Sulindac sulfide selectively inhibits growth and induces apoptosis of human breast tumor cells by phosphodiesterase 5 inhibition, elevation of cyclic GMP, and activation of protein kinase G

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Abstract
Sulindac displays promising antineoplastic activity, but toxicities from cyclooxygenase inhibition limit its use for chemoprevention. Previous reports suggest that its anticancer properties may be attributed to a cyclooxygenase-independent mechanism, although alternative targets have not been well defined. Here, we show that sulindac sulfide (SS) induces apoptosis and inhibits the growth of human breast tumor cells with IC50 values of 60 to 85 μmol/L. Within the same concentration range, SS inhibited cyclic GMP (cGMP) hydrolysis in tumor cell lysates but did not affect cyclic AMP hydrolysis. SS did not induce apoptosis of normal human mammary epithelial cells (HMEC) nor did it inhibit phosphodiesterase (PDE) activity in HMEC lysates. SS increased intracellular cGMP levels and activated protein kinase G in breast tumor cells but not HMEC. The guanylyl cyclase (GC) activator, NOR-3, and cGMP PDE inhibitors, trequinsin and MY5445, displayed similar growth-inhibitory activity as SS, but the adenylyl cyclase activator, forskolin, and other PDE inhibitors had no effect. Moreover, GC activation increased the sensitivity of tumor cells to SS, whereas GC inhibition reduced sensitivity. By comparing PDE isozyme profiles in breast tumor cells with HMEC and determining the sensitivity of recombinant PDE isozymes to SS, PDE5 was found to be overexpressed in breast tumor cells and selectively inhibited by SS. The mechanism of SS binding to the catalytic domain of PDE5 was revealed by molecular modeling. These data suggest that PDE5 inhibition is responsible for the breast tumor cell growth-inhibitory and apoptosis-inducing activity of SS and may contribute to the chemopreventive properties of sulindac. [Mol Cancer Ther 2009;8(12):3331–40]

Introduction
Breast cancer remains the most commonly diagnosed cancer and the second leading cause of cancer-related deaths for women in the United States (1). Despite ongoing efforts to develop novel therapeutics, mortality rates for breast cancer have only recently begun to decline but only slightly and likely as a result of early detection of disease. Chemoprevention is widely believed to be an effective strategy for reducing cancer-related mortalities. However, with the exception of estrogen receptor antagonists and aromatase inhibitors, which have limited efficacy and potentially severe toxicities, no other drugs have received Food and Drug Administration approval for breast cancer chemoprevention.

Epidemiologic studies have shown that nonsteroidal anti-inflammatory drugs (NSAID) display promising breast cancer chemopreventive efficacy. For example, the Women's Health Initiative, a large observational study that followed >80,000 postmenopausal women for >8 years, found that long-term regular use of any NSAID reduced breast cancer risk by 28%. This study also showed that nonaspirin NSAIDs display stronger chemopreventive efficacy because aspirin use was associated with a 21% reduction of breast cancer risk, whereas ibuprofen use was associated with a 49% reduction of breast cancer risk (2). In addition to preventing primary occurrence of breast tumors, NSAIDs have also been shown to reduce the recurrence of breast cancer (3).

NSAIDs are a chemically diverse family of drugs used to treat a variety of inflammatory conditions and chronic pain associated with arthritis. The pharmacologic basis for their activity involves inhibition of the cyclooxygenase (COX) enzymes. Inhibition of COX blocks the conversion of arachidonic acid to prostaglandins, prostacyclins, and thromboxanes that play an important role in inflammation and other physiologic processes, including renal function, clot formation, and gastrointestinal protection (4). Unfortunately, the depletion of physiologically important prostaglandins by NSAIDs and COX-2 inhibitors can result in sometimes fatal gastrointestinal, renal, and cardiovascular toxicities that preclude their use for chemoprevention (4–6).
Several different lines of evidence suggest that a COX-independent mechanism may be fully or partially responsible for the antineoplastic activities of NSAIDs and COX-2–selective inhibitors (7–12). Most notably, much higher doses are required to inhibit tumor growth in vivo and in vitro compared with dosages required to inhibit COX-1 or COX-2 (13, 14), which suggest that a low-affinity, off-target effect may be responsible for their chemopreventive activity. In support of this possibility, the non–COX-inhibitory sulfone metabolite of the NSAID sulindac has been shown to inhibit tumor cell growth and induce apoptosis in vitro (8, 9) and prevent chemically induced tumor formation in several animal models, including mammary tumorigenesis in the rat (15–21). Other studies have shown that sulindac sulfone can inhibit cyclic GMP phosphodiesterase (cGMP PDE; refs. 18, 22, 23). This effect may be shared that sulindac sulfone can inhibit cyclic GMP phosphodiesterase (cGMP PDE; refs. 18, 22, 23). This effect may be shared with the specific isozyme(s) involved has not been identified.

