Cyclooxygenase inhibitors in urinary bladder cancer: \textit{in vitro} and \textit{in vivo} effects

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Abstract

More than 14,000 people die from invasive transitional cell carcinoma (TCC) of the urinary bladder yearly in the United States. Cyclooxygenase (COX)-inhibiting drugs are emerging as potential antitumor agents in TCC. The optimal \textit{in vitro} or \textit{in vivo} systems to investigate COX inhibitor antitumor effects have not been defined. The purpose of this study was to determine COX-1 and COX-2 expression and antitumor effects of COX inhibitors in human TCC cell lines (HT1376, RT4, and UMUC3 cells) and xenografts derived from those cell lines. COX-2 expression (Western blot, immunocytochemistry) was high in HT1376, modest in RT4, and absent in UMUC3 cells \textit{in vitro}. Similarly, COX-2 expression was noted in RT4 but not UMUC3 xenografts. COX-2 expression in HT1376 xenografts was slightly lower than that observed \textit{in vitro}. None of four COX inhibitors evaluated (celecoxib, piroxicam, valeryl salicylate, and NS398) reduced TCC growth in \textit{standard in vitro} proliferation assays at concentrations that could be safely achieved \textit{in vivo} (\leq 5 \mu mol/L). Higher celecoxib concentrations (\geq 50 \mu mol/L) inhibited proliferation and induced apoptosis in all three cell lines. Cilecoxib or piroxicam treatment in athymic mice significantly delayed progression of HT1376 xenografts, which express COX-2, but not UMUC3 xenografts that lack COX-2 expression. In conclusion, standard \textit{in vitro} assays were not useful in predicting COX inhibitor antitumor effects observed \textit{in vivo}. Athymic mice bearing TCC xenografts provide a useful \textit{in vivo} system for COX inhibitor studies. Results of this study provide justification for further evaluation of COX inhibitors as antitumor agents against TCC. [Mol Cancer Ther 2006;5(2):329–36]

Introduction

Urinary bladder cancer is diagnosed in \textgreater 54,000 people yearly in the United States, and \textsim 14,000 people die from the disease each year (1). Most of these deaths are due to invasive transitional cell carcinoma (TCC) of the urinary bladder that is resistant to chemotherapy (2, 3). More effective treatment for TCC is greatly needed. Cyclooxygenase (COX)-inhibiting drugs have had chemopreventive effects in rodents with chemically induced bladder tumors, and have had antitumor activity and chemotherapeutic-enhancing activity against naturally occurring invasive TCC in pet dogs where the disease closely mimics the human condition (4–9). COX inhibitors are being investigated in human clinical trials.

The mechanisms by which COX inhibitors exert antitumor effects and strategies to optimize the use of these drugs against TCC have not been well defined. Understanding mechanisms involved could lead to more effective application of the drugs and possibly to the identification of other downstream (downstream from COX) novel targets for TCC therapy. Much of the current evidence suggests that COX inhibitors have antitumor activity through blocking COX-2 enzyme activity (9–12). COX-2, which is overexpressed in human and canine TCC (13–15), catalyzes the rate-limiting step in prostaglandin synthesis. Increased synthesis of prostaglandins, especially prostaglandin E\textsubscript{2}, is thought to play an important role in tumor cell proliferation, induction of angiogenesis, and inhibition of apoptosis (9, 16–18). Induction of apoptosis and changes in angiogenic factors by COX inhibitors have been associated with antitumor activity of these drugs in dogs with TCC (17). Multiple mechanisms independent of COX activity and of prostaglandin synthesis, however, have also been proposed (19–21). The interpretation of \textit{in vitro} studies has been complicated by the use of excessively high drug concentrations (i.e., concentrations much higher than those that could be safely achieved in serum in humans). In fact, COX inhibitors have failed to block the growth of multiple cell lines when applied at relevant concentrations (22). With the recent finding that COX inhibitors, specifically COX-2 inhibitors, may increase the risk of thromboembolic and cardiovascular events (increased risk compared with placebo; refs. 23, 24), it is more important than ever to carefully determine how to optimize COX inhibitor treatment and to accurately assess the risk-to-benefit ratio of COX inhibitor...
treatment in cancer. Possible benefits could include increased likelihood and longer duration of remission and prolonged survival (along with possibly improved quality of life).

