Effects of Dietary N-(4-Hydroxyphenyl)retinamide on
N-Nitrosomethylbenzylamine Metabolism and Esophageal
Tumorigenesis in the Fischer 344 Rat

Ashok Gupta, Ron Nines, Kapila A. Rodrigo, Robeena A. Aziz, Peter S. Carlton,
Deborah L. Gray, Vernon E. Steele, Mark A. Morse, Gary D. Stoner

Background: 9-cis-Retinoic acid (9-cis-RA) and N-(4-hydroxyphenyl)retinamide (4-HPR) are effective chemopreventive agents against epithelial tumors in the oral cavity, breast, and prostate. We tested the inhibitory activity of these retinoids against N-nitrosomethylbenzylamine (NMBA)-induced tumorigenesis in the rat esophagus. Methods: Male Fischer 344 rats were randomly assigned to receive diets either lacking or containing 9-cis-RA or 4-HPR for 1 week before tumor initiation with NMBA and then for the duration of the study. NMBA metabolism, O6-methylguanine adduct formation, and cytochrome P450 messenger RNA (mRNA) expression in the esophagi of the rats were studied to investigate the mechanisms by which dietary 4-HPR affects tumorigenesis. All statistical tests were two-sided. Results: Dietary 4-HPR resulted in a dose-dependent and statistically significant enhancement (P < 0.05) of tumorigenesis in response to NMBA. In two different tumor bioassays, the mean tumor multiplicity for rats fed the highest concentration of dietary 4-HPR (0.8 g/kg diet) was increased by 5.9 tumors (95% confidence interval [CI] = 1.7 to 10.1 tumors) and 6.7 tumors (95% CI = 5.6 to 7.8 tumors) compared with the mean tumor multiplicity for rats that received the control diet lacking 4-HPR. Animals fed diets containing 9-cis-RA displayed no statistically significant increase in tumorigenesis. Compared with animals fed a diet lacking 4-HPR, animals fed 4-HPR had increased NMBA metabolism in esophageal explant cultures and had higher levels of O6-methylguanine DNA adducts and CYP2A3 mRNA in their esophagi. Conclusions: Dietary 4-HPR enhances tumorigenesis in response to NMBA in the rat esophagus by increasing tumor initiation events. Dietary 4-HPR may exert paradoxical effects at some sites, such as the aerodigestive tract, by modulating the bioactivation of carcinogens in target tissues. [J Natl Cancer Inst 2001;93:990–8]
Investigations into the molecular mechanisms of retinoid action in aerodigestive cancers (24) have shown that these agents affect cell growth and apoptosis, cell differentiation, and gene expression. Such studies have also led to the proposal that aerodigestive cancers result from a field cancerization process, whereby the whole tissue field, including the oral cavity, lung, and esophagus, is at risk for tumor development because it is exposed to similar extrinsic and/or intrinsic etiologic factors (24). Therefore, it is conceivable that retinoids, which are effective inhibitors of carcinogenesis in the oral cavity and lung, may also inhibit the development of esophageal cancers.

The role of retinoids as potential chemoprevention agents in esophageal cancers has not been fully studied. We have evaluated the efficacy of two retinoids, 9-cis-retinoic acid (9-cis-RA), a naturally occurring retinoid, and 4-HPR, a synthetic amide of all-trans-RA, as inhibitors of NMBA-induced esophageal tumorigenesis in the Fischer 344 rat.

**Materials and Methods**

**Animals**

Male Fischer 344 rats (5–6 weeks old) were purchased from Harlan Sprague-Dawley (Indianapolis, IN). They were housed three per cage at 20°C ± 2°C ambient temperature, at 50% ± 10% relative humidity, and in a 12-hour light/dark cycle. Because retinoids are light sensitive, yellow lights were used in the rooms where the animals were housed for the duration of the experiments. Cages were changed twice a week to provide hygienic conditions. The rats were maintained on a modified AIN (American Institute of Nutrition)-76A diet containing 20% casein, 0.3% Na-methionine, 52% cornstarch, 13% dextrose, 5% cellulose, 3.5% AI salt mixture, 1% AI vitamin mixture, and 0.2% choline bitartrate (Dyets Inc., Bethlehem, PA). Neobee M-5 oil (Stepan Co., Northfield, IL) was added at 5% (wt/wt) to the AIN-76A diet by mixing in a Hobart mixer (Hobart Corp., Grove City, OH) for 20–30 minutes. Food and water were provided ad libitum. Animal care was in accordance with the guidelines of the Animal Care and Use Committee of The Ohio State University, Columbus. Measurements of body weight and food consumption were recorded weekly for the duration of the studies.

**Chemicals**

NMBA was obtained from Ash Stevens (Detroit, MI) and was greater than 98% pure when analyzed by reverse-phase high-performance liquid chromatography (HPLC). Tritiated NMBA [3H]NMBA, provided by Dr. Lisa A. Peterson (University of Minnesota Cancer Center, Minneapolis), was greater than 97% pure when analyzed by HPLC and had a specific activity of 2.477 Ci/mmol. 4-HPR and 9-cis-RA were obtained from the Division of Cancer Prevention and Control Repository, National Cancer Institute (Rockville, MD). HPLC-grade acetonitrile and other laboratory reagents were purchased from Fisher Scientific (Pittsburgh, PA).

