Inhibitory effects of 1,3-diaminopropylene, an ornithine decarboxylase inhibitor, on rat two-stage urinary bladder carcinogenesis initiated by N-butyl-N-(4-hydroxybutyl)nitrosamine

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Overexpression of ornithine decarboxylase (ODC) has been shown to be characteristic of tumor development and progression in humans and experimental animals. Therefore, we have examined the effects of 1,3-diaminopropylene dihydrochloride (DAP), a potent inhibitor of ODC, on rat two-stage urinary bladder carcinogenesis initiated with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN). In experiment 1 (36 weeks), 6-week-old F344 male rats were administered 0.05% BBN in drinking water for 4 weeks and then divided into four groups. Animals of groups 1 and 2 received basal diet and drinking water supplemented with or without DAP (2 g/l). Groups 3 and 4 were given diet containing 5% sodium L-ascorbate (NaAsA), a typical urinary bladder tumor promoter, and drinking water with or without DAP. Administration of DAP to group 1 significantly reduced tumor size, multiplicity and incidence, particularly of papillomas, when compared with group 2 values. DAP together with NaAsA (group 3) also decreased tumor size relative to the group 4 case. To determine the effects of DAP on the early stages of bladder carcinogenesis and its mechanisms, a similar protocol was conducted (experiment 2) with death after 20 weeks. DAP treatment caused complete inhibition (0% incidence) of papillary and/or nodular hyperplasia in group 1 but was without influence in group 3, as compared with the respective controls. Moreover, the ODC activity, bromodeoxyuridine labeling indices and mRNA expression levels of cyclin D1 in the urinary bladder mucosa, determined by northern blotting, were markedly lower in group 1 than in group 2, but values were comparable for both groups administered NaAsA. Assessment of mRNA expression levels of the angiogenic vascular endothelial growth factor suggested no involvement in the inhibitory effects of DAP on urinary bladder carcinogenesis. The results indicate that inhibition of ODC could reduce urinary bladder carcinogenesis in rats, particularly in the early stages, through antiproliferative mechanisms.

Introduction

Urinary bladder cancer is one of the leading causes of cancer deaths in the urological field world wide. Lifestyle and environmental factors, such as particular occupations, cigarette smoking, alcohol intake, consumption of foodstuffs containing nitrosamines, certain infectious diseases (such as schistosomiasis) and stone formation, have been suggested to be associated with urinary bladder cancer formation (1,2). All these factors could elevate the chance of transformation of preneoplastic into malignant lesions or accelerate recurrence after an apparently successful surgical operation for bladder cancer. The development of new chemopreventive and chemotherapeutic agents to counter their effects is a high priority.

Enzymes involved in the biosynthesis of the polyamines are considered as targets in this respect (3). Ornithine decarboxylase (ODC) is a key regulatory enzyme in the biosynthesis of polyamines, which are essential for cell growth and differentiation (4). Accumulating evidence indicates that ODC overexpression may play a critical role in neoplastic transformation (5) and ODC activity has been shown to be moderately elevated divided 0.05% BBN in drinking water for 4 weeks and then overexpression of the polyamine biosynthesis pathway, has been shown to significantly reduce tumor size, multiplicity and incidence, particularly of papillomas, when compared with group 2 values. DAP together with NaAsA (group 3) also decreased tumor size relative to the group 4 case. To determine the effects of DAP on the early stages of bladder carcinogenesis and its mechanisms, a similar protocol was conducted (experiment 2) with death after 20 weeks. DAP treatment caused complete inhibition (0% incidence) of papillary and/or nodular hyperplasia in group 1 but was without influence in group 3, as compared with the respective controls. Moreover, the ODC activity, bromodeoxyuridine labeling indices and mRNA expression levels of cyclin D1 in the urinary bladder mucosa, determined by northern blotting, were markedly lower in group 1 than in group 2, but values were comparable for both groups administered NaAsA. Assessment of mRNA expression levels of the angiogenic vascular endothelial growth factor suggested no involvement in the inhibitory effects of DAP on urinary bladder carcinogenesis. The results indicate that inhibition of ODC could reduce urinary bladder carcinogenesis in rats, particularly in the early stages, through antiproliferative mechanisms.

