Exisulind, a Novel Proapoptotic Drug, Inhibits Rat Urinary Bladder Tumorigenesis


ABSTRACT

Exisulind (Aptosyn) is a novel antineoplastic drug being developed for the prevention and treatment of precancerous and malignant diseases. In colon tumor cells, the drug induces apoptosis by a mechanism involving cyclic GMP (cGMP) phosphodiesterase inhibition, sustained elevation of cGMP, and protein kinase G activation. We studied the effect of exisulind on bladder tumorigenesis induced in rats by the carcinogen, N-butyl-N-(4-hydroxybutyl) nitrosamine. Exisulind at doses of 800, 1000, and 1200 mg/kg (diet) inhibited tumor multiplicity by 36, 47, and 64% and tumor incidence by 31, 38, and 61%, respectively. Experiments on the human bladder tumor cell line, HT1376, showed that exisulind inhibited growth with a GI0 of 118 μM, suggesting that the antineoplastic activity of the drug in vivo involved a direct effect on neoplastic urothelium. Exisulind also induced apoptosis as determined by DNA fragmentation, caspase activation, and morphology. Analysis of phosphodiesterase (PDE) isoforms in HT1376 cells showed PDE5 and PDE4 isoforms that were inhibited by exisulind with IC50s of 112 and 116 μM, respectively. Inhibition of PDE5 appears to be pharmacologically relevant, because treatment of HT1376 cells increased cGMP and activated protein kinase G at doses that induce apoptosis, whereas cyclic AMP levels were not changed. Immunohistochemistry showed that PDE5 was localized in tumor foci in HT1376 cells. Immunohistochemistry showed that PDE5 was overexpressed in human squamous and transitional cell carcinomas compared with normal urothelium. The data lead us to conclude that future clinical trials of exisulind for human bladder cancer treatment and/or prevention should be considered and suggest a mechanism of action involving cGMP-mediated apoptosis induction.

INTRODUCTION

Urinary bladder cancer is one of the most common malignancies in the world, with the highest incidence in developed countries. In the United States, ~55,000 new cases of bladder cancer were diagnosed in 1999, and there were >12,000 deaths attributable to the disease (1). More than 90% of urinary bladder tumors are derived from the epithelium, and the majority are TCCs.3 Approximately three-fourths of bladder tumors are low grade, papillary, and superficial, with about one-fourth being invasive. However, ~70% of patients with superficial tumors will show one or more recurrences of lesions after the initial diagnosis, and approximately one-third of these patients will die from bladder cancer as a result of disease progression. If invasive disease is detected at the time of diagnosis, there is only a 6% chance of survival within a 5-year period. Available treatment involves either systemic or intravesical delivery of chemotherapeutic drugs, which produce relatively modest efficacy and are associated with serious side effects and/or delivery complications. The high rate of mortality from urinary bladder cancer and the high incidence of disease recurrence emphasize the need for new therapeutic agents alone or in combination with existing therapies. Research to identify the specific molecular defects involved in bladder tumorigenesis has identified mutations in a number of genes (i.e., ras and p53) or altered expression of proteins (cyclin D and p21 WAF1/CIP1), which are known to regulate cell cycle progression and/or apoptosis (2).

Exisulind is a p.o. active drug that is a member of a new class of drugs called selective apoptotic antineoplastic drugs (3) being developed for the treatment and prevention of precancerous and malignant disease. Exisulind was developed as a result of cell culture studies investigating the growth-inhibitory and apoptosis-inducing properties of sulindac metabolites (3–5) and rodent studies showing COX-independent inhibition of colon tumorigenesis (6). Exisulind does not inhibit either the constitutive or inducible forms of COX (6–8). The biochemical mechanism by which tumor cells undergo apoptosis in response to exisulind has been investigated in human colon tumor cell lines (9). In these studies, exisulind inhibited cGMP PDE of either the PDE2 or PDE5 isozyme families to cause a sustained increase in cGMP and the activation of cGMP-dependent protein kinase. Certain other cGMP PDE inhibitors and guanylate cyclase activators also induced apoptosis, although most other PDE inhibitors, including PDE4- and PDE5-specific inhibitors, failed to induce apoptosis and did not cause a sustained increase in cGMP levels in colon tumor cell lines. Other investigators have also reported that such specific PDE inhibitors, particularly in the absence of an agonist, do not lead to a sustained increase in either cAMP or cGMP in other cell systems (9–11). Thus, exisulind is a novel proapoptotic drug with a mechanism involving cGMP PDE inhibition and PKG activation.