A key question that remains to be answered is what types of studies could be used to determine the antitumor activity and mechanisms involved of COX inhibitors against TCC before human clinical trials. Antitumor activity of COX inhibitors has been observed in pet dogs with naturally occurring TCC where the cancer very closely mimics human invasive TCC (6–9, 17). Antitumor activity or chemopreventive activity has been observed in rodents with chemically induced bladder tumors (4, 5). It would expedite studies, however, if the antitumor effects of COX inhibitors could be reproduced in vitro in cell culture or possibly in vivo in mice bearing human TCC xenografts. Studies in cell culture and in mice bearing TCC xenografts could be conducted more rapidly, in a more controlled setting, and in most instances with less expense than studies in humans or in pet dogs. Similarly, studies in mice bearing human TCC xenografts may be done more rapidly than studies in mice bearing chemically induced tumors. In addition, chemically induced bladder tumors often remain superficial and relatively low grade (4, 5), thus not mimicking advanced bladder cancer in humans. The effects of COX inhibitors in TCC cell culture or in mice bearing TCC xenografts have not been well defined. Studies were undertaken here to determine the effects of COX inhibitors in vitro and in vivo in athymic mice bearing human TCC xenografts (evaluating s.c. and orthotopic tumors). Confirmation of COX-2 expression and assessment of COX inhibitor effects on viable cell number (cultured cells), tumor size (xenografts), and apoptotic index were determined. The goal of these studies was to determine the extent to which these in vitro and in vivo study systems would be useful for further evaluation of the antitumor effects of COX inhibitors against TCC and the mechanisms of the antitumor activity.

Materials and Methods

Study Design

This work included three areas of investigation: (a) assessment of COX-2 expression in human TCC cell lines in vitro and in xenografts (s.c. and orthotopic sites) formed from the cell lines in athymic mice, (b) determination of COX inhibitor effects in suppressing TCC cell proliferation and inducing apoptosis in standard in vitro assays, and (c) evaluation of the effects of COX inhibitors in suppressing tumor growth and inducing apoptosis in TCC xenografts in athymic mice. The cell lines used for these studies (HT1376, RT4, and UMUC3) were obtained from the American Type Culture Collection (Rockville, MD). Athymic mouse studies were done with approval of the Purdue Animal Care and Use Committee in the Purdue Cancer Center Drug Testing Shared Resource.

Western Blot Analysis

Western blotting was done as previously described (13). Cell cultures were rinsed with PBS, lysed with 1% Triton lysis buffer, collected, and centrifuged. The protein concentrations were determined by Pierce Coomassie Protein Assay (Pierce, Rockford, IL) and equal amounts of protein were loaded and separated by SDS-PAGE under reducing conditions and then transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Transfer of protein and equal loading in all lanes was verified using reversible staining with Ponceau S (Sigma Chemical Co., St. Louis, MO). Membranes were blocked with 5% nonfat dry milk. Equal loading of protein was confirmed by blotting for actin (Sigma Chemical). The procedure included 1 hour of incubation with COX-1 monoclonal antibody (Cayman, Ann Arbor, MI) or COX-2 polyclonal antibody (Oxford, MI), followed by incubation with secondary antibody (either goat anti-mouse or goat anti-rabbit conjugated with horseradish peroxidase;1:5,000 dilution) for 1 hour. Protein was detected on Kodak BioMax MR film (Rochester, NY) using chemiluminescence (SuperSignal, Pierce).

