaluated for their binding affinities to CRABP (3, 15, 26, 27, 33). These studies indicate that analogues that possessed high biological activity in hamster tracheal (17, 29) or chick embryo skin (36) organ cultures also possessed high binding affinity for CRABP. Those that are biologically less active were poor binders to the binding protein. Likewise, Jetten and Jetten (15) observed a correlation between the capacity of various retinoic acid analogues to compete with [3H]retinoic acid for CRABP binding sites and their ability to stimulate differentiation of an embryonal carcinoma cell line. Similarly, a correlation between the binding affinities to cellular retinol binding protein and vitamin A activity has been established with retinol and its analogues (19). Thus, it is likely that, in order to project a measure of the chemopreventive potential of a retinoid, the data on its binding affinity to cellular binding proteins might be helpful.

A new synthetic derivative of retinoic acid, 4-[2-(1,2,3,4-tetrahydro-1,1,4,4-tetramethyl-6-naphthyl)-1E-propen-1-yl]benzoic acid, exhibited activity equal to or greater than that of all-trans- and 13-cis-retinoic acid (17). We have synthesized a series of retinoids in relation to their binding affinities and biological activities have been discussed.

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1The abbreviations used are: CRABP, cellular retinoic acid-binding protein; CMPS, p-chloromercuriphenylsulfonic acid; PBS, phosphate-buffered saline (0.03 M sodium phosphate buffer, pH 7.2, in 0.1 M NaCl).

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3920-59, and 4445-40) the bonds corresponding to the 9,11-double bond system of retinoic acid are restricted to a cisoid conformation by the aromatic ring. In 4529-19, the bonds corresponding to the 7,9- and 11,13-double bond systems of retinoic acid are conformationally restricted by the biphenyl ring to cisoid conformations; whereas in 4445-75, those bonds corresponding to the 9,11,13-double bond system are similarly restricted. In 4875-20, a larger, more electonegative phenyl group replaces the 19-methyl group of retinoic acid. Analogues 3204-91 and 3618-35 are analogues of 13-cis-retinoic acid in which the bond corresponding to the 13-cis double bond is restricted to a cis configuration by the phenyl ring. This article provides data on these retinoids with respect to their binding affinities to CRABP and biological potency in the hamster tracheal organ culture assay. Since we find a general correlation between these 2 properties, it was also desirable to derive data for comparative purposes on their binding capacity to serum albumin, the plasma transport protein of retinoic acid (27, 28).

MATERIALS AND METHODS

Retinoic acid and [11,12-3H]retinoic acid (1.28 Ci/mmol) were supplied by the National Cancer Institute, Bethesda, Md. The synthesis of 4-[2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E-butanadien-1-yl]benzoic acid (4657-57), 5-[2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E-butanadien-1-yl]-2-thiophenecarboxylic acid (3920-59), 5-[2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E-butanadien-1-yl]-2-furoic acid (4445-40), 6-[2-(2,6,6-trimethyl-1-cyclohexen-1-yl)-E-ethenyl-1-yl]-2-naphthalene carboxylic acid (4445-75), 4-[4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E-hexatrien-1-yl]benzoic acid (3204-91), and 2-[4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E-hexatrien-1-yl]phenol were synthesized by the methods reported by Dawson et al. (5-8, 10). 4-[4-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-1-phenyl]benzoic acid (4529-19) and (E)-3-methyl-7-phenyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6-nonatetraenoic acid (4875-20) were also synthesized (9). Defatted bovine serum albumin, CMPS, and diethiothreitol were purchased from Sigma Chemical Company, St. Louis, Mo.

