Inhibition of Urinary Bladder Cancer by \( N \)-(Ethyl)-all-trans-retinamide and \( N \)-(2-Hydroxyethyl)-all-trans-retinamide in Rats and Mice

Henry J. Thompson,2 Peter J. Becci,3 Clinton J. Grubbs,4 Y. Fulmer Shealy, Edward J. Stanek, Charles C. Brown, Michael B. Sporn, and Richard C. Moon5


ABSTRACT

The chemopreventive activity of two synthetic retinamides of relatively low toxicity against \( N \)-butyl-\( N \)-(4-hydroxybutyl)nitrosamine (OH-BBN)-induced urinary bladder cancer was studied in F344 rats and C57BL/6 \( \times \) DBA/2 \( F_1 \) mice. Female and male rats were given a total dose of either 1800 or 3200 mg OH-BBN over a period of 6 or 8 weeks, respectively. Male mice were given a total dose of either 90 or 180 mg OH-BBN over a period of 9 weeks. Seven days after the final intubation of OH-BBN, animals were fed either a placebo diet or a diet supplemented with the following retinoids: for rats, 0.8 mmol \( N \)-(ethyl)-all-trans-retinamide, or 2 mmol \( N \)-(2-hydroxyethyl)-all-trans-retinamide per kg diet; and for mice, either 0.5 or 1.0 mmol \( N \)-(ethyl)-all-trans-retinamide or \( N \)-(2-hydroxyethyl)-all-trans-retinamide per kg diet. Animals were killed 6 months after the initial gastric intubation. In comparison to male and female rats fed placebo diets, all three retinamides reduced the incidence, number, and severity of the low-grade papillary transitional cell carcinomas of the urinary bladder. Similarly, treatment of mice with either of the two retinamides reduced the incidence of highly invasive urinary bladder carcinomas. The chemopreventive effect of the less toxic retinamides was equal to or greater than that of 13-cis-retinoic acid.

INTRODUCTION

Although it is possible to identify individuals who are at high risk to either recurrent bladder cancer or the induction of bladder neoplasms due to environmental or occupational exposure to potential carcinogens, no effective prophylactic therapy is available to the clinician. A new approach for the prevention of cancer is the use of pharmacological doses of nontoxic synthetic derivatives of vitamin A (retinoids) as agents which inhibit the development of epithelial neoplasms (1, 3–5, 8, 9, 14, 17–19). The synthetic retinoid, 13-cis-retinoic acid, has been shown to have a marked chemopreventive effect against chemically induced urinary bladder cancer in both rats and mice (1, 3, 4, 9, 17, 18). This retinoid has been reported to reduce the incidence, number, and severity of low-grade papillary and highly invasive sessile transitional cell carcinomas, as well as squamous cell carcinomas. The present study was undertaken to determine the chemopreventive effect of 2 new retinoids, \( N \)-(ethyl)-all-trans-retinamide and \( N \)-(2-hydroxyethyl)-all-trans-retinamide (Chart 1), against the induction of urinary bladder carcinogenesis by OH-BBN\(^6\) in F344 rats and C57BL/6 \( \times \) DBA/2 \( F_1 \) (hereafter called B6D2F\( _1 \)) mice. Like 13-cis-retinoic acid, these 2 new retinoids are significantly less toxic than the natural forms of vitamin A (10, 11).

MATERIALS AND METHODS

Male and female Fischer 344 rats (ARS/Sprague-Dawley Division, The Mogul Corp., Madison, Wis.) and male B6D2F\( _1 \) mice (Simonsen Laboratories, Inc., Gilroy, Calif.) were 6 to 7 weeks of age at the time of the first carcinogen intubation. Rats were housed 3/polycarbonate cage, and mice were housed 5/cage. Animals were held in an environment-controlled room illuminated 12 hr each day and maintained at 22 ± 1°. All animals received Wayne Laboratory Animal Chow 8604-00 (Allied Mills, Inc., Chicago, Ill.) and sterilized tap water ad libitum.

