

# Celecoxib Exhibits the Greatest Potency amongst Cyclooxygenase (COX) Inhibitors for Growth Inhibition of COX-2-negative Hematopoietic and Epithelial Cell Lines<sup>1</sup>

Chris Waskewich, Rosalyn D. Blumenthal, Honglan Li, Rhona Stein, David M. Goldenberg, and Jack Burton<sup>2</sup>

Garden State Cancer Center, Belleville, New Jersey 07109

## ABSTRACT

Cyclooxygenase-2 (COX-2) is an important cellular target for both therapy and/or prevention of inflammatory disorders and cancer. The advent of selective COX-2 inhibitors now allows a more precise and safer treatment approach. The screening of an array of cancer cell lines for growth inhibitory effects of COX-2-selective and -nonselective inhibitors, including celecoxib (Celebrex) and rofecoxib (Vioxx), produced two unanticipated findings. Firstly, the antiproliferative effects of celecoxib were noted to be of very similar magnitude for both hematopoietic and epithelial cancer cell lines. Most hematopoietic cell lines had no detectable COX-2 expression by reverse transcription-PCR, and none expressed COX-2 protein. In addition, COX-2-negative epithelial lines were found to have IC<sub>50</sub>s for celecoxib that were very similar to their COX-2+ counterparts. Thus, important antiproliferative effects were observed that were independent of both the cell lineage and COX-2 status. Secondly, it was also observed that COX-2 inhibitor drugs, celecoxib and rofecoxib, with similar COX-2-selectivity and clinical efficacy for inflammatory indications, differed significantly in their *in vitro* antiproliferative effects on cancer cell lines. IC<sub>50</sub>s of 35–65 μM were observed for celecoxib across this entire panel of cell lines. Finally, no difference in the mode or degree of cytotoxicity was apparent between cell lines, because similar levels of apoptosis were observed in COX-2+ and -negative cell lines after treatment with celecoxib, with correspondingly lower levels after rofecoxib treatment. These data are important in that they provide the first direct comparison of epithelial and hematopoietic cancer cell lines, as well as a direct comparison of the *in vitro* anticancer effects of the two clinically available COX-2 inhibitors.

## INTRODUCTION

COX-2<sup>3</sup> was identified in the early 1990s as a distinct enzyme, which is 60% identical to the COX-1 (1, 2). *Cox-2* is an immediate-early gene, which is up-regulated in stromal and inflammatory cells by cytokines and other mediators and is expressed constitutively in many human carcinomas, including colon, non-small cell lung, breast, gastric, esophagus, prostate, head/neck, and bladder (3–5). This enzyme isoform, like COX-1, catalyzes the rate-limiting initial step in the formation of an array of PGs from the substrate, arachidonic acid. The anticancer potential of COX inhibition was first revealed by preclinical studies in carcinoma model systems using nonselective COX-2 inhibitors, such as aspirin and NSAIDs (3), which were shown to inhibit the *in vitro* and *in vivo* growth of cancer cell lines (6, 7). The *in vivo* effect appeared to be in part related to the antiangiogenic activity of these drugs (8). The clinical potential of COX inhibitors was suggested by epidemiological studies, which showed a lower incidence of a range of epithelial cancers, such as colon, breast, lung,

gastric, and esophageal in cohorts with higher levels of NSAID use (9, 10). The clinical efficacy of COX inhibitors in a precancerous condition, FAP, was demonstrated initially in a single-arm study with the nonselective COX-inhibitor, sulindac, which was later confirmed in a randomized study using this agent (11). Prolonged treatment of FAP patients with this drug was shown to be significantly more effective than placebo in reducing both the number and average size of colonic polyps. A later, multicenter, and randomized trial in this hereditary disorder showed that the selective COX-2 inhibitor, celecoxib, also had a significant impact on these clinical parameters and had a favorable toxicity profile during the 6-month, continuous, and twice-daily dosing period of this study, which led to supplemental Food and Drug Administration approval for this indication (12). COX-2-selective inhibitors have also been shown to have antiproliferative effects for human carcinoma-derived cell lines *in vitro* (13), as well as carcinoma xenografts established in immunodeficient mice (14). The mechanism underlying this effect has not been elucidated, because COX-2 expression by these tumor xenografts has not always been confirmed. Pharmacological inhibition of COX-2 with consequent decreases in PGs might inhibit tumor neovascular function and angiogenesis, thus contributing to the overall antitumor effect (5, 15). COX-2 expression has, indeed, been demonstrated in tumor-associated endothelial cells in both clinical cancer specimens, as well as human tumor xenografts (5). Because certain PGs, such as PGE<sub>2</sub>, have been shown to promote both cancer cell growth and motility, COX-2 expression and consequent PG production by tumor endothelium, infiltrating macrophages, or other accessory cells in tumors could result in paracrine growth-stimulatory effects (16). It should be noted, however, that in the vast majority of tumors, the malignant tumor cells constitute the bulk of the cellular mass. Thus, the tumor cell production of COX-2 would be predicted to have the greatest impact on local PG production.

