Effects of p-Methoxyphenol and Diet on Carcinogen-induced Neoplasia of the Mouse Foreestomach

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ABSTRACT

Previously, p-methoxyphenol fed in the diet was found to be the most potent inhibitor of benzo(a)pyrene-induced neoplasia of the mouse forestomach of 18 phenols investigated. In the present study, the effects of p-methoxyphenol on the direct-acting carcinogen, \( \beta \)-propiolactone (BPL), were determined. p-Methoxyphenol administered at 1 or 4 hr prior to BPL or fed in the diet markedly inhibited BPL-induced neoplasia of the mouse forestomach. Of 10 phenols tested by p.o. intubation, it was the only one that exerted a significant inhibitory activity. Thus far, p-methoxyphenol appears to be an effective inhibitor only when given prior to carcinogen administration. During these studies, it was found that the nature of the diet markedly altered the neoplastic response of the mouse forestomach to BPL but not to benzo(a)pyrene.

INTRODUCTION

In previous work, 2(3)-BHA\(^5\) was found to inhibit the effects of a variety of chemical carcinogens (18). These inhibitory properties focused attention on phenols as putative inhibitors of carcinogen-induced neoplasia. In further investigations, the inhibitory capacities of 18 phenolic compounds on BP-induced neoplasia of the mouse forestomach were determined. Eight of these compounds inhibited the occurrence of forestomach tumors. The most potent inhibition was obtained with p-methoxyphenol (20). This finding was the initial evidence that p-methoxyphenol could have strong inhibitory effects against carcinogens acting upon the forestomach. The mechanism(s) by which phenols inhibit the occurrence of neoplasia has not been established. Induction of increased activities of detoxification systems is postulated to be of importance for the inhibitory effects obtained (18, 19). In this regard, p-methoxyphenol has been shown to be a potent inducer of increased GSH S-transferase activity of the mouse forestomach and also the esophagus (13, 14).

BP, the carcinogen used in the structure-activity experiments in which p-methoxyphenol was found to be a highly potent inhibitor, requires complex metabolic activation to its ultimate carcinogenic form. Accordingly, inhibition could occur as a result of alterations of metabolism or detoxification reactions at any one of a number of steps in the metabolism of this carcinogen as well as by other means. In the present investigation, the carcinogen used in most of the experiments was BPL. Like BP, BPL will cause neoplasms of the forestomach when administered by p.o. intubation. However, unlike BP, BPL is a direct-acting carcinogen. It does not require metabolic activation (1, 3, 6, 9, 10, 16). As in the experiments with BP, p-methoxyphenol proved to be a potent inhibitor of BPL-induced forestomach tumor formation. However, in this instance, p-methoxyphenol appeared to be unique among the phenols tested in terms of its inhibitory capacities against this carcinogen. During the course of this work, 2 diets were used, i.e., a crude diet and a semisynthetic diet. The carcinogenic potency of BPL was found to be quite different depending upon the diet fed, an effect that did not occur with BP.

MATERIALS AND METHODS

Mouse Tumor Experiments. Tumor formation in the forestomach of female ICR/Ha mice (Hartan-Sprague-Dawley, Madison, Wis.) was studied using procedures similar to those described previously (17, 20). Unless otherwise specified, the mice were fed Purina rat chow (Ralston Purina Co.) during the entire experiment. The mice were randomized at 8 weeks of age into groups of 20 mice each in studies in which the test compounds were given by p.o. intubation. In these experiments, BPL (2 mg in 0.1 ml propylene glycol) was administered by p.o. intubation twice per week for 12 weeks starting when mice were 9 weeks of age. The test compound, dissolved in 0.1 ml propylene glycol, was given by p.o. intubation at the designated time (1 or 4 hr) prior to BPL. Two control groups were used: one given vehicle and the other given nothing. In some studies, the test compound was administered in the diet, or the effects of different diets were investigated. In these experiments, the mice were randomized by weight at 7 weeks of age and then placed on the experimental diets. These diets were continued until 24 hr after the last dose of carcinogen. At that time, the mice were fed Purina chow. Two control groups were used, one pair fed with mice given p-methoxyphenol in the diet and the other fed ad libitum. p-Methoxyphenol did not alter diet intake, so that the results from the 2 groups were combined. In some experiments, BP was used as the carcinogen. The dose and schedule of administrations are described in the text where these experiments are presented. Upon termination of all experiments, the stomachs were fixed in an expanded state produced by intragastric injection of formalin. Subsequently, they were split longitudinally. Tumors of the forestomach were counted using the technique of Shimkin (11, 12).