Cyclic nucleotide PDEs are an important enzyme superfamily responsible for regulating second messenger signaling by hydrolyzing the 3′,5′-phosphodiester bond in the cyclic nucleotides 3′,5′-cGMP and/or 3′,5′-cyclic AMP (cAMP). There are 11 PDE families with different substrate specificity, regulatory properties, tissue localization, and inhibitor sensitivity. Due to the expression of multiple genes, alternative mRNA splicing, and posttranslational protein modifications, it is estimated that humans can express >100 distinct PDE isoforms or splice variants (24). Depending on the PDE isozyme content of the cell and the chemical selectivity of the inhibitor, PDE inhibition can increase the magnitude and/or the duration of the cAMP and/or cGMP signal(s). Increasing cyclic nucleotide levels activates specific signaling pathways, which, in the case of cAMP, can lead to activation of cAMP-dependent protein kinase G (PKG), cyclic nucleotide–gated ion channels, or certain cGMP binding PDEs, resulting in protein phosphorylation, ion fluxes, or cyclic nucleotide hydrolysis to affect gene expression or other aspects of cellular activity (25).

Both cAMP and cGMP have been shown to have antiproliferative and proapoptotic effects (26, 27). In addition, altered expression of one or more PDE isoforms has been reported in various carcinomas and hematologic malignancies (18, 23, 28–33). However, little is known about whether cyclic nucleotides regulate proliferation and/or survival of breast tumor cells or which PDE isoforms are expressed. Here, we show that cGMP elevation can inhibit growth of breast tumor cells, that this pathway is activated by the COX inhibitor sulindac sulfide (SS), and that SS preferentially inhibits PDE5, which is overexpressed in breast tumor cells.

Materials and Methods

**Drugs and Reagents**

SS, trequinsin, and forskolin were purchased from Sigma-Aldrich. NOR-3 was purchased from BioMol. LY83583 was purchased from Cayman Chemical. Sildenafil was a generous gift from Pfizer. Recombinant PDE isoforms were purchased from BPS Biosciences. The family-specific anti-PDE antibodies were purchased from GeneTex, antivasoactivator-stimulated phosphoprotein (VASP) antibody was from BD Transduction Laboratories, and anti–phospho-VASP-Ser239 was from Cell Signaling Technology. Anti-rabbit and anti-mouse horseradish peroxidase–conjugated secondary antibodies were also obtained from Cell Signaling Technology. All compounds were solubilized in DMSO and diluted to a final concentration of 1% in enzyme-based and 0.1% in cell-based experiments, which did not interfere with the assays. Unless otherwise specified, all other reagents were purchased from Sigma-Aldrich.

**Cells and Cell Culture**

The human breast cancer cell lines MDA-MB-231 and SKBR-3 were obtained from the American Type Culture Collection and grown under standard cell culture conditions in RPMI 1640 containing 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO2. Assays were done using the same growth conditions, except serum content was decreased to 1.5% fetal bovine serum. Human mammary epithelial cells (HMEC) were obtained from Lonza and grown according to the specifications of the supplier in MEM complete growth medium. Cell counts and viability were determined by trypan blue exclusion followed by hemacytometry. 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. Cells were incubated for 18 to 24 h, treated with the specified compound or vehicle control, and incubated for an additional 72 h. The inhibition of cell growth caused by treatment was determined 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 with a maximum DMSO concentration of 0.2%. Luminescence was measured using a VictorV (Perkin-Elmer) plate reader.

