Selective cyclooxygenase-2 inhibitors inhibit growth and induce apoptosis of bladder cancer

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Abstract. Selective COX-2 inhibitors such as celecoxib and NS-398 are being evaluated as chemopreventive and therapeutic agents for bladder and other cancers. We investigated the effects of these nonsteroidal anti-inflammatory agents on a panel of bladder cancer cell lines, and assessed their effects on anchorage-dependent and -independent growth, cell cycle, apoptosis and morphology. The human bladder cancer cell lines UM-UC-1, -3, and -6 were assayed for COX-2 expression by Western analysis using a monoclonal antibody to COX-2. UM-UC-1, -3, and -6 cells were grown in the presence of increasing concentrations of NS-398 and celecoxib, and cell growth was quantitated over 7 days by crystal violet elution. The cell lines were treated with NS-398 and celecoxib for 48 h and analyzed by flow cytometry with propidium iodide staining and Br-dUTP staining for apoptosis. Anchorage-independent growth was assessed using an agarose growth assay. Western analysis demonstrated that COX-2 expression in UM-UC-1, -6, and -3 was high, low, and undetectable, respectively. NS-398 and celecoxib produced dose-dependent growth inhibition of UM-UC-1 and -6. Both NS-398 and celecoxib also inhibited anchorage-dependent and -independent growth of UM-UC-3 in a dose-dependent fashion, despite the low basal expression of COX-2 in this cell line. Cell cycle analyses of UM-UC-1 and -6 revealed a 50% reduction in S-phase in the presence of 100 μM NS-398 whereas a smaller reduction in S-phase was noted in UM-UC-3 cells. Furthermore, treatment with 100 μM celecoxib resulted in significant apoptosis in all three cell lines, which was associated with downregulation of Bcl-2. COX-2 selective inhibitors NS-398 and celecoxib produced dose-dependent growth inhibition of bladder cancer cells associated with a significant reduction in S-phase. Induction of apoptosis in all three cell lines by celecoxib was associated with downregulation of Bcl-2. These changes occur independently of COX-2 expression levels suggesting the presence of a COX-2 independent pathway.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) have shown great promise as chemopreventive agents for colon and other cancers. Patients who use NSAIDs on a regular basis have a lower relative risk of adenomatous polyps and colorectal cancer (1,2). Regular intake of NSAIDs has also been associated with a decreased risk of bladder cancer (3).

NSAIDs are thought to exert their anti-inflammatory and anti-neoplastic effects by inhibiting the cyclooxygenase enzyme of which there are two isoforms, COX-1 and COX-2. The COX-1 gene is expressed in normal tissues and contributes to homeostasis. COX-2 is not normally expressed, but it is upregulated by inflammation and is associated with neoplasia. COX-2 may contribute to tumor growth and progression by increased production of prostaglandins and downstream mediators that enhance growth, angiogenesis, invasion, and resistance to apoptosis (4,5).

Nonselective NSAIDs such as aspirin and ibuprofen inhibit both COX-1 and COX-2. COX-1 inhibition can result in gastritis and bleeding secondary to platelet dysfunction. To limit COX-1-related toxicity, there has been interest in the use of selective COX-2 inhibitors for chemoprevention (6-8). Recent publicity regarding cardiovascular toxicity of COX-2 inhibitors has curtailed the evaluation of this class of agents in prevention studies (9). However, the COX-2 selective inhibitor celecoxib has been found to be relatively safer than other agents in its class such that it has remained on the market and continues to be evaluated in a prospective chemoprevention trial for superficial bladder cancer (10). More recently, celecoxib has also been evaluated in combination with standard chemotherapeutic agents in multiple tumor types (11-13), and in this setting, any additional long-term cardiovascular risk should be weighed against the benefits of treatment in patients with advanced disease. Here, we examine the biologic effects of two COX-2 selective inhibitors, NS-398 and celecoxib, on bladder cancer cell lines that exhibit various levels of COX-2 expression.

