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New drug targets for cholera therapy

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Intestinal infection with *Vibrio cholerae* results in secretory diarrhea with potentially massive fluid losses and volume depletion. Morbidity and mortality associated with cholera remain a major problem in the developing world despite the success of oral rehydration therapy. New research aiming to inhibit cholera toxin binding to receptors in the intestine provides an attractive strategy for cholera therapy. Together with anti-secretory agents, including inhibitors of enkephalinase and of the cystic fibrosis transmembrane conductance regulator, new treatment options for managing severe diarrhea in cholera could soon be available.

Cholera: from bug to bedside

Cholera is a disease that causes watery diarrhea as a result of intestinal infection by the gram-negative bacillus *Vibrio cholerae*. Cholera remains a major public health problem in Africa, Asia and Latin America, with 200 000–500 000 reported new cases each year, although the actual number of cases is likely to be much greater [1]. Cholera is transmitted through contaminated food and water and, when untreated, leads to severe dehydration and shock. Without medical treatment, mortality associated with cholera infection is 20–50% [2]. The ultimate goal in cholera prevention is the maintenance of clean water supplies, as has been successful in some regions of Southeast Asia. Current management of cholera is based on rehydration therapy with oral rehydration solutions (ORS), which has substantially reduced mortality [3]. Although ORS treatment is the mainstay of cholera therapy, there remains a need for pharmacological approaches to treat cholera, particularly in pediatric, elderly and immunocompromised subjects where dehydration can be complicated by malnutrition, pneumonia and other factors. Effective drug therapy would also be useful to treat rapid outbreaks where medical facilities are limited, as in the refugee crisis in Central Africa in 1994

and 1995 where >40 000 cases were reported or in the current crisis in the Dafur region of Sudan. Cholera also poses a potential bioterrorist threat, being classified by the US government as a Category B biopathogen (http://www2.niaid.nih.gov/Biodefense/bandc_priority.htm). Although there are several anti-diarrheal drugs on the market at present, they have proved to be ineffective in cholera and in some cases contraindicated in cholera treatment [4]. In this article, we discuss recent progress in new approaches to treat cholera.

Intestinal fluid loss in cholera is caused primarily by the release of a heteromeric toxin [cholera toxin (CT)], in addition to related enterotoxins such as zona occludens toxin (ZOT) and accessory cholera toxin (ACE). Cholera toxin consists of an 'A' subunit coupled to a 'B' subunit that contains five identical peptides assembled in a pentameric ring [5]. On release from the bacterium, CT binds to intestinal enterocytes through interaction of the B subunit with the GM1 ganglioside receptor, and is then internalized through retrograde endocytosis. Within the cell the A subunit causes constitutive activation of adenylyl cyclase by activation of the stimulatory G protein $G_{s\alpha}$, resulting in elevated levels of intracellular cAMP [6]. Elevation of cAMP produces active secretion of salt and fluid through activation of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel in the apical plasma membrane of enterocytes, in addition to inhibition of electroneutral Na^+ absorption [7]. Once intestinal infection occurs, there are at least four potential pharmacological strategies to reduce intestinal fluid loss in cholera (Figure 1).

Cholera vaccines and antibiotics

Cholera vaccines have been used for >100 years with generally limited efficacy. However, recent advances in the understanding of *V. cholerae* virulence and immunogenicity have led to a new generation of oral vaccines that have proved promising in clinical field studies. The currently available vaccines are based on either killed whole-cell bacteria including the non-toxic B subunit of CT (WC-BS),

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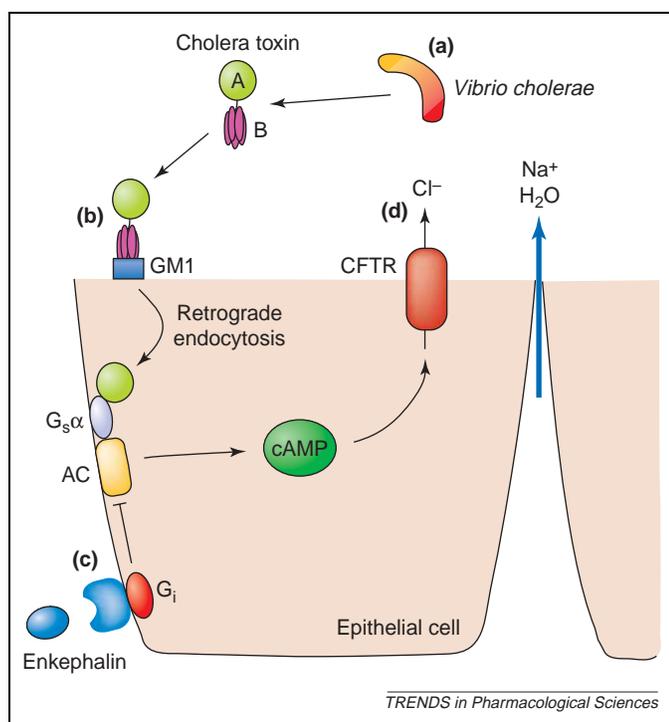