Abbreviations: N1-AcSPD, N1-acetylsporamide; BrdU, bromodeoxyuridine; BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; DAP, 1,3-diaminopropane; DFMO, α-difluoromethylornithine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NEL, nick-end labeling; ODC, ornithine decarboxylase; PN, papillary or nodular; Rb, retinoblastoma; NaAsA, sodium L-ascorbate; SAT, spermidine/spermine N1-acetyltransferase; TBS, Tris-buffered saline; VEGF, vascular endothelial growth factor.
application of NaCl, a gastric tumor promoter, to rats was recently shown to induce ODC and SAT activities with a peak after 5–7 h (22). Also, ODC overexpression is apparently intimately involved in tumor promotion in mouse skin (23). To test the possibility that suppression of ODC might inhibit carcinogenesis in the bladder, the post-initiation effects of DAP with and without NaAsA were assessed in the present experiment. Studies in vitro and in vivo have shown that ODC inhibition may be a way to control cell proliferation (24) and specific inhibitors can prevent neoplastic development associated with antiproliferative potential in a number of organs (3). In animal models, DAP may inhibit cancer while reducing proliferation (13–17). Homma et al. (25) earlier showed that the inhibitory characteristics of the ODC inhibitor DFM0 on rat urinary bladder carcinogenesis were accompanied by a remarkable inhibition of cell proliferation. Induction of cell proliferation and inhibition of apoptosis are suggested to be one mechanism underlying tumor promotion (26), with significant roles for growth factors (27,28).

Moreover, studies in human cancer have shown that G1 cyclins and their cyclin-dependent kinases (Cdks) are consistently altered cell cycle regulatory proteins, with accumulating evidence that deregulated expression of cyclin D1 and Cdk4 is associated with malignancy (29,30). Although a number of genetic changes and cell cycle alterations have been detected in urinary bladder cancer in humans and rodents (31,32), it is not yet clear whether these alterations in cell cycle regulatory proteins and growth factors could also be involved in chemopreventive processes in this organ. To examine mechanisms in vivo, assessment of mRNA expression levels of the angiogenic vascular endothelial growth factor (VEGF), cyclin D1 and Cdk4, ODC and SAT activities and polyamine levels as well as apoptotic indices have been included as parameters in the present study.

Materials and methods

Animals and diet

A total of 157 male F344 rats (Charles River Inc., Hino, Japan), 5 weeks old at commencement, were used. The animals were housed four or five to a plastic cage, with wood chips for bedding, and were given water and MF powdered diet (Oriental Yeast Co., Tokyo, Japan) ad libitum. They were kept for a 1 week acclimation period before the start of the experiments. The animal room was controlled at a temperature of 22 ± 1°C, at 55 ± 5% humidity and with a 12 h/12 h light/dark cycle.

Chemicals

BBN was obtained from Tokyo Kasei Co. (Tokyo, Japan). DAP (Sigma, St Louis, MO) and NaAsA were from Wako Industries Co. (Osaka, Japan).

Experiment I

Two-stage bladder carcinogenesis study. A total of 67 rats were divided into four groups taking body weights into account, in order to decrease the standard errors in each group. All animals were given 0.05% BBN in drinking water into S phase DNA in representative PN hyperplasias and tumors (papillomas and carcinomas). A total of 67 rats were divided into four groups taking body weights into account, in order to decrease the standard errors in each group. All animals were given 0.05% BBN in drinking water into S phase DNA in representative PN hyperplasias and tumors (papillomas and carcinomas).

Fig 1. Experimental protocol (experiment I). ☐ 0.05% BBN in drinking water; ☐ 2 g/l DAP in drinking water; ☐ no treatment; ☐ 5% NaAsA in diet + 2 g/l DAP in drinking water; ☐ 5% NaAsA in diet; U, urinalysis and pH measurement; ▼, killing.

After evaluation of the pH value of each sample (model F-15 pH meter; Horiba, Kyoto, Japan), all samples were kept frozen at –80°C until the urinary electrolyte levels of Na, K, Cl, Ca and P (710 electrolyte analyzer; Hitachi, Tokyo, Japan) and the urinary protein and creatinine levels (ARC 950; Aloka, Tokyo, Japan) were determined.

Tumor evaluation. All animals were killed under deep ether anesthesia. Urinary bladders from all groups were ligated at the neck, inflated by intraluminal injection of 10% phosphate-buffered formalin, removed quickly and immersed in fixative. After fixation, bladders were bisected longitudinally and excess moisture was absorbed with tissue paper. After being weighed, sketches of all the bladders, including any tumors, were drawn to facilitate histological quantitation and counting. To evaluate tumor volume (v), the long (l) and short (w) dimensions (mm) of each tumor mass (length and width) were measured with calipers and cell count was obtained by the equation $v = (l \times w^2)/2$. Livers and kidneys of all animals were removed and weighed and all organs were examined macroscopically for any changes.

Tissue processing. Directly after measurement of tumor volume, bladders were cut into eight strips and embedded in paraffin. Tissue processing was performed for histopathological examination (stained with hematoxylin and eosin) and immunohistochemical staining (4 μm) for BrdU and nick-end labeling (NEL) to detect cell proliferation and apoptosis, respectively. Epithelial lesions of the bladder were histologically classified into simple hyperplasia, papillary or nodular (PN) hyperplasia, papilloma and carcinoma (33). For quantitation, the bladder lesions were counted under a light microscope, the total length of the base membrane was measured with a color image analyzer (IPAP; Sumika Technos Corp., Osaka, Japan) and multiplicities of lesions were calculated as numbers per 10 cm.