**Diet Preparation**

Retinoid-containing diets were freshly prepared every 2 weeks. The unused portions of the diets were stored in black bags at −20°C and were discarded when fresh diets were prepared. 9-cis-RA and 4-HPR were weighed in the dark on an analytic balance to provide the quantities required for the preparation of the various diets. The low- and high-9-cis-RA diets contained 0.06 g and 0.12 g 9-cis-RA/kg AIN-76A diet, respectively. The diets with the low and high concentrations of 4-HPR contained 0.4 g and 0.8 g 4-HPR/kg AIN-76A, respectively. The required amount of 9-cis-RA for each diet was dissolved initially in 5 mL of ethanol, and that solution was mixed with the Neobee M-5 oil portion of the diet. That mixture was then added to the AIN-76A diet by mixing in a Hobart mixer for 20–30 minutes. 4-HPR was added as a dry ingredient to the AIN-76A diet. The diet in each cage was replaced three times during each 2-week period, on Monday and Friday of the first week and on Wednesday of the second week. Previous analyses in our laboratory, as well as other published reports (25, 26), have found that 9-cis-RA and 4-HPR are stable in the diet when they are stored and dispensed in this manner.

**Rat Esophageal Tumor Bioassays**

We performed two bioassays to evaluate the efficacy of retinoids as inhibitors of NMBA-induced esophageal tumorigenesis. In the first bioassay, groups of six rats each were randomly assigned to receive either the modified AIN-76A diet or the modified AIN-76A diet containing either low or high concentrations of 9-cis-RA or 4-HPR. One group of rats, which was randomly assigned to receive the modified AIN-76A diet and NMBA injections, contained three additional animals. One week after the initiation of the respective diets, one group of rats on each type of diet began to receive NMBA (0.5 mg/kg body weight) in 20% dimethyl sulfoxide (DMSO) or 20% DMSO only (vehicle) by subcutaneous injection thrice weekly for 5 weeks. At 15 weeks after the NMBA injections were initiated, the animals were killed by CO2 asphyxiation and subjected to gross necropsy. Their esophagus were removed, opened longitudinally, and examined for the presence of tumors. Tumors measuring greater than or equal to 0.5 mm in length, width, and depth were counted and recorded in a blinded fashion.

In the second bioassay, only the effects of 4-HPR were evaluated. Groups of 25 rats were randomly assigned to receive either the modified AIN-76A diet or the modified AIN-76A diet containing either low or high concentrations of 4-HPR. Two groups of rats, one on the modified AIN-76A diet and the other on the modified AIN-76A diet containing the high concentration of 4-HPR, had three additional animals. One week after the initiation of the respective diets, one group of rats on each type of diet began to receive NMBA (0.5 mg/kg body weight in 20% DMSO or 20% DMSO only (vehicle) by subcutaneous injection thrice weekly for 5 weeks. At 15 weeks after the NMBA injections were initiated, three rats each from the NMBA-alone and NMBA-plus-0.8-g/kg 4-HPR diet groups were killed to provide a preliminary assessment of tumor induction in response to NMBA. The bioassay was terminated at 20 weeks after initiation of NMBA or vehicle injections. At that time, the esophagus from each animal was removed and examined for tumors. Tumors were measured, counted, and recorded as described for the first bioassay.

**In Vitro Assay for NMBA Metabolism**

Groups of three animals were randomly assigned to receive either the modified AIN-76A diet or the modified AIN-76A diet containing 9-cis-RA or 4-HPR at low or high concentrations. The animals received their respective diets for 1 week, after which they were killed and their esophagi were aseptically removed and immersed in Leibovitz’s L-15 medium (Life Technologies, Inc. [GIBO BRL], Rockville, MD). Individual esophagi were opened longitudinally under sterile conditions, divided into halves, and placed mucosal side up in 60-mm tissue culture dishes containing 3 mL of PFMR-4 culture medium (BRFF, Ijamsville, MD) supplemented with epidermal growth factor, hydrocortisone, insulin (Sigma Chemical Co., St. Louis, MO), dialyzed fetal bovine serum (Life Technologies, Inc.), and 1 μM [3H]NMBA. These explants were then placed in an incubation chamber under 50% oxygen and maintained at 37°C with a gentle rocking motion to bathe the cultures in media (15). After 4 hours of incubation, 250-μL aliquots of media were collected from each explant culture, filtered through Acrodiscs (0.2-μM pore size; Gelman Sciences, Ann Arbor, MI), and analyzed by reverse-phase HPLC. The HPLC system used for these analyses consisted of a gradient controller, two 510 pumps, a C18 column, a 484 UV detector, an Eppendorf column heater (all from Millipore Corp., Bedford, MA), and a β-Ram radioflow detector (INUS Systems Inc., Tampa, FL). Fifty microliters of each sample of filtered medium was analyzed, along with authentic standards for NMBA, benzyl alcohol, benzaldehyde, and benzoic acid. Samples were eluted with 2.4% acetic acid (pH 3.7) in 20% acetonitrile. The eluent flow rate was 1.5 mL/minute. The scintillation cocktail flow rate through the radioflow detector was 3.0 mL/minute. Detection of eluates was by UV absorption at 254 nm and flow-through radioactivity. Major peaks of radioactivity were tagged, and the area under each peak was expressed as a percent of total radioactivity in the 50-μL sample (27). The results from three independent explant culture experiments were averaged.