Figure 1. Strategies for drug therapy of cholera. Cholera toxin, released from the bacterium *Vibrio cholerae*, consists of an 'A' subunit coupled to a 'B' subunit that contains five identical peptides assembled in a pentameric ring [5]. Cholera toxin binds to intestinal enterocytes through interaction of the B subunit with the GM1 ganglioside receptor. It is then internalized through retrograde endocytosis. The internalized A subunit causes constitutive activation of adenylyl cyclase (AC), resulting in elevated levels of intracellular cAMP [6], which causes active secretion of salt and fluid through activation of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel in the apical plasma membrane of enterocytes, in addition to inhibition of electroneutral Na^+ absorption [7]. Prevention of diarrhea in cholera can be achieved by intervention at different stages of the mechanism by which *V. cholerae* causes intestinal fluid secretion: (a) elimination of *V. cholerae* bacteria through host immunity and/or antibiotics; (b) inhibition of cholera toxin binding to intestinal epithelial cells; (c) inhibition of increased cAMP levels by the action of enkephalins on opioid receptors; and (d) inhibition of Cl^- secretion by CFTR Cl^- channels.

or live attenuated bacteria consisting of the recombinant strain CVD 103 HgR, which lacks the gene encoding the A subunit [8]. Both types of vaccines have shown efficacy in endemic areas such as Peru, Bangladesh and Micronesia [9–12], but follow-up studies have indicated that protection is relatively short-lived, particularly against the *V. Cholerae* El Tor biotype, and is highly age dependent with low efficacy in children of <5 years of age. It remains unclear whether a vaccination strategy will be completely effective in combating cholera because of the short length of protection, particularly in high-risk patients such as infants, and the consequent uncertainty about cost-effectiveness in endemic areas.

Antibiotic therapy is currently used to reduce the severity of diarrhea in cholera, with the use of tetracycline and furazolidane usually recommended. The use of antibiotics in the treatment of diarrhea, however, is not a viable solution because of the rapid increase in antibiotic resistance, particularly in endemic areas [13].

Inhibition of cholera toxin–receptor binding

A novel and potentially useful strategy to reduce diarrhea in cholera is the inhibition of CT binding to intestinal enterocytes. CT binds to the GM1 surface receptor

through its pentameric B subunit. In a recent study, Pickens *et al.* reported the synthesis and characterization of several bivalent ligands that inhibit CT receptor binding by targeting the B subunit [14]. Using competitive surface binding assays they found significantly greater potency of multivalent ligands compared with monovalent ligands. Previous reports from the same research group showed that pentavalent and decavalent ligands inhibited CT binding with nanomolar potency (~ 40 nM) [15,16]. In the more recent study [14], bivalent ligands, which were unable to occupy multiple toxin binding sites, exhibited unexpectedly large increases in surface binding inhibition compared with monovalent ligands. In a series of elegant solution and crystallographic experiments the authors concluded that the observed increase in potency is probably due to steric blocking effects at the toxin–receptor interface. These studies provide an important first step in the design of potent inhibitors of CT binding, and support the development of multivalent ligands with large steric bulk.

Inhibition of toxin binding is a particularly attractive approach to preventing diarrhea for several reasons. Binding inhibitors can be administered prophylactically in high-risk areas or as therapy after the infection has begun. Furthermore, binding inhibitors can be of any size or charge and designed for oral administration with little systemic absorption. However, although the initial studies show considerable promise, the compounds discovered so far are at a preliminary stage in development and it remains to be demonstrated that this approach is effective *in vivo* to prevent excessive fluid secretion in cholera.

