Gene modulation by Cox-1 and Cox-2 specific inhibitors in human colorectal carcinoma cancer cells

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Introduction

The non-steroidal anti-inflammatory drugs (NSAIDs), are inhibitors of cyclooxygenase (Cox), which is responsible for the formation of prostaglandins (1). Two distinct forms of Cox exist, the constitutively expressed Cox-1, and the inducible Cox-2. Numerous animal studies (2–7), population-based studies (8, 9), and in vitro studies (10–13) with human colorectal carcinoma cells provide evidence that NSAIDs have chemo-preventative activity directed against colorectal cancer as illustrated in several recent reviews (14–18). Colorectal cancer is the third most common cancer in the USA. In humans, the prodrug sulindac, which is converted to sulindac sulfide, is used in a chemo-preventative manner to suppress the development of adenomatous polyps in patients with familial adenomatous polyposis (FAP) (14, 19). In APC (adenomatous polyposis coli) mice, which serve as a genetically defined model of FAP, sulindac, in drinking water inhibits colorectal tumorigenesis (23). Sulindac also causes a rapid regression of pre-existing tumors in APC mice (20), however sulindac sulfone, which lacks NSAID activity does not inhibit tumor formation in vivo suggesting the inhibition of Cox is involved (4, 5). Many studies on the chemo-preventative activity of Cox inhibitors used classical NSAIDs that inhibit both Cox-1 and Cox-2. However, classical Cox inhibitors such as sulindac sulfide and indomethacin are more potent inhibitors of Cox-1 than Cox-2.

Recently, a concerted effort has been focused on the Cox-2 specific inhibitors since Cox-2 is highly expressed in tumor tissues [for a review see ref. (21)]. Celecoxib, a Cox-2 specific inhibitor, both prevents tumor formation and caused regression of pre-existing tumors in APC mouse models (22). SC-58125, also a Cox-2 specific inhibitor, induces apoptosis in vitro (23), and blocks tumor formation in vivo (24). However, other data with Cox-1 knockout APC mice indicate that deletion of Cox-1, in addition to Cox-2, reduces the number of intestinal polyps in these mice (25). Mofezolac, a Cox-1 specific inhibitor, was equally as effective as the Cox-2 specific inhibitor nimesulide, at inhibiting the number of aberrant crypt foci in rats fed azoxymethane, a model for chemoprevention, indicating both Cox-1 and Cox-2 contribute to tumor formation (26). In addition, APC gene knockout mice (APC1309) fed these drugs also showed a similar reduction in the number of colorectal polyps indicating Cox-1 is also important in tumorigenesis (26). Furthermore, Cox-1, but not Cox-2 mRNA and protein is over-expressed in human ovarian cancers when compared with normal ovarian tissue (27), SC-560, but not the Cox-2 specific inhibitor Celecoxib, inhibited arachidonic acid-induced vascular endothelial growth factor (VEGF) expression in the ovary (27). Therefore, specific

Abbreviations: ATF3, activating transcription factor 3; APC, adenomatous polyposis coli; Cox, cyclooxygenase; C/EBPb, C/EBPb; CCAAT enhancer binding protein-β; FACS, fluorescence-activated cell sorting; NF-κB, nuclear factor kappa B; FBS, fetal bovine serum; INSG1, insulin induced gene 1; MSX1, Msh homeo box homolog 1; MAD2, mitotic arrest deficient like 1; NAG-1, NSAID activated gene-1; NSAIDs, non-steroidal anti-inflammatory drugs; NRG-1, NSAID regulated gene-1; PGE2, prostaglandin E2; RT-PCR, reverse transcription-polymerase chain reaction; TBS-T, Tris-buffered saline Tween-20; VEGF, vascular endothelial growth factor.
Cox-1 inhibitors such as SC-560 and mofezolac may have chemo-preventative effects in the colon (26,28), breast (29) and potentially in the ovary (27).

