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Discovery and Development of Antisecretory Drugs for Treating Diarrheal Diseases

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Abstract

Diarrheal diseases constitute a significant global health burden and are a major cause of childhood mortality and morbidity. Treatment of diarrheal disease has centered on the replacement of fluid and electrolyte losses using oral rehydration solutions (ORS). Although ORS has been highly successful, significant mortality and morbidity due to diarrheal disease remains. Secretory diarrheas, such as those caused by bacterial and viral enterotoxins, result from activation of cyclic nucleotide and/or Ca^{2+} signaling pathways in intestinal epithelial cells, enterocytes, which increase the permeability of Cl^- channels at the lumen-facing membrane. Additionally, there is often a parallel reduction in intestinal Na^+ absorption. Inhibition of enterocyte Cl^- channels, including the cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+}-activated Cl^- channels, represents an attractive strategy for antisecretory drug therapy. High-throughput screening of synthetic small molecule collections has identified several classes of Cl^- channel inhibitors that show efficacy in animal models of diarrhea but remain to be tested clinically. In addition, several natural-product extracts with Cl^- channel inhibition activity have shown efficacy in diarrhea models. However, a number of challenges remain to translate the promising bench science into clinically useful therapeutics, including efficiently targeting orally administered drugs to enterocytes during diarrhea, funding development costs, and carrying out informative clinical trials. Nonetheless, Cl^- channel inhibitors may prove to be effective adjunctive therapy in a broad spectrum of clinical diarrheas, including acute infectious and drug-related diarrheas, short-bowel syndrome, and congenital enteropathies.

Keywords

diarrhea; small molecules; chloride channels; CFTR; CaCC; rotavirus

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Diarrheal disease – a global health burden

Diarrheal disease is a major health burden worldwide and still represents a leading cause of mortality and morbidity. This burden, as with many major diseases, falls disproportionately on the very young and the elderly. At present, diarrheal disease is the second leading cause of mortality globally in children under age five, and repeated episodes of dehydration from diarrhea are associated with impaired physical and mental development [1, 2]. In developing countries, the major causes of diarrheal diseases are primarily infectious, including enterotoxin-producing bacteria such as *Vibrio cholerae* and enterotoxigenic *E. coli*, viruses such as rotavirus, enteroinvasive bacteria such as *Shigella* and *Salmonella*, and parasites such as *Entamoeba histolytica* and *Cryptosporidium parvum* [1]. Major non-infectious causes of diarrhea include drug side-effects, particularly with certain cancer and HIV therapeutics, and diarrhea secondary to intestinal inflammatory/autoimmune diseases such as ulcerative colitis, Crohn's disease and celiac disease [3, 4]. Diarrhea is also a major problem in patients with short-bowel syndrome, in rare congenital disorders such as microvillus inclusion disease and tufting enteropathy, and with peptide-secreting neuroendocrine tumors [5]. Oral rehydration solution (ORS) to replace fluid losses and promote intestinal fluid absorption has been the primary therapy for infectious diarrheas, reducing mortality four-fold over the last 30 years [6]. However, there remains an unmet need for alternative and adjunctive antidiarrheal therapeutics to complement ORS, in part because of limitations in ORS availability, acceptability and adequate administration, as well as for symptom relief.

Technological primer: Transporter drug targets for diarrheal disease

Diarrhea results from excessive secretion and/or impaired absorption of fluid and electrolytes across the intestinal epithelium. Movement of fluid across the intestinal epithelium is driven by active transport of ions, mainly Na^+, Cl^+ and K^+, and solutes, mainly glucose. Fluid absorption in the small intestine involves several luminal transporters, including a Na^+/H^+ exchanger (NHE3), Na^+-glucose cotransporter (SGLT1), and Cl^-/HCO_3^- exchanger (DRA) [7,8]. As in epithelia in general, the electrochemical driving force is established by a basolateral Na^+K^+-ATPase pump. In the colon, fluid absorption is also facilitated by the epithelial Na^+ channel (ENaC) and short-chain fatty acid transporters (SMCT1) [9]. Targeting of fluid absorptive pathways for diarrheal therapy is the subject of a companion review.

