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Authors

Ko, Eun-A
Jin, Byung-Ju
Namkung, Wan
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Chloride channel inhibition by a red wine extract and a synthetic small molecule prevents rotaviral secretory diarrhoea in neonatal mice

Eun-A Ko[1], **Byung-Ju Jin**[1], **Wan Namkung**[2], **Tonghui Ma**[3], **Jay R. Thiagarajah**[4], and **A. S. Verkman**[1]

[1]Departments of Medicine and Physiology, University of California, San Francisco, California, USA

[2]College of Pharmacy, Yonsei Institute of Pharmaceutical Sciences, Yonsei University, Incheon, Korea

[3]Department of Physiology, Dalian Medical University, Dalian, China

[4]Department of Gastroenterology, Hepatology and Nutrition, Children's Hospital, Boston, Harvard Medical School, Boston, Massachusetts, USA

Abstract

Background—Rotavirus is the most common cause of severe secretory diarrhoea in infants and young children globally. The rotaviral enterotoxin, NSP4, has been proposed to stimulate calcium-activated chloride channels (CaCC) on the apical plasma membrane of intestinal epithelial cells. We previously identified red wine and small molecule CaCC inhibitors.

Objective—To investigate the efficacy of a red wine extract and a synthetic small molecule, $CaCC_{inh}$-A01, in inhibiting intestinal CaCCs and rotaviral diarrhoea.

Design—Inhibition of CaCC-dependent current was measured in T84 cells and mouse ileum. The effectiveness of an orally administered wine extract and $CaCC_{inh}$-A01 in inhibiting diarrhoea in vivo was determined in a neonatal mouse model of rotaviral infection.

Results—Screening of ~150 red wines revealed a Cabernet Sauvignon that inhibited CaCC current in T84 cells with IC_{50} at a ~1:200 dilution, and higher concentrations producing 100% inhibition. A >1 kdalton wine extract prepared by dialysis, which retained full inhibition activity, blocked CaCC current in T84 cells and mouse intestine. In rotavirus-inoculated mice, oral administration of the wine extract prevented diarrhoea by inhibition of intestinal fluid secretion without affecting rotaviral infection. The wine extract did not inhibit the cystic fibrosis chloride

Correspondence to: Dr Alan S Verkman, 1246 Health Sciences East Tower, University of California San Francisco, CA 94143-0521, USA; Alan.Verkman@ucsf.edu.

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channel (CFTR) in cell cultures, nor did it prevent watery stools in neonatal mice administered cholera toxin, which activates CFTR-dependent fluid secretion. $CaCC_{inh}$-A01 also inhibited rotaviral diarrhoea.

Conclusions—Our results support a pathogenic role for enterocyte CaCCs in rotaviral diarrhoea and demonstrate the antidiarrhoeal action of CaCC inhibition by an alcohol-free, red wine extract and by a synthetic small molecule.

INTRODUCTION

Rotavirus is the leading cause of severe secretory diarrhoea in infants and young children worldwide, resulting in an estimated 0.5 million deaths annually in children under age 5 years, which represents about one-third of deaths attributed to diarrhoea.[1] Older children and adults are rarely affected by rotaviral diarrhoea, which is thought to be due to the development of immunity and changes in intestinal physiology.[2,3] In the past 5 years, rotavirus vaccines have substantially reduced the incidence of rotaviral diarrhoea in developed countries. In developing countries, however, considerable morbidity and mortality remains because of limited vaccine availability and rotavirus strain differences.[4] The principal treatment for acute rotaviral diarrhoea is fluid replacement by oral rehydration solution. The incidence of other viral diarrheas, notably those caused by norovirus and other calciviruses and astroviruses, has recently increased, and may be replacing rotavirus as the leading cause of childhood viral diarrhoea in developed countries.[5]

Secretory diarrhoea, such as that caused by rotavirus infection, results from a combination of excessive secretion of fluid and electrolytes into the intestinal lumen and reduced fluid absorption. Excessive fluid secretion is caused by active chloride secretion into the intestinal lumen, which drives secondary movement of sodium and water.[6,7] Chloride secretion involves activation of chloride channel(s) on the apical plasma membrane of intestinal epithelial cells (enterocytes). The electrochemical driving force for apical membrane chloride secretion is established by basolateral membrane transporters, including the sodium-potassium ATPase, sodium-potassium-chloride cotransporter (NKCC1) and potassium channel(s). In secretory diarrhoeas caused by bacteria, such as cholera (*Vibrio cholerae*) and Travelers' diarrhoea (enterotoxigenic *Escherichia coli*), secreted bacterial enterotoxins activate apical membrane chloride channels, resulting in chloride secretion. The primary chloride channel activated by bacterial enterotoxins is the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated chloride channel.[7,8] In rotaviral diarrhoea, it is believed that a non-structural rotaviral protein (NSP4) acts as a secreted viral enterotoxin, activating calcium-activated chloride channel(s) (CaCCs) at the luminal membrane of enterocytes.[9,10] Though the cellular details remain to be fully elucidated, NSP4 activation of CaCC appears to involve elevation of cytoplasmic calcium, either directly, or through the enteric nervous system, and/or through epithelial galanin receptors (figure 1).[3,11,12]

Here, the potential therapeutic efficacy of CaCC inhibition in rotaviral diarrhoea was investigated. We previously discovered that red wines containing polyphenolic gallotannins strongly inhibit intestinal CaCC.[13] We also previously identified small molecule inhibitors

of the intestinal CaCC by a phenotype-based high-throughput screen.[14] Here, we report that a red wine extract and a small molecule CaCC inhibitor reduce chloride secretion in cell cultures and in intestinal fragments ex vivo, and prevent rotaviral diarrhoea in neonatal mice. The results support the proposed enterotoxin/CaCC activation mechanism for rotaviral diarrhoea, and provide proof-of-concept for antisecretory therapy using a readily available and inexpensive red wine extract.

MATERIALS AND METHODS

Cell culture

Fisher rat thyroid (FRT) cells stably transfected with the halide sensor YFP-F46 L/H148Q/I152 L and human TMEM16A or CFTR were used as described.[15,16] FRT cells were cultured in F-12-modified Coon's medium (Sigma-Aldrich, St Louis, Missouri, USA) supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 500 μg/mL G-418. T84 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) medium supplemented with 10% foetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin.

Red wines and preparation of extracts

Approximately one hundred and fifty red wines from the USA, Europe and South America were collected and tested. A Carbernet Sauvignon (Solovino 2007, Napa Valley, California, USA), which was found to have strong CaCC inhibition activity, was dialysed (Spectrum Laboratories, Rancho Dominguez, California, USA) against a large excess of distilled water over 24 h with 3–4 changes of the bath to produce fractions of molecular size >1 kDa, >10 kDa and >50 kDa. Following lyophilisation, dry weights from 10 mL wine were: 340 mg (original wine), 95 mg (>1 kDa fraction), 62 mg (>10 kDa fraction) and 18 mg (>50 kDa fraction). As a control, a Zinfandel (Dutcher Crossing 2009, Dry Creek Valley, California, USA), which was found to have minimal or no CaCC inhibition activity, was dialysed similarly to produce a >1-kDa fraction (dry weight 85 mg).

