Role of 6-Gingerol in Reduction of Cholera Toxin Activity In Vitro and In Vivo

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Vibrio cholerae is one of the major bacterial pathogens responsible for the devastating diarrheal disease called cholera. Chemotherapy is often used against V. cholerae infections; however, the emergence of V. cholerae with multidrug resistance (MDR) toward the chemotherapeutic agents is a serious clinical problem. This scenario has provided us with the impetus to look into herbal remediation, especially toward blocking the action of cholera toxin (CT). Our studies were undertaken to determine the antidiarrheal potential of 6-gingerol (6G) on the basis of its effect on CT, the virulence factor secreted by V. cholerae. We report here that 6G binds to CT, hindering its interaction with the GM1 receptor present on the intestinal epithelial cells. The 50% inhibitory concentration (IC50) was determined to be 10 μg/ml. The detailed mechanistic study was conducted by enzyme-linked immunosorbent assay (ELISA), fluorescence spectroscopy, and isoelectric focusing. These results were validated with in vitro studies performed with the CHO, HeLa, and HT-29 cell lines, whereas a rabbit ileal loop assay was done to estimate the in vivo action, which confirms the efficacy of 6G in remediation of the cholera-generating effects of CT. Thus, 6G can be an effective adjunctive therapy with oral rehydration solution for severe CT-mediated diarrhea.

Cholera is a severe and potentially life-threatening diarrheal disease caused by toxigenic strains of a Gram-negative organism, Vibrio cholerae, claiming almost 2 million lives per year (1). In addition to severe cholera epidemics on the Indian subcontinent, major outbreaks have occurred during the last 2 decades in several countries (2). The ongoing cholera epidemic in Haiti, which began in October 2010, has signaled the return of cholera to the Western Hemisphere (3) and has raised significant concern for development of improved therapeutic strategies.

The hallmark symptom of cholera is profuse water loss, and this is caused primarily by cholera toxin (CT), an AB5 toxin, which is released into the intestinal lumen of the human carrier after colonization by V. cholerae in the small intestine (4). The five B subunits of CT together form a pentamer that has 5 identical receptor binding sites through which CT interacts with receptor ganglioside GM1 on the surface of the human intestinal epithelial cells. The A subunit is then internalized, and in the cytosol it ADP-ribosylates G protein Gαs, which continuously stimulates the production of cyclic AMP (cAMP). The resulting persistent elevation in cAMP level activates cystic fibrosis transmembrane conductance regulator (CFTR) (5–7), causing dramatic efflux of ions and water from infected enterocytes, leading to severe “rice-water” diarrhea, which is the attribute of cholera.

The principal method for management of cholera is oral rehydration solution (ORS) (8). Though ORS can effectively aid in recovery from dehydration and loss of electrolytes, it cannot reduce the severity and duration of diarrhea. Use of antibiotics is therefore recommended to decrease the duration and volume of diarrhea (9).

Unfortunately, through mutation and the selective pressure exerted by the antimicrobial agents, V. cholerae strains increasingly have been becoming resistant to commonly used antimicrobial agents. In the past 2 decades, most of the V. cholerae strains in countries where cholera is endemic have become resistant to many antimicrobial agents, including tetracycline, ampicillin, nalidixic acid, streptomycin, sulfonamides, trimethoprim, gentamicin, ciprofloxacin, etc. (10, 11). The emergence and spread of multidrug-resistant (MDR) pathogenic bacteria have created the need for the development of novel therapeutic agents. This led to the development of new therapeutic strategies inhibiting bacterial virulence factors. Since the binding of cholera toxin B subunit (CT-B) to the GM1 receptor is the key step in the translocation of toxin to intestinal cells, it is an attractive target for the development of drugs that would prevent the action of CT (12).

Several natural products from plant sources have been isolated and used as pharmaceutical agents (13). Ginger, the rhizome of Zingiber officinale, is a common herb with a history of medicinal use as an antiemetic, diuretic, and antipyretic and has broad beneficial effects on the gastrointestinal tract (14). Among the pungent components of ginger, 6-gingerol (6G) exhibits diverse pharmacological effects of ginger (15, 16).

