Exisulind and guanylyl cyclase C induce distinct antineoplastic signaling mechanisms in human colon cancer cells

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Abstract

The nonsteroidal anti-inflammatory drug sulindac is metabolized to sulindac sulfone (exisulind), an antineoplastic agent that inhibits growth and induces apoptosis in solid tumors. In colon cancer cells, the antineoplastic effects of exisulind have been attributed, in part, to induction of cyclic guanosine 3′,5′-monophosphate (cGMP) signaling through inhibition of cGMP-specific phosphodiesterases, which elevates intracellular cGMP, and novel expression of cGMP-dependent protein kinase (PKG) Iβ, the presumed downstream effector mediating apoptosis. Here, inhibition of proliferation and induction of cell death by exisulind was dissociated from cGMP signaling in human colon cancer cells. Accumulation of intracellular cGMP produced by an exogenous cell-permeant analogue of cGMP or a potent agonist of guanylyl cyclase C yielded cytostasis without cell death. Surprisingly, the antiproliferative effects of induced cGMP accumulation were paradoxically less than additive, rather than synergistic, when combined with exisulind. Further, although exisulind induced expression of PKG Iβ, it did not elevate intracellular cGMP and its efficacy was not altered by inhibition or activation of PKG I. Rather, PKG I induced by exisulind may mediate desensitization of cytostasis induced by cGMP. Thus, cytotoxic effects of exisulind are independent of cGMP signaling in human colon cancer cells. Moreover, combination therapies, including exisulind and agents that induce cGMP signaling, may require careful evaluation in patients with colon cancer. [Mol Cancer Ther 2006;5(5):1190–6]

Introduction

Beyond their conventional role as anti-inflammatory, antipyretic, and analgesic agents, nonsteroidal anti-inflammatory drugs have emerged as important chemotherapeutics for preventing and treating various tumors (1, 2). Although the biological effects of nonsteroidal anti-inflammatory drugs are mediated, in part, by inhibition of cyclooxygenase (COX) and prostaglandin synthesis (3), COX- and prostaglandin-independent mechanisms are also central to their antineoplastic activities (4). Indeed, the antitumor nonsteroidal anti-inflammatory drug sulindac is metabolized to the biologically active sulindac sulfide, a COX inhibitor and effective antineoplastic drug (5), and sulindac sulfone (exisulind), which does not inhibit COX but induces apoptosis in human cancers (6, 7). In human colon cancer cells, exisulind inhibits cyclic guanosine 3′,5′-monophosphate (cGMP)–specific cGMP-regulated phosphodiesterases 2 and 5 (8) and induces novel expression of cGMP-dependent protein kinase (PKG) Iβ (9), presumably promoting cGMP-dependent activation of that kinase (10) underlying exisulind-induced cytotoxicity (8–10). However, the precise role of cGMP signaling in mediating the antineoplastic effects of exisulind in human colon cancer cells remains to be defined.

cGMP mediates discrete cellular functions by interacting with specific binding motifs in target proteins, including PKG, cyclic nucleotide–gated (CNG) channels, and phosphodiesterases (11). In intestinal epithelial cells, cGMP-dependent activation of PKG II induces fluid and electrolyte secretion (12, 13). In contrast, in colon cancer cells, cGMP inhibits proliferation by activating CNG channels, promoting Ca^{2+} influx (14, 15). The principal source of cGMP in these cells is guanylyl cyclase C (GCC; ref. 11), the receptor for the endogenous hormones, guanylin and uroguanylin, and the diarrheagenic bacterial heat-stable enterotoxins (ST), which is selectively expressed by intestinal epithelial cells in adult mammals (16, 17).

Although GCC and cGMP regulate proliferation of normal and neoplastic epithelial cells in intestine, the contribution of apoptosis to this process remains unclear. Thus, loss of GCC has been associated with increased apoptosis in intestine (18). Further, targeted inactivation of the mouse guanylin gene increased proliferation without altering apoptosis along the crypt-villus axis (19).

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Moreover, activation of GCC with ST inhibited colon cancer cell proliferation by inducing a cGMP-dependent cell cycle delay, without arrest or apoptosis (14). These observations suggest that GCC ligands represent a new class of agent for treating primary and metastatic colorectal cancer with cytostatic, but unclear cytotoxic, activities (14, 15, 19).