Skins from 12- to 13-day-old chick embryos and mouse colon tumor 26 on the 14th day after s.c. implantation into BALB/c mice (23, 27) were homogenized in PBS and centrifuged at 100,000 × g for 60 min, and the supernatants were stored at -80°C. Serum albumin that binds [3H]retinoic acid and forms a 5S peak (25) was eliminated by passing the protein extracts through Affi-Gel Blue columns as described earlier (22). Aliquots (3 mg protein in 0.3 ml PBS) of these preparations were incubated with 300 pmol of [3H]retinoic acid (1 µM) in the presence or absence of a 100-fold molar excess of the unlabeled test compounds, and the preparations were dialyzed (23). The 2S binding protein peaks were determined from the radioactivity profiles obtained after sedimentation through 5 to 20% sucrose density gradients at 180,000 × g for 18 hr.

For determination of the binding affinities of the new retinoids to serum albumin, 50 µg defatted bovine serum albumin in 0.3 ml PBS was mixed with 2 nmol [3H]retinoic acid (6.6 µM) with or without a 100-fold molar excess of unlabeled retinoids. After incubation and dialysis as above, 500 µg of albumin were added to the incubation mixture to minimize denaturation due to high dilution. These were centrifuged on 5 to 20% sucrose gradients as above and fractionated, and the 5S albumin peaks were determined.

The procedure for assay of reversal of keratinization in organ cultures of tracheal epithelium from retinoid-deficient hamsters was essentially the same as described before (4, 17, 30). Tracheal epithelium, which showed only occasional patches of squamous metaplasia, from hamsters of early vitamin A deficiency was cultured in a serum-free medium in an atmosphere of 50% oxygen-45% nitrogen-5% CO2. They were main-

tained in retinoid-free medium for 3 days at 37°C, which resulted in keratinized lesions in 60 to 70% of the cultures. The cultures were treated with different concentrations of the retinoids dissolved in dimethyl sulfoxide. Control cultures were incubated with equal concentrations of dimethyl sulfoxide alone. The tracheas were harvested at the end of a total of 10 days in culture, fixed in 10% buffered formalin, and embedded in paraffin. Cross-sections through the midportion were stained with hematoxylin and eosin and examined for keratinization. The control cultures and those treated cultures that contained both keratin and keratohyaline granules were scored retinoid "inactive"; those cultures in which neither keratin nor keratohyaline granules were seen, or if the granules alone were absent were scored retinoid "active." Compounds were usually assayed twice at either 10-8, 10-9, and 10-10 M or 10-9, 10-10, and 10-11 M. Retinoic acid controls at 10-10, 10-11, and 10-12 M were run concurrently and served as an active control. Either 6 or 7 cultures were treated at each concentration. Results were reproducible for each concentration tested. The percentage of cultures is calculated from the ratio of active cultures to the total number of cultures tested. The 50% effective dose value is the estimated concentration of retinoid that led to reversal of keratinization in 50% of the cultures.

RESULTS

Structures and code numbers of the retinoids that were presently studied are shown in Table 1. [3H]Retinoic acid binding to CRABP from chick embryo skin and colon tumor 26 was challenged by 100-fold molar excesses of these retinoids as illustrated in Chart 1. The Affi-Gel Blue column virtually eliminated the interfering 5S albumin-[3H]retinoic acid peak and is used as the control in these competition experiments. As shown by the size of the 2S peaks, unlabeled retinoic acid and the retinoid 4657-57 showed similar competitive effects on [3H]retinoic acid binding, whereas retinoid 4445-40 was without any effect on the 2S radioactive peak.