The chemo-preventative activity of NSAIDs directed against colorectal cancer may, in part, be independent of Cox (28,30–32). Inhibition of cell proliferation and the induction of apoptosis are believed to be responsible, in part, for the chemo-preventative effects of NSAIDs illustrated in many recent reviews (14–18). SC-560 and Celecoxib were both effective at inhibiting the growth of Cox-deficient HCT-15 colon cancer xenografts in nude mice and induced apoptosis in vitro (28). Furthermore, Zhu et al. examined the molecular and structural requirements for the induction of apoptosis by Cox-2 specific inhibitors in prostate cells (33). They concluded that the induction of apoptosis was independent from the structural requirements for Cox inhibition. Therefore, both Cox-dependent and independent mechanisms are probably involved in the chemo-preventative activity of these compounds.

This laboratory has proposed the hypothesis that the chemo-preventative activity of Cox inhibitors is mediated, in part, by altering gene expression (12). Sulindac sulfide is a potent chemo-preventative drug against colorectal cancer (2–6). It is also a significant modulator of gene expression in colorectal cancer cells and these effects are probably linked to the chemo-preventative activity of sulindac sulfide at least in vitro (12). In this report, we have investigated the effect of selective Cox-1 and Cox-2 specific inhibitors on apoptosis, growth of colorectal cancer cells on soft agar, and the expression of the same genes modulated by sulindac sulfide in colorectal cancer cells (12). We used the Cox-1 specific inhibitor, SC-560 and the Cox-2 specific inhibitor, SC-58125 (Table I), which have reported chemo-preventative activity against colorectal (24,28,34) and breast cancer cells (29) in xenograft models.

Materials and methods

Chemicals

Chemicals were purchased from Sigma Chemical Company (St Louis, MO) unless otherwise noted. SC-560 and SC-58125 were from Cayman Chemical Company (Ann Arbor, MI) and were dissolved in DMSO and prepared fresh weekly.

Cell line and reagents

Cell lines were purchased from ATCC (Rockville, MD) and were maintained at 37°C/5% CO₂. Cell culture reagents were from Life Technologies (Rockville, MD). Human colorectal carcinoma HCT-116 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 10 mg/l gentamicin (complete media). Human colorectal carcinoma SW-480 cells were maintained in EMEM medium, which contained 15% fetal bovine serum (FBS), 10 mg/l gentamicin and sodium pyruvate (complete media).

Cell culture treatments

Cells were plated at 50% confluency in complete media overnight and treated in serum-free media containing vehicle, SC-560 or SC-58125 followed by RNA or protein isolation. Vehicle treatments consisted of 0.1% DMSO in serum-free media.

Cell proliferation assay

Cell proliferation was measured using the MTS colorimetric assay by Promega (Madison, WI), which estimates the number of viable cells in proliferation. Briefly, 500 cells/well were plated in complete media in 96-well tissue culture dishes overnight. Cells were treated with various concentrations of vehicle or NSAID in complete media in a final volume of 0.1 ml complete media. Cell viability was measured daily for 5 days at 490 nm in an ELISA plate reader following the addition of 0.02 ml MTS ‘Aqueous One’ solution per well and a 1 h incubation at 37°C/5% CO₂. Each experiment was carried out in quadruplicate and repeated two times. Percent viability is calculated relative to vehicle treated controls using the mean OD 490 ± SEM. A representative experiment from day 5 is shown.

Cell death detection

Cell death was measured using the Nuclear Matrix Protein ELISA kit from Oncogene Research Products (San Diego, CA). Briefly, 0.1 ml of media from cells treated for 30 h in media containing 2% FBS and vehicle, SC-560 or SC-58125 were spun down at 2000 r.p.m. for 10 min. The supernatant was collected and used undiluted according to the protocol provided.