Intestinal fluid secretion is driven by active transepithelial Cl^- secretion, creating the electrochemical force for paracellular Na^+ secretion and the osmotic driving force for transcellular water secretion (Figure 1). Cl^- is transported into the cell at the basolateral membrane by a $Na^+/K^+/2Cl^-$ cotransporter (NKCC1), which is driven by Na^+ and Cl^- concentration gradients produced by the Na^+K^+-ATPase and basolateral K^+ channels. The electrochemical gradient drives Cl^- secretion across the luminal membrane Cl^- channels, primarily the cAMP-activated channel CFTR and Ca^{2+}-activated Cl^- channels (CaCC) [12]. Enterotoxin-producing bacteria such as *Vibrio cholerae* and enterotoxigenic *E. coli* produce secretory diarrhea primarily by activation of CFTR-mediated Cl^- secretion [13]. Viral diarrheas such as caused by rotavirus are thought to result in secretion by causing elevation in cytoplasmic Ca^{2+} and consequent activation of luminal CaCCs [14]. Drug-related diarrhea caused by HIV protease inhibitors is also thought to involve CaCCs [15]. However, the contribution of Cl^- secretion in the pathogenesis of most drug-related diarrheas, congenital pediatric enteropathies, and many bacterial, viral and parasitic infections remains untested. Despite these limitations in our current knowledge, inhibition of luminal CFTR and CaCC Cl^- channels represent an attractive target for potential antidiarrheal therapeutics.

Findings: Discovery and development of chloride channel inhibitors

High-throughput screening for discovery of small-molecule CFTR and CaCC inhibitors

Our lab developed and carried out cell-based high-throughput screens to identify Cl^- channel modulators using genetically encoded, cytoplasmic fluorescent halide sensors, including the yellow fluorescent protein YFP-H148Q/I152L, whose fluorescence is strongly reduced by I^- [17]. Target-based assays utilized epithelial cells expressing YFP-H148Q/I152L and CFTR [17] or the CaCC TMEM16A [18]. The high-throughput screens involved addition of test compound and Cl^- channel activation (by cAMP agonists for CFTR, Ca^{2+} agonists for TMEM16A), followed by extracellular I^- addition to drive cellular I^- influx. Potential inhibitors were identified as compounds reducing I^- influx as monitored by the kinetics of YFP-H148Q/I152L fluorescence decrease. Because the identity of the major enterocyte CaCC is not clear, phenotype-based screening was done to identify intestinal CaCC inhibitors, utilizing a human intestinal epithelial cell line (HT-29) stably expressing YFP-H148Q/I152L by lentiviral transfection [19].

Small-molecule CFTR inhibitors

Three chemical classes of nanomolar-potency small-molecule CFTR inhibitors have been identified from screening of synthetic small molecule collections. The thiazolidinone $CFTR_{inh}$-172 (Fig. 2A) inhibits CFTR Cl^- conductance by binding near arginine-347 on the cytoplasmic side of CFTR and stabilizing the channel closed-state [20]. Studies on $CFTR_{inh}$-172 analogs have identified the chemical structural determinants of CFTR inhibition and have provided analogs with a range of activities and aqueous solubilities [21]. $CFTR_{inh}$-172 has shown antisecretory efficacy in rodent diarrhea models, including a closed-intestinal loop model in which fluid accumulation is measured in response to luminal cholera toxin (Fig. 2A). A more recently identified class of CFTR inhibitors targeting the cytoplasmic surface of CFTR are the PPQ/BPO compounds, with the best compound (R-BPO-27) having IC_{50} ~ 4 nM [22]. The PPQ/BPO compounds have shown efficacy in models of polycystic kidney disease in which cyst expansion involves CFTR Cl^- secretion, but have not been tested in diarrhea models [23].

