Sulindac Selectively Inhibits Colon Tumor Cell Growth by Activating the cGMP/PKG Pathway to Suppress Wnt/β-Catenin Signaling


Abstract

Nonsteroidal anti-inflammatory drugs (NSAID) display promising antineoplastic activity for colorectal and other cancers, but toxicity from COX inhibition limits their long-term use for chemoprevention. Previous studies have concluded that the basis for their tumor cell growth inhibitory activity does not require COX inhibition, although the underlying mechanism is poorly understood. Here, we report that the NSAID sulindac sulfide inhibits cyclic guanosine 3′,5′-monophosphate phosphodiesterase (cGMP PDE) activity to increase intracellular cGMP levels and activate cGMP-dependent protein kinase (PKG) at concentrations that inhibit proliferation and induce apoptosis of colon tumor cells. Sulindac sulfide did not activate the cGMP/PKG pathway, nor affect proliferation or apoptosis in normal colonocytes. The mechanism by which sulindac sulfide and the cGMP/PKG pathway inhibits colon tumor cell growth involves the transcriptional suppression of β-catenin to inhibit Wnt/β-catenin T-cell factor transcriptional activity, leading to down-regulation of cyclin D1 and survivin. These observations suggest that safer and more efficacious sulindac derivatives can be developed for colorectal cancer chemoprevention by targeting PDE5 and possibly other cGMP-degrading isozymes. Mol Cancer Ther; 12(9); 1–12. ©2013 AACR.

Introduction

Colorectal cancer is the third most common malignant disease in the western world (1). In the United States, colorectal cancer accounts for 12% of all newly diagnosed cancers (2) in which approximately 6% of Americans will die each year from this form of cancer (3). Despite the benefits of endoscopic screening to reduce the risk of developing colorectal cancer, there is an unmet medical need to develop safe and effective drugs for colorectal cancer chemoprevention.

Epidemiologic studies have reported that the long-term use of nonsteroidal anti-inflammatory drugs (NSAID) is associated with a significant reduction in the incidence of colorectal cancer and associated mortality (4, 5). Certain prescription strength NSAIDs such as sulindac also cause the regression of precancerous adenomas in patients with familial or sporadic adenomatous polyposis (6–10). These observations are consistent with preclinical studies that have shown the ability of sulindac and other NSAIDs to inhibit tumorigenesis in various animal models of colorectal cancer (11–13). Unfortunately, their COX-inhibitory activity is associated with potentially fatal gastrointestinal, renal, and cardiovascular toxicities that preclude use for cancer chemoprevention. Another limitation is that NSAIDs do not completely protect from disease progression in all individuals. Case reports, for example, have described individuals with familial adenomatous polyposis (FAP) who developed colorectal cancer despite long-term treatment with sulindac (14).

Although COX-2 is well established to play an important role in tumorigenesis, multiple investigators have concluded that the biochemical basis for the antineoplastic activity of NSAIDs is unrelated to their COX-1 or COX-2 inhibitory activity (15, 16). It may therefore be feasible to develop safer drugs for colorectal cancer chemoprevention by designing derivatives that do not disrupt prostaglandin biosynthesis, but retain or have improved anticancer activity. In support of this possibility, the non-COX
inhibitory sulfone metabolite of sulindac was shown to inhibit tumor formation in the azoxymethane-induced rat model of colon tumorigenesis (13). Sulindac sulfone (exsulind) was also reported to be effective in clinical trials involving individuals with familial or sporadic adenomatous polyposis (17, 18), but did not receive U.S. Food and Drug Administration approval because of hepatotoxicity. The mechanism for the antineoplastic activity of sulindac sulfone was previously described to involve the inhibition of cyclic guanosine 3',5'-monophosphate phosphodiesterase (cGMP PDE), which can increase intracellular cGMP levels to activate cGMP-dependent protein kinase (PKG), leading to the suppression of tumor cell growth and the induction of apoptosis (19, 20). More recently, we and others have shown that the COX inhibitory metabolite of sulindac, sulindac sulfide, as well as other NSAIDs from various chemical families, including indomethacin, meclofenamic acid, and celecoxib, also inhibit cGMP PDE and that this activity is closely associated with their tumor cell growth inhibitory activity (21, 22).

