Inhibition of PDE5 by sulindac sulfide selectively induces apoptosis and attenuates oncogenic Wnt/β-catenin mediated transcription in human breast tumor cells

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Abbreviations: COX, cyclooxygenase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; FP, fluorescence polarization; GC, guanylyl cyclase; NSAID, nonsteroidal anti-inflammatory drug; PDE, phosphodiesterase; PKG, cGMP dependent protein kinase; SS, sulindac sulfide; VASP, vasoactivator-stimulated phosphoprotein.
Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as sulindac sulfide (SS) display promising antineoplastic properties, but toxicities resulting from cyclooxygenase (COX) inhibition limit their clinical use. While COX inhibition is responsible for the anti-inflammatory activity of SS, recent studies suggest that phosphodiesterase (PDE) 5 inhibition and activation of cGMP signaling are closely associated with its ability to induce apoptosis of tumor cells. However, the underlying mechanisms responsible for apoptosis induction, factors that influence sensitivity of tumor cells to SS, and the importance of PDE5 for breast tumor cell growth have not been established. Here we show that SS can induce apoptosis of breast tumor cells, which predominantly rely on PDE5 for cGMP hydrolysis, but not normal mammary epithelial cells, which rely on PDE isozymes other than PDE5 for cGMP hydrolysis. Inhibition of PDE5 and activation of PKG by SS was associated with increased β-catenin phosphorylation, decreased β-catenin mRNA and protein levels, reduced β-catenin nuclear localization, decreased Tcf/Lef promoter activity, and decreased expression of Wnt/β-catenin regulated proteins. Suppression of PDE5 with siRNA or known PDE5 inhibitors was sufficient to selectively induce apoptosis and attenuate β-catenin mediated transcription in breast tumor cells with minimal effects on normal mammary epithelial cells. These findings provide evidence that SS induces apoptosis of breast tumor cells through a mechanism involving inhibition of PDE5 and attenuation of oncogenic Wnt/β-catenin mediated transcription. We conclude that PDE5 represents a novel molecular target for the discovery of safer and more efficacious drugs for breast cancer chemoprevention.
Introduction

As the most commonly diagnosed cancer and the second leading cause of cancer related mortality in women in the United States, breast cancer remains a major public health concern [1]. Unfortunately, the rate of breast cancer incidence has remained relatively unchanged in the past 30 years, and more than 40% of breast cancer cases worldwide continue to result in death [1, 2]. Breast cancer develops through a process that occurs over a number of years and involves the accumulation of genetic mutations, which promote progression of normal tissue to hyperplastic lesions and ultimately metastatic disease. Because there are multiple opportunities for early intervention, chemoprevention is widely accepted as a promising approach for reducing the incidence, morbidity, and mortality rates associated with breast cancer [3].

Recent epidemiological studies have shown that the nonsteroidal anti-inflammatory drugs (NSAIDs) can markedly reduce the risk of breast cancer recurrence in patients previously diagnosed with ductal carcinoma in situ (DCIS) or invasive disease [4]. The NSAIDs have also been shown to significantly reduce the risk of de novo disease development with no apparent discrimination between ER+ or the more difficult to treat ER- forms of the disease [5]. Commonly used to treat various inflammatory conditions, NSAIDs suppress the formation of pro-inflammatory prostaglandins by inhibiting the cyclooxygenase (COX) enzymes [6]. While COX inhibition is responsible for their anti-inflammatory efficacy, this mechanism is also associated with potentially fatal side effects, including gastrointestinal ulcers and bleeding, renal toxicity, and increased risk of heart attack and stroke [7]. Consequently, these toxicities have precluded the widespread use of NSAIDs and COX-2 selective inhibitors for cancer chemoprevention.
Because inflammation is closely associated with tumorigenesis and COX-2 has been shown to be overexpressed in precancerous and malignant lesions [8, 9], COX-2 inhibition and the suppression of prostaglandin synthesis is widely accepted as being the primary mechanism responsible for the anticancer activity of the NSAIDs. However, numerous studies have concluded that a COX-independent mechanism may either contribute to or be fully responsible for the chemopreventive activity of NSAIDs [10, 11]. For example, the sulfone metabolite of sulindac has been shown to inhibit tumorigenesis in various experimental models, including chemical-induced mammary tumorigenesis, despite its inability to inhibit COX [12-15].

