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Developing novel antisecretory drugs to treat infectious diarrhea

Diarrhea, a disease of poverty and poor sanitation, kills an estimated two million children each year. Oral rehydration therapy is a very simple and inexpensive treatment that has significantly reduced mortality from secretory diarrhea caused by rotavirus, cholera and enterotoxigenic *Escherichia coli*. The efficacy and adoption of oral rehydration therapy would be enhanced by a drug that reduces fluid loss associated with these diseases and alleviates disease symptoms. Secretion and absorption by the intestine offer a number of potential drug targets to reduce fluid loss. Among these, the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel is the most attractive because it is the primary driver of secretion in cases of diarrhea caused by enterotoxigenic bacteria. CFTR can be inhibited by both natural products and synthetic small molecules. iOWH032 is a synthetic CFTR inhibitor that has recently entered clinical trials for this indication.

Diarrheal diseases are a leading killer in the developing world

Every year, an estimated 2.5 billion cases of infectious diarrhea occur among children under 5 years of age [1,101], leading to the death of an estimated two million children. After respiratory infections, diarrhea is the second leading cause of mortality in that age group and kills more young children than AIDS, malaria and measles combined. It also causes significant morbidity and recent studies suggest that repeated bouts of diarrhea have a significant impact on cognitive development [2,3] and malnutrition [4–6].

Infectious diarrhea can be largely prevented by adequate sanitation and a clean water supply; aid to developing countries is, therefore, often focused in these areas. However, much of the world's population still lacks access to reliable supplies of clean water and/or is at risk of infection when natural disasters strike. Whereas increasing access to clean water is a long-term solution, there is also an immediate need for more effective therapies to treat infectious diarrhea. Even in wealthy countries outbreaks of infectious diarrhea continue to occur, although the etiology is usually different from those in poorer countries, and the mortality rate is lower.

Diarrhea is generally classified according to the time course of the symptoms (acute or chronic) and to whether the etiology is infectious or non-infectious. Non-infectious diarrhea, such as that caused by irritable bowel syndrome, results from a complex interaction of

immune and neuronal factors [7] but is beyond the scope of this review. The mechanisms of diarrhea caused by various pathogens can be classified as inflammatory or non-inflammatory. Dysentery, a typical example of an inflammatory, invasive diarrhea, is marked by visible blood in the stools and is associated with intestinal damage and nutrient losses in an infected individual. The bacterial pathogen *Shigella* is the most common cause of dysentery [8], but amoebic dysentery caused by *Entamoeba histolytica* is a common problem in some regions [9–11]. In contrast, non-inflammatory infectious diarrheas are caused by pathogens that disrupt the absorptive and/or secretory processes of the gut epithelium without causing acute inflammation or mucosal destruction.

This review will focus on the development of therapeutics for non-inflammatory **acute watery diarrhea** (AWD), or secretory diarrhea. AWD can last from hours to days and is associated with copious fluid loss and rapid, life-threatening dehydration. The pathogens most commonly responsible for AWD are **rotavirus**, *Vibrio cholerae* and **enterotoxigenic *Escherichia coli*** (ETEC) bacteria, which are associated with poor sanitation and contaminated water in developing countries [12–14]. There are hotspots for **cholera**, such as flood-prone Bangladesh, but ETEC is widespread and is a common cause of traveler's diarrhea. According to the WHO, rotavirus is the most common cause of severe diarrhea and dehydration of infants in both wealthy and developing countries.

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Key Terms**Acute watery diarrhea:**

Fluid loss due to hypersecretion across the intestinal lumen.

Rotavirus: Most common cause of acute watery diarrhea, responsible for more than 500,000 deaths per year.

Enterotoxigenic

Escherichia coli: Most common infection leading to traveler's diarrhea, but also causes approximately 400,000 deaths per year.

Cholera: Acute watery diarrhea caused by infection with the Gram-negative bacterium *Vibrio cholerae*. Cholera outbreaks worldwide cause over 100,000 deaths each year.

Oral rehydration therapy:

Glucose/electrolyte solution routinely used to treat acute watery diarrhea in developing countries.