Cyclic nucleotide phosphodiesterases are a superfamily of enzymes that hydrolyze the 3',5'-phosphodiester bond in cGMP and/or cyclic AMP (cAMP). There are 11 PDE isozymes having different substrate specificity, regulatory properties, tissue localization, and inhibitor sensitivity (23). PDE1, 2, 3, 10, and 11 are dual substrate-degrading isozymes, whereas PDE5, 6, and 9 are selective for cGMP, and PDE4, 7, and 8 are cAMP selective. PDE inhibitors have the potential to cause a transient or sustained increase in intracellular levels of cAMP and/or cGMP to activate cAMP-dependent protein kinase and/or PKG, as well as other cyclic nucleotide-regulated processes. Although several PDE inhibitors have been developed for various disease indications, there has been relatively little attention given to cancer, despite the well-known regulation of cell growth by cyclic nucleotides (24).

While sulindac sulfide can inhibit several cGMP-degrading isozymes, including PDE2, 3, 5, and 10, PDE5 appears to be a critical target for sulindac sulfide as previously reported (21, 25, 26). For example, sulindac sulfide can inhibit recombinant PDE5 within a concentration range that can suppress tumor cell growth and induce apoptosis in vitro. PDE5 is also widely expressed in colon tumor cell lines and levels of this enzyme are elevated in colon adenocarcinomas and adenomas compared with normal colonic mucosa (21). In addition, we recently described a novel non-COX inhibitory derivative of sulindac that selectively inhibits PDE5 and potently inhibits colon tumor cell growth (27). Here we provide additional evidence that the antitumor activity of sulindac sulfide involves PDE5 inhibition and that this isoform plays an important role in colon tumor cell proliferation and survival. We also describe a novel regulatory influence of the cGMP/PKG pathway over Wnt/β-catenin–T1–cell factor (TCF) transcriptional activity that appears to fully or partially account for the antiproliferative and proapoptotic properties of sulindac.

Materials and Methods

Drugs and reagents

Sulindac sulfide was purchased from Sigma-Aldrich. 8-Bromo-cGMP was purchased from Biolog. Tadalafil and sildenafil were extracted from CIALIS and VIAGRA tablets, respectively. All antibodies were purchased from Cell Signaling Technologies. Dimethyl sulfoxide was used as vehicle for all compounds unless otherwise noted.

Cells and cell culture

Human colon tumor cell lines HCT116, HT-29, SW480, and Caco-2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown under standard cell culture conditions in RPMI 1640 medium containing 5% serum at 37°C in a humidified atmosphere with 5% CO2. The human colonocyte line, NCM460, that is derived from normal human colon mucosa (28) was obtained from INCELL and grown in INCELL’s enriched M3:10 medium with 10% serum as recommended by the supplier. All cell lines were expanded upon delivery, and numerous aliquots of low-passage cells were preserved in liquid N2. Cells were passed no longer than 2 months. Tumor cell lines obtained from ATCC were characterized by short tandem repeat profiling as conducted by ATCC. The NCM460 line was characterized by INCELL as described previously (e.g., tumorigenicity testing; ref. 28). No additional authentication of the cell lines was conducted except for experimental reasons (e.g., confirmation of cell doubling time, morphology, sensitivity to sulindac sulfide, PDE5 expression levels, etc.).

siRNA transfection

Two different sequences of siRNA targeting human PDE5 and scrambled control siRNA were purchased from Qiagen. The siRNA target sequences were as follows: siPDE5-1, 5'-GCCATCTGCTTGCAACTGTAT-3'; siPDE5-2, 5'-CCAGCTTTACTGCCATCAA-3'. The scrambled control siRNA, containing nonspecific sequences do not have homology in the human genome. siRNA duplexes were transfected into NCM460 and HCT116 cells using Hiperfect transfection reagent (Qiagen) and HT29 cells using RNAligMAX transfection reagent (Invitrogen) according to manufacturer’s specifications and incubated at 37°C for 72 hours.

