

# A novel extract SB-300 from the stem bark latex of *Croton lechleri* inhibits CFTR-mediated chloride secretion in human colonic epithelial cells

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## Abstract

An oligomeric proanthocyanidin (SP-303) extracted from the bark latex of the tree *Croton lechleri* (family Euphorbiaceae) is a potent inhibitor of cholera toxin-induced fluid accumulation and chloride secretion. The manufacturing process for SP-303 was optimized and simplified to produce an increased yield of the herbal extract. The novel extract (named SB-300) contained on average  $70.6 \pm 7.2\%$  SP-303 by weight (mean  $\pm$  S.D.;  $n = 56$  lots). Here, we describe the effectiveness of SB-300 on cAMP-regulated chloride secretion, which is mediated by the cystic fibrosis transmembrane conductance regulator  $\text{Cl}^-$  channel (CFTR) in human colonic T84 cells. Exposure of the apical surface to SB-300 blocked forskolin-stimulated  $\text{Cl}^-$  secretion by  $92.2 \pm 3.0\%$  with a half-maximal inhibition constant ( $K_B$ ) of  $4.8 \pm 0.8 \mu\text{M}$ . For SP-303, stimulated  $\text{Cl}^-$  currents were decreased by  $98.0 \pm 7.2\%$  and  $K_B$  averaged  $4.1 \pm 1.3 \mu\text{M}$ . There was no significant difference between the blocking kinetics of SP-303 and SB-300. Forskolin-stimulated whole cell  $\text{Cl}^-$  currents were effectively blocked by extracellular addition of SB-300 ( $63 \pm 8.5\%$ ;  $n = 3$ ) and to a similar extent by SP-303 ( $83 \pm 0.6\%$ ;  $n = 2$ ; at  $50 \mu\text{M}$  each). Both extracts inhibited a time- and voltage-independent  $\text{Cl}^-$  conductance, which indicated the involvement of CFTR  $\text{Cl}^-$  channels. We conclude that both SP-303 (used in Provir®) and SB-300 (used in NSF Normal Stool Formula™) are novel natural products that target the CFTR  $\text{Cl}^-$  channel. SB-300 is a low cost herbal extract and may present a complementary and alternative medicine approach for the treatment of fluid loss in watery diarrhea. © 2004 Elsevier Ireland Ltd. All rights reserved.

**Keywords:** *Croton lechleri*; Proanthocyanidin; Herbal extract; Dietary supplement; Diarrhea; Cystic fibrosis transmembrane conductance regulator chloride channel; Chloride channel blocker

## 1. Introduction

Secretory diarrhea and the associated massive loss of salt and water are a worldwide problem of enormous magnitude. It is the leading cause of death in infants in the developing world (Giannella, 1981) and currently accounts for an estimated 2.5 million deaths in children under 5 years of age (Kosek et al., 2003). Childhood diarrhea is most commonly caused by rotavirus infections (World Health Organization, 1999) of both the epithelium and the enteric nervous system that leads to secretion of both salt and water (Lundgren et al., 2000). Coliform bacteria (such as *Escherichia coli*, *Bacillus cereus*, *Campylobacter fetus* and *Vibrio parahemolyticus*) also cause watery diarrhea primar-

ily by release of bacterial enterotoxins. It has been shown that both the heat-stable and the heat-labile *Escherichia coli* enterotoxins increase intracellular concentrations of adenosine 3',5'-cyclic monophosphate or guanosine 3',5'-cyclic monophosphate which upregulate protein kinase A (PKA) activity (Forte et al., 1992; Chao et al., 1994). PKA is the prime regulator of the cystic fibrosis transmembrane conductance regulator (CFTR)  $\text{Cl}^-$  channel (Cheng et al., 1991; Berger et al., 1991).

CFTR functions as an apical membrane  $\text{Cl}^-$  channel in epithelia and modulates cAMP-dependent fluid secretion. CFTR is mutated and dysfunctional in the autosomal recessive inherited disease cystic fibrosis (CF). Messenger RNA encoding for CFTR and localization of CFTR protein to the apical membrane have been detected in the duodenum, jejunum, ileum and colon (Riordan et al., 1989; Crawford et al., 1991). The small intestine of CF patients

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exhibits decreased  $\text{Cl}^-$  and fluid secretion that results in meconium ileus in 10% of CF newborns and accumulation of mucus and intestinal obstruction in 20% of adult CF patients (Grubb and Boucher, 1997). In contrast, excessive activation of the CFTR  $\text{Cl}^-$  channel in the intestinal epithelia causes secretory diarrhea. It has been proposed that mutations in the CFTR gene and its resulting defective  $\text{Cl}^-$  channel function presented an advantage for heterozygous carriers during episodes of diarrhea, thereby promoting the retention and spread of CFTR mutations in the population (Rodman and Zamudio, 1991; Gabriel et al., 1994).