OneWorld Health: Nonprofit pharmaceutical research and development organization that discovers, develops and delivers safe, effective and affordable new treatments and interventions for people suffering from neglected diseases in the developing world, with an emphasis on diseases that disproportionately affect children.

Current treatment & new therapeutic opportunities

In AWD due to cholera infection, the onset of diarrhea is rapid and, if left untreated, the mortality rate is 50–60%, with death occurring within hours to days. However, with immediate, appropriate treatment the mortality rate can be reduced to 1% [15]. Regardless of etiology, the common symptom of fluid loss in cases of AWD makes the replenishment of lost fluid by **oral rehydration therapy (ORT)** the cornerstone of treatment. Oral rehydration solution (ORS) used in ORT simply consists of electrolytes (sodium and potassium chloride) and glucose, which promotes water absorption and costs only a few cents per treatment. Low-osmolarity ORT (a slightly less concentrated and more effective version of the original ORS recipe) is actively promoted by the WHO and the United Nations Children's Fund, and is credited with saving millions of lives worldwide since its introduction in the 1970s [16].

Variations to the standard ORS recipe include the use of rice water or rice syrup instead of glucose or sucrose, which can deliver more sugar without simultaneously increasing the osmolarity of the ORT [17,18]. Amylase-resistant starches are also being investigated as a supplement to ORS because they are fermented in the colon into short-chain fatty acids such as butyrate, which stimulate local blood flow and, thus, fluid and electrolyte uptake [19]. To complement ORT, the WHO recommends the administration of zinc for 10 days during and after the treatment. This treatment has been shown both to alleviate symptoms of diarrhea and reduce recurrence in the ensuing few months [20]. Similarly, vitamin A supplementation during early childhood has been shown to improve prognosis and reduce mortality due to diarrhea and is being promoted by the WHO [21].

Acute watery diarrhea usually resolves on its own as long as adequate hydration is maintained but in certain cases when specific pathogens are identified (e.g., *V. cholerae*), antibiotics are used in addition to ORS to speed up resolution as well as to reduce shedding of infectious bacteria that can cause further infection. However, prolonged or indiscriminate use of antibiotics runs the risk of driving the spread of resistance in pathogen populations and is not recommended.

Opioid drugs, such as loperamide (Imodium®) or diphenoxylate (in Lomotil®), which decrease diarrheal discharge by reducing peristalsis, are discouraged in the treatment of AWD. The

effect of these drugs is mostly palliative as they do not improve rehydration, and the reduction in discharge can mask the underlying disease. They are contra-indicated because they can cause paralytic ileus [22] and blocking discharge while fluid continues to accumulate in the bowel can have lethal consequences. Furthermore, these drugs can negatively impact disease resolution by reducing the expulsion of bacteria.

Although ORS salts, as well as vitamin A and zinc supplements, are widely available, AWD continues to kill millions. Despite its proven efficacy, low cost and promotion by public health authorities around the world, the adoption of ORT as the standard of care has slowed down in recent years and its use may even be declining in many developing countries [23]. The reason for this is still under investigation, but one postulated explanation is that ORT does not provide quick clinical relief of diarrheal symptoms (in fact, during the acute phase it increases output as the patient rehydrates), leading to its premature discontinuation. Although ORT is very effective, its clinical and perceived efficacies could be improved by an antisecretory drug. An agent that reduces enterotoxin-induced hypersecretion used in conjunction with ORT could provide more rapid relief of symptoms and facilitate the adoption of ORT.

Racecadotril (acetorphan, Bioprojet) is the only currently marketed antisecretory drug. It acts by inhibiting neprilysin (enkephalinase) and thus the degradation of enkephalin, an endogenous peptide that inhibits intestinal secretion [24]. Racecadotril received regulatory approval in Europe and is readily available in India, but some clinical studies have called into question its efficacy [25,26] and as a result it has not been widely adopted. For the past 4 years, **OneWorld Health (OWH)** has been developing a novel antisecretory compound that targets an ion channel that is critical for AWD.