Cell viability assay

Cells were plated in 96-well microtiter plates at a density of 5,000 cells per well. For drug treatment, cells were treated with compound or vehicle, and incubated at 37°C for 72 hours. For siRNA knockdown experiments, cells were transfected with siRNA under the same condition described earlier. All viability assays were conducted in 5% serum using either tumor cells or normal colonocytes, with the exception of those involving sildenafil and tadalafil that were conducted in 1.5% serum. The effect
of treatment on cell viability was measured using the Cell Titer Glo Assay as specified by the manufacturer (Promega).

**Apoptosis assay**
Cells were plated in 96-well microtiter plates at a density of 10,000 cells per well, and allowed to attach overnight. Cells were treated with compounds or vehicle, and incubated at 37°C for 6 hours. For siRNA assays, cells were plated in a 96-well plate (10,000 cells per well) and transfected with siRNA under the same conditions as above. All apoptosis assays were done in 5% serum using either tumor cells or normal colonocytes. The induction of apoptosis caused by treatment was determined using the Caspase 3/7 Glo Assay (Promega).

**5-Ethynyl-2'-deoxyuridine cell-proliferation assay**
The antiproliferative activity of sulindac sulfide and PDE5 siRNA was determined by measuring 5-ethynyl-2'-deoxyuridine (EdU) incorporation during DNA synthesis. Cells were plated at a density of 1.5 × 10^5 cells per 10-cm tissue culture dish and incubated overnight at 37°C. After growing the cells in serum-free media overnight, the cells were treated with sulindac sulfide or vehicle in media with 10% serum for 24 hours. A final concentration of 10 μmol/L EdU was added to each dish after 6 hours treatment. For siRNA assays, cells were plated at a density of 2 × 10^5 cells per well in 6-well tissue culture plates, transfected with siRNA, and incubated at 37°C for 54 hours before the addition of EdU. After another 18 hours of incubation with EdU, cells were harvested and analyzed using the Click-IT EdU Alexa Fluor 488 Proliferation Assay (Invitrogen) according to the manufacturer’s specifications. The percentage of proliferating cells was quantified using a Guava EasyCyte Plus flow cytometer.

**PDE assay**
PDE activity in cell lysates was measured using the IMAP fluorescence polarization PDE assay (Molecular Devices) as described previously (26). For experiments involving siRNA, cells were plated at a density of 2 × 10^5 cells per well in 6-well tissue culture plates and transfected with siRNA for 72 hours before cell lysis.

**cGMP assay**
Cells were plated at a density of 1 × 10^6 cells per 10-cm tissue culture dish, incubated for 48 hours, and treated with sulindac sulfide or vehicle control. After 45 minutes of treatment, cells were lysed and assayed for cGMP content using the cGMP Direct Biotrak ELISA kit (GE Healthcare Life Sciences). The assay was conducted according to the manufacturer’s specifications.

**Cell lysis**
Cells were lysed and protein concentrations were determined as described previously (26).

**Western blotting**
Western blotting was conducted as described previously (26). The band intensities of the images were quantified by ImageJ software.

**Luciferase reporter assay**
Cells were plated at a density of 5 × 10^4 cells per well in 24-well tissue culture plates. After 24 hours of incubation, cells were transiently transfected with 0.1 μg TOP-FLASH/FOP-FLASH constructs (Millipore) or CTNNB1 construct (generously provided by Dr. Darren Browning from Georgia Regents University Cancer Center) and 0.1 μg β-galactosidase-expressing vector (Promega). The CTNNB1 reporter construct was previously shown to subclone the essential promoter region (–2760 to +27) of β-catenin gene into the luciferase reporter vector pGL3Basic (29). After 42 hours of transfection, cells were treated with compound or vehicle for 6 hours. For siRNA assays, cells were cotransfected with reporter constructs and β-galactosidase-expressing vector 24 hours after an initial siRNA transfection and incubated for another 48 hours. At the end of treatment, cells were lysed and both luciferase and β-galactosidase activities were measured using kits from Promega. All luciferase activity was normalized to β-galactosidase activity. For TCF activity, the normalized FOP-FLASH values were further subtracted from the normalized TOP-FLASH values.