The presumed importance of phosphodiesterase inhibition, which increases intracellular cGMP, and novel induction of PKG, as the downstream effectors mediating cytotoxicity, suggests one paradigm in which combining exisulind and GCC ligands should produce synergistic antineoplastic effects on human colon cancer cells. Specifically, cGMP accumulation induced by GCC ligands should be potentiated by exisulind inhibition of phosphodiesterases resulting in improved cytostatic efficacy (14, 15). Similarly, induction of PKG by exisulind should provide a novel downstream effector by which cGMP induced by GCC ligands mediates cytotoxicity (9). However, recent observations suggest that GCC ligands and exisulind could induce mutually antagonistic signaling mechanisms (20). Thus, induction of cGMP signaling by GCC tonically activates phosphodiesterase mediating bradyphylaxis to cytostasis, which would oppose exisulind signaling (20, 21). Similarly, PKG, whose expression is induced by exisulind, mediates homologous desensitization in cGMP signaling, which opposes GCC-mediated cytostasis (20). In the context of these conflicting mechanisms, the present study examined the interaction between exisulind and cGMP signaling in mediating cytotoxicity in human colon cancer cells, including inhibition of proliferation and induction of cell death.

Materials and Methods

Materials

Fetal bovine serum, DMEM, and the DMEM/F12 were obtained from Mediatech, Inc. (Herndon, VA). The CellTiter 96 AQueous One Solution Cell Proliferation Assay containing the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) was purchased from Promega (Madison, WI). Antibody to human PKG Iβ was obtained from Stressgen Biotechnologies (Victoria, British Columbia, Canada), whereas antibody to human α-tubulin was from Santa Cruz Biotechnology (Santa Cruz, CA). Exisulind and RP-8-br-cGMPS were from EMD Biosciences, Inc. (La Jolla, CA). Native ST was prepared as described (14). Zaprinast, 3-isobutyl-1-methylxanthine, 8-br-cGMP, propidium iodide, DMSO, and all other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Caco-2 (passage 20–40), T84 (passage 50–70), and SW480 (passage 100–120) human colon carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cell cultures were maintained at 37°C (5% CO_2) in DMEM (Caco-2 and SW480) or DMEM/F12 (T84) containing 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum. Cells were fed every 3rd day and split when subconfluent.

Tumor Cell Proliferation

Colon cancer cells were plated at ~70,000/mL and grown in their culture medium supplemented with reagents for the indicated times. Treatments were refreshed every other day. For cell counting, cells were seeded in 24-well plates and the number of adherent cells was quantified on a hemocytometer following trypsinization. Cell proliferation was calculated as the percentage of parallel control cultures containing the corresponding amount of vehicle. For MTS-based proliferation assays, cancer cells were plated in 96-well plates and the number of viable cells was determined by adding 20 μL CellTiter 96 AQueous One Solution Reagent directly to the culture well, incubating for 2 hours, and recording absorbance at 490 nm in a Spectra Count 96-well-plate reader.

Flow Cytometry

Cancer cells were plated in six-well plates (~80,000/mL) and processed as for proliferation studies above. After harvesting by trypsinization, cells were pelleted by centrifugation, washed with PBS, and fixed in ice-cold 75% ethanol for 30 minutes. Then, cells were washed again with PBS, resuspended in 500 μL staining solution (50 μg/mL propidium iodide, 100 μg/mL RNase A, 1 mmol/L EDTA, and 0.1% Triton X-100), and analyzed on a Coulter EPICS XL-MCL flow cytometer. The distribution of cells in different phases of the cell cycle was analyzed using Win MDI software (version 2.8.8) provided by Joseph Trotter (Scripps Research Institute, La Jolla, CA). Cell death was quantified as the number of cells in the sub-G_1 fraction of the cell cycle.

Immunoblot Analyses

Cells treated for 5 days (six-well plate) were washed with cold PBS and lysed in 400 μL cold modified radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl, 1% NP40, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L Na_3VO_4, 1 mmol/L NaF, and 500 μmol/L 3-isobutyl-1-methylxanthine) containing the Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Palo Alto, CA). Cell supernatants were obtained by centrifugation of cell lysates at 15,000 rpm for 20 minutes, and proteins were quantified by the bicinchoninic acid–based method (Pierce Biotechnology, Inc., Rockford, IL). Then, 80 μg proteins per each sample were submitted to 10% PAGEr Gold precast gels (BioWhittaker Molecular Applications, Rockland, ME) before being electrophoretically transferred to nitrocellulose membrane (Osmonics, Inc., Minnetonka, MN). Blots were first probed with affinity-purified rabbit polyclonal antibody for detection of the specific 80 kDa band of human PKG Iβ and then stripped with the Western Blot Stripping Buffer (Pierce Biotechnology) and reprobed using affinity-purified rabbit polyclonal antibody for detection of the specific 60 kDa band corresponding to the human α-tubulin.

cGMP Assay

cGMP was quantified in cancer cells (six-well plate) treated for 5 days with the indicated reagents by RIA.

