

Published in final edited form as:

*Anticancer Res.* 2009 October ; 29(10): 3769–3775.

## Cyclin-mediated G1 Arrest by Celecoxib Differs in Low-versus High-grade Bladder Cancer

JASON R. GEE<sup>1,2</sup>, CORRIE B. BURMEISTER<sup>2</sup>, THOMAS C. HAVIGHURST<sup>3</sup>, and KYUNG MANN KIM<sup>3</sup>

<sup>1</sup> Department of Urology, William S. Middleton Memorial Veterans Hospital, Madison, WI

<sup>2</sup> Department of Urology, School of Medicine and Public Health, University of Wisconsin - Madison, Madison, WI, U.S.A

<sup>3</sup> Department of Biostatistics and Informatics, University of Wisconsin - Madison, Madison, WI, U.S.A

### Abstract

**Background**—Celecoxib and other non-steroidal anti-inflammatory drugs (NSAIDs) are being evaluated in the prevention of bladder and other cancers. Here we investigate molecular effects of celecoxib independent of cyclooxygenase (COX)-2 expression levels in urothelial carcinoma of the bladder.

**Materials and Methods**—Low-grade RT-4 and high-grade UM-UC-3 bladder cancer cells were treated with 0–50  $\mu$ M celecoxib. Growth, cell cycle and apoptosis were measured by crystal violet elution and flow cytometry. Western analysis was performed for COX-2, Rb, cyclin B1/D1, and phosphocyclin B1/D1. COX-2 induction was achieved with phorbol ester.

**Results**—Celecoxib inhibited growth of RT-4 and UM-UC-3, with G<sub>1</sub> cell cycle arrest and altered cyclin B1/D1 expression in RT-4, whereas Rb up-regulation occurred in UM-UC-3. Apoptosis occurred in both cell lines.

**Conclusion**—Celecoxib induces G<sub>1</sub> cell cycle arrest in low- and high-grade bladder cancer by different pathways. This heterogeneous molecular response supports combination approaches to prevention and treatment.

### Keywords

Cyclooxygenase (COX)-2; celecoxib; bladder cancer; chemoprevention

---

Aberrant expression of cyclooxygenase (COX)-2 has been identified in multiple tumor types including bladder cancer. This enzyme is thought to contribute to the malignant phenotype and has been implicated in increased growth, invasion, angiogenesis, and resistance to apoptosis. Non-steroidal anti-inflammatory drugs (NSAIDs) are known to inhibit this enzyme, and selective COX-2 inhibitors have been developed in an attempt to limit toxicity associated with the inhibition of COX-1 by non-selective COX inhibitors (1). More recently, COX-2 selective inhibitors have been the focus of much scrutiny and publicity in terms of cardiovascular risk such that COX-2 inhibitors and NSAIDs in general are now being critically evaluated. Of such inhibitors, celecoxib is thought to have a better safety profile than other COX-2 selective inhibitors, and has been evaluated in an NCI-sponsored study in the secondary prevention of

high-risk bladder cancer (2,3). Furthermore, recent clinical trials of celecoxib in the prevention of adenomatous intestinal polyps have demonstrated the efficacy of celecoxib as a preventive agent but found a trend toward elevated risk primarily in those patients with a cardiac history and when celecoxib was given in a *b.i.d.* dosing schedule. In contrast, a recent meta-analysis of multiple recent large clinical trials failed to identify elevated cardiovascular risk with celecoxib (4), supporting further study of this agent in cancer prevention for select patients at lower risk (5). Recent studies have also supported the use of celecoxib as an adjunct to chemotherapy in patients with advanced cancers (6–8). Its efficacy in growth inhibition and potency as an inducer of apoptosis has been found to be better than that of other COX-2 selective drugs and to be independent of COX-2 (9–13). The COX-2-independent activity of celecoxib and other NSAIDs has also been described in multiple tumor types, although the specific pathways involved have been largely unexplored in bladder cancer. We have previously demonstrated that celecoxib inhibits growth and induces apoptosis in the UM-UC-3 cell line, which lacks COX-2 expression (14). Given the finding of heterogeneous expression of COX-2 in bladder cancer (15), an agent such as celecoxib that features both COX-2-dependent and -independent activity may be more effective than other NSAIDs. This study focuses on the mechanism by which celecoxib inhibits growth in bladder cancer. The effects of celecoxib in bladder cancer cells that express COX-2 or lack COX-2 expression will be evaluated.

## Materials and Methods

### Cell culture

Two human bladder cancer cell lines representing either low or high grade malignant phenotypes are the RT-4 urothelial cell line derived from low-grade papillary urothelial carcinoma as well as the poorly differentiated urothelial cell line UM-UC-3 (ATCC). RT-4 and UM-UC-3 were maintained respectively in either MEM containing 10% fetal calf serum along with penicillin-streptomycin or McCoy's medium supplemented with 1% fetal bovine serum, insulin, transferrin, hydrocortisone, L-glutamine, dextrose, and non-essential amino acids.

