Prevention of Breast Cancer in the Rat with 9-cis-Retinoic Acid as a Single Agent and in Combination with Tamoxifen

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Abstract

We show that 9-cis-retinoic acid (9cRA) is a potent inhibitor of mammary carcinogenesis induced by N-nitroso-N-methylurea in Sprague-Dawley rats. Rats were first treated with a single dose of N-nitroso-N-methylurea (50 mg/kg body weight) and then fed non-toxic levels of 9cRA (120 or 60 mg/kg of diet). 9cRA was highly effective in reducing tumor incidence, average number of tumors per rat, and average tumor burden, as well as extending tumor latency. The combination of 9cRA with low levels of tamoxifen (TAM; fed at either 1.0 or 0.5 mg/kg of diet) was particularly effective; addition of 9cRA to a TAM regimen doubled the number of animals that were tumor-free at autopsy and significantly diminished tumor number and tumor burden. For suppression of carcinogenesis in vivo, 9cRA was much more potent than all-trans-retinoic acid, both as a single agent or in combination with TAM, although both retinoids had equivalent inhibitory effects on DNA synthesis in cultured human breast cancer cell lines. Both 9cRA and all-trans-retinoic acid induced the expression of the adhesion molecule, E-cadherin, in the SK-BR-3 cell line. We suggest that clinical evaluation of the combination of 9cRA and TAM, either for chemoprevention or for adjuvant therapy, should be considered.

Introduction

The current controversy over the safety and efficacy of the use of tamoxifen to prevent breast cancer in women at high risk emphasizes the need to develop new agents for suppression of carcinogenesis. Since retinoids are useful agents for chemoprevention of cancer (1–3), one candidate for this purpose is 9cRA2 or one of its analogues. The recent findings that 9cRA is a high-affinity ligand for both the RAR and RXR families of retinoid receptors (4, 5) and that RXRs form heterodimers with other members of the steroid receptor superfamily (6), focus attention on the critical role of 9cRA in retinoid physiology. To date, there have been no reports on the use of 9cRA to prevent breast cancer, either in experimental animals or women. We now show that 9cRA is highly effective for preventing mammary cancer in the standard rat model which uses a single dose of NMU as carcinogen. Furthermore, we show that 9cRA is superior to arRA for this purpose and that 9cRA enhances the chemopreventive activity of low doses of tamoxifen. Finally, we show that both 9cRA and arRA can increase expression of the adhesion molecule, E-cad, at cell-cell contact sites in breast cancer cells. Since loss of functional E-cad has been implicated in breast cancer progression (7–13), these results suggest that retinoids may be useful, not only in the prevention of breast cancer, but also in its treatment.

Materials and Methods

Mammary Carcinogenesis Studies. A standard protocol for induction of breast cancer in Sprague-Dawley rats was used (14, 15), with a single i.v. dose of NMU, 50 mg/kg body weight. All palpated tumors were confirmed at autopsy. Retinoids and TAM were incorporated into powdered lab chow as described previously (15) and fed ad libitum, beginning 1 week after injection with NMU. 9cRA (Kuraray Company, Osaka, Japan) or arRA (Hoffmann-La Roche, Nutley, NJ) were fed at either of 2 doses, 120 mg (high dose) or 60 mg (low dose)/kg of diet; TAM (Sigma Chemical, St. Louis, MO) was fed at either 1.0 mg (high dose) or 0.5 mg (low dose)/kg of diet. Methods for statistical analysis have been reported previously (15).

Cell Culture Studies and Immunohistochemistry of E-cad. Human breast cancer cells were cultured as described (15). To measure induction of E-cad, SK-BR-3 cells were treated with either 9cRA or arRA (10−7 M) for 48 h prior to fixation in cold methanol. Immunocytochemical staining of E-cad has been described previously (12) using an antibody (anti-GP-84) directed against the extracellular domain of E-cad.

