Cyclic-GMP-Elevating Agents Suppress Polyposis in Apc<sup>Min</sup> mice by Targeting the Preneoplastic Epithelium

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Abstract

The cGMP signaling axis has been implicated in the suppression of intestinal cancers, but the inhibitory mechanism and the extent to which this pathway can be targeted remains poorly understood. This study has tested the effect of cGMP-elevating agents on tumorigenesis in the Apc<sup>Min/+</sup> mouse model of intestinal cancer. Treatment of Apc<sup>Min/+</sup> mice with the receptor guanylyl-cyclase C (GCC) agonist linaclotide, or the phosphodiesterase-5 (PDE5) inhibitor sildenafil, significantly reduced the number of polyps per mouse (67% and 50%, respectively). Neither of the drugs affected mean polyp size, or the rates of apoptosis and proliferation. This was possibly due to increased PDE10 expression, as endogenous GCC ligands were not deficient in established polyps. These results indicated that the ability of these drugs to reduce polyp multiplicity was primarily due to an effect on non-neoplastic tissues. In support of this idea, Apc<sup>Min/+</sup> mice exhibited reduced levels of endogenous GCC agonists in the nonneoplastic intestinal mucosa compared with wild-type animals, and this was associated with crypt hyperplasia and a loss of goblet cells. Administration of either sildenafil or linaclotide suppressed proliferation, and increased both goblet cell numbers and luminal apoptosis in the intestinal mucosa. Taken together, the results demonstrate that targeting cGMP with either PDE5 inhibitors or GCC agonists alters epithelial homeostasis in a manner that reduces neoplasia, and suggests that this could be a viable chemoprevention strategy for patients at high risk of developing colorectal cancer. Cancer Prev Res; 11(2); 81–92. ©2018 AACR.

Introduction

Colorectal cancer is the third most commonly diagnosed cancer in the United States with 1 in 20 being diagnosed each year (1). Early screening and treatment is the cornerstone of colorectal cancer prevention; but the high mortality rate associated with diagnosis at advanced stages makes it one of the leading causes of cancer-related deaths. Chemoprevention has therefore emerged as a complementary strategy for patients that are predisposed to colon cancer because of hereditary disease, ulcerative colitis, or family history (2, 3). The most widely investigated chemopreventive agents are nonsteroidal anti-inflammatory drugs (NSAID). They have been shown to reduce the incidence of colorectal cancer when used long-term, but side effects in the average-risk population of predisposed patients limits their utility (4).

A derivative of the NSAID sulindac called Exisulind, lacks COX-inhibitory activity, but was found to block growth in colon cancer cell lines by inhibiting phosphodiesterases (PDE; refs. 5, 6). The underlying mechanism was shown to involve inhibition of β-catenin signaling, leading to apoptosis (7, 8). In clinical trials, Exisulind suppressed tumorigenesis in familial adenomatous polyposis (FAP) patients (9). Although Exisulind induced regression of rectal polyps in FAP patients, it did not get FDA approval due to harmful side effects reported in phase III trials (10). Although Exisulind was ultimately not successful, it did highlight the therapeutic potential of cyclic guanosine monophosphate (cGMP) signaling.

The central source of cGMP in the intestinal epithelium arises from the activity of guanylyl cyclase C (GCC), which is activated by the endogenous peptide secretagogues uroguanylin and guanylin (11). The well-established role of cGMP in the intestine is ion channel regulation to promote fluid secretion (12), but a growing body of evidence has...
shown that it also controls epithelial homeostasis. Analysis of knockout mice suggests that the cGMP signaling axis suppresses proliferation, and apoptosis, while promoting differentiation in the intestinal epithelium (13–15). In addition to its role in regulation of epithelial homeostasis, growing evidence also suggests that activating cGMP signaling in the colon could have therapeutic potential for colon cancer. GCC knockout mice exhibit increased tumorigenesis in both AOM and Apcc−/− mouse models of colon cancer (16, 17). The endogenous GCC peptides uroguanylin and guanylin are commonly lost gene products in most human colon cancers suggesting that supplementing with exogenous hormone could be a potential treatment strategy (18, 19). In support of this idea, administration of the exogenous uroguanylin reduced tumorigenesis in Apcc−/− mice, but the protective mechanism was not addressed (20). More recent studies with the GCC agonist plecanatide (21), and the PDE5 inhibitor sildenafil demonstrate that cGMP elevation can promote barrier integrity, and protect from inflammation-driven colorectal cancer (22–24). However, the relative efficacy of the two approaches to cGMP elevation, and the role of suppressing colitis in the protective mechanism remain unclear. This study sought to compare the tumor prevention effects of orally administered sildenafil and linaclotide in a colitis-independent intestinal polyposis model.