Targeting the cystic fibrosis transmembrane conductance regulator

The cystic fibrosis transmembrane conductance regulator (CFTR) lies at the heart of hypersecretion associated with AWD caused by enterotoxigenic bacteria. The CFTR gene was identified in 1989 as the gene mutated in cystic fibrosis [27]. CFTR belongs to the ABC family of transporters but, unlike other members of the family, it is not involved in active transport. Rather, it allows chloride and bicarbonate to

flow down their electrochemical gradient, usually out of cells. CFTR channels are located primarily at the apical (i.e., luminal) surfaces of epithelial cells in the airways, submucosal glands, intestine, pancreas, kidney and testis, where they modulate chloride secretion, and in the sweat glands, where they are involved in the reabsorption of chloride from sweat [28]. CFTR plays a crucial role in transepithelial fluid homeostasis by controlling the flow of chloride ions and thus the movement of water in and out of cells. The clinical features of CF include chronic lung infection with progressive deterioration of lung function, pancreatic exocrine insufficiency, male infertility, meconium ileus and various less-common gastrointestinal complications [29]. Chronic bacterial lung infections are the result of both impaired fluid secretion (leading to the formation of very thick mucus), as well as improper buffering due to the impaired secretion of bicarbonate. More than 1500 loss-of-function mutations in CFTR have been identified that cause CF [102]. Unlike CF, where a mutation causes loss of function, AWD is caused by the inappropriately sustained activation of wild-type CFTR in intestinal epithelial cells by bacterial enterotoxins produced by *V. cholerae* (cholera toxin) and ETEC (heat-stable toxin) [30].

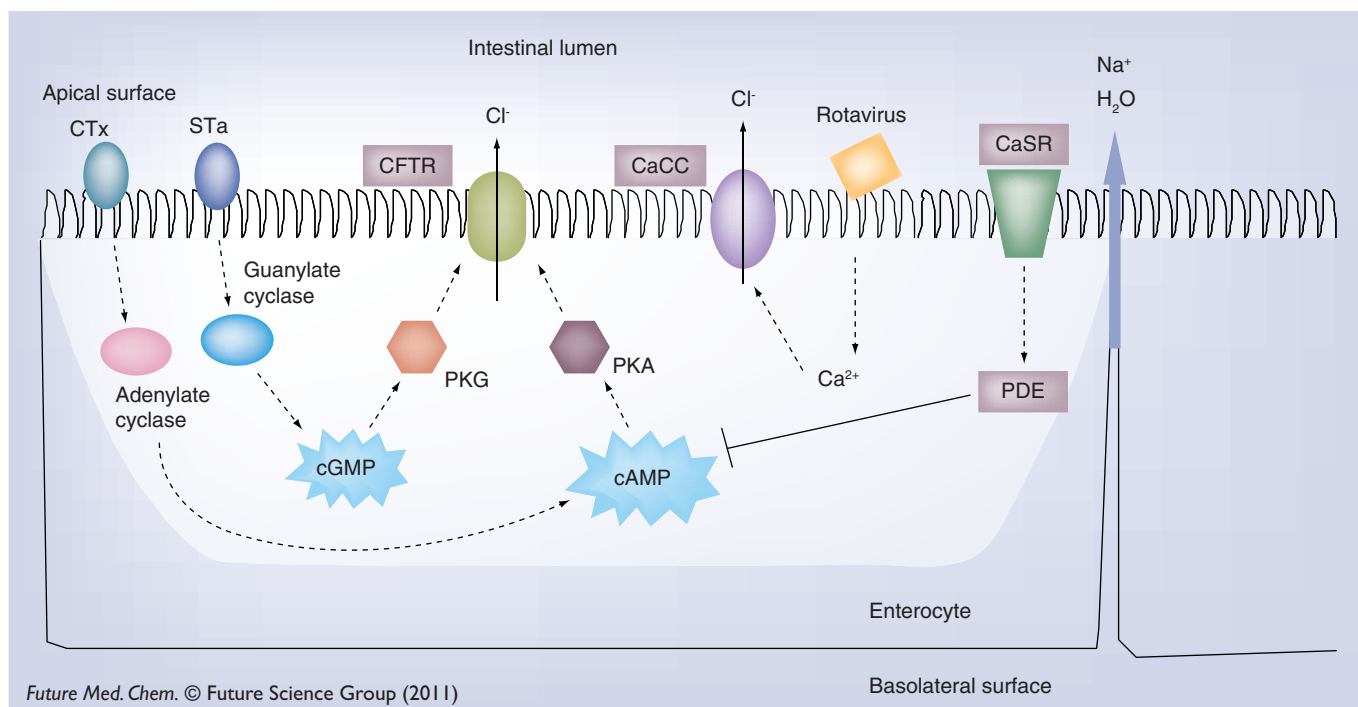
The mechanism of CFTR activation involves an increase in the concentration of cyclic nucleotides (cyclic adenosine monophosphate [cAMP] and cyclic guanosine monophosphate [cGMP]) in affected cells (**FIGURE 1**). Cholera toxin ADP-ribosylates, a $G\alpha_s$ protein that constitutively activates adenylate cyclase, thus inducing an increase in cellular cAMP levels. Heat-stable toxin binds and activates guanylate cyclase, thus inducing an increase in cellular cGMP levels. Higher levels of cAMP and cGMP activate protein kinases A and G, respectively, which phosphorylate CFTR and promote opening of the channel and the efflux of chloride from the cells [31]. Paracellularly, sodium follows the chloride to maintain charge balance and water escapes from the cells to maintain osmotic balance. This efflux of water and electrolytes is manifested as watery diarrhea.