Measurement of DNA content and apoptosis

The DNA content for vehicle or NSAID-treated SW-480 and HCT-116 cells were determined by fluorescence-activated cell sorting (FACS). Cells were plated at 50% confluency in 6-well plates overnight then treated in media containing 2% FBS in the presence of various concentrations of NSAIDs or vehicle for various time points in triplicate repeated two or more times. Living cells were stained with Annexin-V followed by propidium iodine (Oncogene Research Products) according to the manufacturer’s instructions and analyzed by flow cytometry using a Becton Dickinson FACSort (Franklin Lakes, NJ) equipped with CellQuest™ software according to the manufacturer’s instructions. Measurements are fold-increase over vehicle-treated time-matched controls from a representative experiment.

Soft agar cloning assay in the presence of NSAIDs

Soft agar assays were performed to compare the clonogenic potential of colorectal cancer cells in semisolid medium. HCT-116 and SW-480 cells were resuspended at 6000 cells in 2 ml of warm, complete media containing 0.35% agarose and the final concentration of NSAID or vehicle tested in the appropriate media and plated on top of 1 ml of 0.5% agarose in 6-well plates in triplicate repeated twice. Plates were incubated for 3 weeks at 37°C/5% CO₂. Cell colonies were visualized following an overnight stain with 0.5 ml of p-iodonitrotetrazolium violet at room temperature then image captured using

Table I. Comparison of cyclooxygenase inhibitors

<table>
<thead>
<tr>
<th>IC-50:</th>
<th>Sulindac sulfide (47)</th>
<th>SC-560 (48)</th>
<th>SC-58125 (49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cox-1</td>
<td>0.2 µM</td>
<td>0.009 µM</td>
<td>&gt;10.0 µM</td>
</tr>
<tr>
<td>Cox-2</td>
<td>14.0 µM</td>
<td>6.3 µM</td>
<td>0.07 µM</td>
</tr>
<tr>
<td>Cox-2/Cox1</td>
<td>70</td>
<td>700</td>
<td>0.007</td>
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Downloaded from https://academic.oup.com/carcin/article-abstract/25/3/349/2390618 by guest on 13 March 2019
a color CCD camera equipped with a personal computer loaded with Adobe® Photoshop® (San Jose, CA). Colony forming units were counted electronically on a personal computer equipped with IPLab version 3.0 (Scanalytics, Fairfax, VA). Values in the text are from a representative experiment.

RNA isolation
Following treatments, cells were rinsed twice with PBS then RNA was isolated using the Qiagen (Valencia, CA) RNeasy MINI kit according to the manufacturer’s instructions. Cell lysis was performed by centrifugation through a Qia-shredder (Qiagen).

Reverse transcription
RNA was treated with 1 U of amplification grade deoxyribonuclease I (Life Technologies) per microgram of RNA at room temperature for 15 min to remove genomic DNA followed by inactivation of the deoxyribonuclease I with 2.5 mM EDTA (pH 8.0). Then the RNA was incubated at 65°C for 5 min followed by quantification. Two micrograms of the RNA was reverse transcribed using 100 U of Superscript-II reverse transcriptase according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). (After RT, cDNA was treated with 1 U RNase H (Life Technologies) μg RNA at 37°C for 20 min. RT was performed using Qiagen’s Omniscript reverse transcription kit according to the manufacturer’s instructions. A negative control containing all of the RT reagents in the absence of RT enzyme (no RT control) was also routinely performed. The cDNA was diluted 5× with RNase-free water (Ambion, Austin, TX) and stored at −80°C until use.

Traditional and real-time RT-PCR using sybergreen detection
Real-time RT-PCR and traditional PCR primers were designed as described previously (12). Real-time and traditional RT-PCR were performed as described previously by this laboratory (12) using an ABI Prism 7700 (Applied Biosystems, Foster City, CA).