**DNA Adduct Study**

To evaluate the effects of dietary administration of 4-HPR on NMBA-induced formation of O’-methylguanine (O’-meGu) adducts in the rat esophagus, we randomly assigned groups of 18 animals each to receive either the modified AIN-76A diet or the modified AIN-76A diet containing 9-cis-RA or 4-HPR. One group of rats, which was randomly assigned to receive either the modified AIN-76A diet or the modified AIN-76A diet containing either low or high concentrations of 4-HPR. Two groups of rats, one on the modified AIN-76A diet and the other on the modified AIN-76A diet containing the high concentration of 4-HPR, had three additional animals. One week after the initiation of the respective diets, one group of rats on each type of diet began to receive NMBA (0.5 mg/kg body weight in 20% DMSO or 20% DMSO only (vehicle) by subcutaneous injection thrice weekly for 5 weeks. At 15 weeks after the NMBA injections were initiated, three rats each from the NMBA-alone and NMBA-plus-0.8-g/kg 4-HPR diet groups were killed to provide a preliminary assessment of tumor induction in response to NMBA. The bioassay was terminated at 20 weeks after initiation of NMBA or vehicle injections. At that time, the esophagus from each animal was removed and examined for tumors. Tumors were measured, counted, and recorded as described for the first bioassay.

**Journal of the National Cancer Institute, Vol. 93, No. 13, July 4, 2001 ARTICLES 991**
NMBA, at 0.5 mg/kg body weight, in 20% DMSO, which was administered by subcutaneous injection 1 week after initiation of the respective diets. Animals in the vehicle control group were fed the modified AIN-76A diet and received a single injection of 20% DMSO. Nine animals from each group were killed by CO2 asphyxiation at both 24 and 48 hours after injection. The esophagus from each animal was excised and opened longitudinally, and the epithelium was separated from the underlying tissues and immediately frozen in liquid nitrogen and stored at –80 °C. Three randomly selected esophageal mucosae from each group of rats were pooled to yield a single sample for the analysis of O6-meGua adducts. Thus, for each treatment group, three samples were tested for each of the two time points. Genomic DNA was isolated from each sample according to protocols published previously (28,29). Seventy-five micrograms of purified DNA was subjected to acid hydrolysis at 80 °C for 45 minutes and analyzed by strong cation exchange HPLC coupled with fluorescence detection as described previously to detect O6-meGua (30). Levels of O6-meGua were normalized to the overall guanine content of each sample.

**Cytochrome P450 Messenger RNA Expression Study**

Groups of four animals each were randomly assigned to receive either the modified AIN-76A diet or the modified AIN-76A diet containing either low or high 4-HPR concentrations. The animals were killed by CO2 asphyxiation after 1 week on their respective diets. The esophagus was removed from each animal, and the mucosa was stripped and frozen immediately in liquid nitrogen. Total RNA was extracted from each esophageal mucosa with the use of the TRIzol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. We confirmed the integrity of the RNA samples by resolving 1μg of total RNA on an agarose gel and visualizing the 18S and 28S ribosomal RNA bands after staining with ethidium bromide. We confirmed the integrity of the RNA samples by resolving 1μg of total RNA on an agarose gel and visualizing the 18S and 28S ribosomal RNA bands after staining with ethidium bromide. Total RNA was extracted from each esophageal mucosa with the use of the TRIzol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. We confirmed the integrity of the RNA samples by resolving 1μg of total RNA on an agarose gel and visualizing the 18S and 28S ribosomal RNA bands after staining with ethidium bromide. Total RNA was extracted from each esophageal mucosa with the use of the TRIzol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. We confirmed the integrity of the RNA samples by resolving 1μg of total RNA on an agarose gel and visualizing the 18S and 28S ribosomal RNA bands after staining with ethidium bromide.

**Statistical Analysis**

Data were analyzed for statistical significance, defined as α < .05, with the use of analysis of variance (ANOVA) followed by the Newman–Keuls multiple-comparisons test. Whenever the group means were statistically significantly different, 95% confidence intervals (CIs) were constructed for the difference between the means. It was assumed that the variance of two groups was unequal. Satterthwaite’s method was used for the calculation of the degrees of freedom for t distribution. Since the tumor sizes were not normally distributed, Kruskal–Wallis one-way ANOVA on ranks test followed by Kruskal–Wallis multiple-comparisons Z-value test were used for their analyses. Differences in the number of tumors larger than 0.5 mm in all dimensions had binomial distribution with variance (1 – p). Since the sample size, n, was large enough to satisfy np(1 – np) > 10, normal approximation was used for the calculation of 95% CIs. All P values were two-sided.

**RESULTS**

**Tumor Bioassays**

In the first bioassay, we evaluated the chemopreventive activities of the natural retinoid, 9-cis-RA, and the synthetic amide of all-trans-RA, 4-HPR, against NMBA-induced tumorigenesis in the rat esophagus. These two retinoids were chosen because they have been effective chemopreventive agents against other epithelial tumors, such as those arising in the mammary gland, prostate, oral cavity, and skin (32). The esophageal tumor incidence and multiplicity data from this bioassay are summarized in Table 1. Dietary administration of 9-cis-RA or 4-HPR did not adversely affect the survival, growth, or food consumption of any of the rats during the 15 weeks of the study (data not shown). Rats that were given injections of vehicle only while on the control diet (group 1) or the test diets containing 9-cis-RA (groups 2 and 3) or 4-HPR (groups 4 and 5) developed no esophageal tumors (Table 1). In contrast, rats that were given injections of NMBA while on the control diet (group 6) had a mean tumor multiplicity of 4.1 tumors (95% CI = 2.9 to 5.3 tumors) per rat. Rats that were given injections of NMBA while on the diets containing low concentrations (group 7) or high concentrations (group 8) of 9-cis-RA did not have statistically significantly different tumor multiplicities compared with rats that were given injections of NMBA and fed the control diet.