Chemicals. The following compounds were obtained from the Aldrich Chemical Co., Milwaukee, Wis. (the designated purity, when provided, is shown in parentheses): p-methoxyphenol (98%); phenol (99%); anisole (99%); hydroquinone (99%); \( \alpha,\alpha,\alpha \)-trifluoro-p-cresol (98%); 4-fluorophenol (99%); pyrogallol; 3,4,5-trimethoxyphenol (97+%); benzoic acid (99%); and p-hydroxybenzoic acid (99%). BPL was obtained from Sigma Chemical Co., St. Louis, Mo.; 2-tert-butyl-4-hydroxyanisole was synthesized, and 3-tert-butyl-4-hydroxyanisole was isolated by fractional crys-

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\(^{5}\) The abbreviations used are: 2(3)-BHA, 2(3)-tert-butylhydroxyanisole; BP, benzo(a)pyrene; GSH, glutathione; BPL, \( \beta \)-propiolactone; NMR, nuclear magnetic resonance.

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tallization of commercial 2(3)-BHA from acetone using techniques described previously (8).

Studies of Reactivity of BPL with p-Methoxyphenol. Several experiments were performed. In one, 1.8 mmol of BPL were mixed with 1.8 mmol of p-methoxyphenol dissolved in chloroform, aceton, or aceton/ water (50/50). The mixtures were shaken at 40° for 1 hr. The test samples were spotted on thin-layer chromatography plates (Analytech Corp.) and developed in a system of ether/petroleum ether (1/2). The chromatograms were viewed under UV (254 nm) and examined for BPL-p-methoxyphenol adducts. In a similar experiment, other solvents were used for the 2 constituents, and then these were mixed; p-methoxyphenol in cottonseed oil/BPL in propylene glycol; p-methoxyphenol in cottonseed oil/BPL in cottonseed oil; and p-methoxyphenol in propylene glycol/BPL in propylene glycol. Since p-methoxyphenol is administered in cottonseed oil and BPL is administered in propylene glycol in in vivo experiments, the preceding combinations of solvents used to detect adducts include those that occur in vivo. A third procedure was used in an effort to detect adduct formation. This entailed studies of the possible reaction of p-methoxyphenol and BPL as monitored by NMR. NMR spectra were recorded on a Varian HFT-80 NMR spectrometer with 3-(trimethylsilyl)tetradecuteroacetic acid as the internal standard. All kinetic experiments were carried out in the NMR spectrometer at 22° with an initial BPL concentration of 0.2 m in 25% dimethyl sulfoxide-D6 in D20. BPL concentrations were monitored by measuring the peak area of the triplet centered at 4.4 ppm (protons on the beta-carbon of BPL) and the peak areas of the triplet centered at 2.6 ppm (protons adjacent to the carbonyl on 3-hydroxypropionic acid). For a typical kinetic run, 0.25 ml of a freshly prepared 0.4 M solution of BPL in dimethyl sulfoxide/D20. BPL concentrations were monitored by measuring the peak area of the triplet centered at 4.4 ppm (protons on the beta-carbon of BPL) and the peak areas of the triplet centered at 2.6 ppm (protons adjacent to the carbonyl on 3-hydroxypropionic acid).

Determination of GSH S-Transferase Activity. The activity of cytosol GSH S-transferase was determined spectrophotometrically at 30° with 1-chloro-2,4-dinitrobenzene as substrate according to the procedure of Habig et al. (5). Tissue levels of acid-soluble sulfhydryl groups were determined by the procedure of Ellman (4).

Statistical Analysis. Student's t test was used to determine the statistical differences in the number of tumors per group between the control and treated groups, and the x2 test was used for the differences determined by the procedure of Ellman (4). Tissue levels of acid-soluble sulfhydryl groups were determined by the procedure of Habig et al. (5).

Effects of p-methoxyphenol on BPL-induced neoplasia of the mouse forestomach are shown in Table 1. Administration of p-methoxyphenol in the diet or by p.o. intubation 4 hr prior to BPL resulted in pronounced inhibitory effects which were of the same magnitude. If p-methoxyphenol was given 1 hr prior to BPL, the inhibition was slightly less. Most of the tumors of the forestomach were papillomas; some carcinomas occurred as well. p-Methoxyphenol markedly inhibited the occurrence of the benign tumors and prevented the appearance of the malignant neoplasms. In a few instances, the carcinomas occupied most of the stomach, and a tumor count could not be done. In Table 1, this is shown by the numbers in parentheses, which designate the total number of mice upon which tumor counts could be performed, being slightly lower than those for the total number of mice at risk. In each experiment in Table 1, 2 control groups were used as described in "Materials and Methods." The tumor response of the 2 controls was almost identical in each case, so these data have been combined.