Previous studies have shown that the apoptosis-inducing properties of exisulind are fundamentally different from conventional chemotherapeutic drugs. For example, apoptosis induction by exisulind does not require cell cycle arrest as it does for chemotherapeutic drugs, such as 5-fluorouracil (5). Consequently, exisulind should induce apoptosis in precancerous or cancerous cells regardless of the rate of proliferation within the lesion. In addition, p53 or bcl-2 do not appear to be necessary for exisulind to induce apoptosis of colon or prostate tumor cell lines, respectively (5, 7). In contrast, apoptosis induction by most chemotherapeutic drugs has been shown to generally involve p53-dependent (12) and bcl-2-dependent (13) pathways. Therefore, it would be expected that tumor cells harboring p53 mutations or overexpressing bcl-2 (which is common in malignant diseases) would undergo apoptosis in response to exisulind. Such tumor cells may otherwise be resistant to chemotherapeutic agents that require these molecular pathways to induce apoptosis.

Studies using rodent models of chemically induced tumorigenesis or xenograft models involving the colon (6, 14), breast (8, 15), lung (16), and prostate (17) tumors suggest that exisulind has a broad spectrum of antineoplastic activity, particularly involving adenocarcinomas. In addition, exisulind and other selective apoptotic antineoplastic drugs have been shown to inhibit cell growth and induce...
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apoptosis in a variety of human tumor cell lines of diverse histological origin, including nonepithelial tumor cells (4, 7, 15). However, there have been no studies of exisulind and its activity against urinary bladder tumors or other squamous cell carcinomas, nor have any mechanistic studies been performed on bladder tumor cells. We have found that exisulind was effective in a chemically induced model of rat urinary bladder tumorigenesis and that human bladder cancer cells are growth inhibited and undergo apoptosis in response to exisulind treatment. We also found that PDE5 and PDE4 isozymes were expressed in human bladder tumor cells and were sensitive to exisulind at doses that inhibited tumor cell growth. PDE5 appears to be a pharmacologically relevant target for exisulind, because treated bladder tumor cells showed increased cGMP levels and activated PKG. PDE5 was overexpressed in human urinary bladder transitional cell and squamous carcinomas compared with normal urothelium showing the presence of the drug target in malignant lesions.

MATERIALS AND METHODS

Drug Synthesis and Diet Preparation. Exisulind was obtained from Medea Laboratories (Port Jefferson Station, NY) and prepared as described previously (6). Exisulind was administered to the animals as a dietary supplement using powdered Teklad (4%) diet (Harlan Teklad, Madison, WI). The drug was analyzed for purity and stability in the diet using high-performance liquid chromatography (18). Drug-containing diets (0.5 g) were homogenized for 15–30 s in EDTA and ascorbic acid (0.5 mg/ml) using a Brinkmann Polytron and extracted by shaking with 23 ml of 50% methanol/50% ethanol. The extract was then centrifuged for 10 min at 5000 rpm, and the supernatant was analyzed by a Hitachi Model L6200 fitted with a Spherisorb ODS-2 3 μm column (150 mm × 4.6 mm; Alltech, Deerfield, IL). The mobile phase consisted of 4% (v/v) aqueous acetic acid and acetonitrile (65:35) pumped at a flow rate of 1 ml/min. Exisulind and an internal standard (indomethacin) were detected with an online Hitachi Model L4200 UV/VIS detector at 328 nm. The retention times were ~11 min for exisulind and 13 min for indomethacin. Diets were analyzed for exisulind after weeks 1, 12, and 25 of the study and consisted of actual concentrations of ±7% of the theoretical dose.

Carcinogen-induced Rat Urinary Bladder Model. The study used the carcinogen, OH-BBN, which has been shown previously to selectively induce bladder tumors with histopathology similar to human bladder tumors (19). The protocol to induce tumors was based on a method described previously that produced a high incidence of urinary bladder cancers in rats within 6 months after carcinogen administration (20). Briefly, female Fischer 344 rats were purchased from Charles River Laboratories (Indianapolis, IN; virus-free colony 218) at 28 days of age. The animals were placed on the Teklad diet on the day of arrival. Dietary supplementation with exisulind (600, 800, 1000, or 1200 mg/kg of diet) was initiated when the rats were 43 days of age. The carcinogen (OH-BBN) was obtained from TCI America (Portland, OR) and administered as a 150-mg/ml solution of ethanol:water (20:80). Beginning when the rats were 49 days of age, OH-BBN was administered by gavage twice/week for 8 weeks (150 mg/gavage) for a total dose of 2400 mg/rat. The rats were weighed weekly and checked daily for signs of toxicity. The study was terminated at 6 months after the initial dose of carcinogen.

