Antidiarrheal Efficacy and Cellular Mechanisms of a Thai Herbal Remedy

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Abstract

Screening of herbal remedies for Cl− channel inhibition identified Krisanaklan, a herbal extract used in Thailand for treatment of diarrhea, as an effective antidiarrheal in mouse models of secretory diarrheas with inhibition activity against three Cl− channel targets. Krisanaklan fully inhibited cholera toxin-induced intestinal fluid secretion in a closed-loop mouse model with ~50% inhibition at a 1:50 dilution of the extract. Orally administered Krisanaklan (5 μL/g) prevented rotavirus-induced diarrhea in neonatal mice. Short-circuit current measurements showed full inhibition of CAMP and Ca2+ agonist-induced Cl− conductance in human colonic epithelial T84 cells, with ~50% inhibition at a 1:5,000 dilution of the extract. Krisanaklan also strongly inhibited intestinal smooth muscle contraction in an ex vivo preparation. Together with measurements using specific inhibitors, we conclude that the antidiarrheal actions of Krisanaklan include inhibition of luminal CFTR and Ca2+-activated Cl− channels in enterocytes. HPLC fractionation indicated that the three Cl− inhibition actions of Krisanaklan are produced by different components in the herbal extract. Testing of individual herbs comprising Krisanaklan indicated that agarwood and clove extracts as primarily responsible for Cl− channel inhibition. The low cost, broad antidiarrheal efficacy, and defined cellular mechanisms of Krisanaklan suggest its potential application for antisecretion therapy of cholera and other enterotoxin-mediated secretory diarrheas in developing countries.

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Introduction

Secretory diarrhea is a major health challenge in developing countries, representing the second leading cause of mortality globally in children under age 5 [1]. Repeated episodes of hypovolemia from diarrhea can produce malnutrition and impaired development [2]. The mainstay of diarrhea therapy is oral rehydration solution (ORS), which consists of an aqueous mixture of salts and carbohydrates [3,4]. Though ORS has reduced mortality from diarrhea four-fold in the last 3 decades, its efficacy is limited, particularly in the young and elderly, and because of practicalities in its availability and compliance [5]. Antisecretory drug therapy for diarrhea may be efficacious when ORS is not available, as during natural disasters, and it may potentiate the efficacy of ORS.

The intestinal epithelium absorbs and secretes large volumes of fluid, with net absorption under normal conditions and net secretion in secretory diarrheas. Intestinal fluid secretion involves Cl− transport from the blood into the intestinal lumen through Cl− channels in the enterocyte apical plasma membrane, which include the cAMP-gated channel CFTR (cystic fibrosis transmembrane conductance regulator) and one or more CaCCs (Ca2+-activated Cl− channels) whose molecular identity is not known [6–8]. CFTR is the primary route for Cl− secretion in secretory diarrheas caused by bacterial enterotoxins in cholera and Traveler’s diarrhea (caused by enterotoxigenic E. coli). CaCCs are likely involved as well in these diarrheas because of cross-talk between cyclic nucleotide and Ca2+ signaling [9,10], and may provide the primary route for Cl− secretion in some viral and drug-induced diarrheas, including childhood rotaviral diarrhea [11,12] and antiretroviral drug-induced diarrhea [13]. The Ca2+-activated Cl− channel TMEM16A is expressed intestinal pacemaker cells, the interstitial cells of Cajal, where it is required intestinal smooth muscle contraction and motility [14,15]. TMEM16A is widely expressed in secretory epithelia in the airways and salivary gland, but probably plays at most a minor role as a CaCC in intestinal epithelium [16].

There is currently no approved antisecretory drug for treatment of major secretory diarrheas such as cholera. Our laboratory has identified, by high-throughput screening, several classes of small-molecule CFTR and CaCC inhibitors [17], and has shown their efficacy in mouse models of secretory diarrheas [18,19]. As an alternative approach to the costly and lengthy development of a new chemical entity, here we investigated the possibility that effective, natural-product antisecretory therapeutics may already be available, but unappreciated. Screening of diarrhea remedies from around the world for enterocyte Cl− channel inhibition identified Krisanaklan, a herbal extract used widely in Thailand for treatment of diarrhea, as effective in inhibiting intestinal Cl− secretion and motility. We previously reported that one component of Krisanaklan, eugenol, inhibited the CaCC TMEM16A [20]. Here, we report here on the...
Author Summary

Secretory diarrhea is a major health challenge in developing countries. Causative agents include bacteria, as in cholera, and viruses, as in childhood rotaviral diarrhea. Though oral rehydration solution (ORS) has reduced mortality from diarrhea four-fold in the last three decades, its efficacy is limited, particularly in the young and elderly, and because of practicalities in its availability and compliance. Antisecretory drug therapy for diarrhea may be efficacious when ORS is not available, as during natural disasters, and it may potentiate the efficacy of ORS. As an alternative approach to the costly and lengthy development of a new chemical entity, in this study we investigated the possibility that effective, natural-product antisecretory therapeutics may already be available, but unappreciated. Screening of diarrhea remedies from around the world for enterocyte chloride channel inhibition identified Krisanaklan, a herbal extract used widely in Thailand for treatment of diarrhea, as effective in inhibiting intestinal chloride secretion. We report the antisecretory efficacy and cellular mechanisms of Krisanaklan, providing proof-of-concept for its potential utility for antisecretory therapy of major, life-threatening diarrheas in developing countries.