In addition to p-methoxyphenol, 11 other compounds administered by p.o. intubation at 4 hr prior to BPL were studied for their inhibitory effects. None of these produced significant inhibition. They did not reduce significantly the number of mice bearing forestomach tumors or the number of tumors per mouse. The chemical structures of the compounds tested are shown in Chart 1.

In the experiments in which p-methoxyphenol has been studied for its inhibitory effects on BPL or BP-induced neoplasia of the forestomach, the phenol comes into direct contact with the target organ. The question arises as to whether p-methoxyphenol would inhibit at a tissue site distant from the gastrointestinal tract. An experiment of this type was performed with the lung as the target tissue. In Table 2, the effects of feeding p-methoxyphenol on BP-induced pulmonary adenoma formation are presented. No significant inhibition was found. A comparable

### Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Material administered</th>
<th>Manner of administration</th>
<th>Dose or concentration</th>
<th>Time of administration</th>
<th>No. of mice at risk</th>
<th>Wt gain (g)</th>
<th>% of mice with tumors</th>
<th>% of mice with carcinomas</th>
<th>No. of tumors/mouse (all)</th>
<th>No. of tumors/mouse (all)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>Intubation p.o.</td>
<td>5.2 mg</td>
<td>-1 hr</td>
<td>39 (37)</td>
<td>14</td>
<td>100</td>
<td>0</td>
<td>0.1 ± 0.05</td>
<td>0.0 ± 0.05</td>
</tr>
<tr>
<td>2</td>
<td>p-Methoxyphenol</td>
<td>Intubation p.o.</td>
<td>5.2 mg</td>
<td>-4 hr</td>
<td>37 (35)</td>
<td>10</td>
<td>97</td>
<td>0</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>Control diet</td>
<td>In the diet</td>
<td>4.5 mg/g</td>
<td>12 to +1 days</td>
<td>32 (29)</td>
<td>19</td>
<td>100</td>
<td>0</td>
<td>4.5 ± 0.5</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

### RESULTS

The effects of p-methoxyphenol on BPL-induced neoplasia of the mouse forestomach are shown in Table 1. Administration of p-methoxyphenol in the diet or by p.o. intubation 4 hr prior to BPL resulted in pronounced inhibitory effects which were of the same magnitude. If p-methoxyphenol was given 1 hr prior to BPL, the inhibition was slightly less. Most of the tumors of the forestomach were papillomas; some carcinomas occurred as well. p-Methoxyphenol markedly inhibited the occurrence of the benign tumors and prevented the appearance of the malignant neoplasms. In a few instances, the carcinomas occupied most of the stomach, and a tumor count could not be done. In Table 1, this is shown by the numbers in parentheses, which designate the total number of mice upon which tumor counts could be performed, being slightly lower than those for the total number of mice at risk. In each experiment in Table 1, 2 control groups were used as described in "Materials and Methods." The tumor response of the 2 controls was almost identical in each case, so these data have been combined.

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In the experiments in which p-methoxyphenol has been studied for its inhibitory effects on BPL or BP-induced neoplasia of the forestomach, the phenol comes into direct contact with the target organ. The question arises as to whether p-methoxyphenol would inhibit at a tissue site distant from the gastrointestinal tract. An experiment of this type was performed with the lung as the target tissue. In Table 2, the effects of feeding p-methoxyphenol on BP-induced pulmonary adenoma formation are presented. No significant inhibition was found. A comparable
experiment with BPL as the carcinogen was not carried out since we have not found conditions under which this carcinogen produced pulmonary adenoma formation. In further work, to define conditions under which p-methoxyphenol inhibits carcinogen-induced neoplasia, an experiment was performed to determine if the phenol would inhibit forestomach tumor formation when given subsequent to all administrations of carcinogen. As will be seen in Table 3, mice fed p-methoxyphenol had slightly fewer tumors of the forestomach, i.e., 28% less, than did the corresponding controls, but this was not statistically significant.

