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3′,5′-Cyclic diguanylic acid (c-di-GMP) inhibits basal and growth factor-stimulated human colon cancer cell proliferation

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Abstract

The novel cyclic dinucleotide, 3′,5′-cyclic diguanylic acid, cGpGp (c-di-GMP), is a naturally occurring small molecule that regulates important signaling mechanisms in prokaryotes. Recently, we showed that c-di-GMP has "drug-like" properties and that c-di-GMP treatment might be a useful antimicrobial approach to attenuate the virulence and pathogenesis of *Staphylococcus aureus* and prevent or treat infection. In the present communication, we report that c-di-GMP ($\leqslant$50 μM) has striking properties regarding inhibition of cancer cell proliferation in vitro. c-di-GMP inhibits both basal and growth factor (acetylcholine and epidermal growth factor)-induced cell proliferation of human colon cancer (H508) cells. Toxicity studies revealed that exposure of normal rat kidney cells and human neuroblastoma cells to c-di-GMP at biologically relevant doses showed no lethal cytotoxicity. Cyclic dinucleotides, such as c-di-GMP, represent an attractive and novel "drug-platform technology" that can be used not only to develop new antimicrobial agents, but also to develop novel therapeutic agents to prevent or treat cancer.

Keywords: 3′,5′-Cyclic diguanylic acid; Cyclic diguanylate; Cyclic di-guanosine-monophosphate; cGpGp; Colon cancer; Acetylcholine; Epidermal growth factor; Drug; Therapy; Therapeutic; Prevention; Treatment

Cells use selected small molecules, commonly referred to as second messengers, to regulate cell function. Besides calcium, the best-known second messengers are cyclic adenosine- and guanosine monophosphates, cAMP and cGMP, respectively. Both prokaryotes and eukaryotes use cAMP, whereas cGMP is used primarily by eukaryotes. Recently, a novel guanosine-based cyclic dinucleotide, 3′,5′-cyclic diguanylic acid, cGpGp (c-di-GMP), was identified as a novel bacterial signaling molecule [1] (Fig. 1). Evidence by us and others suggests that c-di-GMP acts as an important signaling molecule in a variety of bacterial species influencing various aspects of physiology and behavior [1–8]. These findings indicate that c-di-GMP signaling is widespread and that c-di-GMP, like cAMP and cGMP, is a ubiquitous signaling molecule. We also showed that c-di-GMP has antimicrobial activity against bacterial pathogens such as *Staphylococcus aureus* and might be used to inhibit and treat infection [9].

Approximately 160,000 Americans develop colon cancer and 60,000 Americans die from the disease each year [10]. Although surgical treatment is effective for early lesions, treatment for advanced, particularly metastatic, disease is not very effective. Survival rates <50%

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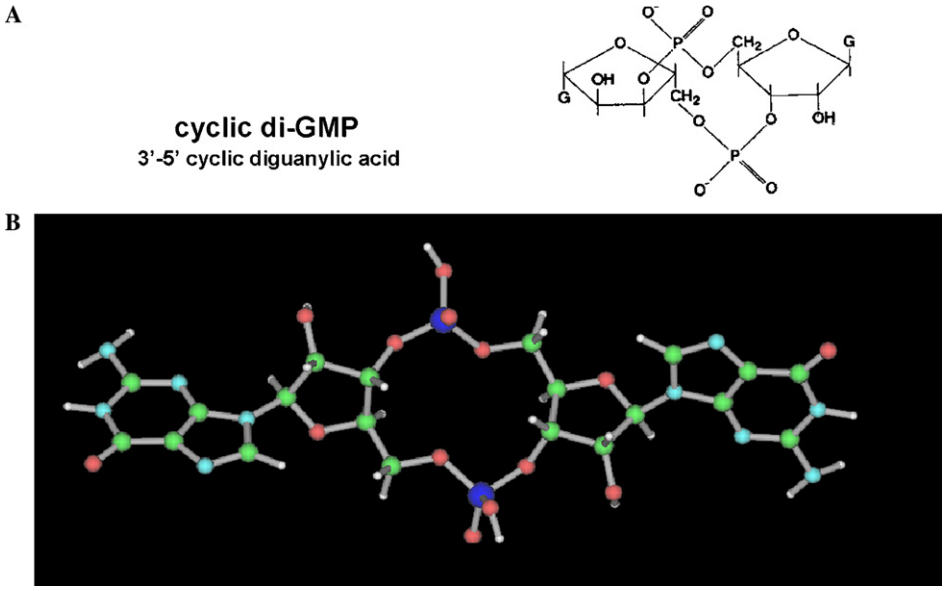