### Cell proliferation

Cells were plated on 96-well plates ( $2 \times 10^3$  cells per well). After 24 h, the cells were treated with 0, 6.25, 12.5, 25, and 50  $\mu\text{M}$  celecoxib (LKT Labs). The cells were then fixed in 1% glutaraldehyde in phosphate-buffered saline (PBS) at 24-h intervals and stained with crystal violet. The dye was eluted with Sorenson's solution (0.03 M sodium citrate, 0.02 N HCl, and 45% ethanol), and absorbance was read at 540 nm.

### Induction of COX-2 expression

RT-4 and UM-UC-3 were grown to semi-confluence in 6-well plates and treated with phorbol ester 0.1  $\mu\text{M}$  6 h prior to protein lysis. Lysates were then evaluated for COX-2 expression by Western analysis.

### Flow cytometry and apoptosis

Cells were grown in 10-cm dishes to 70% confluence and treated with celecoxib (0–50  $\mu\text{M}$ ) for a range of fixed time points (30 min, 1 h, 3 h, 5 h, and 24 h). The cells were then harvested by trypsinization. Floating cells were recovered from the supernatant. The cells were then washed twice with PBS and fixed in 1% paraformaldehyde in PBS at 4°C for 20 min. They were again washed twice with PBS, resuspended in 70% ethanol and stored at  $-20^\circ\text{C}$  overnight. Prior to flow cytometry, the cells were washed twice with PBS, resuspended in a 1-ml solution containing 50  $\mu\text{g}/\text{ml}$  propidium iodide and 20  $\mu\text{g}/\text{ml}$  RNase A in PBS, and incubated for 30

min at 37°C. Apoptosis was determined with the APO-BRDU kit (Phoenix Flow Systems, San Diego, CA, USA). All experiments were repeated in triplicate.

### Western blotting

RT-4 and UM-UC-3 cells were grown to near confluence in 6-well dishes prior to treatment with celecoxib (0–50  $\mu$ M) for 24 h or phorbol ester (0.1  $\mu$ M) for 6 h. Cells were then washed with PBS and extracted by scraping the cells from each well into 100  $\mu$ l of lysis buffer (PBS with 1% Triton X-100, 1 mM PMSF, 0.1 mM leupeptin and 1% aprotinin). After incubation on ice for 30 min, these lysates were centrifuged at 11,000  $\times$ g at 4°C. For Western blotting, the extracts were mixed with non-reducing 6 $\times$  Laemmli sample buffer, and 20  $\mu$ g of sample was applied to a 10% polyacrylamide gel. Following electrophoresis at 140 V, protein was transferred to a nitrocellulose membrane. Membranes were then probed with specific antibodies. Samples were run in parallel with prestained molecular weight standards (Bio-Rad Prestained Standards). The nitrocellulose was blocked overnight at 4°C while shaking in PBS containing 0.05% Tween 20 and 10% nonfat dry milk. The nitrocellulose sheet was incubated while shaking at room temperature for 1 h with primary antibodies to either COX-2 (Cayman), Cyclin D1 and Cyclin B1 (Upstate), phospho-Cyclin D1 [Thr 286] and phospho-Cyclin B1 [Ser 147] (Cell Signaling Technology), or Rb (Abcam), washed three times with PBS, and incubated 1 h with peroxidase-conjugated goat anti-mouse immunoglobulin (Santa Cruz Biotechnology) diluted in blocking solution. The nitrocellulose sheet was then washed in PBS and developed using chemiluminescence with the SuperSignal West Femto Maximum Sensitivity Substrate kit (Pierce Chemical, Rockford, IL, USA).

### Statistical methods

For cellular proliferation, regression analysis was used to test whether celecoxib inhibited the growth of UM-UC-3 and RT-4 in a dose-dependent fashion. For cyclin and Rb expression, the relationships of celecoxib dose to protein expression in the different cell lines were determined as follows. The log of the ratio of the densitometry value divided by the vehicle control values was calculated for each dose level. This was regressed on the log of dose. Regression equations were fit so the intercept was forced to be 0—that is, at dose 0, the log of the dose ratios was assumed to be (and is by definition) 0. Computations were performed with R software (R Development Core Team, Vienna Austria; <http://www.R-project.org>).

## Results

### COX-2 expression

Low levels of COX-2 expression were identified in the RT-4 cell line by Western blotting, as compared with the UM-UC-3 cell line, which lacks COX-2 expression. Induction of COX-2 in RT-4 was achieved by treatment with phorbol ester for 6 h, whereas no induction of COX-2 was detected in UM-UC-3 (Figure 1).

### Cellular proliferation

Cellular proliferation was measured at 24, 48, and 72 h in the presence and absence of celecoxib. Celecoxib inhibited the growth of RT-4 and UM-UC-3 in a dose-dependent fashion ( $p < 0.0001$ ). The  $IC_{50}$  for celecoxib was 12.5  $\mu$ M and 25  $\mu$ M for RT-4 and UM-UC-3, respectively (Figure 2).

