Inhibition by Retinoids of Anthralin-induced Mouse Epidermal Ornithine Decarboxylase Activity and Anthralin-promoted Skin Tumor Formation

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ABSTRACT

The retinoids all-trans-retinoic acid, 13-cis-retinoic acid, 4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1E-propen-1-yI]-benzene 4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1E-propen-1-yI]-benzene 4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1E-propen-1-yI]-benzene 4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1E-propen-1-yI]-benzene are weak tumor promoters in the two-stage carcinogenesis model. The mechanism of action of anthralin has not yet been established; however, evidence indicates interaction with DNA (4, 12-15). The mechanism of action is different from that of the tumor-promoting phorbol ester constituents of croton oil, a powerful skin irritant. Anthralin was subsequently found to act as a weak carcinogen and a weak tumor promoter in mouse epidermis by Bock and Burns (9), Segal et al. (10), Van Duuren and Goldschmidt (8), DiGiovanni et al. (11), Viluksela et al. (6), and Männistö et al. (4).

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INTRODUCTION

For 70 years anthralin (1,8-dihydroxy-9-anthracene; Fig. 1, I3) has been used for the topical treatment of psoriasis (1-3) although it is not without side effects (4-6). Its skin irritancy and its identification as a tobacco smoke constituent (7, 8) led investigators to compare the properties of anthralin with those of the tumor-promoting phorbol ester constituents of croton oil, a powerful skin irritant. Anthralin was subsequently found to act as a weak carcinogen and a weak tumor promoter in mouse epidermis by Bock and Burns (9), Segal et al. (10), Van Duuren and Goldschmidt (8), DiGiovanni et al. (11), Viluksela et al. (6), and Männistö et al. (4).

The mechanism of action of anthralin has not yet been established; however, evidence indicates interaction with DNA (4, 12-15). The mechanism of action is different from that of the tumor-promoting phorbol esters (16). Whereas the phorbol ester, TPA1, is a potent tumor promoter in the two-stage carcinogenesis model and a potent inducer of ODC activity in mouse epidermis (17, 18), anthralin is a weak tumor promoter and inducer of ODC (18). In addition, anthralin appeared to inhibit tumor promotion and ODC induction by TPA (19). DiGiovanni et al. (11) compared the time courses for the induction of ODC in SENCAR mouse epidermis by anthralin, DMBA, and TPA and found that the effect of anthralin more closely resembled that of DMBA than TPA. Treatment with TPA down-modulated TPA-receptor proteins (16, 20-22), whereas treatment with anthralin decreased the level of ODC mRNA (16). Receptor-binding studies indicate anthralin did not compete with [20-3H]phorbol-12,13-dibutyrate for binding to the phorbol ester receptor (23-25).

Gensler and Bowden (26) found that in mice initiated with 400 nmol of benzo[a]pyrene twice-weekly applications of 17 nmol of 13-cisRA, 4, 0.5 h before promotion with 444 nmol of anthralin did not influence the rate of papilloma formation or the number of papillomas per mouse compared with the control group of animals not receiving the retinoid. These results led them to suggest that a TPA-induced promotion step that is inhibited by retinoids is not affected by retinoids in anthralin-induced promotion. In contrast, Kruszewski et al. (27) reported that RA, 3, inhibited tumor promotion by chrysarobin (2, the 3-methyl analogue of anthralin) in SENCAR mice initiated with DMBA.

Because O'Brien et al. (17, 28) showed a positive correlation between the ability of compounds to induce epidermal ODC and their ability to promote epidermal tumors and because Verma and Boutwell (29) and Verma et al. (30, 31) established that the inhibitory effect of retinoids on the induction of ODC correlated with their ability to inhibit papilloma formation in the two-stage carcinogenesis model, we decided to investigate the apparently conflicting reports of Gensler and Bowden (26) and Kruszewski et al. (27) on the effects that retinoids had on tumor promotion by anthralin. In our studies we elected to use CD-1 mice because of their ready availability and our prior long-term use of this strain in promotion studies. We first investigated the ODC induction profile by anthralin in this mouse strain and then the effects that retinoids had on ODC induction. Next we studied the effects that retinoids had on the inhibition of papilloma formation initiated by DMBA and promoted by anthralin. A 444-nmol (100-μg) dose of anthralin was used to conform to the dose used by Gensler and Bowden (26). DMBA was selected as the tumor initiator because it has been successfully used in two-stage carcinogenesis studies with anthralin as the promoter (9, 10, 14). A 200-nmol dose of this initiator was used because we have found that this dose is effective at initiating tumors in CD-1 mice promoted with TPA. We selected five retinoids (3 to 7) having a wide range of activities in inhibiting the induction of ODC by TPA in CD-1 mouse epidermis (32) and in inhibiting the formation of papillomas in these mice initiated by DMBA and promoted by TPA (33). In addition to RA and 13-cisRA, three conformationally restricted aromatic retinoids (5 to 7) were tested. The benzoic acid 5 is one of the most active and toxic retinoids reported (34). The dihydrobenzo[b]pyran 6 (32, 33) and the naphthalene carboxylic acid 7 (33) are analogues of 5 that we synthesized previously.