Immunohistochemistry

Immunohistochemistry was done as described previously (13). Two investigators reviewed all slides independently. The percentage of cancer cells with immunoreactivity to COX-1 or COX-2 (positive staining) was determined. The intensity of COX-1 and COX-2 immunostaining was graded on a scale of 0 to 3, where 0, no staining; 1, equivocal staining; 2, moderate to intense staining; and 3, highest intensity staining.

Assessment of COX-2 Expression in TCC Cell Lines

COX-2 protein was detected in cultured TCC cells by Western blot and by immunocytochemistry. Briefly, HT1376 and UMUC3 cells were cultured in DMEM, and RT4 cells were cultured in McCoy 5A medium with 10% fetal bovine serum and 1% l-glutamine at 37°C. Cells were plated at a density of 5 × 10⁶ per 100 mm plates and incubated for 72 hours. Cells were collected with lysis buffer and Western blot was done as described previously. For immunocytochemistry, cells were grown on Lab-Tek II Chamber slide system (Nalge, Nunc, Naperville, IL) until they were 80% to 90% confluent. The cells were then fixed with formalin (10%) overnight and immunocytochemistry was done as described above for immunohistochemistry.

Assessment of COX-2 Expression in TCC Xenografts

COX-2 protein was detected in TCC xenografts by Western blotting and by immunohistochemistry. Athymic mice (male, 3–4 weeks old) were purchased from Harlan (Indianapolis, IN). Urinary bladder cancer cells, HT1376, UMUC3, and RT4 cells, were injected s.c. (inoculum size 3 × 10⁶ for HT1376 cells, 1 × 10⁶ for RT4 cells, and 2.5 × 10⁶ for UMUC3 cells) near the right axilla or in the urinary bladder wall (inoculum size 1 × 10⁶ cells in 10 μL for HT1376 cells, 1 × 10⁶ cells in 10 μL for RT4 cells, and 5 × 10⁵ cells in 10 μL for UMUC3 cells). Inoculum sizes had been determined in a preliminary study. Mice were sacrificed when s.c. tumors reached a size of ~200 to 300 mm³ or when orthotopic tumors became easily palpable. Tumor tissues were collected and immediately frozen in liquid nitrogen (for Western blot analysis) or immersed in 10% formalin (for immunohistochemistry).
**Effects of COX Inhibitors on TCC Cell Proliferation**

*In vitro*

Celecoxib (selective COX-2 inhibitor, Celebrex) was provided by Pharmacia/Pfizer (St. Louis, MO). Piroxicam (nonselective COX inhibitor that blocks activity of COX-1 and COX-2, Feldene) was provided by Pfizer (Groton, CT). NS-398 (selective COX-2 inhibitor, Sigma Chemical) and valeryl salicylate (selective COX-1 inhibitor, AG Scientific, Inc., San Diego, CA) were purchased. Celecoxib, piroxicam, and NS-398 were solubilized in DMSO. Valeryl salicylate was diluted in ethanol. HT1376, RT4, and UMUC3 cells were plated in quadruplicates at a concentration of 5 × 10^3 per well in 96-well plates, and incubated for 24 hours at 37°C and 5% CO₂. Following 24 hours, cells were then treated with 1, 5, 10, 25, 50, or 100 μmol/L of each drug or with vehicle control for 24, 48, and 72 hours. Numbers of viable treated and control cells were determined by CyQUANT Cell Proliferation Assay kit (Molecular Probes, Eugene, OR) and 96-well plate reader (Perkin-Elmer LS HTS 7000 fluorescent Bio Assay Reader, Molecular Probes). Fluorescence was measured using 485 nm (±10 nm) excitation and 530 nm (±12.5 nm) emission filters. For each experiment, a standard curve was generated from measured fluorescence values of known numbers of cells. Experiments were repeated thrice.