**Caspase Assays**

Tissue culture microtiter 96-well plates were seeded at a density of 10,000 cells per well. Cells were incubated for 18 to 24 h, treated with the specified compound or vehicle control, and incubated for an additional 6 h. The induction of apoptosis caused by treatment was determined using the luminescent Caspase-Glo 3/7 Assay (Promega), which measures cleavage of a substrate for caspase-3 and caspase-7. The assay was done according to the manufacturer’s specifications with a maximum DMSO concentration of 0.2%. Luminescence was measured using a VictorV plate reader.

**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 at pH 7.4), and clarified by centrifugation at 10,000 × g for 10 min at 4°C. Protein content was determined using the bichinchoninic acid protein assay (Pierce) following the manufacturer’s specifications.
PDE Assays
PDE activity in cell lysates was measured using the IMAP fluorescence polarization PDE assay (Molecular Devices) in which binding of hydrolyzed fluorescent cyclic nucleotide substrate to the IMAP reagent increases fluorescence polarization. The assay was modified to use fluorescein-cAMP and tetramethylrhodamine-cGMP as substrates, allowing for simultaneous measurement of cAMP and cGMP hydrolysis. Each well of a 96-well nonbinding plate contained 0.25 mg/mL of whole-cell lysate or recombinant enzyme preparations. Enzymes were incubated with SS for 30 min at 30°C before the addition of a substrate mixture containing 25 nmol/L of each fluorescein-cAMP and tetramethylrhodamine-cGMP. After 90 min of incubation at 30°C, the reaction was terminated by the addition of binding reagent. The maximum DMSO concentration for each experiment was 2%. Fluorescence polarization was measured using a Synergy4 (BioTek) plate reader.

cGMP Assay
Cells were seeded at a density of 1 × 10⁶ per 10-cm tissue culture dish, incubated for 48 h, and treated with the specified compound or vehicle control. After 30 min of treatment, cells were lysed and assayed for cGMP content using the cGMP Direct Biotrak EIA kit (GE Biosciences). The assay was done according to the manufacturer’s specifications. Absorbance was measured at 630 nm using a Synergy4 plate reader.

Western Blots
Whole-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. Protein bands were visualized on HyBlot CL (Denville Scientific) autoradiography film using SuperSignal West Pico Enhanced Chemiluminescence Reagent (Pierce).

Molecular Modeling
Molecular modeling was done using the Schrödinger Suite 2008 (Schrödinger, LLC). The PDE5 protein structure was obtained from the protein databank (PDB ID: 1UDT PDE5). The induced fit docking protocol, which takes into consideration the ligand-induced receptor conformational change, was used for all docking studies. Residues within 5 Å from the ligand were allowed to be flexible. The docking results were scored using the Extra Precision mode of Glide version 4.5 (Schrödinger, LLC). The induced fit docking protocol and parameters were first validated by docking sildenafil in the PDE5 catalytic site. The docking result excellently reproduced the sildenafil-PDE5 crystal complex conformation. The same protocol and parameters were then used to study the docking of SS to PDE5.

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. For the PDE assays, the IC₅₀ value was determined by testing a range of 10 concentrations with a minimum of two replicates per dose. Dose-response curves were constructed using Prism5 software (GraphPad), which calculates IC₅₀ values using a four-parameter logistic equation. All experiments were repeated a minimum of two times 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 SE. Calculation of P values was done by comparing the specified treatment group with vehicle-treated controls using a Student’s t test.