MATERIALS AND METHODS

Chemicals. RA was a gift from Dr. Michael B. Sporn, National Cancer Institute, Bethesda, MD, and was recrystallized from anhydrous ethanol under argon before use; 13-cisRA was obtained from Dr. Y. Fulmer Shealy, Southern Research Institute, Birmingham, AL; and 4-
[2-(5,6,7,8-tetrahydro-5,8,8-tetramethyl-2-naphthalenyl)-1E-propen-1-yl]benzoic acid (Ro19-7410, 5) (34) was obtained from Hoffmann-La Roche, Nutley, NJ. 6-[1-(4-Carboxyphenyl)-1E-propen-2-yl]-3,4-dihydro-4,4-dimethyl-2H-1-benzothiopyran (SRI-5896-39, 6) (32, 33) and 6-(5,6,7,8-tetrahydro-5,8,8-tetramethyl-2-naphthalenyl)-2-naphthalene carboxylic acid (SRI-5898-52, 7) (33) were retinoids synthesized at SRI. Each retinoid was found to have greater than 99% purity by high-performance liquid chromatographic analysis on a Waters Associates ALC 210 instrument equipped with an RCM-100 module containing a reverse-phase RadialPak A column cartridge (methanol eluant at a flow rate of 2.0 ml/min with detection at 260 nm using a Schoeffel Model 770 variable-wavelength UV monitor). Anthralin was purchased from City Chemical Corp., New York, NY; DMBA from Sigma Chemical Co., St. Louis, MO; L-[1-14C]ornithine hydrochloride (specific activity, 54 mCi/mm) from Amersham, Arlington Heights, IL; and reagent grade acetone from Mallinckrodt, Inc., Paris, KY. Oxygen-sensitive retinoids were sealed under argon in glass ampules until use. RA and 13-cisRA were handled under subdued light. Anthralin and DMBA were handled in double-walled containers as a safety precaution.

Animals. Female Charles River CD-1 mice 5 to 7 weeks old were purchased from Charles River Laboratories, Inc., Wilmington, MA. The mice were housed 10 to a plastic cage, with softwood chips as bedding, in a controlled-environment room with a light period from 7:00 am to 6:00 pm. Rodent Laboratory Chow No. 5001 (Ralston Purina Co., Gray Summit, MO) and water were provided ad libitum.

RESULTS

Time of Maximum Induction of ODC by Anthralin in CD-1 Mouse Epidermis. O'Brien (18) reported that a 2200-nmol dose of anthralin applied to the epidermis of CD-1 mice resulted in a 20-fold increase (0.372 nmol of CO2/mg of protein/30 min) in the level of ODC activity over that in the control. This maximal level was reached 48 h after anthralin application. For our studies, a 444-nmol dose of anthralin was used for the following reasons: (a) this dose was used by Gensler and Bowden (26) in their tumor promotion studies; (b) it was within the range found by DiGiovanni et al. (11) to promote tumors in SENCAR mice initiated with DMBA; and (c) earlier studies showed that a 444-nmol dose of anthralin promoted papillomas in CD-1 mice treated with 200 nmol of DMBA, whereas doses of 111 and 333 nmol did not.6 A single topical application of 444 nmol of anthralin to the dorsal epidermis of CD-1 mice resulted in a 40- to 90-fold increase in ODC activity over the basal level of 0.030 ± 0.005 (SE) nmol of CO2/mg of protein/30 min. In one experiment, shown in Fig. 2, the time course of ODC activity was measured. Enzyme activity was significantly elevated over basal levels at 24 h, reached a maximum (2.50 ± 0.27 nmol of CO2/mg of protein/30 min) at 48 h, and then declined to baseline levels by 80 h. The largest increase in activity occurred between the 44- and 48-h measurement points, and the largest decrease between the 48- and 52-h points.