Reactions were terminated with ice-cold 100% ethanol, and supernatants were separated from pellets by centrifugation (12,000 × *g*, 15 minutes at 4°C) and processed for cGMP determinations (16).

Statistics

Unless otherwise indicated, data are expressed as the mean ± SE of a representative of at least three experiments done in triplicate. Data were analyzed using the unpaired two-tailed Student's *t* test and significance was assumed for $P \leq 0.05$.

Results

Exisulind and cGMP Independently Regulate the Proliferation of Human Colon Carcinoma Cells

ST, a potent GCC agonist (Fig. 1A), and 8-br-cGMP, a membrane-permeant and phosphodiesterase-resistant analogue of cGMP (Fig. 1B), suppressed the proliferation of Caco-2 human colon carcinoma cells, which express GCC (22), confirming that cGMP signaling is coupled with antiproliferation in colon cancer (14, 15). Inhibition of proliferation by GCC signaling was time dependent (Fig. 1A and B) and reflected cytostasis, but not cell cycle arrest or cell death (Fig. 1C; refs. 14, 15). In that context, the antineoplastic effects of exisulind, presumably mediated by activation of cGMP signaling through inhibition of cGMP-specific phosphodiesterases (7, 8), should also induce sustained inhibition of proliferation in those tumor cells (20). Indeed, exisulind eliminated proliferation of Caco-2 cells in a concentration-dependent fashion, with an estimated IC_{50} of 271.21 ± 16.54 μmol/L (Fig. 2B; ref. 8), consistent with concentrations at which this agent produces *in vitro* cytotoxicity (8–10), but higher than those inducing clinically relevant anticancer effects (23). Surprisingly, 8-br-cGMP, which inhibited in a dose-dependent manner the growth of Caco-2 cells (IC_{50}, 4.56 ± 0.21 mmol/L by MTS assay after treatment for 5 days; Fig. 2A), did not potentiate the antineoplastic effects of exisulind (IC_{50} in the presence of 8-br-cGMP, 324.03 ± 32.41 μmol/L; Fig. 2B). Rather, addition of 8-br-cGMP resulted in a less-than-additive increase of exisulind-induced inhibition of proliferation (Fig. 2C). Similar results were obtained in T84 cells (data not shown). The inability of cGMP to potentiate the effects of exisulind (Fig. 2B) and their less-than-additive cumulative effects (Fig. 2C) suggest that these agents regulate distinct antiproliferative pathways.

cGMP Signaling Does Not Mediate Exisulind-Induced Cytotoxicity

Treatment-induced cytotoxicity, including growth arrest and activation of cell death programs, was assessed examining cell cycle progression of tumor cells by flow cytometry. Exisulind inhibited the proliferation of Caco-2 cells by altering their cell cycle distribution (Fig. 3A), inducing a 3-fold increase in sub-G_1 cell fragments associated with cell death and a concomitant decrease in cells in G_0-G_1 (Fig. 3B). In contrast, 8-br-cGMP induced cytostasis (Fig. 3A) without altering the cell cycle distribution of Caco-2 cells (Fig. 3B). Importantly, 8-br-cGMP failed to increase the sub-G_1 fraction of cell death or further alter the proportion of cells in G_0-G_1 induced by exisulind (Fig. 3A and B). Because PKG Iβ may mediate cGMP-dependent apoptosis induced by exisulind in colon tumors (8–10), its role was examined with a specific membrane-permeant inhibitor, RP-8-br-cGMPS (24, 25), which blocks activation of PKG I (9, 10). Exisulind increased PKG Iβ protein expression similarly in various human colon cancer cells (Fig. 4A1; fold induction: Caco-2, 2.01; SW480, 1.76; T84, 1.44; Fig. 4A2). However, RP-8-br-cGMPS, added at least 30 minutes before any other treatment, failed to alter exisulind-induced cell death in those cells (Caco-2 cells, Fig. 4B; T84 cells, Fig. 4C; SW480 cells, data not shown). Moreover, 8-br-cGMP, an effective activator of PKG I (26), did not promote cell death in colon cancer cells (Caco-2 cells, Fig. 4B; T84 cells, Fig. 4C; SW480 cells, data not shown), even in the context of exisulind-induced PKG Iβ expression (Figs. 3 and 4A1). Conversely, in T84 cells, 8-br-cGMP significantly reduced cell death (Fig. 4C). Thus, cell death programs induced by exisulind in colon cancer cells are not mediated by cGMP or activation of PKG.