Prior reports have demonstrated COX-2-independent antiproliferative effects of NSAID-like compounds on epithelial cancer cell lines. Another study found nearly identical *in vitro* growth inhibition curves of *Cox-2* (+/+), (+/-), and (-/-) mouse embryo fibroblasts of C57BL/6J background in response to celecoxib treatment. These results in cells of identical genetic background, and differing only in *Cox-2* status, confirmed prior results in COX-2+ and -negative carcinoma cell lines. In the same study, significant differences were observed in the antiproliferative effects of two COX-2 inhibitors with very similar COX-2 enzymatic inhibitory potency on the Lewis lung carcinoma cell line (14). We have found recently a significant circadian variation in the efficacy of celecoxib therapy of three human breast carcinoma xenografts, two of which are COX-2 negative (17). In groups of mice synchronized to differing light-dark cycles, therapeutic efficacy was not correlated with COX-2 levels in any of these breast tumor xenografts, as assessed by Western blotting and confirmed by RT-PCR. In fact, no COX-2 protein or mRNA was detected in two of these cell lines or in their derivative xenografts at any of the circadian time points tested. Another study found no detectable COX-2 in the tumor cells of both a murine and a human tumor xenograft model, both of which showed pronounced antitumor responses to treatment with celecoxib. COX-2 expression was, however, detected in the vasculature of these tumor xenografts (5). These data suggest that significant preclinical antitumor effects of celecoxib may be seen *in vitro* and *in vivo* in the absence of COX-2.

Because we and others have observed preclinical antiproliferative

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<sup>2</sup> To whom requests for reprints should be addressed, at Garden State Cancer Center at the Center for Molecular Medicine and Immunology, 520 Belleville Avenue, Belleville, NJ 07109. Phone: (973) 844-7024; Fax: (973) 844-7020; E-mail: jburton@gscancer.org.

<sup>3</sup> The abbreviations used are: COX, cyclooxygenase; FAP, familial adenomatous polyposis; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2 H-tetrazolium bromide]; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin; PI, propidium iodide; RT-PCR, reverse transcription-PCR; PPAR, peroxisome proliferator-activated receptor.

effects in the absence of COX-2 expression, we performed additional *in vitro* studies to address the effects of celecoxib and related compounds in a wider range of COX-2-negative human cancer cell lines, including lines of hematopoietic origin. On this broad panel of cell lines, we tested the effects of three COX-2-selective, one nonselective COX-inhibitor, and a control compound, thalidomide. To our knowledge, this is the first report addressing the effects of these COX-2 inhibitor drugs on hematopoietic cell lines. Our findings indicate that celecoxib's growth inhibitory effects are similar in COX-2+ and -negative lines, whether they be of epithelial or hematopoietic origin. In addition, the *in vitro* antiproliferative activity of the two COX-2 inhibitors that are currently registered in the United States, celecoxib (Celebrex) and rofecoxib (Vioxx), were compared. We found that these two COX-2-selective inhibitor drugs of similar efficacy for inflammatory indications showed substantial differences in their antiproliferative activity. Thus, the studies reported herein indicate the presence of other important cellular targets of compounds, such as celecoxib, which are expressed in both epithelial and hematological cancer types. These studies also provide an initial rationale for the application of celecoxib to the therapy of hematological cancers. The favorable toxicity profile of celecoxib, as well as the additive or better interaction between both conventional chemotherapy and external beam radiation with this class of compounds, would, thus, facilitate the addition of such drugs to existing therapeutic regimens for an even wider range of cancers.

## MATERIALS AND METHODS

**Cell Lines.** The human epithelial cancer cell lines tested were: A549, CALU-3 (non-small cell lung carcinoma), MCF7, ZR75-30 (breast carcinoma), ALVA-31, TSU-Pr1 (prostate carcinoma), 2008, and OV-1063 (ovarian carcinomas). The hematopoietic lines tested were: BALL-1 (18), BL60, WSU-CLL (three B-cell lines of varying stages of differentiation), U937, and K562 (human myeloid leukemias). Some of the cell lines were the generous gifts of the following individuals: ALVA-31 (Dr. R. Ostenson), TSU-Pr1 (Dr. J. Chiao), 2008 (Dr. S. Howell), BL60 (Dr. J. Wolf), and WSU-CLL (Dr. A. Al-Katib). The remaining cell lines were obtained from American Type Culture Collection (Manassas, VA). The growth media used for all of the cell lines was RPMI 1640 supplemented with 10% fetal bovine serum, 1% concentrations of stock solutions of both L-glutamine (Irvine Scientific, Santa Ana, CA; 200 mM) and penicillin-streptomycin (Irvine Scientific; 10,000 units/ml–10,000  $\mu$ g/ml).