Effect of Time of Topical Application of Retinoids on Anthralin-induced ODC Activity. The maximal inhibition of TPA-induced ODC activity in CD-1 mouse epidermis occurred when...
RA was applied 1 h before the application of either 8.5 nmol or 17.0 nmol of TPA (29). However, we found that when RA and other retinoids were applied 1 h before a 444-nmol dose of anthralin, the degree of inhibition of enzyme induction varied. For example, pretreatment with 170 nmol of RA inhibited ODC activity by 11 to 63%. Retinoid transport and metabolism in the epidermis over the 49-h period from retinoid treatment to maximal induction of ODC activity might have affected the concentration of retinoid in the skin that was available for inhibition. To determine whether the time of application of retinoid influenced the level of anthralin-induced ODC activity, the time-course experiment shown in Fig. 3 was conducted. The naphthalenecarboxylic acid 7 was used as the active retinoid because this compound would not be metabolically deactivated by the usual oxidative metabolism pathways available for retinoids (39). A 3.4-nmol dose of 7 was applied immediately (0 h) and at 12, 24, and 36 h after anthralin treatment. ODC activity was determined at 48 h after anthralin application. Enzyme activity was not significantly different from that of the anthralin-alone treatment group at the 0-h point but was significantly reduced at other times (P < 0.02 to 0.05). The maximum inhibition of induction occurred at the 24- and 36-h points.

The effects, with time, of RA and the naphthalenecarboxylic acid 7 on anthralin-induced ODC activity when retinoid treatment occurred 1 h before and 24 h after the application of anthralin are illustrated in Fig. 2. ODC activity was assayed at the times indicated. The naphthalenecarboxylic acid 7 (170 nmol) was more effective than RA (170 nmol) at inhibiting the induction of ODC when both retinoids were applied 1 h before anthralin. Both retinoids were equally effective inhibitors when applied at 24 h after anthralin. It is interesting to note that the ODC activities in the 1-h retinoid pretreatment groups were significantly elevated over the activity in the anthralin-alone treatment group at the 64- and 76-h assay points for the naphthalenecarboxylic acid 7 (P < 0.001 and < 0.02, respectively) and at the 76-h point for RA (P < 0.05).

Effect of Retinoids on Anthralin-induced Epidermal ODC Activity When Applied 24 h after Anthralin Treatment. All five retinoids inhibited the induction of ODC by anthralin (Table 1). The benzoic acid 5 was the most effective inhibitor, having a calculated ID50 of 0.020 nmol. The next most active retinoid inhibitor was RA, followed in order by the naphthalenecarboxylic acid 7, the dihydrobenzothiopyran 6, and 13-cisRA. Linear

Table 1 Effect of retinoids on anthralin-induced ODC activity in CD-1 mouse epidermis

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>Dose (nmol)</th>
<th>% ODC inhibition</th>
<th>ID50 (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA (J)</td>
<td>0.17</td>
<td>52 ± 5*</td>
<td>0.096</td>
</tr>
<tr>
<td></td>
<td>1.70</td>
<td>60 ± 4*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17.0</td>
<td>68 ± 3*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>170.0</td>
<td>86 ± 1*</td>
<td></td>
</tr>
<tr>
<td>13-cisRA (4)</td>
<td>1.70</td>
<td>20 ± 9</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>17.0</td>
<td>44 ± 2*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>170.0</td>
<td>86 ± 2*</td>
<td></td>
</tr>
<tr>
<td>The benzoic acid 5</td>
<td>0.017</td>
<td>49 ± 6*</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>0.17</td>
<td>67 ± 2*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.70</td>
<td>94 ± 7*</td>
<td></td>
</tr>
<tr>
<td>The dihydrobenzothiopyran 6</td>
<td>1.70</td>
<td>14 ± 10</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>17.0</td>
<td>71 ± 3*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>170.0</td>
<td>80 ± 3*</td>
<td></td>
</tr>
<tr>
<td>The naphthalenecarboxylic acid 7</td>
<td>1.70</td>
<td>39 ± 3*</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>17.0</td>
<td>75 ± 3*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>170.0</td>
<td>92 ± 1*</td>
<td></td>
</tr>
</tbody>
</table>

* Determined by polynomial extrapolation (3/interpolation (4 to 7) of the data.
* Mean ± SE.
* Significantly different from control, at P < 0.025.
* Significantly different from control, at P < 0.005.
* Significantly different from control, at P < 0.001.
* Significantly different from control, at P < 0.01.
* Significantly different from control, at P < 0.05.
* Significantly different from control, at P < 0.02.