Given the central role of CFTR in AWD, there are two approaches to antisecretory therapy: to inhibit secretion at the 'business end', that is, CFTR, the ion channel responsible for secretion, or to target the upstream signal transduction cascade leading to the activation of the channel.

Cystic fibrosis transmembrane conductance regulator has been targeted in previous drug-discovery efforts [32–34], but more frequently to identify compounds that restore the function of the mutant forms of CFTR that are responsible for CF, rather than inhibiting wild-type CFTR. The first CFTR inhibitor to enter clinical trials was crofelemer, a heterogeneous proanthocyanidin fraction derived from the sap of the Amazonian tree *Croton lechleri* currently under development by Napo Pharmaceuticals and its licensee Glenmark Pharmaceuticals (**FIGURE 2A**) [35]. Crofelemer is in Phase III clinical trials for the treatment of AIDS-related diarrhea and Phase II trials for AWD. In a clinical trial with cholera patients at the International Centre for Diarrhoeal Disease Research in Dhaka, Bangladesh, crofelemer only yielded a modest reduction in fluid loss [36]. In addition to crofelemer, numerous other natural products have been reported to inhibit CFTR using *in vitro* and *in vivo* assays including penta-m-digalloyl-glucose [37], lysophosphatidic acid [38] and cocoa-related flavonoids [39]; however, no clinical data have yet been reported with these compounds.

The first large-scale screens for synthetic CFTR inhibitors were conducted in Alan Verkman's laboratory at the University of California, San Francisco (USA), and identified two chemical series with micromolar potency [40]. The lead compound from the thiazolidinone series, INH-172, is now widely used as a tool compound in studies involving CFTR function, but is not considered as a good starting point for optimization through medicinal chemistry (**FIGURE 2B**). Both electrophysiological and pharmacological studies suggest that this compound acts from the intracellular side of the channel rather than the luminal side. Consistent with this evidence is the observation that INH-172 has an antisecretory effect when administered systemically. On the other hand, the inhibitors of the glycine hydrazide (GlyH) (**FIGURE 2C**) series are believed to be pore-blocking inhibitors acting from the luminal side of the channel [41]. More recently, Verkman's laboratory identified a third series of pyrimidopyrrolo-quinoxalinedione CFTR inhibitors with nanomolar potency aimed at the treatment of polycystic kidney disease, in which CFTR plays a role [42]. Nonabsorbed analogs of this series could be useful for treating AWD.

The possibility that the GlyH inhibitors work from the luminal side of the channel made them attractive as the starting point



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Basolateral surface

Figure 1. Secretory pathways in the gut epithelium disrupted by diarrhea-causing pathogens.