Results
SS Inhibition of Breast Tumor Cell Growth and Apoptosis Induction Is Associated with cGMP PDE Inhibition, Intracellular cGMP Elevation, and PKG Activation
SS inhibited the growth of the human SK-BR-3, ZR75-1, and MDA-MB-231 breast tumor cell lines with IC₅₀ values of 59, 76, and 84 μmol/L, respectively (Fig. 1A). By comparison, primary cultures of normal HMECs were appreciably less sensitive with an IC₅₀ value of 163 μmol/L. Tumor cell growth inhibition by SS was associated with the induction of apoptosis as measured by the activation of effector caspase-3 and caspase-7, which are specific biochemical markers of apoptotic cell death. As shown in Fig. 1B, treatment with SS (100 μmol/L, 5 hours) resulted in a 2- to 8-fold increase in the activity of caspase-3 and caspase-7 when compared with vehicle treatment in MDA-MB-231, SK-BR-3, and ZR75-1 cells. HMECs were resistant to SS-induced activation of caspases but were capable of undergoing apoptosis as evidenced by increased caspase activity following treatment with the nonselective apoptosis-inducing agent staurosporine. These results suggest that the reduced sensitivity of HMEC to growth inhibition by SS is attributed to the inability of SS to induce apoptosis. SS also increased terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling of breast tumor cells, although it did not have a significant effect on cell cycle distribution under the conditions used for the growth assays (data not shown).

A fluorescence polarization assay that we developed to simultaneously measure cGMP and cAMP hydrolysis was used to determine if SS can inhibit cyclic nucleotide PDE activity. As shown in Fig. 1C, SS inhibited cGMP hydrolysis in lysates from SK-BR-3 and MDA-MB-231 cells with IC₅₀ values within the same range as concentrations that inhibited tumor cell growth and induced apoptosis but did not significantly affect cAMP hydrolysis in breast tumor cell lysates (Fig. 1D). Additionally, SS did not affect cGMP or cAMP hydrolysis in lysates from HMEC. These observations suggest that SS can preferentially inhibit cGMP-degrading PDE isoforms and that there may be differences in the expression of such isoforms in normal and neoplastic cells.
To determine if the inhibition of cGMP degradation by SS can increase intracellular cGMP concentrations, cell-associated cGMP levels were measured following 30 minutes of treatment with SS. As shown in Fig. 2A, SS increased cGMP levels by 2- to 3-fold in MDA-MB-231 cells over a broad concentration range with levels peaking at 100 μmol/L, which is comparable with the concentration range necessary for growth inhibition and caspase activation in this cell line. Higher concentrations of SS were less effective and may be attributed to necrotic cell death as reported previously (8), which resulted in the leakage of cGMP from the cells before the assay. Although basal levels of cGMP in MDA-MB-231 tumor cells and HMEC were comparable, SS failed to induce cGMP levels in HMEC.

Treatment effects of SS on the phosphorylation of VASP, a known substrate for phosphorylation by the cGMP-activated PKG (34), were measured to determine if the elevation of intracellular cGMP levels by SS is sufficient to activate cGMP signaling in breast tumor cells. As described previously, VASP is preferentially phosphorylated at the Ser239 residue by PKG, thereby allowing the measurement of phosphorylation at this residue to serve as an indicator of PKG activity and cGMP signaling within cells (34). Treatment of MDA-MB-231 cells with SS increased the levels of phosphorylated VASP in a dose-dependent (Fig. 2B) and time-dependent (Fig. 2C) manner without affecting the expression of total VASP. After 30 minutes of treatment, increases in VASP phosphorylation were apparent with 50 μmol/L SS. With 100 μmol/L SS treatment, the level of VASP phosphorylation peaked after 1 hour of treatment. Importantly, the concentrations and time required for SS to increase VASP phosphorylation paralleled those required for cGMP elevation.

To determine if cGMP elevation is necessary for the tumor cell growth-inhibitory activity of SS, the effects of modulating basal cGMP levels were studied using guanylyl cyclase (GC) activators and inhibitors. By pretreating with the GC inhibitor LY83583, significantly higher concentrations of SS were required to suppress the growth of MDA-MB-231 breast tumor cells as evidenced by almost a 2-fold increase in the IC50 value (Fig. 2C, left). As a control, LY83583 was tested for its ability to affect the sensitivity of the MDA-MB-231 cells to growth inhibition by the nonselective cytotoxic agent, doxorubicin, and was found to be ineffective (data not shown). In another series of experiments, cells were pretreated with the nitric oxide donor and GC activator, NOR-3. NOR-3 significantly increased the sensitivity of breast tumor cells to the growth-inhibitory activity of SS as evidenced by a significant reduction in the IC50 value (Fig. 2C, right). These experiments provide evidence that cGMP elevation is necessary for SS to inhibit the growth of human breast tumor cells.