In the present study, we investigated the effect of 6G on V. cholerae-mediated infection and analyzed the mechanism of action of 6G on CT leading to reduction of cholera. Finally, we studied its effects on CT-mediated actions in vitro, such as cell morphology, short-circuit current alteration, and intracellular cAMP levels, and in vivo using fluid accumulation in the ligated rabbit ileal loop model. Collectively our findings indicate that 6G is effective against CT intoxication both in vitro and in vivo in conjunction with specific antibiotic therapy.

MATERIALS AND METHODS

Materials. CT, CT-B, GM1, streptomycin, and 6G were purchased from Sigma-Aldrich. CT-containing V. cholerae supernatant was prepared as follows. V. cholerae O395 was grown for 16 h, and the cells were collected...
by centrifugation, washed and suspended in phosphate-buffered saline (PBS), and sonicated. The debris was further removed by centrifugation, and the supernatant was used for further experimentation.

**Bacterial strains and growth conditions.** *V. cholerae* O395 cells carrying a streptomycin resistance marker were grown in Luria-Bertani (LB) medium with 1 mg/ml streptomycin and maintained at −70°C in LB medium containing 20% (vol/vol) glycerol (Qualigens, India) or Mueller-Hinton (MH) broth supplemented with 1 mg/ml streptomycin.

**Susceptibility testing and growth rate analysis.** *V. cholerae* cells were grown for 16 h, 100 μl of culture (equivalent to 5 × 10⁷ CFU) was incubated at 37°C in the presence or absence of 6G at the indicated concentrations in a 96-well microtiter plate (Nunc, Denmark), and the absorbance was recorded at 600 nm (A₆₀₀) at every 2 h up to 24 h in an enzyme-linked immunosorbent assay (ELISA) reader (Emax precision microplate reader; Molecular Devices). Cell viability was assayed as CFU on Luria agar plates. A bacterial concentration of 5 × 10⁵ CFU/ml corresponded to an absorbance of 1.0. For further confirmation, the susceptibility of *V. cholerae* to 6G was determined using the EUCAST standard protocol (17). From overnight culture in Mueller-Hinton (MH) broth (HiMedia, India) supplemented with streptomycin, about 5 × 10⁵ cells were inoculated in fresh MH broth against different concentrations of 6G (10, 50, and 100 μg/ml) and incubated for different periods (0, 3, 6, 18, and 24 h) at 37°C. The number of CFU was determined by plating in MH agar plates.

**Cell culture and infection.** The human intestinal epithelial cell line Int407, human cervical cancer cell line HeLa, and Chinese hamster ovary (CHO) cell lines used in this study were purchased from NCCS, Pune, India. The colon adenocarcinoma cell line HT-29 was a gift from S. S. Das, National Institute of Cholera and Enteric Diseases, Kolkata, India. Int407 and CHO cells were grown and maintained in minimal essential medium (MEM) (GIBCO-BRL, Gaithersburg, MD), and HeLa and HT-29 cells were grown in Dulbecco’s modified Eagle medium (DMEM). All of these cells were supplemented with 10% fetal bovine serum (GIBCO-BRL) containing penicillin-streptomycin and gentamicin in the presence of 5% CO₂ at 37°C. HT-29 cells needed high-glucose conditions for growth, and the growth medium was supplemented with Na-pyruvate (SRL). Cells were seeded in canted-neck T-75 tissue culture flasks (Falcon) or tissue culture petri dishes (Falcon) as required. Two days later when the cells were confluent, the medium was removed, the cells were washed with PBS, and fresh MEM, DMEM, or high-glucose DMEM with or without antibiotic (whichever was appropriate for the cell line) was added. Bacteria were grown overnight and suspended in fresh cell culture medium without antibiotic and then were added at a multiplicity of infection (MOI) of ~100. Control plates were replenished with fresh medium, and infected plates were incubated at 37°C under 5% CO₂.

**MTT assay.** The *in vitro* growth inhibition effect of 6G (50 or 100 ng/ml or 1, 10, 50, or 100 μg/ml on Int407, HeLa, and HT-29 cells was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, cells were seeded in a 96-well microtiter plate. Exponentially growing cells were exposed to various concentrations of 6G for 24 h. MTT solution was added to each well, and the optical density was measured at 550 nm (OD₅₅₀) using a microtiter plate reader (Emax precision microplate reader; Molecular Devices). Cell viability was calculated as cytotoxicity = (1 − A₆₀₀/ A₉₆₀₀) × 100.