Cyclic guanosine monophosphate phosphodiesterases (cGMP PDE), a group of enzymes responsible for negatively regulating cGMP signaling by catalyzing the hydrolysis of the second messenger, cGMP, have previously been reported to be inhibited by sulindac sulfone as well as certain NSAIDs, which suggests that this family of isozymes may be an important off-target effect that is responsible for or contributes to the antineoplastic properties of this important class of chemopreventive drugs [16-18]. Recently, our laboratory has shown that the COX-inhibitory sulfide metabolite of sulindac can preferentially inhibit the cGMP-specific PDE5 isozyme, resulting in elevation of intracellular cGMP levels and activation of protein kinase G (PKG). The PDE5 inhibitory activity of SS was closely associated with its ability to inhibit tumor cell growth and induce apoptosis [18, 19]. However, neither the mechanism by which activation of PKG promotes apoptosis of tumor cells nor the role of PDE5 expression in breast tumor cell growth and survival has been well defined.

Here we show that siRNA knockdown of PDE5 is sufficient to induce apoptosis of human breast tumor cells and that selective inhibition of PDE5 activity through use of either siRNA or pharmacological inhibitors can suppress β-catenin transcriptional activity. In addition,
we show that PDE5 expression is associated with the sensitivity of breast tumor cells to SS. These studies demonstrate an important role of PDE5 in breast tumor cell survival and suggest that targeting this isozyme could lead to the discovery of new breast cancer chemopreventive drugs with enhanced efficacy and reduced toxicity.
Materials and Methods

Drugs and Reagents: Sulindac sulfide and milrinone were purchased from Sigma-Aldrich (St. Louis, MO); EHNA and MY5445 from Enzo Life Sciences (Farmingdale, NY). Sildenafil was a generous gift from Pfizer. Tadalafil was extracted from pharmacy-obtained tablets with DMSO following pulverization. DMSO was used as vehicle for all compounds unless otherwise noted. SureFECT transfection reagent and PDE5 specific siRNA constructs were obtained from SA Biosciences (Frederick, MD).

Cells and Cell Culture: The human breast tumor cell lines MDA-MB-231, and ZR75-1 were obtained from ATCC and grown under standard cell culture conditions in RPMI 1640 medium containing 5% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO₂. Human mammary epithelial cells (HMEC) were obtained from Lonza (Basil, Switzerland) and grown according to the manufacturer’s specifications.

Growth Assay: 96-well microtiter plates were seeded at a density of 5,000 cells/well. For drug assays, cells were incubated at 37°C for 72 hours following drug treatment. For siRNA assays, cells were transfected with OptiMEM media containing 0.5% SureFECT transfection reagent and 200nM of either negative control or PDE5A siRNA and incubated at 37°C for 24 hours prior to treatment or 96 hours if untreated. The effect of treatment on growth was measured with the Cell Titer Glo Assay (Promega; Madison, WI).

Apoptosis Assay: 96-well microtiter plates were seeded at a density of 10,000 cells/well. For drug assays, cells were incubated at 37°C for 6 hours following treatment. For siRNA assays, cells were transfected with OptiMEM media containing 0.5% SureFECT transfection reagent and 200nM negative control or PDE5A siRNA and incubated at 37°C for 96 hours. Caspase 3 and 7 activity was measured using the Caspase 3/7 Glo Assay (Promega).
**Proliferation Assay:** Cells were seeded at a density of 1x10⁶ cells per 10 cm tissue culture dish and transfected with OptiMEM media containing 0.5% SureFECT transfection reagent and 200 nM of either negative control or PDE5A siRNA then incubated at 37°C for 72 hours prior to the addition of 10 µM EdU. After 24 hours of incubation with EdU, cells were harvested and analyzed using the Click-iT EdU Alexa Fluor 488 Proliferation Assay (Invitrogen) according to manufacturer’s specifications. The percentage of proliferating cells was quantified using a Guava EasyCyte Plus flow cytometer. A minimum of 5,000 events were collected for each treatment group with use of minimal electronic compensation.