BrdU staining and evaluation of labeling indices. Immunohistochemical staining for BrdU was performed using an avidin–biotin complex (ABC) kit in combination with an anti-BrdU monoclonal antibody, according to the manufacturer’s instructions (code no. M0744; Dako A/S, Denmark). Tissue sections were deparaffinized with xylene, hydrated through a graded ethanol series and incubated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. They were then incubated with 10% normal horse serum at room temperature for 30 min to block background staining and overnight at 4°C with BrdU diluted 1:1000 in Tris-buffered saline (TBS). After exposure for 30 min at room temperature to biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA), sections were incubated with avidin–biotin complex at 1:25 dilution. Each step was followed by washing with TBS. Peroxidase activity was visualized by treatment with a 0.02% solution of diaminobenzidine tetrahydrochloride containing 0.05% hydrogen peroxide. Then nuclei were counterstained with hematoxylin.

The labeling indices were evaluated as percentages of cells incorporating BrdU into S phase DNA in representative PN hyperplasias and tumors (papillomas and carcinomas).

In situ detection of apoptosis. To assess possible relationships between metabolic stress caused by DAP or NaAsA and programmed cell death in tumors, direct immunoperoxidase detection of digoxigenin-labeled genomic DNA fragmentation in situ was performed according to the manufacturer’s instructions by the NEL technique (transfer of biotinylated nucleotide to the 3’–OH end), using an ApoTag in situ apoptosis kit (catalog no. S7100; Oncor, Gaithersburg, MD). Apoptotic indices were generated by counting NEL apoptotic bodies in PN hyperplasias and tumors, expressed as percentage values.

Experiment II

Two-stage bladder carcinogenesis study. The same two-stage bladder carcinogenesis protocol as in experiment I with an experimental period of 20 weeks
was carried out to evaluate the effects of DAP on the early stages of bladder carcinogenesis. A total of 80 rats were divided into the same four groups (20 per group). An additional group of 10 non-treated rats were kept separately as a control group for collection of urinary bladder mucosa for northern blotting analysis. Animal killing, bladder preparation, determination of tumor incidences and multiplicities, histological evaluation and immunohistochemical analysis for BrdU labeling indices were performed as described above.

Tissue preparation for ODC and SAT activities, polyamine levels and northern blotting. Whole urinary bladders of 15 randomly chosen rats from each group and from the control non-treated group were opened longitudinally and incubated in phosphate-buffered saline (pH 7.5) containing 25 mM EDTA for 10 min at 37°C. The urinary bladder mucosa were scraped off with sterilized clean scissor blades immediately before frozen at −80°C until assayed for ODC and SAT activities and total polyamine levels and northern blot analysis.

ODC and SAT activities and polyamine assays. The activities of ODC and SAT were measured by a radiometric technique from release of ^14CO_2 from [t-^14C]ornithine, as described previously (34). Briefly, frozen pieces of urinary bladder epithelium from 10 samples were suspended separately in 0.5 ml of 50 mM Tris (pH 7.5) containing 0.25 M sucrose and disrupted with a homogenizer for 30 s. The homogenized suspensions were centrifuged at 100,000 g for 30 min and the supernatants were assayed for ODC and SAT by measurement of the amount of radioactive putrescine produced from [5-^3H]ornithine and of acetyl moiety transfer from [1,4-^14C]acetyl-CoA to spermidine, respectively.

The concentrations of the polyamines, spermine, spermidine and N1-acetyl spermidine (N1-AcSPD) were analyzed by HPLC with a fluorescence detector as described previously by Matsui-Yuasa et al. (21) and separated on an STB ODS-II column (2)×150 mm, particle size 5 μm (Shimadzu Techno Research, Kyoto, Japan). Calf thymus DNA (Sigma, St Louis, MO) was used as a control for the determination of DNA polyamine content.

Extraction of total RNA. Total RNA was extracted from the urinary bladder epithelium of five rats from each group. Briefly, frozen mucosal tissues were homogenized with a polytron homogenizer (PCU-11; Kinematica AG) for 60 s at dial speed 10 in denaturing solution (4 mol/l guanidinium thiocyanate, 25 mMol/l sodium citrate, pH 7.0, 1.0 mol/l 2-mercaptoethanol and 0.5% sodium lauryl sarcosine). The homogenate was added to 1/10 vol of water, 1 vol of phenol and 1/2 vol of chloroform and centrifuged at 10,000 g at 4°C for 20 min. The resulting upper aqueous phase was transferred to a fresh tube and precipitated by addition of 1 vol of isopropanol followed by centrifugation. The precipitate was dissolved in the above solution, incubated with 3.5 mol/l lithium chloride at 4°C for 18 h and then centrifuged. The RNA pellet was washed with 3 mol/l lithium chloride and then with 70% ethanol. Finally, it was dissolved in 0.1% diethylpyrocarbonate-treated water and stored at −80°C until applied for northern blot analysis. RNA concentrations were spectrophotometrically determined at 260 nm.