### Cell cycle and apoptosis

To determine the mechanism by which growth inhibition occurs independent of COX-2 expression, we then evaluated the effects of celecoxib on cell cycle distribution. We found that for celecoxib concentrations ranging from 0 to 50  $\mu$ M, a dose-dependent increase was observed

in the G<sub>0</sub>/G<sub>1</sub> cell population in the UM-UC-3 and RT-4 cell lines. Apoptosis was also observed in response to celecoxib for both cell lines (Figure 3).

### Cyclin and Rb expression

In evaluating cell cycle regulatory checkpoints, we determined whether cyclin expression was altered in the presence of celecoxib for these cell lines by western analysis (Figure 4). Cyclin and Rb expression was measured with doses of 0 (vehicle), 3.125, 6.25, 12.5, 25, and 50  $\mu$ M celecoxib. The highest dose, 50  $\mu$ M, was considered likely to cause apoptosis, so was excluded from this analysis.

For each set of data points, the densitometry values are normalized to vehicle control values. Therefore, control densitometry values are by definition 1, and regression analysis was performed for these values. Table I gives the slopes for change in expression of the cyclins and Rb as well as p-values indicating statistical significance. Expression of cyclin and Rb was measured for doses of 0, 3.125, 6.25, 12.5, 25, and 50  $\mu$ M celecoxib. However, for the above analysis, the highest dose (50  $\mu$ M) was considered likely to cause apoptosis, and therefore was excluded from analysis.

For the cell line RT-4, cyclin D1 ( $p=0.004$ ) and cyclin B1 ( $p<0.001$ ) expression show a significant relationship with dose, the first having a positive relationship, and the second having a negative relationship.

For the cell line UM-UC-3, cyclin expression was not significantly altered, although Rb shows a significant result ( $p=0.012$ ) with a positive relationship. In terms of phosphorylated cyclins, phospho-cyclin D1 shows a positive but also only marginally significant relationship in RT-4 ( $p=0.071$ ). Overall, phosphorylated cyclins (data not shown) did not change significantly in relationship to celecoxib dose, implying that inhibition of phosphorylation is not a major mechanism of growth inhibition in these bladder cancer cell lines.

### Discussion

The cyclooxygenase enzyme mediates prostaglandin production and consists of two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in normal tissues. COX-2 is inducible in the setting of neoplasia and inflammation. COX-2 expression is generally absent in normal tissue, but up-regulation of this enzyme has been reported in multiple tumor types. These include colon, breast, and pancreatic tumors as well as transitional cell carcinoma (16,17). COX-2 facilitates production of prostaglandins and downstream mediators that enhance growth, angiogenesis, invasion, and resistance to apoptosis. For instance, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP) have been identified with the prostaglandin pathway (18–20). Furthermore, increased expression of the anti-apoptotic factor Bcl-2 has been observed with COX-2 up-regulation (21). It is also known that *in vitro* treatment of colon cancer cells with prostaglandin (PGE<sub>2</sub>) causes activation of the phosphatidyl 3-kinase (PI3K)/Akt pathway (22) with resulting increased cellular proliferation and motility.

We have also explored the biologic potential of COX-2 in urothelium. Utilizing a premalignant urothelial model, we have demonstrated that forced COX-2 overexpression in SV-HUC cells increases PGE<sub>2</sub> production with associated enhanced cellular growth and invasion (23). COX-2 activity may be counterbalanced by metabolism of prostaglandins by the prostaglandin dehydrogenase (PGDH) enzyme (24). More recently, we have also reported the loss of PGDH in TCC cell lines and in bladder tumors, which may contribute to higher prostaglandin levels (25,26).

Celecoxib is a selective inhibitor of COX-2. COX-2 inhibitors decrease growth and promote apoptosis in multiple tumor types *in vitro*. We have previously shown that celecoxib reduces PGE<sub>2</sub> levels, inhibits growth, and induces apoptosis in a panel of bladder cancer cell lines (14). Celecoxib is often found to be more effective than other COX-2 inhibitors such as rofecoxib (9–11) and NS-398 (13). This would suggest additional COX-2-independent mechanisms of activity. Celecoxib has been shown to inhibit growth independently of COX-2 expression levels with a G<sub>0</sub>/G<sub>1</sub> cell cycle arrest and decreased levels of cyclins A and B1 or cyclin D1 depending on tumor type (27,28). Furthermore, both celecoxib and NS-398 can induce apoptosis independently of COX-2 with release of cytochrome C, poly (ADP-ribose) polymerase (PARP) cleavage, and activation of caspases 3 and 9 (27,29). Other possible COX-2-independent effects have also been identified with NSAIDs, involving the 3-phosphoinositide-dependent kinase 1 (PKB1) and the serine/threonine protein kinase B (PKB)/Akt pathways in colon and prostate cancer cell lines (30–32). The mechanism by which celecoxib inhibits growth of bladder cancer cell lines remains uncertain and is the focus of this study.