**Semi-quantitative RT-PCR:** MDA-MB-231 cells were seeded at a density of 1.5x10⁶ cells in T-75 flasks. After 24 hours, media was replaced with serum free RPMI. After 18 hours of serum starvation, cells were treated with compound or vehicle control in serum free media. After 4 hours of incubation with compound, cells were lysed and RNA was extracted using the AxyPrep Multisource Total RNA Miniprep Kit (Axygen; Union City, CA) according to manufacturer’s protocol. RNA (500 ng) was then reverse transcribed to cDNA using the RT² First Strand Synthesis kit (SA Biosciences) according to manufacturer’s specifications. The expression of Wnt related genes such as those encoding β-catenin and cyclin D1 was then determined using the Wnt Signaling PCR Array (SA Biosciences; Frederick, MD) in 96-well format. With the exception of performing 60 amplification cycles, the assay was performed according to manufacturer’s specifications using an Eppendorf Real Plex thermal cycler.

**Cell Lysis:** Cells were lysed and protein concentrations determined as described previously [18].

**PDE Assay:** PDE activity in cell lysates was determined using the IMAP PDE assay as previously described [18]. For experiments involving siRNA, 6 well tissue culture treated plates
were seeded at a density of 200,000 cells per well, immediately transfected as described above, and incubated at 37°C for 96 hours prior to cell lysis.

**cGMP Assay:** Intracellular cGMP levels were measured as previously described [18].

**Immunoblotting:** PDE primary antibodies were purchased from GeneTex (Irvine, CA). All other primary and secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Cell lysates (15 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% non-fat dry milk (p-VASP<sup>Ser239</sup>) or 5% BSA in TBS containing 0.05% Tween-20. Membranes were incubated with primary antibodies at 4°C overnight then with secondary horseradish peroxidase conjugated antibody for 2 hours at room temperature. Protein bands were visualized using Super Signal West Pico Enhanced Chemiluminescence Reagent (Pierce).

**β-catenin Imaging Assay:** Cells were seeded in 96-well optical bottom microtiter plates at a density of 10,000 cells per well. After 18-24 hours, the growth media was replaced with serum free media. After an additional 18-24 hours, cells were treated with compound or vehicle control for 45 minutes. β-catenin signaling was then stimulated with Wnt3A conditioned media from Wnt3A secreting L cells (ATCC). After 4 hours, cells were fixed with 4% formalin and blocked with 5% BSA. Cells were incubated with β-catenin primary antibody (BD Transduction Labs) for 2 hours followed by 1 hour incubation in secondary Alexa Fluor 488 conjugated antibody (Invitrogen). Nuclei were counterstained with Draq5. β-catenin expression was visualized using an Evotec Opera confocal microscope with a 20X objective lens. Co-localization of Alexa Fluor 488 fluorescence with the nuclear stain was measured using Acapella image analysis software (Perkin Elmer).
**Tcf/Lef Promoter Activity Assay:** ZR75-1 cells were plated in 24-well tissue culture treated plates. After 24 hours of incubation, cells were transiently transfected with 0.1 µg of Super8XTOPFlash construct (kindly provided by Dr. Randall T. Moon, University of Washington) and 0.1 µg β-galactosidase-expressing vector (Promega). After 24 hours of transfection, cells were treated with compound or vehicle control for 24 hours. After treatment, cells were lysed and luciferase and β-galactosidase activities were measured using assay systems from Promega. Luciferase activity was normalized to β-galactosidase activity.