**Reagents and Chemicals.** Celecoxib (Celebrex) was obtained from Pharmacia (Peapack, NJ); rofecoxib (Vioxx) was obtained from Merck (West Point, PA); NS-398 was obtained from Calbiochem (San Diego, CA); thalidomide (Thalomid) was obtained from Celgene (Warren, NJ); and indomethacin was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). All compounds were dissolved initially in DMSO.

**Cellular Proliferation Assay.** The *in vitro* effects of the compounds listed above were assessed in cell growth assays in 96-well, flat-bottomed plates, in which  $1.5\text{--}3 \times 10^4$  cells/well were added to triplicate wells. Varying concentrations of the test compounds were added in a final volume of 200  $\mu$ l of standard growth medium/well. DMSO was added to control wells at equal volumes to those used for the test compounds. The plates were then incubated at 37°C in a 5% CO<sub>2</sub>-supplemented atmosphere until the control wells showed 60–80% cellular confluence. At this point, for adherent cells, the media were removed and replaced by 100  $\mu$ l of 0.5 mg/ml of MTT (Sigma-Aldrich Chemical Co.) in growth medium, and the plates were put back in the 37°C incubator for 4–6 h. For nonadherent cells, 25  $\mu$ l/well of a 1:1 dilution of MTT stock solution (5 mg/ml); growth medium were added directly to the wells, and plates were placed back in the incubator for 4–6 h. Plates were then centrifuged at  $400 \times g$  for 10 min. Supernatants were removed from the wells, and the reduced MTT dye was solubilized with 100  $\mu$ l/well DMSO. Absorbance was determined on a Spectramax 250 plate reader at 570 nm. Percentage of inhibition was calculated using the formula: % inhibition =  $1 - (\text{OD}_{\text{test}} / \text{OD}_{\text{control}}) \times 100$ . IC<sub>50</sub>s were calculated by plotting the log of the percentage of inhibition values versus drug concentrations.

**Reverse Transcriptase PCR.** The starting material for RT-PCR was total RNA isolated from PBS-washed cells. Cells were solubilized with a guanidine isothiocyanate-based buffer (Tri-Reagent; Sigma-Aldrich Chemical Co., St. Louis, MO), and RNA was isolated according to the modified method of Chomczynski (19). Total RNA (5  $\mu$ g) was used as template for cDNA synthesis, using the First Strand kit of Novagen (Madison, WI) according to

manufacturer's instructions, with 10% of the resulting cDNA product used in each PCR reaction. *Cox-2* forward primer-ATC CTT GCT GTT CCC ACC CA (0.5  $\mu$ M), 0.5  $\mu$ M *Cox-2* reverse primer-CTT TGA CAC CCA AGG GAG TC, 200  $\mu$ M deoxynucleotide triphosphates (Sigma-Aldrich Chemical Co.), 3  $\mu$ l of  $10 \times$  PCR buffer (Sigma-Aldrich Chemical Co.), 2 mM MgCl<sub>2</sub>, 1 unit of Red Taq polymerase (Sigma-Aldrich Chemical Co.), and 2  $\mu$ l of cDNA were added to a 30- $\mu$ l PCR mix.  $\beta$ -actin forward primer-TGA CGG GGT CAC CCA CAC (0.5  $\mu$ M) and 0.5  $\mu$ M  $\beta$ -actin reverse primer-CTA GAA GCA TTT GCG GTG GA were used as a positive control for each cDNA. Thermocycling conditions were as follows: 35 cycles at 94°C denaturation for 30 s, 53°C annealing for 45 s, and 72°C extension for 1 min. PCR products were separated on 1.5% agarose gels containing 0.5  $\mu$ g/ml ethidium bromide and photographed on a UV transilluminator.