**Adherence to intestinal epithelial cells.** For adherence assays, intestinal epithelial cells were seeded in 6-cm petri dishes (Falcon) containing small glass coverslips at a density of about 5 × 10⁵ cells per dish. Two days later, the medium was removed and the cells were washed with PBS. 6G (100 μg/ml) was applied on test dishes, and the mixture was incubated for 4 h prior to infection. Bacteria suspended in fresh MEM (at an MOI of 100) without antibiotic were added to each dish. A petri dish incubated with 6G only served as the negative control. The infected plates along with the control were incubated for 3.5 h at 37°C under 5% CO₂. Cells were then washed vigorously three times with PBS to remove the nonadherent bacteria. Cells were fixed in methanol (5 min at room temperature) and stained with Giemsa stain for 16 h. After being washed with acetone and acetone-xylene solution, the coverslips were mounted on a glass slide. Slides were examined at 100× (oil immersion) with a light microscope (Leica, Germany) (18).

The number of cell-associated CFU was determined after lifting off the monolayer by scraping and vortexing the cells to dissociate bacteria. The percentage of adhesion was calculated as [cell-associated CFU/(cell-associated CFU + CFU present in the supernatant)] × 100.

**GM1 ganglioside-dependent ELISA.** Samples containing CT, CT-B, or *V. cholerae* supernatant without or with different concentrations of 6G were used for estimation of CT by GM1 ELISA. The samples were serially diluted and added to microtiter plates containing immobilized GM1. ELISA was carried out as described previously (19) using polyclonal rabbit antiserum directed against purified B subunit of CT and anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Gibco-BRL). Dilutions of CT-B (Sigma) of known concentrations (100 to 1,000 ng/ml) were used to estimate the amount of CT in the samples. The color intensity was measured at 492 nm in an ELISA reader (Bio-Rad).

**IC₅₀ determination.** The GM1 ELISA was performed with CT or CT-B (500 ng/ml) incubated with or without increasing concentrations of 6G for 15 min at room temperature. The concentration of 6G required for 50% inhibition of CT binding to GM1 (IC₅₀) was estimated. The IC₅₀ was calculated from duplicate sets of data for at least 10 different concentrations of 6G as described above. The values reported are the averages of at least three independent determinations.

**Isoelectric focusing.** Isoelectric focusing of CT (6 μg) and CT complexed with 6G (10 μg/ml) was carried out by using a Protein isolectric focusing cell and 17-cm linear (pH 3 to 10) immobilized-pH-gradient (IPG) strips (Bio-Rad) according to the manufacturer’s instructions. A rehydration loading protocol was used to load the samples onto the IPG strips, and isoelectric focusing was performed according to the instructions provided by the manufacturer. The IPG strips were stained with Coomassie blue for visualization of proteins.

**SDS-PAGE and immunoblotting.** Samples were loaded on SDS-polyacrylamide gel (12%), and electrophoresis was carried out as described earlier (20). Gel was then electrotransferred to polyvinylidene difluoride (PVDF) membrane (Millipore) and immunoblotted using a polyclonal anti-CT rabbit antibody (Sigma) at a dilution of 1:20,000 followed by incubation with alkaline phosphate-conjugated mouse anti-rabbit IgG (Genei, Bangalore, India) at 1:1,000 dilution. The alkaline phosphatase-positive bands were visualized after treatment with developing solution containing 5-bromo-4-chloroindol-2-yl phosphate–nitroblue tetrazolium (Genei), 1.5 mM Tris-HCl (pH 8.8), and water in the dark at room temperature for 10 min.

**Fluorescence spectroscopy.** The fluorescence spectrum of CT with or without 6G was monitored on a PerkinElmer fluorescence spectrophotometer (LS-55) equipped with a 150-W xenon flash lamp and using a fluorescence-free quartz cell with a 1-cm path length at room temperature. The excitation was at 282 nm, and the emission spectra were recorded from 300 nm to 400 nm. For quenching studies, solutions of CT were titrated with increasing concentrations of 6G. The intensities of emission maxima were used for the calculation of binding constant using the equation: log (F₀ − F)/F = log Kᵦ + nQ F, where Kᵦ is the binding constant and n is the number of binding sites, Q is the quencher (6G), F₀ is the fluorescence intensity of free CT, and F is the consecutive fluorescence on addition of 6G. The plots of F₀/F against the concentration of 6G were used to determine the values of Kᵦ and n from the intercept and the slope, respectively. Corrections were made for the inner filter effect for the ligand.