Methods

Ethics statement

This study was approved by the UCSF Institutional Animal Care and Use Committee (IACUC approved protocol AN089748), and was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Cell lines and herbal preparation

FRT cells stably expressing human CFTR or TMEM16A were generated and cultured as described [16,21]. T84 cells (ATCC CCL-248) were cultured as described [22]. The Thai herbal formulation Krisanaklan was purchased from Osotspa Inc. (Bangkok, Thailand).

Short-circuit current

Snapwell inserts containing T84 or FRT cells were mounted in Ussing chambers (Physiologic Instruments, San Diego, CA), as described [16,23]. Activators and inhibitors were added to the apical solution and an equal volume of vehicle was added at the basolateral solution. Symmetrical HCO₃⁻-buffered solutions were used for T84 cells. For FRT cells, the hemichambers were filled with a half-Cl⁻ solution (apical) and the HCO₃⁻-buffered solution (basolateral), and the basolateral membrane was permeabilized with 250 μg/mL amphotericin B. Under these conditions short-circuit current is a direct measure of apical membrane Cl⁻ conductance. Cells were bathed for a 10 min stabilization period and aerated with 95% O₂/5% CO₂ at 37°C. Short-circuit current was measured using an EVC4000 Multi-Channel V/I Clamp (World Precision Instruments, Sarasota, FL).

Transepithelial transport of Krisanaklan

T84 cells were grown on 12-mm diameter collagen-coated transwell inserts (0.4-μm pore size Costar, Corning, Tewksbury, MA). Cells were cultured for 5–7 days to form tight monolayers with transepithelial resistance 900–1,000 Ω cm². Krisanaklan (1.5 ml of 6% solution) in Ringers bicarbonate buffer was added into the basolateral chamber, and 0.5 ml of Ringers bicarbonate alone was added into the apical chamber. Apical chamber fluid (200 μL) was collected at 0, 30 and 60 min (and replaced with the identical volume of buffer). The fluid samples were bioassayed for Cl⁻ transport inhibition by short-circuit current measurement on T84 cells as described above. The percentage transport of inhibitory substance(9) was computed from activities of apical samples versus the original basolateral fluid, correcting for dilution.

Fluid secretion and absorption in mouse intestinal closed-loops

Mice (CD1 strain, weight 25–35 g) were deprived of food for 24 h and anaesthetized with intraperitoneal 2,2,2-tribromoethanol (Avertin, Sigma-Aldrich, St. Louis, MO) (125 mg/kg). Body temperature was maintained at 36–38°C using a heating pad. Following a small abdominal incision, three closed mid-jejunum loops (length 20–30 mm) were isolated by sutures, as described [18]. Loops were injected with 100 μL of PBS or PBS containing cholera toxin (1 μg) without or with Krisanaklan. The abdominal incision was closed with suture and mice were allowed to recover from anesthesia. At 4 h the mice were anaesthetized, intestinal loops were removed, and loop length and weight were measured to quantify net fluid secretion. Fluid absorption was measured separately, from the reduction in loop weight/length ratio at 30 min after injection of 200 μL PBS. PBS containing 10 mM glucose was used as a positive control for fluid absorption. Mice were killed by an overdose of Avertin.

Gastrointestinal motility

Mice (CD1 strain, weight 25–35 g) were deprived of food for 24 h before experiments. Krisanaklan (3% in 100 μL PBS) was administered either orally or by intraperitoneal injection. Fifteen min later mice were orally administered a charcoal meal (0.2 ml of 10% activated charcoal suspended in 5% gum acacia) with or without 3% Krisanaklan. Thirty minutes later the mice were sacrificed and the small intestine was isolated. The peristaltic index was calculated as the percentage of distance traveled of the charcoal meal relative to the total length of small intestine.

Rotaviral diarrhea studies

Neonatal C57bl/6 mice (age 5–7 days, weight 1.8–2.5 g) were inoculated with 30 μL (1.2×10⁷ pfu/mL) of Simian SA-11 rotavirus (ATCC VR 1739) by oral gavage, as modified from prior reported models [10,24]. The treated group received 10 μL Krisanaklan one day after rotavirus infection. Stool specimens were collected by gentle palpation of the mouse abdomen 2 day after rotavirus inoculation. For quantification of stool water content we fabricated a polydimethylsiloxane slab of 1.5-mm thickness with a 1.91-mm diameter hole to contain a cylindrical 4.3-mm³ volume of stool, as described [24]. The stool plug was expelled onto absorbent tissue in a humidified atmosphere and allowed to contact the tissue for 1 min. The wetted area was measured and related to absolute water content using stool standards. In some studies the mid-jejunum was perfusion-fixed at 2 days after rotavirus inoculation for preparation of 5-μm thick, hematoxylin and eosin-stained, paraffin-embedded sections.

Ca²⁺ and cAMP measurement

For measurement of cytosolic Ca²⁺, FRT-TMEM16A cells were plated in 96-well black-walled microplates. After removal of
growth medium 100 μl of 10 μM Fluo-4 NW (Invitrogen, Carlsbad, CA) was added and incubated at 37°C for 30 min, then at room temperature for an additional 30 min. Fluo-4 fluorescence was measured with a plate reader at excitation/ emission wavelengths of 493/538 nm. cAMP was assayed in T84 cells treated for 30 min with 0 or 10 μM forskolin, without or with Krisanaklan, lysed by repeating freeze/thaw, centrifuged, and the supernatant was assayed (Parameter cAMP immunoassay kit; R&D Systems, Minneapolis, MN).