**Western Blotting.** Cell lines were harvested and washed with PBS, and whole cell lysates were prepared using a solution of 0.05 M Tris-HCl (pH=8)/0.15 M NaCl/1.5% Zwittergent 3–12 (Calbiochem)/protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Protein was quantitated using the Noninterfering Protein Assay (GenoTech, St. Louis, MO). Total protein (200  $\mu$ g) in 100  $\mu$ l was immunoprecipitated with 0.5  $\mu$ g of COX-2 monoclonal antibody (Cayman Chemical, Ann Arbor, MI). Immunoprecipitates were isolated using Protein G-agarose, which were washed with cell lysis buffer and eluted with SDS sample buffer. Eluates were separated on 10% SDS-PAGE gels (Gradipore, Frenchs Forest, NSW, Australia) and electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA) at 20 V for 16 h. The membrane was preincubated in blocking buffer (0.2% Tween 20/1% casein in PBS). Polyclonal COX-2 antibody (Cayman Chemical) at a 1:1000 dilution in blocking buffer was used to probe the membrane (2 h at room temperature). After washing, peroxidase-conjugated AffiniPure donkey antirabbit IgG (Jackson Labs, West Grove, PA) was used at a 1:20,000 dilution in blocking buffer for 1 h at room temperature. As control, 50 ng of ovine COX-2 enzyme (Cayman Chemical) was used. SuperSignal West Dura substrate (Pierce, Rockford, IL) was added to the membrane, and blots were exposed to film (Kodak MR, Rochester, NY) for 30 s, 1 min, and 5 min and then were developed.

**In Vitro Apoptosis Assays.** A549 (COX-2+ non-small cell lung carcinoma line) and BALL-1 (COX-2-negative B-leukemia line) were treated with varying concentrations (10–135  $\mu$ M) of celecoxib or rofecoxib. Thalidomide, DMSO, or no drug treatment were the control conditions. Treatment was for 16 h under standard cell culture conditions at 37°C. Cells were harvested and washed with PBS and  $\sim 5 \times 10^5$  cells from each flask were fixed with 70% ethanol (30 min at 4°C). PI (100  $\mu$ g/ml) and RNase A (50  $\mu$ g/ml; both from Sigma Chemical Co.-Aldrich) were added, and the cell suspensions were incubated for 30 min in the dark. Stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA), and data were analyzed using Cell Quest software (Becton Dickinson). To confirm the PI staining results, the same cell lines and conditions were assessed by Annexin V staining. For this, the Annexin V-EGFP apoptosis detection kit (Alexis Biochemicals, San Diego, CA) was used. Staining was carried out according to manufacturer's instructions, and cells were analyzed on the flow cytometer described above.

## RESULTS

**Celecoxib Has Similar *In Vitro* Antiproliferative Effects on Both Epithelial and Hematopoietic Cancer Cell Lines.** Celecoxib and other COX-inhibitory and control compounds were tested against a panel of human epithelial and hematopoietic cancer cell lines using the MTT cellular proliferation assay. Celecoxib was demonstrated to have the greatest *in vitro* antiproliferative potency of the group of compounds tested, which also included rofecoxib, NS-398, indomethacin, and an unrelated control drug, thalidomide. IC<sub>50</sub>s for celecoxib in both epithelial and hematopoietic lines were quite similar, as shown in Fig. 1, A and B. These values ranged from 35 to 65  $\mu$ M, which is in the same range as has been observed previously for COX-2+ epithelial lines.

**Celecoxib Has Greater *In Vitro* Antiproliferative Potency Compared with Other COX-inhibitory Compounds, Including Rofecoxib.** The COX-2-selective inhibitors, celecoxib, rofecoxib, and NS-398, were tested in the MTT assay, as well as the COX-nonselective inhibitor, indomethacin, and an unrelated compound, thalidomide. These agents were tested over the same concentration range, and only celecoxib consistently demonstrated >50% inhibition for the vast majority of the cell lines in the overall panel. Interestingly, in this

experimental system, thalidomide demonstrated very little inhibitory activity for either epithelial or hematopoietic cell lines at concentrations  $\leq 100 \mu\text{M}$  (data not shown). The COX-inhibitory drugs, indomethacin, NS-398, and rofecoxib, all showed attenuated antiproliferative activity that was usually  $< 40\%$ , irrespective of the lineage or the COX-2 levels in the cell lines of the panel (thus,  $\text{IC}_{50}\text{s}$  could not be estimated). Dose-response effects of rofecoxib and celecoxib were compared directly in a series of experiments using the COX-2+ epithelial line, A549, and the COX-2-negative, hematopoietic line, BALL-1. These results are shown in Fig. 2. Celecoxib exposure resulted in growth inhibition  $\geq 80\%$  in both lines, whereas rofecoxib exposure resulted in  $\leq 20\%$  inhibition over the entire concentration range tested. Direct statistical comparisons of the two highest concentrations of these drugs in the A549 line showed these differences to be significant at the  $P < 0.005$  level. The same comparison of the three highest concentrations in the COX-2-negative BALL-1 line also showed significant differences, each of which was at the  $P < 0.01$  level. Thus, despite the very similar potency of these two drugs for enzymatic inhibition of COX-2, there were very significant differences in their *in vitro* antiproliferative effects on both COX-2+ and -negative lines of epithelial and hematopoietic origin, respectively.

