Inhibition of colon cancer cell growth by phosphodiesterase inhibitors is independent of cGMP signaling

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CRC = Colorectal Cancer

VASP = Vasodilator Stimulated Phosphoprotein

PKG = cGMP-dependent Protein Kinase

GC-C = Guanylyl Cyclase C

ATCC = American Type Culture Collection

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Abstract

There is growing interest in the potential use of phosphodiesterase (PDE) inhibitors for colorectal cancer (CRC) prevention and treatment. The present study has tested the idea that PDE inhibitors inhibit growth and viability of CRC cell lines by increasing cyclic guanosine monophosphate (cGMP) and activating cGMP-dependent protein kinase (PKG). Colon cancer cell lines and those with ectopic PKG2 expression were treated with membrane permeable 8Br-cGMP or inhibitors of PDE5, 9, 10a. Levels of cGMP capable of activating PKG were measured by immunoblotting for phosphorylation of vasodilator-stimulated phosphoprotein (VASP). The effects of treatment on CRC cell proliferation and death were measured using hemocytometry with trypan blue. Treatment with 8Br-cGMP had no effect on CRC cell proliferation or death. Endogenous PKG activity was undetectable in any of the CRC cells, but expression of ectopic PKG2 conferred modest inhibition of proliferation but did not affect cell death. Extremely high concentrations of all the PDE inhibitors reduced proliferation in CRC cell lines, but none of them increased cGMP levels, and the effect was independent of PKG expression. The inability of the PDE inhibitors to increase cGMP was due to the lack of endogenous cGMP generating machinery. In conclusion, PDE inhibitors that target cGMP only reduce CRC growth at clinically unachievable concentrations, and do so independent of cGMP signaling through PKG.

Significance Statement

A large number of *in vitro* studies have reported that PDE inhibitors block growth of colon cancer cells by activating cGMP signaling, and that these drugs might be useful for cancer treatment. Our results show that these drugs do not activate cGMP signaling in colon cancer cells due to a lack of endogenous guanylyl cyclase activity, and that growth inhibition is due to toxic effects of clinically unobtainable drug concentrations.

Introduction

Cancers of the colon and rectum (CRC) are some of the most commonly diagnosed, and are responsible for 9% of all cancer deaths due to the late stage of diagnosis where treatments are largely ineffective. It is therefore widely recognized that both primary prevention, and the development of new therapeutics to treat advanced CRC are a high priority to reduce the health burden. Phosphodiesterases (PDE's) are a large family of enzymes that degrade cyclic-nucleotides, and are established therapeutic targets owing to diverse expression and functions in a variety of biological processes (Lugnier, 2006; Maurice et al., 2014). A large and growing body of literature supports the idea that PDE inhibitors have anti-tumor effects in the gastrointestinal tract.

Some of the earliest studies demonstrated that a weak PDE5 inhibitor called sulindac sulphone (exisulind) could cause regression of colorectal polyps in human patients (Stoner et al., 1999; van Stolk et al., 2000). While exisulind was not approved for use in patients owing to toxicity at levels required for therapeutic effects (Stoner et al., 1999; van Stolk et al., 2000), efforts continued in order to understand the anti-tumor mechanism. Exisulind was reported to inhibit growth and induce apoptosis of colon cancer cell lines grown *in vitro* by increasing cGMP and activating type-1 cGMP-dependent protein kinase (PKG1) (Thompson et al., 2000; Joe et al., 2003). PKG1-dependent suppression of Wnt/ β -catenin signaling, and activation of cJun-N-terminal kinase were shown to mediate the pro-apoptotic effects of PDE5 inhibition (Soh et al., 2000; Thompson et al., 2000; Whitt et al., 2012; Li et al., 2013). While PDE5 is specific for cGMP, more recently, inhibition of the dual-specificity enzyme PDE10A has also been reported

to inhibit colon cancer cell growth by a cGMP-dependent inhibition of β -catenin/T-Cell-Factor signaling (Li et al., 2015a; Li et al., 2015b; Lee et al., 2016).

