A novel plant-derived inhibitor of cAMP-mediated fluid and chloride secretion

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1Department of Pediatric Gastroenterology and the 2Cystic Fibrosis/ Pulmonary Research and Clinical Treatment Center, University of North Carolina, Chapel Hill, North Carolina 27599; and 3SHAMAN Pharmaceuticals Inc., South San Francisco, California 94080

Gabriel, S. E., S. E. Davenport, R. J. Steagall, V. Vimal, T. Carlson, and E. J. Rozhon. A novel plant-derived inhibitor of cAMP-mediated fluid and chloride secretion. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G58–G63, 1999.—We have identified an agent (SP-303) that shows efficacy against in vivo cholera toxin-induced fluid secretion and in vitro cAMP-mediated Cl\(^-\) secretion. Administration of cholera toxin to adult mice results in an increase in fluid accumulation (FA) in the small intestine (FA ratio = 0.63 vs. 1.86 in control vs. cholera toxin-treated animals, respectively). This elevation in FA induced by cholera toxin was significantly reduced (FA ratio = 0.70) in animals treated with a 100 mg/kg dose of SP-303 at the same time as the cholera treatment. Moreover, when SP-303 was administered 3 h after cholera toxin, a dose-dependent inhibition of FA levels was observed with a half-maximal inhibitory dose of 10 mg/kg. In Ussing chamber studies of Caco-2 or T84 monolayer preparations, SP-303 had a significant effect on both basal current and forskolin-stimulated Cl\(^-\) current. SP-303 also induced an increase in resistance that paralleled the observed decrease in current. These data suggest that SP-303 has an inhibitory effect on cAMP-mediated Cl\(^-\) and fluid secretion. Thus SP-303 may prove to be a useful broad-spectrum antidiarrheal agent.

Cystic fibrosis (CF) is a disease characterized by defective cAMP-regulated Cl\(^-\) secretion of the apical membrane of epithelial cells (12, 14). Cloning of the CF gene (10, 16) and expression studies of the protein product, the cystic fibrosis transmembrane conductance regulator (CFTR), reveal that it is a cAMP-regulatable Cl\(^-\) channel (1, 2, 18). CFTR is widely expressed in many epithelial tissues and is prevalent in the mammalian small intestinal epithelium in both crypt and villus cells (20). Gene-targeting approaches have successfully been used to create several CF mouse models (reviewed in Ref. 9), all of which show that a primary pathophysiology occurs in the gastrointestinal tract. This pathophysiology can be explained by the finding that CFTR is the predominant mechanism for apical Cl\(^-\) secretion in the murine gastrointestinal tract (5). Thus a functional absence of CFTR in the CFTR(−/−) mouse results in a severely dehydrated intestinal epithelium that is the catalyst for constrictions, obstructions, and ultimately death (17).

SP-303 is derived from the latex of the plant Croton lechleri and comprises a mixture of naturally occurring proanthocyanidin oligomers with an average molecular mass of 2,200 Da (21). This naturally occurring latex has been used by the indigenous people of South America to treat various kinds of watery diarrheas, including diarrhea caused by cholera. In this study, we examine the ability of SP-303 to inhibit CT-induced fluid secretion in the murine intestine. We also test whether this compound is capable of inhibiting Cl\(^-\) secretion across a human intestinal epithelial cell monolayer.

**MATERIALS AND METHODS**

Fluid accumulation. Fluid accumulation (FA) was measured using the sealed adult mouse model for secretory diarrhea as described previously (8, 15). Briefly, 8- to 10-wk-old C57Bl/6 mice of either sex with body masses that ranged from 12.1 to 36.0 g were used. Initially, mice were given single doses of SP-303 dissolved in 7% wt/vol NaHCO\(_3\) delivered by an orogastric feeding tube, followed immediately by 10 µg CT (this is referred to as simultaneous addition). Subsequent experiments involved administration of 10 µg CT at time 0 followed by administration of varying doses of SP-303 at 3 h (one set of experiments, involving SP-303 prepared as an enteric-coated bead). Under either of the experimental conditions, mice were killed 6 h after the administration of CT. The entire small intestine, from the pylorus to the cecum, was