High performance liquid chromatography (HLPC) and dialysis
Fractionation was performed on an AKTA Explorer 10 system (GE Healthcare Life Science, Piscataway, NJ) equipped with a C18 reversed-phase column (Vianar Pursuit XRs, 250×10 mm, 5 mm particle size, Waldbronn, Germany), as described [20]. In separate studies Krisanaklan was dialyzed using 1-, 10-, and 50-kDa cut-off membranes (Float-A-Lyzer G2, Spectrum Laboratories, Rancho Dominguez, CA).

Intestinal smooth muscle contraction

Wild-type CD1 mice (age 7–10 weeks) were killed by avertin overdose (200 mg/kg). The ileum was isolated and washed with (in mM): 120 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, 10 glucose and 10 HEPES, and 25 NaHCO3 (pH 7.4). The ends of the ileal segments were tied and connected to a force transducer, as described [25]. Ileal segments were stabilized for 60 min with a resting force of ~1 mN, with changes of the bathing solution every 20 min.

Whole-cell patch-clamp

Whole-cell recordings were made at room temperature on T84 cells, and CFTR- and TMEM16A-expressing FRT cells. The bath solution contained (in mM): 140 N-methyl-D-glucamine-Cl, 1 CaCl2, 1 MgCl2, 10 glucose and 10 HEPES (pH 7.4) for the TMEM16A and CFTR. The pipette solution contained (in mM): 130 CsCl, 0.5 EGTA, 1 MgCl2, 1 Tris-ATP and 10 HEPES (pH 7.2). TMEM16A was activated by 400 nM free Ca2+ in the pipette solution. CFTR currents were recorded by test pulse from ~80 to +80 mV from a holding potential of 0 mV in the presence of forskolin. Cl− currents in FRT-TMEM16A cells were elicited by applying voltage pulses from a holding potential of 0 mV to potentials between −100 mV and +100 mV with increases of 20 mV. CaCC was activated by 1000 nM free Ca2+ in T84 cells. To record CaCC in T84 cells, external solution contained (in mM): 150 NaCl, 6 CsCl, 2 CaCl2, 1 MgCl2, 10 glucose and 10 HEPES (pH 7.4) were used. The pipette solution contained (in mM): 40 CsCl, 100 Cs-aspartate, 5 EGTA, 1 MgCl2, 4.33 CaCl2, 4 Na2-ATP and 10 HEPES (pH 7.2). The currents in T84 cells were evoked by test pulse from −100 mV to 100 mV with increases of 20 mV from a holding potential of −50 mV. Pipettes (3–4 MΩ) were fabricated on a model P-97 electrode puller (Sutter Instrument, Novato, CA) and polished with a MF-900 Micro Forge (Narishige Scientific Instruments Laboratories). Whole-cell currents were recorded using an Axopatch-200B (Axon Instruments) and currents were filtered at 1–2 kHz and digitized at 2–4 kHz.

Statistical analysis
Statistical analysis was done with Prism 5 software (GraphPad Software Inc., San Diego, CA) using 2-tailed Student’s t test, Mann-Whitney rank-sum test, or one-way analysis of variance (ANOVA), where appropriate. Data are presented as the mean ± S.E.M. A P value of 0.05 or less was considered significant.

Results

A Thai herbal remedy inhibits intestinal cAMP and Ca2+-activated Cl− channels

The Thai herbal medicine Krisanaklan (Fig. 1A) was identified from testing of diarrheal remedies for inhibition of intestinal Cl− channels. Fig. 1B shows inhibition of CFTR Cl− current in a human intestinal epithelial cell line (T84 cells) in response to stimulation by the CFTR agonist forskolin, an adenylyl cyclase activator, and IBMX, a phosphodiesterase inhibitor. The IC50 for inhibition of CFTR Cl− current was <0.01% Krisanaklan (1:10,000 dilution), with complete inhibition at higher concentrations. CFTR Cl− current was inhibited by the CFTR inhibitor CFTRinh-172 (red curve in Fig. 1B). Krisanaklan also inhibited CaCC Cl− current in T84 cells following stimulation by ATP, with IC50 ~0.02% Krisanaklan (Fig. 1C). The CaCC measurement was done in the presence of a CFTRinh-172 to eliminate ATP-dependent CFTR Cl− currents that arise from cross-talk between cAMP and Ca2+ signaling. CaCC Cl− current was inhibited by the non-selective CaCC inhibitor tannic acid (red curve in Fig. 1C).

Krisanaklan did not inhibit cAMP or Ca2+ signaling in T84 cells. Addition of Krisanaklan up to 0.1% did not reduce cytoplasmic cAMP accumulation in response to forskolin (Fig. 1D), nor did it reduce cytoplasmic Ca2+ elevation in response to ATP (Fig. 1E). These results suggest direct action of component(s) of Krisanaklan on CFTR and CaCC Cl− channels.

Whole-cell patch-clamp was done to further investigate Krisanaklan effects on CFTR and CaCC currents. CFTR Cl− current was measured in CFTR-expressing FRT cells following forskolin addition (Fig. 2A). Approximately linear Cl− currents were seen before and after CFTR inhibition by addition of a 1:2000 dilution of Krisanaklan. CaCC Cl− current was measured in T84 cells following activation by high pipette Ca2+ in the presence of CFTR inhibitor CFTRinh-172 (Fig. 2B). Outwardly rectifying Cl− currents were seen before and after Krisanaklan addition, which were fully inhibited by the CaCC inhibitor CaCCinh-A01. Cl− current was also measured in FRT cells expressing TMEM16A (Fig. 2C). The outwardly rectifying currents elicited by high pipette Ca2+ were ~50% inhibited by a 1:2000 dilution of Krisanaklan, and fully inhibited by the TMEM16A inhibitor T616Ainh-A01.