At the time of sacrifice, the urinary bladder of each rat was processed for gross and histological analysis. At necropsy, the empty urinary bladder from each rat was tied off and inflated with 10% neutral formalin. After fixation, the bladders were examined under a high-intensity light for lesions. The approximate location and size of each lesion was recorded. Each lesion was separately embedded in a paraffin block, and random transverse sections (5 μm) from two different levels were cut and stained with H&E for histopathology. For diagnosis, a pathologist read the slides from each rat randomized so that the nature of the treatment received by the rat was not known. The term “carcinoma” was applied to both squamous and transitional cell urinary bladder cancers because most of the lesions contained both components. The statistical analysis used to compare urinary bladder tumors between the different treatment groups and the control group was the χ² test.

Cell Culture Conditions and Treatments. Human bladder HT1376 tumor cells were obtained from American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 supplemented with 5% FCS, 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.25 μg/ml amphotericin. Exisulind was solubilized in 100% DMSO and diluted with medium to obtain a final concentration of 0.1% DMSO or less.

Growth Inhibition. Cells were plated at a density of 1000 cells/well in 96-well microtiter plates and allowed to grow for 24 h before adding the drug in a 10× stock solution of medium and 1% DMSO. The growth-inhibitory activity of exisulind on HT1376 cells was determined colorimetrically by the SRB binding assay after 6 days of treatment, as described previously (4).

Apoptosis. Apoptosis induction of HT1376 cells by exisulind was determined based on DNA fragmentation, cytokeratin cleavage, and morphology. Fragments of DNA fragmentation, cells were plated in 96-well microplates at a density of 10,000 cells/well and allowed to grow for 24 h prior to the addition of the drug. The amount of histone-associated DNA (nucleosomes) contained within cell lysates after 48 h of treatment was quantified by a double-antibody ELISA method using a manufactured kit (Boehringer Mannheim, Indianapolis, IN) as described previously (5). In addition, cells were labeled with an antibody specific for a proteolytic product of cytokeratin 18 that is generated by caspase cleavage of a VEVD/A sequence (21). HT1376 cells were plated on coverslips, allowed to attach for 24 h, and treated with 350 μM exisulind for an additional 24 h. The cells were fixed in ~−20°C methanol for 30 min, air dried, and rehydrated in D-PBS for 5 min at room temperature. Cells were incubated for 1 h in a humidified chamber with a mouse monoclonal cytokeratin 18 antibody (M30 clone) at 1:100 (Boehringer Mannheim). The primary antibody was labeled with a FITC-conjugated secondary antibody (Jackson Immuno Research Labs, West Grove, PA). Cells were washed for 15 min in D-PBS between each incubation step and counterstained with DAPI mounted in Vectashield (Vector Laboratories, Burlingame, CA) to visualize morphological features of apoptosis. Digital images were collected using an Olympus IX70 fluorescence microscope and overlaid using a Spot 2 camera (Diagnostic Instruments) and software. Images were annotated with Photoshop 5.0 (Adobe). The number of M30-positive cells was determined by counting 500 cells/sample.

PDE Isozyme Fractionation and Assay. HT-1376 cells were grown to confluence in 20 150-cm² flasks. Approximately 130 million cells were manually homogenized in a buffer containing 20 mM Tris acetate, 5 mM magnesium acetate, 0.1 mM EDTA, 1.0% Triton X-100, and protease inhibitors (10 mM benzamidine, 10 μM TLCK, 2000 units/ml aprotinin, 2 μM leupeptin, and 2 μM pepstatin A) at pH 7.5 using a glass tissue grinder with a Teflon pestle. After ultracentrifugation at 100,000 × g for 3 h at 4°C, the supernatant was diluted 5-fold with the buffer without Triton (5 mM Tris acetate) and loaded at 1 ml/min onto an 18-ml DEAE Trisacryl M column (BioSepra) using a Pharmacia AKTA/fast protein liquid chromatography. The column was washed with 8 mM Tris acetate, 5 mM magnesium acetate, and 0.1 mM EDTA (pH 7.5), and PDEs eluted with a gradient of 0–1 M sodium acetate in Tris acetate buffer at a flow rate of 1 ml/min into 1.5-M fractions. [3H]cAMP or [3H]cGMP substrates (0.25 μM; 300,000 cpm) were used to differentiate isozymes, as described previously (22).

