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PIROXICAM SELECTIVELY INHIBITS THE GROWTH OF PREMALIGNANT AND MALIGNANT HUMAN ORAL CELL LINES BY LIMITING THEIR PROGRESSION THROUGH THE S PHASE AND REDUCING THE LEVELS OF CYCLINS AND AP-1

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Studies have shown that nonsteroidal antiinflammatory drugs (NSAIDs) reduce the risk of and mortality from a variety of cancers. Although cyclooxygenase (COX)-dependent and -independent pathways may be involved, the mechanisms responsible for these effects remain unknown. In our study, we found that piroxicam inhibited cell growth in premalignant and malignant, but not normal, human oral epithelial cell lines in a concentration- and time-dependent manner. After 6 days of exposure, the concentration that inhibited growth by 50% was 181 and 211 μM for premalignant and malignant cells, respectively. Piroxicam did not induce apoptosis. The growth inhibitory effect was COX and PGE_2 independent. Adding PGE_2 or infecting cells with a COX-1 transgene did not abrogate piroxicam-induced growth inhibition. After treatment of the premalignant and malignant cell lines with piroxicam, cells accumulated in the S phase of the cell cycle. Upon removal of piroxicam, cells entered the G_2 phase. The S phase block was accompanied by a reduction in the protein levels of cyclin A, cyclin B1, cyclin D1, cdc2, PCNA and the c-jun AP-1 component. Therefore, piroxicam may exert its growth inhibitory effects selectively on the premalignant and malignant human oral epithelial cells lines via signaling pathways regulating the progression of cells through the S phase of the cell cycle.

Key words: *piroxicam; oral cancer; S phase; cyclins; cyclin-dependent kinase; AP-1*

Epidemiologic studies indicate a strong association between nonsteroidal antiinflammatory drugs (NSAIDs) and a reduced risk of colorectal cancers.[1–3] Clinical trials have shown dramatic regression of colonic adenomas in patients with familial adenomatous polyposis. Other epidemiologic and animal studies have extended this protection to many other types of cancers, including oral cancer.[4–6] In animal studies, NSAIDs have been observed to be among the most potent agents discovered for the reduction of tumors in both genetic and carcinogen-induced cancer models.[1,3] These and cell culture studies have provided mechanistic insights into the antiproliferative and apoptotic effects of NSAIDs.[1,3–7] Among these, aspirin, piroxicam and sulindac, nonspecific inhibitors of cyclooxygenase (COX), and Celecoxib, a specific inhibitor of COX-2, have been demonstrated to be highly effective chemopreventive agents.[1–6] While NSAIDs are potent inhibitors of COX-1 and/or COX-2, the exact molecular mechanisms in chemoprevention are not clearly understood.[3,8,9] COX enzymes are responsible for the metabolism of arachidonic acid to prostaglandin (PG) metabolites, which are thought to provide growth signals to the cell. COX-1 is constitutively expressed, while COX-2 is induced in inflammatory states and elevated in many cancers, including oral.[10–13] Early studies associated the chemopreventive effectiveness of many COX-2-specific inhibitors with inhibition of elevated levels of mitotic stimulating PGs in cancer cells.[14,15] More recent data with non-COX-inhibiting NSAIDs and the effectiveness of NSAIDs in COX-deficient cell lines indicate that NSAID-induced growth inhibition and apoptosis may be occurring through COX-independent mechanisms.[3,7,9,16] Together, these data support the hypothesis that NSAIDs, depending upon structure and concentration and cell type, may target multiple upstream and downstream components of signaling pathways regulating cell growth and apoptosis.

As with other types of human cancers, the development of oral cancer is a multistep process, progressing through a series of discrete, irreversible, and complementary alterations in genes that control cell growth, differentiation and apoptosis.[17,18] During the premalignant stage, dysplastic tissues usually contain cells with greatly reduced cell generation times, and exhibit one or more dysregulated tumor suppressor and oncogenes regulating cell growth and apoptosis. When the premalignant phenotype converts to malignant and metastatic phenotypes, the cells exhibit aggressive and invasive growth and exhibit additional changes in growth regulatory genes controlling cell cycle progression, including cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors.[19–21] These changes may provide cells with selective growth and survival advantages resulting in aberrant cellular proliferation, enhanced survival and selection of cells that are invasive and metastatic.

Most chemopreventive studies have focused on defining mechanisms in premalignant and malignant cell types, with few studies comparing the effects to those in normal cell phenotypes. It is important to include this comparison to better identify and understand molecular mechanisms leading to agents that selectively target premalignant and malignant cells. We have identified a number of chemopreventive agents that are selective growth inhibitors of premalignant and malignant oral epithelial cell lines.[22,23] Among these agents, piroxicam, a nonselective COX inhibitor, was one of the more selective growth inhibitory agents. The aim of the present study was to further evaluate molecular mechanisms and the involvement of COX in the selective growth inhibition of premalignant and malignant human oral epithelial cell

Abbreviations: cdc2, cell division cycle gene 2; CDK, cyclin-dependent kinase; COX, cyclooxygenase; cpe, cytopathic effect; EIA, enzyme immunoassay; GFP, green fluorescent protein; HRP, horse radish peroxidase; NSAIDs, nonsteroidal antiinflammatory drugs; PCNA, proliferating cell nuclear antigen; PG, prostaglandin.