Western blotting
Proteins (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoreses (SDS-PAGE) and transferred onto nitrocellulose membranes. Blots were blocked overnight with 10% skim milk in TBS containing 0.1% Tween-20 (TBS-T), and probed for 1 h at room temperature in TBS-T with 0.1% milk with Cox-1, Cox-2 (Cayman), activating transcription factor 3 (ATF3), mitotar arrest deficient-like 1 (MAD2), CCAAT/enhancer binding protein-β (C/EBPβ), Msh homeo box homolog 1 (MSX1) or actin (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were washed in TBS-T then treated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature in TBS-T containing 5% milk and washed several times in TBS-T. NSAI activated gene-1 (NAG-1) was detected as reported previously in this laboratory (35). Antibodies were not readily available for NAG-3 (an EST with the Genbank accession number AI45439), insulin induced gene 1 (INSIG1) and NSAI regulated gene-1 (NRG-1) at the time of this study. The signal was detected by enhanced chemiluminescence purchased from (Amersham Biosciences) and followed by autoradiography. Where necessary, blots were stripped of antibody before reuse while sealed in a plastic bag containing a solution of 62.5 mmol/l Tris-HCl (pH 6.8), 2% (w/v) SDS and 100 mmol/l beta-mercaptoethanol for 30 min with constant agitation in a 30°C water bath followed by washing in TBS-T. The presence or absence of Cox-1 and Cox-2 in SW-480 and HCT-116 was evaluated at the mRNA level by traditional PCR and at the protein level in SW-480 cells by western analysis using Cox-1 and Cox-2 specific antibodies (Cayman) as reported previously (13). This cell line was chosen because it contains Cox-1, and the expression of at least some of these genes is attenuated by PGE2 (12).

Northern blotting
Clones were purchased from Research Genetics (Invitrogen) and were sequence verified then used to generate cDNA probes for northern blots as described previously by this laboratory (12). The signal was detected by autoradiography using Biomax MS film and intensifying screen for several hours as appropriate (Kodak, Rochester, NY). Each membrane was hybridized with a GAPDH probe (Ambion) after stripping in 0.1% SDS to verify equal loading.

Statistical analyses
Real-time RT-PCR was performed in triplicate two or more times with individual time-matched vehicle-treated controls for each gene tested. Statistical significance was determined according to a two-sided t-test with a 0.05 level of significance on Ct values following adjustment for actin. For soft agar assays, the mean number of colonies, and cell proliferation assays, the mean OD 490 values were analyzed using ANOVA with Fisher’s LSD method for pairwise comparisons at the P < 0.05 level of significance.

Results

Cell proliferation and apoptosis
To determine if selective Cox inhibitors inhibit cell proliferation, HCT-116 cells were treated with vehicle or various concentrations of SC-560 or SC-58125 in complete media for 5 days. At 100–200 μM concentrations, SC-560 and SC-58125 significantly inhibited cell growth in a concentration-dependent manner (Figure 1A). To verify that the concentrations and conditions used to treat cells with SC-560 and SC-58125 in subsequent experiments were non-toxic to the cells, cell death using an NMP ELISA assay, which measures the concentration of a nuclear protein released into the media, was performed. Both compounds were not toxic according to NMP ELISA at or below 50 (SC-560) or 100 μM (SC-58125) (data not shown).

HCT-116 cells were treated with various concentrations of SC-560 or SC-58125 for 24 and 30 h in media containing 2% serum and apoptosis was greater at 30 h (data not shown). Relative fold-increase in apoptosis was measured by Annexin-V followed by propidium iodine staining using FACS analysis. SC-560 and SC-58125 induced apoptosis at the low, non-toxic concentrations chosen. A significant induction of apoptosis was seen in HCT-116 cells treated for 30 h with 25 and 50 μM SC-560, which occurred in a concentration-dependent manner, as compared with vehicle-treated controls (Figure 1B). SC-58125 also induced apoptosis in HCT-116 cells after a 30 h treatment albeit to a lesser extent than SC-560 even at higher (100 μM) concentrations.

