Patterns of DNA Adduct Formation in Liver and Mammary Epithelial Cells of Rats Treated with 7,12-Dimethylbenz(a)anthracene, and Selective Effects of Chemopreventive Agents

Alberto Izzotti, Anna Camoimano, Cristina Cartiglia, Clinton J. Grubbs, Ronald A. Lubet, Gary J. Kelloff, and Silvio De Flora

Department of Health Sciences, University of Genoa, I-16132 Genoa, Italy [A. I., A. C.; C. C., S. D. F.]; Chemoprevention Center, University of Alabama, Birmingham, Alabama 35294-3561 [C. J. G.]; and National Cancer Institute, Rockville, Maryland 20829 [R. A. L., G. J. K.]

ABSTRACT

7,12-Dimethylbenz(a)anthracene (DMBA) is a prototype carcinogen that induces a high yield of mammary tumors in rats after a single feeding. We investigated the induction and chemoprevention of DNA adducts in female Sprague Dawley rats receiving DMBA by gavage according to a variety of treatment schedules. The patterns of [32P]-postlabeled DNA adducts in liver and mammary epithelial cells were similar to those produced by the in vitro reaction of metabolically activated DMBA with calf thymus DNA. There was a high and statistically significant correlation between dose of DMBA administered to rats (0, 0.6, 2.4, and 12 mg/kg body weight) and levels of DNA adducts in both types of cells. The regression lines relating DMBA doses to total DNA adduct levels were significantly divergent and crossed at 1.5 mg/kg body weight, indicating that, at lower doses, the formation of DNA adducts is more intense in target mammary cells, whereas at higher doses, DNA adduct levels are more elevated in liver cells, presumably due to the greater metabolic capacity of this organ. When the rats were sacrificed 7 days rather than 2 days after DMBA administration, DNA adduct levels were approximately halved in both liver and mammary cells. The observed patterns can be interpreted based on toxicokinetic factors, local and distant metabolism, removal of DNA adducts by excision repair, and cell proliferation rate. Of three chemopreventive agents given with the diet to rats treated with 12 mg of DMBA, 5,6-benzoflavone (1650 ppm) was the most effective, inhibiting DNA adduct formation in liver and mammary cells by 96.5 and 83.5%, respectively. Feeding of 1,2-dithiole-3-thione (600 ppm) inhibited this biomarker by 68.5 and 50.2%, whereas butyl hydroxyanisole (BHA; 5000 ppm) showed a significant inhibition in the liver (46.5%) but was ineffective in mammary cells (29.0%, not significant). These data correlate nicely with the results of a parallel study in which 5,6-benzoflavone and 1,2-dithiole-3-thione, and BHA inhibited formation of hemoglobin adducts of DMBA metabolites tested in calf thymus DNA. There was a high and statistically significant correlation between dose of DMBA and after in vitro formation of DMBA-induced DNA adducts in liver and mammary epithelial cells of rats treated by gavage with varying doses of DMBA; (c) to evaluate the persistence of these molecular lesions; and (d) to investigate modulation of DNA adduct formation in liver and mammary cells of DMBA-treated rats receiving a diet supplemented with chemopreventive agents that, thus far, had not been tested in this model. One of them was 5,6-BF (or β-naphthoflavone), a broad-spectrum inducer of metabolic activities involved in the metabolism of xenobiotics (13). The other two chemicals are monofunctional agents, which primarily induce phase II enzymes. They are the phenol BHA, a synthetic antioxidant (14) used as a food preservative, and 1,2-D3T, which, like other dithiolethiones, was shown to possess antigenotoxic and anticarcinogenic properties in experimental test systems (15). A further goal was to evaluate the relationships between inhibition by chemopreventive agents of DNA adduct formation in liver and mammary cells, the hemoglobin adducts of DMBA metabolites tested in the same animals, and the mammary tumor yield, which, along with the modulation of metabolic factors, was investigated in parallel groups of rats treated with DMBA and chemopreventive agents according to comparable treatment schedules.