Materials and Methods

Animals and drug administration

All procedures were approved by the Augusta University Committee on the Care and Use of Animals. For tumorigenesis studies, male C57/BL6J-Apc−/− mice and female C57/BL6J mice were obtained from Jackson Laboratory (Bar Harbor, ME) at age 6 weeks for breeding. Mice were housed in the Augusta University animal facility in a temperature- and humidity-controlled room with free access to food and water. Pharmaceutical grade sildenafil citrate (Revatio) was ground with a mortar and pestle, and stored in suspension with diH2O at −80°C until use. Stock sildenafil was diluted in drinking water to a concentration of 5.7 mg/kg. Linaclotide was prepared by grinding the contents of Linzess capsules in diH2O using a tissue homogenizer. Aliquots were stored at −80°C and were diluted fresh for each experiment. Mice were gavaged daily with 100 μL of the linaclotide suspension at a concentration of 2.07 μg/mL.

Tumorigenesis studies

Mice were genotyped by PCR from tail-clip DNA at 3 weeks of age and drug treatment started at 4 weeks. Body weight was recorded weekly to monitor disease progression. At the end of 14 weeks, the animals were sacrificed and the entire gastrointestinal tract from stomach to rectum was removed and flushed with ice-cold PBS. The small intestine was then divided into three sections, opened longitudinally, and spread on a light box for the acquisition of high-resolution images. The images were subsequently enhanced and enlarged for identification and selection of polyps, which were quantitated and sized using ImageJ software (NIH, Bethesda, MD). After capturing images, representative polyps were collected for RNA, protein, or histologic analysis.

Histopathology

Intestinal tissues from age-matched mice were formalin-fixed, embedded in paraffin, and sectioned at 5-μm thickness by the Augusta University Histology Core. Sections were processed as follows: deparaffinization with xylene, rehydration with decreasing concentrations of ethanol (100%–50%), and antigen retrieval by boiling in citrate buffer. Tissues were stained directly by the histology core with H&E and Alcian blue/periodic acid Schiff to visualize polyps and goblet cells. The tissues were probed using antibodies against Ki-67 (1:100; Dako Cytomation) and cleaved caspase-3 (1:500; Cell Signaling Technology). Antibody visualization was done using an anti-rat/anti-rabbit ImmunoCruz ABC kit (Santa Cruz Biotechnology) to enhance DAB staining. Mucus density and proliferative and apoptotic indices were quantitated using ImageJ software. Equivalent regions of intestine were compared between groups for all quantitative histologic studies. Briefly, for surrounding tissue, 10 different sections containing approximately 8 crypts per section were analyzed for each mouse. For polyps, three sections from four different polyps per mouse were analyzed.

Analysis of gene expression

Polyps were removed with scissors and forceps, and the mucosa was scraped from the underlying connective tissues. Tissue was vortexed briefly in TRIzol reagent (Life Technologies) and flash-frozen. RNA was DNase treated (TURBO DNA-free kit, Life Technologies) and converted to cDNA using M-MLV reverse transcriptase (Invitrogen). Quantitative PCR (qRT-PCR) analysis of the cDNA was performed using SYBR Green PCR Master Mix (Applied Biosystems). Relative expression levels were calculated using the 2−ΔΔCt method with β-actin (ACTB) as a reference. Amplifications were performed in triplicate wells and melt curve analysis was done to confirm the specificity of the primers used. Primers were designed using Primer Blast Software (NCBI; Supplementary Table S1).