However, rats that were given injections of NMBA while on diets containing either low (group 9) or high (group 10) 4-HPR concentrations had statistically significant increases (P<.05 for each; difference between group 9 and group 6 = 4.1 tumors [95% CI = 0.5 to 7.7 tumors]; difference between group 10 and group 6 = 5.9 tumors [95% CI = 1.7 to 10.1 tumors]) in their tumor multiplicities compared with rats that were given injections of NMBA and fed the control diet. This unexpected increase in tumor multiplicity was greater than twofold for rats that received the diet containing the highest concentration of 4-HPR (10.0 tumors [95% CI = 6.8 to 13.2 tumors]) compared with rats that received the control diet (4.1 tumors [95% CI = 2.9 to 5.3 tumors]). We did not observe a dose-dependent effect of the chemopreventive agents on tumor development.
response for this enhancement, although there was a trend toward increased tumor multiplicity with higher concentrations of 4-HPR in the diet.

On the basis of these preliminary observations, we performed a second bioassay to further evaluate the effects of 4-HPR on NMBA-induced esophageal tumorigenesis. For this bioassay, 15 animals were randomly assigned to receive vehicle injections while on the control diet (group 1), and 25 animals were randomly assigned to each of the remaining treatment groups consisting of vehicle or NMBA injections and control or test diets (groups 2–6). Six additional animals were randomly assigned to receive NMBA injections; three of those animals were fed the control diet (group 4), and the other three were fed a diet containing the high concentration of 4-HPR (group 6). Those six animals were killed at 15 weeks after the initiation of NMBA injections, and their esophagi were examined to provide a preliminary assessment of tumor response. Whereas the three animals that received dietary 4-HPR had multiple measurable tumors in their esophagi, the three animals that received the control diet had very small esophageal nodules, most of which were less than 0.5 mm in all three dimensions.

Because the tumors in the NMBA-alone group were too small to measure at this point, we decided to maintain the remaining animals on their respective treatments for an additional 5 weeks, thereby continuing the study for a total of 20 weeks after the initiation of NMBA or vehicle injections. As we observed in the first bioassay, dietary 4-HPR had no adverse impact on the growth or on the food consumption of animals in any of the treatment groups studied in the second bioassay (data not shown). Tumor incidence and multiplicity data from this bioassay, which are summarized in Table 2, exclude data from the six animals killed at 15 weeks. Animals that received NMBA injections while on diets containing low (group 5) or high (group 6) concentrations of 4-HPR had statistically significant increases (P < .05 for each; difference between group 5 and group 4 = 4.7 tumors [95% CI = 4.0 to 5.4 tumors]; difference between group 6 and group 4 = 6.7 tumors [95% CI = 5.6 to 7.8 tumors]) in their tumor multiplicity compared with rats that were given NMBA injections and fed the control diet (group 4). This increase in tumor multiplicity following 4-HPR administration was dose dependent and was greater than 3.5-fold for rats that received the diet containing high concentrations of 4-HPR (0.8 g/kg) (9.2 tumors [95% CI = 8.2 to 10.2 tumors]) compared with rats that received the control diet lacking 4-HPR (2.5 tumors [95% CI = 2.1 to 2.9 tumors]).

In the second bioassay, we also evaluated the effects of dietary 4-HPR on the average tumor size among animals that received NMBA injections (Table 3). We observed a wide variation in individual tumor size within a treatment group and did not observe an association among tumor sizes within an animal. We, therefore, used tumor as the unit of analysis for these data. A total of 63 tumors with an average size of 6.86 mm³ (95% CI = 4.8 to 8.82 mm³) were recorded and measured in the esophagi of animals that were given injections of NMBA while on the control diet (group 4). By comparison, animals that were given injections of NMBA and fed diets containing low and high concentrations of 4-HPR (groups 5 and 6, respectively) had statistically significantly larger (P < .001 for each; difference between group 5 and group 4 = 3.42 mm³ [95% CI = 0.97 to 5.87 mm³]; difference between group 6 and group 4 = 3.39 mm³ [95% CI = 1.11 to 5.67 mm³]) average tumor sizes. The percentages of tumors that measured larger than 0.5 mm in all dimensions were 85.6% (95% CI = 84.1% to 87.0%) and 87.3% (95% CI = 86.0% to 88.7%), respectively, for animals fed diets containing the low (group 5) and high (group 6) concentrations of 4-HPR. These percentages were statistically significantly increased (P < .001 for each; difference between group 5 and group 4 = 26.9% [95% CI = 22.1% to 31.6%]; difference between group 6 and group 4 = 28.6% [95% CI = 24.0% to 33.3%]) compared with the percentage of tumors that measured larger than 0.5 mm in all dimensions in animals that received NMBA while on the control diet (group 4). Thus, compared with rats fed a control diet, rats exposed to dietary 4-HPR before, during, and after injection of NMBA had statistically significant increases in both the number and the size of tumors in their esophagi.