Short-circuit current measurements

Cells were cultured on Snapwell inserts (Costar Corning, Horseheads, New York, USA) until confluence and mounted in Ussing chambers. For T84 cells, symmetrical HCO_3^--buffered solutions were used in both hemichambers. The HCO_3^--buffered solution contained (in mM): 120 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose, 5 HEPES, and 25 $NaHCO_3$ (pH 7.4). The solutions were aerated with 95% O_2/5% CO_2 and maintained at 37°C. Short-circuit current was measured in the presence of $CFTR_{inh}$-172 to inhibit CFTR. For FRT cells, half-Cl^- solution in the HCO_3^--buffered solution was replaced with Na gluconate in the apical side and HCO_3^--buffered solutions in basolateral side. The basolateral membrane was permeabilised with 250 μg/mL amphotericin B. Short-circuit current was measured using an EVC4000 multichannel voltage clamp (World Precision Instruments, Sarasota, Florida, USA). For measurement of short-circuit current in intestine, strips of mouse ileum were mounted in Ussing chambers (area 0.7 cm^2) and incubated for 1 h in Krebs-bicarbonate solution containing 10 μM indomethacin and 0.1 μM tetrodotoxin at pH 7.4 at 37° C

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(bubbled with 95% O_2/5% CO_2). Short-circuit current was measured after inhibition of Na^+ current by amiloride (50 μM) for 10 min.

Intracellular calcium and cAMP measurements

FRT-TMEM16A cells were plated in 96-well black-walled microplates. Confluent cells were loaded with Fluo-4 NW (Invitrogen, Carlsbad, California, USA) after 48 h after plating. Fluo-4 fluorescence was measured with a FluoStar fluorescence plate reader (BMG Lab Technologies, Durham, North Carolina, USA) at excitation/emission wavelengths of 485/538 nm. For cAMP assay, T84 cells were grown in 24-well plates, treated for 30 min with 1-kDa wine fraction (1% v/v) together with 20 μM forskolin, lysed by repeating freeze/thaw, centrifuged to remove cell debris, and the supernatant was assayed for cAMP (Parameter cAMP immunoassay kit; R&D Systems, Minneapolis, Minnesota, USA).

Rotavirus culture and titre

MA-104 cells, derived from African green monkey kidney (ATCC CRL2378.1, Manassas, Virginia, USA), were grown to 80–90% confluence in T75 flasks and washed three times with Eagle's minimal essential medium (EMEM; Invitrogen, Carlsbad, California, USA). SA-11 simian rotavirus (ATCC VR 1739) was mixed with trypsin to a final concentration of 10 μg/mL and incubated for 1 h at 37°C. The media was removed and the cells were inoculated with 3 mL of rotavirus diluted with serum-free media. Cells were incubated with rotavirus for 1 h at 37°C in a humidified 5% CO_2 atmosphere with rocking every 20 min to redistribute the inoculum. Excess rotavirus was removed by washing with serum-free EMEM. The MA104 cells were maintained for 2–3 days in serum-free EMEM containing 0.5 μg/mL trypsin. The rotavirus-infected cells were frozen and thawed three times at −70°C, and the suspension was centrifuged at 500 g for 5 min to obtain a supernatant containing rotavirus, which was stored at −70°C.

Plaque assays were done using confluent monolayers of MA104 cells in 6-well plates. Rotavirus was activated with trypsin (10 μg/mL), serially diluted in EMEM without serum, and added to MA104 cells. After absorption of rotavirus for 1 h at 37°C in a 5% CO_2 incubator, inoculated cells were washed with EMEM and gently overlaid with 1.5% SeaKem ME agarose (Cambrex Bio Science, Rockland, Maine, USA) in EMEM with trypsin (0.5 μg/mL). After 3–4 days at 37°C in 5% CO_2 plates were stained with neutral red, and viral plaques were counted. Viral titres were expressed as plaque-forming units per millilitre of medium (pfu/mL). In some studies, the 1-kDa wine fraction or $CaCC_{inh}$-A01 (synthesised as described, ref. [14]) was included in the MA104 culture medium during rotavirus inoculation and replication.

Mouse model of rotaviral diarrhoea

Neonatal C57/BL6 mice (age 4–7 days, weight 1.8–2.5 g) were inoculated with 30 μL (1.2×10^7 pfu/mL) of the rhesus rotavirus by oral gavage using polyethylene tubing (0.61 outer diameter, 0.28 inner diameter, Warner Instruments, Hamden, Connecticut, USA) and a Hamilton syringe. After rotavirus inoculation, the mice were returned to their mothers and allowed to suckle. Stool samples were collected daily by gentle palpation of the abdomen.

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Animal protocols were approved by the University of California, San Francisco Committee on Animal Research.

In one set of experiments, a wine-treated group received 20 μL of the 1-kDa wine extract in water (20% w/v) together with the rotavirus, and twice daily until day 3. Control mice received 20 μL water without the wine extract. In another set of experiments, the wine-treated group received 20 μL wine extract starting one day after rotavirus inoculation and continued twice daily. In $CaCC_{inh}$-A01 studies, the treated group received 1.7 μg $CaCC_{inh}$-A01 by intraperitoneal injection at the time of rotavirus inoculation and every 8 h thereafter.

Cholera toxin model

Neonatal C57/BL6 mice of the same age used for rotavirus studies were administered a single dose of 0.1 μg cholera toxin (Sigma) in 30 μL of 7% bicarbonate buffer by oral gavage and then returned to their mothers and allowed to suckle. A wine-treated group received the 1-kDa wine extract (same dose as in rotavirus studies) at the time of cholera toxin administration and twice daily thereafter. Stool samples were collected at 8 h and daily thereafter.

Measurement of stool water content

Stool samples were collected by gentle palpation of the mouse abdomen. Faeces were scored using an established scale: dry brown faeces=0; loose brown faeces=1, soft yellow faeces=2; watery yellow faeces=3). Stools with a score of 2 or greater were considered as diarrhoea. For quantification of stool water in very small specimens we fabricated a polydimethylsiloxane (PDMS) slab of 1.5 mm thickness and punctured a 1.91 mm diameter hole to contain a cylindrical 4.3 mm^3 volume of stool. After loading with stool, the cylindrical stool plug was expelled onto a tissue wipe (Kimberly-Clark Professional, Roswell, Georgia, USA) in a humidified atmosphere and allowed to contact the paper for 1 min. The wetted area was quantified by digital imaging. To generate a standard curve relating the wetted area to stool water content, stool samples of specified water content were prepared by thoroughly mixing oven-dried stool with specified quantities of water.

In vivo intestinal fluid absorption in mouse models

C57/BL6 mice (age 8–10 weeks, body weight 25–35 g) were given access to water but not food for 24 h. Mice were anaesthetised with avertin (200 mg/kg), and body temperature was maintained during surgery at 36–38°C using a heating pad. A small abdominal incision was made to expose the small intestine, and closed mid-jejunum loops (length 2–3 cm) were isolated by sutures. Loops were injected with 100 μL PBS alone or PBS containing the 1-kDa wine fraction (1% w/v). The abdominal incision was closed with sutures, and mice were allowed to recover from anaesthesia. Intestinal loops were removed at 30 min and loop length and weight were measured to quantify fluid absorption.

Histology and immunofluorescence

At 3 days after rotavirus inoculation, the ileum and jejunum were isolated, rinsed with PBS, and fixed for 24 h in 10% (w/v) neutral buffered formalin in PBS. Specimens were embedded in Paraplast X-Tra Tissue Embedding Media (Fisherbrand, Houston, Texas,

USA), and paraffin sections were stained with haematoxylin and eosin. For immunostaining with an antirotavirus antibody, paraffin-embedded tissues were sectioned at 5 μm, deparaffinised with xylene and rehydrated in graded ethanol solutions, rinsed with PBS, and incubated in blocking solution (containing 1% BSA and 0.2% Triton X-100) for 1 h. Slides were then incubated overnight at 4°C with a goat antirotavirus antibody (1:100; Millipore), rinsed in PBS, and then incubated with Alexa Fluor 555–labeled donkey antigoat IgG secondary antibody (1:200; Invitrogen) for 1 h.