Observations made by several independent groups are incongruent with the idea that activation of cGMP/PKG signaling by PDE inhibitors inhibit colon cancer cell growth and viability. It has been suggested that the anti-cancer effect of exisulind is independent of cGMP signaling (Pitari et al., 2006). Others have shown that PDE5 inhibitors can synergize with the cytotoxic effects of chemotherapeutic drugs, but do not affect growth of colon cancer cell lines when used as a single agent (Booth et al., 2014; Booth et al., 2016; Booth et al., 2017). The importance of PKG downstream of PDE5 inhibitors has also been questioned, since both isoforms (PKG1 and PKG2) have been shown to be downregulated in colon cancer tissue compared to normal colon epithelium, and are undetectable in colon cancer cell lines (Hou et al., 2006; Wang et al., 2012). In normal colon epithelium, cGMP is produced upon activation of the receptor guanylyl-cyclase (GC-C) by the peptide hormones guanylin and uroguanylin (Forte, 2004). Epithelial PDE5 antagonizes the effects of GC-C agonists by degrading cGMP (Bakre et al., 2000). Treatment of mice with PDE5 inhibitors has been shown to increase epithelial cGMP levels and suppress the proliferation in the colon crypt in a PKG2-dependent manner (Wang et al., 2014). In addition, it was recently reported that treatment with the PDE5 inhibitor sildenafil can inhibit carcinogenesis in two different models of colon cancer in mice (Islam et al., 2017; Sharman et al., 2018). In contrast to *in vitro* studies with colon cancer cell lines, the *in vivo* inhibition of carcinogenesis was shown to result from suppression of polyp initiation, and that sildenafil treatment did not affect the growth of existing tumors (Islam and Browning, 2018).

The present study has tested the idea that treatment of colon cancer cells with PDE inhibitors *in vitro* can inhibit growth by increasing cGMP and activating PKG signaling. PDE inhibitors were shown to reduce proliferation of colon cancer cell lines at extremely high concentrations that are clinically irrelevant. However, even at the high levels of PDE inhibitors, treatment did not activate cGMP signaling because the colon cancer cell lines lack endogenous cGMP-generating machinery. Taken together, it is concluded physiological doses of PDE inhibitors do not activate cGMP signaling or affect the proliferation of colon cancer cell lines grown *in vitro*.

Materials and Methods

Tissue culture and reagents

The colon cancer cell lines LS174T, Caco2, HCT116, HT29 and SW480 were obtained from the American Type Culture Collection (ATCC) and maintained in 5% CO₂ in RMPI-1640 medium containing 10% FBS, and supplemented with 2 mM L-glutamine, 10 IU/ml penicillin and 10 µg/ml streptomycin. ATCC-derived colon cancer cell lines were made inducible for type 2 PKG expression using a second generation tetracycline-inducible system based on the Tet-On-Advanced and pLVX-puro vector systems (Takara Bio, Mountain View, CA). Packaging was with pLP1, pLP2 and pLP/VSVG vectors, and virus production using 293FT cells was carried out as detailed in the manufacturer instructions (ViraPower; ThermoFisher Scientific). High titers of both virus were mixed and used to transduce cell lines using polybrene, after which populations of lentiviral-infected cells were selected for using cell-optimized G418 concentrations and 2 µg/ml puromycin. Parallel transductions using GFP-expressing virus indicated close to 100% expression in all cell lines. Inducible cell lines were subsequently cultured using maintenance medium supplemented with 200 µg/ml of G418 and 0.2 µg/ml of puromycin. Before experimentation, the cells were grown up for one passage in the absence of antibiotics.

The doxycycline, puromycin, G418 and 8Br-cGMP were from Sigma Chemical (St. Louis MO) and all other chemicals were from ThermoFisher Scientific. The sildenafil citrate (Revatio) and Linacotide (Linzess) were extracted from pharmacy-grade pill/capsule in water, and stored as

aliquots at -80°C. TAK-063 (PDE-10a inhibitor) and PF-04447943 (PDE-9 inhibitor) were from MedChemExpress (Monmouth Junction, NJ).

Cell proliferation and viability assays

To measure cell growth, triplicate wells of 12-well plates were seeded with 5×10^4 cells, and the next day a baseline cell number (day 0) and viability were determined using a TC-20 cell counter with trypan blue (BioRad, Hercules CA). For all cell lines, the density was estimated to be less than 10% confluent on day 0 and sub-confluent on day 3. To quantitate the cells the medium was removed, and after 5 min in trypsin the medium and any floating cells was added back to the well. The cells in the mixture were then pelleted by centrifugation and resuspended in PBS. Drugs to be tested were added to the wells on day 0 and cell counting was carried out on day 3. For quantitation of cell lines with inducible PKG2, doxycycline (0.2 $\mu\text{g/ml}$) was added prior to seeding, and was replaced on day 0. Each experiment containing triplicate wells for each condition was repeated at least three times. Cell numbers measured at day 3 were normalized as a percentage of the mean control value obtained for untreated cells in each replicate. This was done to minimize the variation in cell counts at day 0, which is at the lower range for the TC-20 hemocytometer.