Activation of cGMP Signaling Is a Growth-Inhibitory Signal in Human Breast Tumor Cells

To determine if cGMP or cAMP elevation is sufficient to inhibit the growth of human breast tumor cells, several known activators of cGMP or cAMP signaling were evaluated for effects on the growth of breast tumor cells. As shown in Fig. 3A, NOR-3 inhibited the growth of SK-BR-3 and MDA-MB-231 breast tumor cell lines. By comparison, the adenylyl cyclase activator forskolin had no significant growth-inhibitory activity (Fig. 3B). In addition, several known PDE inhibitors were also tested for tumor cell growth–inhibitory activity, including 8-methoxymethyl-IBMX (PDE1), EHNA (PDE2), milrinone (PDE3), rolipram (PDE4), sildenafil (PDE5), zaprinast (PDE5, PDE6, PDE9, PDE10), and trichostatin A (PDE11). The results of these experiments are shown in Fig. 3D. As shown in Fig. 3D, the PDE4 inhibitor rolipram significantly inhibited the growth of MDA-MB-231 and SK-BR-3 cells. However, the PDE5 inhibitors sildenafil and zaprinast had no significant growth-inhibitory activity. These results indicate that cGMP elevation is necessary for SS to inhibit the growth of human breast tumor cells.
and PDE11), dipyridamole (PDE7, PDE8, PDE10, and PDE11), and the nonselective PDE inhibitor IBMX, but none were found to be active (data not shown), with the exception of two inhibitors, trequinsin and MY5445, as shown in Fig. 3C and D, respectively. Although the PDE isozyme specificity of trequinsin and MY5445 has not been well characterized, both inhibitors were found to selectively inhibit cGMP hydrolysis as described below (Table 1).

Normal and Neoplastic Breast Cells Display Differential Expression of cGMP PDE Isozymes

The ability of SS to selectively inhibit growth and induce apoptosis of human breast tumor cells may be attributed to differential expression of SS-sensitive cGMP-degrading PDE isozymes in breast tumor cells compared with HMEC. To study this possibility, we compared the expression of the cGMP-hydrolyzing enzymes PDE1A, PDE1B, PDE1C, PDE2A, PDE5A, and PDE9A in breast tumor cells and HMEC by Western blotting using PDE family-specific antibodies. As shown in Fig. 4, HMEC expressed a variety of PDE isozymes capable of cGMP hydrolysis, including two isoforms each of PDE1A, PDE5A, and PDE9A and a single isoform each of PDE1B and PDE1C. By comparison, breast tumor cells expressed fewer isozymes, with PDE1B and PDE5A being the major isozymes detected. PDE1B was expressed at a comparable level in all three cell lines. On the other hand, a high molecular weight isoform of PDE5A,

Figure 2. SS induced activation of cGMP signaling in MDA-MB-231 human breast tumor cells. A, dose-dependent increase in intracellular cGMP levels after 30 min of SS treatment in MDA-MB-231 (left) and HMEC (right). B, time-dependent (top) and dose-dependent (bottom) increases in VASP phosphorylation at Ser239 after SS treatment. C, dose-dependent decrease in growth-inhibitory potency of 48 h of SS treatment after 1 h of pretreatment with the GC inhibitor LY83583 (left) and dose-dependent increase in growth-inhibitory potency of 72 h of SS treatment after 1 h of pretreatment with the GC activator NOR-3 (right).
which corresponds to the size of PDE5A1, was overexpressed in breast tumor cells compared with HMEC. Interestingly, the lower molecular weight isoform of PDE5A, which corresponds to PDE5A3, displayed decreased expression in breast tumor cells compared with HMEC. None of the cell lines expressed detectable levels of PDE2A.