Antiproliferative Mechanisms Induced by GCC Ligands and Exisulind Negatively Interact in Colon Cancer Cells

Suppression of tumor growth by exisulind may, in part, reflect inhibition of cGMP-specific phosphodiesterases, inducing cGMP accumulation in colon cancer cells (8, 9). However, exisulind failed to significantly increase cGMP in

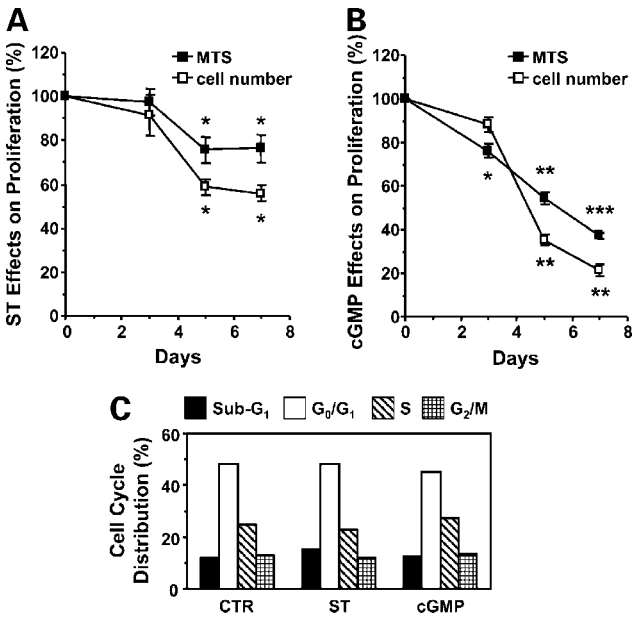


Figure 1. cGMP signaling inhibits colon cancer cell proliferation. Caco-2 cells were treated with 1 μmol/L ST (**A**) or 5 mmol/L 8-br-cGMP (*cGMP*; **B**) for the indicated days. Proliferation was assessed by quantifying either the number of adherent cells or the ability of viable tumor cells to metabolize MTS, as described in Materials and Methods. Results are expressed as the percentage of parallel control incubations treated with the vehicle (PBS). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus respective controls. **C,** a representative experiment quantifying the cell cycle distribution by flow cytometry (see Materials and Methods) of Caco-2 cells treated for 5 d with PBS (*CTR*), ST (1 μmol/L), or 8-br-cGMP (5 mmol/L).