MATERIALS AND METHODS

Reagents for in Vitro Experiments. DMBA was purchased from Fluka (Buchs, Switzerland), and calf thymus DNA was purchased from Sigma.

Received 3/12/99; accepted 7/6/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by the National Cancer Institute (Master Agreement NO1-CN-75008).

2 To whom requests for reprints should be addressed, at Department of Health Sciences, Section of Hygiene and Preventive Medicine, University of Genoa, Via A. Pastore 1, I-16132 Genoa, Italy. Phone: (39) 010-3538500; Fax: (39) 010-3538504; E-mail: sdf@unige.it.

3 The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; PAH, polycyclic aromatic hydrocarbon; 5,6-BF, 5,6-benzoflavone; BHA, butylated hydroxyanisole; 1,2-D3T, 1,2-dithiole-3-thione; GSH, glutathione.

4 L. L. Song et al., manuscript in preparation.
overnight fast, the 12 rats used for the preliminary experiment received a single dose of DMBA by gavage, according to various administration schedules.

### Table 1: Formation of $^{32}$P postlabeled DNA adducts in liver and fat-free mammary epithelial cells of Sprague Dawley rats receiving a single dose of DMBA by gavage, according to various administration schedules

<table>
<thead>
<tr>
<th>DMBA dose (mg/kg body weight)</th>
<th>Time of sacrifice (days)$^a$</th>
<th>Inhibitors (dietary dose)</th>
<th>Organ or cells</th>
<th>DNA adducts/10$^6$ nucleotides$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Spot 1</td>
</tr>
<tr>
<td>0</td>
<td>—</td>
<td>—</td>
<td>Liver</td>
<td>0.01</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>5,6-BF (1650 ppm)</td>
<td>Mammary$^c$</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>BHA (5000 ppm)</td>
<td>Liver</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1,2-D3T (600 ppm)</td>
<td>Liver</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Liver</td>
<td>Mammary</td>
<td>6.5 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
<td>Liver</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>0.6</td>
<td>2</td>
<td>—</td>
<td>Liver</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

$^a$After the last DMBA administration.

$^b$DNA adduct levels are means ± SD of the results obtained in five rats, each analyzed in either duplicate (mammary glands) or triplicate (liver).

$^c$Fat-free mammary epithelial cells.

$^d$P < 0.001, as compared with the values recorded in rats receiving the same dose of DMBA, in the absence of chemopreventive agents.

$^e$P < 0.01, as compared with the values recorded in rats receiving the same dose of DMBA, in the absence of chemopreventive agents.

$^f$P < 0.05, as compared with the values recorded in rats receiving the same dose of DMBA, in the absence of chemopreventive agents.

### Chemicals and Animals

Chemical Co. (St. Louis, MO). S9 mix is a NADPH-generating mixture containing 10% liver S12 fractions from Arocoura 1254-pretreated Sprague Dawley rats (16).

**Animals.** Female Sprague Dawley rats were used throughout the study. Twelve 12-week-old rats (Morini, S. Polo d’Enza, Italy) were used in a preliminary experiment carried out at the University of Genoa. Forty-five 4-week-old rats (viruses-free colony no. 218; Harlan Sprague Dawley, Inc., Indianapolis, IN) were received at the University of Alabama at Birmingham for the final experiment. The rats, maintained on Teklad (4%) diet and given drinking water ad libitum, were housed in a climatized environment at a temperature of 22 ± 2°C with a relative humidity of 45 ± 10%. Ventilation accounted for 15 air renewal cycles/h, and a 12-h light/dark cycle was used. Animal care was in accordance with the institutional guidelines of each university where this study was performed.