To investigate whether the active Cl− inhibitory component(s) in Krisanaklan might act from the inside or outside of cells, we used a bioassay to measure transepithelial transport in T84 cells grown on a porous filter. Following addition of Krisanaklan to the basolateral membrane bathing solution, the apical solution was sampled at 30 and 60 min and assayed for CFTR and CaCC activity by short-circuit current in T84 cells. While the component(s) of Krisanaklan responsible for CFTR inhibition were cell permeable, those responsible for CaCC inhibition were not (Fig. 2D). Therefore, different components of Krisanaklan are responsible for CFTR and CaCC inhibition activities, as investigated further below. The results also suggest an intracellular site of action for CFTR inhibition and an extracellular site of action for CaCC inhibition.

Krisanaklan inhibits intestinal fluid secretion in mouse models of cholera and rotaviral diarrhea

Krisanaklan was tested for antisecretory activity in a mouse model of CFTR-dependent secretory diarrhea caused by cholera toxin and of CaCC-dependent secretory diarrhea caused by rotavirus infection. An established model of cholera toxin-induced intestinal fluid secretion was used in which fluid accumulation is
measured in closed loops of mouse mid-jejunum in vivo at 4 hours after injection of choler toxin into each loop. Fig. 3A shows marked fluid accumulation in a cholera toxin-injected loop compared to a control (PBS-injected) loop. Inclusion of small quantities of Krisanaklan reduced loop fluid accumulation. Fig. 3B shows a dose-dependent reduction in intestinal fluid accumulation, with IC50 of 1–2 μl Krisanaklan per loop, with near complete inhibition of loop fluid accumulation at higher concentrations. The determinants of intestinal fluid accumulation include fluid secretion and absorption. To verify that Krisanaklan did not affect intestinal fluid absorption, measurements of fluid absorption were made in closed, mid-jejunal loops at 30 min after injection of 200 μl PBS, in which ~65% of the injected fluid was absorbed. Fig. 3C shows no significant effects of Krisanaklan on loop fluid absorption.

The prevention of watery stool by Krisanaklan could be a result of its antisecretory action and/or inhibition of rotaviral infection of the intestine. Fig. 4B shows the most characteristic finding of rotaviral infection of the small intestine, prominent enterocyte vacuolization [29]. Similar pathological changes were seen in intestine from Krisanaklan-treated mice, suggesting that Krisanaklan did not prevent the rotavirus infection.

Krisanaklan inhibits intestinal smooth muscle contraction

Based on our prior study of TMEM16A inhibition by Krisanaklan [20], we postulated that the antidiarrheal action of Krisanaklan may also involve a third mechanism – inhibition of intestinal smooth muscle contraction, as TMEM16A is expressed in interstitial cells of Cajal, where it is required for intestinal smooth muscle contraction [14]. Fig. 5A shows Krisanaklan inhibition of TMEM16A Cl− current in TMEM16A-expressing FRT cells, with IC50 ~0.06% Krisanaklan, and complete inhibition at higher concentrations.
Krisanaklan inhibition of intestinal smooth muscle contraction was measured in ex vivo mouse ileal strips using a force transducer and a 37°C physiological bath. Fig. 5B (top) shows spontaneous ileal contractions with amplitude, 1.5 mN. In agreement with our prior data [20], addition of Krisanaklan to the bath produced a concentration-dependent reduction, to near zero, of contraction amplitude, without effect on contraction frequency. Krisanaklan also reduced the amplitude of intestinal contractions following application of the agonist carbachol (Fig. 5B, bottom).

To investigate whether Krisanaklan inhibition of intestinal smooth muscle contraction found ex vivo may be relevant to gastrointestinal motility in vivo, we used a standard assay of intestinal motility involving transit of an orally administered activated charcoal meal. While intraperitoneal Krisanaklan at a dose similar to that used in humans significantly reduced peristaltic index, oral Krisanaklan did not (Fig. 5C). The difference is likely due to minimal accumulation of TMEM16A-inhibiting components in Krisanaklan in T84 cells grown on a porous transwell insert. Percentage transport of Krisanaklan inhibitory compound(s) at 30 and 60 min measured by bioassay of Cl− channel inhibition (mean ± S.E., n = 3).

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Distinct components of Krisanaklan are responsible for Cl− channel inhibition

We investigated the nature of the component(s) responsible for Cl− channel inhibition by Krisanaklan. Initial studies showed that the Cl− channel inhibition activities of Krisanaklan were heat-insensitive (100°C for 2 min, data not shown). Several rough size fractions of Krisanaklan were prepared by dialysis using 1-, 10- and 50-kDa cut-off membranes and tested for Cl− channel inhibition. Fig. 6A shows inhibition of CFTR by the <1 kDa fraction, but little effect of the >1, >10 and >50 kDa size fractions, suggesting that the CFTR inhibitor molecule(s) have molecular size <1 kDa. Similar CaCC inhibition was seen for <1 and >1 kDa size fractions, whereas the >10 and >50 kDa showed little inhibition (Fig. 6B). Strong TMEM16A inhibition was seen for the <1 kDa fraction, with less inhibition for the higher molecular size fractions (Fig. 6C), suggesting that the TMEM16A inhibitor molecule(s) have a molecular size <1 kDa.

