Colon Tumor Cell Growth–Inhibitory Activity of Sulindac Sulfide and Other Nonsteroidal Anti-Inflammatory Drugs Is Associated with Phosphodiesterase 5 Inhibition

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAID) display promising antineoplastic activity, but toxicity resulting from cyclooxygenase (COX) inhibition limits their clinical use for chemoprevention. Studies suggest that the mechanism may be COX independent, although alternative targets have not been well defined. Here, we show that the NSAID sulindac sulfide (SS) inhibits cyclic guanosine 3’,5’-monophosphate (cGMP) phosphodiesterase (PDE) activity in colon tumor cell lysates at concentrations that inhibit colon tumor cell growth in vitro and in vivo. A series of chemically diverse NSAIDs also inhibited cGMP hydrolysis at concentrations that correlate with their potency to inhibit colon tumor cell growth, whereas no correlation was observed with COX-2 inhibition. Consistent with its selectivity for inhibiting cGMP hydrolysis compared with cyclic AMP hydrolysis, SS inhibited the cGMP-specific PDE5 isozyme and increased cGMP levels in colon tumor cells. Of numerous PDE isozyme–specific inhibitors evaluated, only the PDE5-selective inhibitor MY5445 inhibited colon tumor cell growth. The effects of SS and MY5445 on cell growth were associated with inhibition of β-catenin–mediated transcriptional activity to suppress the synthesis of cyclin D and survivin, which regulate tumor cell proliferation and apoptosis, respectively. SS had minimal effects on cGMP PDE activity in normal colonocytes, which displayed reduced sensitivity to SS and did not express PDE5. PDE5 was found to be overexpressed in colon tumor cell lines as well as in colon adenomas and adenocarcinomas compared with normal colonic mucosa. These results suggest that PDE5 inhibition, cGMP elevation, and inhibition of β-catenin transcriptional activity may contribute to the chemopreventive properties of certain NSAIDs. Cancer Prev Res; 3(10); 1303–13. ©2010 AACR.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAID) and cyclooxygenase-2 (COX-2)–selective inhibitors display promising chemopreventive efficacy, especially for colorectal cancer. Epidemiologic studies, for example, have shown that long-term use of NSAIDs is associated with reduced incidence of colorectal cancer in the general population by about 30% to 50% (1, 2). Commonly prescribed for the treatment of inflammatory conditions, traditional NSAIDs inhibit both COX-1 and COX-2 enzymes, which catalyze the production of prostaglandins from arachidonic acid. Unfortunately, the depletion of prostaglandins by NSAIDs is associated with significant side effects, including gastrointestinal ulcers, bleeding, and intestinal perforation. COX-2–selective inhibitors display reduced gastrointestinal toxicity but are associated with increased risk of myocardial infarction. These potential fatal adverse effects outweigh the benefits of NSAIDs or COX-2 inhibitors for preventing colorectal cancer, which tend to require high dosages administered over a long period of time.

The mechanism of action that is responsible for the chemopreventive activity of NSAIDs is widely attributed to COX-2 inhibition, although numerous reports have suggested that a COX-independent mechanism may contribute to or be fully responsible for their antineoplastic activity (3–8). For example, studies have shown that the non–COX-inhibitory sulfone metabolite of the NSAID sulindac can inhibit human colon tumor cell growth and induce apoptosis in vitro (7, 8). Sulindac sulfone also inhibits tumorigenesis in the azoxymethane-induced rat model of colon carcinogenesis without suppressing prostaglandin levels in colonic mucosa (7–9). In clinical trials, sulindac sulfone (exisulind) caused regression of adenomas in patients with either familial (10) or sporadic polyposis (11) but did not receive Food and Drug Administration...
approval due to hepatotoxicity. Mechanistic studies have shown that sulindac sulfone and several structurally related analogues can inhibit cyclic guanosine 3′,5′-monophosphate (cGMP) phosphodiesterase (PDE) activity at concentrations that are comparable with those required to inhibit colon tumor cell growth and induce apoptosis (12–14). However, the specific PDE isozyme(s) responsible for its tumor cell growth–inhibitory activity has not been identified, nor has this effect been well studied with regard to the chemopreventive efficacy of conventional NSAIDs.