Current antidiarrheal drugs largely rely on reduction of smooth muscle motility and contractility. Currently, no drug treatments are available that specifically target and block the CFTR chloride ion channel. However, the primary cause of diarrhea is due to the cAMP-dependent hyper-activation of CFTR and drugs directed at this site should show greater effectiveness and selectivity. The red viscous bark latex of the tree *Croton lechleri* Muell.-Arg. (Euphorbiaceae) (“sangre de drago”) is known for its medicinal properties in the treatment of diarrhea, inflammation, insect bites, viral infections and wounds (Jones, 2003). The sap of sangre de drago has been taken orally by indigenous people of the Amazon basin of South America to treat different types of diarrhea, including cholera (Carlson and King, 2000). Previously, a compound, termed SP-303, was isolated from the bark latex of *Croton lechleri* by Ubillas et al. (1994), using a bioassay-guided fractionation and selection procedure. SP-303 is a purified, heterogeneous proanthocyanidin oligomer. The basic monomers are mainly (+)-gallocatechin and (–)-galloepicatechin, and to a smaller amount (+)-catechin and (–)-epicatechin. The oligomer consists of linearly linked monomers (on average heptamers, ranging from penta- to 11-mers) of varying ratios (Ubillas et al., 1994). SP-303 was recently shown to inhibit cAMP-mediated  $\text{Cl}^-$  and fluid secretion by T84 and Caco-2 cells, as well as in intact loop studies in cholera toxin treated mice (Gabriel et al., 1999).

The safety and efficacy of orally administered SP-303 for the symptomatic treatment of diarrhea has been evaluated in travelers (DiCesare et al., 2002) and in patients with AIDS (Holodniy et al., 1999). In a double blind, randomized, placebo-controlled study among travelers to Jamaica and Mexico, SP-303 shortened the duration of acute secretory diarrhea by 21% without causing post-treatment constipation (DiCesare et al., 2002). An estimated 50–60% of patients with human immunodeficiency virus infection experience diarrhea during their illness which is caused by medications, including protease inhibitors (Sherman and Fish, 2000). In a double blind, randomized, placebo-controlled phase II human clinical trial for treatment of HIV-associated diarrhea, SP-303 reduced stool weight and abnormal stool frequency (Holodniy et al., 1999). SP-303 was safe, well tolerated and had a significant impact on quality of life (Holodniy et al., 1999).

The conventional isolation procedure of SP-303 from the tree bark resulted in a relatively low yield (~2.5%) which prompted us to optimize and, at the same time, simplify the manufacturing process. This resulted in the novel extract termed SB-300, which is now commercially available as a dietary supplement (NSF/Normal Stool Formula™), developed by Shaman Pharmaceuticals Inc. SB-300 contains on average 70% SP-303 by weight. The remaining 30% of SB-300 contain, at least in part, proanthocyanidins that have not been specifically characterized. The purpose of the present study was to compare the efficacy and potency of SB-300 and SP-303 on cAMP-regulated  $\text{Cl}^-$  secretion in human intestinal T84 epithelial cells.

## 2. Methodology

### 2.1. Preparation of SB-300

SB-300 is derived from liquid bark latex of the *Croton lechleri* tree. Basification of the liquid latex is achieved by addition of NaOH to produce a neutral to slightly alkaline pH that results in a precipitate that is filtered out. The remaining liquid filtrate goes through ultrafiltration to produce a retentate that is mixed with CM sepharose resin and then filtered to yield a filtrate that is tray dried to produce the solid SB-300. By HPLC analysis, SB-300 (Lot SL-1315) contained 65% purity of SP-303 by weight. SP-303 is an oligomeric proanthocyanidin compound in the polyphenolic chemical class with a MW of approx. 2200 Da (Ubillas et al., 1994). Polyphenol and Karl Fisher analyses showed that the remaining 30% of the SB-300 contain other non SP-303 polyphenolic compounds that have not been specifically characterized, as well as moisture.