Inhibition of colorectal cancer cell growth on soft agar
Subsequently, we determined if SC-560 and SC-58125 would inhibit the growth of cells on soft agar. Growth of HCT-116 cells on soft agar was inhibited following treatment with various concentrations of SC-560. SC-58125 also inhibited the growth of these cells on soft agar, however, to a lesser degree even at higher concentrations (Figure 2). Thus, SC-560 was more effective than SC-58125, which required higher concentrations.

NSAI ds regulate the expression of several genes related to cell growth and apoptosis
One explanation for NSAI ds chemo-preventative activity is alterations in gene expression. Previously, we reported that the potent chemo-preventative drug sulindac sulfide, a NSAI d that inhibits the growth of HCT-116 cells on soft-agar, altered the expression of eight genes in a concentration-dependent manner that may be linked to this drug’s chemo-preventative activity (12). They include the induced genes: NAG-1, ATF3, C/EBPβ, NAG-3 and the repressed genes INSIG1, MSX1, MAD2 and NRG-1. We determined if SC-560 and SC-58125 also regulated the expression of these genes at the mRNA level as measured by real-time RT-PCR. The real-time RT-PCR data presented for each gene is from the time point with the greatest fold induction or repression measured following treatment with SC-560 (8 h genes include C/EBPβ, NAG-1, ATF-3 and INSIG1, which were similarly induced at 24 h, while the 24 h genes include NAG-3, MSX1, MAD2 and NRG-1). This is consistent with the results observed by microarray analysis following treatment of HCT-116 cells with sulindac sulfide as reported previously (12). To ascertain if changes in gene expression were dependent on the concentration used, we treated HCT-116 cells with various concentrations of SC-560...
and SC-58125. As estimated by real-time RT-PCR, all of these genes were regulated by SC-560 in a concentration-dependent manner and was nearly optimal at 10 μM except for ATF3, which was significantly more induced at 25 μM (Figure 3A and B). In particular, the transcription factors C/EBPβ, ATF3 and MSX1 plus NAG-1, a gene belonging to the TGF-β superfamily linked to the pro-apoptotic and antitumorigenic activity of NSAIDs in vitro (36) and chemo-preventative activity in animal models (37), were all regulated by SC-560 in this study. ATF3 and NAG-1 were the two most avidly expressed genes, while INSIG1 and NRG-1 were the most repressed. Only NAG-3, an EST, was poorly expressed following treatment with SC-560.

In contrast, except for ATF3, SC-58125 poorly altered the expression of these genes indicating that SC-560 is less effective than SC-560 at modulating the expression of these genes (Table II), which is in general agreement with the in vitro pro-apoptotic and antitumorigenic effects of these drugs in this study. At higher concentrations of SC-58125, which had minimal effect on apoptosis and growth on soft agar relative to SC-560, modulation of these genes did occur (Figure 3C and D).

**Effects of SC-560 on protein expression**

Western blots were performed to determine if SC-560 significantly altered protein expression of the five genes that had antibodies available. NAG-1, ATF3 and C/EBPβ protein expression were induced in a concentration-dependent manner and protein expression appeared to be in general agreement with mRNA levels measured by real-time RT-PCR. Similarly, MAD2 protein expression was repressed according to western blotting and real-time RT-PCR in HCT-116 cells (Figure 4).

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**Fig. 1.** Cox specific inhibitors inhibit cell proliferation and induce apoptosis. (A) HCT-116 cells were treated for 5 days in media containing 10% FBS plus vehicle, 10, 100 or 200 μM SC-560 (left panel) or vehicle, 10, 100 and 200 μM SC-58125 (right panel) as indicated. Following treatment, cells were measured for cell proliferation at OD 490 as illustrated in the Materials and methods. (B) HCT-116 cells were treated for 30 h in media containing 2% FBS plus vehicle, 10, 25 or 50 μM SC-560 (left panel) or vehicle, 10, 50 or 100 μM SC-58125 (right panel) as indicated. Apoptosis was then measured using FACS analysis as illustrated in the Materials and methods. *Statistical significance is according to ANOVA with Fisher’s LSD method for pairwise comparisons (P < 0.05) level of significance from a representative experiment.
Gene expression by selective Cox inhibitors