PDE is a metallophosphohydrolase that specifically hydrolyzes the 3′,5′-cyclic phosphate moiety on cyclic nucleotides to a 5′-monophosphate, thereby deactivating cyclic AMP (cAMP) or cGMP. PDE terminates second messenger signaling by degrading cyclic nucleotides, whereas inhibition of PDE activity blocks cyclic nucleotide degradation to mimic or amplify cyclic nucleotide signaling. The PDE superfamily consists of 20 distinct genes divided into 11 protein families (15). Despite the heterogeneity within the PDE superfamily, the catalytic domain is highly conserved across family members, yet minor changes within this domain determine the specificity of each isozyme family member for cAMP (PDE4, PDE7, and PDE8), cGMP (PDE5, PDE6, and PDE9), or both cAMP and cGMP (PDE1, PDE2, PDE3, PDE10, and PDE11) as substrates. Both cAMP and cGMP have been shown to have antiproliferative and proapoptotic effects in many cell types (15, 16), and numerous studies have described the growth-inhibitory and apoptosis-inducing effects of various PDE inhibitors on certain tumor cell types, including colorectal cancer cells (17–20).

Sulindac is perhaps the most effective among the NSAIDs with regard to colon cancer chemoprevention, and its efficacy in numerous preclinical and clinical studies has been well documented. Most notably, sulindac has been shown to cause 60% to 70% reduction of adenoma size and number in patients with familial adenomatous polyposis (21). As reviewed previously (22), sulindac is a prodrug that requires metabolic activation for its antitumor activity. The sulfide moiety is reversibly oxidized to the chemopreventive efficacy of conventional NSAIDs. Mechanistic studies have shown that sulindac sulfide (SS) can selectively inhibit PDE5 activity in colon tumor cells compared with normal colonocytes.

**Materials and Methods**

**Drugs and reagents**

MY5445 and NOR-3 were purchased from BioMol. Recombinant PDE enzymes were purchased from BPS Biosciences. Anti-PDE antibodies were purchased from GeneTex. Anti-rabbit and anti-goat horseradish peroxidase–conjugated secondary antibodies were obtained from Cell Signaling Technology. DMSO was used as the vehicle for all compounds unless otherwise specified. All other drugs and reagents were purchased from Sigma-Aldrich.

**Cells and cell culture**

The human colon cancer cell lines HT-29, SW480, and HCT116 and the primary culture of normal fetal human colonocytes (FHC) were obtained from the American Type Culture Collection and grown under standard cell culture conditions as recommended by the American Type Culture Collection. Cell counts and viability were determined by trypan blue exclusion using a hemocytometer. Only cultures displaying >95% viability were used for experiments.

**Growth assays**

Tissue culture microtiter 96-well plates were seeded at a density of 5,000 cells per well. The inhibition of cell growth caused by treatment was determined after 72 hours of treatment using the luminescent CellTiter-Glo Assay (Promega), which measures viable cells based on ATP content. The assay was done according to the manufacturer’s specifications.

**Proliferation assays**

The antiproliferative activity of NSAIDs was determined by a reduction of DNA synthesis, which was measured by a radioactive thymidine incorporation assay. After treatment, cells were incubated at 37°C for 56 hours, at which time a 1 μCi aliquot of [3H]thymidine (Amersham Biosciences) was added to each well. Cells were incubated for another 16 hours for a total of 72 hours of treatment. Plates were harvested with a semiautomatic cell harvester onto filters that bind the DNA. Samples were counted in an automated scintillation counter to determine the amount of [3H]thymidine uptake.

**Apoptosis assay**

Cells were seeded in 10-cm tissue culture dishes at a density of 1 x 10^6 per dish. After 48 hours of treatment, cells were collected and fixed with 4% formalin on ice for 15 minutes. Samples were stained for DNA strand breaks using the APO-BrdU TUNEL Assay kit (Invitrogen). The assay was done according to the manufacturer’s specifications. The percentage of terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)–positive cells was quantified using a Guava EasyCyte Plus.
flow cytometer. A minimum of 10,000 events was collected for each treatment group with use of minimal electronic compensation. Data were analyzed using CytoSoft 5.0 software (Guava Technologies).

Cell lysis

Cells were harvested, vortexed in ice-cold lysis buffer [20 mmol/L Tris-acetate, 5 mmol/L magnesium acetate, 1 mmol/L EGTA, 0.8% Triton X-100, 50 mmol/L NaF, and protease inhibitor cocktail (pH 7.4)], and clarified by centrifugation at 10,000 × g for 10 minutes at 4°C. Protein content was determined using the bicinchoninic acid protein assay (Pierce) following the manufacturer's specifications. The colon of normal rats was excised and scraped with a glass microscope slide to obtain normal colonocytes, which were then lysed as described.

Tissue homogenization

HT-29 tumor cells were grown s.c. in athymic NCr-mu/nu mice. Tumors weighing 1 to 2 g each were homogenized in 100 mmol/L Tris-HCl (pH 7.5), 10 mmol/L NaCl, 1 mmol/L EGTA, 0.5 mmol/L DTT, 0.1% Triton X-100, and a protease inhibitor cocktail (Sigma). The extract was clarified by centrifugation at 20,000 × g for 1 hour at 4°C. Protein content was determined using the bicinchoninic acid protein assay following the manufacturer's specifications.