Western blotting

Cells were lysed by incubation in ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% deoxycholate) supplemented with protease and phosphatase inhibitor cocktail (ThermoFisher Scientific). Clarified protein extracts were quantified using a BCA protein quantification kit (Thermo Scientific). Proteins were then separated on 10%

polyacrylamide minigels (BioRad, Hercules CA), transferred to nitrocellulose membranes and blocked with 5% BSA in PBS containing 0.025% Tween 20. Antibodies used for immunoblotting: β -actin (1:2000, Sigma), PKG2 (mAb E7; 1:200, Santa Cruz Biotechnology), β -catenin C-terminus (BD Biosciences, San Jose, CA). Phospho-VASP (Ser239), VASP, ERK-P, ERK, AKT-P, AKT were all from Cell Signaling (1:1000; Danvers, MA). Blots were visualized on film using Pierce-ECL substrate (ThermoFisher Scientific). Select film exposures were scanned and bands on the grayscale images were quantitated using ImageJ software. For semi-quantitative analysis the band intensities were normalized to β -actin (β -catenin) or to the total protein level (ERK-P and AKT-P) for each treatment.

Statistical Analysis

The cell proliferation data were calculated as a percentage of the control (untreated) cells for each experiment, and are expressed as mean $\pm$ SD. All cell proliferation and death data were analyzed for significance using a one-sided ANOVA followed by Dunnett's multiple comparisons test comparing to the untreated control. The differences were considered to be significant when $p < 0.05$.

Results

Intracellular cGMP does not inhibit colon cancer cell proliferation in the absence of ectopic PKG2 expression.

Several studies have reported that PDE inhibitors inhibit colon cancer cell proliferation, with the implication that elevated cGMP levels and activation of PKG mediate the effect. In order to directly interrogate the effects of increased intracellular cGMP on colon cancer cell growth, the membrane-permeable, PDE-resistant analog 8Br-cGMP was used. Treatment of parental LS174T colon cancer cells with 8Br-cGMP did not cause phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at the PKG-preferred site Ser239, which demonstrates the absence of endogenous PKG. Expression of ectopic PKG2 in these cells (LS174T-PKG2) conferred a dose-dependent phosphorylation of VASP in response to 8Br-cGMP (Fig. 1A). The lower mobility band observed at higher concentrations of 8Br-cGMP represents additional phosphorylation of Ser157 that causes a change in mobility on SDS-PAGE gels (Smolenski et al., 1998). Treatment with 8Br-cGMP did not affect proliferation or viability of the parental LS174T cells. However, 8Br-cGMP dose-dependently inhibited proliferation of the LS174T-PKG2 cells, with a maximum effect of 30% at 10 μ M but did not affect cell death (Fig. 1B, C). This effect was not unique to LS-174T cells as similar studies using Caco2 and HCT116 cells were also unable to detect PKG activity, and there was no observed effect of cGMP on proliferation or cell death in the parental cell lines (Figure 2). Ectopic expression of PKG2 conferred reduced proliferation of HCT116 cells and viability in Caco2 cells in response to 8Br-cGMP treatment, but these effects were modest.