Northern blot hybridization. For RNA hybridization, 10 μg samples of total RNA were denatured by incubation with 1 mol/l deionized glyoxal and 50% dimethylsulfoxide at 50°C for 1 h and electrophoresed on 1% agarose gels at 50 V. The 28S and 18S rRNAs in gels were stained with ethidium bromide to determine the integrity of applied RNA and to verify that the same amounts of RNA were applied to each lane. RNAs in the gels were then transferred to nylon membranes (GeneScreen Plus; E1 du Pont de Nemours & Co., France). cDNA probes were labeled with [γ-^32P]dCTP (sp. act. 3000 Ci/mmol/l; Du Pont) using a Random Primer DNA Labeling Kit (Takara). The membranes were prehybridized in a solution containing 50% formamide, 5× Denhardt’s solution, 1% SDS and 200 μg/ml denatured salmon sperm DNA by heating for 4 h at 42°C and hybridized with probes for cyclin D1, Cdk4 and VEGF as well as ^32P-labeled (1-18^{10^6}d.p.m./ml) gliceraldehyde 3-phosphate dehydrogenase (GAPDH) at 42°C for 24 h in fresh hybridization solution that was identical to the prehybridization solution except for the absence of salmon sperm DNA. After washing, membranes were exposed to Kodak XAR-5 film between two intensifying screens at −70°C. To strip off the hybridized probe, the nylon membranes were boiled in 0.1× SSC solution containing 1% SDS for 30 min and then rehybridized with other cDNA probes. The densities of the autoradiograms in band in digitized images were measured using the public domain National Institutes of Health IMAGE program and a personal computer (Power Mactintosh G3; Apple Computers). For all RNA samples, the density of individual mRNA bands was divided by that of the GAPDH mRNA band to correct for differences in RNA loading and/or transfer.

cDNA probes. The cDNA probes used were as follows. The probe for cyclin D1 was produced in the First Department of Pathology, Osaka City University, as previously described (32). Briefly, a cyclin D1 fragment was synthesized by PCR amplification (45 cycles) of cDNA extracted from liver of a control rat with primers 5'-AACAGCTCTTCTGTGTGGCAAA-3' (upstream) and 5'-CAGGACCTCTTCTGCAAC-3' (downstream) obtained from the genebank of the National Centre for Biotechnology Information home page. The primer sequences were assayed using the software GENETEX MAC v.8.0. The resultant PCR products were inserted into plasmid pCR-Script SK (+) using a PCR-Script SK (+) cloning kit (Stratagene, La Jolla, CA). cDNA clones containing the template sequence for sense and antisense RNA probe synthesis were propagated and plasmid DNA was recovered with a plasmid extraction kit (Plasmid Midi; Qiagen, Chatsworth, CA). cDNA clones containing the template sequence for sense and antisense RNA probe synthesis were propagated and plasmid DNA was recovered with a plasmid extraction kit (Plasmid Midi; Qiagen, Chatsworth, CA). The sense probes were generated as 1.0 kb HindIII–HindIII fragments and RNA synthesis was with T3 RNA polymerase. The rat Cdk4 cDNA was a 1.2 kb EcoRI–EcoRI fragment (35), the VEGF cDNA was a 1.8 kb Self–Self fragment (36) and the GAPDH DNA was a 1.3 kb Pst–Pst fragment (37).

Statistical analysis

Significances of differences between group mean values were analyzed using the paired two-tailed Student’s t-test and values for bladder lesion incidence were analyzed using the χ^2 or exact probability test (StatView v.4.5; Abacus Concepts).

Results

Experiment I

Two stage bladder carcinogenesis. Clinical observation did not reveal any abnormalities among the groups and no deaths occurred during the treatment period. Data on final body weight and relative and absolute liver, kidney and urinary bladder weights are presented in Table I. Animals treated with BBN alone had the highest body weights among the groups. Groups treated with DAP (groups 1 and 3) showed slight body weight decreases, although this was not significant, compared with the other groups. The bladder weights of animals in group 3 were significantly less than those in group 4. Water intake tended to be decreased slightly in groups administered DAP but food consumption was not essentially changed.