**COX-2 Is Expressed in Only Some of the Cell Lines Used.** All of the cell lines tested by the MTT assay were also analyzed for COX-2 expression at the mRNA and protein level. As shown in Fig. 3A, only some of the epithelial cancer cell lines were positive for COX-2 by RT-PCR, with most of these RT-PCR+ lines also being positive by Western blotting (Fig. 3C). In contrast, the hematopoietic cell lines were all negative by Western blotting (Fig. 3D), with only the two myeloid lines, K562 and U937, showing weakly positive signals by RT-PCR (Fig. 3B). Thus, despite similar sensitivity to the growth inhibitory effects of the COX-2 inhibitor, celecoxib, the cell lines of the panel analyzed in this study showed major differences in COX-2 expression.

**Celecoxib Induces Apoptosis in Both COX-2+ and -negative Cell Lines.** One strongly COX-2+ epithelial line (A549) was compared directly to one COX-2-negative hematopoietic cell line (BALL-1), both of which showed similar celecoxib  $\text{IC}_{50}\text{s}$ , for their apoptotic

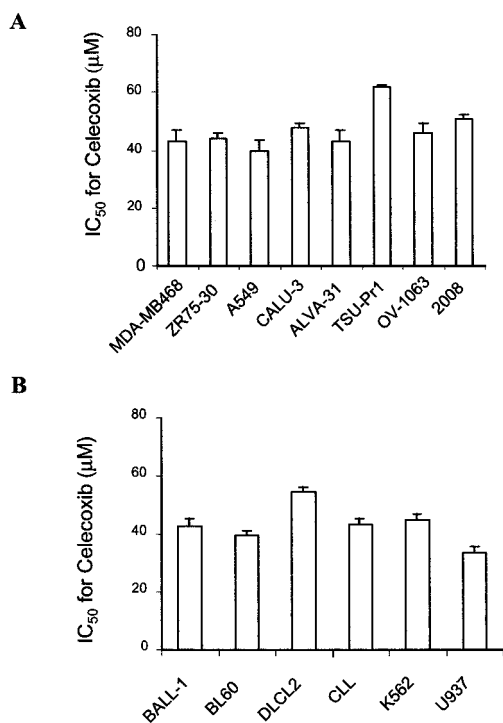


Fig. 1.  $\text{IC}_{50}\text{s}$  for celecoxib from *in vitro* MTT proliferation assays on human cancer cell lines. A, results from the epithelial lines. B, the results from the hematopoietic cell lines.

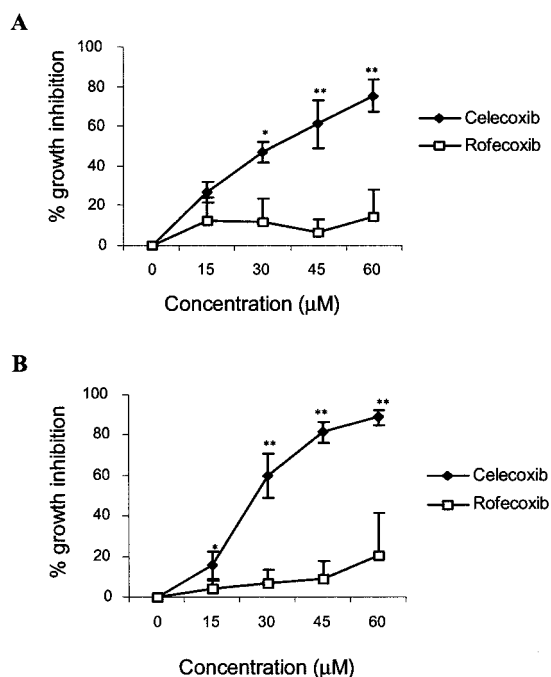


Fig. 2. Dose-response curves for growth inhibition as assessed by MTT assay for celecoxib and rofecoxib. A, plots results for the COX-2+ A549 epithelial line. B, plots results for the COX-2-negative hematopoietic line, BALL-1. \*\*,  $P < 0.01$ ; \*,  $P < 0.05$  by Student's *t* test.