The binding efficiency and biological potency of the retinoids presently evaluated are listed in Table 1. In order to measure the relative binding affinity of retinoids, the inhibition of [3H]retinoic acid binding caused by 100-fold molar excess of unlabeled retinoic acid is regarded as 100% inhibition. The inhibition caused by similar excesses of the new retinoids is expressed as relative inhibition to the above standard. The biological activities of these retinoids, as a percentage of the active cultures to the total cultures tested and their 50% effective dose values in the reversal of keratinization in hamster tracheal organ culture are also included in the same table. Of the 5 retinoids conformationally restricted to cisoid conformations by aromatic ring systems, the benzoic acid analogue 4657-57, the thiophene carboxylic acid 3920-59, and the naphthalene carboxylic acid 4445-75 showed high binding affinity to CRABP and high activity in the reversal of keratinization assay. In contrast, the more polar and more labile furoic acid 4445-40 exhibited no binding and low activity in the keratinization assay. The biphenyl analogue 4529-19 showed only low binding to CRABP and low activity in the organ culture assay. Evidently, a cisoid conformational restriction on the 7,9-double bond system reduces biological activity. The same result was found for the cis conformationally locked retinoids 3204-91 and 3618-35. However, the benzoic acid 3204-91 did exhibit slightly more activity than did the corresponding phenol 3618-35. Replacement of the 19-methyl group by a phenyl group also reduced activity. Thus, the binding affinities of the retinoids directly correlate with their biological activity. Retinoids such as...
**Table 1**

Structures, binding efficacy to CRABP, and biological potency to reverse keratinization

<table>
<thead>
<tr>
<th>Retinoid code</th>
<th>Structure</th>
<th>Chick embryo skin CRABP</th>
<th>Colon tumor 26 CRABP</th>
<th>% Inhibition of [3H]-retinoic acid to CRABP</th>
<th>Reversal of keratinization</th>
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<tr>
<td></td>
<td></td>
<td>m</td>
<td>Active/total cultures</td>
<td>%</td>
<td>50% effective dose</td>
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<td>92</td>
<td>10^-6</td>
<td>15/15</td>
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<tr>
<td>3920-59</td>
<td><img src="image" alt="Structure" /></td>
<td>95</td>
<td>90</td>
<td>10^-8</td>
<td>14/15</td>
</tr>
<tr>
<td>4445-40</td>
<td><img src="image" alt="Structure" /></td>
<td>0</td>
<td>0</td>
<td>10^-10</td>
<td>7/15</td>
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<td>38</td>
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<td>100</td>
<td>100</td>
<td>10^-9</td>
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<tr>
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<td>40</td>
<td>10^-10</td>
<td>5/7</td>
</tr>
<tr>
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<td>25</td>
<td>22</td>
<td>10^-6</td>
<td>2/7</td>
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<tr>
<td>3618-35</td>
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<td>0</td>
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<td>All-trans-retinoic acid</td>
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<td>100</td>
<td>100</td>
<td>10^-10</td>
<td>224/249</td>
</tr>
</tbody>
</table>

*The 50% effective dose value is quoted from Ref. 17.*

|M. B. Sporn, private communication.

4445-75, 3920-59, and 4657-57, the relative percentage of inhibition of which in the binding assays ranged between 90 and 100%, showed appreciable biological activity in the range of from 10^-10 to 10^-9 M. About 50% of the tracheal cultures exhibited reversal at about 10^-10 M. The 2 retinoids, 4445-40 and 3618-35, which showed only 0% inhibition of retinoic acid binding to CRABP, did not express any considerable biological activity even at a concentration of 10^-8 M. The 3 retinoids, 3204-91, 4529-19, and 4875-20, which showed intermediary binding affinity for CRABP, also displayed maximal biological potency in the middle range, 10^-9 to 10^-8 M, in the tracheal organ culture assays.

Inhibition studies with CMPS and iodoacetamide had revealed that thiol functions may exist in the binding of [3H]retinoic acid to CRABP (21). The mercurial-inhibited binding sites were recovered by further treatment with dithiothreitol (21). We wanted to test whether such a sensitivity to the mercurial is involved in the binding of a retinoid under present study. Since radioactive retinoid was not available for direct inhibition reactions, we performed these studies using competitive experiments. Chart 2 shows that 1 mM CMPS completely inhibited the retinoic acid-binding sites on CRABP and about 90% of which was recovered by treatment with dithiothreitol. On further incubation of the dithiothreitol-exposed chick embryo skin preparations with [3H]-retinoic acid plus 100-fold molar excess of the retinoid 4657-57, the unlabeled retinoid efficiently competed for the recovered thiol binding sites of the protein.