Urinalysis. Table II shows data for urinary pH values and concentrations of urinary electrolytes. Elevation of pH was associated with NaAsA treatment. Data on the concentrations of Na and P were significantly elevated by NaAsA treatment as compared with group 2 and the concentration of K was lowered. DAP treatment was without significant effects on ion concentrations except that Cl in group 3 was increased compared with groups 2 and 4.

Urinary bladder lesions. Grossly, papillary tumors were seen in the urinary bladders of all groups. The degrees of lesion development in rats receiving NaAsA (groups 3 and 4) were markedly greater than in groups 1 and 2. Histopathological findings are summarized in Table III. Most of the carcinomas were of the papillary transitional cell type, with rare invasive or squamous metaplasia. Administration of DAP to group 1 caused a significant reduction in the incidence of papillomas and significantly decreased the multiplicity of PN hyperplasia and papillomas. The incidence of carcinomas also showed a tendency to decrease, but this did not reach statistical significance. The tumor sizes (of papillomas and carcinomas), expressed as volume, were also significantly lower in group 1 as compared with group 2 (Figure 2). DAP administration together with NaAsA in group 3 slightly reduced the multiplicities of papillomas and carcinomas, when compared with group 4, and significantly decreased their sizes (Figure 2).

Cell proliferation in bladder tumors. Labeling indices (%) for bladder tumor cells as assessed by incorporation of BrdU into DNA in rats of all groups are shown in Figure 3. PN hyperplasia and bladder tumors (papillomas and carcinomas) in the DAP-
Table I. Data for final body, liver, kidney and urinary bladder weights and water intake of F344 rats treated with BBN followed by DAP and/or NaAsA (experiment I)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Treatment</th>
<th>Final body wt (g)</th>
<th>Body wt (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Kidney wt (g)</th>
<th>Bladder wt (g)</th>
<th>Water consumption (g/rat/day)</th>
<th>Food consumption (g/rat/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17</td>
<td>BBN→DAP</td>
<td>351 ± 22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.9 ± 0.8 (2.6)</td>
<td>1.0 ± 0.1 (0.3)</td>
<td>0.2 ± 0.2 (0.1)</td>
<td>20.9 ± 0.6</td>
<td>17 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>BBN control</td>
<td>373 ± 12</td>
<td>10.1 ± 0.8 (2.7)</td>
<td>1.0 ± 0.2 (0.3)</td>
<td>0.4 ± 0.4 (0.1)</td>
<td>21.8 ± 0.3</td>
<td>17 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>BBN→NaAsA + DAP</td>
<td>344 ± 12</td>
<td>10.3 ± 0.5 (2.9)</td>
<td>1.1 ± 0.1 (0.3)</td>
<td>0.8 ± 0.3&lt;sup&gt;c&lt;/sup&gt; (0.2)</td>
<td>19.2 ± 0.9</td>
<td>16 ± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>BBN→NaAsA</td>
<td>353 ± 26</td>
<td>10.1 ± 0.7 (2.9)</td>
<td>1.1 ± 0.1 (0.3)</td>
<td>1.1 ± 0.5 (0.3)</td>
<td>23.5 ± 0.4</td>
<td>17 ± 0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ratio (%) to body weight.
<sup>b</sup>Values are means ± SD.
<sup>c</sup>Significantly different from the group 4 value, *P* < 0.001 (Student’s *t*-test).

Table II. Urinary levels of electrolytes and urinary pH values for F344 rats treated with BBN followed by DAP and/or NaAsA (experiment I)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of samples</th>
<th>Urinary protein (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
<th>Na (mEq/l)</th>
<th>K (mEq/l)</th>
<th>Cl (mEq/l)</th>
<th>Ca (mg/dl)</th>
<th>P (mg/dl)</th>
<th>pH value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BBN→DAP</td>
<td>10</td>
<td>362 ± 358&lt;sup&gt;a&lt;/sup&gt;</td>
<td>181 ± 39</td>
<td>132.0 ± 54.0</td>
<td>334.4 ± 48.5</td>
<td>182.0 ± 83.9</td>
<td>7.2 ± 1.0</td>
<td>127.6 ± 39.8</td>
<td>7.1 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>BBN control</td>
<td>10</td>
<td>288 ± 216</td>
<td>174 ± 27</td>
<td>120.7 ± 40.6</td>
<td>367.9 ± 90.5</td>
<td>126.0 ± 54.8</td>
<td>10.5 ± 2.3</td>
<td>107.2 ± 34.4</td>
<td>7.3 ± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>BBN→NaAsA + DAP</td>
<td>10</td>
<td>445 ± 173&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>450.4 ± 46.1a</td>
<td>216.1 ± 49.9a</td>
<td>261.3 ± 29.6b</td>
<td>8.2 ± 2.9</td>
<td>189.9 ± 39.1b</td>
<td>7.8 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>BBN→NaAsA</td>
<td>10</td>
<td>387 ± 56</td>
<td>81 ± 7</td>
<td>453.6 ± 88.3b</td>
<td>220.2 ± 54.6b</td>
<td>168.3 ± 41.5</td>
<td>8.3 ± 3.4</td>
<td>201.9 ± 41.8b</td>
<td>7.9 ± 0.2b</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are means ± SD.
<sup>b</sup>Significantly different as compared with group 2, *P* < 0.001 (Student’s *t*-test).
<sup>c</sup>Significantly different as compared with groups 2 and 4, *P* < 0.001 (Student’s *t*-test).