Fig. 1. Molecular structure of c-di-GMP. (A) Line diagram. (B) Ball and stick structure.

are observed at 5 years after diagnosis for patients with invasive and metastatic colon cancer. Hence, a safe therapeutic (anti-cancer) agent that inhibits colon cancer progression, and possibly causes cancer involution and regression would be extremely beneficial. Based on our recent findings with c-di-GMP on *S. aureus* [9] and studies on T-cell regulation that showed that c-di-GMP causes cell cycle arrest in lymphoblastoid cells [11], we speculated that cyclic dinucleotides, like c-di-GMP, could be used as therapeutic agents for the prevention or treatment of cancer. The present study shows that c-di-GMP treatment significantly inhibits basal and growth factor-stimulated colon cancer cell proliferation in vitro at concentrations that are not lethally cytotoxic.

Materials and methods

Chemicals and reagents. c-di-GMP was synthesized in pure form using a recently described novel method that produces a pure, high yield preparation of c-di-GMP diammonium salt [12,13]. Structurally related nucleotides [guanosine 3′,5′-cyclic monophosphate (cGMP) and guanosine 5′-monophosphate (5′-GMP); both from Sigma)] were also examined. Dulbecco's modified Eagle's medium, MEM non-essential amino acids, penicillin, streptomycin, and G418 were from Gibco-BRL. All other chemicals were obtained from Sigma or Fisher.

H508 colon cancer cell culture. H508 human colon cancer cells were grown in RPMI 1640 (ATCC) supplemented with 10% fetal bovine serum (FBS) (Biowhittaker). Adherent cultures were passaged weekly at subconfluence after trypsinization. Cultures were maintained in incubators at 37 °C in an atmosphere of 5% CO_2 and 95% air.

H508 colon cancer cell proliferation assay. Cell proliferation was determined using the sulforhodamine B (SRB) colorimetric assay [14]. Cells were seeded in 96-well plates (Corning Glass Works, Corning, NY) at approx. 10% confluence and allowed to attach for 24 h. Growth medium was removed, and fresh medium without FBS and containing the indicated concentration of test agent was added. Cells were incubated for the indicated periods of time at 37 °C in an atmosphere of 5% CO_2 and 95% air. After incubation, cells were treated for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Protein-bound dye was extracted with unbuffered 10 mM Tris base. Absorbance was measured at 560 nm using a computer-interfaced, 96-well microtiter plate reader.

Toxicity assays in normal rat kidney cells. We tested the toxicity of c-di-GMP in independent cell lines. In these studies, acute (24 and 72 h) toxicity in NRK 52 E cells (normal rat kidney cells) was determined using the Alamar blue assay. NRK 52E cells are a rat renal tubule epithelial cell line originally cloned from a mixed culture termed NRK. The Alamar blue assay is designed to measure the proliferation of cell lines and is used routinely to determine the cytotoxicity of various chemicals. Alamar blue dye functions as a REDOX indicator and exhibits both fluorescence and colorimetric change in the appropriate oxidation-reduction range relating to cellular metabolic reduction. It is minimally toxic to most living cells.

Chemical toxicity on normal rat kidney cells. To assess chemical toxicity, the Alamar blue assay was performed using NRK 52E cells plated at near confluence in 96-well tissue culture plates (Costar). Cells were maintained in DMEM containing 10% FBS and penicillin/streptomycin in tissue culture flasks. Cells were harvested by trypsinization, resuspended in the basal medium, and counted. NRK 52E cells were plated at a high density of $4–5 \times 10^4$ cells/well. After a 24-h attachment/acclimation interval, the tissue culture media were aspirated and replaced with the appropriate test solution. For most experiments, eight wells were used for each solution, i.e., each solution was tested in 1 column of eight replicate wells. In general, each experiment utilized the following design: 1 column served as a baseline control for the plate (no cells, plastic surface only), 1 column served as the negative control (the basal, serum-containing medium), 1 column served as the solvent control (DMEM, serum-free), 1 column served as a positive cell death control (sterile, deionized water, which resulted in osmotic destruction of the cells), and eight columns were used to test varying concentrations of c-di-GMP (2–400 μM). Plates were incubated for 24 or 72 h and observed under phase contrast microscopy. All solutions were aspirated and replaced with fresh DMEM containing Alamar blue dye (10% vol/vol). Plates were incubated at 37 °C for an additional 6 h after which fluorescence was measured using a fluorimetric plate reader (Cytofluor). Data were collected as raw fluorescence units and expressed as a percentage of the solvent control