PDE inhibitors suppress colon cancer cell growth independent of cGMP/PKG2

The inability of high levels of intracellular cGMP to significantly affect proliferation or viability in several colon cancer cell lines is incongruent with published reports describing the growth-inhibitory effects of cGMP elevating PDE inhibitors (Thompson et al., 2000; Li et al., 2013; Li et al., 2015b; Lee et al., 2016). To investigate this discrepancy, inhibitors of cGMP-specific PDE5 (sildenafil), PDE9 (PF-04447943) and dual-specificity PDE10A (TAK-063) were used to treat parental and PKG-expressing LS174T cells (Fig. 3). Surprisingly, all three inhibitors inhibited cell proliferation at concentrations higher than 10 μ M in both parental and PKG2 expressing cells (Fig. 3A). At the highest dose (200 μ M), the inhibition of proliferation in the parental LS174T cells was least for sildenafil (50%), maximal for Pf-04447943 (80%), with TAK-063 in between (65%). Results were similar in the LS174T-PKG2 cells, except that sildenafil was less effective (25% inhibition). Trypan blue staining showed that these drugs had marginal effects on cell viability that were typically less than 20% at all concentrations except for the highest concentration of TAK-063 that was toxic (Fig. 3B). The ability of the PDE inhibitors to suppress proliferation in the parental cells contrasted with the effect of 8Br-cGMP, which did not affect parental cells, and only moderately inhibited growth in PKG2-expressing cells. These observations indicated that the anti-proliferative effect of PDE inhibitors might be independent of cGMP and PKG. To test this, the ability of the PDE inhibitors to activate PKG in LS174T cells was determined using VASP phosphorylation as a readout (Fig. 3C). While 8Br-cGMP treatment caused robust VASP phosphorylation in the LS174T-PKG2 cells, none of the PDE inhibitors were effective except for 50 μ M TAK-063 that caused a faint band in some experiments. This effect was not unique to LS174T cells, as similar results were obtained using Caco2 and HCT116 cells (Fig. 4). Treatment of HCT116 or Caco2 with 50 μ M of the PDE

inhibitors reduced proliferation to varying degrees in both parental and PKG2-expressing cells, with TAK-063 as the most effective in both cell lines. However, none of the PDE inhibitors caused VASP phosphorylation, indicating that the anti-proliferative effect was independent of both cGMP and PKG.

Colon cancer cell lines lack endogenous cGMP-generating machinery

The inability of the PDE inhibitors to elicit a functional cGMP response as measured by PKG-dependent phosphorylation of VASP was unanticipated based upon previous studies (Thompson et al., 2000; Li et al., 2013; Lee et al., 2016). Phosphodiesterases antagonize guanylyl-cyclases by hydrolytically cleaving the cGMP to produce GMP. The source of cGMP in the colon epithelium is membrane-bound GC-C following activation by guanylin (Forte, 2004). GC-C is maintained in colon tumors (Carrithers et al., 1996; Cagir et al., 1999; Birbe et al., 2005), but expression is more variable in colon cancer cell lines (Waldman et al., 1998). However, the GC-C agonist guanylin is rapidly downregulated during tumorigenesis (Cohen et al., 1998; Steinbrecher et al., 2000; Wilson et al., 2014). In order to determine whether functional GC-C is expressed in colon cancer cell lines, we measured VASP phosphorylation following treatment with the GC-C agonist linacotide in combination with the PDE5 inhibitor sildenafil in different colon cancer cell lines that we engineered to express ectopic PKG2 (Fig. 5A). Out of four cell lines that were used in previous studies of PDE inhibitors, only LS174T and Caco2 produced phospho-VASP bands that were comparable to positive controls treated with 8Br-cGMP. LS174T cells expressing ectopic PKG2 were subsequently treated with different doses of linacotide to identify the lowest concentration capable of activating cGMP/PKG signaling (Fig. 5B). The cells were then treated with the PDE inhibitors together with the low dose of linacotide

(10 nM) to determine whether these drugs could amplify the basal cGMP production (Fig. 5C). Sildenafil produced a robust VASP phosphorylation at both 1 and 10 μ M, whereas the PF-04447943 and TAK-063 were much less effective, with weak bands appearing only at the higher 10 μ M dose. Taken together, these results strongly support that idea that the growth-inhibitory effects of PDE inhibitors in colon cancer cells does not involve activation of cGMP signaling, and is more likely to result from off-target effects at higher doses.

The effect of PDE inhibitors on survival signaling in colon cancer cells.

The mechanisms that have been proposed to mediate the anti-proliferative effects of PDE inhibitors and cGMP in colon cancer cells have centered upon growth and survival pathways downstream of receptor-tyrosine kinases, and Wnt signaling. The effect of PDE inhibitors on these pathways was examined in the GC-C expressing LS174T and Caco2 cell lines (Fig. 6). These studies showed that phospho-ERK levels were transiently suppressed by treating cells with 200 μ M of Sildenafil or 100 μ M of the PDE9, 10 inhibitors in both cell lines. This inhibition was more pronounced in Caco2 cells treated with PF-04447943 and TAK-063 (40-70% inhibition), compared to sildenafil (10% inhibition), but in all cases the phospho-ERK normalized after 24 h. Phospho-AKT and total β -catenin levels were not inhibited in either cell line by any of the drugs.