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Materials and methods

Cell culture. The bladder cancer cell lines UM-UC-1, UM-UC-3, and UM-UC-6 (14) were maintained in modified Eagle's medium containing penicillin-streptomycin and 1% fetal bovine serum. The UM-UC-1 cell line was derived from metastatic bladder cancer whereas UM-UC-3 and UM-UC-6 were derived from bladder TUR specimens. Dimethyl sulfoxide (DMSO) was used as the vehicle for both NS-398 and celecoxib.

PGE_2 production. Cells were plated on 6-well plates at a density of 2×10^5 cells per well. After 24 h, the medium was exchanged with media containing either 100 μM of NS-398, 50 μM of celecoxib, or DMSO alone. Media was harvested 48 h following treatment and analyzed by ELISA (Assay Designs, Ann Arbor, MI) in triplicate.

Cell proliferation. Cells were plated on 96-well plates (2×10^3 cells per well). After 24 h, the cells were treated with 0, 12.5, 25 or 50 μM of celecoxib; with 0, 50, 100 or 200 μM of NS-398; or with DMSO alone. The cells were fixed in 1% glutaraldehyde in phosphate-buffered saline (PBS) at 24-h intervals and stained with crystal violet. The dye was then eluted with Sorenson's solution (0.03 M sodium citrate, 0.02 N HCl, and 45% ethanol), and absorbance was read at 540 nm.

Cell cycle. Cells were grown in 10 cm dishes to 70% confluence and treated with 50-100 μM of celecoxib, 100-200 μM of NS-398, or DMSO alone for 48 h. The cells were then harvested by trypsinization. Floating cells were recovered from the supernatant. The cells were washed twice with PBS and fixed in 1% paraformaldehyde in PBS at 4°C for 20 min. They were then washed twice with PBS, resuspended in 70% ethanol, and stored at -20°C overnight. The cells were then washed twice with PBS, resuspended in a 1 ml solution containing 50 μg/ml of propidium iodide and 20 μg/ml of RNAse A in PBS, and incubated for 30 min at 37°C prior to flow cytometry. Apoptosis was determined with the APO-BRDU kit (Phoenix Flow Systems, San Diego, CA).

Clonigenic assay. Cells (1×10^4) were plated on 6-well dishes in triplicate in 0.4% seaplaque agarose (Biowhittaker, Rockland, ME) containing 10% fetal bovine serum with a 0.53% agarose bottom layer. NS-398, celecoxib, or DMSO was distributed in the agarose solution prior to plating. Growth was assessed at 14 days. Colonies were photographed with digital microscopy using an inverted microscope and were manually counted in triplicate wells. Counting was confirmed independently using digital image analysis software (NIH image version 1.61).

RT-PCR. Cells were grown to subconfluence in 10 cm dishes. Total RNA was harvested according to standard protocol (15), and RT-PCR was performed as previously described (16) using primers for COX-1. The 5' primer was 5'-CAGACGACCC GCCTCATCCTCATAG-3', and the 3' primer was 5'-GCC TCAACCCCATAGTCCACCAACA-3'. Amplified DNA fragments were resolved on a 1% agarose gel and visualized with ethidium bromide.
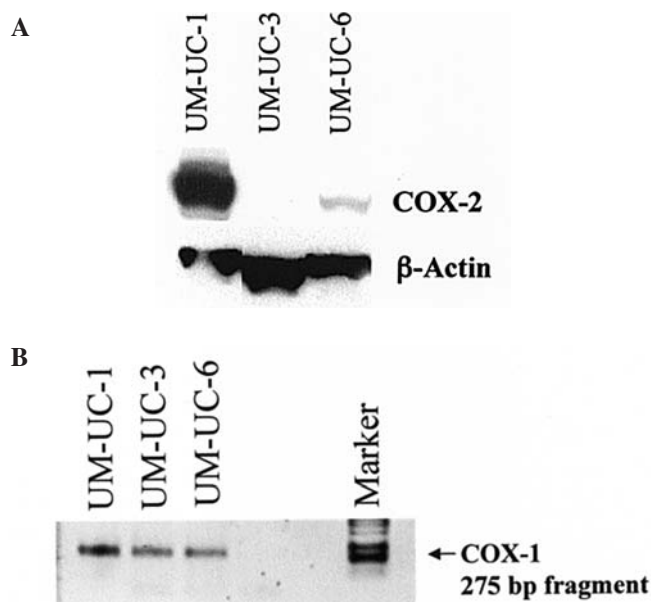