**TER.** Barrier function of monolayers of HT29 was assessed by measurement of transepithelial electrical resistance (TER). The cytotoxicity of the IC₅₀ dose was monitored in HT-29 cells, and 10 μg/ml was found to be
less than 20% cytotoxic. Then HT-29 cells were grown in the upper compartment of 12-mm-diameter Transwell inserts (Millipore) at 37°C in 5% CO₂. Following the application of supernatant samples (preincubated for 1 h with 6G) to the upper compartments of Transwell inserts, TER was measured with the use of an epithelial volt-ohmmeter (Millipore) at 0, 4, 24, 48, and 72 h and expressed as the resistance over 1.5 and 9 V: i.e., Ω cm² (21).

Cytotoxicity in HeLa cells. HeLa cells were seeded in a 35-mm dish at a concentration of ~1.2 × 10⁶ cells per well. Cells were permitted to reach confluence by allowing them to grow for 24 h. After aspiration of the spent medium and supplementation with fresh medium, CT (100 ng) alone or with various concentrations of 6G (cytotoxicity of <40% by the MTT assay) was added to the cells, and the mixture was incubated for 24 h in a CO₂ incubator. Cells were examined for morphological changes with a Leica DM3000 phase-contrast microscope (Leica, Germany) (22).

CHO cell assay. CHO cells cultured in a 35-mm dish (1 × 10⁶ cells/well) were incubated with 100 ng CT alone or 100 ng CT preincubated with either 100 µg GM1 (4 h, 25°C) or 10 µg 6G. CHO cell assays were measured using the cAMP activity assay kit (Biovision, Milpitas, CA) (23).

Ligated rabbit ileal loop assay. The in vivo effect of 6G on CT was examined by using the ligated rabbit ileal loop model, essentially as described by De and Chatterje (24). Briefly, the rabbits fasted for 24 h were anesthetized, and the small intestine was tied into consecutive 7-cm and 2-cm segments proximally to the mesoappendix. CT (500 ng) in PBS or V. cholerae O395 supernatant (equivalent to an MOI of 300) was injected alone or with 10 µg 6G into the rabbit ileal loops. After 16 h, the animals were sacrificed and the small intestines were removed. The fluid that had accumulated in each loop was collected separately, measured, and expressed as a ratio of the amount (ml) of fluid per unit length (cm) of loop. A drop of fluid from each loop was tested on thiosulfate-citrate-bile salt-sucrose (TCBS) agar plates for the presence of V. cholerae. The animal experimental protocol met the National Guidelines on the Proper Care and Use of Animals in Laboratory Research (Indian Science Academy, New Delhi, India) and was duly approved by the Animal Ethics Committee of the CSIR-Indian Institute of Chemical Biology.

RESULTS

Drug susceptibility test. A kinetic study was conducted to investigate the growth of V. cholerae O395 strain in LB media in the absence and presence of different concentrations of 6G. No reduction in growth (data not shown) as well as viability was observed over a period of 24 h from the time of inoculation, as determined by absorbance and CFU (Fig. 1). This implied clearly that 6G could not induce an inhibitory effect on the growth of V. cholerae O395 strain under normal growth conditions. Results were confirmed by determining CFU in LB agar plates. Growth inhibition was calculated as follows: % growth inhibition = [(untreated - treated)/untreated] × 100.

Effect of 6G on the adherence of V. cholerae O395 to the intestinal epithelial cells. It was checked whether the effect of 6G as an anti-diarrheal agent could be due to inhibition of the localized adherence of bacteria to the epithelial cell. Therefore, various concentrations of 6G were incubated with intestinal epithelial cells for 4 h and then infected with V. cholerae O395. After incubation for 3.5 h, the O395 strain did not show any reduced adherence to the intestinal epithelial cell (Fig. 2), thus implying the anti-diarrheal effect of 6G depends on a distinctively different mechanism.