### Treatment of Animals

After 1 week of acclimatization and following an overnight fast, the 12 rats used for the preliminary experiment received a single administration by gavage of DMBA (25 mg/kg body weight) in corn oil, in a volume of 200–300 μl. For the final experiment, at 60 days of age, the rats were divided into nine groups, each composed of five animals, and treated by gavage either with corn oil (controls) or DMBA at three dose levels (0.6, 2.4, or 12 mg/kg body weight), according to the schedule indicated in Table 1. Three groups of rats received a diet supplemented with either 5,6-BF (1650 ppm), BHA (5000 ppm), or 1,2-D3T (600 ppm) for 7 days prior to DMBA administration. The rats were sacrificed by CO2 asphyxiation, either 2 or 7 days after the last DMBA administration (see Table 1). The liver and tissue fragments containing mammary glands were collected and immediately frozen in liquid nitrogen and then stored at −80°C. The samples were packaged in dry ice and shipped by international courier from the University of Alabama at Birmingham to the University of Genoa, where they arrived still frozen, and were maintained at −80°C until use. The treatment groups were blind-coded, and their identity was only disclosed after having reported to the National Cancer Institute the results of DNA adduct analyses.

### Preparation of Rat Mammary Epithelial Cells

Two procedures were used for isolating mammary epithelial cells from the thawed tissue fragments containing mammary glands. The first procedure, described by Moon et al. (17), is aimed at preparing fat-free mammary epithelial cells by eliminating mammary gland adipose tissue by treatment with collagenase. This method maintains a histological state in which mammary ducts and alveoli can be readily identified. The second procedure, described by Moore et al. (18), involves digestion with collagenase and DNase I, plating into culture dishes to remove stromal cells, and filtration through 53-μm pore-size filters to trap the mammary epithelial cells.

### DNA Extraction

Aliquots of liver (100 mg) and mammary cells (50 mg) were thawed and homogenized in a Potter-Elvehjem apparatus at 4°C in 250 mM sucrose, 5 mM 1,4-DTT, and 50 mM Tris-HCl (pH 7.6). DNA was isolated by solvent extraction using an automatic extractor (Genepure 341; Applied Biosystems, Foster City, CA) according to the method of Gupta (19), with some modifications as described previously (20). Homogenized cells were sequentially treated first with a mixture of RNase A and T1 for 1 h and then with proteinase K for 5 h at 55°C, followed by two extractions with a phenol-water-chloroform mixture and two extractions with chloroform only. To increase DNA recovery from mammary epithelial cells, we added 30 μl of a silica-based DNA binding reagent (Quiq-Precip; Edge-Biosystems, Gaithersburg, MD) during alcoholic precipitation. The whole procedure was performed in a helium atmosphere by using phenol of the highest available quality to avoid induction of oxidative DNA damage during extraction. The extracted DNA was analyzed and checked for purity by spectrophotometric analysis. The following absorbance ratios were obtained: A$_{260}$nm/A$_{280}$nm ≥ 1.95; A$_{260}$nm/A$_{230}$nm ≥ 1.80; and A$_{260}$nm/A$_{220}$nm ≥ 1.70 and ≥ 1.95. The amounts of DNA obtained from 1 mg of wet tissue were, on average, 1.87 and 0.27 μg for liver and mammary cells, respectively.

### Detection of DNA Adducts by $^{32}$P-Postlabeling

Postlabeling procedures were basically performed as reported by Gupta (21), with some modifications. Six μg of DNA were hydrolyzed to deoxyribonucleoside 3′-monophosphate by micrococcal nuclease (0.04 units/μg DNA) and spleen phosphodiesterase (1 million/μg DNA) in 20 mM sodium succinate-10 mM calcium chloride (pH 6.0; 37°C for 3.5 h). Normal nucleotides were digested by adding 5 μl of nucleases P1 (2.77 units) in 3 mM zinc chloride-250 mM sodium acetate (pH 5.0; 37°C for 40 min). Polynucleotide kinase (8 units) in 6 μl of 200 mM bicine, 100 mM magnesium chloride, 100 mM 1,4-DTT, and 10 mM spermidine (pH 9.5) was added. The $^{32}$P-Postlabeling reaction was performed by including 4 μl of [γ-$^{32}$P]ATP (16 μCi/μl, specific activity, ≤0.600 Ci/mmol; ICN Biomedicals, Irvine, CA) in the mix and incubating at 22°C for 40 min.