We previously identified activity of celecoxib in the UM-UC-3 cell line, which lacks detectable COX-2 expression (14). Whereas COX-2 expression was undetectable for UM-UC-3 by Western analysis or by RT-PCR, we sought to demonstrate whether COX-2 enzyme expression could be induced in these cells by exposure to phorbol ester. The mechanism by which this occurs is thought to involve activation of protein kinase C with subsequent up-regulation of COX-2. Whereas significant up-regulation of COX-2 was observed for RT-4, COX-2 expression remained undetectable for UM-UC-3 following exposure to phorbol ester. This suggests either a COX-2 promoter mutation or other defect of this pathway that interferes with COX-2 gene expression, such that even low, undetectable basal levels of COX-2 in this cell line probably do not exist. Ironically, this cell line, which lacks COX-2 expression, is the most aggressive of the two, having been derived from an invasive urothelial carcinoma (33). Whereas this may represent an aberration of cell culture, certainly it is known that heterogeneous expression of COX-2 occurs in urothelial tumors (15).

It is conceivable that inhibition of low basal levels of COX-2 in UM-UC-3 could account for the activity of celecoxib. However, COX-2 expression is undetectable by Western analysis for UM-UC-3 (14), and induction of COX-2 does not occur with UM-UC-3 in this study, further confirming that low basal levels of COX-2 are not expressed by UM-UC-3. Therefore, it is most likely an actual COX-2-independent mechanism of action that we observe with celecoxib in UM-UC-3. COX-2-independent activity of NSAIDs could play an important role in which COX-2 is not expressed homogeneously throughout the tumor.

Given the increase in G<sub>0</sub>/G<sub>1</sub> cell population implying G<sub>1</sub> cell cycle arrest, we chose to evaluate cyclin and Rb expression levels in response to celecoxib. An interesting observation for the RT-4 cell line is that up-regulation of cyclin D1 accompanied G<sub>1</sub> arrest in a dose-dependent fashion with celecoxib, whereas cyclin B1 levels decreased, suggesting that modulation of cyclin expression occurs in response to celecoxib in this cell line. Whereas down-regulation of cyclin B has been observed previously in association with G<sub>1</sub> arrest with celecoxib (27), we also identified paradoxical up-regulation of cyclin D1. This would be inconsistent with prior findings of cyclin D down-regulation with celecoxib in other tumor types, which may be anticipated with G<sub>1</sub> arrest (28,34). One possible explanation for this is the reported induction of the cell cycle inhibitor p27 with celecoxib (27), which may, in turn, trigger up-regulation of cyclin D as part of a positive feedback mechanism (35). In this sense, induction of cyclin D may represent a homeostatic feedback response to celecoxib in bladder cancer. While a trend revealing up-regulation of phospho-cyclin D1 also exists, neither levels of phospho-cyclin D1 nor phospho-cyclin B1 change significantly in response to celecoxib, implying that modulation

in cyclin expression plays a greater role than inhibition of cyclin phosphorylation in the RT-4 cell line.

In this study, we also found that celecoxib inhibits growth independently of COX-2 expression in UM-UC-3 with an increase in the G<sub>0</sub>/G<sub>1</sub> cell population accompanied by intense induction of apoptosis. In contrast to RT-4, no significant change in cyclin or phospho-cyclin levels occurs in the COX-2-null UM-UC-3 cell line, implying that cyclin dependent regulation may not be intact in these high grade cells. Nevertheless, up-regulation of Rb was observed in UM-UC-3, indicating that cell cycle arrest may be achieved with celecoxib through alterations in the Rb pathway as a COX-2 independent mechanism. One explanation for this discrepancy is that RT-4, derived from a low-grade tumor, may harbor intact cell cycle machinery and therefore displays changes in cyclin cell cycle mediator expression, whereas UM-UC-3 is a poorly differentiated cell line. In a study by Delia *et al.*, dissociation between cell cycle arrest and apoptosis has been reported for cells of the same tumor type, which differ in whether or not they have an intact G<sub>1</sub>/S checkpoint (36). Alternatively, there may be a necessary role for the COX-2 pathway in modulation of the cyclins by celecoxib in bladder cancer. Further studies are necessary to explore these hypotheses, as these observations appear to be cell line-dependent.

While differences exist in cell cycle regulation in low-*versus* high-grade bladder cancer cells, apoptosis occurs in response to celecoxib for both cell lines and may be a more reliable and consistent indicator of biologic activity of this agent in bladder cancer. We have previously reported that apoptosis occurs independent of COX-2 in association with Bcl-2 down-regulation in bladder cancer (14). In a recent study by Song *et al.*, celecoxib induced apoptosis more effectively than other COX-2 agents independent of COX-2 inhibition (12). What we can conclude from this study is that celecoxib inhibits growth of both low- and high-grade bladder cancer, by cell cycle arrest and apoptosis, and that the primary molecular mechanism of growth inhibition and cellular susceptibility may be ultimately dependent on the degree of cellular differentiation.