Results

Inhibition of Mammary Carcinogenesis. In our laboratory, greater than 90% of Sprague-Dawley rats treated with NMU alone (50 mg/kg body weight) develop invasive mammary adenocarcinomas within 3 to 4 months of a single i.v. injection. We report here results obtained on a total of 252 rats treated with NMU, using either 9cRA or arRA alone or in combination with TAM, to inhibit the development of mammary carcinoma. We chose TAM because of the current ongoing clinical trials being conducted in thousands of women worldwide, evaluating the efficacy of this agent for prevention of breast cancer. At optimal doses, TAM is highly effective in the NMU rat model (16). To evaluate additive or synergistic effects of the combination of either 9cRA or arRA with TAM, we deliberately used suboptimal doses of TAM.

Two separate experiments were performed, and the results are shown in Table 1 and Fig. 1. In all cases, treatment with chemopreventive agents began 1 week after a single i.v. injection of NMU. The data indicate that 9cRA is highly effective in this animal model, and in particular, that it is markedly superior to arRA, both as a single agent and in combination with TAM. Chronic feeding of 9cRA caused significant suppression of carcinogenesis, as measured by four end points, i.e., tumor incidence, average number of tumors per rat, average tumor burden (the total weight of all of an animal’s tumors), and tumor latency. The effect of 9cRA on the number of rats that were tumor free at autopsy is particularly striking; in the two experiments (Table 1), only 1 of 48 (2%) of the control rats treated with NMU alone was tumor free, while 24 of 48 (50%; P < 0.001) of the rats treated with NMU plus 9cRA had no grossly detectable tumors. In addition, statistical analysis indicates that the effects of 9cRA on inhibition of tumor number and tumor burden are also highly significant (P < 0.001 at the higher dose of 9cRA and 0.002 or less at the lower dose of 9cRA). Fig. 1A also shows that 9cRA greatly increases...
tumor latency. In contrast, only 3 of 24 (13%) of the rats treated with 
9c-RA (Experiment 1, Table 1) were tumor free at autopsy, and 
9c-RA had no significant effect on tumor number and tumor burden or on 
tumor latency (Fig. 1C).

In addition to studying its efficacy as a single agent, we also 
determined whether 9c-RA would provide additional benefit when 
combined with TAM. Tables 1 and 2, as well as Fig. 1B, show that 
9c-RA adds a statistically significant benefit to a TAM regimen, using 
the same four end points that were evaluated for single agents. Pooling 
the results of Experiments 1 and 2 (as shown in Table 2) indicates that 
the higher dose of 9c-RA, when added to TAM, doubles the number of 
rats that are tumor free at autopsy (\( P = 0.009 \) for the comparison of 
the combination of 9c-RA plus TAM against TAM alone), decreases 
the average number of tumors/rat from 1.3 to 0.2 (\( P < 0.001 \)), and 
decreases the average tumor burden from 3.5 to 0.3 g (\( P = 0.001 \)), or 
the 75th percentile of the tumor burden distribution from 5.0 to 0.1 g 
(\( P = 0.001 \)). Smaller but highly significant effects are also seen for 
the addition of the lower dose of 9c-RA to the low dose of TAM. 
Finally, when all 72 animals treated with the combination of 9c-RA (at 
either dose) plus TAM (at either dose) are compared with all 36 
animals treated only with TAM alone (at either dose), the \( P \) values for 
the efficacy of adding 9c-RA to TAM are all 0.001 or less, whether one 
measures effects on inhibition of tumor incidence, inhibition of average 
number of tumors/rat, or on average tumor burden. None of the 
chemopreventive agents caused any grossly evident toxicity. Al-
though there was some slightly diminished weight gain in animals fed 
9c-RA, 
a9RA, or TAM, the average weights of rats on chemopreven-
tive regimens were always 85% or greater of those fed chow diets 
alone. Furthermore, since rats fed 
a9RA had equivalent growth curves, the 
beneficial effects of 9c-RA cannot be ascribed to effects on total 
caloric intake.