Fig. 6D shows that the >1 kDa fraction produce little inhibition of intestinal smooth muscle contraction, whereas the original Krisanaklan showed strong inhibition. Fig. 6E shows reverse-phase HPLC fractionation of Krisanaklan, done as reported previously [20]. Testing of individual fractions reveals distinct fractions as responsible for the CFTR, CaCC and TMEM16A inhibition actions of Krisanaklan. CaCC inhibition activity was found in several fractions, suggest a heterogeneous mixture of relatively large molecules as responsible. To determine which of the four herbal constituents of Krisanaklan are responsible for its Cl− channel inhibition activities, extracts were prepared from each individual herb and tested in T84 and FRT-TMEM16A cell cultures. Concentrations were adjusted to correspond to the original Krisanaklan formulation consisting of an ethanol/water (54:46) extract in which each
100 mL is extracted from 10 g Aquilaria crassna bark (agarwood), 33.3 g clove flower bud, 2 g Terminalia triptera Stapf bark and 4.8 g camphor. CFTR inhibition activity was found in the agarwood and clove tracts, but not in the camphor and Terminalia triptera extracts (Fig. 7A). CaCC inhibition activity was found in the agarwood and clove extracts, but not in the camphor and Terminalia triptera extracts (Fig. 7B). TMEM16A inhibition activity was found mainly in the agarwood and clove extracts (Fig. 7C).

Discussion

There is an unmet need for effective drug therapy for secretory diarrheas, especially in developing countries where cholera and other enterotoxin-mediated secretory diarrheas remain a major cause of morbidity and mortality. Potential targets for antisecretory therapy include the causative bacterial or viral agent (vaccines and antibiotics), elaborated endotoxins and endotoxin-enterocyte interactions, as well as enterocyte signaling effectors (cAMP, cGMP, Ca$^{2+}$) and membrane transporters involved in fluid secretion (Cl$^{-}$ and K$^{+}$ channels, NKCC1) and absorption (NHE3, SGLT1) [6]. Cl$^{-}$ channels are attractive targets for antisecretory therapy because they are the final, rate-limiting step in Cl$^{-}$ (and hence Na$^{+}$ and water) secretion. Unlike vaccines and antimicrobials that target the causative microbial agent, therapies targeting host secretory mechanisms, such as enterocyte Cl$^{-}$ channels, are not subject to the emergence of resistance. Here, we identified a widely used Thai herbal remedy, Krisanaklan, as having broad antidiarrheal efficacy in bacterial and viral models of secretory diarrhea, which, at the cellular level, inhibits the two major enterocyte Cl$^{-}$ channels, CFTR and CaCC.

CFTR and CaCCs are responsible for Cl$^{-}$ secretion across the luminal membrane of enterocytes in the intestinal epithelium. Several lines of evidence support the conclusion that CFTR is the major apical membrane Cl$^{-}$ pathway in secretory diarrheas caused by the bacterial enterotoxins in cholera and Traveler’s diarrhea; (i) The small intestine and colon show robust cAMP-activated CFTR Cl$^{-}$ currents [30]; (ii) intestinal Cl$^{-}$ and fluid secretion are reduced in CFTR-deficient mice and humans [31–33]; and (iii) CFTR inhibitors are effective in various rodent models of cholera [18,19]. CaCC(s) are likely involved as well in diarrheas caused by bacterial endotoxins, as experimental evidence supports cross-talk in cAMP and signalling mechanisms in which cAMP elevation increases cytoplasmic Ca$^{2+}$ [9] and Ca$^{2+}$ elevation increases cytoplasmic cAMP [34]. CaCC(s) are proposed to be the primary route for Cl$^{-}$ secretion in diarrheas caused by rotaviral and other viral enterotoxins [24,35] and various anti-retroviral and chemotherapeutic agents [13,36]; however, definitive quantification of the involvement of CaCC(s) in diarrheas awaits their molecular identification. From these considerations therapeutics targeting
Figure 4. Krisanaklan prevents watery diarrhea in rotavirus-inoculated neonatal mice. A. (left) Neonatal mice were inoculated with rotavirus by oral gavage, followed by Krisanaklan (or saline control) at day 1, and stool water was determined at day 2. Photographs of stool obtained from rotavirus-inoculated mice without and with Krisanaklan treatment. Stool was contacted with absorbent paper for 1 min to allow wetting (demarcated by dashed line). (right) Stool water content deduced from the wetted area on absorbent paper following deposition of a defined stool volume (mean ± S.E., 8 mice per group, * P<0.005). B. Hematoxylin and eosin-stained sections of ileum and jejunum from control mice, and untreated and Krisanaklan-treated rotavirus-inoculated mice.

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both enterocyte CFTR and CaCC(s) are predicted to have the greatest and broadest efficacy in secretory diarrheas.