Of note, the concentration at which these events occur *in vitro* is significantly higher than the physiologic concentration reported for celecoxib with oral administration. Similarly, Williams *et al.* (37) reported that *in vitro* concentrations of celecoxib as well as other NSAIDs ranging from 25 to 100 μM were necessary to produce growth inhibition and apoptosis in colon carcinoma cells. Furthermore, serum concentrations of only 2–3 μM celecoxib were required to inhibit the growth of colon carcinoma xenografts in nude mice, illustrating the importance of *in vivo* factors in this process (37). An antiangiogenic effect may also account for increased efficacy of these agents *in vivo* (38). Indeed, celecoxib has been shown to be effective in reducing bladder cancer incidence and growth in an *in vivo* model of bladder carcinogenesis (39). An effect on multiple molecular pathways other than COX-2 is suggested as antitumor effects including apoptosis, cell cycle inhibition, and inhibition of angiogenesis occur at NSAID concentrations 100- to 1,000-fold higher than that necessary to inhibit PGE<sub>2</sub> synthesis (40). For instance, celecoxib has been reported to inhibit the COX-2 enzyme with an IC<sub>50</sub> of less than 1 μM (40). Whether the tissue concentration of celecoxib in *in vivo* models of bladder cancer is sufficient to activate this COX-2-independent pathway remains to be determined.

In summary, celecoxib inhibits the growth of both low-grade and high-grade bladder cancer cells with an accompanying increase in the G<sub>0</sub>/G<sub>1</sub> cell population. Whereby modulation of cyclin cell cycle mediators occurs in the low-grade, COX-2-expressing RT-4 cell line, essentially no change in these cyclins is observed for the poorly differentiated UM-UC-3 cell line, in which cell cycle regulatory pathways are less likely to be intact. However, apoptosis occurs for both RT-4 and UM-UC-3, implying that apoptotic mediators may be more important and universal downstream mediators of celecoxib in terms of prevention and treatment for

patients with low- and high-grade bladder cancer. In this sense, markers of apoptosis may most reliably indicate biologic activity in clinical trials of celecoxib in the prevention and treatment of patients with bladder cancer.

As we continue to study the role of NSAIDs and molecular mechanisms of COX-2 and inhibition of this enzyme, hopefully these studies will permit us to better select patients who may benefit from preventive or treatment strategies utilizing these agents. In balancing risks associated with NSAIDs, these findings support the further study of celecoxib and other NSAIDs in the prevention and treatment of bladder cancer.

## Acknowledgments

Terry Oberley, MD, Ph.D., assisted with editing of this manuscript.

This work is supported by the Office of Research and Development, Biomedical Laboratory R&D Service, Department of Veterans Affairs (J.R. Gee) and by P30 CA 14520 (T. Havighurst & K. Kim).

## Abbreviations

NSAIDs	Non-steroidal anti-inflammatory agents
COX	Cyclooxygenase
PI	Propidium Iodide