using average values from all eight wells for each group (mean fluorescence test/mean fluorescence control × 100 = % control fluorescence).

Cell proliferation assay on normal rat kidney cells. To assess potential effects on cell proliferation, the Alamar blue assay was performed using NRK 52E cells. Cells were harvested as described for the toxicity assay, seeded in 96-well tissue culture plates at a low density of about 3×10^3 cells/well, and evaluated over a 72-h time frame. Cells were allowed to attach for 3 h in the basal, serum-containing DMEM. After attachment, the media were aspirated and replaced with test solutions according to the design described for the toxicity assay with the addition of the Alamar blue dye to each solution (as 10% vol/vol). The reduction of Alamar blue dye was measured in a fluorimetric plate reader (Cytofluor) at 24, 48, and 72 h. Morphological observation was also performed to ensure that the cell patterns matched the fluorescence data. Data were collected as raw fluorescence units and expressed as a percentage of the solvent control using the average values from all eight wells used for each group (mean fluorescence test/mean fluorescence control × 100 = % control fluorescence) at each time period. As a further check to ensure that Alamar blue was not itself exerting some growth or inhibitory effects, the experiments were also performed by plating cells at the lower density with exposures for 72 h without Alamar blue in the media. After the 72-h growth period, the media were aspirated and fresh DMEM containing Alamar blue (10% vol/vol) was added to all wells. Cells were further incubated in the Alamar blue solution for 6 h and fluorescence was measured, and data were collected and expressed as described.

Toxicity assays in human neuroblastoma cells. SH-SY5Y human neuroblastoma cells were cultured in DMEM/Ham's F-12 (1:1) media, supplemented with 10% FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM); the cell culture medium was replaced every two days. Cultures were maintained at 37 °C in 95% air–5% CO_2 in a humidified incubator. A standard curve established the linear range for the assay of approximately 1000–7000 cells/well for a 96-well plate. All data reported were within this range. MTS assay concentration–response curves were performed as follows: 1800 cells/well were plated in 96-well plates. After 30 h, media were changed to include no toxicant (control) or toxicant at varying concentrations. The cells were then exposed for 48 h, the media were removed, and 100 μl minimum essential medium without phenol red and 20 μl MTS reagent were added. Cells were incubated, and 2 h later, plates were measured at 490 nm on an ELx 808 microplate reader (Bio-Tek Instruments, Winooski, VT). EC_{50} values were determined via nonlinear regression using Prism software (GraphPad, San Diego, CA).

Statistical analysis. All graphs show the mean ± SEM of three separate experiments. Statistical calculations were performed using Student's two-tailed unpaired *t* test assuming normal distribution with equal variance. Statistical significance is given by the number of *asterisks* (*$p < 0.05$; **$p < 0.005$).

Results and discussion

We showed recently that c-di-GMP has antimicrobial activity against *S. aureus*, thereby suggesting potential therapeutic applications of the cyclic dinucleotide in the prevention or treatment of bacterial infection [9]. A study investigating T-cell regulation showed that c-di-GMP promotes cell cycle arrest in the lymphoblastoid CD4+ Jurkat cell line [11]. In the current study, we hypothesized that c-di-GMP could be used clinically as a novel therapeutic agent in the treatment of cancer and found that c-di-GMP significantly inhibits the proliferation of human colon cancer cells in vitro.