SS Inhibits PDE5

To determine PDE isozyme selectivity of SS, the sensitivity of catalytically active preparations of recombinant PDE isozymes from each of the 11 PDE isozyme families to inhibition by SS, trequinsin, and MY5445 was determined. Initially, the recombinant enzymes were characterized for substrate selectivity. As summarized in Table 1 and consistent with the known substrate specificity of PDE isozyme families (24), PDE1, PDE2, PDE3, PDE10, and PDE11 were capable of hydrolyzing both cAMP and cGMP, whereas PDE4, PDE7, and PDE8 displayed cAMP specificity, and PDE5, PDE6, and PDE9 displayed cGMP specificity. Among all isozymes evaluated, PDE5 was found to be the most sensitive to inhibition by SS, which resulted in an IC50 value of 38 μmol/L and is consistent with the ability of SS to selectively inhibit cGMP hydrolysis in tumor cell lysates. SS also

Table 1. Substrate selectivity and sensitivity of PDE isozymes for inhibition by SS, trequinsin, or MY5445

<table>
<thead>
<tr>
<th>Purified PDE isoform</th>
<th>Substrate selectivity</th>
<th>SS sensitivity</th>
<th>Trequinsin sensitivity</th>
<th>MY5445 sensitivity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>cAMP</td>
<td>cGMP</td>
<td>cAMP</td>
<td>cGMP</td>
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<tr>
<td>1A</td>
<td>+</td>
<td>++</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>1B</td>
<td>+</td>
<td>++</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>1C</td>
<td>+</td>
<td>+</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>2A</td>
<td>+</td>
<td>+</td>
<td>Inactive</td>
<td>38</td>
</tr>
</tbody>
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NOTE: Substrate selectivity is indicated by +/−, whereas sensitivity to inhibitors is indicated by an IC50 value (μmol/L). SS was tested at concentrations up to 200 μmol/L, trequinsin up to 50 μmol/L, and MY5445 up to 100 μmol/L. A dash (−) represents no detectable enzyme activity. A plus sign (+) represents detectable enzyme activity, with the number of plus signs corresponding to relative level of activity. If the level of inhibition was not significant and an IC50 value was undeterminable, “inactive” is indicated. If a drug was not evaluated, “n.d.” is indicated.
inhibited cGMP hydrolysis by PDE2 and PDE3 with IC₅₀ values of 97 and 84 μmol/L, respectively, yet this activity seems to be irrelevant to its growth-inhibitory activity because PDE2 was not expressed in the breast tumor cells and PDE3 displayed strong selectivity for cAMP hydrolysis. PDE1, PDE4, and PDE6 to PDE11 were insensitive to SS at concentrations up to 200 μmol/L. Trequinsin also inhibited PDE2, PDE3, and PDE5 but, unlike SS, was appreciably more selective for inhibiting PDE3. By comparison, MY5445 was highly selective for PDE5. These results show that PDE5 is a common isozyme target of each of the cGMP PDE inhibitors that were capable of inhibiting breast tumor cell growth.

Molecular Modeling Studies

Molecular modeling studies were done to determine the mode of SS binding to PDE5 and to compare it with the highly potent inhibitor sildenafil. Induced fit docking was used to mimic the conformational change of the enzyme in response to drug binding. Figure 5 shows the crystal structure of the sildenafil-PDE5 complex (Fig. 5A) and SS-docked PDE5 structure (Fig. 5B). Both drugs were found to occupy essentially the same central core formed by Val782, Leu765, Tyr612, Phe786, and Phe820. The face-to-face π-π interaction with the phenyl ring of Phe820 was also well maintained. The hydrogen bonding between sildenafil and Gln817 was substituted with a halogen bonding in SS (DistF-H: 2.46 Å). The methylthiophenyl of SS aligned with the sulfonyl-piperazinyl group of sildenafil and extended into the so-called “lid” region composed of Met816, Phe864, Ala823, and Gly819 (35). The carboxylic acid moiety of SS extended into the metal-binding site and directly interacted with the zinc ion (DistZn-O: 2.10 Å), which further stabilized the SS binding to PDE5. These results show the ability of SS to bind the catalytic region of PDE5 in a manner similar to sildenafil.