RESULTS

Inhibition of intestinal CaCC by a red wine extract and a synthetic small molecule

We screened ~150 red wines from the USA, Europe and South America, using a plate reader fluorescence assay developed previously,[14] to select a wine with high potency for inhibition of CaCC-mediated chloride transport (figure 2A). From this initial screening, a Cabernet Sauvignon (Solovino 2007, Napa Valley, California, USA) was found to have strong CaCC inhibition activity. For further studies, we crudely fractionated the red wine by dialysis and assayed dialysed fractions for inhibition of ATP-stimulated CaCC Cl^- current in T84 intestinal epithelial cells. Short-circuit measurements showed 50% inhibition of Cl^- current at ~0.5% (v/v) of the original red wine (figure 2B). Subsequent dialysis using 1-kDa cut-off dialysis tubing showed similar inhibitory activity to the original wine, whereas inhibitory activity was greatly reduced in the 10-kDa and 50-kDa fractions (figure 2B), indicating that the inhibitory compound(s) have a molecular size >1 kDa. Further studies were done using the wine fraction prepared by dialysis with 1-kDa dialysis tubing, referred to as '1-kDa wine fraction'. Additionally, short-circuit current studies were done using the small molecule inhibitor $CaCC_{inh}$-A01, which was identified previously in a phenotype-based high-throughput screen in HT-29 cells.[14] Figure 2C shows that $CaCC_{inh}$-A01 inhibited ATP stimulated Cl^- current. Figure 2D shows that the 1-kDa wine fraction and $CaCC_{inh}$-A01 inhibited CaCC Cl^- current in mouse ileum following carbachol stimulation.

To investigate the specificity of inhibition by the 1-kDa wine fraction, studies were done looking at two known apical membrane chloride channels involved in transepithelial chloride secretion, CFTR and TMEM16A. Figure 3A shows that the wine fraction did not inhibit CFTR chloride current in T84 cells in response to forskolin, whereas the current was fully inhibited by the CFTR inhibitor $CFTR_{inh}$-172. Figure 3B shows that the wine fraction inhibited the CaCC chloride channel TMEM16A, which is in agreement with prior data showing red wine inhibition of both the intestinal CaCC (unknown molecular identify) and TMEM16A.[13] The 1-kDa wine fraction did not alter cytoplasmic calcium concentration or inhibit its increase in response to ATP (figure 3C), nor did it inhibit the increase in cAMP in response to forskolin (figure 3D).

Neonatal mouse model of rotaviral diarrhoea

A neonatal mouse model of rotaviral diarrhoea was established using the SA-11 simian rotavirus strain. Following preliminary studies using mice of different ages, strains, rotavirus concentrations and administration methods, we found that robust and reproducible diarrhoea was produced by a single oral inoculation of 30 μL (1.2×10^7 pfu/mL) SA-11 rotavirus in

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C57/BL6 mice of age 4–7 days. Figure 4A shows that using this protocol, all mice exhibited diarrhoea, as judged by stool scores at 1–2 days after rotavirus inoculation.

As red wine pigment darkens the stool from the neonatal mice, we developed an alternative, quantitative measure of stool water content that does not involve qualitative assessment of stool colour and consistency. As shown in figure 4B (left), the method involved deposition of a fixed stool volume onto absorbent paper, and measurement of the wetted area after contact for 1 min. A small, fixed stool volume of 1.63 μL was created by filling a cylindrical hole fabricated in a slab of PDMS, the synthetic polymer used in microfluidics. To calibrate wetted area with stool water content, stool specimens of known water content were created by mixing different proportions of oven-dried stool and water. Figure 4B (right) shows wetted area as a function of percentage stool water content.

Red wine extract prevented watery stools in rotavirus-infected neonatal mice

The activity of the red wine extract was tested in the rotaviral mouse model using two administration protocols. One set of studies was done in which mice were inoculated with rotavirus on day 0, and the 1-kDa wine extract was given at the same time and twice daily thereafter. Preliminary studies showed the efficacy of a 4 mg dose of the wine extract. Figure 5A (top) shows grossly watery stools at days 1–3 following rotavirus inoculation. Images are shown of the absorbent paper at 1 min after deposition of the 1.63 μL stool specimen, just before and after physical removal of the stool mass. Mice receiving the wine extract had dark stools with low stool water content. Figure 5A (bottom) summarises the wetted area data, showing significantly reduced wetted area at days 1, 2 and 3 in mice receiving the wine extract. In a second protocol, the wine extract was effective when administered starting at day 1, after significant diarrhoea had begun, with significantly lower water content in stools obtained at days 2 and 3 following rotaviral inoculation (figure 5B).

Intraperitoneal administration of $CaCC_{inh}$-A01 also greatly reduced stool water content following rotavirus inoculation (figure 5C). Initial studies established an effective dose of 34 μg $CaCC_{inh}$-A01 given intraperitoneally three times a day starting at day 0. Due to the small effective plasma volume of neonatal mice it was not possible to measure $CaCC_{inh}$-A01 concentration in blood for pharmacokinetic analysis.

As a control, a Zinfandel (Dutcher Crossing 2009, Dry Creek Valley, CA), which was found to have very weak CaCC inhibition activity, was dialysed to produce a >1-kDa fraction. Short-circuit measurements showed that 1-kDa Zinfandel wine extract caused little inhibition of ATP-stimulated Cl^- current (figure 6A). Mice receiving the wine extract, under the same conditions as in figure 5A, showed red-coloured stools but no reduction in stool water (figure 6B and C).

Rotavirus infection of the intestine was verified by histology, showing marked vacuolisation of enterocytes in rotavirus-infected rodents, similar to prior studies.[17–19] At day 3 after rotavirus inoculation, large vacuoles were seen in enterocytes lining most of the surface of villi, with swelling of the villus tips (figure 7A). Vacuoles were not seen in control (untreated) mice. Rotavirus-infected mice receiving the wine extract showed vacuolisation similar to that in the untreated, rotavirus-infected mice. Immunofluorescence using an

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antirotavirus antibody was similar in the untreated and wine extract-treated rotavirus-infected ileum (figure 7B). We also verified that the wine extract did not inhibit rotavirus replication in cell cultures. Inclusion of the wine extract during inoculation and replication in plaque assays did not significantly reduce rotavirus titre (figure 7C). Together, these studies support the conclusion that the wine extract reduces intestinal fluid secretion but not the rotavirus infection.

Red wine extract did not prevent watery stools in cholera toxin-treated neonatal mice

In order to verify the specificity of the red wine extract for rotavirus-induced diarrhoea, studies were done for cholera toxin-induced diarrhoea. Diarrhoea was produced by cholera toxin in neonatal mice of the same age used in the rotavirus study and treated with the same amount of the 1-kDa wine extract. Initial studies showed that a single administration of 3-μg purified cholera toxin produced watery stools at 8 h and 1 day, which largely resolved by day 2. Figure 8A and B shows that the wine extract did not significantly reduce stool water content in cholera toxin-induced diarrhoea, consistent with the in vitro data showing that the red wine extract did not inhibit CFTR. Effect of the wine extract on intestinal fluid absorption was studied in closed intestinal loops in adult mice that were injected with 100 μl PBS alone or PBS containing 1-kDa wine fraction. Figure 8C shows no significant effect of the wine extract on active intestinal fluid absorption at 30 min, which was inhibited by the sodium/proton exchange (NHE3) blocker amiloride.

DISCUSSION

Viral gastroenteritis remains one of the most common diseases in children in developed and developing countries. We found here that an alcohol-free extract from red wine, as well as a small molecule CaCC inhibitor, prevented intestinal fluid loss in a neonatal mouse model of rotaviral diarrhoea, supporting a central role for CaCC-dependent chloride secretion in rotaviral diarrhoea and suggesting a novel therapeutic strategy. The lack of effect of the wine extract on cholera toxin-induced diarrhoea in neonatal mice, and on CFTR chloride conductance in cell cultures, provides evidence for the CaCC specificity of red wine action, as does the lack of antisecretory action in rotaviral diarrhoea of a red wine lacking CaCC inhibition activity. The lack of effect of the wine extract on rotaviral infection of the intestine supports the conclusion that the inhibition action of red wine targets the secretory mechanism rather than the infectious process.