c-di-GMP inhibits proliferation of human colon cancer cells

We examined the potential therapeutic actions of c-di-GMP on basal and growth factor-stimulated proliferation of cells derived from a moderately differentiated human cecal adenocarcinoma (H508 cells) that express M_3 muscarinic (M_3R) and epidermal growth factor (EGF) receptors (EGFR) [15]. Cell proliferation was determined using the validated sulforhodamine B (SRB) colorimetric assay [14]. H508 cells were incubated in the absence or presence of a growth stimulant [acetylcholine (300 μM) and EGF (1 ng/ml)] alone or with increasing concentrations of c-di-GMP (0.5–50 μM). After a 5-day incubation, the highest concentration of c-di-GMP tested, 50 μM, reduced even basal H508 cell proliferation by approx. 15% ($p < 0.005$ compared to control, Student's unpaired *t* test, Fig. 2). Strikingly, increasing concentrations of the cyclic dinucleotide progressively inhibited acetylcholine- and EGF-induced cell proliferation ($p < 0.05$–0.005 compared to stimulant alone). Moreover, with 50 μM c-di-GMP, acetylcholine- and EGF-induced proliferation was reduced to basal levels ($p < 0.005$, Fig. 2). Since the lower concentrations of c-di-GMP did not significantly alter basal proliferation, we believe that these lower concentrations do not induce apoptosis. However, at 50 μM c-di-GMP, basal proliferation was inhibited, indicating that apoptosis might be responsible although it was not observed by microscopic examination of the cells (Fig. 2).

We then tested whether structurally related guanosine nucleotide analogs [guanosine 3′,5′-cyclic monophosphate (cGMP) and guanosine 5′-monophosphate

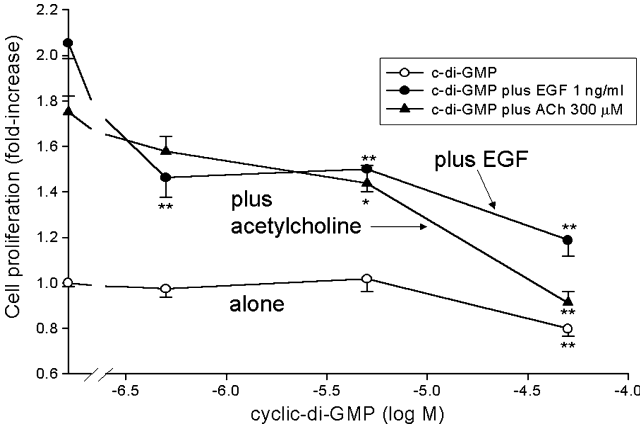

Fig. 2. Increasing concentrations of c-di-GMP inhibit basal, and acetylcholine- and EGF-stimulated colon cancer cell proliferation. H508 cells were treated with water, acetylcholine (300 μM), and EGF (1 ng/ml) for 5 days at 37 °C. Cell proliferation was determined by the sulforhodamine blue (SRB) colorimetric assay [14]. Results are means ± SEM of three separate experiments. * and ** indicate values significantly different than those observed in the presence of water, acetylcholine, and EGF alone ($p < 0.05$ and $p < 0.005$, respectively, unpaired Student's *t* test).

(5′-GMP)] inhibited colon cancer cell proliferation. These experiments were performed to test the specificity of c-di-GMP in the inhibition of cancer cell proliferation and to rule out the possibility that effects on cancer cells were due to the presence of extracellular nucleotides in general or guanosine nucleotide analogs. 5′-GMP and cGMP were also chosen as the structure of c-di-GMP is somewhat similar to those of two cGMP molecules being linked by a 3′–5′ phosphodiester bond and because 5′-GMP is a breakdown product of c-di-GMP. At a concentration of 50 μM, neither of the guanosine analogs tested, cGMP and 5′-GMP, altered basal proliferation of H508 cells after 5 days incubation. However, both analogs reduced acetylcholine- and EGF-stimulated proliferation. Both analogs were less potent in this action than c-di-GMP (Fig. 3).

In H508 colon cancer cells, post-M_3R and EGFR signaling requires activation of the p44/42 (ERK1/2) mitogen-activated protein kinase (MAPK) cascade [15]. c-di-GMP did not alter acetylcholine- and EGF-induced activation of the p44/42 MAPK signaling cascade (not shown). This finding indicates that the actions of the cyclic dinucleotide are not mediated by inhibition of ligand–receptor interaction, but, as observed in Jurkat cells, are more likely mediated by intra-nuclear effects of the agent on cell cycle regulation.