Labeled samples were developed on either 10 × 10 cm (preliminary in vivo experiment) or 12.5 × 10 cm (preliminary in vitro experiment and final in vivo experiment) polyethyleneimine-coated cellulose sheets (Macherey & Nagel, Düren, Germany) according to a multidirectional TLC system. The first development (D1; downward direction in Fig. 1) was performed in 1 m sodium phosphate (pH 6.0) overnight by stapling a wick paper at the top edge of the sheet. The second development (D2) was performed in 3 LiOH formate-8.5 m urea (pH 3.8) at 180°, as compared with D1 (from bottom to top in Fig. 1). A third development (D3) was performed in 0.6 m lithium chloride-8.5 m urea (pH 8.0) at 90° clockwise, as compared with D2 (from left to right in Fig. 1). The final development was in 1.7 m sodium phosphate in the same direction as...
D3. The detection of radioactive spots was achieved by electronic autoradiography, using a 32P imager (Instant Imager, Packard, Meriden, CT). TLC sheets were scanned until the statistical error obtained in counting was <1% (1–3 h). Adduct quantification was performed by calculating the relative adduct labeling index (21) and was expressed as adducts/10^9 normal nucleotides. For the detection of normal nucleotides, an aliquot of each DNA depolymerized sample was developed in a unidirectional TLC system using 170 mM sodium phosphate (pH 6.8; Ref. 22). In each labeling experiment, a benzo[a]pyrene diol epoxide–N2-dGp reference standard (National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute, Kansas City, MO) was used as a positive control, and a DNA-free sample was used as a negative control.

**Statistical Analyses.** Comparisons of mean (± SD) DNA adduct levels in different experimental groups were performed by Student’s t test for unpaired data. Correlations were calculated using Spearman’s and simple regression tests. The significance of differences in the slope of dose-response curves was evaluated by parallel test of regression lines (23).

**RESULTS**

**Preliminary Experiments.** Two preliminary experiments were carried out. The first was an in vitro experiment in which we mixed either 1 mM DMBA or its solvent (DMSO), 100 μl of S9 mix, and 300 μg of calf thymus DNA in 1.3 ml of PBS (pH 7.4). After contact for 20 min at 37°C, DNA was purified, and DNA adducts were measured. No adduct was detected in DMBA-free calf thymus DNA, at a sensitivity level of 0.1 adducts/10^9 nucleotides. Conversely, five well-distinguished autoradiographic spots, the patterns of which were comparable with those shown in Fig. 1, were detected in DMBA-treated DNA. The levels (mean ± SD of triplicate determinations) were 198 ± 43, 110 ± 51, 10.1 ± 3.4, 5.5 ± 2.8, and 2.4 ± 1.8 adducts/10^9 nucleotides for the five individual spots and 326 ± 48 adducts/10^9 nucleotides for total DNA adducts.

The other preliminary experiment aimed at comparing two methods for isolating mammary epithelial cells. For this purpose, 12 female Sprague Dawley rats were treated by gavage with DMBA in corn oil (25 mg/kg body weight). After 48 h, the animals were sacrificed, and tissue fragments containing mammary glands were frozen in liquid nitrogen and then stored at −80°C. The thawed specimens were divided into two groups and comparatively processed by following the protocols described by Moon et al. (17) and Moore et al. (18) for isolating mammary epithelial cells. On average, the two methods yielded 81 and 28 mg of epithelial cells, respectively, from 1 g of s.c. tissue containing mammary glands. 32P-postlabeling of the DNA extracted from the mammary epithelial cells isolated by the two methods yielded a total of 35.1 ± 12.9 and 37.9 ± 10.3 adducts/10^8 nucleotides, respectively (means ± SD of three samples each). We decided to use the procedure described by Moon et al. (17) for the definitive study, due to the higher yield of epithelial cells and the greater simplicity of this method.