Plasma transport of retinoic acid is facilitated by serum albumin (28). It was therefore important also to examine whether the binding affinities of the new retinoids to serum albumin paralleled their affinities to CRABP and correlated with their biological activity. The presence of a 100-fold molar excess of retinoic acid reduced the 5S radioactive peak to about 20% (Chart 3). Although retinoids 4445-40 and 3204-91 showed little or no competition for CRABP and were biologically less active, they showed more affinity for albumin binding than did retinoic acid itself. Also, retinoid 4657-57, which was biologically quite active in the tracheal organ culture assay and possessed high binding affinity for CRABP, exhibited lower albumin-binding affinity than did retinoic acid (Chart 3). Thus, the correlation that exists between the binding affinity and the biological potency of these
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Chart 1. Sucrose density gradient pattern showing the effect of competition of 100-fold molar excess of unlabeled retinoids on the binding of [3H]retinoic acid to chick embryo skin CRABP. O and △, controls with 300 pmol of [3H]retinoic acid plus skin cytosol (3.0 mg protein in 0.3 ml PBS), respectively, with and without Affi-Gel Blue chromatography. All the other radioactivity profiles are the control with Affi-Gel Blue chromatography plus 100-fold molar excess of the following retinoids: •, retinole acid; x, 4657-57; •, 4445-40; D, 4529-19.

Chart 2. Sedimentation patterns on sucrose gradients of chick embryo skin extracts after Affi-Gel Blue chromatography with 300 pmol of [3H]retinoic acid. Before incubation with retinoic acid, the extracts (3.0 mg protein in 0.3 ml PBS) were treated as follows: x, nontreated controls; •, 1 mm CMPS (1 hr at 4°C); O, same as • but incubated with 5 mM dithiothreitol (2 hr at 4°C); △, same as O but a 100-fold M excess of 4657-57 was added with [3H]retinoic acid.

Chart 3. Sucrose density gradient sedimentation profiles of [3H]retinoic acid (2 nmol) plus defatted bovine serum albumin (50 μg in 0.3 ml PBS) with or without 100-fold M excesses of unlabeled retinoids. A, albumin plus [3H]retinoic acid (control); O, control plus retinoic acid; •, control plus 4445-40; △, control plus 4457-47.

Retinoids is not maintained by their binding affinity to serum transport protein.

DISCUSSION

A major hypothesis for the molecular mode of retinol and retinoic acid action in differentiation and control of tumorigenesis is based on the specific interactions of retinol and retinoic acid with the cell nucleus mediated by their specific cellular binding proteins (15, 16, 20, 24, 35). Such interactions with genome are thought to stimulate transcription and thus initiate the sequence of biochemical events leading to the overall morphological and physiological changes produced by the vitamin. The fact that a correlation exists between the binding affinity of retinoic acid and its analogues to CRABP and their biological potency adds further importance and support to the above theory. Retinoic acid analogues evaluated previously contained only minor modifications in the side chain and ring (3, 17, 26, 27, 33, 36). Thus, a retinoic acid-like structure with a free carboxyl group, side chain and ring was believed to be essential for maximal binding to CRABP (27). The aromatic ring containing retinoids presently evaluated for binding to CRABP and tested for biological activity, however, belong to a series of unusual analogues of retinoic acid. Some of these analogues also possess appreciable activity in the reversal of keratinization in hamster tracheal organ culture assay (this report) and in the inhibition of the induction of ornithine decarboxylase (34) by tumor-promoting phorbol esters.5 Those analogues that possessed high biological activity were also good binders to CRABP. Such an extension of the structure-activity relationship into this series marks a new beginning in the design and development of potential chemopreventive agents. As a predictive measure of chemopreventive potential, combined results of these 2 relatively simple assay procedures, namely, the binding affinities to CRABP and the reversal of keratinization in hamster tracheal organ culture, may be considered as a reasonably dependable index.