**Semiquantitative RT-PCR**
HCT116 cells were plated at a density of 1.5 × 10^6 cells per 10-cm tissue culture dish and allowed to adhere overnight. After 24 hours, growth media was replaced with serum-free media. After 18 hours of serum starvation, cells were treated with compound or vehicle for 6 hours. At the end of incubation period, cells were lysed and RNA was extracted using the RNAqueous-4PCR Kit (Ambion) according to manufacturer’s instructions. Semiquantitative RT-PCR was conducted using BluePrint RT-PCR Kit (Takara) according to manufacturer’s instructions with 23 cycles at an anneal temperature of 60°C. The primers (Invitrogen) were as follows: β-catenin forward, 5’-ATCCCCACTGGGCCCTCTGATAAA-3’ and reverse, 5’-CAATAGCTTCTGCAGCCTTCTCCT-3’; GAPDH forward, 5’-TGATGACATCAAGAAGTTGCTGAAG-3’ and reverse, 5’-TCCCTGGAGCCCATGTTGGCCC-3’. The band intensities were quantified by ImageJ software.

**Experimental design and data analysis**
Drug effects on cell growth and IC_{50} values were determined as described previously (26). Experiments were conducted with a minimum of 3 replicates per data point. Each experiment was conducted a minimum of 3 times to verify reproducibility. All error bars represent SEM. Calculation of P values was done by comparing the specified treatment group with vehicle-treated controls using a Student t test. A P value of <0.05 was considered statistically significant.
Results

Growth and cGMP PDE inhibitory activity of sulindac sulfide

Sulindac is a nonsteroidal anti-inflammatory drug from the aryalkanoic acid class in which the sulfide metabolite as shown in Fig. 1A is responsible for its antineoplastic activity. Initial experiments were conducted to quantify the inhibitory effect of sulindac sulfide on the viability of colon cells derived from either malignant or normal tissues. As shown in Fig. 1B, sulindac sulfide inhibited the viability of human HCT116, HT29, and Caco2 colon tumor cell lines with IC50 values ranging from 75 to 83 μmol/L following 72 hours of treatment. By comparison, human NCM460 normal colonocytes were appreciably less sensitive to sulindac sulfide with an IC50 value of 141 μmol/L. Sulindac sulfide also induced apoptosis of HCT116 tumor cells as evident by an increase in caspase activity that occurred within 6 hours of treatment, but did not induce caspase activity in colonocytes (Fig. 1C). In addition, sulindac sulfide inhibited the proliferation of HCT116 tumor cells as measured by EdU incorporation following 24 hours of treatment (Fig. 1D). Colonocytes were again less sensitive in which 100 μmol/L sulindac sulfide inhibited proliferation by only 20% compared with 75% inhibition of colon tumor cell proliferation.

A fluorescence polarization assay that can simultaneously measure cGMP and cAMP hydrolysis was used to determine if sulindac sulfide can inhibit PDE activity in whole cell lysates. As shown in Fig. 1E, sulindac sulfide inhibited cGMP PDE activity in colon tumor cell lysates with IC50 values equivalent to those required for inhibition of viability, but did not significantly affect cAMP hydrolysis (Fig. 1F). Sulindac sulfide also did not inhibit cGMP or cAMP hydrolysis in lysates from colonocytes, which paralleled their reduced sensitivity to the effects of sulindac sulfide on viability, apoptosis, and proliferation.
Sulindac sulfide activates cGMP/PKG signaling in colon tumor cells

Intracellular cGMP levels were next measured to determine if sulindac sulfide can inhibit cGMP PDE activity in intact cells. As shown in Fig. 2A, sulindac sulfide treatment of HCT116 colon tumor cells for 45 minutes increased intracellular cGMP levels within the same concentration range that inhibited cGMP PDE activity in cell lysates. In accord with its lack of cGMP PDE inhibitory activity in lysates from colonocytes, sulindac sulfide did not affect intracellular cGMP levels in colonocytes (Fig. 2B).