**General Patterns of 32P-Postlabeled DNA Adducts.** Table 1 provides an outline of the results obtained in the nine experimental groups. The results are means ± SD of DNA adduct levels measured in five rats per group, each generated in either two separate experiments (mammary epithelial cells) or three separate experiments (liver cells). Therefore, Table 1 synthesizes the results of 90 32P-postlabeling analyses with mammary epithelial cells and of 135 analyses with liver samples.

Table 2. Correlation between dose of DMBA (0, 0.6, 2.4, or 12 mg/kg body weight) and DNA adduct levels in liver and mammary epithelial cells of Sprague-Dawley rats sacrificed 2 days after DMBA administration

<table>
<thead>
<tr>
<th>Organ or cells</th>
<th>Autoradiographic spot</th>
<th>r</th>
<th>P</th>
<th>Regression line&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>1</td>
<td>0.995</td>
<td>&lt;0.05</td>
<td>y = 0.587 + 1.817x</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.997</td>
<td>&lt;0.01</td>
<td>y = 0.079 + 0.73x</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.989</td>
<td>&lt;0.05</td>
<td>y = -0.353 + 0.462x</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.992</td>
<td>&lt;0.01</td>
<td>y = -0.105 + 0.222x</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.986</td>
<td>&lt;0.05</td>
<td>y = -0.312 + 0.385x</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>0.998</td>
<td>&lt;0.01</td>
<td>y = -0.127 + 3.588x</td>
</tr>
<tr>
<td>Mammary epithelial cells</td>
<td>1</td>
<td>0.987</td>
<td>&lt;0.05</td>
<td>y = 0.695 + 1.522x</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.932</td>
<td>0.07</td>
<td>y = -0.435 + 0.293x</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.972</td>
<td>&lt;0.05</td>
<td>y = 0.155 + 0.193x</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.962</td>
<td>&lt;0.05</td>
<td>y = 0.187 + 0.131x</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.974</td>
<td>&lt;0.05</td>
<td>y = 0.182 + 0.259x</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>0.978</td>
<td>&lt;0.05</td>
<td>y = 1.617 + 2.403x</td>
</tr>
</tbody>
</table>

<sup>a</sup> x is the dose of administered DMBA (mg/kg body weight), and y is the level of DNA adducts/10<sup>8</sup> nucleotides.

With an assumed sensitivity threshold of 0.1 adducts/10<sup>9</sup> nucleotides, no DNA adduct was detected in either tissue of untreated rats (controls). Conversely, DNA adducts were formed in both liver and mammary cells of all eight groups of rats treated with DMBA, with varying intensity depending on the experimental group. As shown in Fig. 1, the general patterns showed the consistent presence of one major adduct (spot 1) and four minor adducts (spots 2–5) in both liver and mammary cells. The size of the spots reproduced in Fig. 1 indicates, on an arbitrary scale, the levels of DNA adducts corresponding to either 1 mg or 10 mg of DMBA/kg body weight, as calculated from dose-response regression lines (see below).

**Dose-Effect Relationships in the in Vivo Formation of DMBA-Induced DNA Adducts.** Dose-effect relationships were evaluated by relating the dose of DMBA administered to rats (single dose of 0.6,
2.4, or 12 mg/kg body weight plus 0 for controls) to the levels of individual spots and total DNA adducts measured in liver and mammary cells of rats sacrificed 2 days after DMBA administration in the absence of chemopreventive agents. As shown in Table 2, the dose-effect correlation coefficients ($r$) were very high, all of them being statistically significant ($r$ was of borderline significance only in the case of spot 2 in mammary cells). Therefore, the regression lines reported in Table 2 could reliably be used for further analyses. The surface area of each of the five spots reported in Fig. 1 reproduces, on a relative scale, DNA adduct levels formed after hypothetical administration of DMBA at either 10 mg or 1 mg/kg body weight, as calculated from the regression lines. As shown in Fig. 2, with an increase in DMBA dose, the regression line for liver cells is steeper than that for mammary cells. This differential trend, evaluated by parallel test of the regression lines, is statistically significant ($P < 0.05$). In other words, this sort of calibration curve indicates that the formation of DNA adducts is higher in mammary epithelial cells at DMBA doses that are <1.5 mg/kg body weight, whereas at DMBA doses exceeding that level, the formation of DNA adducts tends to be increasingly higher in liver cells.