Sulindac sulfide is currently used to suppress the development of adenomatous polyps in patients with FAP (19) and because Cox-2 is often elevated in tumors, most of the attention has been focused recently on the use of Cox-2 specific inhibitors as chemo-preventative agents. However, Cox-1 specific inhibitors have recently gained increased attention as they also inhibit tumor formation in animal models. Most traditional Cox inhibitors are more potent inhibitors of Cox-1 than Cox-2. Thus, it appears both Cox-1 and Cox-2 play a role in the development of tumors. This conclusion is supported by Narko et al., who demonstrated that cells transfected with Cox-1 became tumorigenic when injected into nude mice (38). Cox-1 knockout mice show reduced intestinal tumorigenesis in APC

Fig. 2. Cox specific inhibitors repress clonogenic growth on soft agar.

HCT-116 cells were treated with various concentrations of SC-560 or SC-58125 as indicated and incubated for 3 weeks. Colony forming units were counted electronically on a personal computer equipped with IPLab version 3.0 (Scanalytics, Inc., Fairfax, VA). * Statistical significance is according to ANOVA with Fisher’s LSD method for pairwise comparisons (P < 0.05) level of significance from a representative experiment.

Gene expression in SW-480 cells

Since HCT-116 cells are devoid of Cox activity, we chose to determine if these genes were also modulated by SC-560 at the mRNA level and protein level in another cell line, SW-480 cells, which express Cox-1 but only low levels of Cox-2. Following treatment of SW-480 cells with SC-560, NAG-1, C/EBPβ and ATF3 protein expression were up-regulated in SW-480 cells (Figure 5A). MAD2 was repressed at the protein level according to western blotting, however, MSX1 protein levels were somewhat increased following treatment with SC-560 (Figure 5B) similar to that seen in the HCT-116 cells. SC-58125 has little effect on the protein expression of these genes even at high concentrations (data not shown).

Changes in mRNA levels by real-time RT–PCR confirmed the fact that the expression of seven of these eight genes was modulated by SC-560 in this cell line (Table III). Only MSX1 was not altered at the mRNA level by real-time RT–PCR in SW-480 cells, following treatment with 25 μM SC-560. Even at high (100 μM) concentrations, SC-58125 was a relatively weak modulator of these genes in SW-480 cells (Table III). Changes in the mRNA expression of these genes by SC-560 were also verified by northern blot analysis (Figure 6). With the exception of MSX1, the northern blot analysis was in agreement with the real-time RT–PCR data indicating that SC-560 modulates these genes at the mRNA level. The reason for this discrepancy is not known.
Fig. 3. Cox specific inhibitors alter mRNA gene expression in HCT-116 cells. Fold induction (A and C) or repression (B and D) following treatment with SC-560 (A and B) or SC-58125 (C and D). Results are fold change over time-matched vehicle-treated controls.

Table II. Comparison of gene modulation by SC-560 and SC-58125 in HCT-116 cells

<table>
<thead>
<tr>
<th></th>
<th>HCT-116 cells</th>
<th>HCT-116 cells</th>
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<tr>
<td>Induced:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAG-3</td>
<td>10 μM SC-560</td>
<td>10 μM SC-58125</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>1.5 ± 0.32</td>
<td>1.0 ± 0.01</td>
</tr>
<tr>
<td>NAG-1</td>
<td>3.3 ± 0.13</td>
<td>0.79 ± 0.11</td>
</tr>
<tr>
<td>ATF-3</td>
<td>2.9 ± 0.12</td>
<td>1.9 ± 0.04</td>
</tr>
<tr>
<td>Repressed:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSX1</td>
<td>0.71 ± 0.06</td>
<td>1.7 ± 0.17</td>
</tr>
<tr>
<td>INSIG1</td>
<td>0.32 ± 0.01</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>MAD2</td>
<td>0.33 ± 0.02</td>
<td>1.1 ± 0.02</td>
</tr>
<tr>
<td>NRG-1</td>
<td>0.26 ± 0.02</td>
<td>0.94 ± 0.05</td>
</tr>
</tbody>
</table>