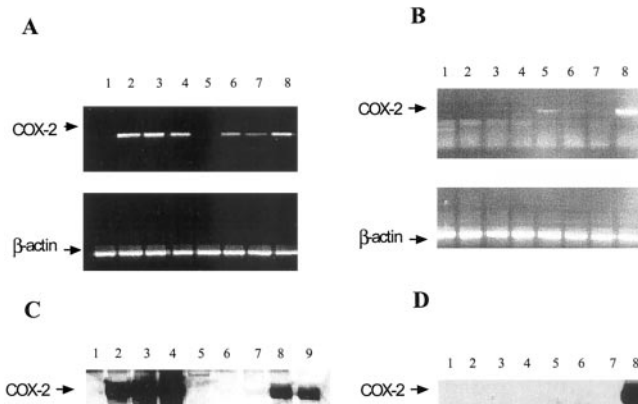


Fig. 3. A, gel photo of RT-PCR reaction products from epithelial lines: 1-MDA-MB468, 2-ZR-75-30, 3-A549, 4-CALU-3, 5-ALVA-31, 6-TSU-Pr1, 7-OV-1063, and 8-2008. B, gel photo of RT-PCR reactions of hematopoietic lines and control epithelial lines: 1-BL60, 2-BALL-1, 3-CLL, 4-DLCL2, 5-K562, 6-U937, 7-MCF7, and 8-A549. C, Western blot for COX-2 in epithelial lines: 1-MDA-MB468, 2-ZR75-30, 3-A549, 4-CALU-3, 5-ALVA-31, 6-TSU-Pr1, 7-OV-1063, and 8-2008; 9-50 ng of ovine COX-2. D, Western blot for COX-2 in hematopoietic lines (plus epithelial control lines, Lanes 7-8): 1-BL60, 2-BALL-1, 3-CLL, 4-DLCL2, 5-K562, 6-U937, 7-MCF7, and 8-A549; 9-50 ng of ovine COX-2.

responses to celecoxib and other compounds. Consistent with its strong antiproliferative effect, apoptotic changes were observed after 16 h of exposure to celecoxib at concentrations near or somewhat above the  $\text{IC}_{50}\text{s}$  for both cell lines, which differed in their COX-2 status and lineage. Rofecoxib, on the other hand, demonstrated much less of an apoptotic effect on all of the cell lines tested, which is also consistent with its lower potency in the *in vitro* proliferation assay. Fig. 4, A and B show the percentage of apoptotic cells (specifically, the sub- $G_1$  fraction) resulting from exposure to celecoxib, rofecoxib, or control conditions. In the A549 (COX-2+) cell line, celecoxib at the highest concentration (135  $\mu\text{M}$ ) gave a mean sub- $G_1$  fraction of  $25.66 \pm 13.07\%$ , whereas rofecoxib at the same concentration gave a



mean value of  $2.81 \pm 0.23\%$  ( $P < 0.02$ ). In this cell line, thalidomide and the solvent control (DMSO) showed similar values of 2.59 and 2.08%, respectively, which were, in turn, very similar to the levels seen with untreated cells. The BALL-1 cell line (hematopoietic and COX-2 negative) was tested in similar fashion. Treatment of this line with  $135 \mu\text{M}$  celecoxib or rofecoxib resulted in sub- $G_1$  percentages of  $20.7 \pm 8.7$  and  $4.2 \pm 2.4$ , respectively ( $P < 0.03$ ). To confirm these results, Annexin V staining was performed. The percentages of Annexin V+ cells after celecoxib and rofecoxib treatment mirrored the results of PI staining. At the  $135 \mu\text{M}$  concentration of celecoxib and rofecoxib, the percentages of Annexin V+ cells were  $43.4 \pm 8.6$  and  $7.2 \pm 1.3$ , respectively, for the A549 cell line and  $60.7 \pm 12.5$  and  $8.2 \pm 4.5$  for the BALL-1 cell line (Fig. 5). For the A549 line, this difference between celecoxib and rofecoxib was significant at the level of  $P = 0.014$ . For the COX-2-negative BALL-1 line, this difference was also significant at  $P = 0.028$ . With the latter, more sensitive assay, apoptotic effects of celecoxib over rofecoxib or control conditions were noted at concentrations down to  $10 \mu\text{M}$ . The most marked differences, however, between celecoxib and rofecoxib were observed at the highest concentration of both drugs ( $135 \mu\text{M}$ ). Although such concentrations are above what can be achieved clinically, this result represents only the early apoptotic effects of celecoxib (*i.e.*, after 16-h incubation). In addition, pharmacodynamic analysis of celecoxib concentrations in human tumor xenografts would be helpful in predicting whether higher drug concentrations are achievable in tumor tissue.