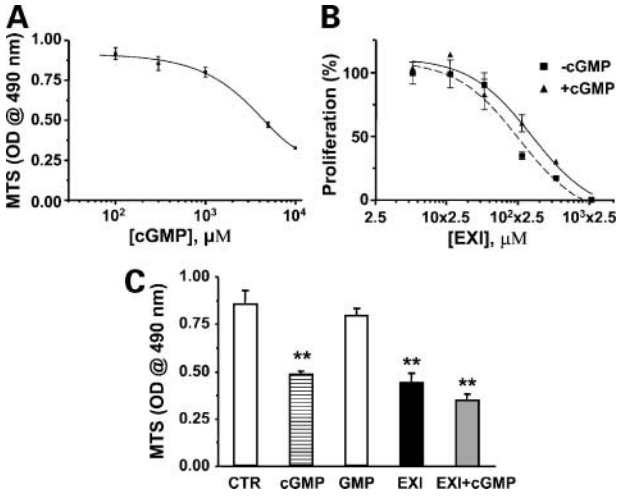


Figure 2. cGMP fails to enhance the antiproliferative effects of exisulind. Concentration dependence of proliferation of human colon cancer cells on 8-br-cGMP (**A**) or exisulind (*EXI*) in the presence or absence of 5 mmol/L 8-br-cGMP (**B**). After 5 d of treatment, Caco-2 cell proliferation was quantified by MTS assay. *OD,* absorbance. In **B**, results are expressed as the percentage of respective controls (the vehicle DMSO for exisulind alone and DMSO plus 8-br-cGMP for exisulind plus 8-br-cGMP) and were subjected to nonlinear regression analyses using sigmoidal dose-response, variable slope (Prism, Graph-Pad). Values of controls were 0.88 ± 0.06 for DMSO and 0.40 ± 0.01 for DMSO plus 8-br-cGMP. **C,** MTS-specific absorbance (490 nm) of Caco-2 cells treated for 5 d with DMSO vehicle (*CTR*), 5 mmol/L 8-br-cGMP, 5 mmol/L 8-br-GMP (*GMP*), 268.53 μmol/L exisulind, or 268.53 μmol/L exisulind plus 5 mmol/L 8-br-cGMP. **, $P < 0.01$, versus controls.

colon cancer cells (Fig. 5A). Zaprinast, a selective phosphodiesterase 5 inhibitor, was also poorly effective, whereas 3-isobutyl-1-methylxanthine, a broad phosphodiesterase inhibitor, significantly elevated (2.4-fold over control) cellular cGMP (Fig. 5A), suggesting that isoforms other than cGMP-specific phosphodiesterases principally mediate cGMP hydrolysis in colon cancer cells. In contrast, activation of GCC by ST produced a striking increase in cellular cGMP (~30-fold; Fig. 5A), demonstrating that synthesis, rather than hydrolysis, plays a dominant role in elevating cGMP in colon cancer cells. Surprisingly, exisulind inhibited the ability of ST to induce maximal intracellular cGMP accumulation (Fig. 5A). In addition, activation of GCC signaling did not alter exisulind-induced cell death (Fig. 5B), demonstrating that intracellular cGMP is unable to support cell death in colon cancer cells. Exisulind also failed to enhance ST-induced inhibition of proliferation, and inhibition of PKG I did not prevent exisulind-mediated inhibition of proliferation (Caco-2 cells, Fig. 5C1; T84 cells, data not shown), underscoring the inability of exisulind to induce productive cGMP signaling coupled to human colon cancer cell proliferation. Finally, the combination of exisulind plus ST produced a less-than-additive inhibition of colon cancer cell proliferation (Fig. 5C2). These observations further underscore the dissociation of exisulind-induced effects on colon cancer cell proliferation from mechanisms mediating cGMP-induced cytostasis.

Discussion

Sulindac prevents carcinogenesis (7) and induces tumor regression (27) in intestine by (*a*) activating death receptor 5 and caspase-8 (28), (*b*) inhibiting peroxisome proliferator–activated receptor δ (29), (*c*) reducing nuclear factor-κB signaling (30), and (*d*) lowering transactivation levels of nuclear β-catenin (31). Although the molecular mechanisms underlying these antineoplastic effects remain unclear, the prodrug sulindac and/or its metabolites contribute to these effects (32). Antineoplastic effects of sulindac (weak) and its reduced metabolite sulfide (strong) result, in part, from their ability to inhibit COX activity by reversibly binding to a hydrophobic channel composing the COX active site (32, 33). In contrast, the oxidative metabolite of sulindac exisulind (sulindac sulfone), which is unable to bind to and inhibit the COX active site, exhibits antineoplastic activity that is COX independent (8, 32, 34).