GM1 ELISA showed a drastic reduction in CT in the presence of 6G. We considered the possibility of alteration, if any, of CT production in the presence of 6G. To check this, V. cholerae O395 was cultured alone or with increasing concentrations of 6G overnight, and then the bacterial supernatant was subjected to GM1 ELISA with anti-CT antibody. Surprisingly, we observed that in the presence of increasing concentrations of 6G, the amount of detectable CT in the culture supernatant was drastically reduced (Fig. 3A). This could be due to (i) reduced CT secretion in the presence of 6G, (ii) hindrance of binding of anti-CT antibody to CT protein by 6G, and/or (iii) reduced binding of CT to the GM1 receptor in the presence of 6G.
6G hindered binding of CT to the GM1 receptor. To examine the possibility that 6G hinders the CT-GM1 binding, which could be due to binding of 6G to CT or GM1, ELISA was carried out at first with GM1 incubated with 6G, washed, and followed by incubation with CT (100 ng/ml). The results clearly showed that prior incubation of GM1 with 6G (100 µg/ml) had no subsequent effect on detection of CT by ELISA, suggesting that GM1 does not interact with 6G (Fig. 3B, column b). However, when CT (100 ng/ml) was preincubated with increasing concentrations of 6G and then added to GM1 immobilized on microtiter plates, CT expression gradually decreased with the increasing concentration of 6G (Fig. 3B), clearly suggesting that 6G interacted with CT and not with
GM1. The IC_{50} was calculated, and 10 μg/ml 6G was found to be causing 50% inhibition of CT-GM1 binding. Taken together, these results suggested that 6G interacted with CT and not GM1 to prevent CT-GM1 binding.

6G did not block the binding of primary antibody to CT. To consider the possibility that 6G blocked the anti-CT antibody binding sites on CT, causing less detection of CT by GM1-ELISA in the presence of 6G, the 6G-treated bacterial supernatant was subjected to SDS-PAGE and then immunoblotted with anti-CT antibody. Clear bands corresponding to the A and B subunits of CT were observed, and the intensities of the bands were comparable in the presence and absence of 6G, suggesting that 6G did not block the anti-CT antibody binding sites on CT (Fig. 3C). Also the detection of comparable amounts of CT in the presence and absence of 6G in the immunoblot suggested that CT secretion is not reduced in the presence of 6G.

Isoelectric focusing. It has been demonstrated previously that protein-ligand binding might change the apparent pl of the protein, probably as a result of surface charge redistribution upon ligand binding. As has been reported previously (25, 26), the pl of CT was determined to be 6.8 by isoelectric focusing on IPG strips (pH 3 to 10). A shift in the apparent pl of CT to 7.5 was observed when CT was incubated with 6G prior to isoelectric focusing (Fig. 4A).

Fluorescence spectroscopic analysis of the binding of 6G to CT. To analyze the interaction of 6G with CT in greater detail, quenching of intrinsic fluorescence of CT by 6G was employed. The steady-state fluorescence emission spectrum of CT was recorded in the presence and absence of 6G. 6G produced a marked quenching of fluorescence intensity of CT, suggesting complex formation of 6G with CT. There was also a blue shift observed in the emission maxima, which increased with increasing concentration of 6G, suggesting the placement of Trp residues in a hydrophobic environment (Fig. 4B). The dissociation constant was estimated from a plot of F_0/F versus 6G concentration, where F_0 and F denote fluorescence intensities of CT in the absence and presence of 6G, respectively. The plot of F_0/F versus concentration of 6G was linear, indicating the occurrence of only one type of quenching by 6G. After correction for the inner filter effect, the apparent K_d was found to be 27.321 ± 3.31 mM^{-1} (R^2 = 0.88) (Fig. 4C).

6G inhibited V. cholerae O395 supernatant-induced loss of epithelial barrier function. In HT-29 monolayers, 6G protected against loss of TER following apical exposure (application to the upper chamber of Transwell inserts) to supernatant samples of the V. cholerae O395 strain. 6G at a concentration of 10 μg/ml inhibited loss of epithelial barrier function induced by supernatant from the O395 culture with an MOI of 300 (Fig. 5A).