PDE assays

cGMP PDE activity was measured using [3H]cGMP scintillation proximity enzyme assay kits (Amersham). The conditions of the assays were 50 mmol/L Tris-HCl (pH 7.5), 8.3 mmol/L MgCl2, 1.7 mmol/L EGTA, 20 μmol/L [3H]cGMP, 2% DMSO, and 0.2 mg/mL of HT-29 lysate in a volume of 100 μL. The reaction was allowed to proceed for 15 minutes at 30°C and terminated by adding scintillation proximity beads, which bind the radiolabeled product. The beads were counted on a scintillation counter. PDE activity was also measured using the IMAP fluorescence polarization PDE assay (Molecular Devices). The assay was modified, as described previously (23), to use fluorescein-cAMP and tetramethylrhodamine-cGMP as substrates, allowing for simultaneous measurement of cAMP and cGMP hydrolysis.

cGMP assay

Cells were seeded at a density of 1 × 10^6 per 10-cm tissue culture dish. After 30 minutes of drug treatment, cells were lysed and assayed for cGMP content using the cGMP Direct Biotrak EIA kit (GE Biosciences) according to the manufacturer's specifications.

Western blots

Cell lysates (30 μg protein) were separated by SDS-PAGE in a 12% polyacrylamide gel followed by electrophoretic transfer to a nitrocellulose membrane. The membranes were blocked with 5% bovine serum albumin in TBS containing 0.05% Tween 20. Incubation of membranes in primary and secondary antibodies was done according to the manufacturers' specifications.

Luciferase reporter assays

Cells were transiently transfected with the TOPFlash luciferase construct (MilliPROBE) and β-galactosidase-expressing vector (Promega). After 24 hours of treatment, cells were lysed and both luciferase and β-galactosidase activities were measured. The luciferase activity was normalized to the β-galactosidase activity.

Tolerance studies

The maximum tolerated dose (MTD) of sulindac was determined using 5- to 6-week-old male NCr-mu/nu mice. Sulindac was administered by oral gavage at dosages of 50, 100, 200, or 400 mg/kg/d as a suspension in 0.5% carboxymethylcellulose (CMC) and 0.25% Tween 80 in water. Mice were observed daily and body weights were measured twice weekly.

In vivo antitumor efficacy

The antitumor efficacy of sulindac was evaluated using the human HT-29 colon xenograft implanted s.c. in 5- to 6-week-old male NCr-mu/nu athymic mice. Sulindac was administered orally once daily for 40 days at a dose of 50 mg/kg as a suspension in 0.5% CMC and 0.25% Tween 80 in water. The experiment consisted of a vehicle-treated control group and a sulindac-treated group, with each group containing 10 mice. Treatment was initiated when the tumors reached a size of 75 to 200 mg. Mice were observed daily for mortality. Tumor dimensions and body weights were measured twice weekly starting on the first day of treatment. For all animal studies described above, mice were housed in microisolator cages, provided tap water and sterile pelleted diet ad libitum, and euthanized as per Institutional Animal Care and Use Committee (IACUC) guidelines. All animal protocols were reviewed by Southern Research IACUC before experimentation.

Immunohistochemistry

Formalin-fixed, paraffin-embedded clinical specimens were obtained from the Fox Chase Cancer Center by Dr. Andre Klein-Szanto and processed for immunohistochemistry by avidin-biotin peroxidase detection. PDE5 antiserum was generated in sheep against a 16-amino acid synthetic peptide sequence contained within the high-affinity cGMP binding domain of the enzyme. Anti-PDE5 IgG was purified using an antigen affinity column. Peptide, antibody, and affinity column were obtained from Bethyl Laboratories.

Experimental design and data analysis

Drug effects on cell growth and PDE activity were measured, and the potency was expressed as an IC50 value, which is the concentration resulting in 50% inhibition when compared with the vehicle control. For growth assays, the IC50 value was determined by testing a range of eight concentrations with a minimum of four replicates.
per dose. Each assay used a minimum of two replicates. Dose-response curves were constructed using Prism5 software (GraphPad), which calculates IC_{50} values using a four-parameter logistic equation. All experiments were repeated a minimum of twice to determine the reproducibility of the results. All values represent a comparison between drug treatment at the specified concentration and vehicle-treated controls. All error bars represent SEM. Calculation of P values was done by comparing the specified treatment group to vehicle-treated controls using a Student’s t test.