Persistence of DMBA-induced DNA Adducts. The persistence of DNA adducts was evaluated in groups of rats receiving a single dose of DMBA, either at 12 mg or 0.6 mg/kg body weight, and sacrificed either 2 or 7 days after DMBA administration (Table 1). Compared with the rats sacrificed 2 days after the DMBA treatment, total DNA adduct levels in liver and mammary cells were 56.0 and 46.8%, respectively, in the animals sacrificed 7 days after administration of the 12-mg dose, and 30.3 and 58.3%, respectively, after administration of the 0.6 mg dose. Although remarkable in terms of percentage decrease, the differences recorded at the 0.6 mg dose were not statistically significant. Those recorded at the 12-mg dose were significant for spots 2 ($P < 0.01$), 3 ($P < 0.05$), 4 ($P < 0.01$), and 5 ($P < 0.05$) and total DNA adducts ($P < 0.01$) in liver cells and for spots 1 ($P < 0.01$) and 3 ($P < 0.05$) and total DNA adducts ($P < 0.05$) in mammary epithelial cells.

Effects of Chemopreventive Agents. Of the three chemopreventive agents under study, 5,6-BF was the most effective inhibitor of DNA adduct formation, decreasing total DNA adducts in liver and mammary cells by as much as 96.5% and 83.5%, respectively. As shown in Table 1, not only total DNA adducts but also all five individual spots were significantly decreased in both liver and mammary cells, as compared with rats receiving 12 mg DMBA, in the absence of chemopreventive agents.

BHA was the less effective inhibitor, decreasing total DNA adducts in liver and mammary cells by 46.3 and 29.0%, respectively. Inhibition was significant for spots 1, 2, and 4 and for total DNA adducts in the liver, whereas it did not reach the statistical significance threshold for DNA adducts in mammary cells.

1,2-D3T had an intermediate efficacy, decreasing total DNA adducts in liver and mammary cells by 68.5 and 50.2%, respectively. The decrease was significant for all spots and total DNA adducts in the liver and for spot 1 and total DNA adducts in mammary cells (Table 1).

DISCUSSION

The analysis of $^{32}$P-postlabeled adducts resulted in the consistent detection of five autoradiographic spots in calf thymus DNA, treated in vitro with DMBA in the presence of an exogenous metabolic system, as well as in liver and mammary epithelial cell DNA of rats receiving DMBA by gavage. As inferred from literature data (24, 25), these spots are likely to reflect reactions between anti-DMBA-dihydriodilepoxide and either dG (spots 1 and 5) or dA (spot 2) and between syn-DMBA-dihydriodileopoxide and either dG (spot 3) or dA (spot 4). Because spot 1 accounted for more than half of the DNA adducts formed in both liver and mammary cells, anti-DMBA-dihydriodilepoxide-dG appears to be the predominant DNA adduct.

A high correlation was found to occur between the dose of administered DMBA and the levels not only of total DNA adducts but also of all five individual spots in both liver and mammary epithelial cells. The regression lines relating the DMBA dose with DNA adduct levels in these two types of cell populations were significantly divergent and crossed at a dose of 1.5 mg/kg body weight DMBA. Thus, by reducing DMBA doses, DNA adduct levels appear to be higher in mammary cells, whereas by increasing DMBA doses, they become higher and higher in liver cells. We observed a similar trend in liver and lung of rats treated i.p. with benzo[a]pyrene (26). Presumably, these patterns depend on the fact that liver cells have a greater capacity of metabolizing PAHs, as compared with the target cells for tumorigenicity, which become more readily saturated. It is noteworthy, in this respect,