### 2.2. Cell culture

The human colonic epithelial cell line T84 (American Type Cell Culture collection, Manassas, Virginia) was grown in a 1:1 mix of Dulbeccos's modified Eagle's medium and Ham's Nutrient mixture F-12 (Life Technologies, Grand Island, New York) supplemented with 10% fetal calf serum (Hyclone, Logan, Utah), 100 U/ml penicillin, 100 mg/ml streptomycin and 4 mM L-glutamine. T84 cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$ . T84 cells were used at passages 65–68. For Ussing chamber studies, T84 cells were seeded on permeable and collagen-coated cell culture inserts with a 0.45  $\mu\text{m}$  pore size and 12 mm diameter (Falcon, Becton Dickinson, Franklin Lakes, NJ) at a density of  $\sim 10^6$  cells/ $\text{cm}^2$ . After seeding, transepithelial resistance ( $R_T$ ) was monitored with an epithelial volttohmmeter (World Precision Instruments, Sarasota, FL) and monolayers with  $R_T > 500 \Omega \text{cm}^2$  were used for Ussing chamber studies after 5–12 days in culture. For patch clamp experiments cells were seeded on glass cover slips and used after 24 h.

### 2.3. Transepithelial Ussing chambers experiments

T84 monolayers grown on cell culture inserts were carefully cut from the plastic cup and mounted in Ussing chambers (World Precision Instruments, Saratoga, FL). Both chamber compartments were separately perfused with Krebs-Henseleit solutions and gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at pH 7.4. The exposed perfusing area was 0.6 cm<sup>2</sup>. Transepithelial voltage was clamped at 0 mV using a standard four-electrode voltage clamp (558C-voltage clamp, University of Iowa, Iowa City, IO). A pair of agar bridges (1 M KCl) connected through half-cell electrodes were used for measuring transepithelial potential. Ag/AgCl pellets connected to agar bridges at the back of the half chambers were used for passing current. Positive currents were defined as anion movement from serosa to mucosa.  $R_T$  was monitored and calculated from current deflections caused by single voltage pulses of 2 mV amplitude and 1 s duration every 20 s using Ohm's law. Data were recorded to a computer through an analog-to-digital board (DataQ Instruments, Akron, OH). Experiments were performed at 37 °C. T84 monolayers were bathed with a Cl<sup>-</sup>-containing solution on the serosal side and a Cl<sup>-</sup>-free solution on the mucosal side. This serosal-to-mucosal Cl<sup>-</sup> gradient was used to increase the electrochemical driving force for Cl<sup>-</sup> secretion across the apical membrane. We determined previously that under these conditions the apical membrane is the rate-limiting step during Cl<sup>-</sup> secretion (Illek et al., 2000). The mucosal Cl<sup>-</sup>-free solution had the following composition: 120 mM Na-gluconate, 20 mM NaHCO<sub>3</sub>, 5 mM KHCO<sub>3</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.6 mM glucose, 2.5 mM Ca(gluconate)<sub>2</sub>, and 1.2 mM MgSO<sub>4</sub>. The serosal Cl<sup>-</sup>-containing solution had the following composition: 120 mM NaCl, 25 mM NaHCO<sub>3</sub>, 5 mM KCl, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, mM 5.6 glucose, 2.5 mM CaCl<sub>2</sub>, and 1.2 mM MgCl<sub>2</sub>. The cAMP-elevating agonist forskolin (Calbiochem, LaJolla, CA) was prepared as a 20 mM stock solution in dimethylsulfoxide and an aliquot was added to the serosal compartment to give a final concentration of 20 μM. *N*-phenyl anthranilic acid (DPC, Aldrich, Milwaukee, WI) was prepared as a 200 mM stock in ethanol and used at 5 mM at the end of all experiments to fully block Cl<sup>-</sup> secretion. SB-300 and SP-303 were dissolved in water as 100 mM stock solutions. Aliquots were added to the mucosal compartment to give final concentrations of 5, 50 and 100 μM.

Dose-dependent block of  $I_{sc}$  was analyzed using Michaelis–Menten (MM) kinetics. Blocked  $I_{sc}$  (in %) was plotted versus concentration ( $c$ ) and fitted to the MM equation of the form  $I_{sc} = (I_{max} \times c)/(K_B + c)$ , which yields the halfmaximal effective blocker concentration,  $K_B$ , and the maximally blocked current,  $I_{max}$ . Data have been expressed as mean ± S.E.;  $n$  refers to the number of experiments. Statistical analysis was performed using ANOVA analysis (StatView, Abacus Concepts, Berkeley, CA). Probabilities of  $P < 0.05$  were considered significant.