These results highlight the importance, novelty, and perhaps specificity of c-di-GMP in its mechanism of action on this colon cancer cell line. The precise molecular mechanism underlying inhibition of H508 cell proliferation is not yet fully understood but is being studied by us. Nonetheless, our findings indicate clearly that c-di-GMP arrests both basal and growth factor-stimulated proliferation of human colon cancer cells. Since neoplastic regulation of cell growth in different organs is similar, we propose that our findings might be applicable to other cancer cell types. Lower concentrations of c-di-GMP did not alter basal cell proliferation. Hence, it is unlikely that these concentrations induce apoptosis. With 50 μM c-di-GMP basal proliferation was inhibited, so apoptosis is a possibility although it was not observed by microscopic examination of cells.

Cytotoxicity tests in normal rat kidney cells

When confluent NRK cells were exposed to c-di-GMP for 24 h, there was little evidence of cytotoxicity (Table 1). When the exposure period was increased to 72 h, inhibition of cell proliferation was observed with 200 and 400 μM c-di-GMP but no lethal cytotoxicity (Table 1). Fluorescence decreased 36% and 14% with

Table 1
Cytotoxicity of cyclic-di-GMP on normal rat kidney cells using the Alamar blue assay

c-di-GMP (μM)	Fluorescence (% control)		
	Confluent		Non-confluent
	24-h	72-h	72-h
20	98.5	98.0	97.0
40	99.4	98.0	99.0
100	99.7	92.2	87.0
200	101.6	64.2	86.0
400	96.2	40.0	3.0

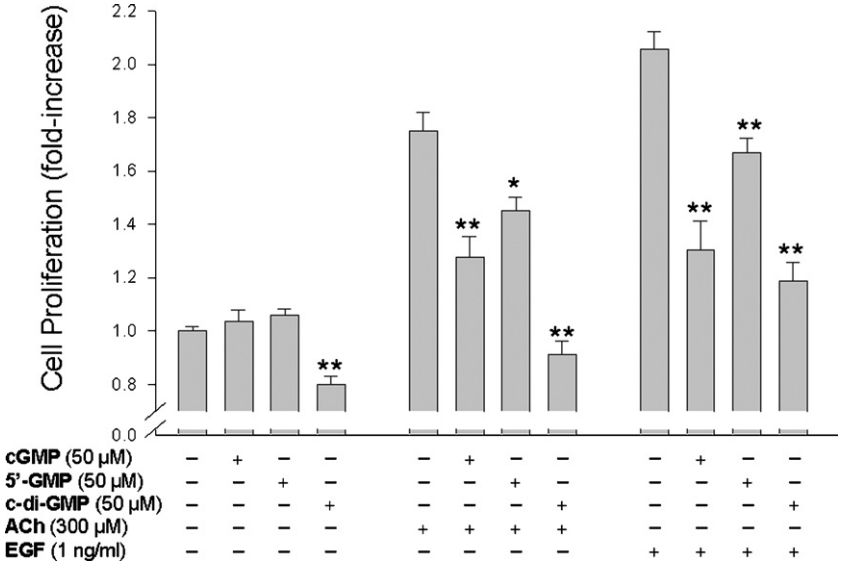

Fig. 3. Effects of c-di-GMP and GMP analogs on basal, and acetylcholine- and EGF-stimulated colon cancer cell proliferation. H508 cells were treated with the indicated concentrations of cGMP, 5′-GMP, and c-di-GMP alone, and in the presence of acetylcholine and EGF for 5 days at 37 °C. Cellular proliferation was determined by the sulforhodamine blue (SRB) colorimetric assay [14]. Results are means ± SEM of three separate experiments. * and ** indicate values significantly different than those observed in the presence of water, acetylcholine, and EGF alone ($p < 0.05$ and $p < 0.005$, respectively, unpaired Student's t test).