Table 3 Inhibition by dietary chemopreventive agents of the formation of DNA adducts and hemoglobin adducts in Sprague-Dawley rats treated with a single intragastric administration of DMBA (12 mg/kg body weight) and of tumor yield in rats of the same strain treated with a single intragastric administration of DMBA (15 mg/kg body weight) (%) inhibition

<table>
<thead>
<tr>
<th>Chemopreventive agent (dietary dose)</th>
<th>DNA adducts&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hemoglobin adducts&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mammary tumors&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Mammary</td>
<td>Incidence</td>
</tr>
<tr>
<td>5,6-BF&lt;sup&gt;c&lt;/sup&gt; (1650 ppm)</td>
<td>96.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>83.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>80.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,2-D3T&lt;sup&gt;c&lt;/sup&gt; (600 ppm)</td>
<td>50.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>44.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>47.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BHA&lt;sup&gt;c&lt;/sup&gt; (5000 ppm)</td>
<td>29.0</td>
<td>0</td>
<td>5.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> This study.
<sup>b</sup> L. L. Song et al., manuscript in preparation (see Footnote 4).
<sup>c</sup> Statistically significant inhibition.
that DMBA metabolites formed in the liver can travel to the mammary gland via the blood circulation, and in addition, DMBA itself can be metabolized in mammary cells (27, 28). Another tentative hypothesis is that differences in constitutive and inducible cytochrome P450 isoforms involved in DMBA metabolism may partially contribute to the organ-specific differences in DNA adduct dose responses observed.

Besides metabolic factors, other mechanisms can account for the selective formation of DNA adducts in different organs and cell types (29, 30). One is toxicokinetics and, in particular, the so-called first-pass effect, in which, after intragastric administration of DMBA, subsequent uptake via the portal system is much more pronounced in the liver than in the mammary gland. On the other hand, the greater first-pass effect and metabolizing capacity is compensated in the liver by a quite efficient removal of DNA adducts via excision repair (30). A further crucial mechanism is represented by the cell proliferation rate, which is higher in mammary epithelial cells than in hepatocytes. More intense cellular turnover tends to dilute DNA adducts accordingly to lower their persistence. At the same time, cell proliferation is an essential mechanism favoring all stages of the carcinogenesis process (30). Due to the balance between these contrasting mechanisms, the persistence of DNA adducts was similar in liver and mammary cells, their levels being approximately halved when measured 7 rather than 2 days after DMBA administration.

The observed linearity of the dose-response curves relating the doses of administered DMBA to the levels of DNA adducts provides an ideal technical premise for the assessment of protective effects. The three investigated chemopreventive agents, all of them given with the diet, displayed a differential ability to inhibit the formation of DMBA-induced DNA adducts. In fact, the efficacy ranked as follows: 5,6-BF > 1,2-D3T > BHA. These data are in agreement with the evaluation of DMBA-hemoglobin adducts in the same animals, which showed inhibitions of 80.0% with 5,6-BF, 44.0% with 1,2-D3T, and 0% with BHA. Moreover, inhibition by these chemopreventive agents of DNA adducts induced by DMBA (12 mg/kg body weight) is well correlated with inhibition of the yield of mammary tumors, as assessed in a parallel study following a very similar treatment schedule, except that DMBA was given at a slightly higher dose (15 mg/kg body weight). In fact, 5,6-BF inhibited tumor incidence by 82.4% and multiplicity by 92.6%; 1,2-D3T inhibited tumor incidence by 47.1% and multiplicity by 80.0%; and BHA did not produce significant effects, decreasing tumor incidence by 5.9% and multiplicity by 7.4% only.