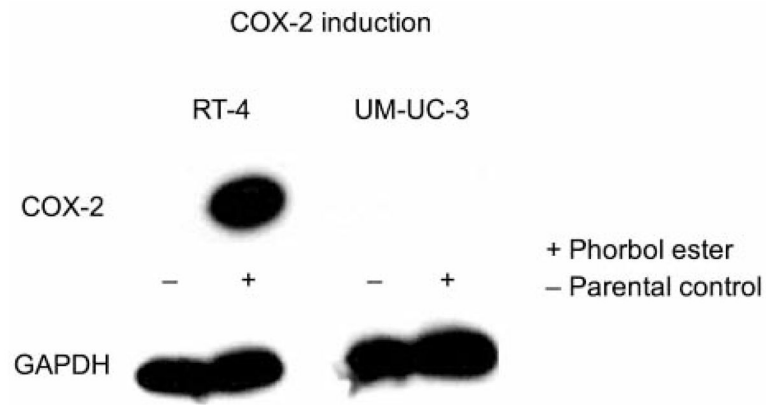
## References

1. Thun MJ, Henley SJ, Patrono C. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. *J Natl Cancer Inst* 2002;94:252–266. [PubMed: 11854387]
2. Okie S. Raising the safety bar – the FDA’s coxib meeting. *N Engl J Med* 2005;352(13):1283–1285. [PubMed: 15800221]
3. Gee J, Sabichi AL, Grossman HB. Chemoprevention of superficial bladder cancer. *Crit Rev Oncol/Hematol* 2002;43:277–288.
4. White WB, West CR, Borer JS, et al. Risk of cardiovascular events in patients receiving celecoxib: a meta-analysis of randomized clinical trials. *Am J Cardiol* 2007;99(1):91–98. [PubMed: 17196469]
5. Bertagnolli MM, Eagle CJ, Zauber AG, et al. Celecoxib for the prevention of sporadic colorectal adenomas. *N Engl J Med* 2006;355(9):873–884. [PubMed: 16943400]
6. Dandekar DS, Lopez M, Carey RI, Lokeshwar BL. Cyclooxygenase-2 inhibitor celecoxib augments chemotherapeutic drug-induced apoptosis by enhancing activation of caspase-3 and -9 in prostate cancer cells. *Int J Cancer* 2005;115(3):484–492. [PubMed: 15688368]
7. Altorki NK, Port JL, Zhang F, et al. Chemotherapy induces the expression of cyclooxygenase-2 in non-small cell lung cancer. *Clin Cancer Res* 2005;11(11):4191–4197. [PubMed: 15930356]
8. Nugent FW, Mertens WC, Graziano S, et al. Docetaxel and cyclooxygenase-2 inhibition with celecoxib for advanced non-small cell lung cancer progressing after platinum-based chemotherapy: a multicenter phase II trial. *Lung Cancer* 2005;48(2):267–273. [PubMed: 15829328]
9. Kazanov D, Dvory-Sobol H, Pick M, et al. Celecoxib but not rofecoxib inhibits the growth of transformed cells *in vitro*. *Clin Cancer Res* 2004;10(1 Pt 1):267–271. [PubMed: 14734479]
10. Yamazaki R, Kusunoki N, Matsuzaki T, Hashimoto S, Kawai S. Selective cyclooxygenase-2 inhibitors show a differential ability to inhibit proliferation and induce apoptosis of colon adenocarcinoma cells. *FEBS Lett* 2002;531(2):278–284. [PubMed: 12417326]
11. Waskewich C, Blumenthal RD, Li H, et al. Celecoxib exhibits the greatest potency amongst cyclooxygenase (COX) inhibitors for growth inhibition of COX-2-negative hematopoietic and epithelial cell lines. *Cancer Res* 2002;62(7):2029–2033. [PubMed: 11929821]

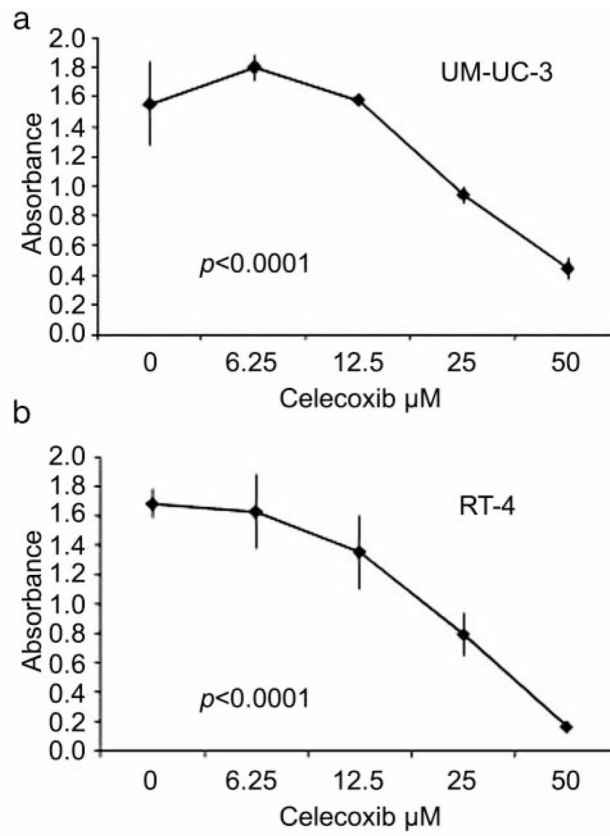
12. Song X, Lin H-P, Johnson AJ, et al. Cyclooxygenase-2, player or spectator in cyclooxygenase-2 inhibitor-induced apoptosis in prostate cancer cells. *J Natl Cancer Inst* 2002;94:585–591. [PubMed: 11959891]
13. Johnson AJ, Song X, Hsu A-L, Chen C-S. Apoptosis signaling pathways mediated by cyclooxygenase-2 inhibitors in prostate cancer cells. *Adv Enzyme Regul* 2001;41:221–235. [PubMed: 11384747]
14. Gee J, Lee I-L, Jendiroba D, et al. Selective cyclooxygenase-2 inhibitors inhibit growth and induce apoptosis of bladder cancer. *Oncol Rep* 2006;15(2):471–477. [PubMed: 16391871]
15. Gee JR, Montoya RG, Khaled HM, Sabichi AL, Grossman HB. Cytokeratin 20, AN43, PGDH, and COX-2 expression in transitional and squamous cell carcinoma of the bladder. *Urol Oncol* 2003;21(4):266–270. [PubMed: 12954496]
16. DuBois RN, Arbramson SB, Crofford L, et al. Cyclooxygenase in biology and disease. *FASEB J* 1998;12:1063–1073. [PubMed: 9737710]
17. Mohammed SI, Knapp DW, Bostwick DG, et al. Expression of cyclooxygenase-2 (COX-2) in human invasive transitional cell carcinoma (TCC) of the urinary bladder. *Cancer Res* 1999;59:5647–5650. [PubMed: 10582676]
18. Fosslie E. Molecular pathology of cyclooxygenase-2 in neoplasia. *Ann Clin Lab Sci* 2000;30:3–21. [PubMed: 10678579]
19. Tsujii M, Kawano S, Tsuji S, et al. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* 1998;93:705–716. [PubMed: 9630216]
20. Tsujii M, Kawano S, Dubois RN. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc Natl Acad Sci USA* 1997;94:3336–3340. [PubMed: 9096394]
21. Tsujii M, DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell* 1995;83:493–501. [PubMed: 8521479]
22. Sheng H, Shao J, Washington MK, et al. Prostaglandin E<sub>2</sub> increases growth and motility of colorectal carcinoma cells. *J Biol Chem* 2001;276:18075–18081. [PubMed: 11278548]
23. Gee J, Lee I-L, Grossman HB, Sabichi AL. Effects of COX-2 overexpression on the immortalized, nontumorigenic urothelial cell line SV-HUC. *Urol Oncol* 2008;26(6):641–645. [PubMed: 18367112]
24. Tong M, Tai HH. Induction of NAD<sup>+</sup>-linked 15-hydroxyprostaglandin dehydrogenase expression by androgens in human prostate cancer cells. *Biochem Biophys Res Commun* 2000;276:77–81. [PubMed: 11006085]
25. Liebert M, Chen I-L, Gebhardt D, et al. Loss of expression of prostaglandin dehydrogenase in human bladder cancers. *Proc Am Assoc Cancer Res* 1998;39:118.
26. Gee JR, Chen I-L, Montoya RG, et al. Down-regulation of prostaglandin dehydrogenase in invasive transitional cell carcinoma. *Proc Am Assoc Cancer Res* 2001;42:264.
27. Maier TJ, Schilling K, Schmidt R, Geisslinger G, Grosch S. Cyclooxygenase-2 (COX-2)-dependent and -independent anticarcinogenic effects of celecoxib in human colon carcinoma cells. *Biochem Pharmacol* 2004;67(8):1469–1478. [PubMed: 15041464]
28. Narayanan BA, Condon MS, Bosland MC, Narayanan NK, Reddy BS. Suppression of *N*-methyl-*N*-nitrosourea/testosterone-induced rat prostate cancer growth by celecoxib: effects on cyclooxygenase-2, cell cycle regulation, and apoptosis mechanism(s). *Clin Cancer Res* 2003;9(9):3503–3513. [PubMed: 12960143]
29. Li M, Wu X, Xu XC. Induction of apoptosis by cyclooxygenase-2 inhibitor NS398 through a cytochrome *c*-dependent pathway in esophageal cancer cells. *Int J Cancer* 2001;93(2):218–223. [PubMed: 11410869]
30. Leng J, Han C, Demetris AJ, Michalopoulos GK, Wu T. Cyclooxygenase-2 promotes hepatocellular carcinoma cell growth through Akt activation: evidence for Akt inhibition in celecoxib-induced apoptosis. *Hepatology* 2003;38(3):756–768. [PubMed: 12939602]
31. Hsu AL, Ching TT, Wang DS, et al. The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. *J Biol Chem* 2000;275(15):11397–11403. [PubMed: 10753955]
32. Arico S, Patingre S, Bauvy C, et al. Celecoxib induces apoptosis by inhibiting 3-phosphoinositide-dependent protein kinase-1 activity in the human colon cancer HT-29 cell line. *J Biol Chem* 2002;277(31):27613–27621. [PubMed: 12000750]