**Effects of COX Inhibitors in Suppressing Growth of TCC Xenografts In vivo**

The antitumor effects of celecoxib and piroxicam were determined in mice bearing s.c. HT1376 and UMUC3 xenografts. S.c. xenografts, rather than orthotopic xenografts, were used because initial studies had shown that s.c. HT1376 xenografts formed consistently, expressed COX-2, were easily monitored for size, and were well tolerated by the mice. An inoculum size of 1 × 10^6 cells was used. Mice whose tumors did not reach 1,000 mm³ by 60 days after the start of treatment were euthanized at that time (60 days). Tumor volume in the treated versus control mice. In a separate experiment, treatment was done as described above and mice were euthanized on the last day of treatment to allow collection of tumor tissues for the assessment of apoptosis and proliferation markers.

**Effects of COX Inhibitors in Inducing Apoptosis in TCC Cell Lines In vitro**

Flow cytometry was used to determine the percentage of cultured TCC cells in the various stages of the cell cycle and to determine the percentage of TCC cells undergoing apoptosis before and after treatment with COX inhibitors. Flow cytometry was done in the Analytical Cytometry Shared Resource of the Purdue Cancer Center, Purdue University. The TACSTM Annexin V-FITC Apoptosis Detection kit (R&D Systems, Minneapolis, MN) as previously described (13). The number of positive stained tumor cells was recorded. Tumor volume was compared using both parametric (ANOVA) and nonparametric (Kruskal-Wallis and Wilcoxon) methods. When appropriate, Dunnett’s multiple comparison procedure was used to compare tumor volume in the treated versus control mice. In a separate experiment, treatment was done as described above and mice were euthanized on the last day of treatment to allow collection of tumor tissues for the assessment of apoptosis and proliferation markers.

**Effects of COX Inhibitors in Inducing Apoptosis in TCC Xenografts In vivo**

Apoptosis was detected in xenograft tissue by terminal deoxynucleotidyl transferase–mediated nick end labeling assay using Apoptag Peroxidase In situ Apoptosis Detection kit (Intergen Co., Purchase, NY) as previously described (13). The number of positive stained tumor cells was recorded in a minimum of five fields at ×400 magnification, and the apoptotic index (percent apoptotic tumor cells) was determined. Two observers examined each section and the results were averaged. Induction of apoptosis was defined as ≥2-fold increase in apoptotic index.

**Effects of COX Inhibitors on Proliferation Markers and Cell Regulatory Proteins**

Antibodies to Ki67, p53, cytochrome c, caspases 3 and 9, cyclin D and E, Bax, and Bcl2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and Western blot analysis was done as described above. Immunohistochemistry to assess the presence or absence of Ki67 in TCC xenografts was done as described above. Proliferative index was defined as the percentage of Ki67 immunoreactive tumor cells.

**Results**

**COX-1 and COX-2 Expression In vitro**

Western blot analyses revealed high amounts of COX-2 protein in HT1376 cells and low amounts of COX-2 protein in RT4 cells (Fig. 1). UMUC3 cells lacked detectable COX-2 protein (Fig. 1). Immunocytochemistry of the
cox-2 expression (Table 1; Fig. 2). Considerable heterogeneity in COX-2 immunoreactivity was noted with immunocytochemistry of HT1376 and RT4 cells (Table 1; Fig. 2). All three cell lines expressed low amounts of COX-1 protein (data not shown).

### COX-1 and COX-2 Expression In vivo

COX-2 protein was detected by Western blotting in snap-frozen tumor tissue specimens from HT1376 and RT4 xenografts (s.c. or orthotopic tumors). Similar to the pattern observed in vitro, the highest expression of COX-2 was observed in HT1376 xenografts, with lower expression of COX-2 in RT4 xenografts. COX-2 expression was somewhat higher in orthotopic HT1376 xenografts compared with s.c. HT1376 xenografts. COX-2 protein was not detected by Western blotting in UMUC3 xenografts. COX-1 protein was detected in tumor cells in HT1376, UMUC3, and RT4 xenografts, with slightly more COX-1 protein in HT1376 cells compared with the other cells. There was no appreciable difference in the expression of COX-1 in the s.c. versus orthotopic xenografts.