Enkephalinase inhibitors

Anti-secretory agents provide an alternative approach to cholera therapy. The enkephalins are endogenous opiate substances that prevent fluid secretion by enterocytes through binding to delta opioid peptide receptors. On receptor binding, enkephalins cause activation of inhibitory G proteins (G_i), resulting in reduced levels of intracellular cAMP and consequent deactivation of apical membrane CFTR Cl^- channels and basolateral K^+ channels [17]. Enkephalins are normally degraded rapidly in the gastrointestinal tract by endogenous enkephalinases. Enkephalinase inhibitors such as racecadotril increase the levels of intestinal enkephalin, resulting in reduced secretion of salt and fluid. Clinical studies have shown that racecadotril can be effective in *V. cholerae*- and *Escherichia coli*-related pediatric diarrhea, although studies in adults suggest that it is not effective, possibly as a result of rapid intestinal transit leading to inadequate tissue concentrations, or inadequate enkephalin-mediated anti-secretory activity [18,19].

CFTR inhibitors

Another potential target for anti-secretory therapy in cholera is the CFTR Cl^- channel. CFTR is the final rate-limiting step for intestinal Cl^- secretion and thus fluid secretion in cholera and other enterotoxin-mediated secretory diarrheas. Recently, small-molecule thiazolidinone (Figure 2a) inhibitors of CFTR Cl^- conductance with submicromolar inhibitory potency (Figure 2b) were

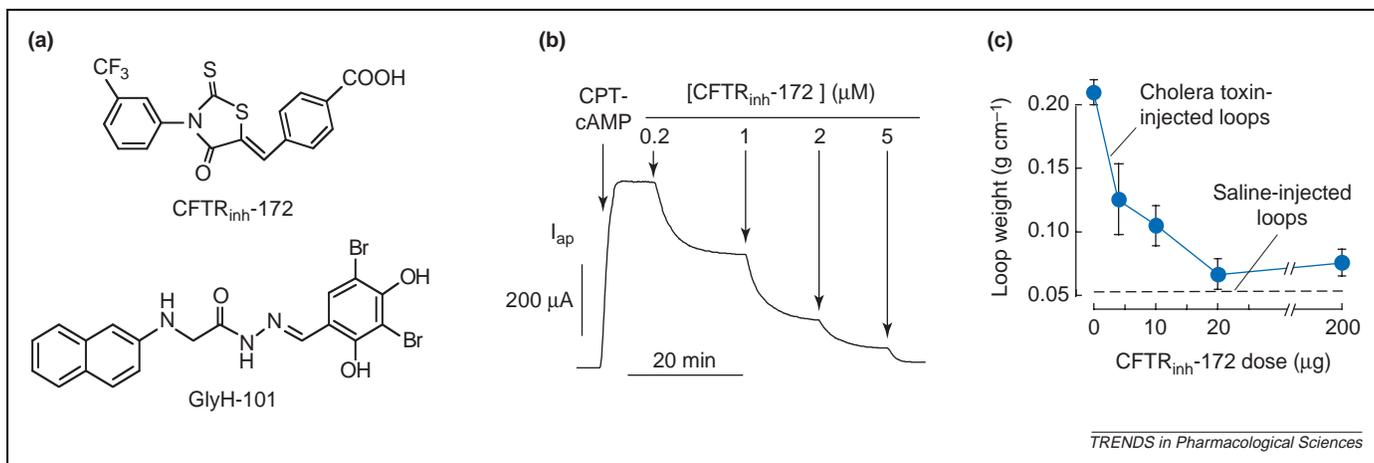


Figure 2. Cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel inhibitors as anti-secretory agents in cholera therapy. **(a)** Structures of the thiazolidinone CFTR inhibitor CFTR_{inh}-172 and glycine hydrazide inhibitor GlyH-101. **(b)** Inhibition of CFTR-dependent apical membrane short-circuit current (I_{ap}) by CFTR_{inh}-172 in Fisher rat thyroid epithelial cells transfected with human CFTR, showing submicromolar inhibitory potency. I_{ap} is shown as μA per cm^2 after stimulation with a permeable cAMP analog (CPT-cAMP) and inhibition with indicated concentrations of CFTR_{inh}-172. **(c)** Dose-dependent inhibition of cholera toxin-induced fluid secretion into closed loops of mouse ileum by CFTR_{inh}-172. CFTR_{inh}-172 was administered by intraperitoneal injection and fluid secretion into ileal loops measured by the ratio of loop weight to length. Data from [19–21].

discovered by high-throughput screening, and shown to be effective in preventing Cl⁻ and fluid secretion by CT in human intestinal cells and rodent models (Figure 2c) [20,21]. Further screening has revealed a glycine hydrazide (Figure 2a) class of CFTR inhibitors that also prevent CT-induced fluid secretion *in vivo* [22]. The glycine hydrazides appear to occlude the CFTR Cl⁻ channel pore at its external surface, suggesting the possibility of developing an orally administered, non-absorbable anti-secretory drug.