The retinamides were synthesized by modifying the method reported in the patent literature (6). All operations involved in the preparation, isolation, purification, and transfer of retinoids were performed in an atmosphere, or under a current, of dry nitrogen. All such operations were also performed in dim or photographic light, and, insofar as possible, with containers wrapped with aluminum foil or with black cloths.

For the synthesis of \( N \)-(ethyl)-all-trans-retinamide, a solution of all-trans-retinyl chloride was prepared by adding phosphorus trichloride (0.94 mol) in dry benzene (12 ml/g) to a suspension of all-trans-retinoic acid (1.33 mol) in dry benzene (10 ml/g). The solution was stirred for an hr after all of the retinoic acid had dissolved (about 3 hr at 25 to 30°), amorphous phosphorus compounds were allowed to settle, and the supernatant solution was decanted and added slowly to a cold (10 to 15°) solution of ethylamine (10 equivalents) in benzene (1.5 ml/ml of amine). The crude retinamide was isolated by dilution with water, evaporation of benzene in vacuo from the resulting mixture, and separation of the precipitated retinoid by filtration. The crude product was purified by recrystallization from aqueous methanol: typical yields, 80 to 85%; high-pressure liquid chromatographic analyses, 99.7 to 100%; m.p., 138 to 140°; UV absorption (absolute ethanol), \( \lambda_{\text{max}} = 347 \text{ nm} \) (\( \epsilon = 50,000 ± 400 \)).

\[
C_{19}H_{20}NO_3
\]

Calculated: C 80.68, H 10.16, N 4.28

Found: C 80.76, H 10.23, N 4.16

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from methanol: typical yields, 70 to 75%; high-pressure liquid chromatographic analyses, 99.5 to 100%; m.p., 140 to 141 °; UV absorption (absolute ethanol), λ_max = 347 nm (ε = 50,400 ± 500).

Male rats received 200 mg OH-BBN (synthesized by the Chemistry Research Division, IIT Research Institute, Chicago, Ill.) via gastric intubation 2 times each week for 8 weeks, for a total dose of 3200 mg OH-BBN. Female rats received 150 mg OH-BBN twice each week for 6 weeks, for a total dose of 1800 mg OH-BBN. Mice were given either 5 or 10 mg OH-BBN via gastric intubation 2 times each week for 9 weeks, for a total dose of 90 or 180 mg OH-BBN, respectively. OH-BBN was diluted with ethanol:water (20:80; v/v) so that each dose was contained in a volume of 0.5 ml for rats and 0.1 ml for mice. All control animals received by intubation either 0.5 or 0.1 ml of the ethanol:water solution. Seven days after the final gastric intubation of solvent or carcinogen, animals were randomized into groups and fed either a placebo diet or a diet supplemented with retinoid. Rats treated with retinoid were given 240 mg (0.8 mmol) 13-cis-retinoic acid, 654 mg (2 mmol) N-(ethyl)retinamide, or 686 mg (2 mmol) N-(2-hydroxyethyl)retinamide per kg of diet. Retinoid-treated mice were fed either 164 or 327 mg (0.5 or 1 mmol) N-(ethyl)retinamide or 172 or 343 mg (0.5 or 1 mmol) N-(2-hydroxyethyl)retinamide per kg of diet.

The retinoid 13-cis-retinoic acid was added to the chow diet in the form of stable gelatinized beadlets provided by Hoffmann-La Roche Inc., Nutley, N. J. The beadlet placebo diet was formulated by blending gelatinized beadlets containing no retinoid into the chow diet. Diets containing retinamides were made as follows. Either crystalline N-(ethyl)-all-trans-retinamide (0.5, 1.0, or 2.0 mmol) or N-(2-hydroxyethyl)-all-trans-retinamide (0.5, 1.0, or 2.0 mmol) was dissolved in 50 g ethanol: trioctanoin, (1:3; v/v) to which 0.5 ml each of antioxidants, Tenox 20 (Eastman Chemicals, Kingsport, Tenn.) and α-tocopherol, was added. The retinoid solution was then incorporated into 950 g of Wayne laboratory chow at the rate of 25 ml/min using a Patterson-Kelly liquid-solid blender. Batches of diet were made fresh each week and stored at −20 °; food cups were changed twice weekly. Analysis of samples of diet showed complete stability of the retinoid under these conditions. The oil placebo diet was formulated by blending all ingredients except retinoid into the chow diet.

