Comparative Activity of Dietary or Topical Exposure to Three Retinoids in the Promotion of Skin Tumor Induction in Mice

David L. McCormick,² Bryan J. Bagg, and Theresa A. Hultin

Life Sciences Research, IIT Research Institute, Chicago, Illinois 60616

ABSTRACT

The activity of dietary and topical administration of three retinoids, all-trans-retinoic acid, 13-cis-retinoic acid, and N-(4-hydroxyphenyl)retinamide (4-HPR), as promoters of skin tumor induction in SENCAR mice was studied. When administered as dietary supplements at their maximum tolerated dose levels, all three retinoids promoted tumorigenesis in mice initiated with a single topical dose of 5 μg 7,12-dimethylbenz(a)anthracene. Maximal promoting activity was observed with dietary 13-cis-retinoic acid; dietary 4-HPR was significantly less active than was either isomer of retinoic acid. When administered via topical application, all-trans- and 13-cis-retinoids both promoted skin tumor induction; 4-HPR did not. HPLC analysis of skin samples from mice receiving dietary 4-HPR showed the parent compound and six metabolites; these metabolites were not found in the skin of mice receiving topical 4-HPR exposure, although 4-HPR itself was present. These data indicate that skin tumor promotion can be induced by systemic administration as well as topical application of the all-trans- and 13-cis-retinoic acids. Substitution of a 4-hydroxyphenylamide terminal group results in a significant reduction in promoting activity. 4-HPR appears to require metabolic activation for tumor promoting activity; this metabolism does not occur in the skin following topical application, but is observed following systemic exposure.

INTRODUCTION

The chemopreventive activity of natural and synthetic analogues of vitamin A (retinoids) has been demonstrated in a variety of experimental carcinogenesis models. Postcarcinogen administration of nontoxic levels of several vitamin A analogues can inhibit cancer induction in the urinary bladder, mammary gland, pancreas, oral cavity, and lung in experimental animals (for reviews, see Refs. 1 and 2). Furthermore, epidemiological data suggest an inverse relationship between vitamin A intake and cancer incidence in several organ sites in humans (3, 4). In addition to their anticarcinogenic activity in vivo, retinoids can also suppress neoplastic transformation in vitro (5).

Although the efficacy of this class of agents as inhibitors of carcinogenesis is well documented, cancer prevention by retinoids is not a universal phenomenon. The activity of individual retinoids in inhibiting cancer induction appears to be organ specific. For example, although 13-cis-retinoic acid is highly effective as an inhibitor of urinary bladder carcinogenesis, it is relatively ineffective in mammary cancer chemoprevention (1). Experimental and epidemiological data also suggest that carcinogenesis in certain tissues, such as the colon, may be refractory to modulation by retinoids (2, 3). Furthermore, in some experimental models, retinoid exposure appears to enhance, rather than inhibit cancer induction (6–8).

One experimental model in which retinoids have diverse effects is multistage tumorigenesis in mouse skin. Retinoids are highly effective inhibitors of skin tumor promotion by croton oil and its active constituent, TPA.² The antipromotional activity of retinoids has been reported by several groups of investigators, and the ability to inhibit tumor promotion extends to a large series of both natural vitamin A compounds and synthetic analogues (9–13). Although antipromotion studies have generally involved topical administration of the retinoid, inhibition of TPA promotion has also been demonstrated using dietary retinoid exposure (14–16).

Paradoxically, in addition to inhibiting tumor promotion, at least one retinoid, all-trans-retinoic acid, can promote skin tumors in mice which have been initiated but are not exposed to TPA (17, 18). While all-trans-retinoic acid is a much less potent tumor promoter than is the phorbol ester, this retinoid has been demonstrated to increase tumorigenesis in mice previously exposed either to the chemical carcinogen, DMBA (17, 18), or to UV light (19).