Table III. Incidences and multiplicity of urinary bladder lesions of F344 rats treated with BBN followed by DAP and/or NaAsA (experiment I)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Number of rats</th>
<th>PN hyperplasia&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Papilloma&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Carcinoma&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Percent</td>
<td>No./10 cm BM&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Percent</td>
</tr>
<tr>
<td>1</td>
<td>BBN→DAP</td>
<td>17</td>
<td>5 (64.6)</td>
<td>1.14 ± 0.88&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>4 (23.5)&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>BBN control</td>
<td>17</td>
<td>13 (76.5)</td>
<td>2.30 ± 1.39</td>
<td>10 (59)</td>
</tr>
<tr>
<td>3</td>
<td>BBN→NaAsA + DAP</td>
<td>18</td>
<td>18 (100)</td>
<td>7.03 ± 3.43</td>
<td>18 (100)</td>
</tr>
<tr>
<td>4</td>
<td>BBN→NaAsA</td>
<td>15</td>
<td>15 (100)</td>
<td>7.10 ± 3.21</td>
<td>15 (100)</td>
</tr>
</tbody>
</table>

<sup>a</sup>PN, papillary or nodular.
<sup>b</sup>Transitional cell papilloma including squamous cell papilloma elements.
<sup>c</sup>TCC including squamous cell carcinoma elements.
<sup>d</sup>BM, basement membrane.
<sup>e</sup>Values are means ± SD.
<sup>f</sup>Significantly different as compared with the BBN control, *P* < 0.01 (Student’s *t*-test).

Table IV. Incidences and multiplicity of PN hyperplasias in the urinary bladder of F344 rats treated with BBN followed by DAP and/or NaAsA (experiment II)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>PN hyperplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Incidence (%)</td>
</tr>
<tr>
<td>1</td>
<td>BBN→DAP</td>
<td>5</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>BBN control</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>BBN→NaAsA + DAP</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>BBN→NaAsA</td>
<td>5</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup>BM, basement membrane.
<sup>b</sup>Significantly different as compared with group 2 at *P* < 0.025 and *P* < 0.001 (*χ<sup>2</sup> test and Student’s *t*-test, respectively).
<sup>c</sup>Values are means ± SD.

The treated group showed significantly less proliferation than in the BBN control group. Labeling indices for bladder tumors in animals treated with DAP and NaAsA also showed a slight decrease as compared with their counterparts in the NaAsA-treated group, but the difference was not statistically significant. Evaluation of apoptosis in bladder tumors. No significant differences were detected in the percentages of apoptotic cells in the papillomas or carcinomas found in groups 1 and 2. The apoptotic indices for PN hyperplasias were low, without variation among the groups. Papillomas and carcinomas had...
Effect of DAP on urinary bladder carcinogenesis

Fig. 2. Average tumor volumes. *Significantly different versus group 2 ($P < 0.005$); **significantly different versus group 4 ($P < 0.001$). Values are means ± SD.

Fig. 3. BrdU indices (%) for urinary bladder lesions of rats treated with BBN followed by DAP and/or NaAsA (experiment 1). *Significantly different versus group 2 ($P < 0.001$). I, PN hyperplasia; i, papilloma; i, carcinoma.

Fig. 4. Apoptotic indices (%) for urinary bladder lesions of rats treated with BBN followed by DAP and/or NaAsA (experiment 1). *Significantly different versus group 4 ($P < 0.001$). □, PN hyperplasia; , papilloma; , carcinoma.

Fig. 5. BrdU labeling indices (%) for urinary bladder lesions of rats treated with BBN followed by DAP and/or NaAsA (experiment II). *Significantly different versus group 2 ($P < 0.001$). I, G1; i, G2; i, G3; i, G4.