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phenotypes by piroxicam. We observed that exposure of the premalignant and malignant, but not normal, epithelial cell lines to piroxicam resulted in growth inhibition due to reversible cell cycle arrest in the S phase. This corresponded to a decrease in the levels of cyclins A, B1 and D1, cdc2, proliferating cell nuclear antigen (PCNA) and c-jun, an AP-1 component. Addition of PGE_2, the major metabolite of COX, did not reverse piroxicam-induced growth inhibition or release the cells from piroxicam-induced cell cycle arrest. These data suggest that piroxicam exerts its growth inhibitory effects toward premalignant and malignant epithelial cell lines via COX/PGE_2-independent mechanisms targeting signaling pathways regulating cell cycle progression.

MATERIAL AND METHODS

Human oral cell lines and treatment

The previously described normal human oral epithelial cell line TE1177 was established from epithelium surgically removed during a tonsillectomy.[23] It was cultured as previously described[24] in a modified alpha MEM with epithelial growth factor, insulin, transferrin, hydrocortisone and 10% defined FBS for growth. Cells were treated in subpassages 3–6 corresponding to population doublings of 4–8. The previously characterized premalignant SCC83-01-82 cell line was isolated from a SCC on the tongue, and the malignant 83-01-82CA cell line used in our study was obtained from the premalignant SCC83-01-82 after treatment with methyl methanesulfonate. Both the premalignant and malignant cells were gifts from Dr. G. Milo of the Ohio State University and maintained at 37°C with 5% CO_2 in MEM with 10% FBS and 100 U penicillin/streptomycin.[25]

Cells were treated in complete medium containing 10% FBS at the indicated concentrations of piroxicam (Sigma, St. Louis, MO) dissolved in DMSO and PGE_2 (Sigma, St. Louis, MO) dissolved in absolute ethanol as previously described. Cells were seeded on the first day; treatment was initiated on the second day and continued for the indicated times. For growth inhibition, 200 premalignant and malignant and 500 normal cells were seeded into each well of a 96-well microtiter tissue culture plate and assayed after 6 days of treatment. For cytotoxicity, 5,000 cells were seeded into the wells for 24 hr of treatment. For cell cycle, protein and mRNA analyses, $1–3 \times 10^5$ cells were seeded into 75 cm^2 flasks and harvested after 24–72 hr of treatment. All experiments included solvent and water controls. The highest final concentrations of DMSO and absolute ethanol were 0.26 and 0.35%, respectively.

Growth inhibition and cytotoxicity analyses

Growth inhibition by piroxicam was measured after 6 days of incubation with agent in complete medium. Due to the degradation of PGE_2 in medium, fresh PGE_2 was added daily and medium containing PGE_2 and piroxicam was replenished on the third day of treatment. On the last day of treatment, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (0.5 mg/ml) was added to the cultures and incubated for 4 hr. The resultant tetrazolium salt was then dissolved by the addition of acidified isopropyl alcohol. Color was measured spectrophotometrically in a microtiter plate reader and used as a relative measure of cell number. The number of cells after treatment was compared to solvent and untreated control cells and used to determine growth inhibition as follows: $GI = (Ab_{treated}/Ab_{control})*100$, where Ab represents the mean absorbance. The concentration that inhibited 50% of cell growth (GI_{50}) was determined from the linear portion of the growth inhibition curve by calculating the concentration of agent that reduced absorbance in treated cells, compared to control cells, by 50%. Cytotoxicity was assayed after 24 hr of treatment with agent using the MTT assay. In this assay, cells were incubated with MTT during the 20–24th hr of treatment. A decrease in MTT activity compared to solvent and untreated controls was used as an indicator and measure of cytotoxicity. Cell viability was determined using the trypan blue dye exclusion assay.

Ad-COX-1 and Ad-GFP (green fluorescent protein) were a gift from Dr. Kenneth K. Wu at the University of Texas. Premalignant and malignant human oral cells were seeded at a density of 1,000 cells/well in 96-well plates. The following day, Ad-COX-1 and Ad-GFP virus were added to the cells equivalent to a titer of 250, 500, 1,000 or 2,000 cytopathic effect (cpe) units/ml and incubated for 24 hr at 37°C. At 2,000 cpe units/ml >77% of the cells expressed GFP, and this titer was used for the growth inhibition experiments. Medium was removed and fresh medium with 600 μM piroxicam was added and incubated for 3 days. MTT was added for the last 4 hr.

Cell cycle analyses

Cells were treated at 70% confluence with piroxicam or DMSO for 24, 48 and 72 hr. At the end of treatment, cells were harvested with trypsin, fixed in 70% ethanol and stored at –20°C. Prior to fluorescence activated cell sorting (FACS) analyses the fixed cells were washed with PBS and incubated with a solution containing 33 μg/ml propidium iodide, 0.2% NP-40 and 7,000 U/ml RNase A for 30 min in the dark. Flow cytometry analysis was performed by the Analytical Cytometry Laboratory of the OSU Comprehensive Cancer Center using a Beckman-Coulter EPICS Elite flow Cytometer equipped with a 488 nm 15 mW air-cooled Argon laser collecting at least 30,000 events. Multicycle DNA modeling software and Modfit software (Verity Software House, Topsham, ME) were used to estimate cell cycle distribution. The DNA profile indicated the relative abundance of the cell population in G_0/G_1, S and G_2/M phases. The coefficient of variance and chi square value for each phase were used in the determination of the significance of differences between the various treatments.