CaCC: Calcium-activated chloride channels; cAMP: Cyclic adenosine monophosphate; CaSR: Calcium-sensing receptor; CFTR: Cystic fibrosis transmembrane conductance regulator; cGMP: Cyclic guanosine monophosphate; CTx: Cholera toxin; PDE: Phosphodiesterase; PKA: Protein kinase A; PKG: Protein kinase G; STa: Enterotoxigenic *Escherichia coli* heat-stable toxin.

for the development of a minimally absorbed inhibitor that would circumvent any potential toxicity resulting with systemic exposure to the compound. In 2007, OWH in collaboration with BioFocus (South Walden, UK) set out to explore this possibility. The inhibitors developed by OWH share the halo-phenol head that is thought to be the pharmacophore in the GlyH series, but the labile acyl hydrazone is substituted by a stable heterocycle.

The activity of the OWH heterocyclic compounds was validated in several cell lines using both electrophysiological and fluorescence-based reporter assays, as well as two animal models of secretory diarrhea [OWH, UNPUBLISHED DATA]. In the mouse 'closed-loop' model, cholera toxin and test compounds were injected directly into intestinal loops in anesthetized animals [43]. OWH heterocyclic compounds inhibited secretion as measured by the weight-to-length ratio of excised loops after a several hours of cholera toxin treatment. In the rat model used, cholera toxin administered orally was used to induce secretion in rats from which the cecum has been previously removed [44]. OWH lead heterocyclic compound **iOWH032** (FIGURE 2D) inhibited secretion by nearly 70% as measured on a semi-quantitative fecal output

scale. IND-enabling studies were recently completed with iOWH032 and it entered clinical trials in 2011.

■ Other targets

While treatment with a CFTR inhibitor is likely to further improve the prognosis of patients with AWD caused by enterotoxigenic bacteria, it is not expected to help pediatric patients affected by rotavirus, which accounts for approximately half of all AWD cases in developing countries [45]. Like cholera and ETEC, rotavirus induces chloride secretion by the gut epithelium, but this efflux appears to be mediated primarily by calcium-activated chloride channels (CaCCs) rather than CFTR [46–48]. Ideally, a single drug would be able to inhibit both CFTR and the relevant CaCCs and thereby serve as a broad-spectrum treatment for AWD. Crofelemer, the proanthocyanidin fraction, has been shown to inhibit CaCCs in addition to CFTR, and in fact had stronger activity on CaCCs [49]. Its clinical efficacy against infectious and AIDS-related diarrhea is currently under investigation.

The process of making a small-molecule inhibitor that can stem the flow of chloride from both CFTR and CaCCs faces a number of

Key Term

iOWH032: Chloride channel blocker with antisecretory activity developed by OneWorld Health and currently in Phase I clinical trials.