Fig. 6. ODC and SAT activities and polyamine levels in the bladder mucosa. Table V summarizes data for ODC and SAT activities in group 1 were signifi- cantly lower than in group 2. Activities in groups 3 and 4 were elevated by NaAsA treatment. No

Fig. 7. Urinary bladder lesions. Grossly, minute nodular lesions (≤2 mm) had developed in the bladder mucosa only in rats receiving BBN + NaAsA (groups 3 and 4) and those given BBN alone (group 2). Histopathological findings in the urinary bladders are summarized in Table IV. DAP administration (group 1) caused complete inhibition (0% incidence) of formation of epithelial lesions in the urinary bladder, in contrast to the 60% incidence observed for group 2. The incidences of PN lesions were 100% in both groups 3 and 4 and numbers/
Table V. ODC and SAT activities of urinary bladder mucosa of F344 rats treated with BBN followed by DAP and/or NaAsA (experiment II)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of samples</th>
<th>ODC activity (pmol/h/mg)</th>
<th>SAT activity (pmol/10 min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BBN→DAP</td>
<td>5</td>
<td>44.75 ± 17.25&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.75 ± 12.77</td>
</tr>
<tr>
<td>2</td>
<td>BBN control</td>
<td>5</td>
<td>147.5 ± 18.02</td>
<td>69.08 ± 14.65</td>
</tr>
<tr>
<td>3</td>
<td>BBN→NaAsA + DAP</td>
<td>5</td>
<td>371.0 ± 128.6</td>
<td>48.82 ± 15.44</td>
</tr>
<tr>
<td>4</td>
<td>BBN→NaAsA</td>
<td>5</td>
<td>162.5 ± 70.44</td>
<td>49.75 ± 15.14</td>
</tr>
</tbody>
</table>

<sup>a</sup>Means ± SD.
<sup>b</sup>Significantly different as compared with group 2 at \( P < 0.05 \) (Student’s t-test).

Table VI. Polyamine levels in urinary bladder mucosa of F344 rats treated with BBN followed by DAP and/or NaAsA (experiment II)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of samples</th>
<th>Spermidine (pmol/100 mg)</th>
<th>Spermine (pmol/100 mg)</th>
<th>N1-AcSPD (pmol/100 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BBN→DAP</td>
<td>5</td>
<td>8.25 ± 4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.8 ± 12.8</td>
<td>0.02 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>BBN control</td>
<td>5</td>
<td>10.9 ± 6.8</td>
<td>43.8 ± 20.6</td>
<td>0.32 ± 0.37</td>
</tr>
<tr>
<td>3</td>
<td>BBN→NaAsA + DAP</td>
<td>5</td>
<td>18.9 ± 5.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.5 ± 10.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>4</td>
<td>BBN→NaAsA</td>
<td>5</td>
<td>17.6 ± 7.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49.3 ± 7.3</td>
<td>0.1 ± 0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Means ± SD.
<sup>b</sup>Significantly different as compared with group 2 at \( P < 0.001 \) (Student’s t-test).

Fig. 6. Quantitation of mRNA levels of cyclin D1, Cdk4 and VEGF in urinary bladder mucosa of rats treated with BBN followed by DAP and/or NaAsA.

*Significantly different versus group 2 at \( P < 0.001 \). Values are means ± SD.

variation in SAT values was evident. The levels of spermine and spermidine were significantly higher in the NaAsA-treated groups over the other two groups, but did not differ between groups 1 and 2 or between groups 3 and 4 at the time of death. Northern blot analysis. Northern blotting data for mRNA expression of cyclin D1, Cdk4 and VEGF processed by image analyzer and compared with expression of GAPDH are shown in Figures 6 and 7. Among the tested mRNA levels, the values of cyclin D1 were markedly reduced by the DAP treatment in group 1, compared with group 2. Although the mRNA levels of cyclin D1 and Cdk4 expressed in the groups treated with NaAsA were higher than those of the other two groups, they did not differ between groups 3 and 4. DAP decreased the cyclin D1 mRNA levels to normal control values. mRNA expression of Cdk4 was slightly decreased by DAP treatment in group 1 as compared with group 2, but VEGF mRNA expression showed no comparable differences between groups 1 and 2 or groups 3 and 4.

Discussion

In the present study administration of DAP for 32 weeks (experiment 1) exerted significant inhibitory effects on BBN urinary bladder carcinogenesis. Interestingly, the administration of DAP alone for 16 weeks resulted in complete inhibition of development of early stage lesions in experiment 2. DAP was also very effective at inhibiting cell proliferation, as indicated by the significant decrease in the BrdU labeling index in line
cyclin D1 overexpression are more likely to progress to interconversion reactions in one tissue and utilized in another, et al. results of Boyle bladder carcinogenesis, probably related to acquisition of Exogenous polyamines from the pool of acetylated forms at catalytic subunits, Cdk4 and Cdk6, which regulate G1 phase experiment 2 found here. On the other hand, polyamine protein kinases, CdKs, which are essential for cell cycle rate of transcription of the gene (54). This might explain the Cyclins are positive regulators of cell cycle progression. They elevated levels of ODC and, in some cases, this overproduction...fi...fi...of the retinoblastoma (Rb) tumor suppressor protein (41). The exempli...fi...fi...effect of DAP on the development of PN hyperplasia after levels, with a slight reduction in Cdk4, as compared with the...suppression of papillomas in mouse skin by DFMO. The...dysplastic epithelia of the oral cavity (42) and...effect of DAP on the development of PN hyperplasia afterlevels, with a slight reduction in Cdk4 and cyclin E proteins and temporally associated with decreased levels of hyperphosphorylated Rb protein without any significant change in c-myc, p21 or p27 expression (45).