Although the promotional and antipromotional activities of all-trans-retinoic acid in mouse skin are well documented, a number of questions concerning retinoid promotion of skin tumor induction have not been addressed. Several of these questions pertain to structure-activity relationships involved in tumor promotion. Is skin tumor promotion by retinoids an effect seen only with all-trans-retinoic acid, or do other analogues also have promoting activity? Is a carboxyl terminal group on the conjugated side chain required for tumor promotion by retinoids? Is promoting activity altered by the substitution of a 13-cis configuration in the side chain? Other questions relate to possible mechanisms of promoting activity. Is skin tumor promotion a local effect requiring topical administration, or can it also be induced by systemic retinoid exposure? Does the promoting activity of individual retinoids correlate with their toxicity, namely, do retinoids which induce less systemic toxicity also have less promoting activity? Finally, can tumor promoting activity be related to patterns of retinoid deposition in mouse skin? It is to these questions which the present report is addressed.

MATERIALS AND METHODS

Experimental Animals. Female SENCAR mice were obtained at 3 to 4 weeks of age from Harlan/Sprague-Dawley, Indianapolis, IN. Animals were housed five per cage on hardwood bedding in a windowless, temperature and humidity controlled room maintained on a 14-h light/10-h dark cycle. Mice had free access to diet and drinking water throughout the study.

Experimental Diets. Basal diet for the experiments was Wayne Lab Chow (Allied Mills, Chicago, IL). This diet contains 8 mg vitamin A per kg, as added retinyl palmitate. All-trans-retinoic acid was purchased from Sigma Chemical Co., St. Louis, MO. 13-cis-retinoic acid was obtained from BASF Aktiengesellschaft, Ludwigshafen-Am-Rhein, West Germany. 4-HPR was synthesized by Dr. Y. Fulmer Shealy, Southern Research Institute, Birmingham, AL. Doses of retinoids used

1 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; 4-HPR, N-(4-hydroxyphenyl)retinamide; DMBA, 7,12-dimethylbenz(a)anthracene.

2 To whom requests for reprints should be addressed, at Life Sciences Department, IIT Research Institute, 10 West 35th Street, Chicago, IL 60616.
in the experiments were the maximum dietary level of each compound which was tolerated without mortality or gross toxicity, as determined in preliminary subchronic feeding studies. As a result of these studies, dietary retinoid levels were (per kg diet): 40 mg (0.133 mmol) all-trans-retinoic acid, 225 mg (0.75 mmol) 13-cis-retinoic acid, and 391 mg (1.00 mmol) 4-HPR. Crystalline retinoids were mixed into basal diet using a trioctanoin vehicle (20). Mice not receiving a dietary retinoid supplement were fed basal diet containing the trioctanoin vehicle only.

Tumorigenesis Protocol. Mice were 7 to 8 weeks old at the initiation of the experiments. Three days prior to initiation, the dorsal skin of all mice was shaved, and those showing regrowth of hair prior to initiation were excluded from the study. Mice were initiated with a single topical dose of 5.0 µg DMBA dissolved in 200 µl reagent grade acetone. Two weeks after initiation, mice were randomized into groups of 25 (Table 1), and dietary or topical administration of retinoids was begun. Retinoids were administered in the diet as indicated above, or were given via twice weekly topical application at a level of 30 nmol per dose in 200 µl acetone. Mice receiving dietary retinoid supplements were painted twice weekly with 200 µl acetone; groups receiving topical retinoid exposure were fed the basal diet supplemented with trioctanoin vehicle. The control group received twice weekly painting with 200 µl acetone, and was fed diet supplemented with trioctanoin vehicle.

Mice were observed twice daily for any signs of overt toxicity, and were weighed once per week; beginning 4 weeks after initiation, animals were observed weekly to monitor tumor appearance. At the termination of the study, sections of representative papillomas were fixed in 10% buffered formalin, cut at 5 µm, and stained with hematoxylin and eosin for histopathological verification.

Calculations of tumor incidence and multiplicity were performed using life table analysis; intergroup comparisons of tumor incidence curves were performed using the logrank test (21). Differences in group mean body weight and tumor multiplicity were tested for statistical significance via analysis of variance.