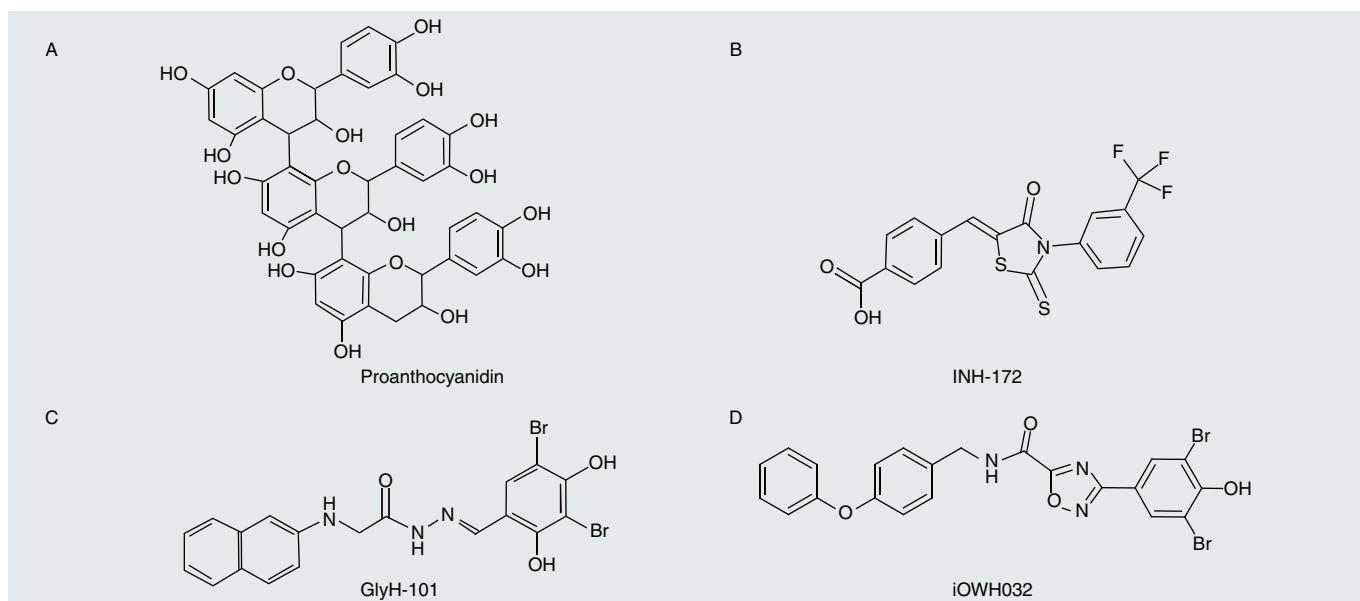


Figure 2. Cystic fibrosis transmembrane conductance regulator inhibitors. (A) Proanthocyanidin oligomer; represents the class of compounds found in crofelemer. Crofelemer oligomers are on average heptamers but range from contain 5–11 monomers. (B) INH-172, a thiazolidinone that was the first synthetic cystic fibrosis transmembrane conductance regulator inhibitor identified by high-throughput screening and is widely used as a tool compound for *in vitro* studies. (C) GlyH-101, a glycol-hydrazone inhibitor that is thought to be lumenally active. This series identified the dihalophenol pharmacophore that is shared with iOWH032. (D) iOWH032, heterocycle derivative of dihalophenol pharmacophore that is currently in Phase I trials.

challenges. CaCCs are a heterogeneous group of channels defined primarily by electrophysiological properties rather than molecular identity. For example, it is not clear which CaCC is the primary one involved in rotavirus-induced chloride secretion. The TMEM16 family of proteins includes two CaCCs (TMEM16A and TMEM16B) [50–52], but they show no sequence homology with CFTR or other chloride channels. Furthermore, TMEM16A is involved in salivary secretion but seems to play only a small role in respiratory and intestinal epithelia and is, therefore, not a good target for antidiarrheal therapy [53]. Verkman and collaborators have identified both small-molecule inhibitors of TMEM16A and nonselective CaCC inhibitors [53,54].

Another approach to inhibiting CFTR and CaCCs would be to target secretion at a common signal-transduction component upstream of the ion channels, but, unfortunately, there are no validated common targets between the cyclic nucleotide and calcium-activated pathways, although some cross talk is likely. However, the intestinal calcium-sensing receptor (CaSR) is involved in the regulation of both secretion and absorption and is thus a potential target for the treatment of secretory diarrhea of various etiologies. This G-protein coupled

receptor is expressed on both the luminal and basolateral membranes of the intestinal epithelium and is sensitive to changes in the concentration of divalent cations (Ca^{2+} and Mg^{2+}), vital nutrients such as polyamines and amino acids, as well as pH and ionic concentration in the GI tract. It is involved in the regulation of calcium homeostasis in the body as well as the secretion and absorption of fluid and electrolytes in the gut [55–57].

John Geibel and the late Steven Hebert at the Yale School of Medicine (USA), in collaboration with Amgen Inc., have shown that stimulating CaSR with either Ca^{2+} or sensitizing (calcimimetic) compounds can stop secretion induced by bacterial enterotoxins as well as increase salt and fluid absorption [57]. They have proposed that the use of a CaSR activator would accelerate rehydration with ORT by both reducing fluid and electrolyte loss and increasing absorption.