**Experimental Design and Data Analysis:** Drug effects on cell growth and PDE activity were measured and the potency expressed as an IC₅₀ value, which is the concentration resulting in 50% inhibition when compared to the vehicle control. Dose response curves were constructed using Prism5 software (Graphpad), which calculates IC₅₀ values using a four parameter logistic equation. Experiments were performed with a minimum of 3 replicates per data point and each experiment was performed a minimum of three times to verify reproducibility. Graphs were constructed from a single representative experiment and values represent a comparison between drug treatment at the specified concentration and untreated controls. Error bars represent standard error of the mean (SEM). Each data set was subjected to a normal quantile plot test for non-normality. Calculation of p values in normally distributed sample sets was done by comparing the specified treatment group to vehicle treated controls using a student’s t test.
**Results**

*Tumorigenicity of human breast cells and sensitivity to SS correlate with PDE5 expression*

To determine the role of PDE5 inhibition in mediating the anticancer properties of SS, we determined the sensitivity of estrogen receptor positive and negative human breast tumor cell lines, MDA-MB-231 and ZR75-1, respectively, to the effects of SS on cGMP PDE activity in cell lysates, intracellular cGMP levels, and the activity of caspases 3 and 7, an early marker of apoptosis. These effects were then compared with the effects on primary cultures of normal human mammary epithelial cells (HMEC). As shown in Figure 1A, SS inhibited cGMP PDE activity in lysates of the breast tumor cell lines with IC$_{50}$ values of approximately 100 µmol/L, which was comparable to concentration required for growth inhibition as previously reported [18]. By comparison, SS caused only marginal inhibition of cGMP hydrolysis in lysates from HMEC, which displayed reduced sensitivity to the growth inhibitory activity of SS. SS treatment also caused a three to four fold increase in the intracellular levels of cGMP in the breast tumor cell lines (Figure 1B). This effect peaked with 100 µmol/L of SS treatment and occurred at concentrations comparable to those necessary for inhibition of cGMP PDE in the lysates from the respective breast tumor cell lines. SS did not increase intracellular cGMP levels in HMEC.

The effects of SS on apoptosis induction as measured by the activity of caspases 3 and 7 closely mirrored its effects on cGMP PDE inhibition in the cell lysates and intracellular cGMP levels in intact cells. SS treatment resulted in as much as a twelve-fold increase in the activity of caspases 3 and 7 in the breast tumor cell lines (Figure 1C), but did not induce caspase activity in HMEC. On the other hand, HMEC were able to undergo apoptosis when treated with the nonselective apoptosis inducer, staurosporine.
We also evaluated the activity and expression of cGMP-degrading PDE isozymes in the breast tumor cell lines and HMEC. First, we measured the sensitivity of lysates from HMEC, MDA-MB-231, and ZR75-1 to inhibition of cGMP hydrolysis by the PDE2 selective inhibitor, EHNA; the PDE3 selective inhibitor, milrinone; and the PDE5 selective inhibitor, sildenafil. As shown in Figure 1D, only sildenafil significantly inhibited cGMP hydrolysis in the lysates from the breast tumor cell lines, yet sildenafil had no effect on cGMP hydrolysis in lysates from HMEC. The effects of calcium and calmodulin, specific activators of PDE1, were also measured and found to cause a twelve-fold increase in cGMP PDE activity in lysates from HMEC but had no effect on cGMP hydrolysis in lysates from the breast tumor cell lines.

Western blot analysis revealed that MDA-MB-231 and ZR75-1 breast tumor cell lines displayed different PDE isozyme expression patterns when compared to HMEC, as shown in Figure 1E. Namely, HMEC expressed a variety of cGMP PDE isozymes, including two splice variants each of PDE1 and PDE9, which were not detected in the tumor cell lines. By comparison, all three breast tumor cell lines expressed high levels of PDE5, which were not detected in the HMEC. Consistent with the insensitivity of cGMP hydrolysis in breast tumor cell or HMEC lysates to PDE2 and 3 isozyme specific inhibitors, neither cell type expressed appreciable levels of PDE2 or PDE3.

Inhibition of PDE5 is associated with attenuation of β-catenin mediated transcription

Previous studies have shown that SS and sulindac sulfone can suppress nuclear levels of β-catenin and that β-catenin can serve as a substrate for PKG in cell-free assays [17, 19-23]. To determine if these effects are related to PDE5 inhibition and if PKG can phosphorylate β-catenin in intact cells, we evaluated the expression of β-catenin phosphorylated at the serine 33, serine 37, or threonine 41 residues after two hours of SS treatment and compared this with overall β-
catenin expression after 48 hours of treatment by Western blotting. As shown in Figure 2A, treatment of the MDA-MB-231 breast tumor cell line with SS caused a two-fold increase in the expression of phosphorylated β-catenin and a 75% decrease in overall β-catenin expression. Conversely, SS treatment did not affect the expression of phosphorylated or total β-catenin in HMEC.