W.-R. Chao, unpublished observations.
regression analysis of the ID₉₀ values (nmol) for these five retinoids in inhibiting anthralin-induced ODC (Table 1) and TPA-induced ODC [3: 0.038, 4: 1.7, 5: 0.03, 6: 0.48, 7: 2.2] and the ID₉₀ values (nmol) for three other retinoids, the dihydropryan analogue of 6 [65 (anthralin); 2.7 (TPA)], the 1-methyl-2-naphthalencarboxylic acid analogue of 7 [2.7 (anthralin); 1.7 (TPA)], and the 5,6,7,8-tetrahydro-8,8-dimethyl-6-naphthalenyl analogue of 7 [200 (anthralin); 6.8 (TPA)], indicated that the correlation between these sets of values was good (r = 0.975; n = 8; P < 0.001).

Effect of Retinoid 7 on ODC Activity Induced in Mouse Epidermis Treated with Multiple Applications of Anthralin. O'Brien (18) reported that multiple applications of anthralin (2200 nmol) at 3- to 4-day intervals over a 3-week period shifted the maximal ODC activity peak in CD-1 mouse epidermis forward to 6 h after the last application. Enzyme activity decreased to control levels by 24 h and appeared to rise again slightly at 48 h. The maximal level of activity after multiple anthralin treatments was approximately 10% of that found after a single treatment. Kruszewski et al. (40) substantiated these findings in the epidermis of female SENCAR mice. For example, five treatments with 220 nmol of chrysarobin over a 2.5-week period gave a major activity peak 4 to 6 h after the last chrysarobin treatment, with a minor peak 24 h after treatment and some increase at 48 h. Activity was less than 10% of that found after a single application. O'Brien (18) noted the similarity between the kinetic patterns of ODC induction by TPA and anthralin.

Because of these reports by O'Brien (18) and Kruszewski et al. (40) and because of the correlation between ODC induction and tumor promotion in mouse epidermis (17, 28-31), we considered that the two-stage tumorigenesis protocol used for TPA promotion was appropriate for studies using anthralin as the promoter. In our studies multiple treatments with anthralin (2200 nmol) resulted in a level of epidermal ODC activity at 6 h after the last anthralin treatment of approximately 10% or less of that found with a single application of anthralin. However, O'Brien (18) noted the similar inhibition of the naphthalenecarboxylic acid 7 displayed symptoms of retinoid toxicity during the 32-week promotion period, as evidenced by redness and scaling of the skin on the ears and the presence of the naphthalencarboxylic acid 7.

Dose groups of the benzoic acid 5 and the 170-nmol dose group of the dihydrobenzothiopyran 6, and the naphthalencarboxylic acid 7. The least active retinoid was 13-cisRA. In all cases, retinoids delayed the onset of tumors at the dose levels listed in the table (data not shown). Higher doses of retinoids were more effective at delaying the onset. The animals in the 0.17- and 1.7-nmol dose groups of the benzoic acid 5 and the 170-nmol dose group of the naphthalencarboxylic acid 7 displayed symptoms of retinoid toxicity during the 32-week promotion period, as evidenced by redness and scaling of the skin on the ears and the appearance of general malaise.

Comparison of the ID₉₀ values presented in Tables 1 and 2 indicated that those retinoids (3 and 5) that were very effective at inhibiting the induction of ODC by anthralin (444 nmol) were also very effective at inhibiting tumor promotion by anthralin (444 nmol), whereas retinoid 4, a poor inhibitor of ODC induction, was also a poor inhibitor of tumor promotion.

DISCUSSION

The present studies in CD-1 mice confirm the results of O'Brien (18) that the time of maximal induction of epidermal ODC is 48 h after a single application of anthralin. However, we did not confirm the reports that multiple applications of anthralin (2200 nmol) (18) and chrysarobin (220 nmol) (40) produced a maximal level of ODC activity that was approximately 10% or less of that found with a single application of the promoter. In our studies multiple treatments with anthralin (444 nmol) resulted in a level of epidermal ODC activity at 6 h after the last application of anthralin that was 65% of the
maximal level (2.7 nmol of CO₂/mg of protein/30 min) found after a single anthralin treatment.

