Production of Urothelial Tumors in the Heterotopic Bladder of Rat by Benzidine Derivatives

Ching Y. Wang, Kim Zukowski, Hideyuki Yamada, Katsumi Imada, and Mei-Sie Lee

Department of Chemical Carcinogenesis, Michigan Cancer Foundation, Detroit, Michigan 48201

ABSTRACT

Male Fischer rats which had been implanted with a heterotrophic bladder were randomly divided into five groups and their heterotrophic bladders were instilled once a week for 20 weeks with 0.5 ml phosphate-buffered saline:dimethyl sulfoxide solution (4:1) or this solution containing 1 μmol benzidine (BZ), N'-hydroxy-N-acetylbenzidine, the N'-glucuronide of N'-hydroxy-N-acetylbenzidine, or the N-glucuronide of N-hydroxy-2-aminofluorene. These bladders were then instilled once a week for an additional 30 weeks with the phosphate-buffered saline without dimethyl sulfoxide. The experiment was terminated at the end of 50 weeks. Transitional cell carcinomas were observed in 1 of 39 (control), 1 of 29 (BZ), 18 of 30 (N'-hydroxy-N-acetylbenzidine), 28 of 28 (N'-hydroxy-N-acetylbenzidine A'-glucuronide), and 24 of 29 (N'-hydroxy-2-aminofluorene N'-glucuronide) rats. No histological alterations were observed in their natural bladders. These results demonstrate the urothelial carcinogenicity of the N-hydroxy metabolites of BZ and suggest that N'-hydroxy-N-acetylbenzidine N'-glucuronide may play a major role in the initiation of urothelial carcinogenesis by BZ in humans.

INTRODUCTION

Exposures to certain industrially utilized arylamines have been associated with a high incidence of urinary bladder cancer in humans (1). Based on data collected from animal studies, it has been proposed that the carcinogenic potential of arylamines may be related to the metabolites produced in or otherwise transported to the susceptible target organ during the process of xenobiotic metabolism. The transport of hydroxylamines, presumably the proximate bladder carcinogen as proposed for 2-AN(1) and 4-ABP, may be facilitated by prior glucuronide conjugation in the liver and, once within the acidic bladder milieu, the hydroxylamines may become liberated from their glucuronide moiety (2, 3). The regenerated hydroxylamines can be activated by conjugations in urothelial cells (4, 5). Since arylamines can be directly activated by PHS to react with nucleic acids (6, 7), peroxidation has been proposed as an additional pathway for their activation (8).

BZ is used in the chemical dye industry, where human exposure during the manufacturing process has resulted in an increased incidence of bladder cancer. It produces various tumors in experimental animals (1, 9). Results presented thus far have suggested that ABZ may be the main metabolite contributing to BZ carcinogenesis in species that are capable of acetylation. For example, BZ is metabolized to ABZ by cultured human hepatocytes (10). Although cultured rat hepatocytes convert BZ to ABZ and DABZ (10), ABZ produces more DNA adducts than does DABZ in rat liver in vivo, and the DNA adducts contain the acetyl group of ABZ (11). Furthermore, BZ is more carcinogenic than DABZ in the rat (12). Dogs cannot acetylate BZ and they are much less susceptible than humans and rats to BZ carcinogenesis (9). It is thus speculated that BZ is metabolized in humans in the liver to N'-OH-N'-GI-ABZ, is transported to the urinary bladder, and upon hydrolysis releases N'-OH-ABZ, which is then absorbed and activated by O-acetylation to initiate bladder carcinogenesis. Metabolism by PHS represents an alternative activation pathway. BZ is the best substrate among known carcinogenic arylamines for PHS (7). Two-electron oxidation by PHS yields benzidine diimine, which is the predominant reactive intermediate for the DNA binding (13). Since human urothelial cells have both PHS (14) and O-acetylase (5), these two activation pathways may contribute to the bladder carcinogenesis of BZ, but only if BZ and N'-OH-N'-GI-ABZ, with activation by urothelial enzymes, can induce bladder tumors. The present study investigated the carcinogenic activities of these two compounds in the heterotopically implanted bladder of rat (15-17), a species whose urothelial cells have PHS activity (18) and are capable of activating hydroxylamines (4, 19).