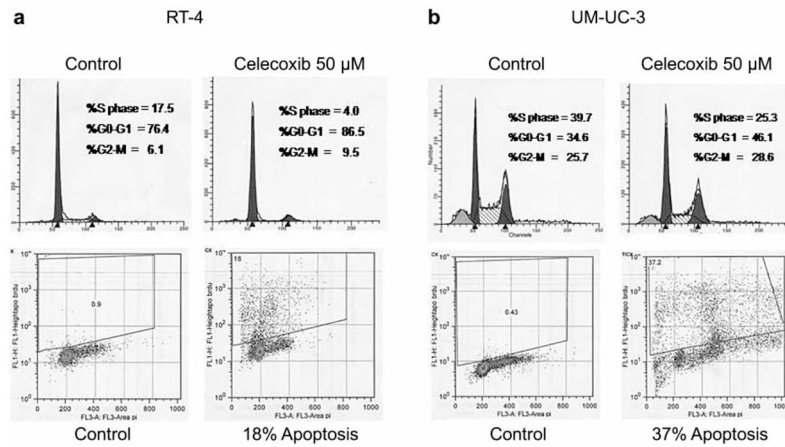
33. Grossman HB, Wedemeyer G, Ren L, Wilson GN, Cox B. Improved growth of human urothelial carcinoma cell cultures. *J Urol* 1986;136:953–959. [PubMed: 3761468]
34. Weinstein IB. Disorders in cell circuitry during multistage carcinogenesis: the role of homeostasis. *Carcinogenesis* 2000;21(5):857–864. [PubMed: 10783304]
35. Yamamoto H, Soh JW, Shirin H, et al. Comparative effects of overexpression of p27<sup>Kip1</sup> and p21<sup>Cip1/Waf1</sup> on growth and differentiation in human colon carcinoma cells. *Oncogene* 1999;18(1):103–115. [PubMed: 9926925]
36. Delia D, Goi K, Mizutani S, et al. Dissociation between cell cycle arrest and apoptosis can occur in Li-Fraumeni cells heterozygous for *p53* gene mutations. *Oncogene* 1997;14(18):2137–2147. [PubMed: 9174049]
37. Williams CS, Watson AJM, Sheng H, et al. Celecoxib prevents tumor growth *in vivo* without toxicity to normal gut: Lack of correlation between *in vitro* and *in vivo* models. *Cancer Res* 2000;60:6045–6051. [PubMed: 11085526]
38. Masferrer JL, Leahy KM, Koki AT, et al. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. *Cancer Res* 2000;60:1306–1311. [PubMed: 10728691]
39. Grubbs CJ, Lubet RA, Koki AT, et al. Celecoxib inhibits *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine-induced urinary bladder cancers in male B6D2F1 mice and female Fischer-344 rats. *Cancer Res* 2000;60(20):5599–5602. [PubMed: 11059745]
40. Tegeder I, Pfeilschifter J, Geisslinger G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J* 2001;15:2058–2072.