Values are fold change ± SEM over time-matched vehicle-treated controls. Cells were treated for 8 or 24 h at the concentration indicated as illustrated in the Materials and methods. Bold values are statistically significant according to a two-sided t-test. A separate control was used for each NSAID.

Fig. 4. SC-560 modulates protein expression in HCT-116 cells. Western blots of NAG-1, ATF3, C/EBPβ and MAD2 were performed as indicated in the experimental procedures. HCT-116 cells were treated in SFM for 24 h containing vehicle (lane 1), or 10, 25, 50 and 100 μM SC-560 (lanes 2-5). Blot was stripped and re-probed with actin.
in HCT-116 cells, respectively. Thus, changes in gene expression by Cox inhibitors appear to be complex and dependent on the structural character of the specific Cox inhibitor rather than its ability to selectively inhibit Cox-1 or Cox-2. This is in agreement with Zhu et al. who reported that NSAIDs induce apoptosis independent of their ability to inhibit Cox (33).

As illustrated here and elsewhere, evidence is mounting that gene regulation may play a part in the chemo-preventative agreement with Zhu et al. who reported that NSAIDs induce apoptosis independent of their ability to inhibit Cox (33).

Several of these genes are believed to be important in the response to NSAIDs including NAG-1, which is induced by NSAIDs (36) and repressed in human colorectal tumors (37). Treatment of mice with the pro-drug sulindac, which is converted to sulindac sulfide, increases the expression of NAG-1 in mice tissues (37). Similarly, NRG-1 is repressed by NSAIDs and induced in tumors (42). Furthermore, the transcription factors ATF3 and MSX1 as well as MAD2, are linked to cell cycle progression. In addition, C/EBPβ binds to the promoter of p21WAF1/CIP1, which is a powerful cell cycle inhibitor, thereby inducing its expression (43). Both C/EBPβ and ATF3 are members of the basic leucine zipper family of transcription factors and are considered immediate early genes most often equated with involvement in the stress response. ATF3 forms heterodimers with C/EBPβ regulating the expression of Gadd153 and several other growth-regulating cellular promoters and even heterodimerizes with Gadd153, resulting in down regulation of ATF3 and C/EBPβ-mediated gene regulation (44). Tetracycline inducible over-expression of ATF3 forms heterodimers with C/EBPβ and ATF3 as seen with SC-560 may be an important event in the apoptotic cascade and they occur prior to the induction of apoptosis, supporting the conclusion that these genes are part of the apoptotic events rather than the result of apoptosis.

### Table III. Comparison of gene modulation by SC-560 and SC-58125 in SW-480 cells

<table>
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<tr>
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<th>SW-480 cells</th>
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<tr>
<td>Induced:</td>
<td>25 µM SC-560</td>
<td>100 µM SC-58125</td>
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<tr>
<td>NAG-3</td>
<td>1.9 ± 0.21</td>
<td>0.7 ± 0.01</td>
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<tr>
<td>C/EBPβ</td>
<td>4.2 ± 0.03</td>
<td>1.7 ± 0.06</td>
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<tr>
<td>NAG-1</td>
<td>3.1 ± 0.07</td>
<td>1.4 ± 0.03</td>
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<tr>
<td>ATF-3</td>
<td>3.1 ± 0.09</td>
<td>0.97 ± 0.09</td>
</tr>
<tr>
<td>Repressed:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSX1</td>
<td>1.2 ± 0.03</td>
<td>1 ± 0.03</td>
</tr>
<tr>
<td>INSIG1</td>
<td>0.2 ± 0.01</td>
<td>1.2 ± 0.08</td>
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<tr>
<td>MAD2</td>
<td>0.8 ± 0.01</td>
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<tr>
<td>NRG-1</td>
<td>0.56 ± 0.02</td>
<td>0.7 ± 0.05</td>
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Values are fold change ± SEM over time-matched vehicle-treated controls. Cells were treated for 8 or 24 h at the concentration indicated as illustrated in the Materials and methods. Bold values are statistically significant according to a two-sided t-test.