**N MBA Metabolism Study**

We next investigated the potential mechanisms through which dietary 4-HPR enhanced NMBA-induced tumorigenesis in the rat esophagus. Because the rats in the two bioassays were exposed to dietary 4-HPR for the entire study period, we hypothesized that 4-HPR could increase the percentage of esophageal tumors that were sufficiently large to be measured with our equipment. We, therefore, used tumor as the unit of analysis for these data. A total of 63 tumors with an average size of 6.86 mm³ (95% CI = 4.8 to 8.82 mm³) were recorded and measured in the esophagi of animals that were given injections of NMBA while on the control diet (group 4). By comparison, animals that were given injections of NMBA and fed diets containing low and high concentrations of 4-HPR (groups 5 and 6, respectively) had statistically significantly larger (P < .001 for each; difference between group 5 and group 4 = 3.42 mm³ [95% CI = 0.97 to 5.87 mm³]; difference between group 6 and group 4 = 3.39 mm³ [95% CI = 1.11 to 5.67 mm³]) average tumor sizes. The percentages of tumors that measured larger than 0.5 mm in all dimensions were 85.6% (95% CI = 84.1% to 87.0%) and 87.3% (95% CI = 86.0% to 88.7%), respectively, for animals fed diets containing the low (group 5) and high (group 6) concentrations of 4-HPR. These percentages were statistically significantly increased (P < .001 for each; difference between group 5 and group 4 = 26.9% [95% CI = 22.1% to 31.6%]; difference between group 6 and group 4 = 28.6% [95% CI = 24.0% to 33.3%]) compared with the percentage of tumors that measured larger than 0.5 mm in all dimensions in animals that received NMBA while on the control diet (group 4). Thus, compared with rats fed a control diet, rats exposed to dietary 4-HPR before, during, and after injection of NMBA had statistically significant increases in both the number and the size of tumors in their esophagi.

**Table 3. Effects of 4-HPR on tumor size***

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment, injection: diet</th>
<th>Mean tumor size, mm³ ‡ (95% CI)</th>
<th>Proportion of tumors larger than 0.5 mm in each dimension § (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>NMBA: control</td>
<td>6.86 (4.8 to 8.82)</td>
<td>37/63 = 58.7%</td>
</tr>
<tr>
<td>5</td>
<td>NMBA: 0.4 g/kg 4-HPR</td>
<td>10.28 (8.93 to 11.63)‡</td>
<td>56.8% to 60.7%</td>
</tr>
<tr>
<td>6</td>
<td>NMBA: 0.8 g/kg 4-HPR</td>
<td>10.25 (9.0 to 11.26)§</td>
<td>84.1% to 87.0%</td>
</tr>
</tbody>
</table>

*4-HPR = N-(4-hydroxyphenyl)retinamide; NMBA = N-nitrosomethylbenzylamine; CI = confidence interval.

†Values for mean tumor size were analyzed by Kruskal–Wallis one-way analysis of variance on ranks test followed by Kruskal–Wallis multiple-comparisons Z-value test to determine statistical significance.

‡Values in this column were analyzed by the chi-squared test to determine statistical significance.

§Values in this column were analyzed by the chi-squared test to determine statistical significance.
pothesized that a component of the enhanced tumorigenicity might reside in the initiation stage of esophageal tumorigenesis. For example, 4-HPR might enhance NMBA metabolism in esophageal tissues, potentially increasing the number of initiated cells and, therefore, contributing to enhanced tumor multiplicity in animals fed 4-HPR.

Using [3H]NMBA, we therefore compared the abilities of esophageal explants derived from animals fed the control diet or the test diets containing 4-HPR to metabolize NMBA in vitro. We analyzed [3H]NMBA metabolism in the explants after 4 hours of incubation because we observed in preliminary experiments that approximately 50% of [3H]NMBA was metabolized in explants from animals fed the control diet at that time point and that adequate levels of NMBA metabolites were detected in the aliquots of media collected from these explants (Table 4). Under the incubation conditions used in our experiment, [3H]NMBA remained stable for up to 24 hours in the culture media in the absence of esophageal tissues (data not shown).

Using HPLC, we detected three major unidentified peaks of radioactivity (i.e., metabolite peaks 1, 2, and 3) in media aliquots collected from all explants after 4 hours of incubation with [3H]NMBA. We also detected radioactive peaks that co-eluted with two known metabolites of NMBA, benzyl alcohol and benzoic acid, as well as unmetabolized NMBA, as was expected on the basis of a previous report (28). The amount of peak 1 in media aliquots collected from esophageal explants from animals fed diets containing either low (group 2) or high (group 3) concentrations of 4-HPR was statistically significantly increased (P<.05 for each; difference between group 2 and group 1 = 4.9% [95% CI = 1.6% to 8.2%]; difference between group 3 and group 1 = 7.3% [95% CI = 4.4% to 10.2%]) compared with the amount of peak 1 in media aliquots from explants established from animals fed the control diet (group 1). A statistically significant increase in the amount of peak 2 was also seen in the animals fed the diets containing low (group 2) and high (group 3) concentrations of 4-HPR (P<.05 for each; difference between group 2 and group 1 = 5.9% [95% CI = 3.7% to 8.1%]; difference between group 3 and group 1 = 8.8% [95% CI = 4.5% to 13.1%]) compared with media aliquots from explant cultures from the group given the control diet (group 1). This finding represented a twofold increase in unmetabolized NMBA present in the media from group 2 and group 1 (9.2% [95% CI = 8.8% to 9.6%]) compared with media from explant cultures from the group fed the control diet (9.2% [95% CI = 8.8% to 9.6%]). Media from animals fed the high dietary concentration of 4-HPR also contained more benzoic acid, the final product of NMBA metabolism, than media aliquots from animals fed the control diet (Table 4). Consistent with the increase in [3H]NMBA metabolites, explant culture media from animals fed the diets containing low (group 2) and high (group 3) concentrations of 4-HPR also had statistically significant decreases in residual, unmetabolized NMBA (P<.05 for each; difference between group 2 and group 1 = −14.6% [95% CI = −25.1% to −4.1%; difference between group 3 and group 1 = −23.4% [95% CI = −32.3% to −14.5%]) compared with residual NMBA in media from animals fed the control diet (group 1). This result represented a twofold reduction in unmetabolized NMBA present in the media from the group given the high concentration of 4-HPR in the diet (23.0% [95% CI = 18.5% to 27.5%]) compared with the group given the control diet (46.4% [95% CI = 39.5% to 53.3%]). Benzaldehyde was not detected in any of the media aliquots, perhaps because it was rapidly converted to benzoic acid by both enzymatic and nonenzymatic means (27). Taken together, these data suggest that dietary administration of 4-HPR increases the metabolism of NMBA in the rat esophagus. This effect of 4-HPR on NMBA metabolism was not dose related. In contrast, esophageal explants from animals fed 9-cis-RA did not differ in their ability to metabolize NMBA compared with explants from animals fed the control diet (Table 4).