Permeabilized Cell Assay of Cyclic Nucleotide PDE. Cultured human bladder cancer cells HT-1376 were grown to confluence in 24-well tissue culture plates. Growth medium was removed, and cells were washed for 5 min with DMEM, followed by cold PBS. Cells were then placed on ice in 700 μl of ice-cold Tris-HCl buffer (20 mM; pH 7.4) containing MgCl₂ (5 mM), 0.5% Triton X-100, and protease inhibitors (10 μM tosyl-l-hysine-chloro-ketone, 2 μM leupeptin, 2 μM pepstatin A, 10 mM benzamidine, and 2000 units/ml aprotinin/ml). The reaction was initiated by adding 100 μl of 0.5 mg/ml snake venom (Sigma Chemical Co., St. Louis, MO) and 0.25 μM cGMP or cAMP, along with [3H]cGMP or [3H]cAMP, respectively. After incubating for 30 min at 30°C, the reactions were terminated by the addition of 1.8 ml of methanol. The extract was then applied to a 1-ml Dowex anion exchange column to remove unreacted substrate. The eluant was collected and counted in 6 ml of scintillation fluid.

Cyclic Nucleotide Measurements. Intracellular levels of cGMP and cAMP were measured by an enzyme-linked immunooassay. HT1376 cells (2 × 10⁶) were plated on 100-mm dishes. Exisulind was added after the 3rd day of growth. After a rapid wash with cold PBS, 1 ml of 0.2 N HCl/50% methanol was added directly to the attached cells, and the extract was dried
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Fig. 1. Gain in body weight of control and exisulind-treated rats after carcinogen administration. The groups (30 rats/group) were fed: control diet (○); exisulind, 1200 mg/kg diet (●); exisulind, 1000 mg/kg diet (■); exisulind, 800 mg/kg diet (▲); and exisulind, 600 mg/kg diet (▼). The unpaired Student t test was used to compare differences in body weights.

Table 1  Effect of exisulind on the incidence of OH-BBN-induced urinary bladder lesions a,b

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Papillomas</th>
<th>Carcinomas</th>
<th>Papillomas + Carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teklad diet only</td>
<td>60%</td>
<td>27%</td>
<td>77%</td>
</tr>
<tr>
<td>Exisulind (600 mg/kg)</td>
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<td>30%</td>
<td>77%</td>
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<td>Exisulind (800 mg/kg)</td>
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<td>53%</td>
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<td>Exisulind (1200 mg/kg)</td>
<td>27%</td>
<td>7%</td>
<td>30% b</td>
</tr>
</tbody>
</table>

a The study involved groups of 30 female Fisher 344 rats and was terminated at 6 months after the initial dose of OH-BBN. b Different from control, P < 0.01, χ2 test.

using a speed vacuum. Samples were reconstituted in 200 μl of water and acetylated as described previously (23), and the cyclic nucleotide content was determined using enzyme-linked immunoassay kits purchased from Linco Research, Inc. (St. Charles, MO). The results were expressed in fmol of cGMP or cAMP per mg of protein.

PKG Activity. PKG activity in lysates from HT1376 cells was measured by an affinity bead substrate assay using PDE5 as a substrate. The GST-PDE5 fusion protein fragment of PDE5 corresponding to Val155-Asp393 of bovine PDE5 (1) was prepared and bound to GSH-Sepharose affinity beads as described previously (24). After treating with 1 h with 0.5% DMSO or 500 μM exisulind, cells were washed with cold PBS and lysed with cold buffer containing 50 mM Tris-HCl, 1% NP40, 150 mM NaCl, 1 mM EDTA, 1 mM Na2VO4, 1 mM NaF, 500 μM 1-methyl-3-isobutylxanthine, and Complete protease inhibitor mixture (Roche Molecular Biochemicals, Palo Alto, CA). Cell lysates (100 μg), substrate (20 μg protein), 0.5 μM protein kinase inhibitor (Refs. 5–24; Calbiochem-Novabiochem Corp., San Diego, CA), 4.5 mM Mg2+, and [γ-32P]ATP (10 μCi; 190 μM), with or without added cGMP (100 μM), were mixed and incubated at 30°C for 30 min. The phosphorylated PDE5 fusion protein was resolved by 7.5% SDS-PAGE and quantified by a phosphorimager.

PDE5 Immunolabeling. The presence and distribution of PDE5 in cultured human bladder tumor cells and specimens from patients with bladder carcinoma were determined using an affinity-purified polyclonal anti-PDE5A IgG. PDE5 antisera was generated in sheep against the peptide antigen AQLYETSLLENKRNQV corresponding to residues 317–332 in PDE5A1, prepared by Bethyl Labs (Montgomery, TX), and purified by an antigen affinity column. Monolayer cultures of HT1376 cells were seeded on cover-slips for 24 h, fixed in 3% paraformaldehyde in D-PBS for 10 min at room temperature, and permeabilized in 0.5% Triton X-100 in D-PBS for 5 min at room temperature. The cells were incubated for 1 h in a humidified chamber at room temperature with affinity-purified anti-PDE5 IgG (50 μl) at 1:200. After three 5-min washes of D-PBS, the cells were incubated with Cy3-conjugated secondary antibody (Jackson Immuno Research Labs) for 30 min at room temperature and analyzed as described above for the cytokeratin cleavage product.