Statistical analysis

All data are expressed as mean ± SEM, unless stated otherwise. A one-way ANOVA was used to test the significant differences between control mice and the two treatment groups, unless otherwise stated. Statistical significance was set at P < 0.05.
Figure 1.
Linaclotide treatment suppresses polyp multiplicity in the Apc\(^{Min/+}\) mouse intestine.

A, Top, representative jejunal sections from the intestines of 14-week-old Apc\(^{Min/+}\) mice, untreated (Ctrl) or treated for 10 weeks with linaclotide (Lin). Bottom, an H&E-stained section containing a representative intestinal polyp from a control animal. B, Plot shows the effect of linaclotide on median polyp number per animal. C, Mean polyp size in Apc\(^{Min/+}\) mice, untreated (Ctrl), or treated for 10 weeks with linaclotide (Lin). D, Average number of polyps in various size classes in Apc\(^{Min/+}\) mice, untreated (Ctrl), or treated for 10 weeks with linaclotide (Lin). E and F, Histologic analysis of proliferative index (Ki-67), and apoptotic index (CC3) within polyps from control and linaclotide-treated animals. Indices were calculated as staining nuclei (or cells for CC3) as a function of the total number of nuclei stained by hematoxylin. Error bars, SEM, and the \(P\) values are from a two-tailed Student \(t\) test. Scale bars, top panel, 10 mm; bottom panel, 50 \(\mu\)m.
Figure 2.
Sildenafil treatment reduces the number but not the phenotype of intestinal polyps from Apc$^{min/+}$ mice. 
A, The effect of sildenafil on median polyp number per animal. B, Mean polyp size in Apc$^{min/+}$ mice, untreated (Ctrl) or treated for 10 weeks with sildenafil (Sild). C, Average number of polyps in various size classes in Apc$^{min/+}$ mice, untreated (Ctrl) or treated for 10 weeks with sildenafil (Sild). D and E, Histologic analysis of proliferative index (Ki-67), and apoptotic index (CC3) within polyps from control and sildenafil-treated animals. Indices were calculated as staining nuclei (or cells for CC3) as a function of the total number of nuclei stained by hematoxylin. Error bars, SEM, and the $P$ values are from a two-tailed Student $t$ test.
Results

cGMP-elevating agents reduce intestinal polyp multiplicity in ApcMin/+ mice

Treatment with the GCC agonist plecanatide or with the PDE5 inhibitor sildenafil has recently been shown to suppress polyp multiplicity in inflammation-driven models of colon cancer in mice (21, 23). To better understand the inhibitory mechanism, this study compared the two approaches to cGMP elevation in a non-inflammatory model of intestinal carcinogenesis. Loss of heterozygosity at the Apc locus causes ApcMin/+ mice to develop numerous adenomatous polyps primarily in the small intestine (Fig. 1A). Daily administration of linaclotide by gastric gavage reduced the number of intestinal polyps per mouse by 67% compared with controls given water alone ($P < 0.001$, Fig. 1B). The suppressive effect of linaclotide on polyp number was more pronounced on smaller polyps ($< 2$ mm), but the mean polyp size was unaffected (Fig. 1C and D). Further analysis of polyp sections by IHC showed that linaclotide treatment of the host did not significantly affect proliferation ($P = 0.07$) or apoptosis ($P = 0.43$) of the polyps (Fig. 1E and F).

Treatment of ApcMin/+ mice with sildenafil in the drinking water resulted in 56% fewer intestinal polyps compared with control mice on water alone ($P = 0.003$, Fig. 2A). As with the linaclotide-treated animals, the smaller polyps from sildenafil-treated mice were also suppressed while mean polyp size did not differ compared with controls (Fig. 2B and C). Histologic analysis showed that the polyps did not differ with respect to proliferative and apoptotic indices (Fig. 2 D and E).