We also measured treatment effects of SS and MY5445, a PDE5 selective inhibitor that can inhibit breast tumor cell growth and induce apoptosis [18], on β-catenin mRNA levels in the MDA-MB-231 breast tumor cell line by semi-quantitative real time PCR. As shown in Figure 2B, both compounds decreased the expression of β-catenin mRNA levels, which resulted in a complete loss of detection of β-catenin mRNA. Thus, SS can increase the phosphorylation of β-catenin to trigger protein degradation and can also suppress the synthesis of β-catenin.

β-catenin mediates gene transcription in response to Wnt stimulation by translocating from the cell surface or cytosol to the nucleus where it can bind to and activate the Tcf/Lef family of transcription factors [24]. To determine treatment effects of SS on the nuclear pool of β-catenin, we developed an imaging assay to measure nuclear β-catenin levels following stimulation with Wnt3A conditioned medium. As shown in Figure 2C (top), Wnt stimulation increased nuclear levels of β-catenin in the MDA-MB-231 breast tumor cell line. Pretreatment with SS or MY5445 resulted in decreased levels of nuclear β-catenin (Figure 2C, bottom and 2D) in the MDA-MB-231 and ZR75-1 breast tumor cell lines at concentrations that inhibited cGMP hydrolysis and increased intracellular cGMP levels. Additionally, the effect of SS on Tcf/Lef transcriptional activity in the ZR75-1 breast tumor cell line was determined using the TOP-flash luciferase reporter assay. As shown in Figure 2E, SS caused a 40-45% reduction in the activity of these transcription factors, which is consistent with reduced β-catenin nuclear levels.
As further evidence that inhibition of PDE5 and activation of PKG are associated with the attenuation of β-catenin mediated transcription, we evaluated the effects of SS on the expression of survivin, which is an important oncogenic protein known to be regulated by β-catenin [25] and compared with the effects on VASP phosphorylation, an intracellular marker of PKG activity [26]. First, we measured the time-dependent effects of SS treatment in MDA-MB-231 and HMEC (Figure 3A). SS reduced survivin protein levels in the breast tumor cells, which is consistent with inhibition of β-catenin transcriptional activity. This effect on survivin expression was inversely proportional to the effects on VASP phosphorylation, reaching its maximum effect after four hours of treatment. HMEC did not express detectable levels of survivin; therefore, we were unable to measure an effect on this protein with SS treatment. Dose-dependent effects of SS treatment on survivin expression were also determined (Figure 3B). Consistent with the time-course studies, SS reduced expression of survivin in the breast tumor cells but not in the HMEC due to lack of survivin expression. Again, these effects were inversely proportional to the effects on VASP phosphorylation, each peaking with 100 µmol/L of treatment.

To determine whether the effects of SS on survivin expression may be related to PDE5 inhibition, the effects of the PDE5 selective inhibitors, MY5445 and tadalafil were also evaluated in the MDA-MB-231 breast tumor cell line (Figure 3C). MY5445 and tadalafil caused a dose-dependent increase in VASP phosphorylation after three hours of treatment. Furthermore, both compounds caused a dose-dependent decrease in survivin protein levels, which was inversely proportional to the effects on VASP phosphorylation. These effects occurred at concentrations comparable to those necessary for inhibition of MDA-MB-231 growth (Figure 3D).
Suppression of PDE5 with siRNA is sufficient to selectively induce apoptosis of breast tumor cells through a mechanism involving attenuation of β-catenin mediated transcription

To further characterize the importance of PDE5 for breast tumor cell growth and survival, siRNA was used to suppress PDE5 expression in HMEC and ZR75-1 breast cells. As shown in Figure 4A, PDE5 siRNA caused a significant reduction in PDE5 mRNA and protein levels in both the ZR75-1 tumor cell line and HMEC. Consistent with its known substrate specificity for cGMP and expression profiling as described above, PDE5 suppression by siRNA resulted in a 40% reduction in cGMP hydrolysis in lysates from the ZR75-1 cell line but had no effect on cAMP hydrolysis or hydrolysis of cAMP or cGMP in lysates from siRNA treated HMEC (Figure 4B).