**Figure 1.** COX-2 induction by 6-h exposure to phorbol ester. COX-2 expression is induced in RT-4 (which normally expresses low basal levels of COX-2), whereas COX-2 up-regulation does not occur in the UM-UC-3 cell line (in which COX-2 expression is undetectable under control conditions).

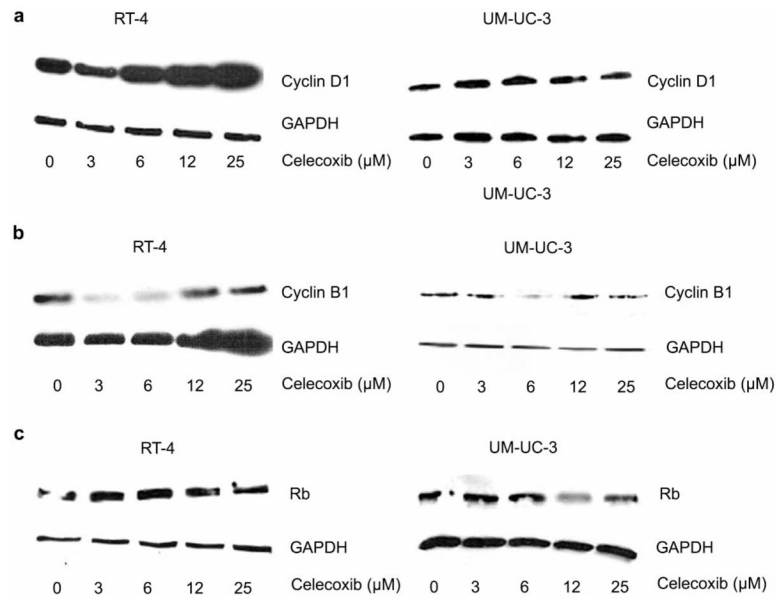


**Figure 2.** Celecoxib effectively inhibits growth in a dose-dependent fashion in the UM-UC-3 and RT-4 cell lines independent of COX-2 expression levels.



**Figure 3.**

An increase in the G<sub>0</sub>/G<sub>1</sub> cell cycle population is observed in the presence of celecoxib 50  $\mu$ M in the UM-UC-3 cell line. G<sub>1</sub> arrest is also observed in the COX-2-expressing cell line RT-4. Apoptosis is also observed for UM-UC-3 and RT-4 treated for 48 h with celecoxib 50  $\mu$ M.



**Figure 4.** Expression of cyclin D1 (A) and cyclin B1 (B), and Rb (C) in the presence of celecoxib (0–25  $\mu\text{M}$ ). Minimal variation in cyclin expression in the high-grade, COX-2-null cell line UM-UC-3 is observed as compared with the low-grade, COX-2 expressor RT-4.

Table 1

Western blot densitometry.

Target	Cell type	Slope	Standard error	R <sup>2</sup>	P-value
Cyclin D	RT-4	0.05483	0.01324	0.71	<b>0.004</b>
Cyclin B	RT-4	-0.10126	0.01558	0.858	<b>&lt;0.001</b>
Phospho-cyclin D	RT-4	0.04152	0.01953	0.392	0.071
Phospho-cyclin B	RT-4	-0.00782	0.01287	0.05	0.562
Rb	RT-4	-0.03815	0.03688	0.133	0.335
Cyclin D	UM-UC-3	0.00633	0.00483	0.223	0.238
Cyclin B	UM-UC-3	-0.00235	0.00909	0.011	0.805
Phospho-cyclin D	UM-UC-3	0.01989	0.01755	0.155	0.294
Phospho-cyclin B	UM-UC-3	-0.04111	0.02283	0.316	0.115
Rb	UM-UC-3	0.0254	0.00756	0.617	<b>0.012</b>

The comparisons that are statistically significant are highlighted in bold.