BrdU DNA incorporation assay

BrdU incorporation was measured according to the supplier's instructions (Oncogene Research Products, Darmstadt, Germany) as an indicator of the number of cells in the S phase of the cell cycle. Briefly, premalignant cells were seeded into 96-well plates at a density of 1,500 cells per well. On the following day 300 or 600 μM piroxicam or solvent DMSO was added and incubated for 24, 48 or 72 hr. BrdU was added and incubated with the cells for the final 22 hr. After immunologic detection of incorporated BrdU, fluorescence was read at 320 nm (excitation) and 420 nm (emission). Relative cell numbers were determined after staining of the cells with methylene blue dye. This was used to normalize the amount of BrdU incorporated by relative cell number. The fold change in the level of BrdU incorporated was calculated by dividing the relative fluorescence units (RFU) of BrdU incorporated after piroxicam treatment by the matched DMSO controls.

Western Blot analysis

Immediately after treatment, cells were washed twice with PBS and removed from the surface of the plate with a trypsin/EGTA solution. Cells were collected by centrifugation, washed twice with PBS and lysed in a buffer containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aproptinin and 50 mM Tris-HCl (pH 8.0). Cell debris was removed by centrifugation, and protein concentration determined using the Bio-Rad assay (Bio-Rad, Hercules, CA). Equal amounts (30–70 μg) of protein were subjected to gel electrophoresis in 10–12% polyacrylamide slab gels (Invitrogen, Carlsbad, CA) in the presence of SDS. The protein was electrophoretically transferred to a nitrocellulose membrane and dried. The membrane was blocked with blocking buffer (5% nonfat milk, 500 mM NaCl, 100 mM Tris and 0.1% Tween-20) and then incubated with primary antibody at 4°C overnight. The blot was probed with primary antibody specific for the following proteins: cyclin A, cdc2, PCNA, c-jun, c-fos and α-tubulin (Oncogene Research Products, San Diego, CA), cyclin D1 (Cell Signaling Technology, Beverly, MA), cyclin B1, COX-1 and COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA). The concentration of antibody was anti-cyclin D1, 1:2,000; anti-cyclin B1, 1:1,000; anti-cyclin A, 1:1,000; anti-cdc2, 1:300; anti-PCNA, 1:1,000; anti-c-jun, 1:200; anti-c-fos, 1:200; anti-COX-1, 1:1,000; and anti-

COX-2, 1:1,000. After washing the membrane 3 times with TBS-T (20 mM Tris, 500 mM NaCl and 0.1% Tween-20) for 10 min, the membrane was incubated with mouse, rabbit or goat secondary antibody labeled with horse radish peroxidase (HRP) at 4°C for 1 hr. Western blots were developed using a chemiluminescence kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The membrane was then exposed to x-ray film and the image documented and analyzed using AlphaEase™ software from Alpha Innotech Corporation (San Leandro, CA). α-Tubulin was detected on the same membrane and used as a loading control.

Semiquantitative RT-PCR

After treatment, cells were lysed on the surface of the dishes and the RNA captured using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was quantified using the fluorescent-based RiboGreen RNA quantitation assay kit (Molecular Probes, Eugene, OR). RNA was evaluated for integrity of 18S and 28S rRNA by resolving 1 μg of RNA on a 1.0% agarose gel. The reverse transcription of 500 ng of RNA was performed using random hexamers with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) in a 20 μl reaction according to the manufacture's protocol. Negative controls with no SuperScript II and water were run at the same time to check for the presence of genomic DNA. A 1 μl sample of the resultant cDNA was used for PCR using Taq DNA polymerase (Invitrogen) in a Biometra PCR system (LABPRPCO, Horsham, PA). Primers for *COX-1* were[26] sense 5′-TGCCCAGCTCCTGGCCCGC-CGCTT-3′ and antisense 5′-GTGCATCAACACAGGCGC-CTCTTC-3′. Primers for *COX-2* were[27] sense 5′-AAGCCT-TCTCTAACCTCTCC-3′ and antisense 5′-TAAGCACATCGCATACTCTG-3′. PCR conditions were 95°C for 1 min, 55°C 1 for min and 72°C for 1 min. After PCR, samples were resolved by electrophoresis in a 1.8% agarose gel. PCR products were visualized and digitally captured after Syber green (Molecular Probes) staining using an Alpha Imager 2200 documentation and analysis system (Alpha Innotech Corp., San Leandro, CA). The expression of *COX-1* and *COX-2* mRNA was normalized against the expression of *18S* (Applied Bioscience, Foster City, CA).

ELISA of PGE_2 production

Cells were seeded at a density of 2×10^4 (premalignant or malignant) or 1×10^5 per well (normal) in 24-well plates in medium containing 10% FBS. After 24 hr, the medium was replaced with medium containing 1% serum, 0.26% DMSO and 300 or 600 μM piroxicam for 24 or 72 hr. The supernatants were collected and stored at −80°C. The cells were removed from the well and counted using a hemacytometer. The concentration of PGE_2 in the medium was determined using the PGE_2 monoclonal enzyme immunoassay (EIA) kit (Cayman, Ann Arbor, MI) according to the manufacture's protocol. The amount of PGE_2 per milliliter per 10^6 cells was calculated.