Discussion

A large body of work spanning two decades has highlighted cGMP-elevating PDE inhibitors for the prevention and treatment of colorectal cancer. Building on this foundation, recent large-cohort retrospective epidemiological studies support their efficacy in humans, and provide the impetus for prospective clinical studies to interrogate their utility in patients (Huang et al., 2019; Sutton et al., 2020). However, there are inconsistencies in the literature regarding the potential utility of cGMP-PDE inhibitors as a primary prevention modality or as chemotherapy for cancer treatment. The present study provides new information that impacts the interpretation of foundational *in vitro* studies of PDE inhibitors in colon cancer cell lines.

A key observation shown here is that high levels of intracellular cGMP do not inhibit proliferation or affect the viability of ATCC-derived colon cancer cell lines. In addition, the colon cancer cell lines do not express detectable PKG activity that has been reported to mediate anti-proliferative signaling. Ectopic expression of PKG2 in colon cancer cells conferred a modest anti-proliferative response to cGMP treatment in some colon cancer cell lines, but did not affect viability. While the number of cell lines used in these studies is not exhaustive, the results were similar between cells of different genetic etiology, and are supported by observations from several independent groups using diverse gastrointestinal cancer cell lines (Wang et al., 2012; Wu et al., 2013; Booth et al., 2014).

Another key observation from the present study is that inhibitors of PDE5, PDE9 and PDE10a all suppressed colon cancer cell proliferation, but only at concentrations exceeding 10 μ M. These results are consistent with observations made by several independent laboratories, which have

reported EC₅₀ values for inhibiting colon cancer cell growth ranging from 20 μ M to over 500 μ M for different PDE inhibitors (Table 1). Notably, these concentrations are between 10,000 to almost 100,000 times the published IC₅₀ for the target PDE enzyme. This massive disconnect in effective concentration suggests that the inhibitory mechanism is due to off-target effects. Most importantly, the EC₅₀ values for inhibiting colon cancer cell growth *in vitro* are almost 50 times the published peak plasma concentrations (C_{max}) of these drugs when administered at high doses to human patients. The results shown here are consistent with the anti-proliferative effects of PDE5 and PDE10a inhibitors on colon cancer cell lines as published by other laboratories, but the doses required are clinically unachievable.

The notion that the anti-proliferative effect of the cGMP-PDE inhibitors is due to off-target effects is supported by the lack of effect of 8Br-cGMP on colon cancer cell growth. The present study measured activation of PKG as a functional readout for cGMP, because the biological significance of absolute cGMP levels is unclear. A surprising observation was that none of the PDE inhibitors studied were able to increase cGMP levels to activate PKG in the colon cancer cell lines. The underlying reason was clearly shown to be due to the lack of endogenous cGMP-generating machinery. Without a basal rate of cGMP production to provide substrate for PDE's, their inhibition had no effect. In agreement with previous studies, it was shown here that functional GC-C was only expressed in only 2 of the cell lines tested (Waldman et al., 1998). GC-C is the main source of cGMP in the colon epithelium that is activated by peptide hormone guanylin, which is universally lost in colon cancer (Wilson et al., 2014). In the presence of GC-C and the synthetic agonist linacotide, a physiological dose (1 μ M) of the PDE5 inhibitor sildenafil potently activated cGMP signaling as measured by VASP-phosphorylation. The lack of effect of PDE9 and PDE10a inhibitors in the linacotide-treated cells demonstrated that PDE5 is

the central enzyme controlling cGMP levels in colon cancer cells as has been reported by others (Bakre et al., 2000; Piazza et al., 2000; Mei et al., 2015). The slight increase in cGMP detected at very high concentrations of the PDE9 and PDE10a inhibitors in the present study likely reflects crossover inhibition of PDE5.