The health benefits of red wine are well described anecdotally and, more recently, in clinical studies.[20–22] The motivation for testing a red wine extract came from our prior identification, by high-throughput screening, of tannic acid and related polyphenol-containing gallotannins as CaCC inhibitors.[13] Tannic acid administration is dose-limited due to toxicity, with early studies showing liver toxicity when it was used to improve the contrast in barium sulfate diagnostic enemas.[23] In rodents, oral administration of high-dose tannic acid results can result in respiratory failure preceded by convulsions and hypothermia, with LD_{50} of 2–3.5 g/kg.[24] Rather than tannic acid itself, polymeric condensed tannins and pigmented tannins constitute the majority of wine phenolics. The typical concentration of hydrolysable tannins (non-flavonoids, from oak), and proanthocyanidins and condensed

tannins (flavonoids) in aged red wines is ~250 mg/L and 1000 mg/L, respectively.[25] Here, we screened red wines and related natural products for CaCC inhibition activity. Though most red wines showed some CaCC inhibition activity, their activities varied widely with their geographic origin and age. White wines were inactive, as were the original grapes/grape skins, as well as extracts from wood specimens used in wine barrel storage containers. Screening of ~150 red wines identified a Cabernet Sauvignon as having strong CaCC inhibition activity. Attempts to identify active components by HLPC and dialysis revealed activity over a wide range of molecular sizes greater than 1 kdalton (data not shown). We therefore used a >1-kdalton wine extract generated by dialysis, which contained all CaCC inhibition activity. Twice daily oral administration of the water-dissolved lyophilised extract prevented rotaviral diarrhoea. The wine extract inhibited CaCC current in intestinal cell cultures and intestinal fragments ex vivo, without inhibition of CFTR, the other major enterocyte chloride channel, and without inhibition of intestinal fluid absorption.

The mechanism(s) by which intestinal rotavirus infection produce diarrhoea remain to be fully elucidated. There is evidence that the secreted protein NSP4 is a viral enterotoxin that causes chloride secretion by CaCCs.[26] NSP4 is thought to bind to integrins on the surface of intestinal cells, resulting in elevation of cytosolic calcium and consequent CaCC activation.[11] Other studies have suggested that galanin, a neuroendocrine peptide that binds to Gal1 receptors, is the initiating event in calcium-dependent chloride secretion.[3] Regardless of the exact signalling pathway, activation of apical membrane CaCCs appears to be the final step in rotaviral-mediated intestinal chloride secretion. The molecular identity of the CaCCs involved in intestinal transepithelial fluid secretion also remains unknown. Candidate channels have been proposed including anoctamins, such as TMEM16A and bestrophins,[26,27] although several studies have provided evidence that these channels are not involved in intestinal chloride secretion.[28,29] The red wine extract and small molecule inhibitor used here provide new pharmacological tools in ongoing work to characterise and identify intestinal CaCC(s).

The introduction of rotavirus vaccines has reduced the disease burden in children, particularly in developed countries where the vaccine is readily available. The effectiveness of rotavirus vaccines has been reported to be as low as 50% in developing countries, compared with >90% in developed countries,[4,30] with corresponding reduced seroconversion rates in vaccinated children. Antisecretory therapy may have clinical benefit in reducing the morbidity and mortality associated with severe viral gastroenteritis, particularly in developing countries where vaccine efficacy is low and infant mortality is high. As antisecretory therapy with the wine extract does not affect rotavirus infection at the cellular level, it should not prevent the development of immunity.

Mouse models of rotavirus infection have been used extensively to study the pathophysiology of diarrhoeas. Both homologous and heterologous host models have been used to induce fluid loss, dehydration and diarrhoea.[9,31–35] Here, we adapted an established heterologous host model involving infection with the simian SA-11 rotavirus strain to produce diarrhoea in neonatal mice. A challenge in mouse models has been accurate measurement of intestinal fluid losses during infection because of the small volumes of excreted stool. The method introduced here to quantify water content in very small stool

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specimens should be widely useful in small-animal studies of diarrhoea, as the current standard, scoring of stool appearance, is descriptive and can be confounded by drugs or manoeuvres that affect stool colour or consistency. The challenge was to quantify stool water in microlitre-volume specimens that are too small for gravimetric measurement of wet:dry weight ratios. After evaluating potential approaches, such as electrical impedance, mechanical deformability, capillary uptake and so on, we adapted a simple blotting procedure in which a fixed volume of stool loaded into a cylindrical hole in a PDMS slab was expelled onto blotting paper. Measurements using stool specimens with specified water contact verified the reproducibility of the method and established a quantitative relationship between stool water content and assay readout (wetted area). Though effects of CaCC inhibition here were large and unambiguous, the quantitative readout afforded by the blotting method should allow unbiased detection of more subtle, submaximal effects of diarrhoea-modifying therapeutics.

As a readily available, inexpensive natural product, red wine, or extracts thereof, are attractive candidates for antidiarrhoeal therapy for CaCC-dependent secretory diarrhoeas, which include rotaviral and other viral diarrhoeas, and as well as diarrhoeas caused by certain antiretroviral drugs and cancer chemotherapeutics.[36,37] The alternative drug approach, notwithstanding considerable cost and time, is the development of a new chemical entity, such as the small molecule $CaCC_{inh}$-A01 studied here. CaCC inhibition would likely not be beneficial in diarrhoeas caused by bacterial enterotoxins, such as cholera toxin and heat-stable *E coli* STa toxin, or diarrhoea produced by some gastrointestinal tumours, which are primarily cyclic nucleotide and CFTR-dependent. CaCC inhibition is also unlikely to be beneficial in chronic inflammatory diarrhoeas where mucosal damage and inhibition of fluid absorption predominate.

In summary, our results support the conclusion that secretory diarrhoea following rotaviral infection is CaCC-dependent, and that antisecretory therapy with CaCC inhibitors can reduce intestinal fluid loss. As dehydration is a major determinant of morbidity and mortality in childhood rotaviral and other viral diarrhoeas, antisecretory therapy may have clinical benefit as stand-alone or adjunctive therapy to oral or intravenous rehydration, particularly in developing countries.

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References

1. Tate JE, Burton AH, Boschi-Pinto C, et al. 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis. Lancet Infect Dis. 2012; 12:136–41. [PubMed: 22030330]
2. Greenberg HB, Estes MK. Rotaviruses: from pathogenesis to vaccination. Gastroenterology. 2009; 136:1939–51. [PubMed: 19457420]