**DISCUSSION**

The experimental results in this report confirm the *in vitro* antiproliferative effect of celecoxib on cancer cell lines. This effect was found to be similar for both COX-2+ and COX-2-negative epithelial cell lines, and the effect extends to COX-2-negative breast, prostate, and ovarian carcinoma cell lines. Importantly, this study also shows for the first time that the effect of celecoxib is very similar for an array of hematopoietic cancer cell lines, all of which were negative for COX-2. Indeed, there were no significant differences in the  $IC_{50}$ s for celecoxib across the entire range of cell lines tested in this study. In contrast to some reports, we found substantially less biological activity with the other COX-inhibitory drugs tested (indomethacin, NS-398, and rofecoxib), whether they be COX-2 selective or nonselective. This difference between celecoxib and the other compounds tested

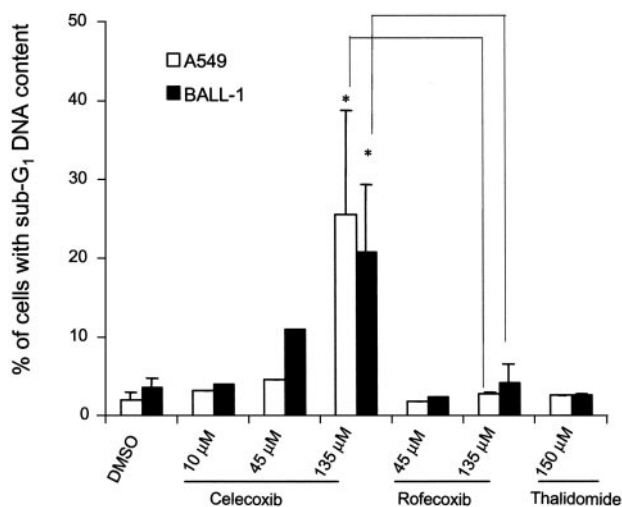


Fig. 4. Shown in this graph are the sub $G_1$  percentages obtained by PI staining of the COX-2+ and -negative cell lines, A549 and BALL-1, respectively, after treatment with selected compounds. The statistical comparison by Student's *t* test within cell lines and between celecoxib and rofecoxib at  $135 \mu\text{M}$  (marked by \*) gave  $P$ s of  $< 0.03$  for both cell lines.

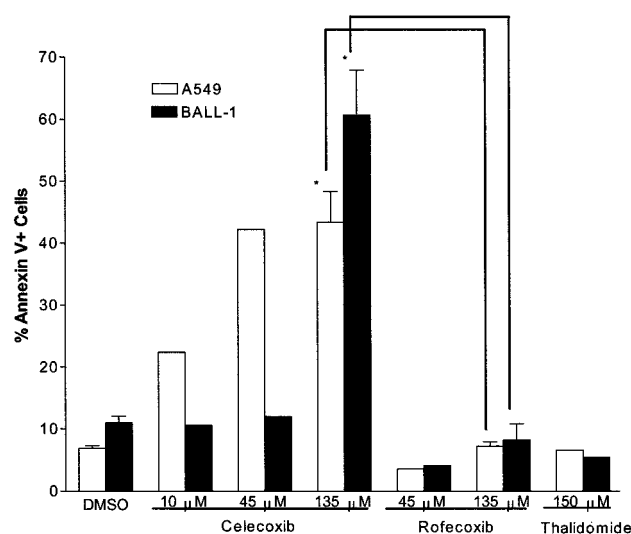


Fig. 5. Shown in this graph are the values for percentage of positivity obtained by fluorescent Annexin V staining of the COX-2+ and -negative cell lines, A549 and BALL-1, respectively, after treatment with selected compounds at three concentrations of celecoxib, two concentrations of rofecoxib, as well as the DMSO and thalidomide controls. The statistical comparison by Student's *t* test within cell lines of the percentage of positivity observed with celecoxib and rofecoxib at  $135 \mu\text{M}$  (marked by \*) gave  $P$ s of  $P = 0.014$  for A549 and  $P = 0.028$  for BALL-1.

was evident in both the MTT proliferation assay, as well as in the apoptotic assays. The difference between both the antiproliferative and apoptotic effects of celecoxib *versus* rofecoxib is particularly noteworthy, because these drugs have quite similar potency for inhibition of the COX-2 enzyme and very similar clinical efficacy in inflammatory indications.