A third chemical class of small-molecule CFTR inhibitors, the glycine hydrazides, target the extracellular CFTR surface in the channel pore itself [24]. These compounds offer the unique opportunity for development as externally acting, non-absorbable antisecretory agents; however, by targeting an external site on the intestinal lumen a potential barrier is accessing CFTR in the deep intestinal crypts against a strong convective washout force during secretory diarrheas. Small-molecule glycine hydrazides, such as the original compound GlyH-101 [25] and an analog being studied clinically (iOWH032 [26]) are unlikely to be effective in significant diarrheas as they are washed off within seconds and have poor IC_{50} (> 10 μM) at the interior-negative enterocyte membrane potential. Following structure-activity analysis, we synthesized ‘sticky’ glycine hydrazide analogs with improved potency down to 50 nM that resist intestinal washout [27]. A macromolecular conjugate containing a CFTR inhibitor moiety linked by a polar spacer to a lectin, which bound strongly to the enterocyte surface glycocalyx, produced improved survival in a suckling mouse model of cholera (Fig. 2B).

Small-molecule CaCC inhibitors

The initial phenotype-based screen in HT-29 cells yielded several small-molecule CaCC inhibitors, the most potent being the 3-acyl-2-aminothiophene $CaCC_{inh}$-A01, which fully inhibited CaCC-dependent halide flux in different intestinal cell lines with IC_{50} ~ 1 μM. $CaCC_{inh}$-A01 likely targets the CaCC directly based on patch-clamp studies and its lack of effect on cytoplasmic Ca^{2+} signaling. $CaCC_{inh}$-A01 was shown recently to prevent watery

diarrhea in a neonatal mouse model of rotavirus [28]. Smallmolecule inhibitors of the CaCC TMEM16A were identified in a target-based screen [18]. However, while TMEM16A is the major CaCC in salivary gland and in interstitial cells of Cajal in the intestine, it probably represents a minor contributor to Cl^- conductance in enterocytes.

Natural-product Cl^- channel inhibitors

Natural products represent a potentially attractive source of antidiarrheal therapeutics, as they are generally inexpensive and have the potential for rapid translation to the clinic. In addition, there is a long history of anecdotal evidence of efficacy of various antidiarrheal remedies in many parts of the world. A drug / natural-product screen done in our lab for CaCC inhibitors revealed, unexpectedly, tannic acid as a general CaCC inhibitor. Subsequent studies showed strong CaCC inhibition by red wines that contain chemically related polyphenolic gallotannins[29]. A wide range of CaCC inhibition activities was found in different red wines, though white wines and various grape extracts showed no inhibition. Motivated by the likely involvement of CaCC activation in rotaviral diarrhea, we recently showed that an alcohol-free >1 kdalton red wine extract prevented rotaviral diarrhea in neonatal mice, without effect on the rotaviral infection [28]. As shown in Fig. 2C, oral administration of the red wine extract stained the stool red but prevented watery diarrhea. In control studies, a red wine extract with minimal CaCC activity in vitro did not prevent rotaviral diarrhea, and the red wine extract used in Fig. 2C did not inhibit CFTR or prevent cholera toxin-induced diarrhea. The use of red wine extracts for CaCC-dependent diarrheas thus warrants potential clinical testing.

In a separate study, we recently screened various diarrhea remedies from sources around the world for those showing Cl^- channel inhibition. A commonly used Thai herbal remedy was identified that fully inhibited both CFTR and CaCC Cl^- conductance in vitro, and was efficacious in mouse models of cholera and rotaviral diarrhea [30]. The remedy also inhibited intestinal smooth muscle contraction and motility. Natural products thus represent a potentially inexpensive and readily available therapy for secretory diarrheas with a defined mechanism of action.

A natural product extract, Crofelemer, was recently approved for treatment of diarrhea associated with HIV drug therapy [31]. Crofelemer is a heterogeneous proanthocyanidin oligomer extracted from the bark latex of South American tree *Croton lechleri.* In vitro studies showed crofelemer as a weak and partial (~60 %) antagonist of CFTR, though a relatively strong inhibitor of CaCC with IC_{50} < 10 μM [32]. It is not clear at this time whether the reported antidiarrheal efficacy of crofelemer in HIV drug-associated diarrhea is related to CaCC inhibition or unrelated actions.