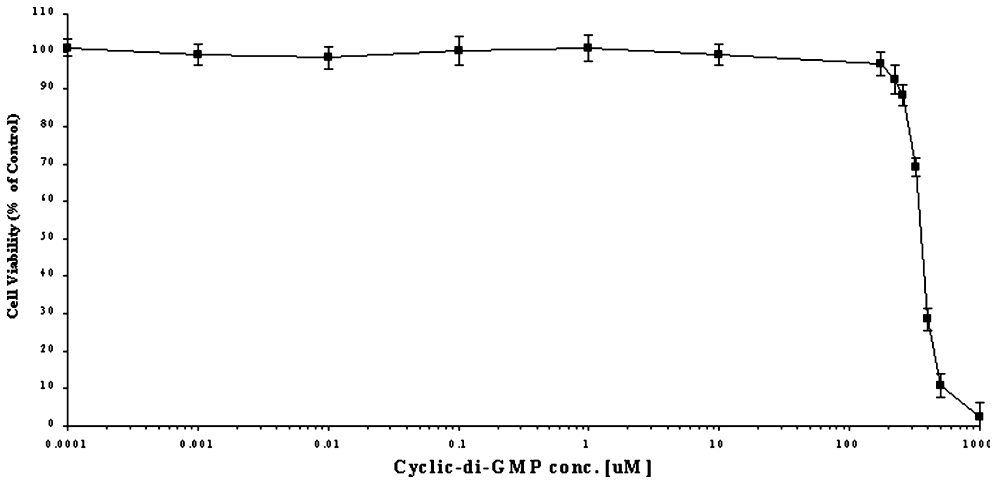

Fig. 4. Concentration-dependent effects of c-di-GMP on cell viability in SH-SY5Y human neuroblastoma cells. Cell viability (% control) was determined by the MTS colorimetric assay. Values represent means ± SEM of three experiments.

200 μM c-di-GMP in 72 h confluent and non-confluent cells, respectively. There was no decrease in fluorescence relative to the solvent control in the cells exposed to 20, 40, and 100 μM of the compound. For comparison, the positive control (deionized water) reduced fluorescence relative to control by 100%, indicating maximal cytotoxicity. Hence, at all relevant biological concentrations, c-di-GMP demonstrates no lethal cytotoxicity on normal rat kidney cells.

When c-di-GMP (200 and 400 μM) was evaluated on non-confluent NRK cell proliferation, the higher dose consistently retarded cell proliferation to the extent that there was little growth over the 72-h period of exposure but no lethal cytotoxicity. The 200-μM dose demonstrated retardation of cell proliferation that was much less pronounced than the 400-μM dose and by 96 h, the fluorescence in the former group was 85% of control. One hundred micromolar c-di-GMP caused a transient reduction in proliferation at 48 and 72 h (75% and 87% of control fluorescence, respectively); however, by 96 h the cells had proliferated to a degree similar to the solvent control (94% control fluorescence) further suggesting no lethal cytotoxicity (data not shown). Cell proliferation was not altered by 5, 25 or 50 μM c-di-GMP (data not shown).

Cytotoxicity tests in human neuroblastoma cells

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay is a quantitative colorimetric assay for measurement of cellular proliferation, cytotoxicity, and viability. MTS is converted to a formazan product only in living cells. MTS is similar to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is widely used in cytotoxicity assays. The difference is that the formazan product for the MTS reagent is soluble in media while the MTT reagent is not.

To study the cytotoxicity of c-di-GMP in SH-SY5Y human neuroblastoma cells, increasing concentrations (0.0001–1000 μM) were added to the culture medium and cell viability was assessed by the MTS assay. The cyclic dinucleotide dose-dependently decreased cell viability after 48 h (Fig. 4). The calculated EC_{50} was 350 μM. Up to 100 μM there was no effect on cell viability; at greater concentrations, a sharp decline in cell viability was observed. Based on these data, the concentration range of 0.0001–100 μM c-di-GMP may be considered safe (Fig. 4).

Our results show clearly that c-di-GMP inhibits basal and growth factor-stimulated proliferation of human colon cancer cells in vitro. Our study also indicates that c-di-GMP is safe and non-cytotoxic at concentrations that inhibit cancer cell proliferation. The striking ability of c-di-GMP to significantly inhibit colon cancer cells in vitro clearly suggests that further studies to test the efficacy of this molecule in an animal cancer model are warranted. We recently suggested that since the structure of c-di-GMP is known and the molecule shows several ideal "drug-like" properties, cyclic dinucleotide analogs can be synthesized and potentially developed into a drug-platform for a variety of diseases or disorders [9]. Based on our earlier findings [9] and the results reported here, we propose that cyclic dinucleotides, such as c-di-GMP, represent a novel small molecule "drug-platform technology" that can be developed as antimicrobial and cancer therapeutic agents.

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