Retinoids were able to inhibit ODC induction caused by single and multiple treatments of anthralin. The correlation between the calculated concentrations of retinoids required to inhibit by 50% the level of ODC activity induced by single applications of anthralin (444 nmol) and of TPA (17 nmol) was good. These results suggest that although previous studies provide convincing evidence that the mechanisms of action of anthralin and TPA are different (11, 16-24), retinoids appear to inhibit a similar step in the pathway leading to the induction of ODC by these promoters.

Retinoids were also able to inhibit tumor promotion by anthralin (44 nmol) in CD-1 mouse epidermis in the two-stage tumorigenesis model using DMBA (200 nmol) as the initiator. These results are in agreement with those of Kruszewski et al. (27) in SENCAR mice. Gensler and Bowden (26) used only 17 nmol of 13-cisRA on SENCAR mouse epidermis to inhibit a similar step in the pathway leading to the induction of ODC by anthralin (444 nmol). Our results (Table 2) indicate that the 23% inhibition in the number of tumors per mouse in the control groups (58 survivors/60 total mice) was not statistically significant. The higher dose (170 nmol) of 13-cisRA on SENCAR mouse epidermis to inhibit number of papillomas per mouse by 50% was determined by polynomial extrapolation (3, 5, 6)/interpolation (4, 7) of the data.

**Table 2**

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>Dose (nmol)</th>
<th>Incidence (%)</th>
<th>Inhibition of tumors/mouse (%)</th>
<th>ID₅₀ (nmol)</th>
<th>No.</th>
<th>S/T (wk of death)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA (3)</td>
<td>1.70</td>
<td>41</td>
<td>56 ± 7*</td>
<td>0.61</td>
<td>0</td>
<td>29/30 (25)</td>
</tr>
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<td></td>
<td>17.0</td>
<td>34</td>
<td>68 ± 10*</td>
<td></td>
<td>0</td>
<td>29/30 (31)</td>
</tr>
<tr>
<td></td>
<td>170.0</td>
<td>28</td>
<td>78 ± 8*</td>
<td></td>
<td>0</td>
<td>29/30 (32)</td>
</tr>
<tr>
<td>13-cisRA (4)</td>
<td>1.70</td>
<td>50</td>
<td>36 ± 32</td>
<td>114</td>
<td>3</td>
<td>30/30</td>
</tr>
<tr>
<td></td>
<td>17.0</td>
<td>55</td>
<td>23 ± 14</td>
<td></td>
<td>0</td>
<td>29/30 (25)</td>
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<tr>
<td></td>
<td>170.0</td>
<td>45</td>
<td>60 ± 11*</td>
<td></td>
<td>0</td>
<td>29/30 (32)</td>
</tr>
<tr>
<td>The benzoic acid 5</td>
<td>0.17</td>
<td>33</td>
<td>68 ± 10*</td>
<td>0.024</td>
<td>0</td>
<td>27/30 (32)</td>
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<td></td>
<td>1.70</td>
<td>12</td>
<td>89 ± 5*</td>
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<td>0</td>
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<td>The dihydrobenzothiopyran 6</td>
<td>1.70</td>
<td>43</td>
<td>56 ± 12*</td>
<td>0.96</td>
<td>0</td>
<td>30/30</td>
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<tr>
<td></td>
<td>17.0</td>
<td>50</td>
<td>59 ± 11*</td>
<td></td>
<td>1</td>
<td>30/30</td>
</tr>
<tr>
<td></td>
<td>170.0</td>
<td>27</td>
<td>80 ± 7*</td>
<td></td>
<td>1</td>
<td>30/30</td>
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<tr>
<td>The naphthalencarboxylic acid 7</td>
<td>1.70</td>
<td>41</td>
<td>37 ± 22</td>
<td>3.0</td>
<td>0</td>
<td>28/30 (28, 29)</td>
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<td>17.0</td>
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<td>80 ± 8*</td>
<td></td>
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<td></td>
<td>170.0</td>
<td>0</td>
<td>100*</td>
<td></td>
<td>0</td>
<td>29/30 (22)</td>
</tr>
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</table>

* Calculated dose of retinoids required to inhibit number of papillomas per mouse by 50%; determined by polynomial extrapolation (3, 5, 6)/interpolation (4, 7) of the data.

**ACKNOWLEDGMENTS**

The authors wish to thank Ronald Seikey for his assistance in the tumor promotion studies and Dr. A. K. Verma for his helpful suggestions.

**REFERENCES**

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