PKG is an important downstream mediator of cGMP signaling, which we predicted would be activated in colon tumor cells treated with sulindac sulfide. PKG activity was measured by the phosphorylation level of vasodilator-stimulated phosphoprotein (VASP) that is preferentially phosphorylated at the serine 239 residue by PKG (30). As shown in Fig. 2C, sulindac sulfide caused a time- and dose-dependent increase in phospho-VASP levels in HCT116 colon tumor cells without affecting total VASP levels.

A role of the cGMP-specific PDE5 isozyme

PDE5 has been reported to be an important target for the anticancer properties of sulindac sulfide based on several lines of evidence (21, 25, 26). The requirement of PDE5 for colon tumor cell proliferation and/or survival is evident by the ability of highly specific PDE5 inhibitors, tadalafil and sildenafil, to selectively inhibit colon tumor cell viability without affecting the viability of colonocytes (Fig. 3A and B). We next determined if the difference in sensitivity between colon tumor cells and colonocytes to sulindac sulfide was associated with differences in PDE5 expression levels. As shown in Fig. 3C by Western blotting, PDE5 levels were appreciably higher in 4 colon tumor cell lines compared with colonocytes. These results are consistent with previously published immunohistochemistry studies using patient specimens in which PDE5 was found to be expressed in colon adenomas and adenocarcinomas at higher levels compared with normal colonic mucosa (21).

PDE5 siRNA selectively inhibits colon tumor cell growth

To further study the requirement of PDE5 for tumor cell proliferation and survival, siRNA knockdown studies were performed in colonocytes and 2 colon tumor cell lines, HCT116 and HT29. Two sequences of PDE5 siRNA, designated as siPDE5-1 and siPDE5-2, were used to avoid potential off-target effects of siRNA. Western blotting showed that transient transfection with both sequences of PDE5 siRNA suppressed PDE5 expression by approximately 90% in all 3 cell lines compared with control transfection using a scrambled sequence (Fig. 4A). Both siRNA sequences of PDE5 resulted in 40% reduction in total cGMP hydrolysis in tumor cells, whereas there was no effect of cGMP hydrolysis in colonocyte lysates (Fig. 4B). The tumor selectivity of the siRNA knockdown is consistent with differences in PDE5 expression levels between colon tumor cells and colonocytes as described earlier and may be attributed to the presence of other
cGMP PDE isozymes in colonocytes that can compensate for PDE5 knockdown. As an additional control for assessing the PDE isozyme specificity of the siRNA knockdown, PDE5 siRNA was confirmed to have no effect on cAMP hydrolysis in all 3 cell lines (Fig. 4C), which is to be expected based on the known substrate specificity of PDE5 for cGMP.

Significant suppression of cell viability was observed in HCT116 and HT29 colon tumor cells transfected with PDE5 siRNA compared with the control scrambled siRNA. In contrast, PDE5 siRNA knockdown did not affect the viability of colonocytes (Fig. 4D). PDE5 siRNA knockdown also increased caspase activity in HCT116 and HT29 tumor cells, but did not affect caspase activity in colonocytes (Fig. 4E). We also observed a significant inhibition of cell proliferation in tumor cells following PDE5 knockdown, whereas there was no effect in colonocytes (Fig. 4F). Thus, PDE5 knockdown by siRNA closely mirrored the tumor cell selective effects of sulindac sulfide on viability, apoptosis, and proliferation.