In previous studies, inhibition of VEGF, a potent angiogenic factor and mediator of vascular permeability, was found to block tumor neovascularization with consequent inhibition of both primary tumor growth and micrometastasis when inhibitors were administered at the time of tumor inoculation (46,47). In the present study, the marked inhibitory effects on the early stages of bladder carcinogenesis by DAP were not linked to any change in VEGF mRNA levels.

Administration of DAP together with NaAsA did not show clear inhibitory effects on tumor development, although late lesion size was significantly reduced, probably due to increased apoptosis. Also, an unequivocal influence on the other parameters investigated was not apparent. The significant increases in DNA synthesis and ODC activity were in line with promotion of tumor development by NaAsA, and a close relationship between tumor-promoting capacity of chemicals and induction of ODC and cell proliferation has been reported (48). Since there were no significant differences in percentages of apoptotic cells between tumors in groups 1 and 2, the inhibitory effects of DAP appeared to be due to inhibition of cell proliferation. The antiproliferative effects of DAP could possibly have been masked when combined with NaAsA. Li et al. (49) recently showed that the ODC inhibitor DFMO can act synergistically with aspirin in inducing apoptosis in colonic lesions in rats. In previous studies, both increased apoptosis and/or decreased cell proliferation have been proposed as mechanisms for the chemopreventive activities of many agents (50,51). It has been demonstrated (52) that non-genotoxic carcinogens may differentially alter molecular pathways involving p53, bcl-2 and bax. Some cell lines show resistance to the antiproliferative effects of ODC inhibitors in vitro. Pegg et al. (53) postulated that this resistance appears to be mediated by the presence of elevated levels of ODC and, in some cases, this overproduction was due to a massive increase in ODC mRNA or an increased rate of transcription of the gene (54). This might explain the relative increase in ODC activity in group 3 over group 4 in experiment 2 found here. On the other hand, polyamine metabolism could be affected by oncogene expression, as exemplified by the observation that N-myc-transfected rat fibroblasts do not exhibit elevated ODC activity but rather accumulate polyamines by increasing their transport into the cell (55).

This present study has demonstrated a particularly strong effect of DAP on the development of PN hyperplasia after BBN initiation alone. Soler et al. (56) also showed complete suppression of papillomas in mouse skin by DFMO. The availability of polyamines from other endogenous sources, and also an exogenous supply (57), could be the reason for the induction of a few tumors after prolonged administration of DAP in the first experiment. Putrescine can be mobilized by interconversion reactions in one tissue and utilized in another, with the circulation functioning as a transport system (58). Exogenous polyamines from the pool of acetylated forms at the cellular level or from the diet or intestinal bacteria may stimulate cell proliferation and growth (59) and depletion of cellular polyamines can elicit compensatory uptake of extracellular polyamines (60).

Spermine turnover occurs in principle through the consecutive action of SAT and FAD-dependent polyamine oxidase (61). No changes in polyamine levels or SAT activities between groups were detected at the time of death, but the levels of N1-AcSPD, an acetylated derivative of spermine and spermidine, were found to be decreased by DAP treatment. Recently,
levels of this metabolite were shown to be increased in malignancies (62). Also, patients with high levels of N1-AcSPD in their tumors show a far worse prognosis, with significantly shorter and less recurrence-free survival than those with tumors containing low levels of the derivative (63). Further investigation of the influence of DAP on metabolites of polyamines thus appears warranted.

DAP did not cause serious toxic effects during treatment in previous experimental animal studies (13–17) or in this experiment. Pegg (3) postulated that ODC inhibitors are not particularly toxic and could be given in drinking water at levels of 1–3%. Again on the other positive side, DFMO was quite well tolerated in clinical trials (particularly when given i.v.) and was not life threatening (64,65). Therefore, the carcinogenesis by ornithine decarboxylase inhibitor was not a serious toxic effect. Therefore, the carcinogenesis by ornithine decarboxylase inhibitor was not a serious toxic effect.

Interpreting Chemical Effects
Carcinogenicity. Testing, Predicting, and Modulation of apoptosis by sulindac, curcumin, phenylethyl-3-growth and as a target for chemotherapy.

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