Antiproliferative and antitumor activity of COX-inhibitory compounds have been reported previously in COX-2-negative epithelial cell lines and tumor xenografts. Recently, Williams *et al.* (14) demonstrated very similar growth inhibition curves for celecoxib in *Cox-2* (+/+), (+/-), and (-/-) mouse embryo fibroblasts. Thus, COX-2-independent effects of celecoxib also were observed in nontransformed, but highly proliferative, cells of identical genetic background. Very little, however, was known about the effect of these agents on hematological cancers. The precise cellular target(s) of these COX-independent effects have not been elucidated, but potential candidates have been identified. One such candidate, the 15-lipoxygenase-1 enzyme, was shown to be up-regulated by the COX-2 inhibitor, NS-398, as well as sulindac sulfone, which is devoid of COX-inhibitory activity (20). 15-lipoxygenase-1 catalyzes the production of the eicosanoid mediator, 13-S-HODE, which has both growth-inhibitory and proapoptotic effects. Sulindac sulfone and other growth inhibitory compounds have been shown to inhibit other cellular targets with potential roles in tumor cell growth, phosphodiesterases-2 and -5 (21).

Another class of potential targets for mediation of the antiproliferative effects of NSAID-like drugs are the PPARs ( $\alpha$ ,  $\gamma$ , and  $\delta$ ). NSAIDs have been shown to bind to both PPAR $\alpha$  and  $\gamma$  and mediate the prototypical biological effect of induction of differentiation in normal adipocytes (22). PPAR $\gamma$  activation has been shown to result in differentiation and growth inhibition in liposarcoma, and, in one study, the antidiabetic drug and PPAR $\gamma$  agonist, troglitazone, was shown to have clinical antitumor effects in liposarcoma (23). Preclinical anticancer effects of PPAR $\gamma$  agonists have also been observed in breast and colon cancer models (24, 25). NSAIDs have also been shown to bind PPAR $\delta$ , a transcription factor that has been well characterized in colon carcinoma. It is up-regulated by the  $\beta$ -catenin signaling pathway, and its overexpression suppresses the apoptotic effect of sulindac in colon carcinoma cell lines. As opposed to the activating effects of NSAIDs on PPAR $\alpha$  and  $\gamma$ , these drugs bind to and inhibit the function of

PPAR $\delta$  (26). The role of these three PPARs in other epithelial, as well as hematological, cancer types has not as yet been determined. Thus, PPARs are another set of potentially relevant targets, which may be associated with the preclinical or clinical anticancer effects of drugs of this general category.

The finding in this report of the substantial difference in activity between celecoxib and other COX-inhibitory compounds has important implications. At a mechanistic level, in light of the COX-2 expression data, it strongly suggests other important cellular targets beyond COX-2. This difference in activity among a single class of compounds provides another means of validating such heretofore undiscovered targets. It also provides insight into potentially important structure-function relationships, *e.g.*, celecoxib has a pyrazole ring, which is substituted with a trifluoromethyl group, as well as having a benzenesulfonamide moiety, both of which are absent in rofecoxib. In addition, based on the results presented herein, novel candidate cellular targets would be predicted to be expressed in both epithelial and hematological cancers.

Another important implication of these findings is that celecoxib (Celebrex) would appear to be the current drug of choice for both solid tumor and hematologic cancer treatment indications. Currently, it is registered for both the inflammatory indications of osteoarthritis and rheumatoid arthritis, as well as the precancerous condition, FAP. Although its clinical anti-inflammatory effects are similar to the other registered drug in this class, rofecoxib (Vioxx), these data indicate a significant difference in potency in these *in vitro* cancer model systems. It should be noted that rofecoxib has also been demonstrated to be active *in vivo* in the Min APC $\Delta^{716}$  mouse model for FAP, where treatment with this agent at clinically relevant doses leads to significant suppression of both intestinal polyp number and size (27). This indicates potential efficacy of this drug in FAP. Because colon cancer progression may be linked to COX-2 expression, potent inhibitors of this target would be predicted to have similar efficacy in this indication. A better assessment of the overall clinical potential of any compound in this general category awaits validation in relevant preclinical model systems. These *in vitro* data for celecoxib indicate that fairly high serum levels (>10  $\mu\text{M}$ ) may be required to exert an effect. Our group, as well as others, however, have already demonstrated preclinical efficacy of celecoxib in COX-2-poor or -negative human cancer xenograft models (5, 14, 17). Available pharmacokinetic data in humans suggest that serum levels do not exceed 10  $\mu\text{M}$ . However, pharmacodynamic data from tumor tissue have not been reported, and it is possible that these levels may be higher. Overall, the *in vitro* results reported herein provide support for additional preclinical evaluation of this class of compounds in other *in vivo* cancer model systems.

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## Celecoxib Exhibits the Greatest Potency amongst Cyclooxygenase (COX) Inhibitors for Growth Inhibition of COX-2-negative Hematopoietic and Epithelial Cell Lines

Chris Waskewich, Rosalyn D. Blumenthal, Honglan Li, et al.

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