### 2.4. Patch clamp recordings

Single T84 cells were whole cell patch-clamped as described previously (Illek and Fischer, 1998). Briefly, cells were patch-clamped in an open, heated (to 37 °C) chamber (volume = 500 μl) on the stage of an inverted microscope. The membrane potential was continuously clamped to -60 mV (EPC7, ALA Scientific Instruments Inc., Westbury, NY) and pulsed every 20 s for 1 s to -50 mV to monitor membrane conductance,  $G_m$ . Bath solution contained (in mM): 140 mM *N*-methyl-D-glucamine (NMDG)Cl, 10 mM glucose, 10 mM sucrose, 10 mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]), 1.7 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, pH 7.3. The pipette (i.e., the intracellular) solution contained: 140 mM NMDGCl, 5 mM NMDG-EGTA (ethyleneglycol-bis-(*b*-aminoethyl ether) *N,N,N',N'*-tetraacetic acid), 1 mM MgCl<sub>2</sub>, 2 mM HEPES, 1 mM glucose, 1 mM Mg-ATP, 0.1 mM Li-GTP, pH 7.3. With these solutions the major current carrier is chloride. Bath solution was hypertonic with respect to pipette solution to prevent cell swelling (Worrell et al., 1989). Cl<sup>-</sup> currents were activated by stimulation of the cell with 10 μM forskolin in the bath solution. SP-303 and SB-300 were added to the bath. Current–voltage step protocols were applied before and after addition of blocker. The membrane potential was clamped to the target voltages (-80 to +30 mV) in 10 mV increments using pClamp software (Axon Instruments Inc., Foster City, CA). Voltage-dependence of the blocked  $G_m$  was determined from the numerical difference of current–voltage-step protocols recorded before and after blocker addition. Currents were reported as current densities normalized to cell membrane capacitance ( $C_m$ , a measure for the membrane area).  $C_m$  was determined from current transients elicited by a voltage pulse. Average  $C_m$  of T84 cells was  $27.1 \pm 2.7$  pF ( $n = 17$ ).

## 3. Results

### 3.1. Inhibition of cAMP-stimulated chloride secretion across monolayers of T84 cells by SB-300

The expedited extraction of the bark latex of *Croton lechleri* yielded a novel extract (named SB-300) which contained on average  $70.6 \pm 7.2\%$  by weight SP-303 ( $n = 56$ ; mean ± S.D.). The efficacy of SB-300 on blocking intestinal Cl<sup>-</sup> secretion was studied on forskolin-stimulated T84 monolayers in Ussing chambers. Under control conditions  $I_{sc} = 36.7 \pm 3.2$  μA/cm<sup>2</sup> and  $R_T = 441 \pm 65$  Ω cm<sup>2</sup> ( $n = 12$ ). Addition of the cAMP-elevating agonist forskolin (10 μM) increased  $I_{sc}$  to  $125.0 \pm 9.4$  μA/cm<sup>2</sup> and decreased  $R_T$  to  $279 \pm 27$  Ω cm<sup>2</sup> ( $n = 7$ ). After the forskolin-stimulated Cl<sup>-</sup> currents had stabilized, cumulative doses of SB-300 were added to the mucosal Ussing chamber reservoir resulting in final concentrations of 5, 50 and 100 μM. The forskolin-stimulated  $I_{sc}$  was dose-dependently blocked by

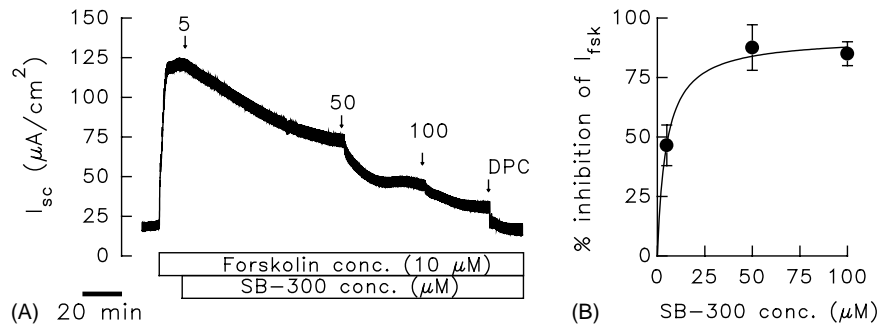


Fig. 1. Effect of SB-300 on cAMP-stimulated  $\text{Cl}^-$  secretion across T84 monolayers. (A) Transepithelial  $\text{Cl}^-$  secretion was stimulated by the cAMP agonist forskolin (10  $\mu\text{M}$ ). Mucosal addition of SB-300 inhibited forskolin-stimulated  $I_{sc}$  dose-dependently. Subsequent addition of DPC (5 mM) further blocked  $I_{sc}$ . On this compressed time scale the single 2-mV pulses are not discernible. (B) Dose-dependent inhibition of forskolin-stimulated  $\text{Cl}^-$  currents. Line represents fit to Michaelis–Menten kinetics yielding a  $K_B$  of  $4.8 \pm 0.8 \mu\text{M}$  and  $I_{max} = 92.2 \pm 3.0\%$ . Values represent means  $\pm$  S.E. of six individual T84 monolayers.