As summarized in Table 3, with the exception of a significant ability of BHA to inhibit DNA adducts in liver cells, which is well explained by distinctive metabolic effects of this phenolic antioxidant in hepatocytes and mammary cells (see below), there is a striking concordance between inhibition of early biomarkers, either in target cells (DNA adducts in mammary epithelial cells) or in the main metabolizing organ (DNA adducts in liver cells) or at a systemic level (hemoglobin adducts) and inhibition of mammary tumor incidence and multiplicity. These findings support the validity of early biomarkers that measure the ability of the biologically effective dose to predict the final pathological event in the DMBA rat mammary tumor model. Such a conclusion is in agreement with the general postulate that inhibition of DNA adducts is a reliable indicator of chemoprevention of tumors. In fact, in most cases, these promutagenic lesions are a necessary step, the down-regulation of which breaks the chain of events leading to the disease (31). A parallelism between inhibition of mammary DNA adducts and tumors in DMBA-treated rats was also observed after dietary administration of sodium selenite (7), 1,4-phenylenebis(methylene)selenoxyanate (8), aliphatic selencyanates (10), or butyl phthalate (6) or after i.p. injections of butylated hydroxytoluene (3), rosemary extract and its constituent carnosol (4), or curcumin (5).

Chemopreventive agents can inhibit genotoxicity and carcinogenicity through a large variety of mechanisms (32, 33). Because the agents under study had parallel effects on the formation of DNA adducts and on the yield of mammary tumors, it is likely that they worked at a level preceding binding of DMBA metabolites to DNA, presumably by altering the metabolism of this PAH. In any case, inhibition by these chemopreventive agents was of the same order of magnitude for the individual DNA adducts in both types of cells, which indicates that there was no preferential effect toward the two DMBA-dihydrodiol-epoxide enantiomers.

All three chemopreventive agents under study are well-known phase II enzyme inducers and have additional effects on the metabolism of xenobiotics. For instance, 5,6-BF binds the Ah receptor and, accordingly, induces a variety of cytochrome P-450 isoforms, including CYP1A1, CYP1A2, and CYP1B1 (13). It was also proposed that 5,6-BF can inhibit DMBA mammary carcinogenesis by interfering with the oxidative metabolism of this carcinogen (34). Interestingly, irrespective of DMBA administration, 5,6-BF had striking effects on phase II activities in Sprague Dawley rats by inducing NAD(P)H quinone reductase and GSH-transferase and by increasing GSH levels in different tissues, including the mammary gland. BHA had no influence on these parameters in the mammary gland, whereas substantial induction of phase II enzymes and GSH content were seen in the liver. Similar conclusions were drawn in the same rat model after dietary administration of butylated hydroxytoluene (35). This differential metabolic effect nicely correlates with our finding that dietary BHA selectively inhibited the formation of DNA adducts in the liver but not in mammary epithelial cells and with the failure of this phenol to modulate the mammary carcinogenesis of DMBA.

There is no doubt that the selective induction of phase II activities by monofunctional inducers is an important mechanism of inhibition of genotoxicity and carcinogenicity (32, 33). However, as shown in this study by the potent inhibition produced by 5,6-BF, broad-spectrum inducers may even be more effective. Indeed, as also reported with other chemopreventive agents (33, 36), the ideal detoxification may be the one provided by agents that increase metabolism of the procarcinogen, while simultaneously inactivating ultimate carcinogenic metabolites by increasing the concentration of nucleophiles and achieving detoxification and excretion via phase II pathways.

REFERENCES

8. el-Bayoumi, K., Chae, Y. H., Upadhyaya, P., Meschter, C., Cohen, L. A., and Reddy, B. S. Inhibition of 7,12-dimethylbenz(a)anthracene (DMBA)-induced tumors and...


21. Gupta, R. C. Enhanced sensitivity of 32P-postlabeling analysis of aromatic carcino-


4290

Downloaded from cancerres.aacrjournals.org on January 22, 2018. © 1999 American Association for Cancer Research.
Patterns of DNA Adduct Formation in Liver and Mammary Epithelial Cells of Rats Treated with 7,12-Dimethylbenz( a )anthracene, and Selective Effects of Chemopreventive Agents

Alberto Izzotti, Anna Camoirano, Cristina Cartiglia, et al.