Figure 1. Basal COX-2 expression in UM-UC-1, -3, and -6 by Western analysis (A). Basal COX-1 expression in UM-UC-1, -3, and -6 by RT-PCR amplification of a 275-bp COX-1 coding region segment using primers: 5'-CAGACGACCCGCCTCATCCTCATAG-3' and 5'-GCCTCAACCCCAT AGTCCACCAACA-3' (B).

Western analysis. Protein was harvested as previously described (17) and was denatured at 100°C for 5 min in a sample buffer containing ß-mercaptoethanol prior to loading on 10% polyacrylamide gels. Following electrophoresis at 80-100 V, protein was transferred to a nitrocellulose membrane. Membranes were then probed with antibodies to Bcl-2 (Santa Cruz Inc., Santa Cruz, CA), COX-1, and COX-2 (Cayman, Ann Arbor, MI).

Results

COX-1 and COX-2 expression levels for UM-UC-1, UM-UC-3, and UM-UC-6 were determined by Western analysis. Whereas, UM-UC-1 and UM-UC-6 had high- and low-levels of COX-2 expression, respectively; COX-2 was undetectable in UM-UC-3. COX-1 expression was undetectable by Western analysis in all three cell lines. However, RT-PCR revealed the presence of COX-1 mRNA in all three cell lines, suggesting a low basal expression of this enzyme (Fig. 1B).

Basal prostaglandin E_2 (PGE_2) levels measured in the medium reflected the levels of COX-2 expression in the cell lines. The highest PGE_2 level was found in UM-UC-1 (12,030 pg/ml), followed by UM-UC-6 (59 pg/ml) and UM-UC-3 (20 pg/ml). Following treatment of cells with either NS-398 or celecoxib for 48 h, PGE_2 levels were significantly reduced for UM-UC-1 and UM-UC-6 (Fig. 2). However, PGE_2 levels for UM-UC-3 were unchanged.