Suppression of PDE5 with siRNA reduced ZR75-1 growth, but had no effect on the growth of HMEC (Figure 4C). This effect appeared to be associated with induction of apoptosis rather than inhibition of proliferation because PDE5 siRNA did not significantly affect proliferation of either ZR75-1 or HMEC, as measured by BrdU incorporation. Conversely, PDE5 siRNA increased caspase activity in ZR75-1 tumor cells but had no effect on caspase activity in HMEC.

To evaluate the effects of PDE5 suppression on β-catenin mediated transcription, we measured the effects of PDE5 siRNA on nuclear localization of β-catenin in the ZR75-1 breast tumor cell line. As shown in Figure 4D, PDE5 siRNA treatment resulted in a significant decrease in nuclear localization of β-catenin in response to Wnt stimulation. Consistent with the selective effect of PDE5 siRNA on apoptosis, PDE5 suppression was associated with reduced survivin expression in the ZR75-1 tumor cell line (Figure 4E).
The effect of PDE5 siRNA on the sensitivity of ZR75-1 breast tumor cell line to the growth inhibitory potency of SS was also evaluated. As shown in Figure 4F, the knockdown of PDE5 by siRNA sensitized the cells to the growth inhibitory effects of SS, as evidenced by a significant reduction in the IC_{50} value. As a control compound, the PDE5 inhibitor MY5445 was also evaluated and siRNA was found to cause a similar reduction in the IC_{50} value.
Discussion

We previously reported that PDE5 inhibition and activation of cGMP/PKG signaling are closely associated with the tumor cell growth inhibitory and apoptosis inducing activity of SS as well as certain other NSAIDs [18, 19]. However, the downstream mechanism responsible for mediating the induction of apoptosis following PKG activation has not been defined, nor has the importance of PDE5 for breast tumor cell growth and survival been recognized. Here we demonstrate that the MDA-MB-231 and ZR75-1 breast tumor cell lines rely predominantly on PDE5 for hydrolysis of cGMP, that selective inhibition of this cGMP-specific isozyme with SS, known PDE5 inhibitors, or siRNA is sufficient to induce apoptosis of breast tumor cells, and that the pro-apoptotic effects of PDE5 inhibition are mediated through attenuation of β-catenin mediated transcription.

Normal mammary epithelial cells, as likely the case with other cell types, appear to rely more heavily on multiple PDE isozymes, such as PDE1 or PDE9, for cGMP hydrolysis, while breast tumor cells appear to rely predominantly on PDE5 for cGMP hydrolysis. This was demonstrated by measuring cGMP hydrolysis in cell lysates, which revealed that breast tumor cell lysates were more sensitive than lysates from HMEC to inhibition by the PDE5 selective inhibitor sildenafil, whereas lysates from HMEC were more sensitive to the PDE1 activators, calcium and calmodulin. PDE isozyme profiling by Western blotting also revealed high levels of PDE5 and low levels of other cGMP PDE isozymes in the breast tumor cells. We therefore conclude that PDE5 inhibition by SS can account for its improved potency to induce apoptosis of breast tumor cell lines, given its specificity to inhibit PDE5 without affecting PDE1 or PDE9 [18]. Furthermore, the PDE5 expression pattern observed here is consistent with a previous study that reported high levels of PDE5 expression in invasive breast tumor cells from clinical
biopsies but not in the surrounding normal tissue [27] as well as other studies that have reported high levels of PDE5 expression in carcinomas from colon [19], bladder [28], and lung [29]. Consistent with these observations, cGMP levels have been reported to be decreased in colon tumors compared with normal colon mucosa [30]. Although PDE5 has not been recognized to play a role in tumor cell survival, our results suggest the possibility that its expression in tumor cells and resulting decreased intracellular cGMP levels may stimulate tumor cell proliferation or inhibit apoptosis, although further studies are needed to fully define a role in tumorigenesis.