The anti-proliferative effect of high-doses of PDE inhibitors was shown here to be an off-target effect of these drugs, but specific signaling pathways have previously been implicated as important. The most prevalent mechanism proposed is the cGMP/PKG-mediated downregulation of β -catenin protein levels, leading to suppression of TCF-target gene expression (Thompson et al., 2000; Whitt et al., 2012; Li et al., 2015b). However, inhibition of AKT and ERK pathways in cells expressing ectopic PKG2 has also been reported (Wu et al., 2012; Wu et al., 2013; Wang et al., 2017). There was no obvious effect on any of these signaling pathways that was common to the inhibitors, or across cell lines with any single inhibitor. A recent study showed that treating SW480 and HCT116 colon cancer cells with 300 μ M sildenafil for 48 h inhibited proliferation, which was associated with reduced β -catenin levels (Mei et al., 2015). The mechanism was shown to involve increased redox stress and G1 arrest, but no specific pathway was indicated. It is possible that higher inhibitor concentrations over a longer time period might have resulted in additional toxicity, but our results demonstrate that direct targeting of specific proliferative signaling pathways is unlikely have a role.

An important conclusion from the present work is that the effective concentration of cGMP-PDE inhibitors used in colon cancer studies must be a critical factor when evaluating the significance of *in vitro* observations. The present study did not address the potential benefits of using PDE5

inhibitors in combination with traditional chemotherapy (Booth et al., 2014; Booth et al., 2017), or as immunomodulatory agents (Serafini et al., 2006; Lin et al., 2017). However, the concentrations of cGMP-PDE inhibitors that are needed to directly suppress colon cancer cell growth are clinically unachievable. Studies using knockout mice with defective cGMP signaling have shown that cGMP activation of PKG2 regulates intestinal homeostasis by slowing epithelial turnover (Steinbrecher et al., 2002; Li et al., 2007; Wang et al., 2012). Increasing cGMP using physiological doses of PDE5 inhibitors or GC-C agonists, inhibits colon carcinogenesis in mice (Chang et al., 2017; Islam et al., 2017; Sharman et al., 2018). The mechanism was proposed to involve the reducing tumor initiation rather than inhibiting the growth of existing tumors, suggesting that PDE5 inhibitors might be useful for colon cancer prevention (Islam and Browning, 2018; Browning, 2019).

In summary, the present results demonstrate that proliferation and viability of colon cancer cell lines is not affected by cGMP or physiological doses of cGMP-elevating PDE inhibitors. Extremely high doses of PDE inhibitors can inhibit colon cancer cell proliferation using an off-target mechanism that does not involve cGMP/PKG signaling.

Authorship Contributions

Participated in research design: YH, AW, DB

Conducted experiments: YH, AW, NM, KB, BI, DB

Contributed new reagents or analytic tools: KV, DB

Performed data analysis: YH, AW, DB

Wrote or contributed to the writing of the manuscript: RW, KV, DB

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Footnotes

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Figure Legends

Figure 1. Ectopic PKG2 is required for cGMP to inhibit the growth of LS174T colon cancer cells. Parental LS174T colon cancer cells and those engineered to express ectopic PKG2 were treated with 8Br-cGMP at the indicated concentrations. **(A)** The cGMP signaling was measured by immunoblotting for VASP phosphorylation at Ser239 (VASP-239P). The effect of 8Br-cGMP of parental and PKG2-expressing LS174T cells on **(B)** cell proliferation and **(C)** cell viability. Data show means, and error bars are SD. *** $p < 0.0001$.

Figure 2. The inability of cGMP to affect proliferation is common among colon cancer cell lines. **(A)** Parental Caco2 and HCT116 colon cancer cells and those with inducible PKG2 expression were treated with 8Br-cGMP (100 μ M) and doxycycline (Dox) to induce PKG2 expression as indicated. The cGMP signaling was measured by immunoblotting for VASP phosphorylation at Ser239 (VASP-239P). The effect of 8Br-cGMP treatment on **(B)** cell proliferation (hemocytometer), and **(C)** cell viability (trypan blue exclusion) is shown. Data show means, and error bars are SD. * $p < 0.05$.

Figure 3. High doses of cGMP-PDE inhibitors suppress growth of LS-174T colon cancer cells by a cGMP-independent mechanism. Parental and PKG2-expressing LS174T colon cancer cells were treated for 3 days with different doses of PDE inhibitors. Sildenafil (Sil)-PDE5, PF-04447943 (PF)-PDE9, and TAK-063 (TAK)-PDE10a. The effect of PDE inhibitor treatment on **(A)** cell proliferation (hemocytometer), and **(B)** cell viability (trypan blue exclusion). **(C)** The effect of PDE inhibitors on cGMP levels in LS-174T-PKG2 cells was measured by

immunoblotting for phosphorylation of VASP (VASP-239P). Data show means, and error bars are SD. *** $p < 0.0001$, ** $P < 0.005$, * $P < 0.05$.

