Peptide inhibitors of chloride channels for treating secretory diarrhea

Jitu Ma¹, Xueqin Ding¹, Yulong Yin¹², Pengfei Huang¹

¹Animal Nutrition and Human Health Laboratory, School of Life Sciences, Hunan Normal University, Changsha City, Hunan, 410081, China, ²Chinese Academy of Science, Institute of Subtropical Agriculture, Research Center for Healthy Breeding of Livestock and Poultry, Hunan Engineering and Research Center of Animal and Poultry Science and Key Laboratory for Agroecological Processes in Subtropical Region, Scientific Observation and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Changsha City, Hunan, 410125, China

TABLE OF CONTENTS
1. Abstract
2. Introduction
3. CFTR and CaCC are anti-secretory targets for treating diarrhea
   3.1. Targeting CFTR
   3.2. Targeting CaCC
4. Mechanisms of infectious secretory diarrhea
   4.1. ETEC diarrhea
   4.2. Rotaviral diarrhea
5. Discovery and development of CFTR and CaCC inhibitors
   5.1. Small-molecule inhibitors
   5.2. Peptide inhibitors
6. Summary and perspective
7. Acknowledgements
8. References

1. ABSTRACT

Morbidity and mortality associated with diarrheal diseases remain significant burdens on global health. In the developing world, the major sources of secretory diarrhea are infectious, including those caused by bacteria such as enterotoxic *Escherichia coli*, and viruses such as rotavirus. In many cases of secretory diarrhea, activation of pathways for cyclic nucleotides and/or Ca²⁺ signaling in the apical membrane of enterocytes increases the conductance of Cl⁻ channels at the enterocyte lumen-facing membrane. Those channels include the cystic fibrosis transmembrane conductance regulator (CFTR) and Ca²⁺-activated Cl⁻ channel (CaCC). Inhibition of enterocyte Cl⁻ channels is an effective strategy for anti-secretory drug therapy. Small molecules and natural peptides with Cl⁻ channel inhibitory activity have shown efficacy in diarrhea models. Screening of natural peptides via the patch-clamp technique provides evidence that such channel inhibition by an extract of black tea may be responsible for its anti-diarrhea benefits.

2. INTRODUCTION

Diarrheal diseases have always been a major global health challenge. Secretory diarrhea is a leading cause of mortality and morbidity in children under age 5 and adults above age 70 (1-3). For these susceptible populations, the risk is often further enhanced by associated enteric infections (4, 5). In developing countries, secretory diarrhea is primarily caused by bacteria such as enterotoxic *Escherichia coli* (ETEC), whereas in developed countries the leading causes are viruses such as rotavirus (6-8). By secreting enterotoxins, ETEC activates apical membrane cystic fibrosis transmembrane conductance regulator (CFTR) and Ca²⁺-activated Cl⁻ channel (CaCC). Infection of enterocytes increases the conductance of Cl⁻ channels at the enterocyte lumen-facing membrane. Those channels include the cystic fibrosis transmembrane conductance regulator (CFTR) and Ca²⁺-activated Cl⁻ channel (CaCC). Inhibition of enterocyte Cl⁻ channels is an effective strategy for anti-secretory drug therapy. Small molecules and natural peptides with Cl⁻ channel inhibitory activity have shown efficacy in diarrhea models. Screening of natural peptides via the patch-clamp technique provides evidence that such channel inhibition by an extract of black tea may be responsible for its anti-diarrhea benefits.
Current anti-diarrheal therapies center around prompt improvement of dehydration, such as through intravenous fluid replacement and oral rehydration solution (20, 21). Although symptomatic treatment effectively ameliorates dehydration, it does not change the secretion of chloride or electrolyte losses, and most contemporary methods for managing and treating infectious secretory diarrhea are decades old (22-25). In the past five years, CFTR and CaCC have emerged as novel drug candidates based on greater understanding of the mechanisms of Cl\(^{-}\) secretion and intestinal electrolyte movement (26). Several Cl\(^{-}\) channel inhibitors have been identified from screening of small-molecule collections or natural peptides (14, 27). The latter are often recognized as an excellent starting point for drug development and, when compared with traditional small molecules, peptides are relatively safe, highly selective and efficacious, and easy to synthesize (27, 28).