The most unusual structural feature of most of these analogues is that an aromatic ring has replaced a portion of the tetraene side chain of the retinoic acid skeleton so that certain bonds corresponding to retinoic acid double bonds are confor-

5 Unpublished observations.
mationally locked in a cisoid configuration. Our results indicate that retinoid activity and CRABP binding affinity are still substantially retained in the cisoid 11,13- and 9,11,13-double bond analogues, 4657-57, 3920-59, and 4445-75. However, activity is very much reduced in 4529-19 in which the bonds corresponding to the 7,9-double bond system are restricted to a cisoid conformation by the phenylene ring. Binding affinity is abolished in the more polar analogue, 4445-40, in which a labile furan ring is used as the aromatic ring substituent. Replacement of the 19-methyl group of retinoid acid by a phenyl group also reduces binding affinity and the ability to reverse keratinization. Restriction of the 13-double bond to a cis configuration, as in analogue 3204-91, leads to loss of binding affinity and activity.

The requirement of a terminal carboxyl on the retinoid skeleton for binding to CRABP is further established by the fact that phenol 3618-35 had no binding affinity, whereas the more acidic carboxylic acid 3204-91 retained some binding affinity. Because of their similarity in pK value, a tetrazole group is sometimes used as a carboxylic acid group equivalent; however, 5-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E,7E-octatetraen-1-yl]tetrazole, an all-trans analogue of retinoid acid, did not show any competition for binding. It also displayed very weak activity in the organ culture assay. In this case, steric hindrance from the more bulky tetrazole group may have interfered with binding. No apparent difference was evident in the binding affinities of the retinoids to the binding protein of a normal tissue or a tumor tissue (Table 1).

It has already been shown that retinoid acid binding to CRABP is sensitive to mercurials and that the inhibition is reversible in the presence of diethiothreitol (21). We therefore postulated that thiol functions may be involved in the protein-ligand interactions. A comparable study using the retinoid 4567-57 (Chart 2) indicates that this retinoid can efficiently compete for the diethiothreitol-recovered [3H]retinoic acid-binding sites from CMPS inhibition. Thus, it is probable that the synthetic retinoid is also sharing the sulfhydryl group for binding to CRABP. An alternate explanation could be that this retinoid binds to CRABP at a second site, which does not depend on thiol groups, and that this binding changes the conformation of the protein, which reduces its affinity for retinoid acid. The exact mode of interaction of these retinoids with CRABP is yet to be elucidated by further studies.

Tissue availability of drugs in vivo is greatly influenced by the extent of their binding to plasma transport proteins. Plasma transport of retinoid acid is accomplished by serum albumin. All of the retinoids presently examined that contained a free carboxylic group showed binding affinity for serum albumin. Retinoids 4445-40 and 3206-91 that were poor binders to CRABP and were biologically less potent showed the maximum binding efficiency to serum albumin. The overall data suggest that the affinities of these retinoids for albumin do not parallel with affinities for CRABP or correlate with their biological activity. The ideal drug for in vivo experiments is probably the one that exhibits a fair degree of affinity for albumin, shows greater affinity for CRABP, and possesses good in vitro biological activity. For example, one such synthetic retinoid which satisfies these parameters (3, 17, 27, 29, 33, 36) and showed pronounced anticarcinogenic properties (32) is 13-cis-retinoid acid, which is also less toxic than its naturally occurring all-trans-isomer (13, 14).

The general correlation observed between the binding efficiency of this series of retinoids to CRABP and their biological activity attributes greater significance to these assays for the evaluation of retinoids. Retinoids 4657-57, 3920-59, and 4445-75 may now be added to the group of the already known active retinoids, 13-cis-retinoid acid, trimethyloxephenyl retinoid acid, dimethylacyclohexenyl analogue of retinoid acid, and RO 13-7410 (2, 3, 15, 17, 27, 29, 33, 36). Toxicological evaluation of these retinoids will reveal their usefulness for chemopreventive studies.

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