SECRETORY DIARRHEA REMAINS one of the major causes of infant morbidity and mortality around the world, accounting for over a billion episodes per year in children under 5 years of age (13). In comparison to other mechanisms of diarrheal disease, secretory diarrheas caused by bacterial enterotoxins [e.g., Vibrio cholerae toxin and Escherichia coli, heat stable (ST), heat labile (LTa), and LTB toxins] are not associated with severe intestinal histopathology. Rather, secretory diarrheas appear to function by stimulating transepithelial Cl\(^-\) secretion, thereby increasing the osmotic impetus for fluid secretion (4). Cholera toxin (CT) is likely the most recognizable enterotoxin that causes secretory diarrhea. After colonization of the small intestine by Vibrio cholerae, binding of the cholera enterotoxin to the intestinal enterocyte leads to ADP-ribosylation of the α-subunit of a stimulatory G protein, G\(_{\alpha}\) (7, 11). This covalent modification activates adenylyl cyclase for the lifetime of the enterocyte during its migration up the villus axis. As a consequence of this irreversible elevation of cAMP, an apical membrane Cl\(^-\) channel is activated that results in a voluminous Cl\(^-\) and fluid secretion that can be fatal if untreated (4, 7).

**Fluid accumulation.** Fluid accumulation (FA) was measured using the sealed adult mouse model for secretory diarrhea as described previously (8, 15). Briefly, 8- to 10-wk-old C57Bl/6 mice of either sex with body masses that ranged from 12.1 to 36.0 g were used. Initially, mice were given single doses of SP-303 dissolved in 7% wt/vol NaHCO\(_3\) delivered by an orogastric feeding tube, followed immediately by 10 µg CT (this is referred to as simultaneous addition). Subsequent experiments involved administration of 10 µg CT at time 0 followed by administration of varying doses of SP-303 at 3 h (one set of experiments, involving SP-303 prepared as an enteric-coated bead). Under either of the experimental conditions, mice were killed 6 h after the administration of CT. The entire small intestine, from the pylorus to the cecum, was

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isolated with care to avoid tissue rupture and loss of fluid. The attached mesentery and connective tissues were removed, and the mass of the tissue and enclosed fluid was determined. The tissue was then opened longitudinally, the fluid was removed, and the tissue was patted dry. The mass of the dry tissue was then weighed. FA was measured as a ratio of the mass of accumulated fluid in the small intestine and cecum vs. the mass of the small intestine minus the mass of the fluid.

Both the CT dose administered and the time of incubation were selected to ensure that a maximal amount of fluid would be secreted into the intestinal lumen. Data were analyzed to determine whether SP-303 administration resulted in a statistically significant reduction in CT-induced FA ratios compared with CT-treated animals given only NaHCO₃ as a control solution. Baseline (e.g., nonsecretory) animals were administered a bolus of 7% NaHCO₃ solution in the absence of CFTR. For all experiments, cells were evaluated for confluence by daily monitoring of transepithelial resistance (Rₑ) and potential difference (PD). Only monolayers generating at least a 0.5 mV PD and a 150 Ω·cm² resistance were used for Ussing chamber studies.

Ussing chamber studies. Electrical measurements, PD, and short-circuit current (Iₑ(sc)) were made on cultured epithelial cell monolayers mounted in Ussing chambers (4). Unless otherwise stated, the bathing medium used for all studies was a standard Kreb-Ringer bicarbonate solution (KRB) on the basolateral side and a modified KRB, high-K⁺ and low-Cl⁻ solution (glucuronate was substituted for Cl⁻) on the apical side (6). In some experiments, an apical Na⁺-free solution (N-methyl-D-glucamine was substituted for Na⁺) was used to eliminate any contribution of absorptive Na⁺ channels. In these experiments, ion solutions were balanced with the exception of Na⁺ channels. In these experiments, ion solutions were balanced with NaCl 140 mM, NMDG 150 mM, KCl 25 mM, and 10–15% FCS, in an atmosphere of 95% air and 5% CO₂.