Pharmacology Protocol. To determine patterns of retinoid deposition in the skin following dietary or topical exposure to individual compounds, additional groups of SENCAR mice were administered the retinoid-supplemented or control diets. After a minimum of 10 weeks of dietary or topical retinoid exposure, mice were killed by cervical dislocation. Dorsal skin was excised, frozen in liquid nitrogen, and stored at −80°C prior to analysis. Tissues were lyophilized, extracted with chloroform/methanol (2:1), and analyzed for retinoid content via reversed-phase high-performance liquid chromatography using the technique of Huitín et al. (22). This method employs a Spectra Physics model 8700 high-performance liquid chromatograph equipped with a model 8440 variable-wavelength detector set at 350 nm. Separations were performed using a 250 x 4.6 mm I.D., 10 µm, bonded octadecylsilane reversed-phase column (Partisil 10 ODS-2; Whatman, Clifton, NJ), and a 30-min linear gradient of methanol/water (70:30) to 100% methanol at a flow rate of 1.2 ml/min. Chromatography continued at the final conditions for an additional 40 min.

Where appropriate, pharmacology studies were conducted using 3H-labeled retinoids, obtained through the National Cancer Institute from Dr. Sung W. Rhee, SRI International, Menlo Park, CA. 13-eis-Retinoic acid (specific activity, 3.55 Ci/mmol) was labeled at the 10 and 11 positions.

## RESULTS

All three retinoids had significant tumor promoting activity when administered as dietary supplements to DMBA-initiated mice. At 30 weeks postinitiation, the control group (DMBA initiated, vehicle fed, and acetone promoted) had a tumor incidence of 4% (Table 1); one animal in this group developed two papillomas, the first appearing at week 26. The most active tumor promoter of the three dietary retinoids was 13-cis-retinoic acid. At 30 weeks postinitiation, tumor incidence was 79%, with a mean of 5.9 tumors per mouse, in the group fed 225 mg 13-cis-retinoic acid per kg diet (Figs. 1 and 2). Dietary exposure to 40 mg all-trans-retinoic acid per kg also promoted skin tumors induced by DMBA: tumor incidence in this group was 68%, with 3.3 tumors per mouse at 30 weeks.

4-HPR was significantly less active in skin tumor promotion than was either all-trans- or 13-cis-retinoic acid. Administration of 391 mg 4-HPR per kg diet to DMBA-initiated mice resulted in a 35% tumor incidence at 30 weeks (P < 0.05 versus both all-trans- and 13-cis-retinoic acid groups). In addition to a lower tumor incidence in mice fed 4-HPR, tumor appearance in this group was delayed in comparison to groups fed either retinoic acid isomer: while the first tumor was observed at 12 weeks in both the 13-cis- and all-trans-retinoic acid groups, time to first tumor was 19 weeks in the 4-HPR group (Fig. 1).

The relative promoting activity of topical application of the three retinoids was significantly different than their activity when administered as dietary supplements. All-trans- and 13-cis-retinoic acids were both active as tumor promoters when applied topically at a dose of 30 nmol twice weekly: at 30 weeks the tumor incidence in the group painted with all-trans-retinoic acid was 58% with 2.5 tumors per mouse, while with 13-cis-retinoic acid the tumor incidence was 24% with 1.4 tumors per mouse (Figs. 3 and 4). By contrast to the effects of the two isomers of retinoic acid, topical application of 4-HPR was without apparent promoting activity. At 30 weeks, the 4% incidence of tumors in the group painted twice weekly with 30 nmol of 4-HPR was identical to that observed in acetone controls.

In order to address possible mechanisms for the differential activity of dietary and topical exposure to 4-HPR, patterns of retinoid deposition in the skin were determined following dietary and topical administration of this compound. As indicated in Fig. 5, dietary exposure to 4-HPR resulted in deposition of several 4-HPR metabolites in mouse skin; prominent among these metabolites is a peak which cochromatographs with N-(4-methoxyphenyl)retinamide, eluting at approximately 27 min