**Activation of cGMP pathway inhibits β-catenin signaling**

Numerous investigators have reported that sulindac and other NSAIDs can inhibit Wnt/β-catenin signaling and have concluded that this effect is important for their cancer chemopreventive activity (25, 31–34). We therefore conducted a series of experiments to determine if PDE5 inhibition and activation of the cGMP/PKG pathway can mediate the inhibitory effect of sulindac sulfide on Wnt/β-catenin signaling. Within the same time period as required for PKG activation (1–2 hours), sulindac sulfide reduced β-catenin protein levels, as well as the expression of cyclin D1 and survivin that are known to be regulated by Wnt/β-catenin-dependent TCF transcriptional activity (Fig. 5A). In addition, 8-bromo-cGMP, a PKG activator, reduced levels of β-catenin, along with cyclin D1 and survivin in colon tumor cells (Fig. 5B). Similar to the effects caused by sulindac sulfide and 8-bromo-cGMP, PDE5 siRNA also reduced β-catenin, cyclin D1, and survivin levels in HCT116 cells (Fig. 5C). Consistent with previous reports that sulindac sulfide and other PDE5 inhibitors (e.g., MY5445) can inhibit TCF transcriptional activity (21), PDE5 siRNA was found to suppress TCF transcriptional activity as measured by the TOPFLASH luciferase reporter construct following transient transfection of HCT116 and HT29 colon tumor cells (Fig. 5D). These results provide evidence for a novel role of the cGMP/PKG pathway in regulating oncogenic β-catenin signaling.

**PDE5 suppression inhibits β-catenin synthesis**

Previous studies have reported that PKG can directly suppress the transcription of β-catenin (35), although other studies have suggested a mechanism involving the phosphorylation of β-catenin to induce ubiquitin-mediated proteosome degradation (20, 36). To further study the mechanism by which sulindac sulfide attenuates Wnt/β-catenin signaling, we measured the effect of sulindac sulfide treatment on steady-state levels of β-catenin mRNA in HCT116 colon tumor cells by RT-PCR. As shown in Fig. 6A, sulindac sulfide treatment reduced β-catenin mRNA levels in a dose-dependent manner at concentrations that can inhibit the growth of colon tumor cells. A reporter construct that encodes for the promoter regions of β-catenin gene (CTNNB1) was used to confirm that reduced levels of β-catenin mRNA were due to transcriptional repression. Figure 6B shows that...
Sulindac sulfide treatment of HCT116 tumor cells caused a dose-dependent suppression of CTNNB1 activity. PDE5 siRNA also significantly inhibited CTNNB1 activity in HCT116 and HT29 colon tumor cells (Fig. 6C). We also measured levels of phosphorylated β-catenin by Western blotting using a phospho-specific antibody with specificity for Ser33/37/Thr41 residues that are known to target β-catenin for degradation, but did not observe significant changes.

Figure 4. Suppression of PDE5 with siRNA is sufficient to selectively inhibit colon tumor cell growth. A, siRNA knockdown of PDE5 protein expression in HCT116 and HT29 colon tumor cells and NCM460 colonocytes (Western blotting). B, selective suppression of cGMP hydrolysis by PDE5 siRNA in HCT116 and HT29 colon tumor cells, but not NCM460 colonocytes. C, no effect of PDE5 siRNA on cAMP hydrolysis in HCT116, HT29, and NCM460 cells. D, selective inhibition of tumor cell viability by PDE5 siRNA. E, tumor cell-specific induction of apoptosis by PDE5 siRNA. F, selective inhibition of tumor cell proliferation by PDE5 siRNA. All treatment effects (A–F) were measured 72 hours posttransfection.
an increase in β-catenin phosphorylation in response to sulindac sulfide treatment (data not shown). Thus, the suppression of PDE5 by either sulindac sulfide or siRNA can inhibit the transcription of β-catenin, which seems to explain their inhibitory effects on TCF-dependent transcriptional activity as well as their effects on viability, proliferation, and apoptosis.

Discussion
Numerous epidemiological studies have reported that NSAIDs can significantly reduce the incidence and mortality from colorectal cancer and other cancers, but toxicities associated with COX-1 and COX-2 inhibition and the suppression of physiologically important prostaglandins limit their long-term use for cancer chemoprevention. However, a few investigators have concluded that the basis for the antineoplastic activity of NSAIDs does not require COX inhibition, which suggest that it may be feasible to develop safer and more efficacious derivatives for colorectal cancer chemoprevention by targeting the underlying mechanism. Studies described here establish strong evidence that the cGMP-specific PDE5 isozyme is a target for sulindac and provide insight as to how the cGMP/PKG pathway can regulate tumor cell proliferation and survival.