## DNA Adduct Study

Metabolic activation of NMBA by esophageal cytochrome P450 (CYP) enzyme(s) generates electrophilic species and results in the methylation of DNA at the N7 and O6 positions of guanine (33,34). The O6-meGua adduct has been implicated in the generation of NMBA-induced mutations and, therefore, has been associated with its carcinogenic effects (35). We measured levels of O6-meGua in the genomic DNA isolated from esophagi of animals that had received a single dose of NMBA while on the control diet or both 4-HPR-containing diets. These results are shown in Table 5. At 24 hours after NMBA dosing, rats fed the diets containing the low concentration of 4-HPR (group 2) or the high concentration of 4-HPR (group 3) had statistically significantly increases (P<.05 for each; difference

### Table 4. Effects of 4-HPR and 9-cis-RA on NMBA metabolism in rat esophageal explant cultures incubated for 4 hours

| Group No. | Addition to diet | Metabolite‡ | Residual NMBA†<sub>|</sub>(% CI) |
|-----------|-----------------|-------------|-------------|
| 1         | None            | Peak 1 (95% CI) | Peak 2 (95% CI) | Peak 3 (95% CI) | Benzoic acid (95% CI) | Residual NMBA†<sub>|</sub>(% CI) |
| 2         | 0.4 g/kg 4-HPR  | 13.1<sub>1</sub> | 9.2<sub>1</sub> | 4.7<sub>1</sub> | 26.6<sub>1</sub> | 46.4<sub>1</sub> |
| 3         | 0.8 g/kg 4-HPR  | 18.0<sup>‡</sup> | 15.1<sup>‡</sup> | 4.5<sup>‡</sup> | 30.4<sup>‡</sup> | 31.8<sup>‡</sup> |
| 4         | 0.06 g/kg 9-cis-RA | 20.4<sup>‡</sup> | 18.0<sup>‡</sup> | 4.2<sup>‡</sup> | 34.6<sup>‡</sup> | 23.0<sup>‡</sup> |
| 5         | 0.12 g/kg 9-cis-RA | 23.0<sup>‡</sup> | 21.3<sup>‡</sup> | 3.4<sup>‡</sup> | 34.2<sup>‡</sup> | 18.5<sup>‡</sup> |

*4-HPR = N-(4-hydroxyphenyl)retinamide; RA = retinoic acid; CI = confidence interval; NMBA = N-nitrosomethylbenzylamine.

†Mean values with 95% CIs from three independent experiments are presented as the percentage of the total radioactivity in the samples.

‡Values are statistically significantly different (P<.05) from those of group 1 within the same column as determined by analysis of variance followed by Newman–Keuls multiple-comparisons test.
Table 5. Effects of dietary 4-HPR on NMA-induced O^6^-methylguanine levels in rat esophageal DNA

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Addition to diet</th>
<th>24 h after NMA dosing (95% CI)</th>
<th>48 h after NMA dosing (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None (control)</td>
<td>16.9 (13.1 to 20.7)</td>
<td>20.0 (19.0 to 21.0)</td>
</tr>
<tr>
<td>2</td>
<td>0.4 g/kg 4-HPR</td>
<td>26.6 (24.4 to 28.8)</td>
<td>26.7 (25.3 to 28.1)</td>
</tr>
<tr>
<td>3</td>
<td>0.8 g/kg 4-HPR</td>
<td>27.5 (25.3 to 29.7)</td>
<td>34.1 (31.8 to 36.4)</td>
</tr>
</tbody>
</table>

*4-HPR = N-(4-hydroxyphenyl)retinamide; NMA = N-nitrosomethylbenzylamine; CI = confidence interval.
†Statistically significantly different from group 1 (P<0.05), as determined by analysis of variance followed by Newman–Keuls multiple-comparisons test.
‡Statistically significantly different from group 1 and group 2 (P<0.05), as determined by analysis of variance followed by Newman–Keuls multiple-comparisons test.
§Statistically significantly different from group 2 and group 3 (P<0.05), as determined by analysis of variance followed by Newman–Keuls multiple-comparisons test.