### Fig. 5. SC-560 modulates protein expression in SW-480 cells. Western blots of (A) induced genes (NAG-1, ATF3 and C/EBPβ) and (B) repressed genes (MAD2, MSX1) were performed as indicated in the experimental procedures. SW-480 cells were treated with vehicle (lane 1), 10 or 100 µM (lanes 2–3) SC-560 for 24 h. Blots were stripped and re-probed with actin.

### Fig. 6. Measurement of mRNA expression in SW-480 cells following treatment with SC-560. Northern blots of (A) ATF3, (B) NAG-3, MSX-1, NRG-1, (C) INSIG1, NAG-1 and MAD2 treated with vehicle (lane 1) or 25 µM (lane 2) SC-560 for 8, 24 and 4 h, respectively, and (D) C/EBPβ treated with vehicle (lane 1) or 10 µM (lane 2) SC-560 for 8 h. Blots were stripped and re-probed with GAPDH.

Several of these genes are believed to be important in the response to NSAIDs including NAG-1, which is induced by NSAIDs (36) and repressed in human colorectal tumors (37). Treatment of mice with the pro-drug sulindac, which is converted to sulindac sulfide, increases the expression of NAG-1 in mice tissues (37). Similarly, NRG-1 is repressed by NSAIDs and induced in tumors (42). Furthermore, the transcription factors ATF3 and MSX1 as well as MAD2, are linked to cell cycle progression. In addition, C/EBPβ binds to the promoter of p21WAF1/CIP1, which is a powerful cell cycle inhibitor, thereby inducing its expression (43). Both C/EBPβ and ATF3 are members of the basic leucine zipper family of transcription factors and are considered immediate early genes most often equated with involvement in the stress response. ATF3 forms heterodimers with C/EBPβ regulating the expression of Gadd153 and several other growth-regulating cellular promoters and even heterodimerizes with Gadd153, resulting in down regulation of ATF3 and C/EBPβ-mediated gene regulation (44). Tetracycline inducible over-expression of ATF3 forms heterodimers with C/EBPβ and ATF3 as seen with SC-560 may be an important event in the apoptotic cascade and they occur prior to the induction of apoptosis, supporting the conclusion that these genes are part of the apoptotic events rather than the result of apoptosis.

In conclusion, the Cox-1 specific inhibitor SC-560 induces apoptosis and inhibits the growth of human colorectal cancer cells on soft agar, an in vitro indicator of tumorigenicity, and is a powerful modulator of gene expression, mimicking the
effects seen by the potent chemo-preventative drug sulindac sulfide. In contrast, the selective Cox-2 inhibitor, SC-58125 was a weak modulator of gene expression although it modestly induced apoptosis and inhibited growth on soft agar in this study. Together, the data lead us to believe that in addition to Cox-2, Cox-1 is important to the inhibition of colorectal cancer by NSAIDs, and one mechanism that probably explains, at least in part, the chemo-preventative activity of NSAIDs is modulation of gene expression. Thus, the mechanisms by which Cox inhibitors exert their chemo-preventative activity in colon are complex and may involve changes in the expression of multiple genes in addition to inhibition of prostaglandin production. Further studies using animal models are required to better understand the chemo-preventative activity of these compounds.

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