Figure 4. The cGMP/PKG-independent inhibition of proliferation by PDE-inhibitors is a common feature of colon cancer cell lines. HCT116 and Caco2 colon cancer cells were either untreated, or treated with the PDE5 inhibitor sildenafil (Sil), the PDE9 inhibitor PF-04447943 (PF), and the PDE10a inhibitor TAK-063 (TAK) as indicated. The ability of the drugs increase cGMP in (A) HCT116-PKG2 and (C) Caco2-PKG2 was measured by immunoblotting for VASP phosphorylation at Ser239 (VASP-239P) using 8Br-cGMP as a positive control. The effect of 50 μ M of the PDE inhibitors on proliferation and viability was measured in both parental and PKG2-expressing (B) HCT116 and (D) Caco2 colon cancer cells. Cells were treated with 50 μ M of Sil, PF, or TAK for 3 days. Cell death of was measured using trypan blue exclusion. Data show means, and error bars are SD. *** $p < 0.0001$, ** $P < 0.005$.

Figure 5. PDE inhibitors do not increase cGMP in colon cancer cell lines due to a lack of endogenous cGMP production. The expression of functional guanylyl-cyclase-C (GC-C) in colon cancer cells engineered to express ectopic PKG2 was determined by measuring VASP phosphorylation in response to treatment with the GC-C agonist linacotide. (A) Different cell lines induced to express PKG2 as indicated below, were either untreated (control, C), treated with 8Br-cGMP (cG), or with the combination of linacotide and sildenafil (L+S). Western blots were probed for β -actin as a loading control for PKG2 expression. (B) Dose-response study of linacotide stimulation of LS174T-PKG2 cells to determine the dose required to generate minimal cGMP/PKG2 activity as measured by VASP phosphorylation. (C) LS174T-PKG2 cells

were treated with PDE inhibitors as indicated, in the presence of 10 nM linaclotide. Relative cGMP levels were then measured by immunoblotting for VASP-239P. All immunoblots were reprobed with anti-total VASP to indicate relative phosphorylation.

Figure 6. The effect of high-dose PDE inhibitors on proliferation/survival signaling in colon cancer cell lines. (A) LS174T and Caco2 colon cancer cells were treated with 200 μ M Sildenafil (Sil), 100 μ M PF-04447943 (PF), or 100 μ M TAK-063 (TAK) for different times as indicated. The effect of treatment on ERK, AKT and β -catenin was determined by immunoblotting. (B) Densitometric analysis of blots, where ratios of band intensities for ERK-P/ERK, AKT-P/AKT, and β -catenin/ β -actin were calculated and expressed as a percent of the basal level (0 hr time point; dashed line).

Table 1: Effective Doses of cGMP-PDE Inhibitors

Target	Drug	IC₅₀	EC₅₀[#]	Human plasma [C_{max}]
PDE5	Sildenafil	3.9 nM (Gupta et al., 2005)	66-271 μM (Li et al., 2013; Mei et al., 2015)	1.2 μM (Gupta et al., 2005) (100 mg dose)
	Tadalafil	1-5 nM (Gupta et al., 2005; Ahmed, 2018)	38 μM (Li et al., 2013)	0.87 μM (Gupta et al., 2005) (20 mg dose)
	Exisulind	113 μM (Soh et al., 2000)	165-557 μM (Thompson et al., 2000)	13-27 μM (van Stolk et al., 2000) (40-200 mg dose)
PDE9	Pf-04447943	12 nM (Charnigo et al., 2019)	90 μM *	0.12-0.63 μM (Charnigo et al., 2019)
	BI-409306	52 nM (Rosenbrock et al., 2019)	ND	1.37 μM (Moschetti et al., 2016) (100 mg dose)
PDE10	TAK-063	0.3 nM (Harada et al., 2015)	22 μM *	0.57 μM (Tsai et al., 2016) (1000 mg dose)
	Pf-2545920	0.16 nM (Lee et al., 2016)	14-20 μM (Lee et al., 2016)	0.1-0.52 μM (Delnomdedieu et al., 2017) (10-30 mg dose)

*From the present study. ND: Not determined. [#]Inhibition of colon cancer cell growth.

Fig.1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6