Results

NSAID inhibition of colon tumor cell growth correlates with cGMP PDE inhibition

To determine whether cGMP PDE represents a potential COX-independent target responsible for the antineoplastic activity of sulindac, we compared the non–COX-inhibitory sulfoxide and sulfone metabolites of the drug with the COX-1- and COX-2–inhibitory sulfide metabolite, the highly selective COX-2 inhibitor rofecoxib, and the COX-1–selective inhibitor indomethacin. These drugs were evaluated for their potency to inhibit purified COX-1 and COX-2 enzymes, cGMP hydrolysis in lysates from HT-29 human colon tumor cells, and proliferation of HT-29 colon tumor cells. As summarized in Table 1, sulindac sulfone and sulfoxide inhibited the proliferation of HT-29 colon tumor cells with IC_{50} values of 89 and 224 μmol/L, respectively, despite lacking COX-1– or COX-2–inhibitory activity at concentrations up to 300 μmol/L. SS more potently inhibited proliferation of the colon tumor cells than either the sulfoxide or sulfone forms with an IC_{50} value of 34 μmol/L. However, this effect required appreciably higher concentrations compared with those needed to inhibit COX-1 or COX-2. In contrast with COX inhibition, all three forms of sulindac inhibited cGMP PDE activity with IC_{50} values that were comparable with their respective IC_{50} values for inhibiting proliferation. Indomethacin also inhibited colon tumor cell growth and cGMP PDE but with reduced potency compared with SS despite its superior potency to inhibit COX-1 or COX-2. Moreover, the highly potent and specific COX-2 inhibitor rofecoxib was unable to inhibit colon tumor cell proliferation or cGMP PDE activity. These results show that the potency for cGMP PDE inhibition, but not the potency for COX-1 or COX-2 inhibition, is closely associated with colon tumor cell growth–inhibitory potency among sulindac and its metabolites.

A series of chemically diverse NSAIDs representative of the acetic acid, fenamate, and propionic acid families of NSAIDs, as well as another COX–2–selective inhibitor, celecoxib, were compared for potency to inhibit COX-2, cGMP PDE, and HT-29 colon tumor cell growth (Fig. 1A). Among these anti-inflammatory drugs, a strong positive correlation was observed between potency for HT-29 cell growth inhibition and potency for inhibition of cGMP PDE activity with an r^2 value of 0.933 (Fig. 1B). However, no correlation was observed by comparing potencies of these NSAIDs for COX-2 inhibition as previously reported (25) with potencies for HT-29 cell growth inhibition (Fig. 1C). The salicylate family, which contains aspirin as well as non-carboxylate NSAIDs from the pyrazole (e.g., phenylbutazone), and oxicam (e.g., piroxicam) families displayed low potency for HT-29 growth inhibition and did not affect cGMP PDE activity (data not shown). These data show that a variety of chemically diverse NSAIDs can inhibit cGMP PDE activity and that this effect is associated with their in vitro growth-inhibitory potency.

SS selectively inhibits cGMP hydrolysis in colon tumor cells

SS was further studied for its ability to inhibit cGMP hydrolysis and growth of the human colon cancer cell lines HT-29, SW480, and HCT116 and were compared with FHCs, which are representative of a nontumorigenic colon cell line. As shown in Fig. 2A, SS selectively inhibited growth of the human colon tumor cells lines with IC_{50} values ranging from 73 to 85 μmol/L compared with the

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**Table 1.** Potency of the sulindac metabolites, the COX-2–selective inhibitor rofecoxib, and the COX-1–selective inhibitor indomethacin to inhibit COX-1, COX-2, cGMP hydrolysis in HT-29 colon tumor cell lysates, and colon tumor cell proliferation

<table>
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<tr>
<th>Compound</th>
<th>IC_{50} (μmol/L)</th>
<th>COX-1</th>
<th>COX-2</th>
<th>cGMP PDE</th>
<th>Proliferation</th>
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<td>SS</td>
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<td>1.8</td>
<td>6.3</td>
<td>19.7</td>
<td>33.9</td>
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<td>Sulindac sulfone</td>
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<td>&gt;300</td>
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<tr>
<td>Sulindac sulfoxide</td>
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<tr>
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<td>&gt;300</td>
<td>&gt;500</td>
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<tr>
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<td>1.0</td>
<td>24</td>
<td>136</td>
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</table>

NOTE: Purified ovine COX-1 and COX-2 were used for the COX assays. cGMP hydrolysis was measured by the scintillation proximity assay using cell lysates obtained from HT-29 human colon tumor cells, which were used as the source of cGMP PDE isozymes. Antiproliferative activity was measured after 72 h of drug treatment of HT-29 cells using a tritiated thymidine assay.
IC50 value of 150 μmol/L necessary for growth inhibition of normal colonocytes. In addition to inhibiting colon tumor cell growth, SS also induced apoptosis as measured by DNA fragmentation (Fig. 2B). After 48 hours of treatment, 100 and 200 μmol/L SS resulted in 6- and 15-fold increases, respectively, in the percentage of TUNEL-positive HT-29 cells.