Discussion

NSAIDs have shown promising antineoplastic activity in experimental models but their use for cancer chemoprevention in humans is not practical because of gastrointestinal, renal, and cardiovascular toxicities that result from COX-1 and/or COX-2 inhibition and the depletion of physiologically important prostaglandins. Previous studies have concluded that a COX-independent mechanism may be responsible for their tumor cell growth-inhibitory and apoptosis-inducing activities, which suggests the feasibility of developing safer and more efficacious drugs for cancer chemoprevention by targeting such mechanisms. The non-COX-inhibitory sulfide metabolite of sulindac has been previously shown to inhibit cGMP PDE, although the specific isozyme(s) involved has not been identified nor has this putative mechanism been studied with regard to the COX-inhibitory sulfide metabolite of sulindac. Here, we show for the first time that the COX-inhibitory sulfide metabolite of sulindac can selectively inhibit cGMP PDE activity in breast tumor cells, leading to the elevation of intracellular cGMP levels and activation of PKG. SS did not inhibit cGMP PDE activity in lysates from HMEC nor did it increase cGMP levels in HMEC. By comparing the expression of cGMP-degrading isozymes in breast tumor cells with HMEC and determining the sensitivity of PDE isozyme family members to SS, we conclude that PDE5 is overexpressed in human breast tumor cells and selectively inhibited by SS. This conclusion is supported by studies from other investigators showing antiproliferative and proapoptotic effects of PDE5 antisense in colon tumor cells (36).

Initial evidence for the involvement of a cGMP-specific isozyme was suggested by experiments showing a high degree of selectivity for SS to inhibit cGMP hydrolysis compared with cAMP hydrolysis in the breast tumor cell lysates. Consistent with these observations, SS did not affect the activity of cAMP-specific PDE enzymes (PDE4, PDE7, and PDE8) but selectively inhibited the cGMP-specific PDE5 isozyme. The biological relevance of SS to inhibit PDE5 was confirmed by experiments involving intact cells, which showed that SS treatment increased intracellular cGMP levels in human breast tumor cells within the same concentration range as required for cGMP PDE inhibition in cell-free assays. Other experiments showed that the magnitude of cGMP elevation by SS was sufficient to activate PKG, as measured by phosphorylation of VASP, a known PKG substrate, which occurred within the same concentration range and time period as required for cGMP elevation in breast tumor cells.

A mechanistic model for the tumor cell growth–inhibitory and apoptosis-inducing activity of SS involving PDE5 inhibition, cGMP elevation, and PKG activation is illustrated in Fig. 5C. Whereas PKG activation seems to play an important role in mediating the apoptosis-inducing activity of SS, the specific substrates that are phosphorylated by PKG are not known by this point. Downstream signaling events responsible for apoptosis induction, as well as the potential involvement of other cGMP-dependent proteins, such as cyclic nucleotide–gated ion channels or cGMP binding PDEs, require further study.

Figure 4. Expression levels of cGMP PDE isozymes in HMEC, MDA-MB-231, and SK-BR-3 human breast cells.
We also showed that disrupting cGMP synthesis by GC inhibition could desensitize breast tumor cells to the growth-inhibitory activity of SS, whereas GC activation enhanced sensitivity to SS. Together, these experiments support a mechanism of growth inhibition that involves cGMP PDE inhibition. In addition, GC activators and the cGMP PDE inhibitors trequinsin and MY5445 inhibited breast tumor cell growth. By contrast, compounds known to activate cAMP signaling, such as the adenylyl cyclase activator, forskolin, and cAMP PDE inhibitors, had no significant effect on growth of breast tumor cells. These data show that the elevation of cGMP is both necessary and sufficient for SS-mediated growth inhibition in human breast cells and that cGMP plays an exclusive role in breast tumor cell survival compared with cAMP. Supporting a role of cGMP in chemoprevention, other investigators have shown that GC-deficient mice display increased susceptibility to colon tumorigenesis (37). In addition, GC agonists such as uroguanylin have been shown to have chemopreventive activity in the APC⁰ mouse model of colorectal tumorigenesis (38), which is a highly sensitive model to sulindac as well.