Both NS-398 and celecoxib inhibited the growth of all three cell lines in a dose-dependent fashion (Fig. 3). The IC_{50} of celecoxib was 25 μM for all three cell lines, and the IC_{50} of NS-398 was 75 μM for UM-UC-1 and 100 μM for UM-UC-3 and UM-UC-6. Growth inhibition was independent of baseline COX-2 expresssion.
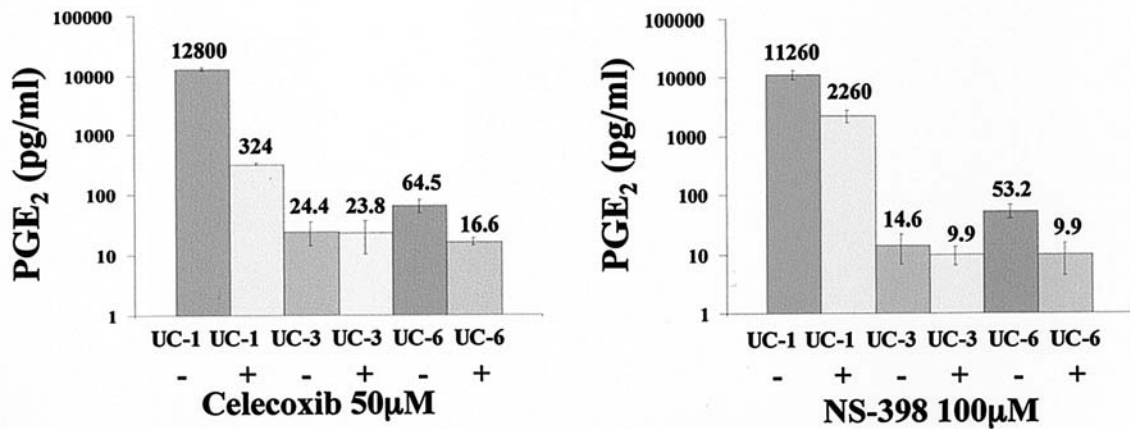
Figure 2. Prostaglandin E$_2$ levels as measured by ELISA in the supernatants of UM-UC-1, -3, and -6.
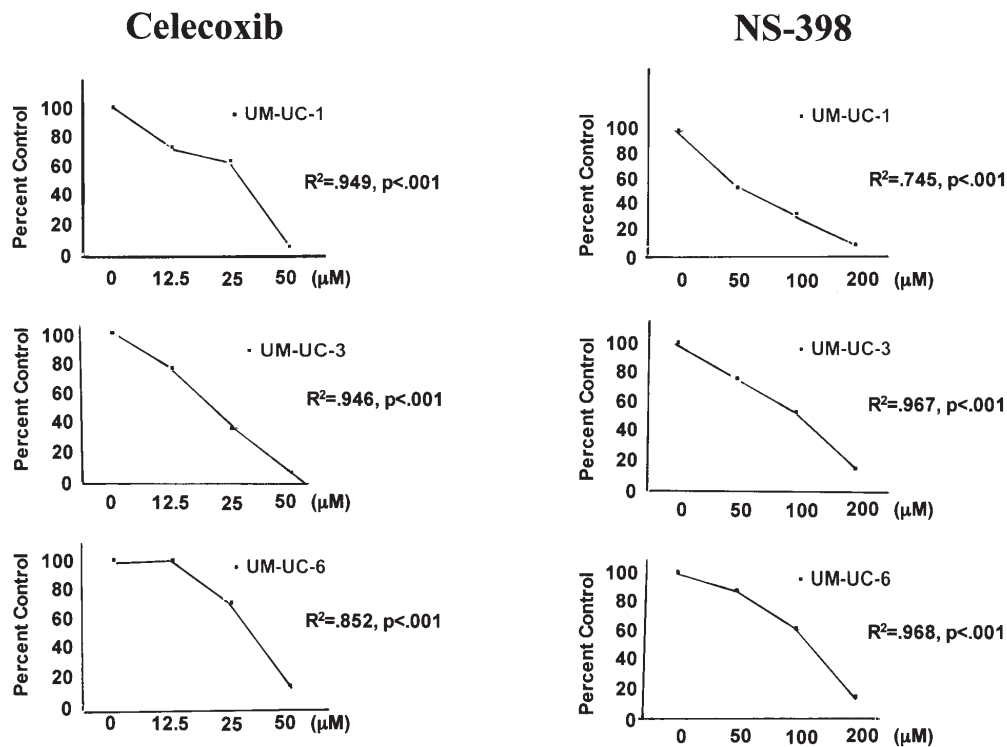

Figure 3. Growth inhibition by celecoxib and NS-398. Graphs represent the percentage of growth relative to control (DMSO only). Linear regression revealed a statistically significant correlation (R^2=0.75-0.97, p<0.001) for each cell line.

Following treatment with 100 μM of NS-398 for 48 h, UM-UC-1, -3, and -6 cells exhibited a decrease in the S-phase population of 44, 25, and 16%, respectively. This decrease in S-phase was associated with a 42 and 53% increase in the G2-M population for UM-UC-1 and UM-UC-3, respectively, whereas a 24% increase in the G0-G1 population was noted for UM-UC-6 (Fig. 4). Apoptosis was not observed in any of these cell lines following treatment with concentrations of NS-398 ≤200 μM for 48 h. NS-398 precipitated out of solution when concentrations >200 μM were used.