RESULTS

Piroxicam inhibits the growth of premalignant and malignant, but not normal, human oral cell lines

A comparison of the growth inhibitory effects of piroxicam on the normal, premalignant and malignant oral cell lines is shown in Figure 1. The normal cell line was highly resistant to growth inhibition with the 600 μM concentration only reducing growth approximately 30%. In contrast, piroxicam induced a concentration-dependent inhibition of cell growth, almost completely arresting cell growth at the 600 μM concentration in premalignant and malignant cells. The IC_{50} for piroxicam was 181 μM in the premalignant and 211 μM in the malignant oral cell lines. Growth inhibition was time dependent with the 100 μM concentration inducing 11, 31 and 44% growth inhibition of the premalignant cell line after 1, 3 and 6 days of treatment, respectively. Consistent with growth inhibition, piroxicam induced distinct morphologic changes in cell shape that could be observed at concentrations greater than 300 μM in the premalignant and malignant, but not in normal, cell lines (data not shown).

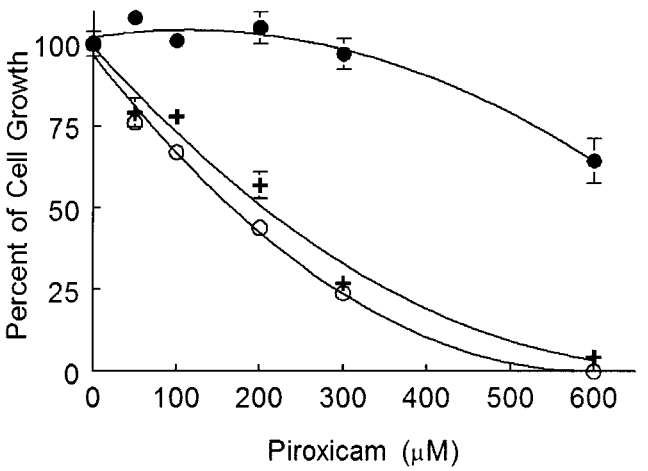


FIGURE 1 – Comparison of the growth inhibition exhibited by normal, premalignant and malignant oral cell lines incubated with piroxicam. The premalignant (○), malignant (+) and normal (●) oral cell lines were treated with the indicated concentrations of piroxicam for 6 days. The relative number of cells was determined using an MTT assay as described in Material and Methods. Cell growth is expressed as a percentage of cell growth relative to the DMSO control. Data represents the mean ± SD from 4 replicates.

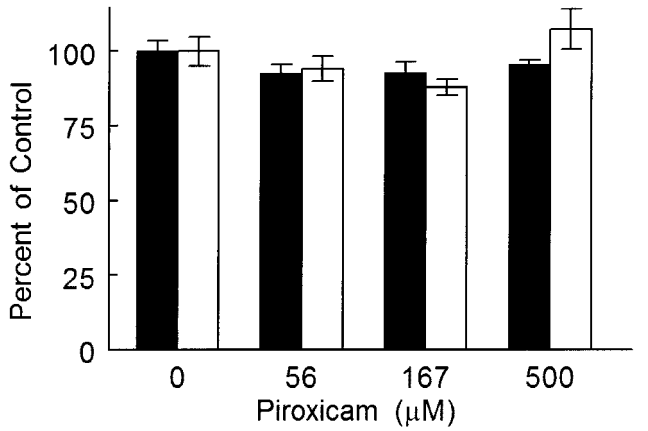


FIGURE 2 – Piroxicam is not cytotoxic to the oral cell lines. Premalignant (black bar) and malignant (white bar) cells were incubated with the indicated concentration of piroxicam for 24 hr. Cell viability was determined using an MTT assay as described in Material and Methods and is expressed as a percent of the DMSO control MTT activity.

Cytotoxicity is not a major factor in piroxicam-induced growth inhibition

The MTT metabolic activity, trypan blue dye exclusion and apoptotic assays were used to ascertain whether growth inhibition was due to piroxicam-induced cytotoxicity. As shown in Figure 2, there was no significant change in MTT activity after 24 hr incubation of the premalignant and malignant cell lines. The trypan blue dye exclusion assay indicated that cell viability was maintained with 95.3 and 97.5% viability of the premalignant and malignant cell lines after 72 hr incubation with 600 μM piroxicam, compared to 98.7 and 99.7% in the solvent-treated controls. There was also no significant induction of apoptosis in the cell lines treated with piroxicam for 24 to 72 hr, as indicated by the absence of a sub-G_0 peak in flow cytometry, the absence of a DNA ladder, nuclear staining indicating intact chromatin, low caspase 3 activity and intact poly (ADP-ribose) polymerase (PARP) (data not shown).