Translation and roadblocks

A number of hurdles remain in the translation of anti-diarrheal drug candidates to widely used therapy. Although a number of compounds have been advanced through pre-clinical testing in murine models, new high-throughput model systems of enterocyte fluid secretion, such as human intestinal enteroids or genetically tractable systems such as zebrafish, warrant development to identify novel compounds and antidiarrheal drug targets. A major translational roadblock, however, is the difficulty in designing and funding informative clinical trials. Field trials in developing countries are logistically difficult to implement and require considerable funding from non-governmental agencies, but have large patient populations with a relatively small number of specific infectious pathogens. In developed countries challenges includes enrollment of adequate and specific patient cohorts that are not confounded by multiple diarrheal etiologies or existing medications. Nevertheless, a number

of specific patient populations, such as norovirus diarrheal infections and congential pediatric enteropathies, may allow testing of new antisecretory drugs.

Barriers to diarrheal drug development in developing countries include the need for very low manufacture cost, high stability in hot / humid environments, as well as obtaining funding to support commercial development of new chemical entities with relatively low profit potential.

For drugs targeting the enterocyte extracellular surface, an additional challenge, as mentioned above, is convective washout in which secreted fluid in intestinal crypts washes away inhibitor drugs. A mathematical model of intestinal convection-diffusion concluded that in severe secretory diarrheas such as cholera the antisecretory efficacy of an orally administered, surface-targeted inhibitor requires: (i) high inhibitor affinity to its target (low nanomolar K_d) in order to obtain sufficiently high luminal inhibitor concentration (> 100-fold K_d); and (ii) sustained high luminal inhibitor concentration *or* slow inhibitor dissociation [33]. Washout is a significant concern for small-molecule CFTR glycine hydrazides such as iOWH032 and potentially for several of the natural-product agents.

Conclusions

Antisecretory drug therapy has considerable potential in reducing morbidity and mortality associated with infectious, drug-induced and other diarrheas. The identification of synthetic small molecules and potent natural products, as well as the repurposing of existing medications, present a promising pre-clinical pipeline of drug candidates. The overall human and economic cost of diarrheal disease globally demands a comprehensive approach that includes pharmacological therapies in addition to existing public health priorities such as improvements in access to ORS, vaccination and sanitation. Although multiple challenges remain in the development of antisecretory drugs, including the funding of informative clinical trials, the next decade may bring the exciting prospect of new drugs to combat diarrheal disease.