between group 2 and group 1 = 9.7 pmol O^6^-meGua/μmol guanine [95% CI = 5.2 to 14.2 pmol O^6^-meGua/μmol guanine]; difference between group 3 and group 1 = 10.6 pmol O^6^-meGua/μmol guanine [95% CI = 6.1 to 15.1 pmol O^6^-meGua/μmol guanine] in the O^6^-meGua content of their esophageal DNA compared with rats that received the control diet (group 1). This result represented a greater than 50% increase in the amount of this mutagenic adduct present in esophageal DNA of rats fed the highest concentration of 4-HPR (27.5 pmol O^6^-meGua/μmol guanine [95% CI = 25.3 to 29.7 pmol O^6^-meGua/μmol guanine]) compared with the levels for rats fed the control diet (16.9 pmol O^6^-meGua/μmol guanine [95% CI = 13.1 to 20.7 pmol O^6^-meGua/μmol guanine]). Similarly, 48 hours after NMA dosing, the levels of O^6^-meGua increased from 20.0 pmol O^6^-meGua/μmol guanine (95% CI = 19.0 to 21.0 pmol O^6^-meGua/μmol guanine) in the animals fed the control diet (group 1) to 26.7 pmol O^6^-meGua/μmol guanine (95% CI = 25.3 to 28.1 pmol O^6^-meGua/μmol guanine) and 34.1 pmol O^6^-meGua/μmol guanine (95% CI = 31.8 to 36.4 pmol O^6^-meGua/μmol guanine) in animals fed the diets containing a low concentration of 4-HPR (group 2) and a high concentration of 4-HPR (group 3), respectively. These increases in O^6^-meGua content in the two groups of animals fed 4-HPR were statistically significant (P<0.05 for each; difference between group 2 and group 1 = 6.7 pmol O^6^-meGua/μmol guanine [95% CI = 4.9 to 8.5 pmol O^6^-meGua/μmol guanine]; difference between group 3 and group 1 = 14.1 pmol O^6^-meGua/μmol guanine [95% CI = 11.5 to 16.7 pmol O^6^-meGua/μmol guanine]). In summary, the results from the metabolism and DNA methylation studies demonstrate that dietary 4-HPR enhances NMA metabolism and increases the levels of O^6^-meGua, a mutagenic adduct, in rat esophageal DNA.

CYP Expression Study

The exact identity of the CYP enzyme(s) involved in NMA metabolism in the rat esophagus is not known. Previous studies (36,37) have suggested that two CYPs, CYP2A3 and CYP2E1, can metabolize NMA in vitro, which makes them candidate enzymes for the in vivo metabolism of NMA in the rat esophagus. Using semiquantitative RT–PCR, we examined the effects of dietary 4-HPR on the steady-state mRNA levels of these two CYPs in the rat esophagus. We found that CYP2A3 mRNA levels in esophagi were consistently higher in animals that had received 4-HPR in their diet for 1 week than in animals that had received the control diet (Fig. 1). The mean increase in CYP2A3 mRNA was 4.2-fold (95% CI = 2.5-fold to 5.9-fold) among three experiments after normalization to the mRNA levels for the housekeeping gene HPRT. We observed no statistically significant differences in CYP2A3 mRNA levels between animals fed low or high concentrations of dietary 4-HPR, nor were any consistent alterations in CYP2E1 mRNA levels found among the different treatment groups in these studies.

**DISCUSSION**

We have examined the efficacy of 9-cis-RA and 4-HPR for their chemopreventive activity against NMA-induced rat esophageal tumorigenesis when each was given in a complete carcinogenesis protocol. Unlike other studies, which have documented the antitumor effects of 4-HPR in animal models, we observed that dietary administration of 4-HPR produced a statistically significant enhancement in tumor response to NMA in the rat esophagus. A potential explanation for our results may lie in the target specificity of retinoids [reviewed in (38)]. For example, 13-cis-RA prevents two-stage carcinogenesis in mouse skin as well as chemically induced cancers of the bladder in mice. However, 13-cis-RA is not effective against DMBA-induced or N-methyl-N-nitrosourea (MNU)-induced mammary carcinogenesis in rats. Similarly, although 4-HPR is highly effective against mammary and urinary bladder carcinogenesis, it is not effective against carcinogenesis in the colon. This tissue specificity may reflect different tissue distributions of these retinoids.
inoids and/or differences in their metabolism in different organs. For example, when administered orally to rodents, 4-HPR is extensively metabolized to yield the primary metabolite 4-methoxyphenylretinamide, which is more lipophilic than the parent compound. Because of the high lipophilicity of its metabolites, 4-HPR has a substantially long half-life of 12 hours. Although 4-HPR accumulates primarily in the bladder, liver, and mammary glands (39,40), our results suggest that 4-HPR may also accumulate in the rat esophagus and can substantially affect tumorigenesis in response to NMBA at this organ site.

Retinoids have been shown to mediate the inhibition as well as the enhancement of chemical carcinogenesis in animal models. For example, Crist et al. (41) found that 4-HPR inhibits the initiation phase of MNU-induced mammary carcinogenesis in rats. In contrast, Grubbs et al. (42) reported a trend toward an increase in the number of mammary cancers when either 4-HPR or retinyl acetate was administered 2 months before MNU treatment. Pretreatment with these retinoids also increased the incidence of benign tumors in DMBA-treated rats without affecting the development of adenocarcinoma. However, other studies of DMBA- and MNU-induced mammary carcinogenesis in the same animal models (33,43,44) have shown that, when given after carcinogen exposure, retinyl acetate and 4-HPR effectively reduce tumor incidence and multiplicity and act as potent anti-promotion/progression agents. A similar situation exists in mouse skin models: Whereas most retinoids inhibit tumor promotion by phorbol esters in a two-stage carcinogenesis protocol (45), retinoic acid failed to prevent DMBA-induced carcinogenesis in the skin when used in a complete carcinogenesis protocol. In some experiments, retinoic acid actually increased the number of papillomas per mouse (46). Similarly, retinoic acid enhanced tumor response when UV irradiation was used as a complete carcinogen (47,48), when given before and during carcinogen exposure, 4-HPR has also been found to enhance the incidence of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung adenomas in A/J mice (49). Taken together with the results from our study, these findings suggest that the timing of retinoid administration may be an important determinant of its effects on carcinogenesis. When given before or simultaneously with the carcinogen, these retinoids can substantially enhance tumor formation in the same target tissues in which they inhibit tumor formation when given subsequent to tumor initiation with the carcinogen. It may, therefore, be necessary to evaluate all retinoids for their effects on both tumor initiation and tumor promotion in preclinical settings before beginning clinical chemoprevention trials.