In contrast, treatment with 100 μM of celecoxib induced apoptosis in UM-UC-1 and UM-UC-3, with a lesser degree of apoptosis observed for UM-UC-6 (Fig. 5). Treatment of UM-UC-6 with 100 μM of celecoxib resulted in a 21% decrease in S-phase for this cell line, with a corresponding 21% increase in the G0-G1 population. Apoptosis was not detected for these cell lines in the presence of 50 μM celecoxib. However, decreased levels of Bcl-2 expression were observed in all three cell lines in the presence of 25-50 μM of celecoxib (Fig. 6) and 100 μM of NS-398 (data not shown).

NS-398 and celecoxib were both effective in reducing the number and size of UM-UC-1 and UM-UC-3 colonies in soft agarose in a dose-dependent fashion. However, only celecoxib significantly reduced UM-UC-6 colony formation (Fig. 7).
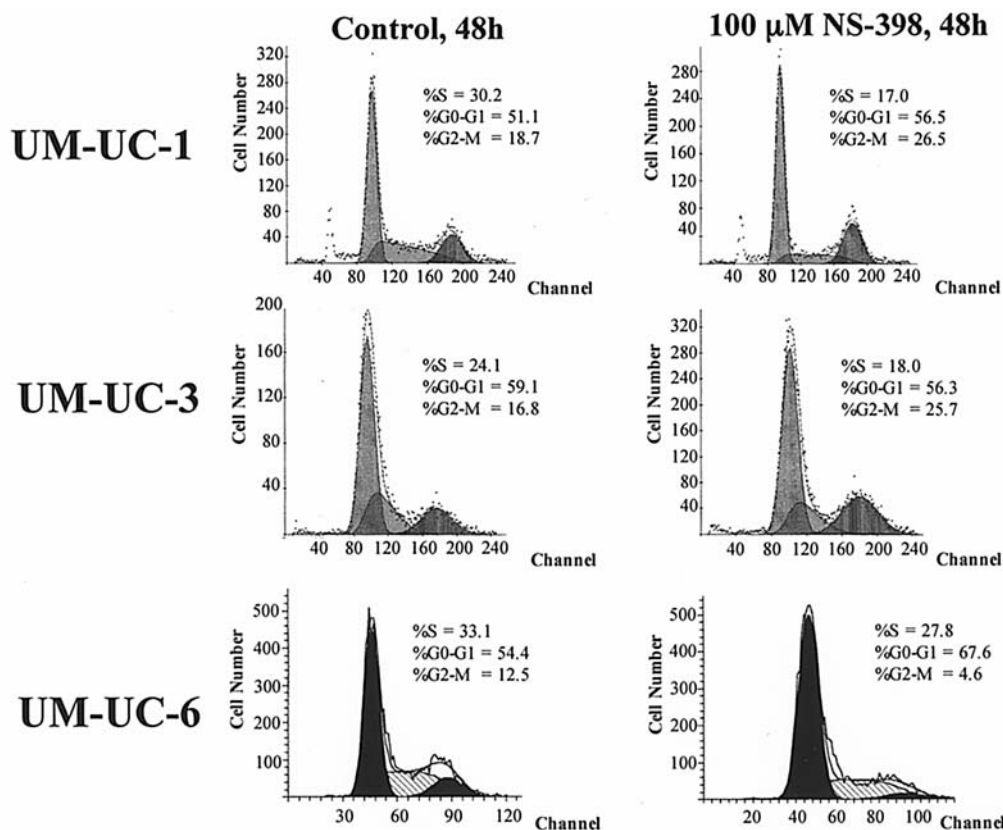
Figure 4. Cell cycle distributions demonstrating a decrease in S-phase following treatment with 100 μM of NS-398.

Discussion

Overexpression of the COX-2 enzyme occurs in a variety of tumors. Forced expression of COX-2 in colon carcinoma cells has been shown to enhance invasion and induce resistance to apoptosis. These findings are associated with changes in the expression of downstream mediators such as Bcl-2, VEGF, matrix metalloproteinases, and E-cadherin (18-20). Excess prostaglandin production by COX-2 is thought to contribute to the malignant phenotype. The treatment of colorectal carcinoma cell lines with PGE_2 results in enhanced growth, motility, invasion, and resistance to apoptosis (21-23).