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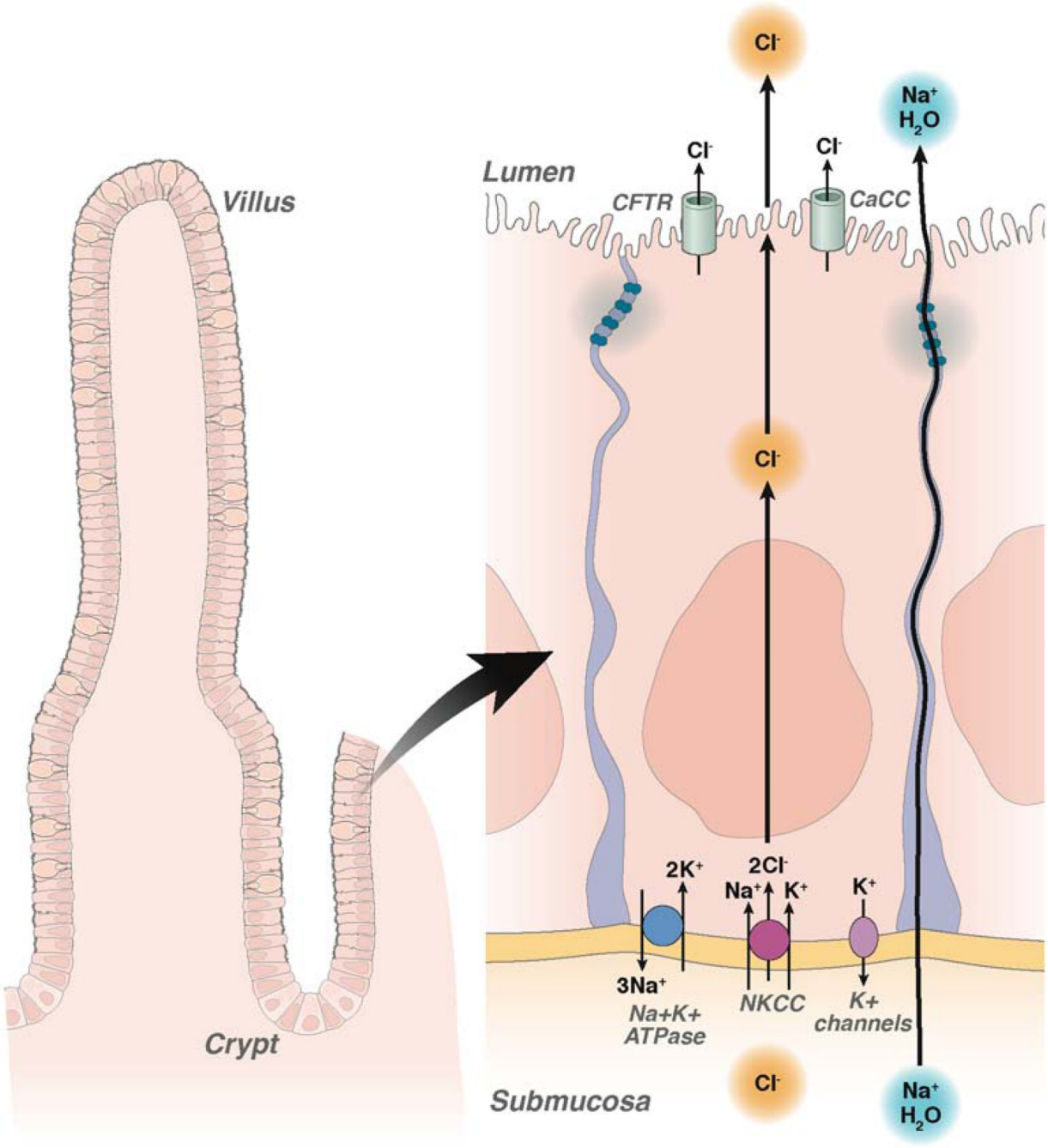

Figure 1. Cl^- channels as targets for therapy of secretory diarrheas

Diagram of fluid secretory mechanism in enterocytes lining intestinal crypts and villi, showing active Cl^- transport from the blood/sub-mucosa to the intestinal lumen facilitated by luminal membrane CFTR and CaCC channels. *Top inset.* CFTR channel pore showing proposed site of action of $CFTR_{inh}$-172 (arginine 347) and external pore blocking action of GlyH-101. N- NBD binding domain, r- regulatory domain.