MATERIALS AND METHODS

Instrumentation. IR spectra were recorded with a Perkin Elmer IR spectrophotometer (using KBr discs), UV spectra with a Varian Cary 219 spectrophotometer, and nuclear magnetic resonance spectra with a GE QE-300 NMR. Melting points were measured with an electrothermal melting point apparatus. Elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN).

Chemicals. N'-OH-N'-GI-ABZ was synthesized as previously described (20). BZ was kindly provided by Dr. E. Zeiger (National Institute of Environmental Health Sciences, Research Triangle Park, NC). BZ was converted to 4-acetamido-4'-nitrobiphenyl (21). Reduction of this product to N'-OH-ABZ was accomplished according to the published procedure (22). Reaction of N'-OH-ABZ with ammonium glucuronate, as described previously (20), yielded N'-OH-N'-GI-ABZ. The yield of the N-glucuronide from N'-OH-ABZ was 60%. The physical properties of the compound were: completely charred at 200°C without a melting point; NMR (in D2O) δ 9.96 (s, 1, amide H), 7.62-7.08 (m, 8, aromatic Hs), 4.78-4.75 (doublet of C9—H of the sugar, J = 8.7 Hz), 3.62-3.08 (m, C2—C5 Hs of the sugar; the signals were partially shielded 10 mvi potassium phosphate, pH 7.0), ε = 26,800; IR: 1600 and 1400 cm⁻¹, that were also found with other N-glucuronides of arylhydroxylamines (20).

Ammonium salt of N'-OH-N'-GI-ABZ·3H2O (C2aH3aN3O11)
Calculated: C 49.08, H 6.33, N 8.58
Found: C 49.31, H 6.09, N 8.45
Reactions of this compound with trisodium pentacyanoferrate, ammoniacal silver nitrate, or \(p\)-dimethylaminobenzaldehyde gave colored products that are characteristic of the \(N\)-glucuronides of arylhydroxylamines (20). These data confirmed that the product was indeed \(N'{-}OH-N'{-}GI-ABZ\). The NMR signal of the \(CH_3OH\) of the sugar, which was a doublet with a coupling constant of 8.7 Hz, is indicative of a \(\beta\)-glucuronide (20).

**Preparation of Injection Port-Bladder Unit.** Male Fischer rats weighing approximately 200 g were obtained from Charles River Breeding Co. (Wilmington, MA). Transplantation of the donor bladder into the recipient animal was performed according to the procedure of Oyasu et al. (15, 16). Briefly, the animals were anesthetized with an i.p. injection of sodium brevital (Eli Lilly; Indianapolis, IN). The donor bladder was removed, ligated to the connecting tube of an injection port unit, and inflated with 0.5 ml of phosphate-buffered saline, pH 7.0, containing 0.15 mg of gentamicin sulfate. The gluteal muscle of the recipient was then removed and the bladder was implanted into the cavity thus produced. One week later, the bladder fluid was removed and then replaced with 0.5 ml of fresh phosphate-buffered saline/gentamicin solution, using a syringe equipped with a 23-gauge needle. This procedure was repeated once weekly for 4 weeks before starting the carcinogen treatment.

**Tumor Induction.** During the entire experiment, animals were kept on Rodent Lab Blox (Continental Grain Co., Chicago, IL) and drinking water. The animals were housed in plastic rat cages with no more than three animals per cage. The animal room was maintained at 22–24°C and 55% relative humidity, with a 12-h light/dark cycle. Animals were weighed once per month. The pH of the fluid removed from the bladder was examined with pH papers (pH range, 4.0–7.0 and 6.5–10.0; EM Laboratories, Inc., Elmford, NY). Monitoring of microbial contamination in the bladder was performed periodically by inoculating the bladder fluid into Difco nutrient broth and incubating overnight at 37°C.