SS inhibited PDE5 with an IC₅₀ value of 38 μmol/L, which is significantly higher compared with IC₅₀ values of 2 and 6 μmol/L to inhibit COX-1 and COX-2, respectively (39). Nonetheless, the higher concentration range of SS to inhibit PDE5 is consistent with the requirement of higher concentrations to inhibit breast tumor cell growth and induce apoptosis compared with COX-1 or COX-2. However, further studies are necessary to determine if clinically relevant dosages of sulindac cause PDE5 inhibition and cGMP elevation in vivo. In support of this possibility, pharmacokinetic studies in humans have shown that a 400 mg dosage 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 and can cause adenoma regression in patients with familial adenomatous polyposis (40, 41). Moreover, the sulfide metabolite can achieve higher levels in certain tissues compared with plasma (42) and is likely a reflection of the lipophilic properties of the drug.

As further evidence for direct binding of SS to PDE5, molecular modeling studies showed the ability of SS to bind the catalytic domain of PDE5 in a manner similar to the highly potent and selective PDE5 inhibitor sildenafil. These studies suggest a mechanism involving competitive inhibition of substrate binding. Molecular modeling also suggested a mechanism of selectivity to bind PDE5 but not other isozymes. For example, overlapping PDE5 with PDE1 showed that their catalytic pockets were structurally similar to each other except for the location of the “lid” region, which consisted of residues 661 to 680 in PDE5 and
residues 271 to 290 in PDE1. However, using the same induced fit docking protocol, SS resulted in very different binding configuration when docked to PDE1. Comparing with SS-PDE5 docking result, the docked SS-PDE1 complex showed a relatively unfavorable docking score of ~4 kcal/mol higher, which is equivalent to a ~800-fold higher dissociation constant and is consistent with results from enzyme assays. Previous studies have concluded that the closed catalytic pocket of PDE5 is responsible for the selectivity of sildenafil binding to PDE5 (35). The wider pocket of PDE1 may also explain the more favorable binding of SS to PDE5 compared with PDE1. The lack of the closed lid loop, especially the phenyl group of Phe^664, to shield the methylthiophenyl group of SS from solvent may prevent SS from binding PDE1. In fact, such a lid-covered pocket is quite unique for PDE5, whereas open pocket structures have been observed in other PDE isozymes, including PDE2, PDE3, and PDE4 (PDB ID: 1Z1L, 1SO2, and 3D3P). This structural difference near the binding pocket might explain the selectivity of SS to bind PDE5.

Although PDE5 seems to be the major cGMP-degrading PDE isozyme expressed in breast tumor cells that is sensitive to SS, we cannot rule out the possibility that additional cGMP PDE isozymes or splice variants may be involved, especially because highly selective and potent PDE5 inhibitors (e.g., sildenafil) were unable to inhibit breast tumor cell growth. However, sildenafil was also unable to significantly increase intracellular cGMP levels or activate PKG in breast tumor cells despite its ability to inhibit the majority of cGMP PDE activity in tumor cell lysates. This suggests that there may be another explanation as to why conventional PDE5 inhibitors do not have anticancer properties. For example, PDE5 in tumor cells may not be accessible to such drugs possibly due to a restricted subcellular localization. Alternatively, such drugs may be effectively transported out of tumor cells by efflux mechanisms such as the multi-drug resistance proteins, which are often upregulated in tumor cells.

We also show novel observations that the expression pattern of cGMP PDE isozymes is distinctly different between normal and neoplastic breast cells. With the exception of PDE5A1, HMECs display increased expression and greater diversity of all other cGMP-degrading PDE isozymes when compared with breast tumor cells. The increased expression of PDE5A1 observed in breast tumor cells compared with HMEC is consistent with other studies reporting PDE5 overexpression in tumors from lung, bladder, and breast carcinomas (18, 23, 43). However, this is the first report showing that normal cells express a greater diversity of other cGMP PDE isozymes. Based on these observations, we speculate that the expression of cGMP PDE isozymes in HMECs, which are insensitive to SS, may provide a compensatory mechanism that allows normal cells to be refractory to SS.

From the data presented here, we conclude that PDE5 inhibition by SS leads to the intracellular accumulation of cGMP and activation of cGMP signaling to result in the selective apoptosis of breast tumor cells. The identification of more potent and selective compounds that can stimulate cGMP signaling in human breast tumor cells and thereby induce apoptosis could potentially yield a chemopreventive agent with sufficiently low toxicity and more complete efficacy for breast cancer chemoprevention compared with conventional NSAIDs and COX-2 inhibitors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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