Sildenafil does not affect inflammation in the ApcMin/+ mouse intestine. Sildenafil has been reported to suppress myeloid-derived suppressor cell (MDSC) function and reduce inflammation in colonic polyps (23, 25, 26). To

Figure 3. Sildenafil treatment does not reduce inflammation within polyps of ApcMin/+ mice. A–C, Real-time qPCR analysis of inflammatory cytokine gene expression in mucosa from C57BL/6 mice and mucosa and polyps from untreated or sildenafil-treated ApcMin/+ mice ($n = 6$). D and E, Representative images and quantification of Cd11b+ cells (red) in intestines of C57BL/6 mice and untreated or sildenafil-treated ApcMin/+ mice. Tissues were counterstained with DAPI, $n = 3$ mice, 3 polyps per mouse. Scale bars, 50 μm.
determine whether this phenomenon played a role in this study, RT-qPCR was used to measure inflammatory cytokine expression in the ApcMin/+ mouse intestinal tissues. These studies showed that the expression of IL1b, TNFα, and IFNγ in the nonpolyp tissue from the ApcMin/+ mouse intestine was comparable with the levels in wild-type mice (Fig. 3A–C). In contrast, the intestinal polyps showed dramatic increases in the mRNA levels of these inflammatory cytokines. Furthermore, treatment of mice with sildenafil did not significantly reduce the cytokine expression in either the polyp or nonpolyp tissue. In support of this, the slightly higher density of CD11b cells associated with polyps did not change in response to sildenafil treatment (Fig. 3D and E). These results support the idea that mucosal inflammation is not a central driver of intestinal carcinogenesis in the ApcMin/+ mouse, and that the suppressive effect of cGMP is not due to an effect on polyp inflammation.

The expression of cGMP signaling components in the ApcMin/+ intestinal polyps. While both cGMP-elevating drugs examined in this study reduced polyp multiplicity in the ApcMin/+ intestine, neither drug affected polyp size, proliferation, or apoptosis. Similar observations were reported recently using the AOM/DSS inflammatory carcinogenesis model, where a dramatic loss of cGMP-generating machinery in the polyps partially explained the phenomenon (23). To determine whether this occurred in the intestinal polyps in ApcMin/+ mice, the expression of cGMP signaling components was examined using RT-qPCR. These studies revealed that the mRNA levels of uroguanylin and GCC were unchanged between nonpolyp epithelium and the polyp tissue (Fig. 4A). Surprisingly, guanylin mRNA levels were significantly higher in the polyps compared with the surrounding tissue (P = 0.007), but the physiologic significance is not clear as the relative transcript levels were much lower than uroguanylin (data not shown). The expression of the effector protein kinases PKG1α, β did not differ significantly between polyp and nonpolyp tissue in the ApcMin/+ mouse intestine, but the relative mRNA level of the PKG2 isofrom was elevated in the polyps (P = 0.02, Fig. 4B). Expression analysis of phosphodiesterases with activity toward cGMP (PDE5, 9, and 10a) revealed little change in PDE5 or PDE9, but significantly higher levels of the dual-specificity PDE10a were observed in the polyps compared with nonpolyp tissue (P = 0.001, Fig. 4C). However, the increased expression of PDE10a in the ApcMin/+ polyps was independent of sildenafil treatment.

cGMP-elevating agents regulate homeostasis in the preneoplastic tissue of ApcMin/+ mice. The suppression of tumorigenesis by both sildenafil and linaclotide in the absence of any effect on initiated polyps suggested that the principal effect of cGMP is on the preneoplastic epithelium. To explore this further, RT-qPCR analysis of cGMP signaling component expression in the nonpolyp epithelium of the ApcMin/+ intestine was compared with wild-type animals. These studies revealed significantly less uroguanylin and guanylin (P = 0.025 and 0.04, respectively), but elevated levels of GCC in the ApcMin/+ intestine (P = 0.03, Fig. 5A). To determine whether the altered
expression of these components affected epithelial homeostasis, histologic analysis of proliferation, apoptosis, and differentiation was examined in the nonpolypl epithelium. These results showed that the intestine of Apc<sup>Min/+</sup> mice exhibited significantly higher crypt proliferation as evidenced by Ki67 staining (Fig. 5A) and total PCNA levels (Supplementary Fig. S1). In addition, Apc<sup>Min/+</sup> mice had fewer goblet cells and more apoptosis at the villus tip compared with wild-type animals (P = 0.003, Fig. 5B–D).