Using a fluorescence polarization assay that we developed to simultaneously measure cAMP and cGMP hydrolysis, the effects of SS on PDE activity in lysates from HT-29 colon cancer cells and FHC were evaluated (Fig. 2C). SS inhibited cGMP hydrolysis with an IC50 value of 74 μmol/L in the HT-29 cell lysate, but appreciably higher concentrations were necessary to inhibit cAMP hydrolysis. Additionally, FHC displayed reduced sensitivity to the cGMP PDE–inhibitory activity of SS, which paralleled its reduced sensitivity to growth suppression by SS.

To determine the relevance of cGMP PDE inhibition by SS in colon tumor cell lysates to the effects of the drug in intact cells, intracellular levels of cGMP were measured after 30 minutes of SS treatment using a cGMP immunoassay (Fig. 2D). SS treatment of HT-29 and SW480 colon

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**Fig. 1.** Colon tumor cell growth, cGMP, and COX-2 inhibitory activity of a group of NSAIDs and COX-2 inhibitors. A, structures of compounds. B, positive association between the potencies of HT-29 growth inhibition and inhibition of cGMP hydrolysis in HT-29 cell lysates. C, no association between the potencies for HT-29 growth inhibition and inhibition of COX-2.
tumor cells resulted in a significant increase in intracellular cGMP levels at concentrations of 100 and 150 μmol/L, which is consistent with concentrations required to inhibit colon tumor cell growth and induce apoptosis.

cGMP elevation causes colon tumor cell growth inhibition
To determine if elevated levels of intracellular cGMP in colon tumor cells are sufficient to inhibit colon tumor cell growth, the effect of the nitric oxide donor and guanylyl cyclase activator NOR-3 on cell growth was evaluated in the HT-29, SW480, and HCT116 lines. NOR-3 inhibited growth in all three cell lines with IC50 values ranging from 59 to 74 μmol/L (Fig. 3A). Conversely, the adenylyl cyclase activator forskolin did not significantly affect growth of any of the cell lines at concentrations up to 100 μmol/L (Fig. 3B), suggesting that cGMP, but not cAMP, can signal events leading to the suppression of colon tumor cell growth.

A panel of PDE isozyme–specific inhibitors, including vinpocetine (PDE1), EHNA (PDE2), milrinone (PDE3), rolipram (PDE4), and MY5445 (PDE5), was evaluated for growth-inhibitory activity in HT-29, SW480, and HCT116 colon tumor cell lines. Among these, only MY5445 was found to inhibit colon tumor cell growth with IC50 values ranging from 8 to 18 μmol/L (Fig. 3C). MY5445 also selectively inhibited cGMP PDE activity in HT-29 cell lysates with an IC50 value of 4 μmol/L without substantially affecting cAMP hydrolysis (Fig. 3D). Similar to SS, MY5445 inhibited proliferation and induced apoptosis of colon tumor cells (data not shown). In addition, highly potent and selective PDE5 inhibitors such as tadalafil and vardenafil suppressed tumor cell growth. However, high concentrations were required relative to concentrations that inhibited purified PDE5, which may be attributed to low uptake or high efflux from cultured tumor cells.

SS selectively inhibits PDE5
To determine which PDE isozyme is responsible for tumor cell growth–inhibitory activity of SS, recombinant isozymes from all 11 isozyme family members were screened for sensitivity to SS using cGMP or cAMP as a substrate. As previously reported, the cGMP-selective PDE5 isozyme was the most sensitive, although cGMP hydrolysis by PDE2 and PDE3 was also inhibited, albeit with reduced sensitivity (23). The difference in sensitivity is shown in Fig. 4A with concentration-dependent inhibition of PDE5 by SS, resulting in an IC50 value of 38 μmol/L. By
comparison, SS inhibited cGMP hydrolysis by PDE2 and PDE3 with IC50 values of 97 and 84 μmol/L, respectively. Consistent with its cGMP selectivity, SS did not significantly inhibit cAMP hydrolysis by PDE2 or PDE3 (data not shown). To assess the potential contribution of PDE2, PDE3, and PDE5 to cGMP degradation in whole cells, their activity and expression in colon cell lines was studied using PDE isozyme–specific antibodies and inhibitors. First, the levels of protein expression in HT-29 cells were determined by Western blotting and compared with the levels of protein expression in FHC. As shown in Fig. 4B, HT-29 cells expressed PDE3 and PDE5, but did not express PDE2. Conversely, FHC expressed PDE3 at levels higher than HT-29 cells, but lacked expression of PDE2 and PDE5. Other human colon cancer cell lines, HCT116 and SW480, also expressed PDE5 (data not shown). To determine the importance of these isozymes to the overall cGMP hydrolytic capacity of the colon tumor cells, the cGMP PDE–inhibitory activity of the PDE2-selective inhibitor EHNA, the PDE3-selective inhibitor milrinone, and the PDE5-selective inhibitor sildenafil were analyzed in HT-29 cell lysates. Consistent with the expression levels determined by Western blotting, milrinone and sildenafil inhibited cGMP PDE activity in the HT-29 lysate by 31% and 42%, respectively, whereas EHNA had only a modest effect of <12% inhibition (Fig. 4C). The overexpression of PDE5 in colon tumor cells compared with FHC is consistent with the selectivity of SS for inhibition of PDE5 compared with other cGMP PDE isozymes and for inhibition of tumor cell growth and cGMP PDE compared with normal colonocytes.