Using immunohistochemistry, COX-2 expression was detected in tumor cells in all sections of HT1376 xenografts (Fig. 2). The percentage of tumor cells with immunoreactivity ranged from <5% to 40% (mean 14.7%). The staining intensity varied from 1 to 3+ with the majority of sections (90%) having 2 to 3+ staining intensity. Immunoreactivity seemed slightly higher in HT1376 orthotopic xenografts than in s.c. xenografts. COX-2 immunoreactivity was lacking in tumor cells in UMUC3 xenografts. COX-2 immunoreactivity was noted in blood vessels on the periphery of UMUC3 and RT4 xenografts and in some sections of HT1376 xenografts.

### Effects of COX Inhibitors on TCC Cell Growth In vitro

Piroxicam, NS398, and valeryl salicylate did not inhibit growth of HT1376, RT4, or UMUC3 cells at any of the concentrations tested (1–100 μmol/L) in vitro (Fig. 3). Celecoxib at concentrations of <25 μmol/L did not inhibit the growth of any of the cell lines. Slight reduction (~5%) was noted in HT1376 cells treated with 25 μmol/L celecoxib. Celecoxib at concentrations of 50 and 100 μmol/L inhibited the growth of all three cell lines by >80% (Fig. 3).

Effects of COX Inhibitors in Suppressing Growth of TCC Xenografts In vivo

The COX-2 inhibitor, celecoxib, and the nonselective COX inhibitor, piroxicam, were evaluated in mice bearing HT1376 (COX-2 positive) and UMUC3 (COX-2 negative) s.c. xenografts.

Celecoxib and piroxicam were well tolerated by mice with no weight loss or signs of toxicity noted. A dose-response relationship between celecoxib and antitumor activity was observed in preliminary studies, with the highest dose having the most antitumor activity. It was difficult, however, to consistently deliver the high celecoxib dose (250 mg/kg total delivered via 125 mg/kg by gavage and 800 ppm in diet) because it was difficult to evenly distribute the celecoxib in the gavage preparation at that concentration.

Further experiments were conducted in which celecoxib was delivered at a dosage of 80 mg/kg by p.o. gavage daily (a concentration that could be uniformly given by p.o. gavage) and 800 ppm in the diet (205 mg/kg total per 24 hours).

At the end of 2 weeks of celecoxib treatment (80 mg/kg p.o. gavage, 800 ppm in diet) or piroxicam treatment, the volume of HT1376 xenografts was significantly less ($P < 0.005$) than the volume of HT1376 xenografts in nontreated control mice (Fig. 4). There was no significant difference between tumor volume in mice receiving celecoxib and mice receiving piroxicam. The average time for tumors to reach 1,000 mm³ was 46.7 days for celecoxib-treated mice, 46.3 days for piroxicam-treated mice, and 37.1 days for control mice.

The growth of UMUC3 xenografts was not inhibited to any extent by celecoxib or piroxicam treatment. On one date during celecoxib treatment, UMUC3 xenografts seemed smaller in mice receiving celecoxib than in control mice. However, very rapid tumor growth followed in treated as well as control mice. The average time for tumors to reach 1,000 mm³ was 20.8 days in celecoxib-treated mice, 23.6 days in piroxicam-treated mice, and 21.7 days in control mice.

### Induction of Apoptosis and Cell Cycle Arrest In vitro

Of the four COX-inhibiting drugs tested (piroxicam, NS398, valeryl salicylate, and celecoxib), growth inhibition was only observed with celecoxib and only with celecoxib concentrations ≥50 μmol/L. Celecoxib was further studied for its effects on apoptotic index and cell cycle proteins. In

### Table 1. COX-2 expression in HT1376, RT4, and UMUC3 urinary bladder cancer cells detected by immunocytochemistry and by Western blot