Our findings show that sulindac sulfide can selectively inhibit cGMP hydrolysis in lysates from colon tumor cells without affecting cAMP hydrolysis. The biological relevance of this observation was confirmed by experiments showing the ability of sulindac sulfide treatment to induce cGMP elevation and PKG activation in intact colon tumor cells. These experiments also show that the concentration range of sulindac sulfide required to inhibit tumor cell growth matched the concentration range required to inhibit cGMP PDE activity and induce cGMP/PKG signaling. Specifically, the 50 to 100 μmol/L concentration range of sulindac sulfide that was required to activate PKG in colon tumor cells was the same range as for cGMP PDE inhibition in tumor cell lysates as well as for the elevation of intracellular cGMP levels. It is notable that all 3 measurements involved different assay formats. Moreover, the IC₅₀ values of sulindac sulfide to inhibit purified cGMP PDE isozymes as described previously (27) were also within the same concentration range to inhibit cGMP PDE activity in whole cell lysates. It is also remarkable that the time required to activate PKG (1–4 hours) followed a relatively short period of time (45 minutes) when a maximal increase in intracellular cGMP levels was observed, which proceeded a longer
duration of time (6 hours) required to induce apoptosis as detected biochemically by an increase in caspase activity.

A unique characteristic of sulindac and likely other NSAIDs that may be highly pertinent to their safety and efficacy for cancer chemoprevention is their ability to selectively inhibit proliferation and induce apoptosis of cancer cells without toxicity to normal proliferating tissues as is the case for conventional chemotherapy. To model the tumor selectivity in vitro, we compared the effects of sulindac sulfide on colon tumor cells with cells derived from normal colonic mucosa. We showed that colonocytes were appreciably less sensitive to sulindac sulfide, especially by using specific assays measuring proliferation and apoptosis. The reduced sensitivity of colonocytes to sulindac sulfide was paralleled by the inability of sulindac sulfide to inhibit cGMP PDE activity in lysates from colonocytes or to induce cGMP signaling in colonocytes.

The ability of sulindac sulfide to selectively inhibit colon tumor cell proliferation and induce apoptosis was associated with increased PDE5 expression levels compared with colonocytes. The role of the PDE5 isozyme is supported by the ability of highly specific PDE5 inhibitors used for the treatment of erectile dysfunction to selectively suppress colon tumor cell growth as well as by a siRNA knockdown approach in which suppression of PDE5 mimicked the tumor cell growth inhibitory activity and selectivity of sulindac sulfide. The high levels of PDE5 as measured in cultured colon tumor cells compared with colonocytes is consistent with studies involving human clinical specimens that have reported higher PDE5 levels...
in colorectal, bladder, lung, and breast carcinomas compared with normal epithelium from these tumor types (19, 21, 37, 38). PDE5 expression may therefore be essential for colon tumor cell growth or survival, although further studies are needed to fully define its role in tumor initiation and progression.

We have previously reported that sulindac sulfide can inhibit recombinant PDE5 with an IC50 value of 38 μmol/L, although slightly higher concentrations in the 50 to 100 μmol/L range are required to activate cGMP/PKG signaling in intact cells. While these potency values matched those required for growth inhibition, they are appreciably higher than those required to inhibit COX-1 or COX-2 in which IC50 values in the 10-5 mol/L range or lower have been described previously (39). Nonetheless, dosages of sulindac (150 mg twice a day) that have been reported to be effective for the treatment of adenomas in FAP patients (7) can achieve blood levels in the 10-5 mol/L range that are sufficient to inhibit PDE5 in vivo (40). Higher dosages of sulindac could be more effective but would be associated with a higher risk of COX-dependent toxicities. Alternatively, it may be feasible to design derivatives that lack COX inhibitory activity and hold the potential to be safer and more efficacious for colorectal cancer chemoprevention. The possibility of uncoupling COX and PDE5 inhibitory activity from sulindac was recently shown by an amine derivative of sulindac that was found to be PDE5 selective, but did not inhibit COX-1 or COX-2, yet potently inhibited colon tumor cell growth and induced apoptosis (27).