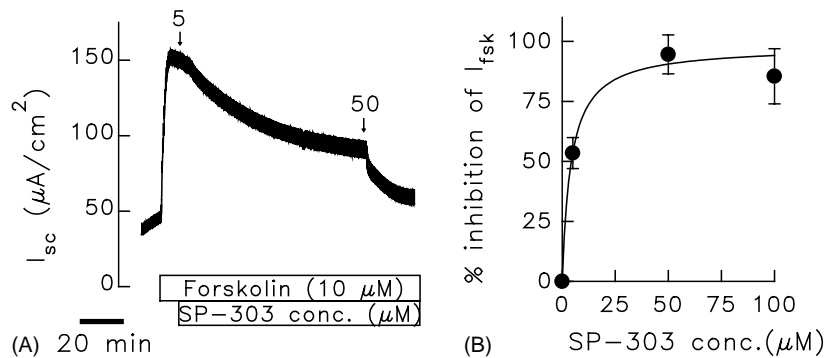


Fig. 2. Effect of SP-303 on cAMP-stimulated  $\text{Cl}^-$  secretion across T84 monolayers. (A) Typical  $I_{sc}$  recording; current was activated with 10  $\mu\text{M}$  forskolin and blocked by SP-303. In this experiment 50  $\mu\text{M}$  SP-303 blocked 93% of the forskolin-stimulated  $I_{sc}$ . (B) Dose-dependent inhibition of forskolin-stimulated  $\text{Cl}^-$  currents by SP-303. Line represents fit to Michaelis–Menten kinetics yielding a halfmaximal inhibitory constant of  $K_B = 4.1 \pm 1.3 \mu\text{M}$  and  $I_{max}$  of  $98 \pm 7.2\%$ . Values represent mean  $\pm$  S.E. of three individual T84 monolayers treated with each dosage.

SB-300 (Fig. 1A). Subsequent addition of the  $\text{Cl}^-$  channel blocker DPC (5 mM) produced a small further decrease in  $I_{sc}$ . Apical exposure of the intestinal cells to 5  $\mu\text{M}$  SB-300 decreased forskolin-stimulated  $\text{Cl}^-$  currents from  $125.0 \pm 9.4$  to  $79.4 \pm 10.6 \mu\text{A}/\text{cm}^2$  ( $n = 6$ ) corresponding to an average inhibition of forskolin-stimulated  $\text{Cl}^-$  currents by  $46.0 \pm 8.8\%$ . Exposure to 50  $\mu\text{M}$  SB-300 blocked  $I_{sc}$  to  $46.1 \pm 10.5 \mu\text{A}/\text{cm}^2$  corresponding to  $90.5 \pm 9.8\%$  inhibition. Maximal blocker effects were seen at 100  $\mu\text{M}$ . Kinetic analysis of dose-dependent current inhibition by SB-300 is shown in Fig. 1B. Data were fitted with MM kinetics yielding a  $K_B$  of  $4.8 \pm 0.8 \mu\text{M}$  and  $I_{max} = 92.2 \pm 3.0\%$ .

### 3.2. Comparison of SB-300 to SP-303 standards

The potency of the novel SB-300 extract was compared to the standard compound SP-303. Fig. 2A shows the inhibition of forskolin-stimulated  $I_{sc}$  by 5 and 50  $\mu\text{M}$  SP-303. As reported previously (Gabriel et al., 1999),  $I_{sc}$  was also effectively blocked by SP-303 in our experiments. The dose-dependent blocker effects of SP-303 are shown in Fig. 2B. Fit of the dose-response relation resulted in  $K_B = 4.1 \pm 1.3 \mu\text{M}$  and  $I_{max}$  of  $98 \pm 7.2\%$ .

Fig. 3 summarizes the inhibitory effects of SB-300 compared to the standard SP-303 at 5  $\mu\text{M}$  (Fig. 3A) and 50  $\mu\text{M}$  (Fig. 3B) concentrations. At both concentrations SB-300 showed effects not different from the SP-303 standards

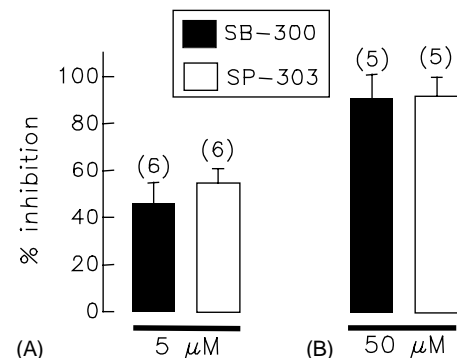


Fig. 3. Comparison of  $\text{Cl}^-$  current inhibition by SB-300 and two different batches of SP-303. (A) Inhibitory effects of SB-300 and SP-303 are expressed as percent block of forskolin-stimulated current ( $I_{fsk}$ ). Inhibitory effects were not different between SB-300 and SP-303 standards at 5  $\mu\text{M}$  (ANOVA,  $P = 0.70$ ). (B) Similarly, at 50  $\mu\text{M}$  blocker effects were not different between SB-300 and SP-303 (ANOVA,  $P = 1.0$ ). Numbers in parentheses note number of experiments.