RESULTS

We have previously shown that CT-induced fluid and Cl⁻ secretion is directly proportional to the expression level of the CFTR Cl⁻ channel (8). In this study, we investigate the effects of SP-303 on CT-induced FA in the small intestine of mice expressing normal levels of CFTR. Control mice treated with only a bolus of 7% NaHCO₃ solution in the absence of either CT or SP-303 show an FA ratio of 0.63 ± 0.07 (Fig. 1, dashed line in A and C). CT (10 µg)-treated animals show an FA level (1.86 ± 0.2) that is nearly three times greater than control values. Simultaneous administration of SP-303 with CT results in an inhibition of FA in a concentration-dependent manner (Fig. 1A). Gavage administration of 100 mg/kg SP-303, simultaneously with CT treatment, restores FA to near normal levels (0.70 ± 0.19). To exclude the possibility that SP-303 may be either interacting with CT or preventing binding of CT to the ganglioside receptor, we studied SP-303-mediated inhibition of FA in mice prestimulated by CT. Mice that received a dose of SP-303 3 h after exposure to CT showed a significant decrease in FA ratios (Fig. 1B). The dose-response curve showed inhibition with an apparent half-maximal inhibitory concentration (IC₅₀) of ~10 mg/kg. The highest SP-303 doses administered brought about a nearly complete inhibition of CT-induced FA levels (i.e., 50 mg/kg SP-303-treated mice had FA values that were not significantly different from untreated control FA values (0.78 ± 0.08 vs. 0.63 ± 0.07)). In an attempt to maximize efficient delivery of SP-303 to the small intestinal lumen and in anticipation of future clinical trials, SP-303 was encapsulated in an alkali-soluble, enteric-coated bead and FA ratios were measured (Fig. 1C). Administration of SP-303 encapsulated in this formulation was likewise capable of significantly inhibiting CT-induced FA (0.75 ± 0.10 vs. 1.51 ± 0.08 for CT and 100 mg/kg SP-303 vs. treatment with CT alone, respectively).

Secretory diarrhea as induced by CT is the result of irreversible activation of adenylate cyclase, leading to elevation of cAMP and finally stimulation of Cl⁻ secretion. Thus we sought to determine if the observed SP-303 inhibition of FA was due to a metabolic inhibition of cellular cAMP levels. We measured the effect of SP-303 on cellular cAMP levels in confluent T84 cell monolayers. Administration of CT (10 µg/ml) resulted in a large increase in cAMP levels sampled 6 h after treatment (1,031.4 ± 64 vs. 4.97 ± 0.3 pmol/ml for CT vs. control, respectively; n = 6 for each condition). Addition of 300 µM SP-303 neither significantly reduced the CT-elevated cAMP levels (1,031.4 ± 64 vs.
972.5 ± 77 pmol/ml for CT vs. CT and SP-303, respectively; \( P = 0.57 \) nor reduced the non-CT-treated basal cAMP levels (4.97 ± 0.3 vs. 5.51 ± 0.3 pmol/ml for control vs. control and SP-303, respectively; \( P = 0.24 \)).

The lack of an effect of SP-303 on cAMP levels suggests that the compound is acting at a site distal to elevation of cAMP (i.e., the CFTR Cl\(^{-}\) channel itself or a regulator of the channel). We therefore investigated the effects of SP-303 on the basal \( I_{sc} \) of Caco-2 epithelial cell monolayers as a measure of Cl\(^{-}\) secretion (Fig. 2). Sequential addition of SP-303 to the solution bathing the apical membrane inhibited the basal \( I_{sc} \) by nearly 50%. Moreover, subsequent addition of forskolin (an activator of adenylate cyclase) resulted in a relatively small Cl\(^{-}\) secretory response (Fig. 2A). Forskolin-stimulated currents had a mean of 16.9 ± 2.5 µA/cm² (\( n = 8 \)) after exposure to 300 µM SP-303, whereas forskolin-stimulated currents in monolayers not exposed to SP-303 showed a mean of 38.3 ± 4.3 µA/cm² (\( n = 16 \)).

Inhibition of basal \( I_{sc} \) was seen at all doses of SP-303, with maximal effects observed following administration of 300 µM SP-303 (Fig. 2B). Importantly, increasing doses of SP-303 resulted in an increase in monolayer resistance that correlated with the decrease in \( I_{sc} \) (300 µM SP-303 showed an increase in resistance of 39 ± 7% of basal values, \( n = 9 \)).

To further define the effect of SP-303 on cAMP-mediated Cl\(^{-}\) secretion, we examined the ability of SP-303 to inhibit the maximum \( I_{sc} \) stimulated by forskolin in Caco-2 cells (Fig. 3). Cl\(^{-}\) secretion maximally stimulated by the addition of forskolin was inhibited in a stepwise manner by sequential additions of SP-303 to the apical solution (Fig. 3A). Forskolin-stimulated currents had a mean of 16.9 ± 2.5 µA/cm² (\( n = 8 \)) after exposure to 300 µM SP-303, whereas forskolin-stimulated currents in monolayers not exposed to SP-303 showed a mean of 38.3 ± 4.3 µA/cm² (\( n = 16 \)).
metanide, an inhibitor of the basolateral Na\(^+\)K\(^+\)2Cl\(^-\) cotransporter, inhibited the residual current to prestimulated levels. A further addition of 50 µM SP-303 to the basolateral bath (total concentration = 100 µM) did not have an effect on the forskolin-stimulated current. We were not able to reverse the inhibitory effect of apical SP-303 on forskolin-stimulated current in T84 monolayers. After stimulation of I\(_{sc}\) by forskolin, addition of 100 µM SP-303 to the apical solution resulted in the expected inhibition of current as previously reported in Fig. 3A. The apical bath solution was then replaced with identical Ringer solution devoid of SP-303. This maneuver did not restore I\(_{sc}\) levels (68.2 ± 7.8 vs. 67.8 ± 10.1 µA/cm\(^2\); I\(_{sc}\) measured after inhibition by SP-303 and after bath replacement of SP-303, respectively; n = 8).