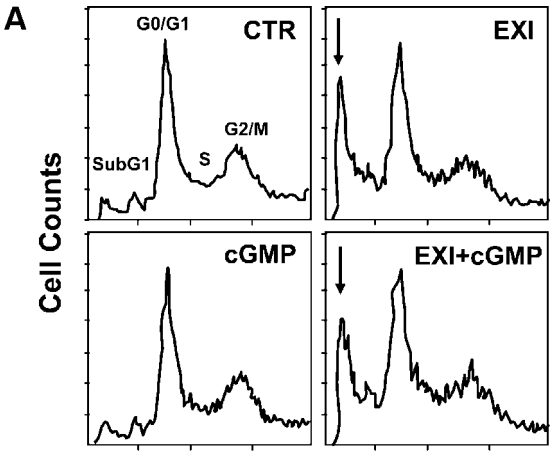
Previous observations suggest that the antineoplastic effects of exisulind may reflect inhibition of cGMP-specific phosphodiesterases, inducing intracellular accumulation of cGMP (7, 8). Activation of cGMP-dependent signaling may mediate apoptosis of colon cancer cells induced by exisulind by inhibiting β-catenin signaling (8) or stimulating c-Jun NH_2-terminal kinase 1 (10). Moreover, in those cells, exisulind induced the novel expression of PKG Iβ, the putative downstream effector of cGMP mediating apoptosis (8–10). These observations suggest that combining exisulind and ligands for GCC, a cGMP-generating cyclase selectively expressed in colorectal tumors, might produce synergistic antineoplastic effects on human colon cancer cells. However, the present observations revealed that cGMP signaling induced cytostasis, rather than cytotoxicity, in colon cancer cells, and administration of GCC ligands or exogenous cGMP failed to enhance exisulind-induced cell death. Further, PKG Iβ did not mediate cytotoxicity induced by exisulind because activation or inhibition of that kinase did not affect the distribution of cells in the sub-G_1 and the G_1-G_0 phases of the cell cycle induced by that agent. Rather, cGMP signaling may oppose cell death programs in colon cancer cells (Fig. 4C), reflecting decreased proliferation associated with cytostasis (18, 20, 35). Conflicting results may reflect the short durations (up to 48 hours) during which the effects of exisulind on PKG expression were examined previously (8–10). Similarly, although exisulind acutely increased intracellular levels of cGMP (9), it did not support sustained elevations of cGMP in colon cancer cells following prolonged incubations (see Fig. 5A). Conversely, cytotoxic effects of exisulind develop over extended incubations (>5 days[1]; ref. 34) and require concentrations in excess of those necessary to block phosphodiesterase activities (8).

Like cell death, GCC signaling failed to enhance inhibition of colon cancer cell proliferation induced by

[1] Personal observation.

exisulind. Rather, combining exisulind and agents inducing cGMP signaling yielded less-than-additive antiproliferative effects. Further, exisulind was fully efficacious in suppressing tumor growth in the presence of a specific inhibitor of PKG I (24, 25), eliminating the involvement of that kinase in mediating the antineoplastic effects of exisulind. Additionally, overexpression of PKG Iβ did not significantly alter the antineoplastic potency of exisulind in colon cancer cells (36). Thus, the present observations support a model in which the antitumor effects of exisulind in colon cancer reflect the sequential recruitment and activation of multiple signaling components, other than exisulind-induced PKG Iβ, which seems to be an epiphenomenon, rather than mechanistically related to exisulind-induced tumor suppression. Indeed, exisulind induced colon cancer cell death by chronically reducing intracellular polyamine levels through specific activation of spermidine/spermine N^1-acetyltransferase (34). Moreover, restoration of intracellular polyamine concentrations prevented sulindac-induced cell death in those tumors (37). Also, inhibition of extracellular signal-regulated kinase 1/2 is absolutely required for the apoptotic effect of exisulind (38, 39). Hence, signaling mechanisms other than those dependent on cGMP mediate the antineoplastic effects of exisulind in colon cancer.

In contrast to exisulind, ligand activation of GCC and exogenous cGMP inhibited proliferation without perturbing the cell cycle distribution of human colon cancer cells, confirming that GCC signaling imposes cytostasis by inducing a generalized cell cycle delay, without cell cycle arrest or apoptosis (14). These effects on cancer cells may recapitulate the physiologic role of GCC in regulating the transition from proliferation to differentiation along the crypt-to-villus axis in intestine. Indeed, mice in which GCC signaling was eliminated by targeted disruption of the genes encoding guanylin or GCC exhibited increased proliferation (19) or apoptosis (18), respectively, in intestinal crypts. In that context, although exisulind induced the expression of the canonical receptor for cGMP, PKG Iβ, this agent did not potentiate the effects of ST and 8-br-cGMP on



B

	G_0/G_1	S	G_2/M	Sub-G_1
CTR	48.5±0.8	24.0±0.8	16.0±1.2	10.3±1.8
cGMP	46.0±3.2	25.4±0.8	16.2±1.4	11.1±1.4
EXI	31.6±3.9	22.2±2.1	13.6±1.7	31.6±5.5
cGMP+EXI	31.2±3.1	21.5±2.3	12.5±1.3	33.6±4.1

Figure 3. cGMP fails to enhance exisulind-induced cytotoxicity. **A,** representative experiment of cell cycle distribution by flow cytometry of Caco-2 cells treated for 5 d with the DMSO vehicle, 5 mmol/L 8-br-cGMP, 268.53 μmol/L exisulind, or 268.53 μmol/L exisulind plus 5 mmol/L 8-br-cGMP. *Arrows,* sub-G_1 fraction of dead cells induced by exisulind. **B,** Caco-2 cells were treated and analyzed as in **A**. Cell cycle distribution from four independent experiments was quantified with the Win MDI software (version 2.8.8). *Columns,* mean; *bars,* SE. **, $P < 0.01$ versus controls.