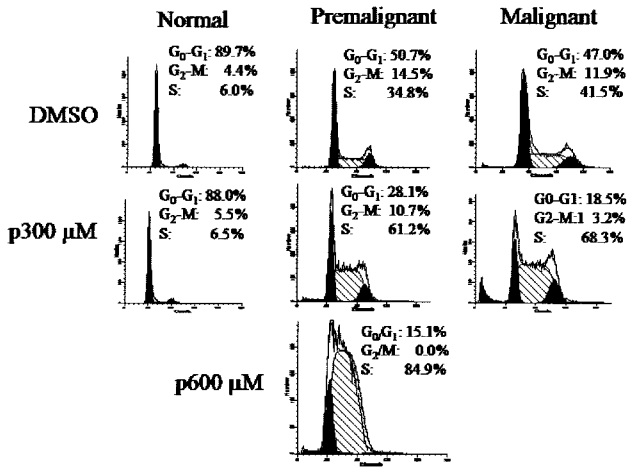


FIGURE 3 – Effect of piroxicam on cell cycle distribution of premalignant, malignant and normal oral cells. Distribution of the asynchronous cell populations is shown in the top panel after incubation with DMSO solvent controls. After the treatment of the cell lines for 72 hr with 300 μM piroxicam (p300), premalignant and malignant, but not normal, cells accumulate in the S phase. Incubation of the premalignant cell line with 600 μM piroxicam (p600) shows the absence of cells in the G_2/M phase. The distribution of cells in the cell cycle was determined using flow cytometry as described in Material and Methods. Results are shown from a representative experiment and are similar to results obtained with 2 other independent experiments.

Piroxicam induces an accumulation of premalignant and malignant cells in the S phase

To gain further insight into the mechanisms of piroxicam-induced growth inhibition, the effect of piroxicam on cell cycle distribution was determined using flow cytometry. Figure 3 compares the distribution of normal, premalignant and malignant oral cell lines after incubation with piroxicam for 72 hr. In the solvent-treated cell lines, 41.5% of the malignant, 50.7% of the premalignant and 89.7% of the normal cells were in the G_0/G_1 phase. A large portion of the malignant (41.5%) and premalignant (34.8%) cells were in the S phase, while only 6.0% of the normal cells were in the S phase. This distribution is consistent with the higher rates of cell proliferation exhibited by the malignant and premalignant *vs.* the normal cell lines. After exposure of the premalignant and malignant cell lines to 300 μM piroxicam, the percentage of cells in the S phase increased 1.6-fold to 61.2 and 68.3%, respectively, while cells in the G_0/G_1 and G_2/M phases decreased to 28.1 and 18.5%, respectively. Treatment of the premalignant cell line with 600 μM piroxicam further increased the percentage of cells in the S phase to 84.9%, with the corresponding reduction of G_0/G_1 and G_2/M phase cells to 15.1 and 0%, respectively. Removal of piroxicam from the cultures allowed cells to exit the S phase and within 24 hr exhibited a cell cycle distribution pattern similar to that of the untreated control (data not shown). There was no significant change in the distribution of the normal cell line treated with piroxicam. To further confirmed the accumulation of piroxicam-treated cells in the S phase, the levels of BrdU incorporation were determined. As shown in Table I, piroxicam increased BrdU incorporation coinciding with the accumulation of cells in the S phase.

Effect of cell cycle regulatory proteins in oral cell lines treated with piroxicam

Since piroxicam was found to selectively alter the distribution of premalignant and malignant oral cells in the cell cycle, we evaluated the effects of piroxicam on the expression of cell regulatory proteins including cyclins A, B1 and D1, the CDK cdc2 and PCNA (Fig. 4). cdc2 was highly expressed in both the premalignant and malignant cell lines and was not detectable in the normal cell line. Cyclin B1, which complexes with cdc2 to form the M phase kinase, was highly expressed in the premalignant cell line. The malignant cell line exhibited reduced levels of cyclin B1, while it was not detectable in the normal cell line. The lack of detectable cdc2 and cyclin B1 in the normal cell line was in agreement with the comparatively low levels of normal cells in the G_2/M phase of the cell cycle. The expressions of both the cdc2 and cyclin B1 proteins were inhibited in the premalignant and malignant cells by increasing concentrations of piroxicam correlating with the piroxicam depletion of G_2/M cells. Cyclin A accumulates prior to cyclin B1 and is associated with S phase cells. There was a low expression of cyclin A in the normal cell line, moderate expression in the malignant cell line and comparatively higher expression in the premalignant cell line. Piroxicam induced a concentration-dependent decrease in the levels of cyclin A in both the premalignant and malignant cell lines. PCNA is highly expressed in premalignant and malignant cell lines and is often used as a biomarker of tumor cell proliferation. Piroxicam significantly reduced the levels of PCNA in both the premalignant and malignant cell lines. Cyclin D1 expression was similar in normal, premalignant and malignant oral cell lines. While piroxicam inhibited the expression of cyclin D1 protein in the premalignant and malignant cell lines, it did not appear to affect cyclin D1 levels in the normal cell line.

TABLE I – COMPARISON OF THE EFFECT OF PIROXICAM ON THE NUMBER OF PREMALIGNANT CELLS IN THE S PHASE AND BRDU INCORPORATION

Concentration (μM)	300			600		
Time (hr)	24	48	72	24	48	72
S phase[1]	1.43	1.70	1.66	1.15	1.71	1.79
BrdU incoporation[2]	1.40	1.47	1.9	1.35	3.33	3.92

[1]The ratio was determined as the number of S phase cells following piroxicam treatment divided by number of S phase cells incubated with the DMSO solvent control.–[2]The ratio was determined by dividing the relative signal (RFU) of BrdU incorporated in cells treated with piroxicam by the DMSO solvent control. BrdU was added during the last 22 hr of incubation with piroxicam.