For immunolabeling of tissues, specimens were obtained from either biopsies or surgically removed human bladder transitional or squamous cell carcinomas. The specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of 4-μm thickness were cut, mounted on poly-lysine-coated slides, deparaffinized, rehydrated in alcohol, and blocked for endogenous peroxidase (0.3% α- Peroxidase in PBS). Sections were then blocked with 1% normal rabbit serum by incubating overnight at 4°C in a humidified chamber. After washing three times in D-PBS (5 min/wash), the sections were incubated with affinity-purified anti-PDE5 IgG (1:500) for 1 h at room temperature. Antibody labeling was detected using the Vector Elite ABC kit and visualized with the peroxidase substrate, diaminobenzene. Slides were counterstained with 5% hematoxylin and analyzed by light microscopy. The specificity of the anti-PDE5 IgG was confirmed in cultured colon cells and tumor specimens. In both cases, preincubating the anti-PDE5 IgG with the peptide antigen or human PDE5 GST-fusion protein (corresponding to the bovine PDE5 (1) sequence Val155–Asp393) for 1 h at room temperature, before incubating with cells or tissue sections, blocked essentially all immunoreactivity. In addition, immunoreactivity was not observed by omitting the primary antibody from the labeling procedure or substituting with affinity-purified preimmune sheep antiserum.

RESULTS

Carcinogen-induced Rat Urinary Bladder Tumorigenesis. Exisulind at doses of 600, 800, 1000, and 1200 mg/kg diet were tested as a dietary supplement initiated 6 days before the first dose of the carcinogen. Rats receiving exisulind at all doses showed a rate of body weight gain similar to untreated rats ±5% (Fig. 1), indicating that exisulind treatment was well tolerated with respect to the growth of the rats. No overt signs of toxicity were observed, and no rats died in either the control or drug-treated groups during the study.

Exisulind treatment inhibited the incidence of urinary bladder papillomas and carcinomas (Table 1). Rats administered the carcinogen without treatment had a 60% incidence of papillomas and 27% incidence of carcinomas, with a combined 77% incidence. A decrease in tumor (papillomas and carcinomas) incidence to 30% (61% reduction) occurred with the 1200 mg/kg exisulind-treated group (P < 0.01), although exisulind at 1000 and 800 mg/kg also showed a trend to reduce tumor incidence to 48% (38% reduction) and 53% (31% reduction), respectively.

Exisulind treatment had a pronounced effect on tumor multiplicity (Table 2). The multiplicity of papillomas was reduced from 0.97 tumors/rat in the untreated group to 0.53, 0.55, and 0.40 tumors/rat in the 1200 (P < 0.01), 1000 (P < 0.05), and 800 (P < 0.05) mg/kg dose groups, respectively. Exisulind also reduced the multiplicity of carcinomas from 0.33 tumors/rat in the control group to 0.07 tumors/rat in the 1200 mg/kg dose group (P < 0.05). A trend toward reduction to 0.14 tumors/rat occurred in the 1000 mg/kg dose group. A dose-response relationship was apparent by tabulating both carcinomas and papillomas

Table 2  Effect of exisulind on the multiplicity of OH-BBN-induced urinary bladder lesions a,b

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Papillomas</th>
<th>Carcinomas</th>
<th>Papillomas + Carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teklad diet only</td>
<td>0.97</td>
<td>0.33</td>
<td>1.30</td>
</tr>
<tr>
<td>Exisulind (600 mg/kg)</td>
<td>0.77</td>
<td>0.37</td>
<td>1.13</td>
</tr>
<tr>
<td>Exisulind (800 mg/kg)</td>
<td>0.53 b</td>
<td>0.30</td>
<td>0.83 b</td>
</tr>
<tr>
<td>Exisulind (1000 mg/kg)</td>
<td>0.50 b</td>
<td>0.14</td>
<td>0.69 b</td>
</tr>
<tr>
<td>Exisulind (1200 mg/kg)</td>
<td>0.40 b</td>
<td>0.07 b</td>
<td>0.47 b</td>
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</table>

a The study involved groups of 30 female Fisher 344 rats and was terminated at 6 months after the initial dose of OH-BBN. Values are the average number of tumors/rat. b Different from control, P < 0.05, χ2 test.

b Different from control, P < 0.01, χ2 test.
papillomas. From the lowest to the highest dose of exisulind, the values were 1.13, 0.83, 0.69, and 0.47 tumors/rat, representing reductions of 13, 36, 47, and 64%, respectively, when compared with the controls. For the 1200, 1000, and 800 mg/kg dose groups, the reduction was statistically significant at $P < 0.01$–0.05.