SB303 (proanthocyanidin oligomer), a polyphenolic polymer extracted from the bark latex of the tree *Croton lechleri*, also inhibits CFTR-mediated Cl⁻ secretion and inhibits CT-induced fluid accumulation [23]. In recent studies, this natural extract has been further purified, resulting in increased potency [24]. Another natural extract that inhibits CFTR-mediated secretion was purified originally from rice, although the chemical identity of the active component remains to be determined [25].

Progress in the development of novel agents to treat diarrheal diseases such as cholera has been slow during the past decade partly because of the success of ORS in reducing mortality and partly because of limited commercial interest in drugs for the developing world. Anti-secretory agents such as enkephalinase inhibitors or potent CFTR inhibitors might become useful additions to the treatment options for cholera. The recent studies of Pickens *et al.* provide an important step in the discovery of strategies to block toxin binding with potential applications to other surface receptor-mediated diseases. Further studies to design more-potent inhibitory ligands are needed, as is *in vivo* testing of anti-diarrheal efficacy.

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Letters

Rediscovery of known natural compounds: nuisance or goldmine?

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We hypothesize that: ‘When a natural compound occurs in unrelated species it must have an important biological function by interacting with a specific molecular target’.

This hypothesis is based on the fact that many neurotransmitters, hormones, pharmacological reference compounds and even some natural drugs occur in unrelated species. By studying literature sources [1,2], we have identified a substantial number of other compounds that are found in multiple species but have as yet unknown mechanisms of action. We predict that some of these compounds are potentially novel pharmacological tools or leads for novel drugs. It is possible that some of these examples are not synthesized in all species but are sequestered through the food-chain [3]. In this letter, some of the many examples are discussed, highlighting chemically distinct compounds that are linked only by the fact that they occur in unrelated species. This letter was triggered by hot debates following the publication of our previous *TiPS* article [4]. From those debates, polygodial, the first pertinent example, emerged.

The sesquiterpenoid polygodial (Figure 1, Compound 1), which is responsible for the peppery taste of the water pepper (*Polygonium hydropiper*), occurs in many different plants, ferns and liverworts, and surprisingly also in marine snails (*Ophistobranchia*). The compound is an insect antifeedant (i.e. repels insects), has antimicrobial activity, inhibits plant growth and is cytotoxic and poisonous to fish. In mammals it has analgesic, anti-inflammatory, anti-allergic and vasorelaxant effects, and was recently shown to inhibit gastric lesions caused by alcohol and other necrotizing agents [5]. The molecular mechanisms that underlie these activities are all unknown. Iridomyrmecin and actinidine, which are structural analogs of polygodial, are also found in unrelated species.

Theanine (Compound 2), a non-proteinogenic amino acid that occurs in high concentrations in tea and certain fungi, has neuroprotective properties (e.g. decreases the size of cerebral infarcts in mice) [6]. Other amino acids that occur in several species include orotic acid, baikiain and hernycin, the latter being related to ergothioneine (Compound 3), which is present in human blood, semen, liver and kidney, the ergot fungus and the king crab (*Limulus polyphemus*). Ergothioneine has anti-apoptotic activity, and potential therapeutic value in Alzheimer’s disease [7]. Trigonelline (Compound 4), which was isolated from mammalian tissues and plants (e.g. alfalfa, *Coffea* spp. and *Cannabis sativa*), is hypoglycaemic in animals [8]. Betaine (Compound 5), originally isolated from *Atriplex spongiosa*, was later found in other plants and mammalian tissues, and was shown to be active in an animal model of alcoholic liver disease [9]. A quinone that occurs in unrelated species is hypericin, which is partly responsible for the antidepressant activity of St John’s wort and is also detected in mealy bugs. Mellein (Compound 6) was first isolated from fungi, but later from wax moths, ants and termites, where it is part of their defense secretions. In the ant *Lasius fuliginosus* mellein is the trail pheromone. Other quinones isolated from unrelated species are islandicin, juglone, canthin-6-one and mimosamycin.

Kinetin (Compound 7) is a plant cytokine that also occurs in yeast; it counteracts defective splicing of pre-mRNA [10], a major cause of human disease. Vanillin not only occurs in *Vanilla planifolia*, but also in other plants, and the bug *Eurygaster integriceps* uses it as insect attractant. It has anti-fungal, anti-mutagen and anti-carcinogen activities. 2-Aminoacetophenone (Compound 8), which dominates the flavor of grapes, also occurs in human milk and is a pheromone that is produced by virgin honeybee queens. In small social groups, it repels and is used to terminate agonistic interactions between

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