**Cell Culture Studies.** We analyzed several human breast cancer 
cell lines to study their response to 9c-RA compared to 
a9RA. As 
reported by others (17, 18, reviewed in 19), we found that 
a9RA inhibited \( ^{3}H \)thymidine incorporation in several differentiated (E-cad-positive) cell lines including MCF-7, T47D, and 
ZR-75B, while it 9c-RA AND TAMOXIFEN IN BREAST CANCER PREVENTION

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor-free ((P_{1}; P_{2}))</th>
<th>Avg. no. tumors ((P_{1}; P_{2}))</th>
<th>ATB ((P_{1}; P_{2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0/24</td>
<td>3.6</td>
<td>10.9</td>
</tr>
<tr>
<td>9c-RA, Hi</td>
<td>9/12 ((&lt;0.001))</td>
<td>0.3 ((&lt;0.001))</td>
<td>0.8 ((&lt;0.001))</td>
</tr>
<tr>
<td>9c-RA, Lo</td>
<td>5/12 ((0.002))</td>
<td>1.6 ((0.002))</td>
<td>4.1 ((0.002))</td>
</tr>
<tr>
<td>a9RA, Hi</td>
<td>2/12</td>
<td>2.8</td>
<td>11.2</td>
</tr>
<tr>
<td>a9RA, Lo</td>
<td>1/12</td>
<td>3.3</td>
<td>12.0</td>
</tr>
<tr>
<td>TAM, Lo</td>
<td>3/12 ((0.03))</td>
<td>1.8 ((0.002))</td>
<td>3.4 ((0.004))</td>
</tr>
<tr>
<td>9c-RA, Hi + TAM, Lo</td>
<td>8/12 ((&lt;0.001; 0.05))</td>
<td>0.3 ((&lt;0.001; 0.003))</td>
<td>0.5 ((&lt;0.001; 0.01))</td>
</tr>
<tr>
<td>9c-RA, Lo + TAM, Lo</td>
<td>8/12 ((&lt;0.001; 0.05))</td>
<td>0.8 ((&lt;0.001; 0.04))</td>
<td>2.1 ((&lt;0.001; 0.05))</td>
</tr>
<tr>
<td>a9RA, Hi + TAM, Lo</td>
<td>5/12 ((0.002; 0.33))</td>
<td>0.9 ((&lt;0.001; 0.05))</td>
<td>2.0 ((&lt;0.001; 0.20))</td>
</tr>
<tr>
<td>a9RA, Lo + TAM, Lo</td>
<td>6/12 ((&lt;0.001; 0.20))</td>
<td>1.2 ((&lt;0.001; 0.17))</td>
<td>1.3 ((&lt;0.001; 0.06))</td>
</tr>
</tbody>
</table>

**Table 1** Prevention of mammary cancer by 9c-RA, a9RA, TAM, and combinations thereof

\* ATB, average tumor burden; the average weight of a rat's tumors at autopsy. In both experiments, rank sum tests were used to compare tumor burdens. \( P_{1} \), value for the comparison of any chemopreventive treatment versus controls treated with NMU alone. \( P_{2} \), value for the comparison of any combined retinoid + TAM treatment versus TAM alone. Doses were as follows: 9c-RA or a9RA, Hi, 120 mg/kg diet; 9c-RA or a9RA, Lo, 60 mg/kg diet; TAM, Hi/Lo, 1.0/0.5 mg/kg diet. In Experiment 1, agents were fed for 3 months, and in Experiment 2, agents were fed for 4.5 months. All rats were injected with NMU 1 week before starting diets.

Discussion

We report here, for the first time, the use of 9c-RA for the preven-
tion of breast cancer in a widely used animal model. In this model, the 
9-cis-isomer of retinoic acid is clearly superior to the all-trans-isomer. 
The mechanism(s) for this difference need to be determined. Inas-
much as 9c-RA has high affinity for RXRs as well as RARs (4, 5), 
breeder receptor activation may contribute to the greater activity of 
9c-RA; studies of the extent of conversion of a9RA to 9c-RA in 
mammary epithelium in vivo thus need to be performed. However, 
we could find no significant difference between 9c-RA and a9RA in our 
cell culture studies. Another possibility is that there are significant 
pharmacokinetic differences between the two isomers, and in partic-
ular, that 9c-RA does not induce its own oxidative inactivation to 
the same extent as a9RA. The ability of a9RA to induce enzymes which 
lower its own concentration is well known (20, 21) and is presumed 
to be an important factor in the relapse which occurs during treatment 
of acute promyelocytic leukemia with a9RA (21). Thus, further studies 
on the comparative pharmacokinetics of 9c-RA and a9RA are critically 
needed.