**Figure 5.** Nonpolypl epithelium from Apc<sup>Min/+</sup> mice appears deficient in cGMP signaling. A, RT-qPCR analysis of uroguanylin, guanylin, and GCC gene expression in C57BL/6 mucosal tissue and nonpolypl mucosal tissue from Apc<sup>Min/+</sup> mice. B–D, Quantification and representative images of goblet cells (AB/PAS), proliferation (Ki-67), and apoptosis (CC3) in stained sections of intestine from C57BL6 mice and Apc<sup>Min/+</sup> mice. A, n = 6; B–D, n = 3. Error bars, SEM; P values are from a two-tailed Student t test. Scale bars, 50 μm (B and C) and 25 μm (D).
It has been reported that increasing cGMP in the intestine with PDE5 inhibitors can suppress proliferation and apoptosis in the mouse colon, but this has not been examined previously in the small intestine (22, 24). To better understand the effect of cGMP-elevating agents in the Apc\(^{min/+}\) mice, the effects of linaclotide and sildenafil on homeostasis in the nonneoplastic intestinal epithelium was measured. Similar to the effect of these agents on the colon, both drugs increased goblet cell density and suppressed proliferation (Fig. 6A and B). In contrast to previously reported effects on the colon, however, both linaclotide and sildenafil treatment increased apoptosis at the tip of the villi. These drugs had a similar effect in the small intestine of wild-type mice as in the Apc\(^{min/+}\) mice, except that the effect on proliferation is much weaker and did not attain significance (Supplementary Fig. S2).

**Discussion**

Recent reports show that the GCC agonist plecanatide can reduce DSS-induced tumorigenesis in the colon of Apc\(^{min/+}\) mice (21), and that sildenafil can inhibit tumorigenesis in colon of AOM/DSS–treated mice (23, 25). However, these two classes of drugs have not been compared in a single model, and the tumor-suppressive mechanism remains poorly understood. Results shown here demonstrate that the GCC agonist linaclotide and the PDE5 inhibitor sildenafil were equally able to suppress polyp multiplicity in the intestine of Apc\(^{min/+}\) mice. Moreover, the magnitude of the tumor-suppressive effect in the intestine was the same as reported for sildenafil in the colon of AOM/DSS–treated mice. Despite differences in tumor initiation and location, these observations underscore a common cGMP-dependent mechanism. PDE5 inhibitors have been shown to suppress DSS-induced inflammation, suggesting that this could play an important role in the tumor prevention effect of sildenafil (22). However, the AOM/DSS study concluded that the tumor-suppressive effect of sildenafil was mostly during the mutagenesis/initiation phase, and that the timing of drug administration was independent of inflammation (23). This study supports the idea that these drugs suppress initiation as it affected the smaller polyps but not the larger ones that presumably were initiated in the 4 weeks prior to drug administration. A separate study that used a very high dose of sildenafil in the AOM/DSS model suggested that the tumor-suppressive effect is due to blockade of myeloid cell function (25). However, the results shown here do not support this idea because endogenous inflammation is not a driver of tumorigenesis in the small intestine of Apc\(^{min/+}\) mice, and in contrast to the colonic polyps in AOM/DSS mice, inflammation in the intestinal polyps was not affected by sildenafil.