6G could restore CT-induced alteration of morphology in HeLa and CHO cells. To determine whether 6G can restore the cytotoxic effect caused by CT supernatant on HeLa cells, the latter were seeded in 3.5-mm dishes (~0.8 × 10^6 cells) overnight, preincubated with 6G (1, 10, and 50 μg/ml) for 4 h, and exposed to purified CT for 3.5 h. The cell morphology was investigated under a phase-contrast microscope and compared with those of control HeLa cells and HeLa cells treated with CT. Rounding of cells revealing a cytotoxic effect was prominent in the case of HeLa cells treated with CT, while the effect was much less in the presence of 6G (Fig. 5C).

To address the in vitro efficiency of 6G against biological activity of CT, the well-established CHO cell assay was used. CHO cell monolayers were treated with CT (100 ng) alone and CT (100 ng) with increasing concentrations of 6G (1, 10, 50, 100, and 200 μg). The CT-induced elongation of CHO cells was inhibited by 6G at as low as 10 μg, suggesting that 6G can inhibit the biological activity of CT. (Fig. 5B). Thus, 6G can restore the alteration of cell mor-
phology induced by *V. cholerae* O395 supernatant or pure CT alone.

**CT-induced cAMP production in the CHO cell line is reduced by 6G.** Another indicator of CT toxicity is an increase in cellular cAMP. The cAMP assay was performed to evaluate the role of 6G in CT-induced intracellular cAMP production in the CHO cell line. Experimental data revealed that CT preincubated with 6G showed a significant reduction in cAMP production compared to that in CHO cells treated with CT only (Fig. 5D). This may be attributed to the binding of 6G with CT, which in turn results in decreased cAMP production in cells.

**Effect of 6G in CT-mediated fluid accumulation in rabbit ileal loop.** Since the major biological function of CT is to promote the loss of fluid from epithelial cells, we used the ligated rabbit ileal loop assay, which is used as an animal model of cholera diarrhea, to examine intestinal fluid accumulation when CT was administered in the ileal loops with or without 6G. Fluid accumulation induced by instillation of CT or *V. cholerae* supernatant was inhibited by simultaneous administration of 6G (Fig. 6A). Although about 1.4 ml/cm and 1.2 ml/cm fluid accumulated in ileal loops inoculated with 500 ng CT and *V. cholerae* supernatant, respectively, the amounts were reduced to 0.6 ml/cm and 1.0 ml/cm when administered together with 10 μg/ml 6G (Fig. 6B). The less inhibitory activity of 6G toward *V. cholerae* supernatant could be attributed to the presence of other toxins, such as Zot and Rtx, in the supernatant administered (Fig. 6).

**DISCUSSION**

Our results indicate that 6G, which is the pharmacologically active ingredient of ginger, one of the most frequently consumed spices, inhibits CT effects, including distortion of cellular morphology, cAMP activation, and accumulation of fluid in intestinal loops. It does so by binding to the CT and in turn disturbing its binding to the GM1 receptor on the cell surface.

Studies were undertaken to investigate the effect of 6G on bacterial adherence to host cells as this is the initial key step toward colonization and establishment of infection within the host. The experimental results revealed that 6G had no effect on the growth and adherence of *V. cholerae* to intestinal epithelial cells. This clearly indicated that 6G does not have any antibacterial effect. We further extended our study to investigate whether 6G has any effect on the activity of CT, the major virulence factor produced by *V. cholerae*, as there are some herbal compounds that have been reported to reduce CT secretion (27).

The inhibitory effect of chemical compounds/natural products on CT activity were found to be due to various mechanisms. For

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**FIG 5** (A) TER of HT-29 cells in the presence of CT and 6G. *V. cholerae* supernatant (equivalent to an MOI of 300) alone or preincubated (1 h) with 10 μg/ml 6G was added on the apical surface of HT-29, and TER was measured (in Ω cm²) at different time points (0, 4, 24, 48, and 72 h). (B) Effects of 6G on CT-induced elongation of CHO cells. CHO cells were incubated alone (a) or with 1, 10, 50, 100, or 200 μg/ml of 6G (b to f, respectively) prior to treatment with CT (100 ng) or with CT (100 ng) alone (g). Magnification, ×20. (C) Effect of 6G on CT-induced changes in HeLa cell morphology. Shown are results from HeLa cells alone (a), HeLa cells incubated with CT (100 ng) alone (b), and HeLa cells incubated with 100 ng CT preincubated (1 h) with 1, 10, or 50 μg/ml 6G (c to e, respectively). Magnification, ×40. (D) Effect of 6G on CT-induced cAMP accumulation in CHO cells. CHO cells were incubated alone (column a) or with 100 ng CT (column b), 100 ng CT and 100 μg GM1 (column c), or 100 ng CT and 10 μg 6G (column d) and assayed for cellular cAMP concentration. Data represent means ± SE from three independent experiments performed under similar conditions. An asterisk (*) indicates significant difference from CT-treated cells (P < 0.05).
example, plant-derived dihydroisosteviol reversibly inhibited cAMP-activated Cl− intestinal secretion in the mouse intestinal closed-loop model by targeting CFTR chloride channels (28). Capsaicin, one of the active compounds of red chili, was reported to be a potent repressor of CT production in V. cholerae by enhancing the transcription of the hns gene coding for the histone-like nucleoid structure protein (27). Recently some common spices such as red chili, sweet fennel, and white pepper were found to show an inhibitory effect on the expression of virulence factors of V. cholerae, including CT (29).