Colon adenomas and adenocarcinomas overexpress PDE5

The expression of PDE5 in normal colonic mucosa, adenomas, and adenocarcinomas from archival clinical specimens was determined by immunohistochemistry using a PDE5–specific antibody. Analysis of 11 specimens containing normal colonic mucosa showed consistent negative labeling in the colonic epithelium, despite labeling of the muscularis mucosa. By contrast, 5 of 5 adenomas and 11 of 13 adenocarcinomas displayed positive labeling. Representative micrographs from normal colon mucosa, adenomas, and adenocarcinomas are shown in Fig. 4D.

Tumor cell growth–inhibitory activity of SS is associated with inhibition of β-catenin transcriptional activity

Previous studies reported that sulindac sulfide and sulfone can activate cGMP–dependent protein kinase (PKG), which can then phosphorylate β-catenin to promote its proteosomal degradation (12, 23). We evaluated the effects of SS and MY5445 on β-catenin signaling in colon cancer cells to determine if this mechanism is involved in the growth-inhibitory activity of these compounds. Both compounds resulted in a significant reduction of T-cell factor/lymphoid enhancer factor (Tcf/Lef)–mediated transcription as measured by a luciferase reporter assay in HT-29, HCT116, and SW480 colon cancer cells (Fig. 4E). Although the length of treatment necessary to cause an effect differed between compounds and between proteins, SS and MY5445 treatment resulted in a significant reduction in expression of the proteins cyclin D1 and survivin, whose transcription is promoted by β-catenin signaling (Fig. 4F).
Fig. 4. PDE isozyme expression and activity. A, inhibition of cGMP hydrolytic activity of recombinant PDE2, PDE3, and PDE5 by SS. B, relative levels of PDE2, PDE3, and PDE5 isozyme expression in HT-29 and FHC as measured by Western blotting with PDE family–specific antibodies. C, relative activities of PDE2, PDE3, and PDE5 families in HT-29 cell lysates as measured by overall inhibition of cGMP hydrolysis by the PDE2-selective inhibitor EHNA, the PDE3-selective inhibitor milrinone, and the PDE5-selective inhibitor sildenafil. D, PDE5 expression in normal colon mucosa, adenoma, and adenocarcinomas from human clinical specimens as determined by immunohistochemistry following avidin-biotin peroxidase labeling. E, effects of SS (top graph) or MY5445 (bottom graph) on Tcf/Lef transcriptional activity as measured by a luciferase reporter assay in HT-29 (black columns), HCT116 (blue columns), and SW480 (red columns) colon cancer cells. F, time-dependent decrease in the expression of cyclin D1 and survivin in SW480 cells after treatment with SS or MY5445, with β-actin as a loading control.
These data are consistent with cGMP-PKG–mediated phosphorylation of β-catenin to induce degradation, thereby suppressing the transcription of key genes that regulate tumor cell proliferation and apoptosis.

SS selectively inhibits cGMP hydrolysis in colon tumors

To further evaluate the tumor selectivity of SS, we measured the sensitivity of cGMP PDE activity in lysates from HT-29 tumors established in athymic mice to SS and MY5445 and compared the sensitivity to lysates obtained from normal rat colonic mucosa. Similar to the effects in cultured HT-29 tumor cells, both SS and MY5445 selectively inhibited cGMP PDE activity in lysates obtained from the HT-29 tumors. However, cGMP hydrolysis in normal colon mucosa displayed reduced sensitivity, comparable with cultured FHC (Fig. 5A and B). SS displayed 4-fold greater potency for inhibition of the tumor cGMP PDE compared with that present in the normal tissue, whereas MY5445 displayed >10-fold greater potency. Both compounds inhibited cGMP PDE in the tumor lysate with IC<sub>50</sub> values comparable with those necessary for cGMP PDE inhibition in cultured tumor cell lysates and for in vitro colon tumor cell growth inhibition. These results are consistent with increased expression and activity of PDE5 in colon tumor cells compared with normal colonocytes.