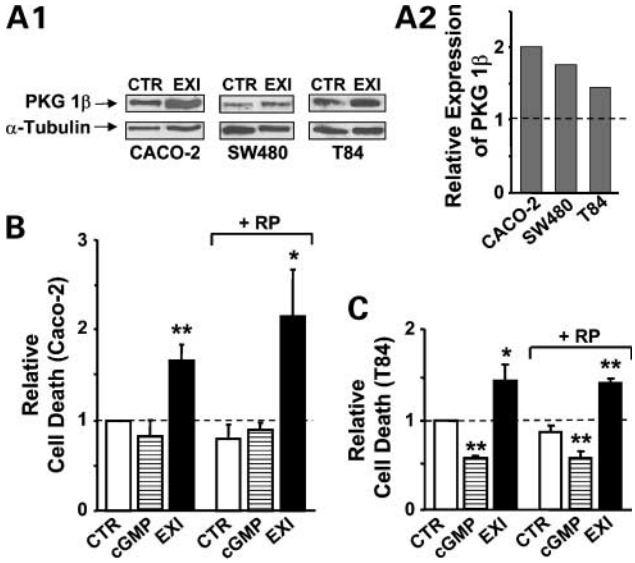


Figure 4. Activation of PKG Iβ does not mediate exisulind-induced cell death. **A1,** exisulind induced the expression of PKG Iβ in different human colon carcinoma cells. Tumor cells were treated for 5 d with the DMSO vehicle or 268.53 μmol/L exisulind. Representative of at least three experiments. **A2,** expression levels of PKG Iβ relative to the respective α-tubulin controls were quantified by densitometry from experiment in **A1**. Caco-2 (**B**) or T84 (**C**) cells were treated for 5 d with DMSO, 5 mmol/L 8-br-cGMP, and 268.53 μmol/L exisulind, in the presence or absence of the membrane-permeant inhibitor of PKG I RP-8-br-cGMPS (*RP*), used at 50 μmol/L to completely inhibit PKG I (24, 25). The sub-G_1 fraction of the cell cycle, reflecting cell death, was quantified by flow cytometry. Data are expressed as cell death relative to sub-G_1 values (7.85 ± 0.69 for Caco-2 in **B** and 18.33 ± 2.10 for T84 in **C**) of DMSO controls. *Columns,* mean of three independent experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$, versus respective controls.

proliferation, indicating that PKG Iβ is not a downstream effector of cytostasis imposed by cGMP in colon cancer cells. Rather, cytostasis induced by cGMP signaling was mediated by CNG channel activation, resulting in Ca^{2+} influx in human colon cancer cells (15).

The present observations underscore the cGMP-independent nature of antineoplastic signaling by exisulind. Recent studies showed that activation of PKG, which is induced by exisulind (8–10), mediates rapid (tachyphylaxis) and delayed (bradyphylaxis) desensitization of cGMP-induced cytostasis through phosphorylation that inhibits CNG channels and activates cGMP-specific phosphodiesterases, respectively, in human colon cancer cells (20). Indeed, inhibition of PKG was required for enduring cytostasis induced by GCC signaling (20). Thus, exisulind, which induces PKG expression (Fig. 4A), and GCC ligands, whose signaling undergoes desensitization mediated by PKG (20),

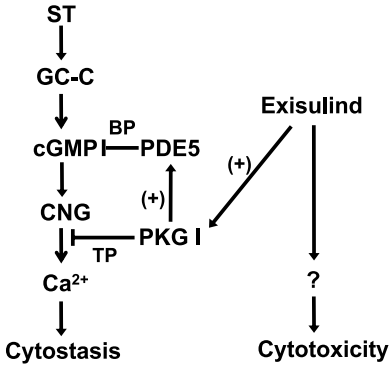


Figure 6. Proposed mechanisms of interfering antineoplastic signaling induced by exisulind and GCC ligands in human colon cancer cells. See Discussion for description. *TP,* tachyphylaxis; *BP,* bradyphylaxis.