In this study, we evaluated the biologic effects of two structurally different COX-2 selective inhibitors, celecoxib and NS-398, on bladder cancer cells. Two of the cell lines, UM-UC-1 and UM-UC-6, expressed COX-2 at high- and low-levels, respectively. PGE_2 levels in the medium paralleled the levels of cellular expression of COX-2. Treatment with cele-coxib and NS-398 significantly reduced the PGE_2 produced by these cell lines and significantly inhibited anchorage-dependent and -independent growth. Celecoxib, but not NS-398, induced apoptosis in these cell lines. NS-398 and celecoxib inhibited the growth of UM-UC-3 cells and celecoxib induced apoptosis in this cell line, which does not express detectable levels of COX-2, suggesting that these agents may also work via a COX-2-independent pathway in bladder cancer. Because COX-2 inhibitors have varying degrees of selectivity for the COX-2 isoform, it is possible that COX-1 inhibition may account for some of this effect (24-26).

At the relatively high doses used in this study, these agents may also have inhibited the COX-1 enzyme.

There is evidence indicating that NSAIDs may work independently of COX. Sulindac sulfone, a metabolite of the NSAID sulindac sulfide, affects neither COX-1 nor COX-2 but inhibits growth and induces apoptosis in colon carcinoma cells (27). Furthermore, celecoxib has been shown to inhibit cell cycle progression and to induce apoptosis in colon cancer cell lines lacking COX-2 (28). Additionally, NS-398 has been shown to induce apoptosis in colon cancer cells lacking COX-2 expression (29). NSAID concentrations at which antitumor effects such as induction of apoptosis, cell cycle inhibition, and inhibition of angiogenesis are seen may be 100- to 1000-fold higher than that necessary to inhibit PGE_2 synthesis, which may indicate an effect on other molecular pathways (30). Structural modifications of celecoxib, in which the terminal phenyl group was replaced with various substituents, demonstrated a wide range of apoptotic activity for these derivative compounds (31). Apoptotic activity was unrelated to COX-2 inhibitory activity of these compounds. NS-398 and celecoxib have been reported to inhibit the COX-2 enzyme (IC_{50}) with a range of 0.1-0.9 μM (30). However, we found that concentrations of NS-398 ranging from 25 to 200 μM were necessary to inhibit the growth of bladder cancer cells, and growth inhibition and induction of apoptosis in the presence of celecoxib occurred at concentrations ranging from 12.5 to 100 μM. Growth inhibition occurred in a dose-dependent fashion with a statistically significant correlation by linear regression analysis (R^2=0.75-0.97, p<0.001). Our findings are consistent with that of Williams