**Growth Inhibition and Apoptosis Induction in Human Bladder Tumor Cell Cultures.** Exisulind inhibited the growth of the human bladder tumor cell line, HT1376, with a GI$_{50}$ of 118 $\mu$m at 6 days of treatment (Fig. 2A). Relative to untreated cells, exisulind at doses 400 $\mu$m or higher inhibited growth by $\sim$90%. Similar potency (GI$_{50}$, 133 $\mu$m) and extent of growth inhibition was observed using another human bladder tumor cell line, 3-HTB (data not shown).

The effect of exisulind on apoptosis of HT1376 cells was measured after 48 h of treatment and showed a dose-dependent increase in DNA fragmentation (EC$_{50}$, 317 $\mu$m; Fig. 2B). Apoptosis induction was also studied by labeling treated cultures with an antibody specific for a cytokeratin-18 proteolytic product and DAPI to visualize characteristic morphological features of apoptotic cells. The photomicrographs in Fig. 3 show examples from HT1376 cultures treated with exisulind (350 $\mu$m; 24 h). In Fig. 3, A–C show an example of an early-stage apoptotic cell (bottom arrow), which displays filamentous labeling from the cleaved cytokeratin-18. No morphological evidence of apoptosis is apparent at this early stage. Panels D–F show an example of a late-stage apoptotic cell that displays globular labeling with the cytokeratin-18 antibody and intense nuclear labeling, indicating nuclear condensation. The top arrow shown in Fig. 3, A–C, shows an example of an intermediate-stage cell. Quantification of apoptosis in the attached cell population after 24 h of treatment demonstrated that exisulind increased the percentage of apoptotic cells (labeling index) from 1 to 9.9%.

**PDE Isozyme Expression in Cultured Human Bladder Tumor Cells and Sensitivity to Exisulind.** Previous studies in colon tumor cell lines have shown that exisulind induces apoptosis by a mechanism involving cGMP PDE inhibition of either or both the PDE2 and PDE5 isozyme families (9). To determine which PDE isoforms are
expressed in human bladder tumor cells, HT1376 cell lysates were fractionated by anion-exchange chromatography (Fig. 4A). HT1376 cell lysates contained two peaks of PDE activity. The first peak of activity hydrolyzed cGMP, and not cAMP, whereas the second peak hydrolyzed primarily cAMP. Most of the cGMP hydrolytic activity in the first peak was inhibited by E4021, a PDE5-selective inhibitor. Essentially all of the cAMP hydrolytic activity in the second peak was inhibited by rolipram, a PDE4 selective inhibitor. Hydrolysis of cAMP assayed in the presence of cGMP to either stimulate or inhibit activity of PDE2 or PDE3, respectively, showed that both peaks were essentially insensitive to cGMP, indicating the fractions were not contaminated with either PDE2 or PDE3. Fractions from the two PDE peaks were pooled, and the IC50s for exisulind were determined (Fig. 4B). Exisulind comparably inhibited PDE5 with an IC50 of 112 μM and inhibited PDE4 with an IC50 of 116 μM.

To further study the PDE isozyme content of bladder tumor cells and to test the possibility that certain PDE enzymes were degraded during fractionation, we measured PDE activity in detergent-permeabilized HT1376 cells (Fig. 5). Consistent with results from fractionation experiments, PDE4 and PDE5 were present, as evident by the sensitivity of cAMP hydrolysis to rolipram and cGMP hydrolysis to E4021, respectively. The addition of cGMP did not affect cAMP hydrolysis in the presence of rolipram to indicate the absence of PDE2 and PDE3 in these cells. Calcium/calmodulin-dependent PDE1 was also absent in these cells, as indicated by the insensitivity of cGMP hydrolysis to exogenous calcium or calcium chelation by EGTA treatment.

**Effect of Exisulind on Cyclic Nucleotide Levels in Intact Cells.** Whereas the above experiments demonstrate the presence of PDE4 and PDE5 in HT1376 cells and comparable sensitivity to exisulind in isolation, additional experiments were performed to determine the effects of the drug on cyclic nucleotide levels in intact HT1376 cells (Fig. 6A). Exisulind at either 100 or 500 μM significantly increased the levels of cGMP within 1 h of treatment. The effect of the drug on cGMP levels was sustained, because comparable induction was observed after 48 h of treatment. Although cAMP levels were ~100-fold higher in HT1376 cells compared with cGMP levels, exisulind treatment did not affect cAMP levels after either 1 or 48 h of treatment at either the low or high dose tested.