Sildenafil only slightly reduced proliferation in the colonic polyps of AOM/DSS–treated mice, but notably increased mucus differentiation (23). This contrasts with the intestinal polyps from Apc\(^{min/+}\) mice, which were largely devoid of goblet cells and this was not altered by treatment with either drug (data not shown). The observation that neither linaclotide nor sildenafil affected proliferation or apoptosis of established polyps is ostensibly at odds with numerous reports describing antitumor effects of cGMP (27–29). The previous study of sildenafil in AOM/DSS–treated mice partly attributed the relatively benign effect of the drug on established polyps to reduced guanylin expression (23). Guanylin levels are also reduced in human colorectal tumors relative to nontumor tissue (18, 19, 30). As GCC ligands have been shown to have cytosstatic effects in colon cancer cell lines (5, 16, 29), “hormone replacement therapy” has been suggested as an approach to use exogenous ligands to treat colon tumors (31–33). The results shown here do not support this idea, as linaclotide did not affect mean size or proliferation of polyps. Moreover, the mRNA levels of uroguanylin and guanylin were not reduced in the intestinal polyps from Apc\(^{min/+}\) mice. These observations indicate that the loss of GCC ligands is not the central reason for a lack of effect of cGMP-elevating drugs on initiated polyps. In support of several previous studies, it was shown here that the PDE10a increased dramatically in the intestinal polyps (23, 34, 35). It is possible that this dual-specificity PDE impedes the activation of cGMP signaling in tumors. As inhibition of PDE10a inhibits proliferation of colon cancer cell lines (35), whether such inhibitors might be used as a treatment strategy for colon cancer will be an important area for future investigation.

The lack of effect of either sildenafil or linaclotide on established polyps strongly suggested that the reduced polyp formation resulting from treatment with these agents was due to an effect on the preneoplastic tissue. A growing body of evidence demonstrates that cGMP signaling controls intestinal homeostasis, and that increasing cGMP can suppress proliferation while increasing goblet cell density (13, 14, 24, 36). It was shown here that treatment with either linaclotide or sildenafil caused an increase in epithelial apoptosis in the intestine, which contrasts with previous reports of reduced apoptosis in the colon epithelium (22, 24). However, because the apoptosis is restricted to the luminal border, and because these agents prevent polyp formation in both colon and intestine, it is unlikely that the apoptosis contributes to the mechanism of tumor suppression.

A novel observation in this study was the reduced expression of GCC ligands in the mucosa of Apc\(^{min/+}\) mice relative to wild-type animals, and increased proliferation that is consistent with reduced cGMP signaling. While further study is needed to determine the significance of this to human FAP patients, both linaclotide and sildenafil were found to reduce proliferation in the intestine of Apc\(^{min/+}\) mice. The carcinogenic mechanisms underlying...
Figure 6. Linaclotide and sildenafil treatment suppress proliferation in the nonpolyp epithelium of ApcMin/+ mice. Nonpolyp tissue from ApcMin/+ mice treated with sildenafil or linaclotide for 10 weeks were collected and prepared for histologic analysis. A–C, Tissue was stained for goblet cell density (AB/PAS), proliferation (Ki-67), and apoptosis (CC3). Representative pictures of the staining are shown at the right of the graph. Arrows on C indicate CC3-positive cells. Error bars, SEM; P values are from a two-tailed Student t test. Scale bars, 50 μm (A and B) and 25 μm (C).
the AOM/DSS and Apc\textsuperscript{Min/+} colon cancer models are different. In the AOM/DSS model, carcinogenesis is due to mutations caused by azoxymethane, whereas in the Apc\textsuperscript{Min/+} model, it is due to loss of heterozygosity (LOH) at the Apc gene locus (37, 38). Assuming an equal rate of DNA repair, the rate of LOH should be proportional to the size of the proliferative compartment. The suppression of proliferation in response to cGMP-elevating agents would affect both the LOH in Apc\textsuperscript{Min/+} mice, and mutagenic efficacy in the AOM/DSS model, and is therefore likely to be part of the tumor preventative mechanism. The mechanism by which cGMP can reduce the proliferative compartment of both the intestine and colon of mice is presently unknown. However, this novel interpretation predicts that increasing epithelial cGMP is also likely to reduce tumorigenesis in sporadic and Lynch syndrome–associated lesions that are also a function of proliferation.

Taken together, the results shown here demonstrate that PDE5 inhibitors and GCC agonists can equally suppress intestinal tumorigenesis in mice. The equal efficacy of the two drugs to suppress polypl multiplicity underscores the common mechanism involving elevation of cGMP in the preneoplastic epithelium resulting in a reduced proliferative compartment. A recent report demonstrated that linaclotide administration can reduce proliferation in the colon epithelium of human patients (39). This preclinical study therefore underscores the potential for targeting cGMP signaling for chemoprevention of colorectal cancer in human patients at increased risk.

Disclosures of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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