<table>
<thead>
<tr>
<th>Immunochemistry (% positive cells)</th>
<th>HT1376</th>
<th>RT4</th>
<th>UMUC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining intensity, 3+</td>
<td>25%</td>
<td>3%</td>
<td>0%</td>
</tr>
<tr>
<td>Staining intensity, 1+ and 2+</td>
<td>17%</td>
<td>4%</td>
<td>0%</td>
</tr>
<tr>
<td>No immunoreactivity</td>
<td>58%</td>
<td>93%</td>
<td>100%</td>
</tr>
<tr>
<td>Western blot (0–3+)</td>
<td>3+</td>
<td>1+</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 1. In vitro COX-2 expression in three human TCC cell lines (HT1376, RT4, and UMUC3) detected by Western blot as described in Materials and Methods. Actin confirms equal loading of protein.
HT1376 cells, the percentage of cells undergoing apoptosis was 0.5% with vehicle control, 4% with 25 μmol/L celecoxib, and 81% with 50 μmol/L celecoxib. In UMUC3 cells and RT4 cells, apoptosis in cells treated with 25 μmol/L celecoxib was not different than that of control cells (<3.0 %). With 50 μmol/L celecoxib, apoptosis was observed in 23% and 14% of UMUC3 and RT4 cells, respectively.

An accumulation in S phase and a decrease in G2-M phase were observed in HT1376 cells treated with celecoxib. There was a dose-dependent increase in the percentage of cells in S phase from 31.7% in untreated HT1376 cells, to 44.7% and 60.1% in cells treated with 25 and 50 μmol/L celecoxib, respectively. This was accompanied by a concomitant decrease in percentage of cells in the G2-M phase of the cell cycle, from 45.0% (control, untreated HT1376 cells) to 33.5% (25 μmol/L) to 9.9% (50 μmol/L celecoxib). This pattern of accumulation of cells in the S phase was also observed in celecoxib-treated RT4 cells but not in UMUC3 cells (data not shown).

Inhibition in cell proliferation by 50 μmol/L celecoxib was associated with slight increases in Bax and cyclins D and E, and slight decrease in Bcl2 (data not shown). No differences in cell number or in the amounts of cell regulatory proteins were detected with celecoxib concentrations ≤25 μmol/L.

Induction of Apoptosis and Inhibition of Proliferation In vivo

Apoptotic and proliferative indices were determined in HT1376 xenografts harvested from mice at the end of 7 or 14 days of treatment with celecoxib. Considerable intratumoral and intertumoral heterogeneity was noted in the apoptotic index (percentage of apoptotic tumor cells). The mean apoptotic index was 4.1% (range 0–11.7%) in control mice, 14.7% (range <1–15.5%) after 7 days of celecoxib treatment, and 8.3% (range <1–15%) after 14 days of celecoxib treatment. The proliferative index also varied within and between xenograft samples. The mean proliferative index was 51.3% (range 20 to >70%) in control mice, 28.9% (range <1–60%) after 7 days of celecoxib treatment, and 31.6% (range <1–50%) after 14 days of celecoxib treatment.

Discussion

The results of these studies provide important information in two areas. (a) The athymic mouse studies provide further evidence that COX inhibitors have antitumor activity against urinary bladder cancer. This, along with compelling studies in pet dogs, and emerging preliminary data from human trials provide justification for further studies of COX inhibitors against TCC. (b) Investigators must carefully consider the relevance and use of in vitro assays when attempting to predict the in vivo efficacy of COX inhibitors and the mechanisms involved. In standard