Animals with functioning injection port-bladder units were randomly divided into four test groups with 30 animals each and a control group with 40 animals. Their heterotopic bladders were treated with injections, once a week for 20 weeks, of 0.5 ml of the phosphate-buffered saline/gentamicin solution:DMEM, 4:1 (control group), or this solution containing 1 \(\mu\)mol of BZ, \(N'{-}OH-ABZ\), \(N'{-}OH-N'{-}GI-ABZ\), or \(N'{-}OH-N'{-}GI-AF\). \(N'{-}OH-N'{-}GI-AF\) was used as a positive control (17). Each of the four test compounds was first dissolved in DMSO and then added directly to 4 volumes of the phosphate-buffered saline/gentamicin solution, which was used within the 10 min needed for the fluid exchange in each group. The heterotopic bladders were then treated with injections of 0.5 ml phosphate-buffered saline/gentamicin solution once a week for an additional 30 weeks. The experiment was terminated at the end of 50 weeks. The animals were sacrificed and necropsied, with their injection port-bladder units being isolated from surrounding tissue. The heterotopic bladder, along with the natural bladder, were filled with a fixative solution (ethanol:formalin:glacial acetic acid:distilled water, 30:9:1:60). The bladders were cut into strips, which were then embedded into paraffin, sectioned, and stained with hematoxylin and eosin for histological examination. Statistical analyses were performed using the Fisher 2 × 2 contingency table.

**RESULTS**

Only one heterotopic bladder was found to have microbial contamination during the experiment. Additionally, four heterotopic bladders failed due to their separation from the connecting tube of the injection port. These animals were not included in the evaluation. The pH of the vesicle fluid was between 7.1 and 7.4. Histological alterations of the heterotopic bladders, including inflammatory polyps, hyperplasia, and tumors, were classified according to the method of Ozono et al. (16). Inflammatory polyps, which developed near the tip of the connecting tube, were frequently found in the control and BZ groups. They were less frequently observed in other groups which had tumors (Table 1). The incidence of transitional cell carcinomas was significantly greater in the \(N'{-}OH-ABZ\), \(N'{-}OH-N'{-}GI-ABZ\), and \(N'{-}OH-N'{-}GI-AF\) groups than in the control group (\(P < 0.001\)). The incidence of bladder tumors in the \(N'{-}OH-N'{-}GI-ABZ\) group was significantly greater than that in the \(N'{-}OH-ABZ\) (\(P < 0.01\)) and the \(N'{-}OH-N'{-}GI-AF\) groups (\(P < 0.05\)). No tumors or hyperplastic lesions were found in the natural bladders of these rats.

In agreement with previously published results (4, 19, 23), \(N'{-}OH-N'{-}GI-AF\) produced UDS in both HCV-29 and rat urothelial cells, but BZ did not (Table 2). \(N'{-}OH-ABZ\) and \(N'{-}OH-N'{-}GI-ABZ\) also produced UDS in these cells (Table 2).

**DISCUSSION**

Development of the HTB system by Oyasu et al. has provided a useful method for characterizing the carcinogenic potential of metabolites believed to be involved in the target organ-specific activation of these agents. Urinary bladder carcinogens have been demonstrated to expose the target tissue via urinary tract transport. The direct administration to a HTB of a metabolite believed to be crucially related to bladder tumor induction circumvents systemic circulation, since absorption of the compound into the circulatory system will result in excretion through the kidney and exposure of only the normal in situ bladder. This mechanism may have been responsible for the observation that \(N\)-butyl-N-(3-carboxypropyl)nitrosamine administered directly into a HTB produced tumors only in the normal bladder, i.e., systemic metabolism of the compound by tissues other than the HTB were required to convert the compound to a metabolite that could initiate the urothelial cells (24).

In attempting to assess the relative importance of a specific metabolite for its carcinogenic potential in the bladder, it is reasonable to compare the tumorigenic response of equal doses of related metabolites. In the present study, the dose administered was 1 \(\mu\)mol/week, which was estimated to be approximately equal to the amount of weekly excretion of free BZ by a rat consuming a diet contained 0.02% BZ. The results of this study clearly establish that, on a molar basis, the \(N\)-hydroxylated BZ metabolites are more carcinogenic than the parent compound. Although this study does not preclude the possibility that higher doses of BZ may be tumorigenic in the HTB, the inability of BZ to elicit UDS in rat urothelial cells argues against such a carcinogenic potential.