Krisanaklan is an inexpensive, natural-product extract containing ingredients that fully inhibit the major enterocyte Cl\textsuperscript{−}2 channels, CFTR and CaCC. There are two antisecretory agents currently under clinical evaluation, one natural product and one synthetic small molecule. Crofelemer, a mixture of proanthocyanidin oligomers extracted from the bark latex of Croton lechleri, was recently approved for HIV-associated diarrhea [37]. Crofelemer is a weak and partial inhibitor of CFTR (IC\textsubscript{50} = 100 μM), though it fully inhibits enterocyte CaCC, albeit with low potency (IC\textsubscript{50} = 10 μM) [23]. Crofelemer is thus unlikely to be beneficial in secretory diarrheas such as cholera and Traveler’s diarrhea in which CFTR is the major Cl\textsuperscript{−} secretory pathway and in which fluid secretion is very high. A small molecule, iOWH032, is in clinical trials for cholera [38]. iOWH032 is a close chemical analog of the glycine hydrazide GlyH-101 [39] that targets the extracellular (lumen-facing) surface of CFTR. However, iOWH032 has low CFTR inhibition potency (IC\textsubscript{50} > 5 μM) and hence rapid (seconds or less) dissociation from CFTR. Mathematical modeling of an orally administered drug targeting the extracellular surface of intestinal crypts predicts little antisecretory efficacy of a micromolar-affinity CFTR inhibitor under conditions of high fluid secretion because of convective washout [40].

Figure 5. Krisanaklan inhibits TMEM16A Cl\textsuperscript{−} current and intestinal contraction. A. Short-circuit current in TMEM16A-expressing FRT cells, showing Krisanaklan inhibition of 10 μM E\textsubscript{act} (a TMEM16A activator)-stimulated TMEM16A Cl\textsuperscript{−} current. Measurements were made following permeabilization of the basolateral membrane and in the presence of a transepithelial Cl\textsuperscript{−} gradient (see Methods). Parallel study done with added Krisanaklan (grey curve); where indicated 5 μM T16A\textsubscript{inh-A01} (a TMEM16A inhibitor) was added. B. (top) Contractile force generated spontaneously by mouse ileal segment showing inhibition by Krisanaklan. (bottom) Krisanaklan inhibition of ileal contraction after stimulation by carbachol. Contraction data are representative of 3 sets of experiments. (inset) Reversibility of Krisanaklan action following washout. C. (top) Peristaltic index in mice receiving 3% Krisanaklan by intraperitoneal injection or oral gavage, compared to water control (mean ± S.E., 4 mice per group, ** P<0.01). (bottom) Representative photographs of small intestine, showing distance traveled of an activated charcoal meal.

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Alternative candidates for CFTR-targeted antidiarrheal therapy include glycine hydrazide conjugates with IC$_{50}$ > 50 nM that resist convective washout [19,41], and thiazolidinones and quinoxalinediones that act on the cytoplasmic surface of CFTR with IC$_{50}$ as low as 4 nM [18,21,42,43].

The three distinct actions of Krisanaklan, including inhibition of CFTR and non-TMEM16A CaCC(s), and TMEM16A, are mediated by different components of the herbal extract. HPLC fractionation showed each of the inhibition activities in different fractions, and testing of size fractions prepared by dialysis indicated that small molecules of <1 kDa molecular size account for the CFTR and TMEM16A inhibition activities, and more heterogeneous, larger molecules for CaCC inhibition. We previously reported that the small molecule eugenol, a major component of clove, as a small-molecule TMEM16A inhibitor that likely accounts, at least in part, for the TMEM16A inhibition activity of Krisanaklan [20]. The molecular identities of the CFTR and CaCC inhibitors in Krisanaklan were not determined in this study, though testing of individual herbs suggest that they arise from two of the four herbal constituents, agarwood and clove. Based on prior studies of Crofelemer [23] and red wines [44], the compounds responsible for CaCC inhibition are probably relatively large, heterogeneous and polyphenolic, whose molecular identities would be very difficult to determine. Agarwood extracts have been shown to contain several classes of phytochemical components including alkaloids, saponins, tannins, anthroquinones, glycosides and triterpenoids [45,46], some of which may be responsible its Cl$^{-}$ channel inhibition activity. Clove is the dried flower bud of Caryophyllus aromaticus L, which contains the volatile compound eugenol, as well as non-volatile tannins, flavonoids, sterols and glycosides [47,48]. Though eugenol and tannins lack CFTR inhibition activity [20,44], flavonoids are known to bind to CFTR and may be responsible for CFTR inhibition.

Our results suggest that Krisanaklan, or extracts/components from its individual herbal constituents, is a potential candidate for antisecretory therapy of life-threatening diarrheas in developing countries. The potential advantages of Krisanaklan over alternative antisecretory agents under development include broad Cl$^{-}$ channel specificity with proven efficacy in mouse models, a long history of use in adults and children, low cost, and immediate availability for clinical testing. However, data from in vitro and animal models should be extrapolated cautiously to human diarrheas because of differences in intestinal anatomy, fluid secretion rates and, potentially, enterocyte signaling mechanisms.
We also note that, as found for vaccines, the efficacy of antisecretory therapeutics may differ in different target populations because of genetic and environment factors. Notwithstanding these caveats, the preclinical data reported here support clinical trials of Krisanaklan for antisecretory therapy of diarrheas.

Figure 7. Herbal constituents responsible for the Cl⁻ channel inhibition activities of Krisanaklan. A. Short-circuit current in T84 cells showing inhibition of forskolin (20 μM) and IBMX (100 μM)-stimulated CFTR Cl⁻ current by extracts from agarwood, Terminalia triptera, camphor and clove. B. Short-circuit current in T84 cells showing inhibition of ATP (100 μM)-stimulated CaCC Cl⁻ current by extracts. C. Short-circuit current in FRT-TMEM16A cells showing inhibition of Eact (10 μM)-stimulated TMEM16A Cl⁻ current by extracts. Data representative of 3 sets of experiments.

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Author Contributions
Conceived and designed the experiments: LT ASV. Performed the experiments: LT EAK. Analyzed the data: LT EAK. Wrote the paper: LT EAK ASV.