A   **UM-UC-1**

B   **UM-UC-3**

C   **UM-UC-6**

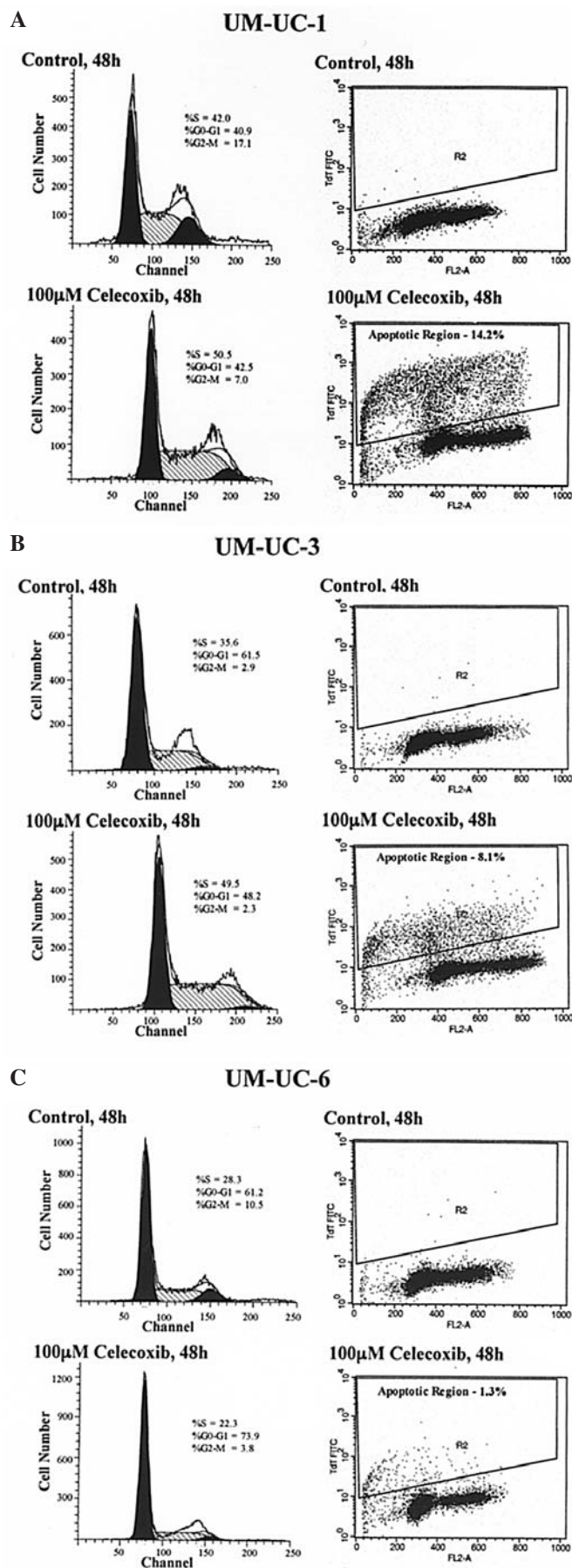Figure 5. Induction of apoptosis in UM-UC-1 in the presence of 100 μM celecoxib (A). Induction of apoptosis in UM-UC-3 in the presence of 100 μM celecoxib (B). Apoptosis is seen to a lesser extent in UM-UC-6 in the presence of 100 μM celecoxib (C).
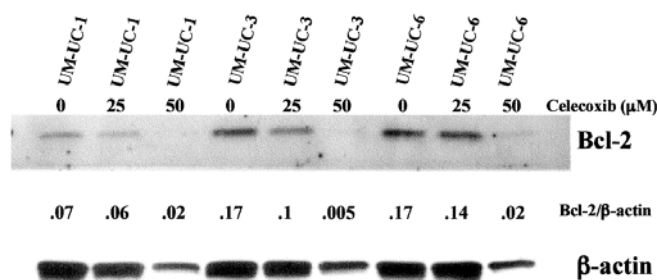

Figure 6. Bcl-2 downregulation in the presence of increasing concentrations of celecoxib.

et al (28), in which *in vitro* concentrations of celecoxib as well as other NSAIDs ranging from 25 to 100 μM were required to produce growth inhibition and apoptosis in colon carcinoma cells. They also found that serum concentrations of only 2-3 μM celecoxib were sufficient to inhibit the growth of colon carcinoma xenografts in nude mice, illustrating the importance of *in vivo* factors in this process (28). Increased efficacy *in vivo* may result from an anti-angiogenic effect (32). The efficacy of celecoxib in reducing bladder cancer incidence and growth *in vivo* has also been reported by Grubbs *et al* (33).

We found that celecoxib downregulates Bcl-2 in bladder cancer cells. Downregulation of Bcl-2 and induction of apoptosis have also been reported following treatment of the prostate carcinoma cell line LNCaP with NS-398 (34). Alternative molecular targets for COX-2 inhibitors that have been identified include NF-κB, AP-1, Akt, PPARδ PPARγ and STAT1 (29,35,36). No significant change in PPARγ expression in the presence of COX-2 inhibitors was identified by Western analysis (data not shown). Furthermore, there has been recent interest in the 15-lipoxygenase enzyme as an alternate mechanism by which NSAIDs may induce apoptosis. Detectable expression of 15-lipoxygenase was not observed in any of the bladder cancer cell lines in this study (data not shown). However, *in vitro* studies of esophageal carcinoma as well as colon carcinoma have demonstrated that NSAID treatment with both COX-2 selective and nonselective inhibitors upregulates the 15-lipoxygenase enzyme (37,38).