When V. cholerae O395 culture supernatant treated with various concentrations of 6G was analyzed for the presence of secreted CT by Western blot analysis, no changes in CT concentration were observed in the presence of 6G, whereas in the ELISA a decreasing CT concentration in culture supernatant was observed with increasing concentration of 6G. This indicates the possibility that 6G does not exert any effect on the secretion of CT but might have some effect on the binding of CT to GM1 receptor, resulting in the reduced detection of CT in ELISA. Since it is the B subunit of CT that binds it to the GM1 receptor, 6G might specifically bind with the B subunit to hinder its association with GM1. This is supported by the dose-dependent decrease in CT-GM1 interaction with increasing concentration of 6G (IC50, 10 μg/ml). A number of galactose derivatives have been screened to develop GM1 receptor antagonists, and m-nitrophenyl-galactoside (MNPG) derivatives were found to be the most effective. Next-generation leads based on MNPG (i.e., functionalization of m-nitrophenyl-α-D-galactopyranoside (MNPG) with 1,4-bis (3-aminopropyl) piperazine (APP-MNPG) yielded an MNPG derivative (IC50 of 200 μM) with efficacy greater than that of MNPG (30). However, our study reveals that 6G is 6 times more effective than MNPG derivatives as a GM1-CT binding antagonist. We further validated the interaction of CT with 6G by fluorescence spectroscopy. The intrinsic fluorescence of CT was quenched with increasing concentrations of 6G. Isoelectric focusing data also corroborated with the fluorescence result. A distinct shift in the isoelectric point was observed for CT incubated with 6G compared to pure CT, which could only be possible if there is remarkable CT-6G interaction. All of these results clearly point out the fact that 6G specifically binds to CT and thus hinders its interaction with GM1, which is the stepping stone for the induction of the CT-induced cholera-genic effect. These results encouraged us to look into the effect of 6G on reduction of pathogenic effects of CT in vitro, in particular with the cAMP assay, cell cytotoxicity assay, and increased epithelial cell permeability (measured by TER). The intestinal barrier serves as the first line of defense against pathogens within the intestinal lumen. HT-29 monolayers maintained a high and steady TER, which decreased drastically in the presence of V. cholerae supernatant and could be attributed to the opening of membrane channels or pores resulting in enhanced permeability due to CT in the supernatant and this effect being suppressed by 6G. This is supported by the evidence that 6G is effective in suppressing CT-induced elevation in cAMP level. Enteroocyte CAMP is the signaling molecule for CT (31). It is one of the intracellular mediators of active ion secretion and plays a central role in the regulation of ion secretion in the enterocyte. Furthermore, the CT-induced rounding of HeLa cells and distention of CHO cells were greatly restored on treatment with various concentrations of 6G. Finally, the effectiveness of 6G was checked in vivo by fluid accumulation in the rabbit ileal loop model, and it was observed that 6G inhibited the fluid accumulation induced by V. cholerae O395. Our findings open up a new area toward the possibility of oral administration of 6G, preferably together with oral rehydration salt and antibiotics, which could be used as a therapeutic modulation for cholera by abolishing the toxin–receptor interaction. Since 6G is a natural herbal product acting on the secreted toxins and not on V. cholerae, it is expected to have no effect on existing resistance or emergence of resistance. Further clinical studies are necessary to confirm the efficacy of 6G for the treatment of cholera.

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