References
1. Walker CL, Rudan I, Liu L, Nair H, Theodoratou E, et al. (2013) Global burden of childhood pneumonia and diarrhoea. Lancet 381: 1405–1416.
2. Moore SR, Lima NL, Noares AM, Orsi RB, Pinkerton RC, et al. (2010) Prolonged episodes of acute diarrhea reduce growth and increase risk of persistent diarrhea in children. Gastroenterology 139: 1156–1164.
3. Guarino A, Dupont C, Gorlov AV, Gottrand F, Lee JK, et al. (2012) The management of acute diarrhea in children in developed and developing areas: from evidence base to clinical practice. Expert Opin Pharmacother 13: 17–26.
4. Harris JB, LaRocque RC, Qadri F, Ryan ET, Calderwood SB (2012) Cholera. Lancet 379: 2466–76.
5. Munos MK, Walker CL, Black RE (2010) The effect of oral rehydration solution and recommended home fluids on diarrhoea mortality. Int J Epidemiol 39: 175–87.
6. Venkatakrishnan J, Meenakshi A, Rako MC (2010) Iod transport in small intestine. Curr Opin Gastroenterol 26: 123–129.
7. Muoz M, Kopie S, Gehel J (2010) Evidence for intestinal chloride secretion. Exp Physiol 95: 471–478.
8. Thiajarajah JR, Vermaan AS (2012) CFTR inhibitors for treating diarrhea disease. Clin Pharmacol Ther 92: 287–290.
9. Hoque KM, Woodward OM, van Rossum DB, Zachos NC, Chen L, et al. (2010) Epac1 mediates protein kinase A-independent mechanism of forskolin-activated intestinal chloride secretion. J Gen Physiol 135: 43–58.
10. Offin PA, Clark HF, Kornstein MJ, Plotkin SA (1984) A murine model for oral infection with a primate rotavirus (simian SA11). J Virol 51: 233–236.
11. Morris AP, Scott JK, Ball JM, Zeng CQ, O’Neal WK, et al. (1999) NSP4 elicits age-dependent diarrhea and Ca²⁺ mediated I(Ca) influx into intestinal crypts of CF mice. Am J Physiol 277: G431–G444.
12. Greenberg HB, Estes MK (2009) Rotaviruses: from pathogenesis to vaccination. Gastroenterology 136: 1939–1951.
13. Ralfe PA, Liu PW, Andrade A, Jiang L, Rampe L, et al. (2004) Diarrhea-associated HIV-1 APLs potentiate muscarinic activation of Cl⁻ secretion by T84 cells via prolongation of cytosolic Ca²⁺ signaling. Am J Physiol Cell Physiol 286: C990–C1000.
14. Hwang SJ, Blair P, Britton FC, O’Driscoll KE, Heunig G, et al. (2009) Expression of anoctamin 1/TMEM16A by interstitial cells of Cajal is fundamental for slow wave activity in gastrointestinal muscles. J Physiol 587: 4807–4804.
15. Huang F, Rock JR, Harfe BD, Cheng T, Huang X, et al. (2009) Studies on expression and function of the TMEM16A calcium-activated chloride channel. Proc Natl Acad Sci USA 106: 21413–21418.

16. Namkung W, Phuan PW, Verkman AS (2011) TMEM16A inhibitors reveal TMEM16A as a minor component of calcium-activated chloride channel conductance in Airways and intestinal epithelial cells. J Biol Chem 286: 2365–2374.

17. Verkman AS, Galii JJJ (2009) Chloride channels as drug targets. Nat Rev Drug Discov 8: 153–171.

18. Thiagarajah JR, Broadbent T, Haich E, Verkman AS (2004) Prevention of toxin-induced intestinal ion and fluid secretion by a small-molecule CFTR inhibitor. Gastroenterology 126: 511–519.

19. Sonawane ND, Zhao D, Zegarra-Moran O, Galii JJJ, Verkman AS (2007) Lecitin conjugates as potent, nonabsorbable CFTR inhibitors for reducing intestinal fluid secretion in cholera. Gastroenterology 132: 1234–1244.

20. Yao Z, Namkung W, Ko EA, Park J, Tradrantip L, Verkman AS (2012) Fractionalization of a human antidiarrheal medicine reveals eugenol as an inhibitor of Ca2+–activated Cl− channel TMEM16A. PLoS ONE 7: e38030.

21. Ma T, Thiagarajah JR, Yang H, Sonawane ND, Folli C, et al. (2002) Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. J Clin Invest 110: 1651–1658.

22. De La Fuente R, Namkung W, Mills A, Verkman AS (2008) Small-molecule screen identifies inhibitors of a human intestinal calcium-activated chloride channel. Mol Pharmacol 73: 758–766.

23. Tradrantip L, Namkung W, Verkman AS (2010) Crofelemer, an antisecretory antidiarrheal prosthecocyanidin oligomer extracted from Croton lechleri, targets two distinct intestinal chloride channels. Mol Pharmacol 77: 69–78.

24. Ko EA, Jin BJ, Namkung W, Ma T, Thiagarajah J, et al. (2013) Chloride channel inhibition by a red wine extract and a synthetic small molecule prevents rotaviral secretory diarrhea in neonatal mice. Sep 19. doi: 10.1136/gutjnl-2013-305663. [Epub ahead of print]

25. Namkung W, Yao Z, Finkbeiner WE, Verkman AS (2012) Small-molecule activators of TMEM16A, a calcium-activated chloride channel, stimulate rotavirus-induced secretory diarrhea in neonatal mice. Mol Pharmacol 78: 148–157.