(ANOVA), resulting in a total average of current inhibition for all three extracts of  $50.3 \pm 5.3\%$  ( $n = 12$ ) at  $5 \mu\text{M}$ , and  $91.2 \pm 6.0\%$  ( $n = 10$ ) at  $50 \mu\text{M}$ . Therefore, SB-300 blocked transepithelial  $\text{Cl}^-$  secretion with a similar efficacy as the SP-303 standard.

### 3.3. Whole cell patch clamp experiments

To identify the biophysical properties of the  $\text{Cl}^-$  conductance blocked by SB-300 and SP-303, single T84 cells were investigated with the whole cell patch clamp technique under conditions where only  $\text{Cl}^-$  current was measured (see Section 2). T84 cells were continuously clamped to  $-60 \text{ mV}$  and pulsed every 20 s to  $-50 \text{ mV}$ . The voltage-dependence of the measured  $\text{Cl}^-$  conductance was determined by clamping the membrane potential from  $-80$  to  $+30 \text{ mV}$  in  $10\text{-mV}$  increments.  $\text{Cl}^-$  currents were continuously recorded. Unstimulated T84 cells expressed a specific membrane conductance ( $G_m$ ) of  $19.9 \pm 5.6 \text{ pS/pF}$  ( $n = 6$ ), which was

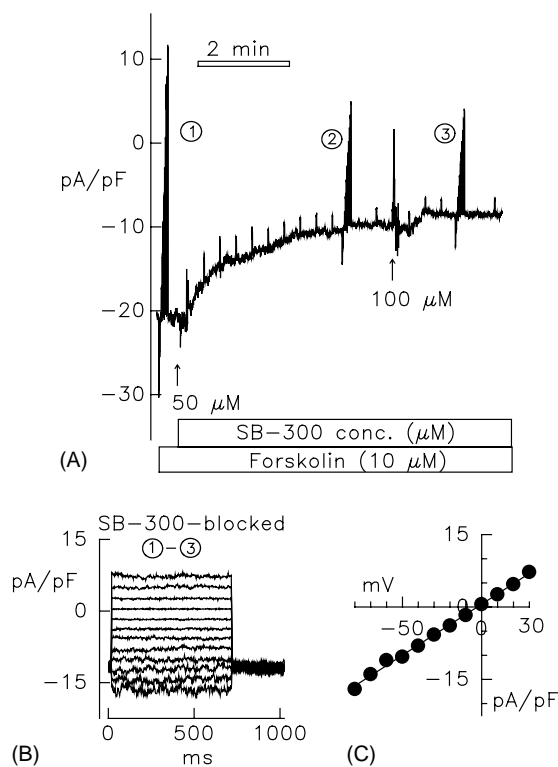


Fig. 4. Effect of SB-300 on forskolin-stimulated whole cell  $\text{Cl}^-$  currents in single T84 cells. (A) Continuous whole cell current recording. Specific current density (in  $\text{pA/pF}$ ) is plotted over time. T84 cell was pre-stimulated with  $10 \mu\text{M}$  forskolin. Membrane potential was clamped to  $-60 \text{ mV}$  and pulsed every 20 s to  $-50 \text{ mV}$ . Current–voltage step protocols (from  $-80$  to  $+30 \text{ mV}$  in  $10 \text{ mV}$  increments) were applied before and after addition of  $50$  and  $100 \mu\text{M}$  drug (at ①, ②, ③). Fifty and  $100 \mu\text{M}$  of SB-300 were added to the bath where indicated. (B) SB-300-blocked currents were calculated by subtracting currents recorded at ③ from currents at ① and the currents are voltage-independent. (C) Current–voltage relation of SB-300-blocked current. Line shows linear regression, which yielded a SB-300-blocked  $G_m$  of  $215 \text{ pS/pF}$  and  $C_m$  of  $13 \text{ pF}$ . In this recording  $G_m$  was  $362 \text{ pS/pF}$  before blocker addition (at ③).