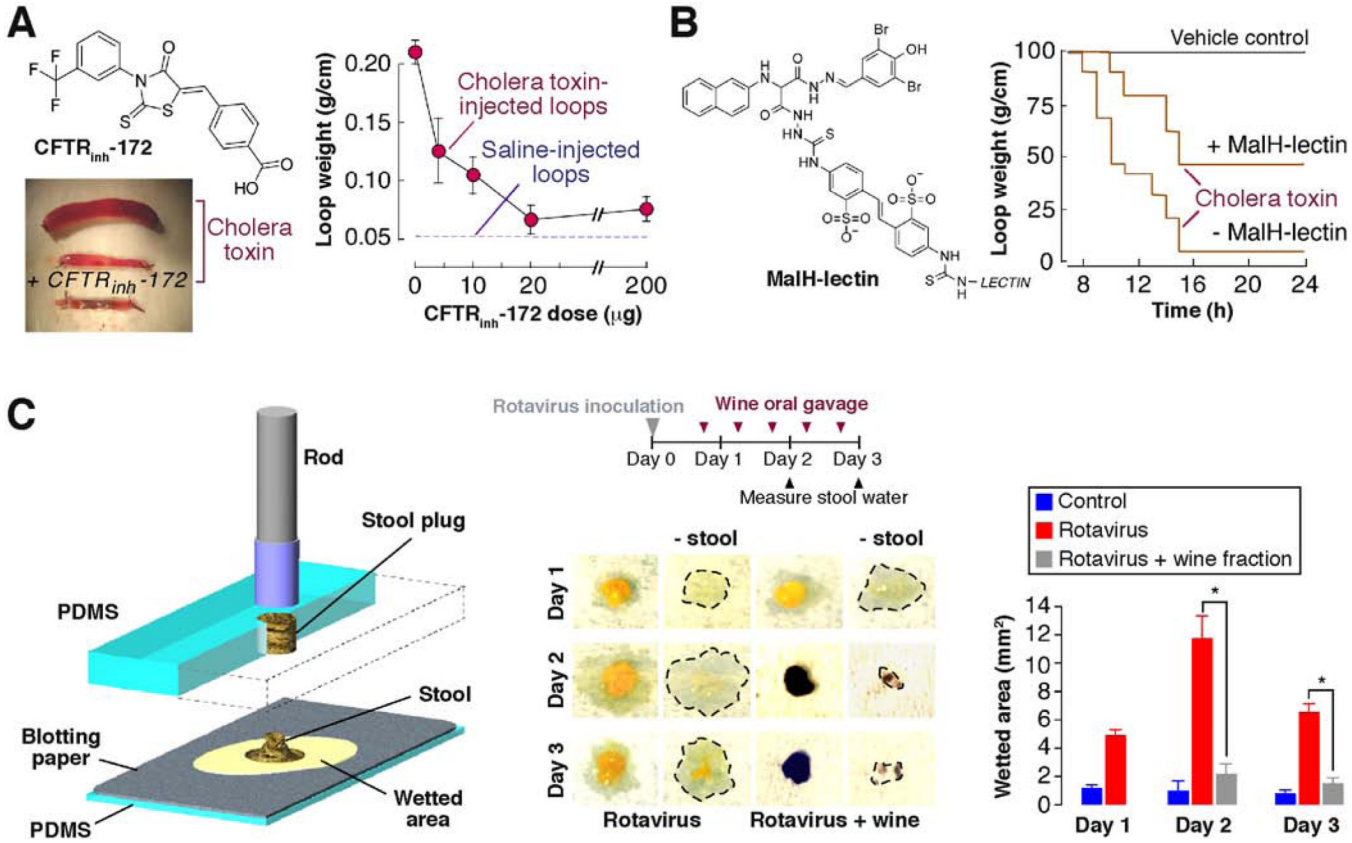

Figure 2. Efficacy of Cl^- channel inhibitors in animal models of secretory diarrheas

A. CFTR inhibition prevents cholera toxin-induced fluid secretion. $CFTR_{inh}$-172 structure (left, top) and photographs of intestinal loops at 6 hours after injection with saline or cholera toxin (left, bottom). Dose-response for inhibition of loop fluid accumulation (right). Mice were given single dose of $CFTR_{inh}$-172 by intraperitoneal injection and loop weight measured at 6 hours. From ref. 13. B. Improved survival of suckling mice following gavage with cholera toxin without vs. with the lectin conjugate MalH-ConA showing chemical structure (left) and survival plot (right). From ref. 27. C. CaCC inhibition by a red wine extract prevents rotavirus-induced fluid secretion in neonatal mice. Schematic showing expulsion of a 1.9 mm-diameter, 1.5 mm-thick cylindrical volume of stool onto absorbent tissue paper in which stool water content is quantified by wetted area (left). (center) Mice were inoculated with rotavirus at day 0 and gavaged with 1-kDa wine extract twice a day. 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. (right) Wetted area at indicated days. From ref. 28.