**PKG Activation by Exisulind.** We reasoned that increased levels of cGMP induced by exisulind may activate PKG to transmit the effect of the drug to apoptosis pathways. To test this possibility, HT1376 cells were treated, and PKG activity was measured in cell lysates using a phosphorylation assay with a GST-PDE5 fusion protein (Val36-Glu529) bound to GSH-Sepharose as a substrate. The
results showed that cell lysates obtained from exisulind-treated cells displayed 2.5-fold greater PKG activity compared with lysates from control cells (Fig. 6, B and C). The addition of cGMP to the reaction mixture enhanced the phosphorylation, indicating that PKG, rather than some other kinase, was activated by exisulind.

**PDE5 Immunolabeling in Cultured Human Bladder Cells and TCCs.** The presence and intracellular localization of PDE5 in human bladder tumor cells was studied by immunofluorescence microscopy using affinity-purified anti-PDE5 IgG. PDE5 labeling was seen primarily in discrete perinuclear foci, although diffuse cytoplasmic labeling was also apparent (Fig. 7).

The PDE5 antibody was also used for immunohistochemistry to determine PDE5 in normal and neoplastic human bladder specimens. In normal bladder epithelium, basal cells were weakly labeled, whereas superficially located normal epithelial cells were not labeled (Fig. 8). By contrast, cells from transitional and squamous cell carcinomas showed strong labeling. Stromal cells showed no or minimal labeling.

**DISCUSSION**

This report describes the preclinical efficacy of exisulind for the treatment and/or prevention of urinary bladder cancer. Using a rat model of chemically induced urinary bladder tumorigenesis, we demonstrated that exisulind inhibited tumor multiplicity and incidence in a dose-dependent manner. The inhibitory effect of the drug on tumorigenesis was observed in the absence of overt toxicity to the animals. These findings are consistent with other rodent studies of exisulind involving chemically induced tumorigenesis in colon (6, 14, 25), mammary (8, 15), and lung (16) models. These studies, as well as those from xenograft models involving malignant prostate cells (17), demonstrate a broad spectrum of activity of exisulind across histologically diverse tumor types.

Exisulind inhibited the growth of cultured human bladder cells. For example, treatment of HT1376 cells with exisulind for 6 days resulted in a GI50 of 118 μM. Exisulind also induced apoptosis, although at higher concentrations, with an EC50 for DNA fragmentation of 317 μM. Differences in treatment time (6 versus 2 days) or other assay requirements probably account for the apparent difference in potency observed in the growth and apoptosis assays. In addition, growth inhibition in the SRB assay is a measure of a cumulative effect of the drug caused by a reduction in the number of live cells, whereas apoptosis assays measure DNA fragmentation of dying cells in the adherent (or intact) cell population.

Previous studies in rats have shown that exisulind at a dose of 1000 mg/kg diet can generate steady-state plasma levels of ~350 μM (6). Because such doses cause marked growth inhibition and apoptosis of bladder tumor cells in vitro, the effects of exisulind in the bladder model involve apoptosis in neoplastic urothelium. Exisulind has also been reported to affect angiogenesis (26), differentiation (27), and cell proliferation (4) in experimental models, which may also contribute to the antineoplastic properties of exisulind. However, an antiproliferative effect was not observed in a clinical trial involving familial adenomatous polyposis patients treated with exisulind (27). In this study, exisulind treatment caused the regression of polyps with a corresponding increase in apoptosis in the adenomatous tissue. Increased mucinous differentiation within adenomatous tissue was also reported, although it is not clear if this effect is responsible for the regression of adenomas or occurs as a consequence of epithelial normalization.

Recently, the COX-2-selective nonsteroidal anti-inflammatory drug, celecoxib, was shown to inhibit chemically induced urinary bladder tumorigenesis in the same rat model that we used to evaluate exisulind (28). The effect of celecoxib on cancer incidence and multiplicity was comparable with what we report here for exisulind. However, celecoxib, unlike exisulind, did not inhibit preneoplastic lesions in this model and indicates that the two drugs have different pharmacodynamic properties. In addition, appreciably higher blood levels of exisulind can be achieved in vivo (6) compared with celecoxib (29).