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Figure 3. Effects of celecoxib, piroxicam, and NS-398 (72-h exposure) on the growth of HT1376 urinary bladder cancer cells. Piroxicam and NS-398 did not inhibit growth. Celecoxib had minimal inhibitory effects at 25 μmol/L and marked inhibitory effects at 50 and 100 μmol/L. Concentrations of celecoxib reached in humans are typically ≤5 μmol/L (25). Results with RT4 and UMUC3 cells were similar to those observed in HT1376 cells (data not shown). Experiments were repeated thrice.
short-term, monolayer culture assays, none of the COX inhibitors (piroxicam, celecoxib, NS398, valeryl salicylate) applied at relevant concentrations (concentrations that could be reached in serum in humans) blocked the growth of any of three urinary bladder cancer cell lines evaluated. Yet, COX inhibitors (piroxicam, celecoxib) had antitumor effects in mice bearing HT1376 xenografts. Moreover, relevant doses of COX inhibitors have been reported to have antitumor activity in pet dogs with naturally occurring TCC and in rodents with chemically induced bladder tumors (4–8). The antitumor effects of COX inhibitors are clearly influenced by the experimental conditions under which they are evaluated.

The in vitro proliferation assays reported here were done in a standard fashion. This included monolayer culture, 10% serum in the medium, and 72-hour treatment period in which cell number in control wells doubled thrice or more. Studies done under these typical conditions were not found to be useful in detecting the antitumor activity of relevant concentrations of COX inhibitors. Much higher drug concentrations were required to inhibit cell growth than concentrations that could be safely achieved in vivo in humans or mice. Celecoxib serum concentrations in humans have been reported to typically range from 3 to 5 μmol/L following approved dosage (25), and mice receiving celecoxib in the diet have had a mean serum concentration of 5.7 μmol/L (19). Concentrations >25 μmol/L were required to inhibit growth of TCC cells in culture to any extent or to induce apoptosis. Studies of such high drug concentrations would only be relevant to the treatment of humans with cancer if strategies were developed to safely deliver COX inhibitors locally to the cancer at these high concentrations. Our laboratory has also observed lack of growth inhibition with COX inhibitors in other types of cancer cell lines (22). The reasons why COX inhibitors do not kill cancer cells at relevant concentrations in standard in vitro assays are not known. Some of the antitumor effects of COX inhibitors have been postulated to involve stimulatory effects on immune function directed against the cancer or possibly inhibition of angiogenesis (16–18). These effects, of course, would only be observed in animals or humans with intact immune system and body processes. It is also possible that specific factors present in the microenvironment of TCC in vivo, which are necessary for the growth inhibitory effects of COX inhibitors, are lacking in standard cell culture. Although this work does not support use of standard in vitro assays for the study of COX inhibitors, it is possible that other types of in vitro assays may be found to be useful for such studies.

The TCC cells in our studies included those that express COX-2 and those that do not express COX-2. The high COX-2 expression in HT1376 cells and the absence of COX-2 expression in UMUC3 cells have also been reported by others (26). The expression of COX-2 was low in RT4 cells in our experiments, whereas other laboratories have reported higher COX-2 expression in RT4 cells (26). One of the unexpected findings was the heterogeneity of COX-2 protein detected by immunocytochemistry in vitro. In HT1376 cells, for example, Western blot revealed a large amount of COX-2 protein. One could presume that this protein would be distributed fairly evenly throughout the cells in the culture. This was not found to be the case. In fact, with immunocytochemistry, only 42% of cells were immunopositive with staining intensity ranging from 1 to 3+. The heterogeneity is not thought to be the cause of lack of COX inhibitor effects in vitro, however, because COX inhibitors have activity in canine and human cancers in vivo, which have heterogeneous COX-2 distribution (13, 17).

Athymic mice studied included those with s.c. tumors and those with orthotopic xenografts. Mice with s.c. xenografts were selected for the drug studies due to the presence (HT1376) or absence (UMUC3) of COX-2 and due to ease of measuring tumors. Disadvantages of orthotopic tumors include the difficulty in measuring the tumors in the bladder and the finding that orthotopic xenografts caused urinary tract obstruction, which could shorten life expectancy and interfere with assessment of drug effects.