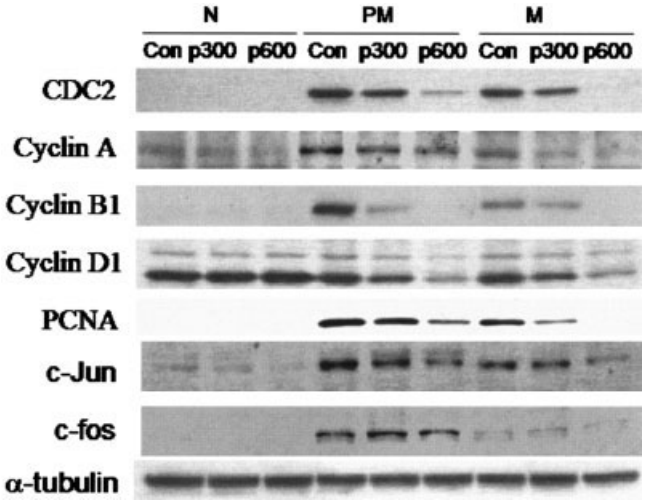


FIGURE 4 – Effect of piroxicam on the expression of cyclins, cdc2, PCNA, c-jun and c-fos. Cell lysates were prepared from the normal (N), premalignant (PM) and malignant (M) oral cell lines incubated with 0 (DMSO control), 300 and 600 μM piroxicam for 72 hr. The indicated proteins were detected by Western blotting analysis. α-Tubulin levels confirmed that similar amounts of protein were loaded on the gels. Representative Western blots are shown and are similar to results obtained with 3 or 4 other independent experiments.

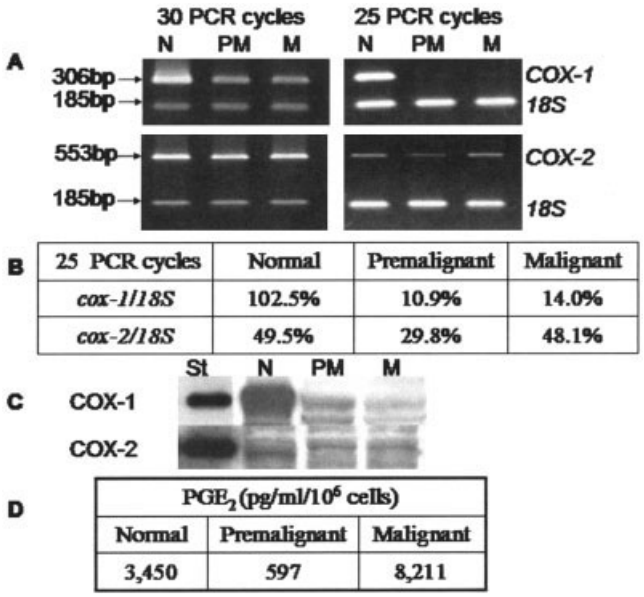
Piroxicam reduces c-jun expression

Since AP-1 may regulate the expression of cyclins A, B1 and D1,[28,29] the expressions of c-jun and c-fos, 2 critical components of AP-1, were determined after treatment of cells with piroxicam. As shown in Figure 4, c-jun was highly expressed in the premalignant and malignant cell lines and was not detectable in the normal cell line. Piroxicam reduced the level of c-jun protein in both the premalignant and malignant cell lines. c-fos was highly expressed in premalignant cells, but not in malignant and normal cells. Piroxicam treatment did not affect the expression of c-fos.

Growth inhibition is independent of cyclooxygenases/PGE_2 activity in premalignant and malignant cells

Since piroxicam is a nonselective COX inhibitor, the expression of COX-1 and COX-2 in these cell lines was examined. RT-PCR indicated that both *COX-1* and *COX-2* mRNA were expressed in the 3 cell lines. The expression of *COX-1* appeared to be 8- to 10-fold greater in normal than in premalignant and malignant cell lines (Fig. 5*a* and *b*). The level of *COX-2* mRNA was lowest in the premalignant cell line. These differences in *COX-1* and *COX-2* gene expression were further confirmed by Western blotting (Fig. 5*c*). To further establish the role of COX-1 and COX-2 in piroxicam-induced growth arrest, the levels of PGE_2, the major metabolite of COX, were measured after treatment. As shown in Figure 5*d*, the levels of PGE_2 in the medium of untreated normal, premalignant and malignant cells were 3,450, 597, and 8,211 pg/ml/10^6 cells, respectively. The inconsistency between the expression of COX and the levels of PGE_2 in the 3 cell lines may be related to the activity of other enzymes involved in PGE_2 synthesis, such as PLA2 and PGE_2 synthase.[30] After exposure to 300 and 600 μM piroxicam for 24 and 72 hr, PGE_2 levels were reduced to the assay's background in all 3 cell lines even though there was a small increase in COX-1 protein in the normal cell line after piroxicam treatment. COX-2 protein level was unchanged in all 3 cell lines (data not shown). The addition of 0.1 and 1.0 μM PGE_2 prior to piroxicam did not reverse the piroxicam-induced growth inhibition in premalignant and malignant cells (Fig. 6). PGE_2 ($<$1.0 μM) stimulated the growth of normal cells. Higher concentrations ($>$1 μM) of PGE_2 inhibited cell growth in all 3 cell lines. This suggests that piroxicam-induced growth inhibition of premalignant and malignant oral cell lines is independent of COX/PGE_2 activity.