Sulindac inhibits colon tumor cell growth in vivo

To assess the in vivo pharmacologic relevance of the above observations, the tumor growth-inhibitory activity of sulindac was determined using the HT-29 colon tumor xenograft mouse model. Dosages of sulindac were selected based on a tolerance study following daily oral gavage for 20 days of treatment. The MTD was determined to be ∼50 mg/kg, where no deaths (Fig. 5A) or significant weight loss occurred. To determine antitumor efficacy of sulindac, two groups of 10 mice each were inoculated s.c. with fragments of HT-29 tumors; one group was treated with vehicle (CMC) and the other was treated with sulindac at 50 mg/kg once daily by oral gavage. Sulindac treatment caused a significant reduction of tumor growth of ∼55% by the end of treatment compared with mice treated with vehicle only (Fig. 5D) without affecting body weight (data not shown). In addition, levels of sulindac and its metabolites were measured in plasma collected 6 hours following a single oral dose of sulindac at 50 mg/kg. Sulindac generated plasma levels of 36.7 ± 10.2 μmol/L for the sulfide, 8.9 ± 1.2 μmol/L for the sulfone, and 23.1 ± 5.5 μmol/L for the parent sulfoxide. These experiments show that SS is the predominant metabolite in plasma and that dosages of sulindac required for in vivo antitumor efficacy can generate plasma levels of SS that are comparable with the concentrations required to inhibit colon tumor cell proliferation and PDE5 activity in vitro.

Discussion

Epidemiologic, preclinical, and clinical studies have shown convincing evidence that NSAIDs and COX-2 inhibitors have cancer chemopreventive efficacy, especially...
with regard to colorectal cancer. Unfortunately, gastrointestinal, renal, and cardiovascular toxicities that result from COX-1 and/or COX-2 inhibition limit their use for cancer chemoprevention. Here, we show that the in vitro colon tumor cell growth–inhibitory activity of a group of NSAIDs is closely associated with cGMP PDE inhibition. For instance, the potency of SS to inhibit HT-29 colon tumor cell growth was nearly identical to the potency for inhibition of cGMP PDE in tumor cell lysates, yet was more than an order of magnitude greater than the potency for inhibition of COX-1 or COX-2. Sulindac sulfone and sulfoxide did not inhibit COX but did inhibit tumor cell growth and cGMP PDE. Additionally, a strong positive correlation existed between the potencies of other chemically diverse NSAIDs for cGMP PDE inhibition and colon tumor cell growth inhibition, yet no correlation was apparent for COX-2 inhibition. Interestingly, the COX-2–selective inhibitor rofecoxib, which did not inhibit tumor cell growth, also did not inhibit cGMP PDE, whereas another COX-2 inhibitor, celecoxib, did inhibit tumor cell growth at concentrations that inhibited cGMP PDE. These data suggest that cGMP PDE inhibition represents a COX-independent mechanism responsible for the in vitro tumor cell growth–inhibitory activity of certain NSAIDs and COX-2 inhibitors that potentially could be targeted for the discovery of safer and more efficacious drugs for chemoprevention.

Because of its high potency to inhibit colon tumor cell growth, SS was used to investigate the role of cGMP in regulating tumor cell growth and to identify the PDE isozyme(s) responsible for its growth-inhibitory activity. Moreover, SS-induced growth inhibition was associated with inhibition of proliferation and induction of apoptosis, which occurred at concentrations comparable with those necessary for inhibition of cGMP hydrolysis in HT-29 cell lysates. The biological relevance of cGMP PDE inhibition in HT-29 cell lysates was confirmed using a cell-based immunoblot assay, which showed the ability of SS to increase cGMP levels in colon tumor cells at concentrations that inhibited growth and induced apoptosis. The ability of other cGMP signaling activators, including the guanylyl cyclase activator NOR-3 and the PDE5-specific inhibitor MY5445, to inhibit growth suggests that activation of cGMP signaling is sufficient for inhibition of colon tumor cell growth. Consistent with previous findings (12), we showed that activation of the cGMP signaling pathway by compounds such as SS and MY5445 is associated with inhibition of β-catenin transcriptional activity, a pathway that is well documented to be associated with tumorigenesis. Therefore, inhibition of cGMP PDE, activation of cGMP signaling, and inhibition of β-catenin–mediated transcription by certain NSAIDs may fully account for their in vitro tumor cell growth–inhibitory and apoptosis-inducing activity and contribute to their antitumor efficacy.