Animals were weighed weekly and observed twice daily for external signs of retinoid toxicity. All animals were killed at 6 months after the first carcinogen intubation. At necropsy, the urinary bladders were distended with 4% formaldehyde:1% glutaraldehyde in a 176 mosm phosphate buffer (13), and a ligature was placed around the neck of each bladder to maintain proper distension. After fixation, each bladder was processed for histopathological evaluation as described previously (2, 3). The criteria used in the present study for scoring epithelial lesions have been reported elsewhere (1).

RESULTS

Rat Studies. All retinoids were found to be effective agents for the prevention of urinary bladder cancer induced in female and/or male F344 rats by the chemical carcinogen OH-BBN. The incidence, average number, and severity of the transitional cell carcinomas induced are shown in Tables 1 and 2. The

### Table 1

<table>
<thead>
<tr>
<th>Sex</th>
<th>Retinoid</th>
<th>No. of rats</th>
<th>No. of rats with transitional cell carcinoma</th>
<th>Av. no. of transitional cell carcinomas/rat</th>
<th>Mean atypia score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>Placebo</td>
<td>80</td>
<td>28 (35)</td>
<td>0.49</td>
<td>2.39</td>
</tr>
<tr>
<td>Female</td>
<td>13-cis-RA</td>
<td>40</td>
<td>7 (19)</td>
<td>0.20</td>
<td>1.93</td>
</tr>
<tr>
<td>Female</td>
<td>ER</td>
<td>40</td>
<td>5 (12)</td>
<td>0.13</td>
<td>1.73</td>
</tr>
<tr>
<td>Female</td>
<td>2-HER</td>
<td>40</td>
<td>5 (12)</td>
<td>0.13</td>
<td>2.05</td>
</tr>
<tr>
<td>Male</td>
<td>Placebo</td>
<td>60</td>
<td>55 (92)</td>
<td>2.25</td>
<td>3.50</td>
</tr>
<tr>
<td>Male</td>
<td>13-cis-RA</td>
<td>30</td>
<td>24 (80)</td>
<td>1.43</td>
<td>3.19</td>
</tr>
<tr>
<td>Male</td>
<td>ER</td>
<td>30</td>
<td>25 (83)</td>
<td>1.76</td>
<td>3.17</td>
</tr>
<tr>
<td>Male</td>
<td>2-HER</td>
<td>30</td>
<td>19 (63)</td>
<td>1.27</td>
<td>2.90</td>
</tr>
</tbody>
</table>

*Atypia was scored on a scale from 0 to 5 depending upon the presence of increased cellularity, hyperchromasia, prominent nucleoli, pleomorphism of size and shape, loss of cell polarity and orientation, loss of epithelial luminal surface differentiation, and presence of mitosis.

Table 2

<table>
<thead>
<tr>
<th>TCC with atypia score equal to or greater than</th>
<th>Placebo</th>
<th>13-cis-Retinoic acid</th>
<th>N-(EthenyL)retinamide</th>
<th>N-(2-Hydroxyethyl)retinamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female rats</td>
<td>5</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.14</td>
<td>0.10</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.45</td>
<td>0.20</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.49</td>
<td>0.20</td>
<td>0.13</td>
</tr>
<tr>
<td>Male rats</td>
<td>5</td>
<td>0.08</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.68</td>
<td>0.26</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.06</td>
<td>1.33</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.25</td>
<td>1.43</td>
<td>1.76</td>
</tr>
</tbody>
</table>

* TCC, transitional cell carcinoma.
* See Table 1, Footnote b.
* Significantly different from respective control; p < 0.05 (one-sided).
* Significantly different from respective control; p < 0.025 (one-sided).
* Significantly different from respective control; p < 0.01 (one-sided).