The primary mechanism for the antisecretory effect of CaSR agonists is thought to be via the activation of intracellular phosphodiesterases that degrade cAMP and cGMP in the cells [57]. But in addition to downregulating CFTR, activation of CaSR also influences net fluid secretion by affecting the function of two other ion channels, through a mechanism that

has not been fully characterized. The Na-K-Cl co-transporter (NKCC1) lets chloride into the cell from the blood, and its downregulation reduces chloride and water secretion [58]. While CFTR and NKCC are downregulated by CaSR activation, the Na⁺/H⁺ exchanger NHE3 [59] is upregulated, thus enhancing sodium and electrolyte uptake from the gut lumen. By affecting the function of multiple players in the disease state, CaSR agonists have the potential to serve as part of a broad-spectrum therapy for infectious diarrhea.

Lessons learned

■ Animal models

As in many drug-development projects, animal models are a potential weak link in the testing of antisecretory compounds for treating AWD. Diarrhea models are not 'off-the-shelf' models, such as some of those used to test anticancer or anti-inflammatory drugs. Establishing and validating them is difficult and costly. Most importantly, their ability to predict efficacy in treating humans with AWD is largely untested.

The mouse closed-loop cholera model is widely used because it is relatively simple and reliable but in this model there is no flow of fluid through the lumen, a key feature of AWD. The cecectomized rat model recapitulates secretion and flux through the gut but it requires a time-consuming and expensive surgical modification. A neonatal mouse model for rotavirus infection [60] and a neonatal rabbit model using live cholera or ETEC [61,62] are available, but technically challenging and the throughput is slow.

■ Target product profile

Having a detailed target product profile (TPP) is very important for any design project and, in the financially constrained world of nonprofit drug development, an accurate TPP is absolutely essential. OWH consulted closely with clinicians and global health thought leaders to develop a TPP for an antisecretory compound. This TPP describes the minimal and optimal characteristics for the compound, including efficacy spectrum, patient population, dosing regime, safety and pharmacological profile, as well as cost.

Drugs for neglected diseases have special requirements and cost is a critical parameter. The medicines developed for neglected diseases are not bought by the relatively generous health insurance programs available in rich countries.

Instead, they are paid for with the meager resources of NGOs, local public health organizations, or out of the pockets of poor patients. As a rule of thumb, treatment should not cost more than US\$1 per patient per day. This places a tight limit on the cost of new treatments and a high bar for the necessary cost/benefit ratio. A new drug for neglected diseases must not only be inexpensive but also provide a marked improvement over the existing therapy. Otherwise, the drug will not be adopted or reach the intended patient population.

Future perspective

A greater understanding of intestinal physiology at the molecular level, combined with advances in high-throughput screening and medicinal chemistry bodes well for the development of antisecretory therapies. OWH is committed to developing a portfolio of drugs against secretory diarrhea. iOWH032 may be just the first of a new class of drugs that will complement ORT. In time, additional molecular targets will be exploited for therapeutic intervention. Drugs that have antisecretory activity in cases of diarrhea caused by a broad range of pathogens will be the most valuable, especially in developing world situations where an accurate diagnosis may be difficult and drug access is limited. A convergence of technical tools, economic factors as well as the emergence of product development partnerships will have a positive impact on the pipeline of drugs for neglected diseases. The coming years are likely to see additional antisecretory drug candidates enter the clinic but the funds for conducting Phase II and III trials will remain scarce and the adoption of a new product will remain a major challenge. In combination with greater availability of clean water, new and improved vaccines against enteric pathogens, and broader use of ORT, the development of antisecretory therapies should help reduce the global burden of diarrheal diseases.

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Executive summary

Diarrheal diseases are a leading killer in the developing world

- Two million deaths occur per year, mainly of children under 5 years old.
- Major causative pathogens are rotavirus, *Vibrio cholerae* and enterotoxigenic *Escherichia coli*.

Current treatment & new therapeutic opportunities

- Oral-rehydration therapy is effective and inexpensive, but adoption has slowed.
- Oral-rehydration therapy adoption could be enhanced by an antisecretory drug.

Antisecretory drug targets

- Cystic fibrosis transmembrane conductance regulator chloride channel blockers have antisecretory activity in preclinical models and are being tested in human trials.
- Other antisecretory targets include calcium-activated chloride channels, calcium-sensing receptor and others.

Lessons learned

- Lack of validated, predictive animal models is a major hurdle for development of antisecretory therapies.
- A detailed target product profile is essential for antisecretory drug development.

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