An important question that remains from these studies is whether targeting PDE5 alone is sufficient or if there are advantages in targeting additional cGMP PDE isozymes. On one hand, we previously reported that sulindac sulfide can inhibit several cGMP PDE isozymes (e.g. PDE2, 3, 5, and 10), but not others such as PDE1, 6, 9, or 11 (27). On the other hand, we show here that highly selective PDE5 inhibitors (e.g., sildenafil and tadalafil) and PDE5 siRNA can inhibit colon tumor cell growth, which implies that the inhibition of PDE5 is sufficient. However, high concentrations of PDE5 inhibitors in the micromolar range were required to suppress tumor cell growth compared with nanomolar concentrations required to inhibit PDE5 in isolated enzyme assays. Therefore, the inhibition of additional cGMP PDE isozymes by sulindac sulfide appears to be necessary for its growth inhibitory activity.

Given that the canonical Wnt/β-catenin signaling pathway plays an important role in colorectal cancer progression (41–43) and the ability of sulindac to suppress this pathway, we investigated the potential for crosstalk with the cGMP/PKG pathway. We report here for the first time that sulindac sulfide and siRNA knockdown of PDE5 can reduce β-catenin protein expression and transcriptional activity of TCF leading to the suppression of important tumor cell cycle and survival regulatory proteins, such as cyclin D1 and survivin. Moreover, the ability of 8-bromo-cGMP, a highly specific PKG activator, to attenuate β-catenin signaling confirmed a role of PKG. These observations are consistent with reports from other investigators showing that sulindac metabolites or activators of the cGMP/PKG pathway can suppress the oncogenic activity of β-catenin (20, 21, 35, 36, 44, 45).

The mechanism by which sulindac sulfide suppresses the oncogenic activity of β-catenin is likely at the transcriptional level given that β-catenin mRNA levels were reduced in colon tumor cells following treatment with sulindac sulfide at concentrations that reduced β-catenin protein expression. PDE5 knockdown by siRNA also resulted in a significant suppression of β-catenin transcription, which suggest that the inhibitory effect of sulindac sulfide on PDE5 and activation of the cGMP/PKG signaling involves the repression of β-catenin transcription. However, the underlying mechanism by which PKG inhibits β-catenin transcription requires further study. A previous report has shown binding sites of numerous transcription factors within CTNNB1 promoter, including AP-1, TCF/LEF, E2F1, NF-kB, MEF1, etc. (29). Moreover, another report showed that PKGII inhibits the transcriptional activity of AP-1, and expression level of components of AP-1, c-Jun, and c-Fos, thereby suppressing the proliferation of gastric cancer cells (46). It is possible that PKG may inhibit transcription of β-catenin through negative regulation of these transcription factors (e.g., AP-1), although further studies are necessary to test this possibility. Other investigators have reported that PKG activation can also downregulate β-catenin expression by promoting its degradation (20, 36). Although sulindac sulfide did not increase the level of phosphorylated β-catenin in our experiments, it is possible that sulindac sulfide stimulated the phosphorylation of β-catenin, but may not have been detectable due to its rapid degradation.

As depicted in Fig. 6D, we conclude that sulindac sulfide can selectively inhibit colon tumor cell proliferation and induce apoptosis by inhibiting PDE5 to increase intracellular cGMP levels and activate PKG signaling. The relevance of this pathway to tumorigenesis was showed by its ability to inhibit Wnt/β-catenin-dependent TCF transcriptional activity, which leads to the suppression of critical proteins that regulate colon tumor cell proliferation and apoptosis. These data suggest that PDE5 is an important target of sulindac sulfide that contributes to its antineoplastic activity, and which can be targeted to develop safer and more efficacious drugs for colorectal cancer chemoprevention.

Disclosure of Potential Conflicts of Interest
M.P. Moyer has ownership interest (including patents) in INCELL Corporation. No potential conflicts of interest were disclosed by the other authors.

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References


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