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3. Hempson SJ, Matkowskyj K, Bansal A, et al. Rotavirus infection of murine small intestine causes colonic secretion via age restricted galanin-1 receptor expression. Gastroenterology. 2010; 138:2410–17. [PubMed: 20197068]
4. Taneja DK, Malik A. Burden of rotavirus in India–is rotavirus vaccine an answer to it? Indian J Public Health. 2012; 56:17–21. [PubMed: 22684168]
5. Payne DC, Vinje J, Szilagyi PG, et al. Norovirus and medically attended gastroenteritis in U.S. children. N Engl J Med. 2013; 368:1121–30. [PubMed: 23514289]
6. Thiagarajah JR, Broadbent T, Hsieh E, et al. Prevention of toxin-induced intestinal ion and fluid secretion by a small-molecule CFTR inhibitor. Gastroenterology. 2004; 126:511–19. [PubMed: 14762788]
7. Thiagarajah JR, Verkman AS. CFTR inhibitors for treating diarrheal disease. Clin Pharmacol Ther. 2012; 92:287–90. [PubMed: 22850599]
8. Chao AC, de Sauvage FJ, Dong YJ, et al. Activation of intestinal CFTR Cl^- channel by heat-stable enterotoxin and guanylin via cAMP-dependent protein kinase. EMBO J. 1994; 13:1065–72. [PubMed: 7510634]
9. Ball JM, Tian P, Zeng CQ, et al. Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein. Science. 1996; 272:101–4. [PubMed: 8600515]
10. Morris AP, Scott JK, Ball JM, et al. NSP4 elicits age-dependent diarrhea and Ca^{2+} mediated I^- influx into intestinal crypts of CF mice. Am J Physiol. 1999; 277:G431–44. [PubMed: 10444458]
11. Seo NS, Zeng CQ, Hyser JM, et al. Integrins alpha1beta1 and alpha2beta1 are receptors for the rotavirus enterotoxin. Proc Natl Acad Sci U S A. 2008; 105:8811–18. [PubMed: 18587047]
12. Lundgren O, Peregrin AT, Persson K, et al. Role of the enteric nervous system in the fluid and electrolyte secretion of rotavirus diarrhea. Science. 2000; 287:491–5. [PubMed: 10642552]
13. Namkung W, Thiagarajah JR, Phuan PW, et al. Inhibition of Ca^{2+}-activated Cl^- channels by gallotannins as a possible molecular basis for health benefits of red wine and green tea. FASEB J. 2010; 24:4178–86. [PubMed: 20581223]
14. De La Fuente R, Namkung W, Mills A, et al. Small-molecule screen identifies inhibitors of a human intestinal calcium-activated chloride channel. Mol Pharmacol. 2008; 73:758–68. [PubMed: 18083779]
15. Namkung W, Phuan PW, Verkman AS. TMEM16A inhibitors reveal TMEM16A as a minor component of calcium-activated chloride channel conductance in airway and intestinal epithelial cells. J Biol Chem. 2011; 286:2365–74. [PubMed: 21084298]
16. Galietta LJ, Springsteel MF, Eda M, et al. Novel CFTR chloride channel activators identified by screening of combinatorial libraries based on flavone and benzoquinolizinium lead compounds. J Biol Chem. 2001; 276:19723–8. [PubMed: 11262417]
17. Katyal R, Rana SV, Vaiphei K, et al. Effect of rotavirus infection on small gut pathophysiology in a mouse model. J Gastroenterol Hepatol. 1999; 14:779–84. [PubMed: 10482428]
18. Osborne MP, Haddon SJ, Spencer AJ, et al. An electron microscopic investigation of time-related changes in the intestine of neonatal mice infected with murine rotavirus. J Pediatr Gastroenterol Nutr. 1988; 7:236–48. [PubMed: 2832583]
19. Coelho KI, Bryden AS, Hall C, et al. Pathology of rotavirus infection in suckling mice: A study by conventional histology, immunofluorescence, ultrathin sections, and scanning electron microscopy. Ultrastruct Pathol. 1981; 2:59–80. [PubMed: 16830450]
20. Gronbaek M, Deis A, Sorensen TI, et al. Mortality associated with moderate intakes of wine, beer, or spirits. Br Med J. 1995; 310:1165–9. [PubMed: 7767150]
21. Pellegrini N, Simonetti P, Brusamolino A, et al. Composition of platelet phospholipids after moderate consumption of red wine in healthy volunteers. Eur J Clin Nutr. 1996; 50:535–44. [PubMed: 8863014]
22. Micallef M, Lexis L, Lewandowski P. Red wine consumption increases antioxidant status and decreases oxidative stress in the circulation of both young and old humans. Nutr J. 2007; 6:27. [PubMed: 17888186]
23. Zboralske FF, Harris PA, Riegelman S, et al. Toxicity studies on tannic acid administered by enema. 3. Studies on the retention of enemas in humans. IV. Review and conclusions. Am J Roentgenol Radium Ther Nucl Med. 1966; 96:505–9.

24. Boyd EM, Bereczky K, Godi I. The acute toxicity of tannic acid administered intragastrically. Can Med Assoc J. 1965; 92:1292–7. [PubMed: 14291458]
25. Waterhouse AL. Wine phenolics. Ann N Y Acad Sci. 2002; 957:21–36. [PubMed: 12074959]
26. Ousingsawat J, Mirza M, Tian Y, et al. Rotavirus toxin NSP4 induces diarrhea by activation of TMEM16A and inhibition of Na^+ absorption. Pflugers Arch. 2011; 461:579–89. [PubMed: 21399895]
27. Barro-Soria R, Schreiber R, Kunzelmann K. Bestrophin 1 and 2 are components of the Ca^{2+} activated Cl^- conductance in mouse airways. Biochim Biophys Acta. 2008; 1783:1993–2000. [PubMed: 18652850]
28. Yu K, Lujan R, Marmorstein A, et al. Bestrophin-2 mediates bicarbonate transport by goblet cells in mouse colon. J Clin Invest. 2012; 120:1722–35. [PubMed: 20407206]
29. D'Andrea L, Lytle C, Matthews JB, et al. Na:K:2Cl cotransporter (NKCC) of intestinal epithelial cells. Surface expression in response to cAMP. J Biol Chem. 1996; 271:28969–76. [PubMed: 8910547]
30. Cortese MM, Immergluck LC, Held M, et al. Effectiveness of monovalent and pentavalent rotavirus vaccine. Pediatrics. 2013; 132:e25–33. [PubMed: 23776114]
31. Offit PA, Clark HF, Kornstein MJ, et al. A murine model for oral infection with a primate rotavirus (simian SA11). J Virol. 1984; 51:233–6. [PubMed: 6328042]
32. Feng N, Kim B, Fenaux M, et al. Role of interferon in homologous and heterologous rotavirus infection in the intestines and extraintestinal organs of suckling mice. J Virol. 2008; 82:7578–90. [PubMed: 18495762]
33. Maeno Y, Shinzato M, Nagashima S, et al. Effect of osteopontin on diarrhea duration and innate immunity in suckling mice infected with a murine rotavirus. Viral Immunol. 2009; 22:139–44. [PubMed: 19327001]
34. Wolber FM, Broomfield AM, Fray L, et al. Supplemental dietary whey protein concentrate reduces rotavirus-induced disease symptoms in suckling mice. J Nutr. 2005; 135:1470–4. [PubMed: 15930454]
35. Rollo EE, Hempson SJ, Bansal A, et al. The cytokine osteopontin modulates the severity of rotavirus diarrhea. J Virol. 2005; 79:3509–16. [PubMed: 15731245]
36. Rufo PA, Lin PW, Andrade A, et al. Diarrhea-associated HIV-1 APIs potentiate muscarinic activation of Cl– secretion by T84 cells via prolongation of cytosolic Ca^{2+} signaling. Am J Physiol Cell Physiol. 2004; 286:C998–C1008. [PubMed: 15075198]
37. Kahn ME, Senderowicz A, Sausville EA, et al. Possible mechanisms of diarrheal side effects associated with the use of a novel chemotherapeutic agent, flavopiridol. Clin Cancer Res. 2001; 7:343–9. [PubMed: 11234889]

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Significance of this study

What is already known about this subject?

- Secretory diarrhoea caused by rotavirus infection results from a combination of excessive secretion of fluid and electrolytes into the intestinal lumen and reduced fluid absorption.
- Chloride secretion involves activation of chloride channels on the apical plasma membrane of intestinal epithelial cells.
- Red wines containing polyphenolic gallotannins strongly inhibit intestinal calcium-activated chloride channels (CaCCs).

What are the new findings?

- A >1 kdalton wine extract prepared by dialysis, which retained full inhibition activity, blocked CaCC current in T84 intestinal epithelial cells and mouse intestine.
- Oral administration of a red wine extract prevented diarrhoea in rotavirus-inoculated neonatal mice by inhibition of intestinal fluid secretion.
- A small molecule CaCC inhibitor identified by high-throughput screening, $CaCC_{inh}$-A01, also prevented rotaviral diarrhoea.

How might it impact on clinical practice in the foreseeable future?