Piroxicam (nonselective inhibitor) and celecoxib (COX-2 inhibitor) caused similar growth inhibition in mice bearing HT1376 xenografts. UMUC3 tumors that lack COX-2 expression were not affected to any extent by either drug. This does not prove that UMUC3 xenografts were resistant to COX inhibitor treatment because of lack of COX-2 or that HT1376 xenografts were sensitive to COX inhibitor treatment because of the presence of COX-2 in these tumors. Although much of the published work relating to COX inhibition and cancer suggest that COX-2 is the major target for treatment (9–12), COX-independent effects of these agents have also been proposed (19–21).

In the athymic mouse studies, tumors were significantly smaller in mice treated with celecoxib or piroxicam for 14 days when compared with control mice. It was somewhat disappointing that the tumors did not actually shrink in size in this study, as has been observed in dogs with TCC treated with COX inhibitors (6–8, 17, 27). Other agents with known antitumor activity against TCC in humans, however, have had similar effects (growth delay but not...
tumor regression) in mice with bladder cancer xenografts; thus, the findings were considered meaningful (28, 29). It is possible that greater antitumor activity and possibly tumor regression would occur with longer COX inhibitor treatment in mice. In mice with human prostate cancer xenografts, celecoxib caused much more dramatic antitumor effects after 25 days of treatment than after 14 days of treatment (19). In dogs with TCC who have tumor remission with COX inhibitor treatment, tumor regression is observed after ~4 weeks of treatment and lifelong COX inhibitor therapy seems necessary. Relapse has been noted in dogs who initially have remission (even complete remission) of their TCC and who then discontinue COX inhibitor treatment (27).

Results of the athymic mouse work suggest that studies in this in vivo system could be useful in further COX inhibitor research and could compliment studies in naturally occurring TCC in pet dogs. Canine TCC has the major advantage of being more similar to human invasive TCC. Canine TCC arises from transformation of transitional epithelial cells in the bladder lining, invades into the bladder wall, and metastasizes to distant sites similarly to that in humans. These events do not occur in athymic mice. Athymic mouse studies, however, have key advantages over canine studies, including availability of subjects, opportunity to perform studies at sites other than major veterinary hospitals, ability to test multiple treatment approaches in a relatively short period of time, and lower costs.

This work provides justification for further study of COX-inhibiting drugs as anticancer agents against urinary bladder cancer. A question that remains is whether to pursue selective COX-2-inhibiting drugs or nonselective COX inhibitors. COX-2 is expressed in the majority of invasive TCC in humans (and dogs), but not in normal bladder epithelium (13–15), and COX-2 has been considered a viable target for bladder cancer treatment (9). Selective COX-2 inhibitors have been associated with less gastrointestinal toxicity than nonselective COX inhibitors (30). Recently, however, widespread concern has been expressed for the increase in risk for cardiovascular disease in patients receiving COX inhibitors, especially selective COX-2 inhibitors (23, 24). Both the nonselective COX inhibitor, piroxicam, and the selective COX-2 inhibitor, celecoxib, had antitumor effects in mice. Further studies must be carefully conducted to determine the risk-to-benefit ratio for the use of nonselective COX inhibitors or COX-2 inhibitors in cancer patients. It is likely that carefully prescribed COX inhibitor treatment may result in beneficial effects, such as increasing the frequency and duration of remission, prolonging survival, and improving quality of life. Such beneficial effects, if they occur, would justify the use of COX inhibitors in the treatment of cancer. The recent concern for the adverse cardiovascular effects of COX-2 inhibitors should not lead to these drugs being abandoned altogether as anticancer agents.

In conclusion, further evaluation of COX-inhibiting drugs as antitumor agents against urinary bladder cancer is justified. Results of this study show that investigators must carefully select the conditions and types of studies to use to investigate the antitumor activity of COX inhibitors. Standard, short-term in vitro culture assays may not be useful for the study of COX inhibitors against TCC. Athymic mouse studies along with work in naturally occurring TCC in pet dogs (6–8, 17, 27) seem to provide useful in vivo study systems to evaluate COX inhibitors against urinary bladder cancer.

References
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