Previously, attention has been drawn to the cleavage of the carbohydrate moiety from \(N\)-glucuronides in acidic media. In the present study, the agents were instilled in a neutral solution. It is possible that the comparatively greater stability of the \(N\)-glucuronide that would be expected at this pH contributes to a longer retention time in the HTB and, consequently, a greater carcinogenic potential because of the extended period over which exposure takes place.

2-AN has been estimated to be approximately 3 times more potent than BZ in inducing human urinary bladder cancer (25). It is much more potent than BZ in the dog (1). However, BZ is much more potent than 2-AN in other species (1). Thus, although it is impractical to use humans to elucidate the mechanisms of BZ carcinogenesis in humans, a reasonable prediction...
Male Fischer rats which had been implanted with a heterotopic bladder were treated once a week for 20 weeks with instillations of 0.5 ml phosphate-buffered saline-DMSO solution (4:1), pH 7.0, or this solution containing 1 μmol of the test compounds. The bladders were then instilled once a week for an additional 30 weeks with 0.5 ml of the buffered saline without DMSO. The experiment was terminated at the end of 50 weeks.

Table 1  Histological findings of heterotopic bladders of rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Effective no. of rats</th>
<th>Carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Hyperplasia</td>
</tr>
<tr>
<td>Control</td>
<td>39</td>
<td>10</td>
</tr>
<tr>
<td>BZ</td>
<td>29</td>
<td>7</td>
</tr>
<tr>
<td>N'-OH-ABZ</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>N'-OH-N'-Gl-ABZ</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>N-OH-N-Gl-AF</td>
<td>29</td>
<td>5</td>
</tr>
</tbody>
</table>

* Only the most severe lesions are given. For example, if a rat has hyperplasia and grade 1 tumor, it is entered only as a grade 1 carcinoma. The number of rats with polyplys is given irrespective of other lesions.

ABZ are derivatives of p-substituted phenyldihydroxylamine and they may have similar activation mechanisms. It has been claimed that BZ produces a urothelial DNA adduct which is consistent with a peroxidation mechanism (13). Thus, if the PHS pathway is involved in the activation of arylamines, it may be important only for BZ and only in the dog.

Arlyhydroxylamines such as N'-OH-ABZ can be activated by O-acetylases (5, 28–33). These enzymes, which are present in cytosols of several species, also catalyze N-acetylation of arylamines and N,O-acetyltransfer of arylhydroxamic acids. Microsomal enzymes also can catalyze O-acetylation of hydroxylamines and N,O-acetyltransfer of arylhydroxamic acids (5, 30, 34). These cytosolic and microsomal enzymes are present in human urothelial cells (5). Dogs cannot N-acetylate ary lamines (9), but their urothelial microsomes can catalyze activation by N,O-acetyltransfer (4) and O-acetyltransfer (19). These ur othelial enzymes may activate N'-OH-ABZ, which may be released in the urine from its glucuronic acid conjugate.

Evidence has been presented that the slow acetylator workers who have been exposed occupationally to BZ have a greater chance of contracting urinary bladder cancer than their rapid-acetylator counterparts (35). If, as we postulate, N'-OH-N'-Gl-ABZ is responsible for the induction of bladder tumors by BZ, then the slow-acetylator phenotype may excrete more of this metabolite than the rapid-acetylators. Thus, the latter may further acetylate ABZ to DABZ. Further metabolism of DABZ may yield the O-glucuronide of N-OH-DABZ. Like the O-glucuronide of N-OH-AAF, which is inactive in producing DNA damage repair in urothelial cells (23) and is a very weak carcinogen in vivo (36), the glucuronide of N-OH-DABZ may not be carcinogenic. The O-glucuronide of N-OH-DABZ has been detected in the urine of rats that had been given BZ (37), and the urinary bladder is not a target organ of BZ in the rat. N'-OH-ABZ has only been identified as an in vitro metabolite of BZ (38). Probably because of their instability, neither N'-OH-ABZ nor its N-glucuronide has been detected in the urine.

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REFERENCES

CARCINOGENICITY OF BENZIDINE METABOLITES


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