We also investigated the potential mechanisms through which 4-HPR affects NMBA-induced esophageal tumorigenesis. For NMBA to be carcinogenic, it must be metabolized to produce electrophilic species that can react with DNA. CYP enzymes in the rat esophageal epithelium catalyze the α-hydroxylation of NMBA as a part of this process. Hydroxylation of the methane carbon of NMBA, in particular, produces the α-hydroxy-derivative 1, which spontaneously decomposes to benzaldehyde and the electrophile, methanediazohydroxide. Methanediazohydroxide methylates DNA at the N7 and O6 positions of guanine [reviewed in (33)]. In our study, dietary 4-HPR increased the metabolic activation of NMBA in the rat esophagus, and this increased metabolism was associated with an increase in O6-meGua adducts in esophageal DNA. Although the specific CYP enzymes involved in NMBA metabolism in the rat esophagus are not known, our preliminary evidence indicates that dietary 4-HPR also increases the steady-state levels of CYP2A3 mRNA. To our knowledge, this is the first report describing the effects of 4-HPR on carcinogen metabolism in an animal model. We suggest that the increased metabolism of NMBA and the subsequent elevations in methylated DNA adducts that result from dietary 4-HPR exposure could cause more frequent genetic mutational events. These events could lead ultimately to a higher number of initiated cells that, in part, would contribute to increased tumor formation.

Our observation that animals fed 4-HPR have statistically significantly larger tumors than animals fed the control diets points to the additional effects of 4-HPR during the promotion and progression phases of carcinogenesis. Luo et al. (50) found that dietary 4-HPR induced extensive cellular proliferation in the esophagi of treated animals. In addition, generation of reactive oxygen species has been found to be an important mechanism of 4-HPR effects in human cervical cancer (51), head and neck cancers (52), and lung cancer cell lines (49). Increased levels of reactive oxygen species have been shown to facilitate tumor promotion and progression (53–55). We postulate that similar mechanisms may contribute to the increased tumor size in animals fed 4-HPR.

It is premature to assess the implications of our findings with respect to the clinical use of 4-HPR. It was reported recently that oral administration of 4-HPR had no effect on squamous metaplasia or dysplasia in the bronchial epithelium of current smokers (56). However, it is not known if these individuals had any esophageal pathologic condition, such as premalignant lesions, that was affected by 4-HPR administration. Similarly, no increase in the incidence of tumors at other sites was noted in a study in which more than 2500 women with breast cancer were randomly assigned to receive either 4-HPR or no treatment (57). However, this cohort may not be the most appropriate one in which to detect the tumor-enhancing effects of 4-HPR because these subjects were not at high risk for esophageal cancer. It is likely that the paradoxical effects of 4-HPR in humans may depend on exposure to certain carcinogens and/or may be limited to specific tissues, as appears to be the case in rodent models of chemical carcinogenesis.

In summary, our data reveal that dietary administration of 4-HPR dramatically enhances tumor response to NMBA in the rat esophagus. In contrast, we found that dietary administration of 9-cis-RA did not statistically significantly alter tumor multiplicity in response to NMBA, although we did observe a trend toward increased tumorigenesis. A larger study will be necessary to fully evaluate the effects of 9-cis-RA on tumor response in this model system. Our mechanistic studies of animals fed 4-HPR indicate that enhancement of initiation events, such as NMBA metabolism and O6-meGua adduct formation, is an important part of sensitization to NMBA-induced rat esophageal tumorigenesis. The increased average tumor size in animals fed 4-HPR points to the additional promotional effects of this synthetic retinoid. Taken together with the published studies on the effects of retinoids on rat mammary carcinogenesis, our observations of the rat esophagus suggest that dietary 4-HPR can exert opposing effects on different target organs in the same species. Our findings are potentially important in light of ongoing human clinical studies of 4-HPR for the chemoprevention of breast and prostate cancers. The ability of 4-HPR to enhance tumor initiation may result in a paradoxical effect of this retinoid in other
sites, such as the aerodigestive tract, where it can possibly enhance nitrosoamine-induced carcinogenesis.

**REFERENCES**


(55) Gupta A, Rosenberger SF, Bowden GT. Increased ROS levels contribute to elevated transcription factor and MAP kinase activities in malignantly progressed mouse keratinocyte cell lines. Carcinogenesis 1999;20:2063–73.


NOTES

Supported in part by Public Health Service grant P01CA46535 (to G. D. Stoner) and P30CA16058–22 (to The Ohio State University Comprehensive Cancer Center) from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

We thank H. Sunny Kim, Ph.D., Senior Consulting Research Biostatistician, Biostatistics Program, The Ohio State University, Columbus, for her assistance in the statistical analyses of the data.

Manuscript received November 8, 2000; revised May 1, 2001; accepted May 8, 2001.