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**Sex**

- Female
- Male

**No. of rats**

- 80
- 40
- 40
- 40
- 60
- 30
- 30
- 30
- 30
- 28
- 7 (19)
- 5 (12)
- 5 (12)
- 55 (92)
- 24 (80)
- 25 (83)
- 19 (63)
- 0.49
- 0.14
- 0.45
- 0.49
- 0.08
- 0.68
- 2.06
- 2.25

**No. of rats with transitional cell carcinoma**

- 28 (35)
- 7 (19)
- 5 (12)
- 5 (12)
- 55 (92)
- 24 (80)
- 25 (83)
- 19 (63)

**Av. no. of transitional cell carcinomas/rat**

- 0.49
- 0.20
- 0.13
- 0.13
- 2.25

**Mean atypia score**

- 2.39
- 1.93
- 1.73
- 2.05
- 3.50
- 3.19
- 3.17
- 2.90

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statistical procedures used to compare the differences between treatment groups were the \( \chi^2 \) procedure for testing the equality of binomial proportions and a nonparametric procedure proposed by Mantel for testing the equality of average number of tumors and the average atypia scores (7, 12).

For female animals treated with carcinogen, the animals not treated with retinoid had a urinary bladder cancer incidence of 35% with an average of 0.49 cancers per animal at the termination of the study. These figures were significantly reduced in each group fed retinoids (Table 1). Furthermore, all retinoids reduced the severity of the transitional cell carcinomas and the average atypia scores (7, 12).

The data obtained at both dose levels of carcinogen for each dietary treatment group were analyzed for statistical homogeneity. No statistically significant differences were found, and the results were thus combined. The combined data are shown in Table 4. The results were statistically analyzed for the one-sided hypothesis that a decrease in tumor incidence occurred in the retinoid-treated animals by a \( \chi^2 \) test (7). Animals treated with carcinogen and fed a placebo diet had a 37% incidence of urinary bladder cancer at the time of the study. Both the low- and high-dose levels of all retinoids reduced the incidence of the highly invasive carcinomas in comparison to animals fed placebo. However, only at the high level of N-(ethyl)retinamide and N-(2-hydroxyethyl)retinamide was a statistically significant reduction in tumor incidence observed, 37 to 21 and 22%, respectively (\( p < 0.025 \)).

Table 5 shows the group mean body weights of mice at the
dose of OH-BBN to male B6D2F1 mice induced a similar incidence of urinary bladder carcinoma, 36 and 38%, respectively. As reported elsewhere (4), the carcinomas induced were similar to the highly invasive variant of transitional cell urinary bladder carcinoma which occurs in humans. The carcinomas which were induced had areas of transitional, squamous, and glandular differentiation, and the majority of them invaded either into or through the urinary bladder muscle wall. Three of the 704 animals which received OH-BBN were observed to have transitional cell carcinomas of the ureter; 2 additional animals had hemangiosarcomas of the urinary bladder. Bladder stones were observed in about 10% of the mice. No tumors were observed in solvent-treated animals.

<table>
<thead>
<tr>
<th>Retinoid Treatment</th>
<th>Placebo</th>
<th>13-cis-RA</th>
<th>ER</th>
<th>2-HER</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>36.1 ± 0.3 (102)</td>
<td>36.0 ± 0.4 (102)</td>
<td>36.7 ± 0.4 (102)</td>
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<td></td>
<td></td>
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<td>36.3 ± 0.8 (100)</td>
</tr>
</tbody>
</table>

\( ^a \) Animals were given no carcinogen or a total dose of either 1800 mg OH-BBN for females or 3200 mg OH-BBN for males starting at 6 to 7 weeks of age as described under "Materials and Methods."

\( ^b \) Mean ± S.E.