### Table 1 Influence of dietary and topical retinoids on skin tumor promotion and animal body weight

<table>
<thead>
<tr>
<th>Group</th>
<th>Retinoid</th>
<th>Dietary retinoid level (mg/kg diet)</th>
<th>Topical retinoid level (nmol/dose, 2×/week)</th>
<th>Tumor incidence (%)</th>
<th>Tumors per mouse</th>
<th>Body weight (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>0 (Vehicle)</td>
<td>0 (Acetone)</td>
<td>4</td>
<td>0.08</td>
<td>37.1 ± 5.8</td>
</tr>
<tr>
<td>2</td>
<td>4-HPR</td>
<td>391</td>
<td>0 (Acetone)</td>
<td>35a</td>
<td>1.87a</td>
<td>36.1 ± 3.6</td>
</tr>
<tr>
<td>3</td>
<td>All-trans-retinoic acid</td>
<td>40</td>
<td>0 (Acetone)</td>
<td>68a, b</td>
<td>3.28a, b</td>
<td>30.9 ± 3.4, a</td>
</tr>
<tr>
<td>4</td>
<td>13-cis-Retinoic acid</td>
<td>225</td>
<td>0 (Acetone)</td>
<td>79a, b</td>
<td>5.88a, c</td>
<td>34.1 ± 4.9d</td>
</tr>
<tr>
<td>5</td>
<td>4-HPR</td>
<td>0 (Vehicle)</td>
<td>30</td>
<td>4</td>
<td>0.04</td>
<td>37.2 ± 5.4</td>
</tr>
<tr>
<td>6</td>
<td>All-trans-retinoic acid</td>
<td>0 (Vehicle)</td>
<td>30</td>
<td>58a, c</td>
<td>2.54a, c</td>
<td>37.5 ± 3.9</td>
</tr>
<tr>
<td>7</td>
<td>13-cis-Retinoic acid</td>
<td>0 (Vehicle)</td>
<td>30</td>
<td>24a, d</td>
<td>1.40a, e</td>
<td>37.9 ± 4.9</td>
</tr>
</tbody>
</table>

* P < 0.01 versus control (group 1).

b P < 0.05 versus appropriate 4-HPR group.

P < 0.01 versus appropriate 4-HPR group.

P < 0.05 versus control (group 1).
Fig. 1. Skin tumor promotion by dietary retinoids: influence of retinoids on tumor incidence. ■, all-trans-retinoic acid [40 mg (0.133 mmol) per kg diet]; △, 13-cis-retinoic acid [225 mg (0.75 mmol) per kg diet]; ★, 4-HPR [391 mg (1.00 mmol) per kg diet]; ●, control.

Fig. 2. Skin tumor promotion by dietary retinoids: influence of retinoids on tumor multiplicity. ■, all-trans-retinoic acid [40 mg (0.133 mmol) per kg diet]; △, 13-cis-retinoic acid [225 mg (0.75 mmol) per kg diet]; ★, 4-HPR [391 mg (1.00 mmol) per kg diet]; ●, control.

Fig. 3. Skin tumor promotion by topical retinoids (30 nmol per dose, twice weekly): influence of retinoids on tumor incidence. ■, all-trans-retinoic acid; △, 13-cis-retinoic acid; ★, 4-HPR; ●, control.

Fig. 4. Skin tumor promotion by topical retinoids (30 nmol per dose, twice weekly): influence of retinoids on tumor multiplicity. ■, all-trans-retinoic acid; △, 13-cis-retinoic acid; ★, 4-HPR; ●, control.

Fig. 5. High-performance liquid chromatographic elution profile of skin sample from mouse receiving chronic dietary exposure to 4-HPR (391 mg/kg diet). Peaks 1–4, unidentified; peak 5, 4-HPR; peak 6, cochromatographs with N-(4-methoxyphenyl)retinamide; peak 7, putative ester of 4-HPR; peak 8, retinyl palmitate.

Fig. 6. Chromatogram from an animal which received twice weekly topical 4-HPR exposure for more than 6 months is presented in this figure. As indicated in this figure, topical exposure to 4-HPR was associated with the presence of only the parent compound (peak 5) and retinyl palmitate (peak 8) in the skin; the metabolites present in animals receiving dietary supplementation with 4-HPR were not detected in mice receiving the compound via local application.