During the course of the studies of BPL-induced neoplasia of the forestomach, mice were fed 2 diets, i.e., powdered Purina chow or a powdered semipurified diet consisting of 27% casein, 59% starch, 10% corn oil, 4% salt mixture, plus a complete vitamin supplement. These diets were started 12 days before the first administration of BPL and were terminated 1 day following the last dose of the carcinogen. At that time, both groups were placed on diets of pellets of Purina chow. The same diets also were fed to mice given BP as the carcinogen. The results of these studies are shown in Table 4. The diet had a marked

Table 2

<table>
<thead>
<tr>
<th>Additions to the diet</th>
<th>No. of mice at risk</th>
<th>Weight gain (g)</th>
<th>% of mice with adenomas/mouse</th>
<th>No. of adenomas/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>15</td>
<td>8</td>
<td>100</td>
<td>20.1 ± 1.7</td>
</tr>
<tr>
<td>p-Methoxyphenol</td>
<td>16</td>
<td>7</td>
<td>100</td>
<td>16.8 ± 1.2</td>
</tr>
<tr>
<td>3-tert-Butyl-4-hydroxyanisole</td>
<td>16</td>
<td>5</td>
<td>100</td>
<td>3.7 ± 0.4</td>
</tr>
</tbody>
</table>

* The compounds indicated were added to Purina chow and fed from 14 days prior to the first dose of BP until 1 day after the last dose. BP (3 mg in 0.2 ml cottonseed oil) was given by p.o. intubation at 2-week intervals with 3 administrations.

Table 3

<table>
<thead>
<tr>
<th>Additions to the diet</th>
<th>No. of mice at risk</th>
<th>Weight gain (g)</th>
<th>% of mice with tumors</th>
<th>No. of tumors/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>34</td>
<td>4</td>
<td>97</td>
<td>6.4 ± 0.61</td>
</tr>
<tr>
<td>p-Methoxyphenol</td>
<td>17</td>
<td>10</td>
<td>100</td>
<td>4.8 ± 0.80</td>
</tr>
</tbody>
</table>

* p-Methoxyphenol was added to Purina chow at a concentration of 0.028 mmol/g. The diets were fed starting 2 weeks after the final dose of BP. BP (1.5 mg in 0.2 ml corn oil) was given by p.o. intubation twice per week for 4 weeks.

Table 4

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Diet</th>
<th>Carcinogen</th>
<th>No. of mice at risk</th>
<th>Weight gain (g)</th>
<th>% of mice with tumors</th>
<th>No. of tumors/mouse</th>
<th>% of mice with carcinomas/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Purina Chow</td>
<td>BPL</td>
<td>(17)</td>
<td>16</td>
<td>100</td>
<td>11</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>Purina Chow</td>
<td>BP</td>
<td>(12)</td>
<td>15</td>
<td>90</td>
<td>0</td>
<td>4.7 ± 0.7</td>
</tr>
<tr>
<td>3</td>
<td>Semipurified</td>
<td>BP</td>
<td>(16)</td>
<td>15</td>
<td>94</td>
<td>0</td>
<td>4.8 ± 0.5</td>
</tr>
</tbody>
</table>

* Mice were fed the diet indicated starting 12 days before the first administration of carcinogen (Experiment 1) or 14 days before the first administration (Experiment 2), and were continued on it until 1 day after the last dose of carcinogen, at which time all mice were placed on a diet of Purina chow.

* BPL (2 mg in 0.1 ml propylene glycol) twice per week for 12 weeks; BP (2 mg in 0.2 ml corn oil) at 2-week intervals with 4 administrations.

* Mice surviving until the termination of the experiment or dying with carcinoma of the forestomach.

* Weight gain from 10 to 36 weeks of age.

* p < 0.001.

Effects of p-Methoxyphenol on BPL-induced pulmonary adenoma formation in female A/JHa mice

Table 1. Compounds studied for their capacity to inhibit BPL-induced neoplasia for the forestomach of ICR/Ha mice. The test compounds were administered by p.o. intubation 4 hr prior to BPL, with the exception of hydroquinone which was given 1 hr prior to BPL. Numbers in parentheses, ratio of the number of tumors occurring per mouse in the group receiving the test compound to that of the corresponding control group. The only compound showing significant inhibition is given 1 hr prior to BPL. Numbers in parentheses, ratio of the number of tumors occurring per mouse in the group receiving the test compound to that of the corresponding control group. The only compound showing significant inhibition is given 1 hr prior to BPL.

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effect on the response to BPL. The number of neoplasms in the mice fed the semipurified diet was considerably greater than that in those fed Purina chow. In contrast, when the carcinogen used was BP, an almost identical neoplastic response was found in the mice fed the 2 diets.