\( ^c \) Numbers in parentheses, percentage comparison of a particular group with the respective control group that received no retinoid.

\( ^d \) 13-cis-RA, 13-cis-retinoic acid; ER, N-(ethyl)retinamide; 2-HER, N-(2-hydroxyethyl)retinamide.

\( ^{a,b} \) Mean ± S.E.

\( ^c \) ER, N-(ethyl)retinamide; 2-HER, N-(2-hydroxyethyl)retinamide.

\( ^d \) Low dose, 0.5 mmol/kg diet.

\( ^e \) High dose, 1.0 mmol/kg diet.

Table 4: Effect of retinoids on urinary bladder cancer incidence in mice

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<thead>
<tr>
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<td>36.3 ± 0.8 (100)</td>
</tr>
</tbody>
</table>

\( ^a \) Number of mice with carcinoma

\( ^{a,b} \) Significantly different from respective control; \( p < 0.025 \).

Table 5: Effect of retinoids on urinary bladder cancer incidence in mice

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<thead>
<tr>
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<th>13-cis-RA</th>
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<th>2-HER</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt (g)</td>
<td></td>
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<td>36.3 ± 0.8 (100)</td>
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<td>36.3 ± 0.8 (100)</td>
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</tbody>
</table>
termination of the study. Feeding of either N-(ethyl)retinamide or N-(2-hydroxyethyl)retinamide did not cause any significant failure to grow; mice fed these retinoids had final weights which were 100 to 106% of those mice fed no retinoid. Testicular and liver morphology of animals fed retinoids (but not OH-BBN) was essentially normal.

DISCUSSION

It has been suggested that synthetic modification of the terminal-end group of the retinoid molecule could alter significantly the toxicological properties of these compounds without loss of biological activity (16). We report here for the first time the use of amide derivatives of all-trans-retinoic acid for prevention of bladder cancer; in these compounds, the polar nature of the terminal carboxyl groups of retinoic acid has been depressed significantly.

N-(Ethyl)retinamide and N-(2-hydroxyethyl)retinamide had significant inhibitory activity against the induction of both low-grade papillary transitional cell carcinomas of the urinary bladder in rats and highly invasive urinary bladder carcinomas in mice. The degree of chemopreventive activity which was exerted by the retinamides was either equal to or greater than that of 13-cis-retinoic acid observed in this and previous investigations (1, 3, 4, 9, 17, 18). In terms of the toxicological properties of these compounds, it has been reported by others (11) that, when fed to mice, both retinamides are significantly less toxic than all-trans-retinoic acid. Furthermore, N-(ethyl)retinamide was found to be significantly less toxic than all-trans-retinoic acid when administered to human patients in Phase I studies (15). The body weight data and the results of morphological evaluation of livers of retinoid-treated rats and mice obtained in this investigation are in agreement with those studies (11, 15). It was possible to feed 20 times more of either N-(ethyl)retinamide or N-(2-hydroxyethyl)retinamide in the diet of animals than the established vitamin A requirement for the rat without any external signs of toxicity or marked reduction in the rate of body weight gain. While greater quantities of both retinamides are better tolerated than 13-cis-retinoic acid by the rat and mouse, the chemopreventive effect of both retinoids against urinary bladder cancer was found to be approximately equal to that of 13-cis-retinoic acid. The chemopreventive effect of 13-cis-retinoic acid against urinary bladder cancer has been well documented (1, 3, 4, 9, 17, 18), and until now, it was the most effective and least toxic retinoid which was available for urinary bladder cancer prevention. The fact that both retinamides reduced cancer incidence, number, and/or severity suggests that amide derivatives of all-trans-retinoic acid may provide chemopreventive agents with suitable pharmacological properties for the investigation of the prevention of human urinary bladder cancer.

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Inhibition of Urinary Bladder Cancer by $N$-(Ethyl)-all-$trans$-retinamide and $N$-(2-Hydroxyethyl)-all-$trans$-retinamide in Rats and Mice

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