increased to  $348 \pm 209 \text{ pS/pF}$  by  $10 \mu\text{M}$  forskolin. Fig. 4A shows a typical recording of the inhibitory effect of SB-300 on forskolin-stimulated whole cell chloride currents. Subsequent additions of  $50$  and  $100 \mu\text{M}$  SB-300 to the bath solution blocked whole cell  $\text{Cl}^-$  currents with a  $t_{1/2}$  of  $\sim 1 \text{ min}$  suggesting that SB-300 acts from the extracellular side. Before and after drug addition current–voltage relations were recorded, as depicted in Fig. 4A with circled 1 to circled 3. The voltage-dependence of the SB-300-sensitive  $\text{Cl}^-$  current is shown in Fig. 4B. The SB-300 blocked  $\text{Cl}^-$  current was time- and voltage-independent and the current–voltage relation was linear (Fig. 4C), which are typical characteristics of  $\text{Cl}^-$  currents mediated by the CFTR  $\text{Cl}^-$  channel. Blocked  $G_m = 215 \text{ pS/pF}$ , which was  $59.6\%$  of total  $G_m$  ( $362 \text{ pS/pF}$ ). On average,  $50 \mu\text{M}$  SB-300 inhibited  $63 \pm 8.5\%$  ( $n = 3$ ) of whole cell  $G_m$ .

Fig. 5 shows the inhibition of forskolin-stimulated whole cell  $\text{Cl}^-$  currents by the standard SP-303. Addition of  $50 \mu\text{M}$  SP-303 decreased  $G_m$  from  $584$  to  $100 \text{ pS/pF}$ , i.e., by  $82.8\%$ . On average,  $50 \mu\text{M}$  SP-303 blocked  $83 \pm 0.6\%$  ( $n = 2$ ) of whole cell  $G_m$ . SP-303-blocked currents were time- and voltage-independent (Fig. 5B), and the current–voltage relation was linear (Fig. 5C). The voltage-dependent properties

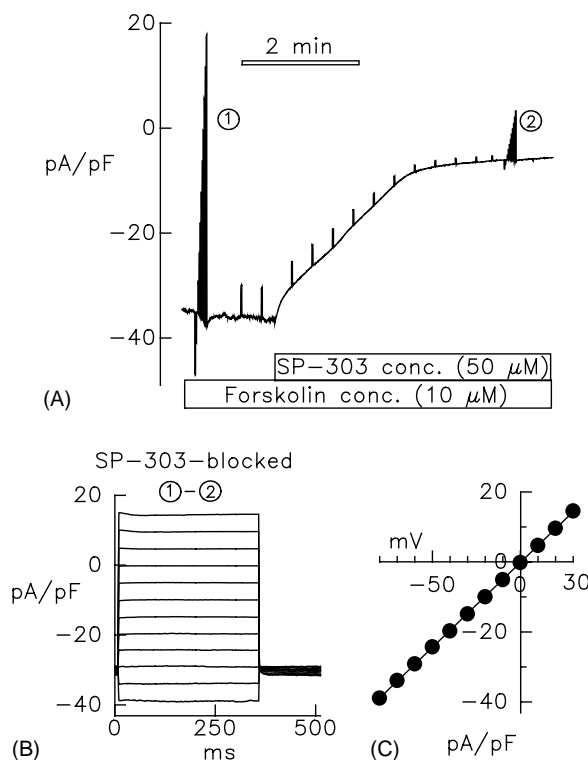


Fig. 5. Effect of SP-303 on forskolin-stimulated whole cell  $\text{Cl}^-$  currents in single T84 cells. (A) SP-303 was added at  $50 \mu\text{M}$  to the bath solution. Conditions as in Fig. 4. (B) SP-303-blocked currents were calculated by subtracting currents recorded at ② from currents at ①. Membrane potential was clamped from  $-80$  to  $+30 \text{ mV}$ . The currents are voltage-independent. (C) Current–voltage relation of SP-303-blocked current; linear regression yielded a SP 303-blocked  $G_m$  of  $484 \text{ pS/pF}$  and  $C_m$  of  $51 \text{ pF}$ . In this recording  $G_m$  was  $584 \text{ pS/pF}$  before blocker addition (at ①).

of the SB-300 and SP-303 blocked currents had identical characteristics indicating that both extracts inhibited the same type of chloride conductance.

#### 4. Discussion and conclusions

The stem bark latex as well as the bark of the tree *Croton lechleri* are widely used by South American indigenous peoples as a general remedy for numerous ailments, including the oral treatment of diarrhea, cough and flu and the topical treatment of Herpes simplex (Orozco-Topete et al., 1997). SP-303 was initially isolated from the bark latex by using bioassays for anti-viral activity (Ubillas et al., 1994). Owing to the historical usage of the bark as an anti-diarrhetic agent, Gabriel et al. (1999) tested the effects of SP-303 on cholera toxin-induced fluid secretion in mice and on forskolin-activated  $\text{Cl}^-$  secretion across intestinal epithelial cultures. They found that both fluid and  $\text{Cl}^-$  secretion were blocked by SP-303.