In addition to showing its efficacy as a single agent, we have also 
found that 9c-RA markedly enhances the chemopreventive activity of 
TAM, particularly when TAM is used at very low doses. The highest
dose of TAM given to a rat in the present study is less than 20% of that given to a 60-kg woman receiving the standard 20 mg daily dose (for this calculation, we have assumed that the rat weighs 250 g and eats 10 g of food daily). There is concern at present regarding the safety of chronic administration of the standard 20 mg daily dose of TAM, which is widely used in the adjuvant setting and is currently under study in long-term human chemoprevention trials. The present data suggest that it may be possible to lower the dose of TAM and still retain efficacy if TAM were used in combination with a second agent, such as 9cRA. Synergistic interaction of TAM and another retinoid, 4-hydroxyphenyl retinamide, in another model of prevention of breast cancer has been shown previously (22), although the dose of the retinoid was very high and the benefit obtained was not as great as in this study. Furthermore, although we have reported previously that synthetic analogues of vitamin D (deltanoids) can synergize with TAM in the NMU breast cancer model (15), the suppressive effects of this combination were not as great as we have obtained here with 9cRA plus TAM. The present data emphasize the importance of obtaining new information about the safety of chronic administration of low doses of 9cRA to human populations at high risk for development of cancer so that it may be clinically evaluated for suppression of carcinogenesis. 9cRA is currently in Phase I human trials as a therapeutic agent. Most notably, the striking effects of the combination of two agents, both given at relatively low doses, on inhibition of carcinogenesis indicate that it will be increasingly important to consider the use of combination chemoprevention (23) in the clinical setting of cancer prevention. Although we have emphasized the use of 9cRA for chemoprevention in the present study, retinoids also inhibit the growth of many overtly malignant breast cancer cells (17–19). Taken together with the ability of 9cRA to increase expression of E-cad, an adhesion molecule often lost in undifferentiated breast carcinomas, the present study suggests testing the addition of 9cRA to the standard TAM regimen that is currently in widespread use for adjuvant chemotherapy for postsurgi
cal treatment of breast cancer in women, especially in a subset that is

W. K. Hoeg, personal communication.

Table 2 Benefit of addition of 9cRA to TAM in prevention of mammary cancer

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor-free (P)</th>
<th>Avg. no. (P)</th>
<th>Top quartile TB (g) (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAM, Lo</td>
<td>9/24</td>
<td>1.3</td>
<td>5.0 (0.001)</td>
</tr>
<tr>
<td>TAM, Lo + 9cRA, Hi</td>
<td>18/24 (0.009)</td>
<td>0.2 (0.001)</td>
<td>0.1 (0.001)</td>
</tr>
<tr>
<td>TAM, Lo + 9cRA, Lo</td>
<td>17/24 (0.021)</td>
<td>0.5 (0.001)</td>
<td>0.2 (0.023)</td>
</tr>
</tbody>
</table>

P, comparison of the effects of adding 9cRA to TAM when compared to TAM alone. Top quartile tumor burden is the 75th percentile of the tumor burden distribution for animals in each particular cohort. The value for the top quartile TB in the control group which received no chemopreventive agent was 17.1 g. Rank sum tests were used to compare tumor burdens. Doses of agents as in Table 1.
known to be at particularly high risk for relapse. The next problem in the laboratory will be to find a third, or possibly even a fourth, agent to add to this already promising combination regimen to approach the ultimate asymptote of total suppression of mammary carcinogenesis.

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References

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