26. Luckege O, Peregine AT, Person K, Cordiali S, Unno I, et al. (2000) Role of the enteric nervous system in the fluid and electrolyte secretion of rotavirus diarrhea. Science 287: 491–495.

27. Ousingsawat J, Mizra M, Tian Y, Roussas E, Schreiber R, et al. (2011) Rotavirus toxin NSP4 induces diarrhea by activation of TMEM16A and inhibition of Na+ absorption. Pflügers Arch 461: 579–589.

28. Seo NS, Zeng CQ, Hyser JM, Utama B, Crawford SE, et al. (2008) Integrins α2β1 and α2β2 are receptors for the rotavirus enterotoxin. Proc Natl Acad Sci USA 105: 8811–8816.

29. Goelho KI, Byrden AS, Hall C, Flewett TH (1981) Pathology of rotavirus infection in suckling mice: A study by conventional histology, immunofluorescence, ultrastructural sections, and scanning electron microscopy. Ultrastruct Pathol 6: 203–210.

30. Chao AC, de Sauvage FJ, Dong YJ, Wagner JA, Goeddel DV, Gardner P (1994) Activation of intestinal CFTR Cl− channel by heat-stable enterotoxin and guanylin via cAMP-dependent protein kinase. EMBO J 13: 1065–1072.

31. Grubb BR (1995) Ion transport across the jejunum in normal and cystic fibrosis mice. Am J Physiol 268: G650–G653.

32. Berschneider HM, Knowles MR, Azizkhan RG, Boucher RC, Tobey NA, et al. (1988) Altered intestinal chloride transport in cystic fibrosis. FASEB J 2: 2625–2629.

33. O’Loughlin EV, Hunt DM, Gaskin KJ, Stiel D, Bruntzak IM, et al. (1991) Abnormal epithelial transport in cystic fibrosis jejunum. Am J Physiol 260: G750–G763.

34. Namkung W, Finkbeiner WE, Verkman AS (2010) CFTR-adenylyl cyclase I association responsible for UTP activation of CFTR in well-differentiated primary human bronchial cell cultures. Mol Biol Cell 21: 2639–2640.

35. Hempton SJ, Markowsky K, Banal A, Tsao E, Habib I, et al. (2010) Rotavirus infection of murine small intestine causes colonic secretion via age restricted gα11 receptor expression. Gastroenterology 138: 2410–2417.

36. Kahn ME, Senderowicz A, Sauvaudille EA, Barrett KE (2001) Possible mechanisms of diarrheal side effects associated with the use of a novel chemotherapeutic agent, flavopiridol. Clin Cancer Res 7: 343–349.

37. MacArthur RD, Hawkins T, Brown SJ, LaMarca A, Chatuvedi P, et al. (2012) ADVENT Trial: Crofelemer for the treatment of secretory diarrhea in HIV+ individuals [poster 889]. Conference on Retroviruses and Opportunistic Infections (CROI) 2012 - Seattle, WA March 5-8, 2012.

38. de Hostos EL, Choy RK, Nguyen T (2011) Developing novel antisecretory drugs to treat infectious diarrhea. Future Med Chem 3: 1317–1325.

39. Muonprasat C, Sonawane ND, Salinas D, Tadieh A, Galii JJJ, et al. (2004) Discovery of glycine hydrizide pore-secluding CFTR inhibitors: mechanism, structure-activity analysis, and in vivo efficacy. J Gen Physiol 124: 125–137.

40. Jin BJ, Thiagarajah JR, Verkman AS (2013) Convective washout reduces the antidiarrheal efficacy of enterocyte surface-targeted antisecretory drugs. J Gen Physiol 141: 261–272.

41. Sonawane ND, Zhao D, Zegarra-Moran O, Galii JJJ, Verkman AS (2008) Nanomolar CFTR inhibition by pore-secluding divalent polyethylene glycol-malic acid hydrazides. Chem Biol 15: 710–720.

42. Tradrantip L, Sonawane ND, Namkung W, Verkman AS (2009) Nanomolar potency pyrimido-pyrrolo-quinoxalinedione CFTR inhibitor reduces cyst size in a polycystic kidney disease model. J Med Chem 52: 6447–6455.

43. Snyder DS, Tradrantip L, Yao C, Kurth MJ, Verkman AS (2011) Potent, metabolically stable benzopyrimido-pyrrolo-oxazine-dione (BPO) CFTR inhibitors for polycystic kidney disease. J Med Chem 54: 5460–5477.

44. Namkung W, Thiagarajah JR, Phuan PW, Verkman AS (2010) Inhibition of Ca2+-activated Cl− channels by gallopamil as a possible molecular basis for health benefits of red wine and green tea. FASEB J 24: 4178–4186.

45. Dash M, Patra JK, Panda PP (2008) Phytochemical and antimicrobial screening of extracts of Aquilaria agallocha Lam. Int J Pharm & Ind Res 2: 416–423.

46. Shan B, Cai YZ, Sun M, Corke H (2005) Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. J Agric Food Chem 53: 7749–7759.

47. Nassar MI, Garaah AH, El-Ghorab AH, Farrag AH, Shen H., et al. (2007) Chemical constituents of clove (Syzygium aromaticum, Fam. Myrtaceae) and their antioxidant activity. Rev Latinoamer Quim 35: 47–57.