- Inhibition of intestinal CaCCs with a red wine extract or a synthetic small molecule may be clinically useful in reducing intestinal fluid losses in rotaviral and other CaCC-dependent diarrhoeas.
- As dehydration is a major determinant of morbidity and mortality in childhood rotaviral and other viral diarrhoeas, antisecretory therapy may have clinical benefit as stand-alone or adjunctive therapy to oral or intravenous rehydration, particularly in developing countries.

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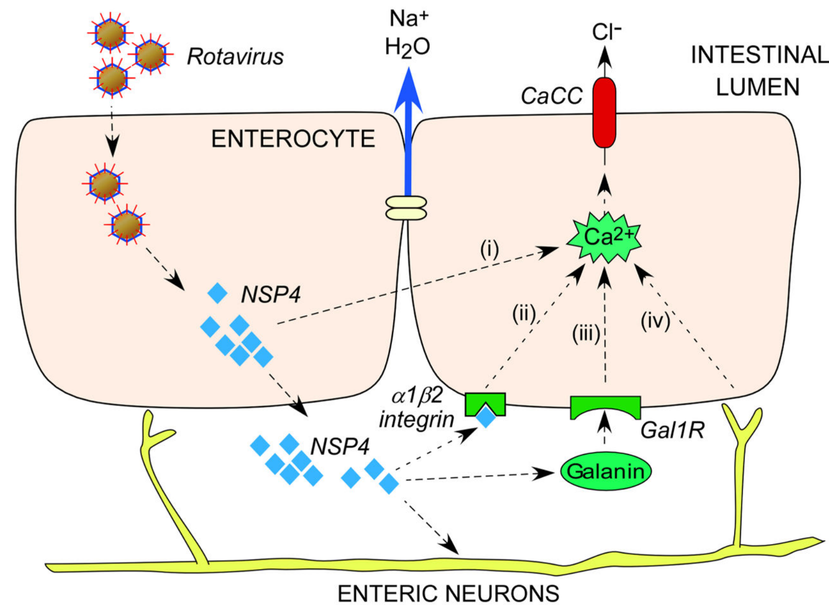

Figure 1.

Proposed mechanisms of intestinal fluid secretion in rotaviral diarrhoea. Infection of epithelial cells by luminal rotavirus results in translation of viral proteins and formation of viroplasms. The non-structural protein 4 (NSP4) is secreted from infected cells. NSP4 causes elevation of cytoplasmic calcium by (i) direct release of calcium stores from ER; (ii) binding to cell membrane integrin receptor α1β2; (iii) release and binding of galanin and/or (iv) signalling via enteric nerves. Elevation of cytoplasmic calcium activates CaCC(s) at the apical membrane resulting in chloride secretion and secondary sodium and water secretion.

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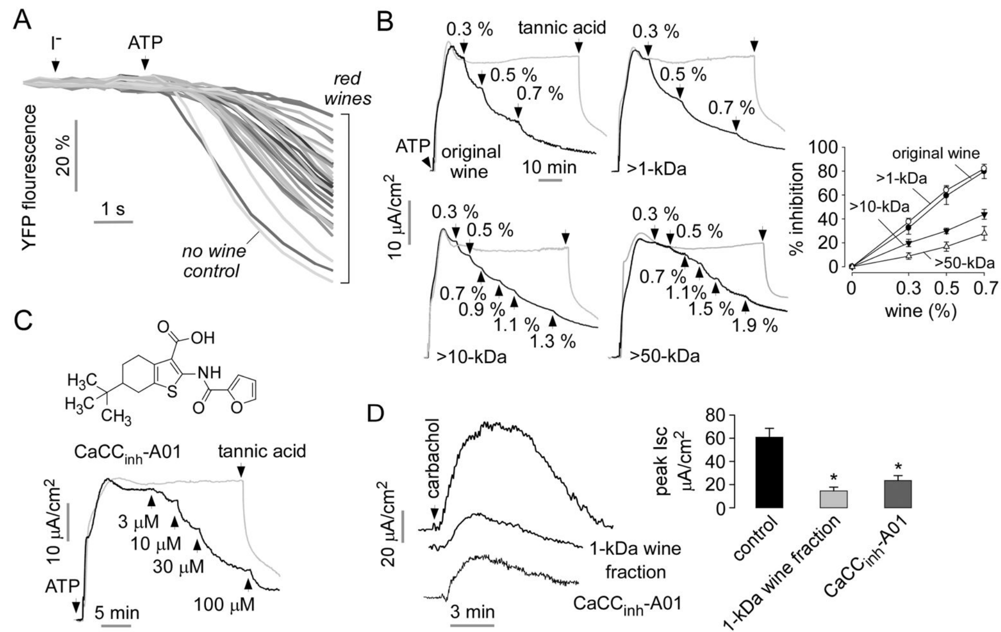

Figure 2.

A red wine fraction inhibits Ca^{2+}-dependent Cl^- current in intestinal cells. (A) Screening of red wines using a cell-based plate reader fluorescence assay in which ATP causes Ca^{2+} elevation and CaCC activation, resulting in reduced YFP fluorescence. (B) Short-circuit current in T84 cells in response to ATP (100 μM) and indicated amounts of the original red wine (directly from bottle) and wine fractions prepared by dialysis. $CFTR_{inh}$-172 (20 μM) was present to inhibit CFTR. Grey curve shows responses without added wine, with 100 μM tannic acid added where indicated. (right) Data summary (SE, n=4–6). (C) Similar study as in B, showing inhibition by $CaCC_{inh}$-A01. (D) Short-circuit current in mouse ileum showing inhibition of carbachol (1 mM)-stimulated Cl^- current by 10 min pretreatment with 1-kDa wine fraction (0.5% v/v) and $CaCC_{inh}$-A01 (50 μM) (SE, n=3–5).

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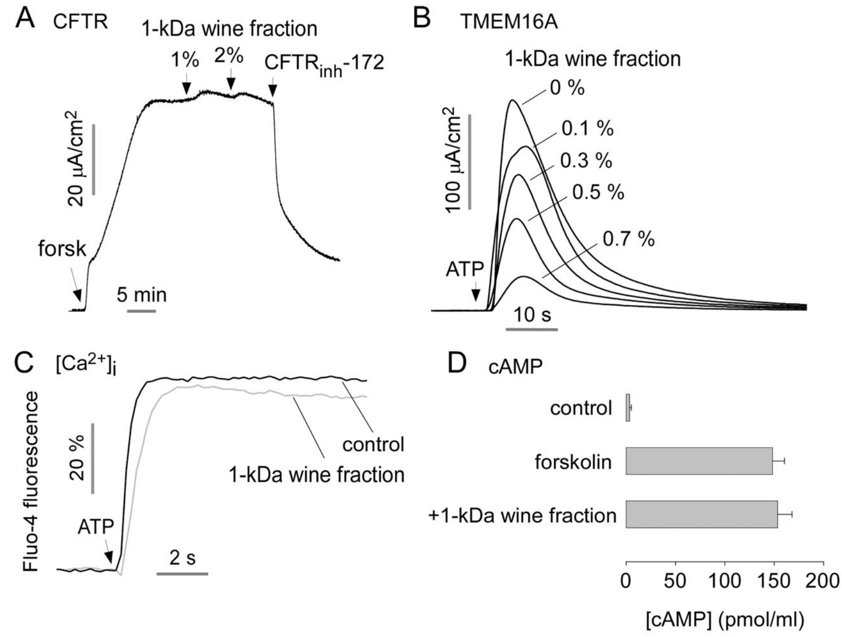

Figure 3.