Although activation of cGMP signaling is sufficient to inhibit colon tumor cell growth, nonselective activation of this pathway is not a plausible pharmacologic approach to develop new chemopreventive agents due to the myriad of roles that cGMP plays in normal physiology and the potential for adverse effects. However, we hypothesized that selective inhibition of one or more of the PDE isozymes expressed in tumor cells may provide a more effective strategy to selectively induce cGMP levels and kill neoplastic cells. Nontumorigenic colon cells were not found to express the PDE5 isozyme that was expressed in colon tumor cell lines. The clinical relevance of this observation was confirmed by immunohistochemical analysis of human colon specimens, which showed increased PDE5 expression in adenomas and adenocarcinomas compared with normal colonic mucosa. Consistent with these observations, SS displayed selectivity for inhibition of cGMP hydrolysis by PDE5 compared with all other PDE isozymes and improved potency for both inhibition of colon tumor cell growth and cGMP PDE activity when compared with the non-tumorigenic colon cells. These results are consistent with previous studies that used PDE5 antisense in HT-29 human colon tumor cells to show that PDE5 activity is necessary for growth and survival of colon tumor cells (24).

Although the known PDE5 inhibitor MY5445 inhibited colon tumor cell growth at concentrations that are comparable to concentrations that can inhibit PDE5, highly potent PDE5 inhibitors used for erectile dysfunction such as tadalafil and vardenafil required micromolar levels to inhibit tumor cell growth compared with nanomolar levels required to inhibit purified PDE5. There are several potential explanations for this discrepancy, including an inability of the drugs to access PDE5 within cancer cells due to limited uptake, confined subcellular localization of the enzyme in cancer cells, or an active efflux of the drugs from cancer cells. Whatever the reason for this, the data presented here strongly suggest that PDE5 inhibition and cGMP elevation are responsible for the tumor cell growth–inhibitory and apoptosis-inducing activity of SS and possibly other NSAIDs.

We also show that SS inhibited cGMP hydrolysis not only in human HT-29 colon tumor cell lysates but also in homogenates from colon tumors grown in vivo. Additionally, SS displayed selectivity for inhibition of tumor tissue cGMP PDE compared with normal colon tissue cGMP PDE, mirroring the observations in cell cultures. Furthermore, the PDE5-selective inhibitor MY5445 exhibited a greater degree of selectivity for inhibition of cGMP PDE activity in tumor tissue homogenates compared with that exhibited by SS. The pharmacological relevance of these observations is supported by other experiments showing that sulindac can suppress colon tumor growth in mice at dosages that can generate plasma levels of SS that are capable of inhibiting PDE5 in vivo. Although more work is necessary to confirm the direct effects of such in vivo treatments on cGMP signaling, these data suggest that inhibition of PDE5 and activation of cGMP signaling may play an important role in the in vivo antineoplastic activity of sulindac and potentially other NSAIDs. In support of this possibility, pharmacokinetic studies in humans have shown that clinically used dosages of sulindac can achieve plasma concentrations of the sulfide that are comparable...
with those required for PDE5 inhibition and cGMP elevation as we report here (21, 22). However, such dosages are unlikely to be sufficiently safe for chemoprevention due to COX-dependent toxicities. New derivatives of sulindac that lack COX inhibitory activity, but have more potent growth inhibitory activity hold more promise for improved efficacy and reduced toxicity (26).

We conclude that PDE5 inhibition and subsequent elevation of intracellular cGMP levels and activation of cGMP signaling are important mechanisms responsible for the chemopreventive efficacy of sulindac and potentially other NSAIDs. The differential expression of PDE5 isoforms between normal and tumorigenic colon cells provides a COX-independent strategy to identify new drug candidates for chemoprevention. As such, further studies are warranted to determine the role of PDE5 and cGMP signaling as a molecular target and pathway for chemoprevention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Andre Klein-Szanto for providing clinical specimens and histopathologic support for analysis of PDE5 expression in tissue sections and Dr. William Thompson for providing the PDE3 antibody.

Grant Support

NIH grants R01 CA131378, R21 NS59509, and R03 CA128021.

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Received 02/11/2010; revised 04/23/2010; accepted 05/13/2010; published OnlineFirst 09/28/2010.

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www.aacrjournals.org Cancer Prev Res; 3(10) October 2010 1313
Colon Tumor Cell Growth–Inhibitory Activity of Sulindac Sulfide and Other Nonsteroidal Anti-Inflammatory Drugs Is Associated with Phosphodiesterase 5 Inhibition

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