As evidence that PDE5 is necessary for the growth and survival of breast tumor cells, selective inhibition of PDE5 with SS, known pharmacological inhibitors (e.g. MY5445, tadalafl) and PDE5 siRNA were sufficient to inhibit growth and induce apoptosis of breast tumor cells, which is consistent with the increased reliance of these cells on PDE5 for cGMP hydrolysis. While SS may have additional targets that are relevant for cancer chemoprevention, especially inhibition of COX and suppression of prostaglandin synthesis [18, 31-33], PDE5 inhibition appears to be more closely associated with its tumor cell growth inhibitory activity compared with COX-2 inhibition [18, 19]. In support of this possibility, PDE5 knockdown by siRNA increased the sensitivity of breast tumor cells to SS.

We also investigated the potential involvement of the Wnt/β-catenin pathway in the pro-apoptotic effects of PDE5 inhibition because deregulation of this pathway is common in breast cancer [34] and attenuation of Wnt signaling at the level of β-catenin has been previously implicated in the pro-apoptotic effects of SS and sulindac sulfone [17, 19-23, 35]. Although previous studies have reported to ability of PKG to phosphorylate β-catenin in cell-free models, we provide here the first evidence that cGMP signaling can increase β-catenin phosphorylation in intact cells and that this effect appears to be tumor cell specific given that this effect was not
apparent in normal mammary epithelial cells. Consistent with these observations, we found that activation of PKG by PDE5 inhibitors or PDE5 siRNA was also associated with attenuation of β-catenin mediated transcription as measured by expression of total or phosphorylated β-catenin, nuclear localization of β-catenin, activity of Tcf/Lef transcription factors, and expression of proteins that are regulated by Tcf/Lef mediated transcription. All of these effects occurred at comparable concentrations necessary for inducing apoptosis of the breast tumor cells, demonstrating that attenuation of β-catenin mediated transcription is an important mediator of cGMP-dependent apoptosis induction in breast tumor cells.

Taken together, these findings demonstrate the importance of PDE5 expression and activity for the survival of breast tumor cells as well as sensitivity to SS. While the concentrations of SS described here are significantly higher than plasma concentrations that can be achieved with standard dosages of sulindac [36], these results suggest that PDE5 inhibition may be an important off-target effect responsible for its anticancer properties observed in vitro, which could lead to the discovery of safer and more efficacious drugs for cancer chemoprevention. As depicted in Figure 5A, we hypothesize that selective inhibition of PDE5 with compounds such as SS induces apoptosis through a mechanism involving elevation of intracellular cGMP levels, activation of PKG, and attenuation of β-catenin mediated transcription. However, the expression of other PDE isozymes (e.g. PDE1 and PDE9) in the normal breast cells compensates for the loss of cGMP hydrolysis by PDE5, ultimately desensitizing these cells to the pro-apoptotic effects of SS (Figure 5B). These findings demonstrate that PDE5 is an important protein necessary for breast cancer cell growth and survival that warrants further study to assess the role of this enzyme and signaling pathway in carcinogenesis.
References

Figure Legends

Figure 1. Sensitivity of human breast cells to SS correlates with PDE5 expression. A, inhibition of cGMP PDE activity by SS in lysates of MDA-MB-231 and ZR75-1 breast tumor cells or HMEC. B, effect of SS on intracellular cGMP levels in HMEC, MDA-MB-231, and ZR75-1 cells after 30 minutes of treatment. C, effect of SS on caspase 3 and 7 activity in HMEC, MDA-MB-231, and ZR75-1 cells after 6 hours of treatment. +, 1 µmol/L staurosporine. D, activity of cGMP PDE isozymes in HMEC, MDA-MB-231, and ZR75-1 lysates as measured by overall percent inhibition induced by the PDE2 selective inhibitor EHNA, the PDE3 selective inhibitor milrinone, or the PDE5 selective inhibitor sildenafil, or as measured by fold increase in cGMP hydrolysis after treatment with the activators of PDE1, calcium and calmodulin. E, expression of cGMP PDE isozymes in MDA-MB-231, SKBR3, and ZR75-1 breast tumor cells or HMEC as measured by Western blotting.