We have reported in cultured human colon tumor cells that the underlying biochemical mechanism of apoptosis induction by exisulind involves inhibition of cGMP PDEs of either the PDE2 or PDE5 isozyme families (9). The experiments described here support a similar mechanism for bladder tumor cells. Analysis of the profile of PDE isozymes present in the bladder cell line HT1376 showed similar PDE5 expression as observed in SW480 colon adenocarcinoma cells, except that the bladder tumor cell line lacked the PDE2. PDE frac-
tionation experiments do not rule out the possibility that other isozymes, such as PDE6-11 or other novel PDE isozymes, may be present in intact cancer cells in lesser quantities or are extremely labile. However, the data do suggest a predominance of one PDE isozyme for degrading cGMP (PDE5) and one for degrading cAMP (PDE4). This profile of PDE expression appears to be a common characteristic of a number of epithelial-derived tumor cell lines that we have analyzed thus far. Exisulind inhibited PDE5 with an IC₅₀ nearly identical to the GI₅₀ for growth inhibition (112 versus 118 μM) and lead to a sustained increased in cGMP levels in bladder tumor cells. PDE4 was inhibited by exisulind with a similar potency as PDE5, but drug treatment did not affect intracellular levels of cAMP. In the absence of an agonist, this is not unexpected, because cAMP levels are tightly regulated by mechanisms involving activation of PDE4 by phosphorylation and transcriptional regulation (11). Thus, sustained elevation of cGMP appears to be sufficient for the growth-inhibitory and apoptosis-inducing properties of exisulind. Accordingly, exisulind treatment of HT1376 cells activated PKG. Two potential substrates of PKG, which mediate the apoptotic response of colon tumor cells to exisulind, are β-catelin (9) and c-Jun NH₂-terminal kinase-1 (30). Future studies will need to determine whether these and/or other substrates of PKG are phosphorylated in bladder tumor cells in response to exisulind and play a role in signaling apoptosis.

PDE5 displayed a unique subcellular distribution pattern in human bladder tumor cells. Immunocytochemistry showed labeling of discrete perinuclear foci. The significance of perinuclear localization of PDE5 awaits further study of its function in tumor cells. Immunohistochemistry using the same antiserum showed that human transitional and squamous cell carcinomas overexpressed PDE5 relative to normal-appearing adjacent urothelium. These results are consistent with previous observations showing the overexpression of PDE5 in colon

Fig. 7. Subcellular localization of PDE5 in cultured human bladder tumor cells. HT1376 human bladder tumor cells were incubated with anti-PDE5 IgG labeled by immunofluorescence as described in “Materials and Methods.” A, PDE5 labeling. B, nuclear labeling by DAPI. C, the merged image. Note discrete focal labeling around the nuclear periphery and diffuse labeling in the cytoplasm.

Fig. 8. PDE5 expression in normal and neoplastic human bladder. Biopsies from normal bladder (A) or a TCC of the bladder (B) from the same patient were incubated anti-PDE5 IgG and labeled for immunohistochemistry as described in “Materials and Methods.” C–E, immunolabeling of surgical specimens from two high-grade TCCs (C and D) and one intermediate grade squamous cell carcinoma of the bladder (E) from three different patients.
adenomas and adenocarcinomas (31). The abnormal expression of PDE5 in neoplastic tissues may contribute to the specificity by which exisulind induces apoptosis. Moreover, the data suggest the involvement of PDE5 in carcinogenesis and are consistent with a number of previous reports showing differences in PDE and/or cyclic nucleotide levels between normal and neoplastic tissues, particularly those that have described increased cGMP PDE activity (32).

An additional factor for the treatment of bladder cancer patients is that high concentrations of exisulind may be reached in the urinary bladder lumen. In pharmacokinetic studies, a single oral dose of radiolabeled exisulind in healthy human volunteers showed that 56 – 78% of the administered dose was excreted unchanged in the urine for up to 96 h. A therapeutic dose of 250 – 300 mg twice per day, as is being used in ongoing clinical trials, has the potential of generating high concentrations of the drug in the urine, which could have a direct effect on surface urothelium in addition to the systemic levels of the drug.

In summary, the effects of exisulind observed in the rat model of chemically induced urinary bladder tumorigenesis, the growth-inhibitory and apoptosis-inducing properties, and the presence of the drug target in human bladder tumors suggest that future clinical trials of exisulind for bladder cancer treatment and/or prevention be considered. In vitro studies support a mechanism of action involving PDE5 inhibition, sustained elevation of cGMP, and activation of PKG. Future studies are needed to define downstream events in bladder tumor cells after PKG activation and to elucidate the mechanism for PDE5 induction during bladder tumorigenesis and how it may influence neoplastic progression.

REFERENCES


Exisulind, a Novel Proapoptotic Drug, Inhibits Rat Urinary Bladder Tumorigenesis