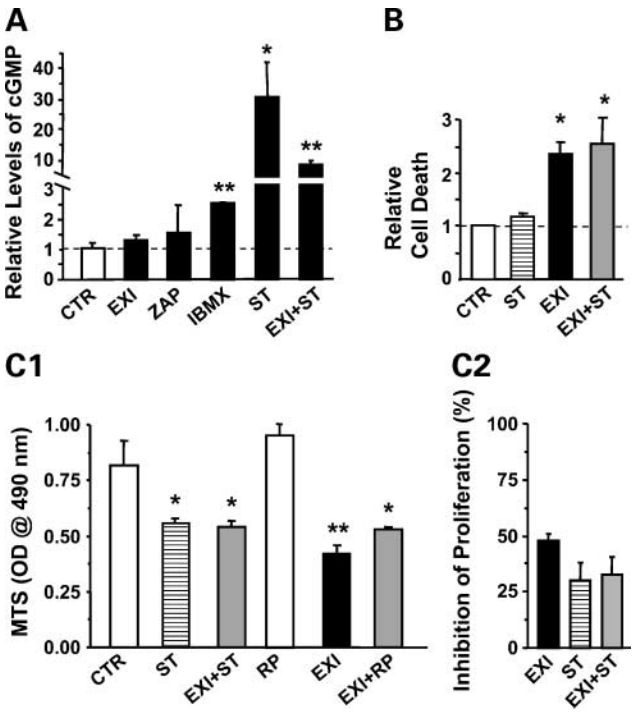


Figure 5. Antineoplastic effects of exisulind and GCC are separated. **A,** cGMP accumulation in Caco-2 cells treated for 5 d with DMSO, exisulind (268.53 μmol/L), zaprinast (*ZAP*, 10 μmol/L), 3-isobutyl-1-methylxanthine (*IBMX*; 1 mmol/L), ST (1 μmol/L), or exisulind plus ST. Data, normalized to milligrams of proteins, are relative levels of cGMP, compared with the control (1.26 ± 0.18 pmol/mg protein). *Columns,* mean of a representative experiment done in duplicate, with RIAs done in triplicate; *bars,* SE. **B,** percentages of Caco-2 cell death in the sub-G_1 phase of the cell cycle quantified by flow cytometry after treatment for 5 d with ST (1 μmol/L) and 268.53 μmol/L exisulind, alone or in combination. Data are expressed as cell death relative to sub-G_1 values (11.66 ± 1.47) of DMSO controls. *Columns,* mean of four independent experiments; *bars,* SE. **C1,** proliferation quantified by MTS assay of Caco-2 cells treated for 5 d. Treatments used are DMSO controls, 1 μmol/L ST, 268.53 μmol/L exisulind, and 50 μmol/L RP-8-br-cGMPS. In **C2**, data obtained in **C1** are expressed as {100 − [(MTS absorbance in the presence of the indicated treatment) / (MTS absorbance in the control incubation) × 100]}. *, $P < 0.05$; **, $P < 0.01$, versus controls.

induce molecular mechanisms with potential negative interactions that yield less-than-additive antiproliferative effects in human colon cancer cells (see Figs. 2C and 5C). Additionally, induction of PKG expression, and the resultant activation of phosphodiesterase 5, likely underlies the inability to produce sustained elevations of cGMP and the inhibition of GCC ligand-induced cGMP accumulation by exisulind in human colon cancer cells (see Fig. 5A). However, the precise mechanisms of interaction between GCC ligands and exisulind with respect to PKG expression and activity remain unclear.

Taken together, these observations show that exisulind and GCC ligands induce signaling mechanisms that mediate separate and distinct antineoplastic effects in human colon cancer cells (Fig. 6). Exisulind induces cytotoxicity through as yet unspecified cGMP-independent mechanisms. Conversely, GCC induces cytostasis specifically by mediating the accumulation of cGMP, which activates CNG channels and induces influx of Ca^{2+} (15). Rather, these signaling mechanisms may be antagonistic, wherein PKG induced by exisulind mediates tachyphylaxis and bradyphylaxis of cytostasis induced by GCC ligands (20). In the absence of synergy and in the context of possible antagonistic interactions, the combination of exisulind and agents that induce cGMP signaling may require careful evaluation for the treatment of patients with colorectal cancer.

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