Figure 2. Inhibition of PDE5 is associated with attenuation of β-catenin mediated transcription. A, effect of SS on expression of β-catenin phosphorylated at the serine 33, serine 37, or threonine 41 residues after 2 hours of treatment or expression of total β-catenin after 48 hours of treatment in HMEC and MDA-MB-231 cells by Western blotting. RhoA was used as a loading control. B, relative levels of β-catenin mRNA in MDA-MB-231 cells after 4 hours of treatment with 100 µmol/L SS or 50 µmol/L MY5445 as measured by semi-quantitative real time PCR. 0.1% DMSO was used as a vehicle control. C, representative images from β-catenin imaging assay depicting the effects of 4 hours of Wnt3A stimulation with (bottom) or without (top) 1 hour of pretreatment with SS in MDA-MB-231 cells. D, quantification of the effects of SS or MY5445 treatment on Wnt3A induced β-catenin nuclear localization in MDA-MB-231 and ZR75-1 breast cancer cells.
tumor cells. $E$, activity of Tcf/Lef transcription factors after 24 hours of SS treatment in ZR75-1 cells as measured by luciferase reporter assay.

Figure 3. Inhibition of PDE5 is associated with decreased survivin expression. $A$, time-dependent effects of 100 µmol/L SS treatment on the expression of p-VASP$^{\text{Ser239}}$ and survivin in HMEC and MDA-MB-231 cells. Rho A was used as a loading control. $B$, dose-dependent effects of 2 hours of SS treatment on the expression of p-VASP$^{\text{Ser239}}$ and survivin in HMEC, MDA-MB-231, and ZR75-1 cells. $C$, dose-dependent effects of 3 hours of MY5445 or tadalafil treatment on p-VASP$^{\text{Ser239}}$ and survivin expression in MDA-MB-231 cells. RhoA was used as a loading control for MY5445 treatment. GAPDH was used as a loading control for tadalafil treatment. $D$, effects of tadalafil and MY5445 on MDA-MB-231 breast tumor cell growth.

Figure 4. Suppression of PDE5 with siRNA is sufficient to selectively induce apoptosis of breast tumor cells. $A$, effect of PDE5 siRNA on PDE5 mRNA (top) or protein (bottom) levels in HMEC and ZR75-1 cells. Actin was used as PCR and loading control for mRNA studies, whereas actin was used as a loading control for the protein studies. $B$, effect of PDE5 siRNA on cAMP and cGMP hydrolysis in HMEC and ZR75-1 cells. $C$, effect of PDE5 siRNA on growth, proliferation, and caspase activity in HMEC and ZR75-1 cells. $D$, effect of PDE5 siRNA on nuclear localization of β-catenin in response to Wnt3A stimulation in the ZR75-1 breast tumor cells. $E$, expression of survivin in HMEC and ZR75-1 cells after treatment with PDE5 siRNA. RhoA was used as loading control. $F$, effect of PDE5 siRNA on sensitivity of ZR75-1 breast tumor cells to growth inhibition by SS or MY5445. All experiments utilized 200 nmol/L siRNA with 0.5% SureFECT transfection reagent. Cells were transfected for 96 hours prior to being
assayed. For experiments involving drug treatment, cells were treated after 24 hours of transfection.

Figure 5. COX-independent anticancer mechanism of SS. A, because breast tumor cells predominantly rely on PDE5 for hydrolysis of cGMP, selective inhibition of PDE5 by SS results in accumulation of cGMP, activation of PKG, and attenuation of β-catenin mediated transcription, ultimately resulting in apoptosis. B, normal breast cells predominantly rely on SS-insensitive PDE isozymes, such as PDE1 and PDE9, for cGMP hydrolysis, which allows for hydrolysis of cGMP even in the presence of PDE5 selective inhibitors and prevents accumulation of cGMP and desensitizes these cells to the pro-apoptotic effects of PDE5 inhibition.
Figure 1, Tinsley, et al.
Figure 2, Tinsley, et al.
Figure 3, Tinsley, et al.
Figure 4, Tinsley, et al.
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