The pattern of retinoid deposition in mouse skin following chronic topical exposure to 4-HPR was significantly different from that observed in animals receiving dietary supplementation with the retinoid. A chromatogram from an animal which had received twice weekly topical 4-HPR exposure for more than 6 months is presented in Fig. 6; this mouse was killed 2 h after administration of the final topical dose. As indicated in this figure, topical exposure to 4-HPR was associated with the presence of only the parent compound (peak 5) and retinyl palmitate (peak 8) in the skin; the metabolites present in animals receiving dietary supplementation with 4-HPR were not detected in mice receiving the compound via local application.
chain alkyl retinamides (26). However, although 13-cis-retinoic acid induces less systemic toxicity than does its all-trans isomer, substitution of the 13-cis configuration does not result in a loss of tumor promoting activity; in fact, when the compounds are administered at their maximum tolerated dietary levels, 13-cis-retinoic acid is a more potent tumor promoter than is all-trans-retinoic acid. Preliminary results from other experiments using the same DMBA dose as that used in the present study indicate that 13-cis and all-trans-retinoic acids have similar promoting activity when administered at equimolar levels in the diet (McCormick et al., unpublished). These data suggest that the differential promoting activity of the 13-cis and all-trans isomers of retinoic acid seen in the present study is related to the increased level of the 13-cis isomer in the diet, and does not appear to be a function of any greater intrinsic promoting activity of 13-cis-retinoic acid.

The importance of a carboxyl terminal group on the retinoid side chain appears to depend on the route of administration of the retinoid. When administered as a dietary supplement, 4-HPR, in which a hydroxyphenylamido moiety is substituted for the carboxyl group of all-trans-retinoic acid, had significant activity as a tumor promoter. However, the activity of 4-HPR administered as a dietary supplement was less than that observed with all-trans-retinoic acid. This reduced activity was observed in spite of the fact that the dietary level of 4-HPR was more than seven times the molar level of all-trans-retinoic acid.

By contrast, when administered via topical application, 4-HPR had no activity as a tumor promoter. The lack of activity of topical 4-HPR is in direct opposition to the effects of topical administration of all-trans-retinoic acid. The divergent effects of dietary versus topical exposure to 4-HPR suggest that systemic metabolism of this retinoid may be required for activity as a modifier of skin tumor induction; such metabolism apparently does not occur locally in the skin following topical application of 4-HPR. Furthermore, the data presented in Figs. 5 and 6 indicate that a metabolite of 4-HPR, rather than the parent compound, is responsible for promoting activity. This conclusion is based on the observation that comparable levels of the parent compound can be detected in mouse skin following either dietary or topical 4-HPR administration, yet no promoting activity was observed in animals painted with 4-HPR.

As indicated in Fig. 5, dietary administration of 4-HPR resulted in the deposition of the parent compound and at least 6 metabolites in mouse skin; by contrast, these metabolites were not observed when 4-HPR was administered via topical application. These data suggest that, although enzyme systems involved in the biotransformation of xenobiotics are present in mouse skin, these enzyme systems may be unable to generate locally sufficient quantities of the metabolite or metabolites of 4-HPR which are responsible for its activity in the modulation of skin tumor induction. The qualitative pattern of 4-HPR metabolites observed in mouse skin is similar to those observed in the mammary gland and urinary bladder, two other target tissues in which this compound has significant biological activity. However, the identity of the metabolite(s) of 4-HPR which have activity as modifiers of carcinogenesis remains unknown.

To address this issue, studies are in progress to isolate and characterize metabolites of 4-HPR which are found in target tissues where the compound has biological activity.

One possible mechanism for 4-HPR activity following systemic administration is that the compound is metabolized to retinoic acid; in fact, metabolism of 4-HPR to retinoic acid has been hypothesized as a possible mechanism of action of this retinoid (27). However, in the present experiment, none of the 4-HPR metabolites observed in mouse skin coeluted with either

**DISCUSSION**

The results of the present experiment indicate that all three retinoids can promote tumors in mice initiated with DMBA. The observation that topical administration of all-trans-retinoic acid can promote skin tumors initiated by DMBA confirms the reports of Hennings et al. (17) and Fischer et al. (18). A significant extension of these reports is the promotional activity of dietary retinoids observed in the present study. These results clearly indicate that retinoid influences on skin tumor induction can be induced with systemic exposure; in all cases studied, dietary administration of a retinoid at its maximum tolerated dose level had greater promoting activity than did twice weekly topical application at 30 nmol per dose.