Specificity of wine inhibition of CaCC Cl^- current. Measurements done using 1-kDa wine fraction. (A) CFTR activity in T84 cells measured following activation by 20 μM forskolin. Where indicated 20 μM $CFTR_{inh}$-172 added. (B) TMEM16A activity in TMEM16A-expressing FRT cells following basolateral membrane permeabilisation. Cells were pretreated for 10 min with indicated concentrations of 1-kDa wine fraction prior to addition of 100 μM ATP. (C) Cytoplasmic [Ca^{2+}] measured by Fluo-4 fluorescence without and in the presence of 1-kDa wine fraction (0.7% v/v). (D) cAMP concentration in T84 cells measured after 30 min incubation with 20 μM forskolin and 1-kDa wine fraction (1% v/v) (SE, n=4).

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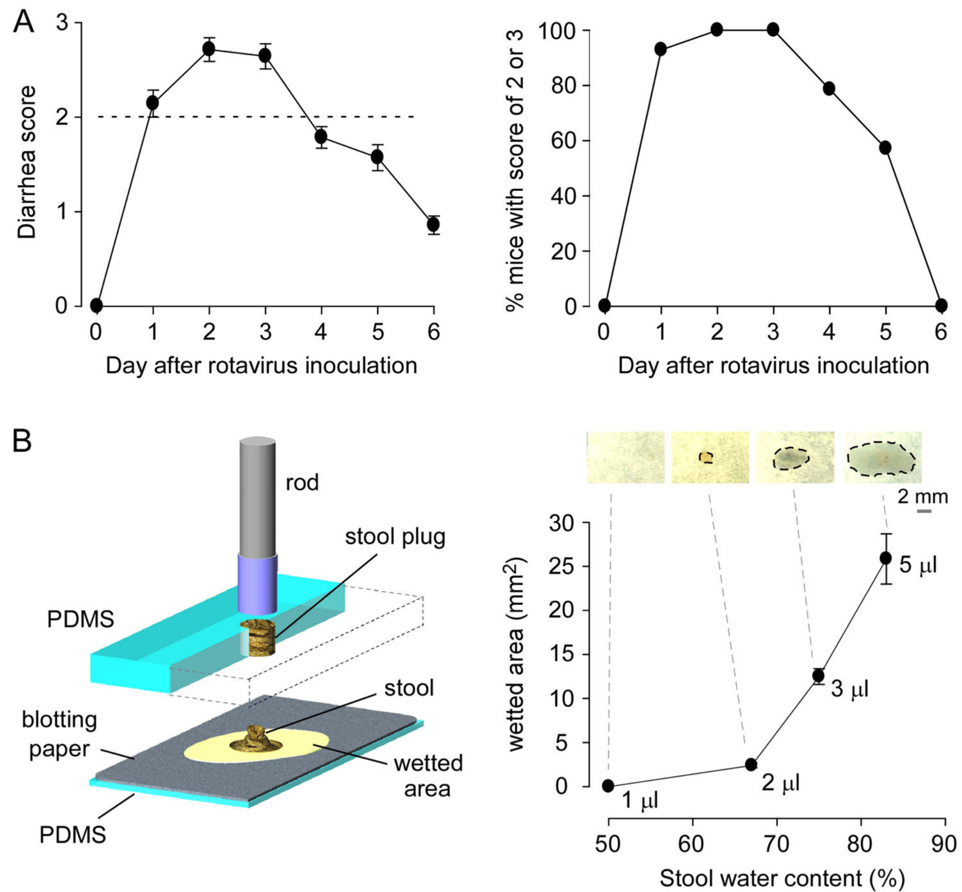

Figure 4.

Stool water content in a neonatal mouse model of rotaviral diarrhoeal. (A) Diarrhoea score (left) and percentage of mice with diarrhoea (right) following inoculated with rotavirus on day 0. A score of 2 was considered diarrhoea (dashed line in panel at left) (mean±SE, 14 mice). (B) (left) Schematic showing expulsion of a 1.91 mm diameter, 1.5 mm-thick cylindrical volume of stool onto absorbent tissue paper. Stool water content quantified by wetted area. (right) Standard curve relating wetted area to stool water content using prepared stool standards of specified water content (SE, n=5–7).

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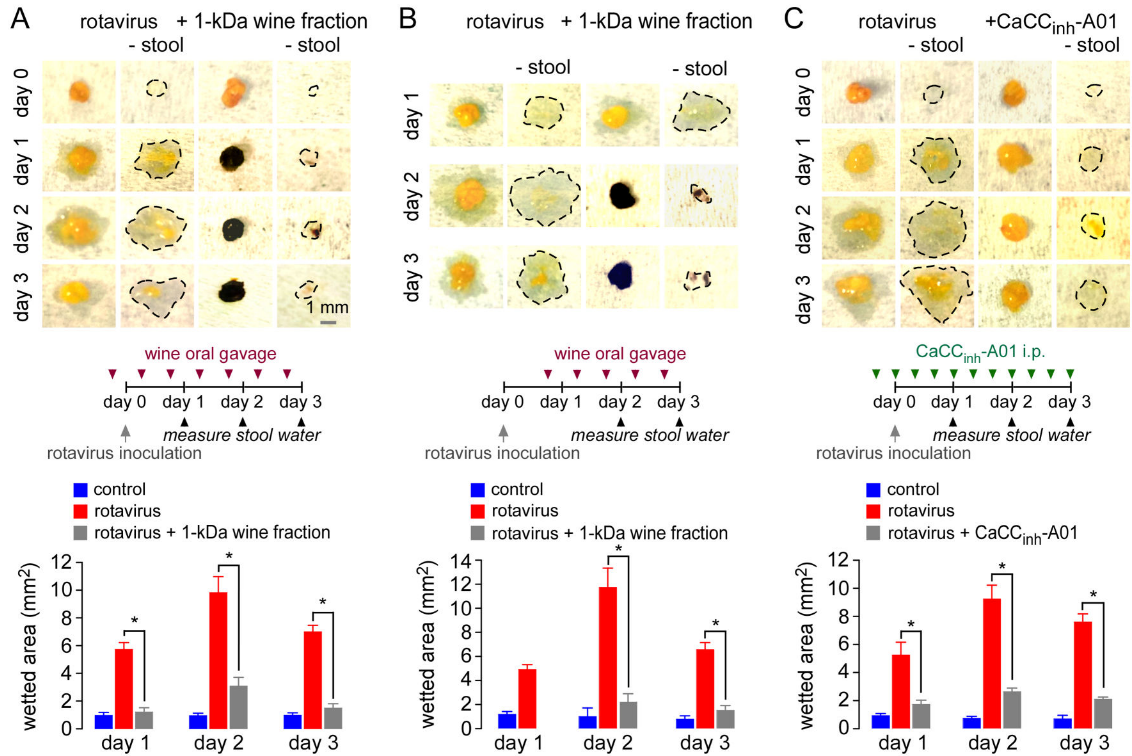

Figure 5.

Wine extract prevented diarrhoea in a neonatal mouse rotaviral model. (A) Mice were inoculated with rotavirus at day 0 and gavaged with 1-kDa wine extract twice a day. (top) Photographs of absorbent tissue at 1 min after contacting stool specimen, just before (photos at left) and after (photos at right) removal of stool mass. Wetted area demarcated by thin line. (bottom) Wetted area at indicated days (SE, 5–14 mice). (B) Similar study as in A, except that wine gavage started at day 1 (SE, 5–12 mice). (C) Similar study as in A, except that the treated group received $CaCC_{inh}$-A01 by intraperitoneal injection (34 μg three times a day) starting on day 0 (SE, n=4–10 mice).