To clarify whether high COX-1 expression protects normal cells from the growth-inhibiting effects of piroxicam, the premalignant and malignant cell lines were infected with Ad-COX-1 and Ad-GFP. COX-1 transgene expression was confirmed by Western blot. Growth inhibition was similar in the infected and controls of the premalignant (55 *vs.* 54%) and malignant (45 *vs.* 43%) cell lines after 3 days of treatment with 600 μM piroxicam.



B

25 PCR cycles	Normal	Premalignant	Malignant
cox-1/18S	102.5%	10.9%	14.0%
cox-2/18S	49.5%	29.8%	48.1%

D

PGE_2 (pg/ml/10^6 cells)		
Normal	Premalignant	Malignant
3,450	597	8,211

FIGURE 5 – Relative levels of COX-1 and COX-2 in the normal, premalignant and malignant cell lines. RNA and protein were extracted from the normal (N), premalignant (PM) and malignant (M) cell lines. mRNA was amplified using a 2-step RT-PCR reaction, and cell protein extract was prepared as described in Material and Methods. *(a)* Representative photograph of Sybr Green-stained gel shows the 306, 553 and 185 bp DNA fragments for *COX-1*, *COX-2* and *18S* RNA (loading control), respectively, after 25 and 30 PCR cycles. No bands were detectable using H_2O and reverse transcriptase minus as negative controls. *(b)* Shows high levels of *COX-1* in the normal cell line compared to the premalignant and malignant cell lines, and low levels of *COX-2* in the premalignant cell line. Data are from 25 PCR cycles normalized to control 18S mRNA. *(c)* Western blot analysis showing the levels of COX-1 and COX-2 proteins. St denotes COX-1 and COX-2 protein standards. *(d)* Comparison of PGE_2 levels in the oral cell lines. Cells were incubated in complete growth medium for 72 hr. The levels of PGE_2 in the supernatant of normal, premalignant and malignant cell lines were determined using PGE_2 ELISA as described in Material and Methods. Results are the mean ± SD of 4 replicates.

DISCUSSION

In the present study, we showed that piroxicam was a highly selective growth inhibitor of premalignant and malignant human oral cell lines. Growth inhibition was not due to apoptosis, but resulted from reversible cell cycle arrest in the S phase. The accumulation of cells in the S phase coincided with an increase in BrdU incorporation and reduction in the levels of cyclins A, B1 and D1, cdc2, PCNA and c-jun proteins regulating the progression of cells through the cell cycle. The growth inhibitory effects of piroxicam were not related to the differences in the levels of COX-1, COX-2 or their mitogenic metabolite, PGE_2. These data suggest that piroxicam exerts its growth inhibitory effect toward premalignant and malignant human oral cell lines via COX/PGE_2-independent mechanisms targeting cell cycle regulatory proteins.

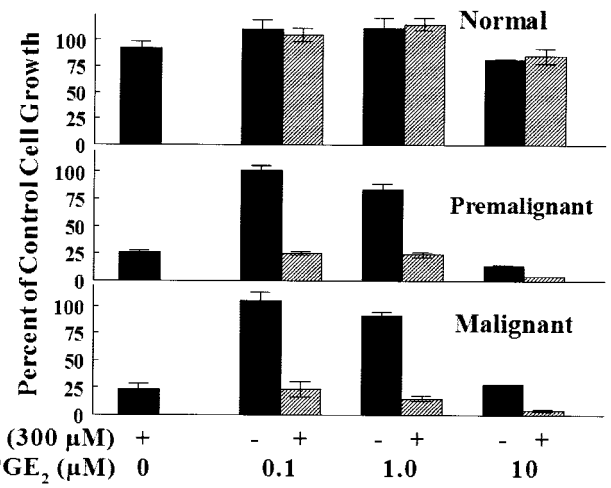


FIGURE 6 – PGE_2 does not reverse piroxicam-induced growth inhibition in premalignant and malignant human oral cells. Normal, premalignant and malignant oral cell lines were treated with 0 (DMSO control) and 300 μM piroxicam (PI) together with the indicated concentration of PGE_2. Fresh PGE_2 was added to the culture every day, while piroxicam was replenished on the third day for a total of 6-day incubation. Results are expressed as a percent of solvent matched control cell growth and represent the mean ± SD of 3 replicates.