Comparisons of the promoting activity of the three retinoids provide structure-activity data relevant to the activity of this class of compounds as modifiers of tumor induction in mouse skin. As indicated by the maximum tolerated dietary levels used in these studies, and the influence of the compounds on animal body weight, replacement of the all-trans side chain of retinoic acid with a 13-cis configuration results in a significant reduction in systemic toxicity. This finding confirms the results of Hixon and colleagues (24, 25) and is in general agreement with our previous report comparing the toxicity and anticarcinogenic activity of the all-trans and 13-cis isomers of a series of short chain alkyl retinamides (26). However, although 13-cis-retinoic acid shows greater activity as a tumor promoter than does all-trans-retinoic acid, the concentration of this compound required for tumor promotion is more than 100-fold greater than that of 4-HPR.

Fig. 6. High-performance liquid chromatographic elution profile of skin sample from mouse receiving chronic topical exposure to 4-HPR (30 nmol per dose, twice weekly; sample taken 2 h after final 4-HPR dose). Peak 5, 4-HPR; peak 8, retinyl palmitate.
all-trans- or 13-cis-retinoic acids. Thus, although it is attractive in its simplicity, this hypothesis is not supported by our experimental data.

The tumorigenesis data obtained in the present study differ from those of our recent report which noted that dietary administration of 4-HPR did not promote tumors in a 20-week study using both SENCAR and CD-1 mice (14). The differences in results can be attributed entirely to the relationship between the latency of tumors promoted by 4-HPR and the duration of the two studies. In the previous study, where no tumor promotion by 4-HPR was found, an initiating dose of 2.5 µg of DMBA was used, and the experiment was terminated after 20 weeks. In the present study, a 5.0-µg initiating dose of DMBA was used, and the time to first tumor in the group fed 4-HPR was 19 weeks. This and other DMBA dose-response data generated in our laboratory suggest that promoting activity may have been observed in the first experiment had it been extended for an additional 10 to 15 weeks.

Consideration of these data together with the existing literature concerning retinoid promotion of skin tumorigenesis provides information relevant both to the identification of structure-activity relationships for such promotion and to the possible relationship between the systemic toxicity and tumor promoting activity of retinoids. Substitution of a 13-cis configuration into all-trans-retinoic acid results in a significant reduction in systemic toxicity (24, 25), but this reduction in systemic toxicity is not accompanied by a parallel decrease in tumor promoting activity. Indeed, when initiated mice received dietary exposure to the two compounds at their maximum tolerated levels, 13-cis-retinoic acid was a more effective promoter of skin tumor induction than was its all-trans isomer. By contrast, substitution of a hydroxyphenylamide moiety into the polar terminal group of all-trans-retinoic acid (thus producing 4-HPR) resulted both in a reduction in systemic toxicity and a significant diminution of promoting activity. Thus, there appears to be no correlation between the systemic toxicity and promoting efficacy of individual retinoids.

Replacement of the carboxyl group of all-trans-retinoic acid with a less polar group (i.e., the hydroxyphenylamide group of 4-HPR) appears to result in a total loss of promoting activity via the topical route. At the present time, the generality of this effect is not known. However, it should be noted that these data are similar to those of Fischer et al.(18), who reported that the synthetic retinoid Ro 10-9359 also lacks promoting activity when administered topically. Like 4-HPR, Ro 10-9359 has a substitution (in this case, an ethyl ester) in the polar terminal group; unlike 4-HPR, however, Ro 10-9359 also has multiple ring substitutions which could be responsible for its lack of promoting activity.

These data indicate that the activity of retinoids as skin tumor promoters is not limited to the effects previously reported for all-trans-retinoic acid, but are also seen to varying degrees with other compounds of this chemical class. Further studies of the patterns of deposition of these agents in mouse skin are in progress in the attempt to identify metabolite(s) which are responsible for this promoting activity.

ACKNOWLEDGMENTS

We thank Dr. R. C. Moon for supplying the unlabeled 13-cis-retinoic acid and 4-HPR and Patricia Moser for secretarial assistance.

REFERENCES

Comparative Activity of Dietary or Topical Exposure to Three Retinoids in the Promotion of Skin Tumor Induction in Mice

David L. McCormick, Bryan J. Bagg and Theresa A. Hultin


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/22/5989

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.