In previous investigations, p-methoxyphenol added to the diet induced a marked increase in GSH S-transferase activity of the mouse forestomach (14). Studies of the effects of single administrations of p-methoxyphenol by p.o. intubation at short time intervals prior to sacrifice were not carried out. Because of the data showing inhibition of neoplasia in experiments in which p-methoxyphenol was administered at 1 or 4 hr prior to BPL, experiments of this type were performed. The effects of p-methoxyphenol on GSH S-transferase activity of the forestomach of mice given this phenol by p.o. intubation 1 or 4 hr prior to sacrifice were determined. No change in activity of this enzyme was found. Likewise, there was no alteration of the acid-soluble sulfhydryl levels of the forestomach at these 2 time intervals.

Since BPL is a direct-acting carcinogen and p-methoxyphenol is a strong nucleophile, a possible mechanism of inhibition is the direct reaction between the carcinogen and the phenol. However, no reaction has been demonstrable in any of the solvent systems used with thin-layer chromatography as the analytical technique. Likewise, efforts at detecting a reaction using NMR were negative. The hydrolysis of BPL occurs in aqueous media to give 3-hydroxypropionic acid. The reaction has been shown to be first order and independent of pH over the pH range of 1 to 7. The hydrolysis of BPL in the presence of the phenol at 22° followed first-order kinetics with a rate constant identical to that found for its hydrolysis in D2O in the absence of the phenol (Kw = 3.4 × 10-3/min), which excludes any catalytic action of p-methoxyphenol on BPL hydrolysis.

DISCUSSION

The results of studies carried out thus far with phenols indicate that p-methoxyphenol has some special characteristics of interest in relationship to inhibition of carcinogen-induced neoplasia. In previous studies of BP-induced neoplasia of the mouse forestomach, this compound was the most potent inhibitor tested. In the present investigation, it showed high potency in inhibiting BPL-induced neoplasia of the forestomach. With BPL, other phenols have little inhibitory effects when administered by p.o. intubation prior to the carcinogen. p-Methoxyphenol inhibits BPL- or BP-induced neoplasia of the forestomach but not BP-induced pulmonary adenoma formation. It inhibits when given either prior to or prior to and simultaneously with carcinogen administration. When fed in the diet subsequent to exposure to BP, p-methoxyphenol had, at most, a small suppressive effect.

The mechanism(s) by which p-methoxyphenol inhibits carcinogen-induced neoplasia has not been established. No evidence has been found for a direct reaction between p-methoxyphenol and BPL. When fed in the diet, p-methoxyphenol is a potent inducer of increased GSH S-transferase activity of the forestomach and esophagus (13, 14). This is an important enzyme system for carcinogen detoxification (2). However, p-methoxyphenol does not increase GSH S-transferase activity or acid-soluble sulfhydryl levels by 4 hr subsequent to administration. Thus, an increase in these parameters cannot account for the inhibitory effects observed when p-methoxyphenol was given by p.o. intubation 4 hr prior to BPL administration. Of possible relevance are studies of responses to a related phenol, 3-BHA; 3-BHA administration produces a coordinated detoxification response entailing enzyme induction and also some modifications of microsomal monoxygenase metabolism that occur within 2 hr after 3-BHA administration (7, 15, 18, 19). This early effect is poorly understood. It is possible that p-methoxyphenol has parallel properties that cause inhibition of carcinogen-induced neoplasia of the forestomach.

A puzzling finding in the studies of the effects of various phenols on BPL-induced neoplasia of the forestomach is the unique inhibitory capacity of p-methoxyphenol; 2-BHA is almost as potent as p-methoxyphenol as an inducer of GSH S-transferase activity of the forestomach and as an inhibitor of BP-induced neoplasia of that tissue (20). One would have anticipated that p-methoxyphenol and 2-BHA would show similar inhibitory properties against BPL. The fact that they do not underscores the special properties of p-methoxyphenol. Exactly what these are remains to be determined. Ultimately, these data could be of considerable importance, since p-methoxyphenol is effective in protecting squamous epithelium of the upper alimentary tract against at least 2 carcinogens, one direct-acting and the other requiring metabolic activation.

The studies of the effects of diet on BPL-induced neoplasia of the forestomach show that the nature of the diet consumed during the period of carcinogen administration can significantly alter the neoplastic response. This alteration is not generic for all carcinogens, as is evident from the finding that the dietary regimens producing differences in the neoplastic response to BPL produced almost no differences when BP was the carcinogen used. Thus, in consideration of data on the relationship of dietary composition to the occurrence of neoplasia in the human, the possibility should be kept in mind that the same dietary factors may provide similar protection against carcinogen-induced neoplasia of that tissue (20).

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