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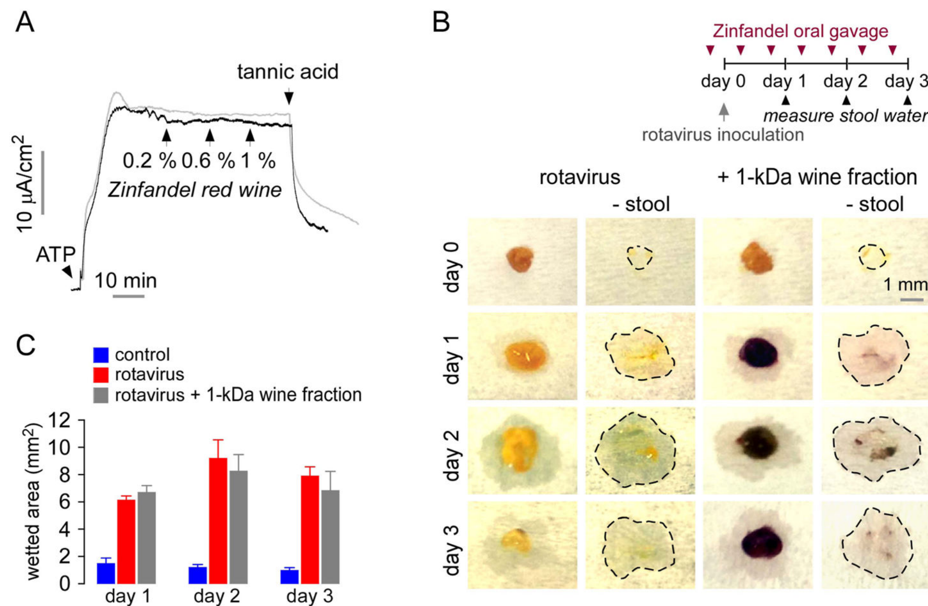

Figure 6.

Extract from a red wine with minimal CaCC inhibition activity did not prevent rotaviral diarrhoea. (A) Short-circuit current in T84 cells in response to ATP (100 μM) and indicated amounts of a 1-kDa Zinfandel wine extract prepared by dialysis. $CFTR_{inh}$-172 (20 μM) was present to inhibit CFTR. Grey curve shows responses without added wine, with 100 μM tannic acid added where indicated. (B) Mice were inoculated with rotavirus at day 0 and gavaged with the 1-kDa Zinfandel wine extract (20% w/v) twice a day. Photographs of absorbent tissue at 1 min after contacting stool specimen, as in figure 5. (C) Wetted area at indicated days (SE, 4–6 mice).

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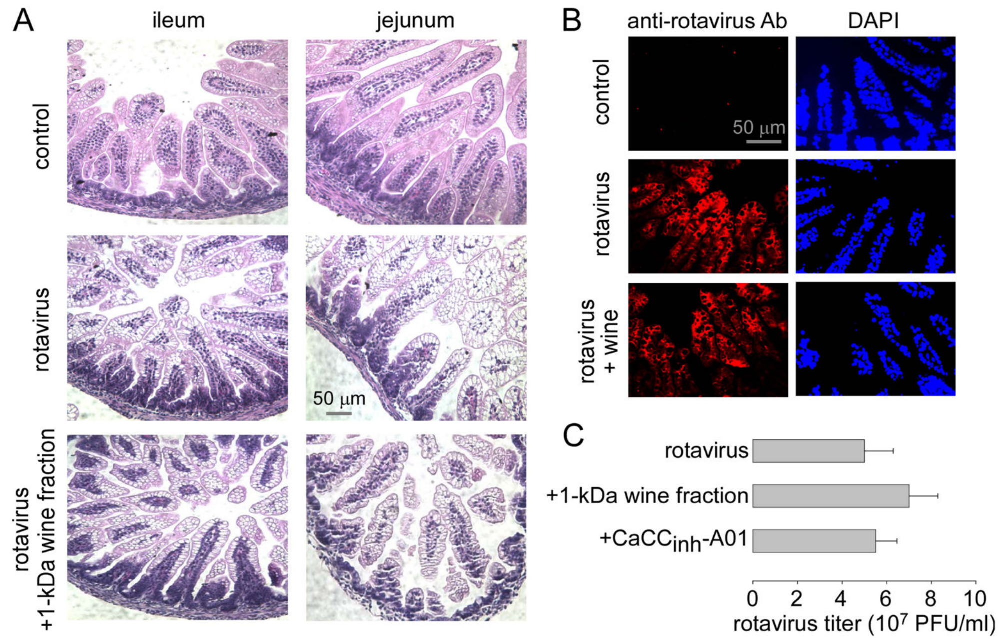

Figure 7.

Wine extract did not prevent rotavirus infection or replication. (A) Histology of mouse ileum and jejunum at day 3 after rotavirus inoculation. Haematoxylin and eosin-stained paraffin sections from control mice (upper) and rotavirus-inoculated control (middle) and wine-treated (bottom) mice. (B) Immunofluorescence of mouse ileum at day 3 after rotavirus inoculation using a polyclonal antirotavirus antibody to detect rotavirus proteins. (C) Rotavirus titre measured by plaque assay in MA104 cells with 1-kDa wine fraction (1% v/v) or $CaCC_{inh}$-A01 (30 μM) present during rotavirus inoculation and growth as indicated (SE, n=4–6).

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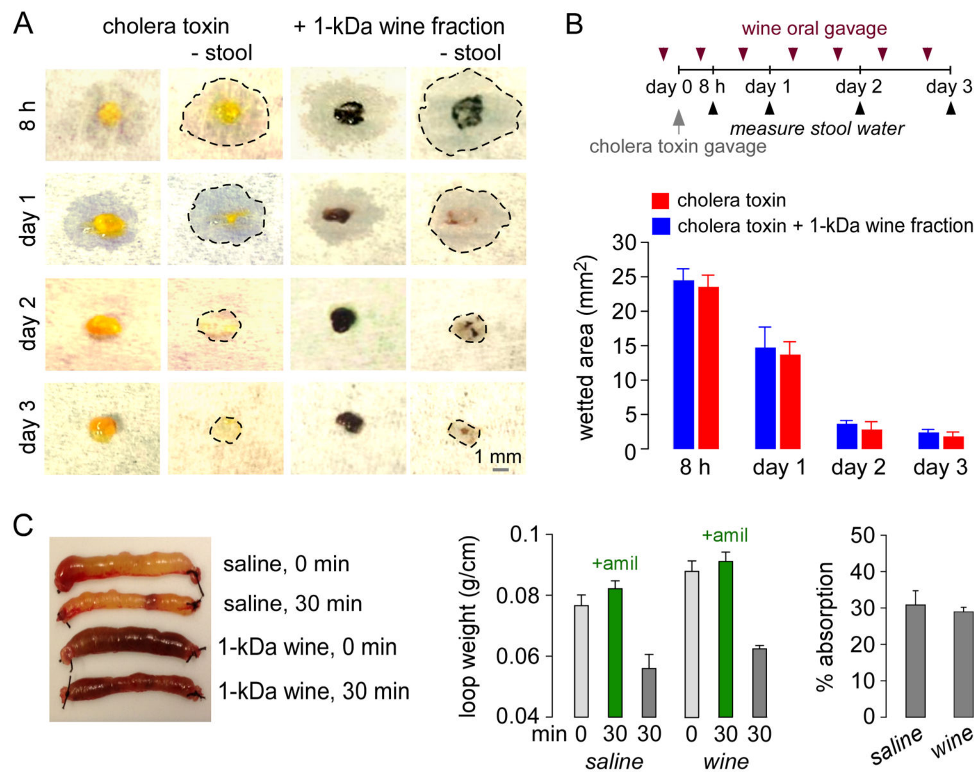

Figure 8.
Wine extract did not prevent cholera toxin-induced diarrhoea in neonatal mice. (A) Mice were inoculated with 3 μg cholera toxin on day 0 and gavaged with 1-kDa wine extract twice daily. (B) Wetted area of stool specimens (SE, 5–7 mice per group). (C) Intestinal fluid absorption. Closed intestinal loops in live adult mice were injected with 100 μL PBS alone or PBS containing 1-kDa wine fraction (1% w/v). Absorption was measured from change in loop weight/length ratio at 30 min (SE, 4–6 mice per group). Where indicated, 200 μM amiloride (amil) was included in the loop fluid.