**DISCUSSION**

We have described effects of SP-303 on CT-induced fluid secretion in an in vivo mouse model and also on CAMP-mediated Cl\(^-\) secretion measured across epithelial cell monolayers. The animal studies demonstrate a potent, nearly complete inhibition of fluid secretion when SP-303 is applied either early (simultaneous to toxin delivery) or late (3 h after CT delivery, when stimulated current, if untreated, was previously shown (6) to be relatively stable for up to 45 min after stimulation. Maximal inhibition of the forskolin-stimulated I\(_{sc}\) was achieved by the addition of 300 µM SP-303 (Fig. 3B). The I\(_{sc}\) for SP-303 was ~50 µM. Bumetanide, an inhibitor of the basolateral Na\(^+\)K\(^+\)2Cl\(^-\) cotransporter responsible for Cl\(^-\) entry, decreased the residual I\(_{sc}\) to prestimulated levels.

To determine that the effect of SP-303 was not restricted to a single intestinal epithelial cell line, we also studied inhibition of Cl\(^-\) secretion in another colonic cell line, T84. The T84 cell line is known to express CFTR at relatively high levels, and, when studied as a polarized monolayer in Ussing chambers, CAMP causes a significant increase in I\(_{sc}\) that is primarily due to Cl\(^-\) secretion (19). Moreover, by bathing the apical surface of the T84 monolayers in an Na\(^+\)-free solution, we more effectively translate I\(_{sc}\) into a direct measurement of apical Cl\(^-\) secretion. Under these conditions, addition of 500 µM SP-303 to the apical solution inhibited nearly all the I\(_{sc}\) stimulated by forskolin (mean inhibition of 88.7%; n = 8) (Fig. 4).

SP-303 did not have any effect on basal or forskolin-stimulated I\(_{sc}\) measured in Caco-2 monolayers exposed to bilateral Cl\(^-\)-free solutions (data not shown), although under these conditions basal currents were decreased before SP-303 addition. Basolateral addition of SP-303 to T84 monolayers was used as an assessment of nonspecific (metabolic) inhibitory effects of SP-303. T84 monolayers were bathed in bilateral KRB solution and exposed to 50 µM SP-303 in the basolateral bath followed by apical addition of forskolin (5 µM). Basolateral addition of SP-303 neither had an effect on basal current (28.4 ± 2.4 vs. 27.4 ± 1.1 µA/cm\(^2\); I\(_{sc}\) measured in Caco-2 monolayers exposed with or without pretreatment by SP-303, respectively; n = 3) nor did it significantly blunt the subsequent increase mediated by forskolin (ΔI\(_{sc}\) of 103.7 ± 11.4 vs. 119 ± 13.8 µA/cm\(^2\); with or without pretreatment by SP-303, respectively; n = 3). A further addition of 50 µM SP-303 to the apical solution resulting in the expected inhibition of current as previously reported in Fig. 3A. The apical bath solution was then replaced with identical Ringer solution devoid of SP-303. This maneuver did not restore I\(_{sc}\) levels (68.2 ± 7.8 vs. 67.8 ± 10.1 µA/cm\(^2\); I\(_{sc}\) measured after inhibition by SP-303 and after bath replacement of SP-303, respectively; n = 8).
Ca^{2+}-activated Cl^{-} channels. If SP-303 does show high specificity to CFTR, by either inhibition and/or binding, this compound will be of great utility as a tool in understanding CFTR structure-function relationships, aside from its use as an antidiarrheal drug.

Although cholera is the classic example of a secretory diarrhea, other bacterial toxins such as E. coli ST and LT are also capable of causing fluid secretion. As shown previously, agents such as E. coli STa, regardless of initial second messenger activation, ultimately result in elevation of cAMP and stimulation of CFTR (3). Therefore, a specific inhibitor acting directly at CFTR could prove to be very useful as it would inhibit the final step associated with most if not all known bacterial agents causing secretory diarrhea.

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