SP-303 is an oligomeric proanthocyanidin. The isolation and purification procedure resulted in a low product yield of ~2.5% of the bark latex. Therefore, the extraction process was modified, which resulted in significantly higher yields. The novel extract SB-300 contained on average 70.6% by weight SP-303. The aim of this study was to investigate the efficacy of SB-300 on cAMP-regulated  $\text{Cl}^-$  secretion in comparison to the SP-303 standard. In Ussing chamber recordings, SB-300 reached on average 85% of the affinity of SP-303 and blocked on average 94% of currents blocked by SP-303. In patch clamp recordings, SB-300 blocked on average 76% of SP-303 blocked currents. Therefore, it appears that the SP-303 standard was slightly more potent than the novel extract SB-300. This can be attributed to the lower content of SP-303 in SB-300 when compared to the purified SP-303. In addition both the SP-303 compound and SB-300 extract blocked the cAMP-activated and CFTR type  $\text{Cl}^-$  conductance with identical biophysical characteristics, suggesting that they both target the CFTR  $\text{Cl}^-$  channel with a common mode of action.

Although a variety of factors may cause diarrhea in humans, uncontrolled  $\text{Cl}^-$  secretion appears to be a key factor leading to the loss of vast amounts of salt and water. Under physiological conditions, the  $\text{Cl}^-$  conductance of the apical cell membrane of intestinal epithelial cells is a critical regulator of transcellular  $\text{Cl}^-$  secretion. Several epithelial  $\text{Cl}^-$  channels have been described in intestinal cells, including swelling-activated, Ca-activated, and voltage-activated  $\text{Cl}^-$  channels, which are distinguished by their physiological regulation and by their biophysical, voltage-dependent characteristics (Worrell et al., 1989; Cliff and Frizzell, 1990; Anderson et al., 1992). However, evidence has accumulated that the major apically located  $\text{Cl}^-$  conductance in intestinal cells is mediated by the CFTR  $\text{Cl}^-$  channel (Anderson et al., 1992; Huflejt et al., 1994). Therefore, the CFTR  $\text{Cl}^-$  channel presents a primary drug target for the pharmacologic

block of  $\text{Cl}^-$  secretion. Several small molecules of different structural classes are commonly used in the laboratory to block CFTR such as stilbenes, arylaminobenzoates (e.g., DPC used in this study), sulfonyleureas (Schultz et al., 1999), polyphenols (Illek et al., 2000) and recently discovered thiazolidinones (Ma et al., 2002). However, with the exception of thiazolidinones all other currently used CFTR blockers have various other cellular targets and are effective only at high concentrations unacceptable for human use.

Our data suggest that SP-303 and SB-300 are direct CFTR blockers acting from the extracellular side. This notion is supported by the observations that both extracts provoked an immediate response upon exposure of the intestinal lumen in Ussing chamber experiments and also upon addition to the extracellular bath solution in patch clamp experiments. Since the average molecular weight of both isolates is high (2200 Da for SP-303 and 3000 Da for SB-300), transport across the membrane at a sufficient rate to reach a potential intracellular target seems unlikely. When given orally, SP-303 could not be detected in the serum of patients, indicating that SP-303 did not permeate the intestinal wall (Holodniy et al., 1999). Yet, SP-303 and SB-300 are effective in the low micromolar range and, thus, are some of the highest affinity CFTR blockers to date. It is intriguing and worth additional investigation to determine the effects of SP-303 and SB-300 on single channel parameters of the CFTR  $\text{Cl}^-$  channel.

The unrefined extract derived from several *Croton* species has been recently shown to impair the capsaicin-stimulated ion transport across guinea pig ileum when added to the serosal bath in Ussing chambers (Miller et al., 2000). The effect was attributed to its ability to directly compromise sensory afferent activation. Thus it is likely that the sap of *Croton* species has additional cellular targets involved in the regulation of intestinal ion transport when applied from the blood side of the epithelium.

In summary we conclude that the expedience of extraction from the liquid stem bark latex of *Croton lechleri* yielded a novel extract (SB-300), which did not compromise the known pharmacologic properties of the herbal extract as a chloride transport inhibitor. Both SP-303 and SB-300 targeted the CFTR  $\text{Cl}^-$  channel suggesting that SB-300 presents a cost-effective alternative to SP-303. The development of a pharmacological formulation of SB-300 may provide a potent anti-diarrhetic agent for turning off excessive CFTR activity during watery diarrhea. SB-300 may serve as a complementary and alternative approach for the treatment of pediatric diarrhea in the developing world, as well as for diarrheal episodes in AIDS patients and for people affected with traveler's diarrhea.

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