NSAIDs have been found to be effective chemoprevention agents due to their antiproliferative and apoptotic effects.[1,3,9] There is good correlation between high levels of COX-2 and tumor cell sensitivity to NSAIDs.[14,15] While these studies point to COX-dependent mechanisms in NSAID chemoprevention, increasing data with non-COX-inhibiting NSAIDs and cells lacking COX-1 or COX-2 indicate that NSAIDs also induce growth inhibition and apoptosis through COX-independent mechanisms.[7] Our data, showing that piroxicam selectively inhibits the growth of premalignant and malignant human oral cell lines, are consistent with a COX/PGE_2-independent mechanism for the following reasons: *(i)* there is a poor association between the levels of COX-1/COX-2/PGE_2 and the growth rate of the 3 oral cell lines and growth inhibitory effects of piroxicam; *(ii)* the levels of COX-2 were similar in both the normal and malignant cell lines and low in the premalignant cell lines; and *(iii)* the normal cell line expressed much higher levels of COX-1 than the premalignant and malignant cell lines, and infection of premalignant and malignant cells with Ad-COX-1 or addition of PGE_2 did not attenuate piroxicam-induced growth inhibition. Some studies have shown that PGE_2 can stimulate the proliferation of colon cancer cells[31] and a human lung cancer cell line, A549.[32] Hansen *et al.*[33] showed that piroxicam and sulindac inhibited tumor growth in $Apc^{Min/+}$ mice and PGE_2 attenuated this inhibition. Sumitani *et al.*[34] found that PGE_2 could reverse the inhibitory effect of NS-398 in oral NA cells. Studies by Minter *et al.*[10] showed that both COX/PGE_2-dependent and -independent mechanisms exist in human oral cells. Many other studies do not support the role of PGE_2, the major metabolite of COX, in tumor growth and progression. A recent study compared the growth-inhibiting effects of 5 COX-2 inhibitors, which markedly suppressed the production of PGE_2 in the HT-29 colon cell line.[35] Among these agents, only celecoxib, NS-398 and nimesulide inhibited HT-29 cell proliferation. Mohammed *et al.*[36] showed that piroxicam inhibition of human invasive urinary bladder cancer in a canine model did not correlate with PGE_2 levels. El Attar and Lin[37] reported that PGE_2 itself inhibited the growth of the human oral squamous carcinoma cell line SCC-25. Our present data also suggest that PGE_2 does not play a critical role in growth inhibition by piroxicam in the premalignant and malignant human oral cell lines. This became evident when low concentrations of exogenous PGE_2 did not reverse the inhibitory effect of piroxicam in premalignant and malignant cell lines. The differences in the responses of cells to PGE_2 and COX as a target of NSAID chemoprevention may be related to the level of COX/PGE_2 and dependency of the tested cells on them. Other factors like PGE_2 receptors and/or signaling pathways in the different cell types may also be players.[38,39]

Aberrant cellular proliferation is a characteristic of many premalignant and malignant tumor types, including those of the oral cavity.[40,41] There is good evidence that many types of chemopreventive agents, including NSAIDs, induce cell cycle arrest in the G_0/G_1, S and G_2/M phases. In our premalignant and malignant oral cell lines, piroxicam reversibly arrested cells in the S phase of the cell cycle. The effect was concentration and time dependent with 600 μM piroxicam treatment for 72 hr, completely arresting cells at the S phase. This suggested that piroxicam was affecting proteins regulating cell cycle progression. Cyclins, cyclin-dependent kinases and onco- and tumor suppressor genes have been identified as controlling the progression of cells through the G_0/G_1, S and G_2/M phases of the cell cycle. These include cyclin D1, cdk4/6, p53, p16, p21, p27 and Rb for G_1; cyclins A and E and cdk2, for S; and cyclin B1 and cdc2 for G_2/M transition during the cell cycle.[42–44] Cyclin A, cyclin B1 and cdc2 are expressed at high levels in both the premalignant and malignant cell lines and at low levels in the normal oral cell lines. This is consistent with other studies showing elevated levels of cyclins in most head and neck tumor cells[45–47] and high levels of cyclins correlating with their malignant and tumorigenic potential.[48–50] Piroxicam-induced reductions of multiple cyclins suggest that there may be upstream targets for cyclins that can be regulated by piroxicam leading to growth inhibition. PCNA is an essential factor in the initiation, processing and completion of DNA replication during the S phase by forming complexes with DNA polymerase δ and DNA ligase I.[51] The reduction of PCNA levels by piroxicam, leading to S phase accumulation of the premalignant and malignant oral cell lines, may be due to piroxicam inhibiting these replication complexes prior to the entrance of cells into the G_2/M phase of the cell cycle. The formation of the cyclin B1 and cdc2 complex is critical for the progression of cells through the G_2/M phase of the cell cycle. Cyclin A is critical for cells to transverse the S phase, requiring the forming of a complex with cdk2 and phosphorylating the replication protein A.[52] Additionally, the suppression of E2F-1 DNA-binding activity by cyclin A-kinase is linked to orderly S phase progression.[53] Since cdc2 can form a complex with both cyclins A and B, it is an important regulator of cells passing through the S phase and may be a target for piroxicam-induced cell cycle arrest in the S phase, leading to growth inhibition.

The inhibition of the upstream transcriptional c-jun transcriptional factor, a component of AP-1, by piroxicam could lead to downstream inhibition of multiple cell cycle regulatory proteins. Recently it was reported that the depletion of functional c-jun by dominant negative c-jun reduced both cyclin A and cyclin B1 in HT1080 cells.[28] The reduction of cyclin D1 protein, but not mRNA, may result from piroxicam-induced S phase accumulation of the cells, as cyclin D1 protein expression is lowest when cells are at the S phase.[54] We are presently investigating these and other mechanisms by which piroxicam reduces cell cycle regulatory proteins and the effect of piroxicam on upstream signaling pathways regulating cell cycle progression.

In summary, piroxicam is a highly selective growth inhibitor of premalignant and malignant human oral epithelial cell lines. Growth inhibition is independent of COX/PGE_2 activity being mediated through signaling mechanisms that alter the distribution of cells in the cell cycle, leading to an accumulation of premalignant and malignant oral cells in the S phase. Corresponding with this accumulation of cells in the S phase is a marked reduction in the levels of cyclins, cdc2, PCNA and AP-1 proteins regulating cell cycle progression.

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