Biologic differences were demonstrated for the two COX-2 selective NSAIDs in this study. Although both agents produced significant growth inhibition of the bladder cancer cells, a decrease in S-phase was observed consistently only for NS-398, suggesting differing mechanisms of growth inhibition for these two agents. Furthermore, apoptosis was observed only in cells treated with celecoxib. In prostate cancer, growth inhibition and rapid cellular changes consistent with apoptosis were observed within 2 h of treatment with celecoxib but were seen only after cells were exposed to NS-398 for 120 h (39). Structural differences of celecoxib and NS-398 may account for the different rates of apoptosis observed.

In conclusion, the COX-2 selective inhibitors NS-398 and celecoxib produce dose-dependent growth inhibition of bladder cancer cell lines. However, biologic differences in terms of cell cycle progression and induction of apoptosis were observed for these two agents. Both celecoxib and NS-398 down-regulated Bcl-2. These changes occurred in cells that do not
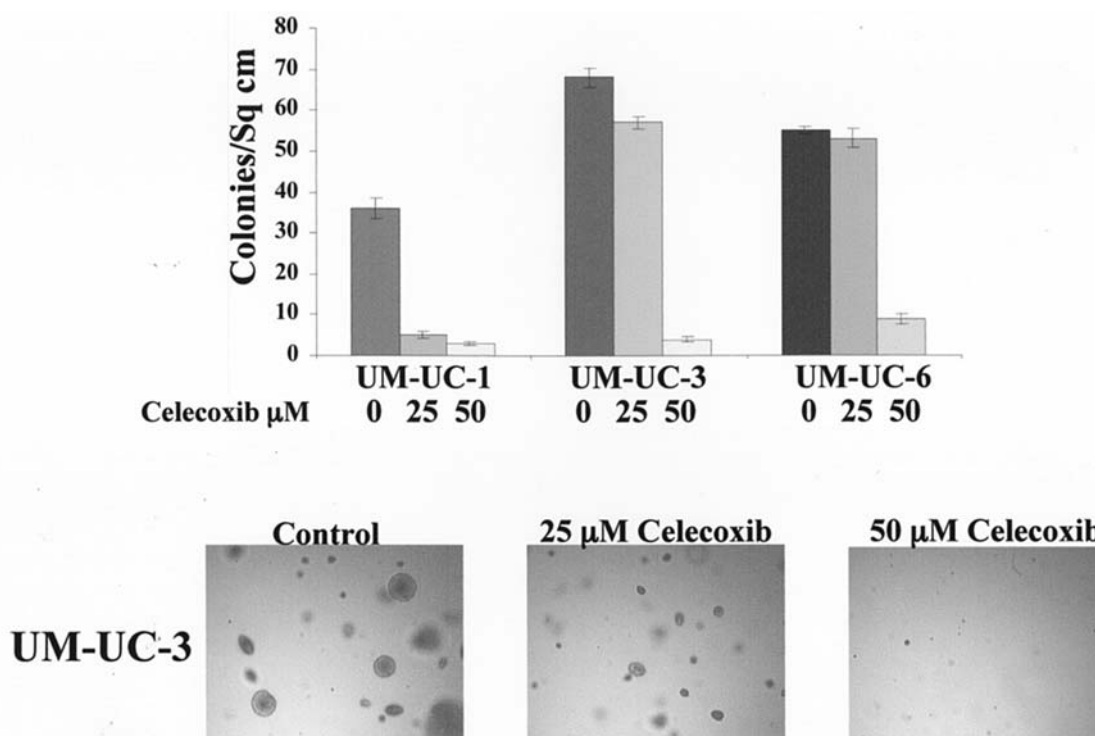
Figure 7. Inhibition of anchorage independent growth-colony formation for UM-UC-3 in the presence of celecoxib treatment.

express COX-2, suggesting an important role for COX-2-independent activity for these agents.

Acknowledgements

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