Here, we describe the major pathophysiology mechanisms of secretory diarrhea and discuss the opportunities that have been revealed from basic research to clinical applications for Cl\(^{-}\) channel inhibitors. We also report that a black tea extract containing peptides strongly inhibits intestinal CaCC, and we present several ways in which peptide inhibitors offer tremendous growth potential as new therapeutic strategies.

3. CFTR AND CACC ARE ANTI-SECRETORY TARGETS FOR TREATING DIARRHEA

Secretory diarrhea results from excessive secretion of fluid and electrolytes into the intestinal lumen (24, 29-31). The movement of sodium and water between the lumen and blood vessel is driven by active trans-epithelial Cl\(^{-}\) secretion. This involves the activation of chloride channels located on the apical (lumen-facing) epithelial membranes (32). The electrochemical driving force for chloride secretion across the luminal membrane through CFTR and CaCC is established by basolateral (circulation-facing) membrane transporters (Figure 1). Thus, CFTR and
Peptide inhibitors of chloride channels for treating secretory diarrhea

CaCC are potential membrane channel targets to reduce intestinal fluid and the secretion of electrolytes.

3.1. Targeting CFTR

As a cAMP-activated chloride channel, CFTR occurs on the apical surface of many mammalian epithelia and fluid-transporting tissues (33-35). In 1989, The CFTR gene was unexpectedly identified when researchers were searching for the cystic fibrosis gene through positional cloning (13, 36, 37). Mutations in that gene cause cystic fibrosis, a hereditary, lethal disease. This transmembrane regulator contains the ATP binding cassette (ABC) transporter structural motif. Members of the ABC superfamily have two six-helix membrane-spanning alpha helices, each followed by a homologous nucleotide binding domain (NBD) (38-40). However, unlike other ABC proteins, CFTR has a regulatory (R) domain linking the first NBD and the second membrane-spanning domain. Activation of CFTR involves ATP binding and hydrolysis at its NBDs, as well as cAMP-dependent phosphorylation of multiple R-domain sites, the details of which remain unknown.

Under normal physiological conditions, CFTR is expressed at the apical epithelial membrane, and plays a major role for chloride and, hence, fluid secretion into the intestine lumen (9). After ETEC infection, enterotoxins up-regulate intracellular cyclic nucleotide levels of intestinal epithelial cells, resulting in activation of the CFTR channel. Pharmacological inhibition of CFTR Cl− conductance has potential therapeutic value in secretory diarrhea initiated by ETEC.

3.2. Targeting CaCC

The CaCC is widely distributed in various tissues of vertebrates and invertebrates, such as neurons, myocardium, skeletal muscle, smooth muscle, epithelial cells, olfactory cells, photoreceptor cells, uterine muscle cells, breast cells, and lymphocytes, where it has a wide range of physiological functions, including neuronal and cardiac excitation, smooth muscle contraction, trans-epithelial fluid secretion, olfactory and sensory signal transduction, and oocyte fertilization (34, 41, 42). Therefore, investigating the molecular basis of CaCC is of great interest to many scientists. The characteristics of early candidates are not consistent with endogenous CaCC properties (43-45). In 2008, three laboratories independently demonstrated that transmembrane protein 16A (TMEM16A) is the molecular basis for CaCC. Strong evidence for this included the similarity of electrophysiological properties between TMEM16A and native CaCC, the presence of CaCC currents in various transfected cell systems, a decrease in CaCC currents after RNAi knockdown, and its tissue distribution (46-48). Therefore, identification of TMEM16A may be helpful to our discovery of targeted inhibitors of CaCC.

Intracellular calcium is a major factor influencing CaCC permeability. Chemical, electrical, or sensory signals can elevate the level of calcium in intestinal epithelial cells and then induce the opening of CaCC, causing chloride outflow to produce an inward current.

4. MECHANISMS OF INFECTIOUS SECRETORY DIARRHEA

Secretory diarrhea that arises from ETEC and rotavirus infections is widespread and prevalent in gastrointestinal diseases. In developing countries, the incidence of infectious diarrhea leads all infectious diseases. Diarrhea is also the main factor in malnutrition, stunted growth, and developmental disorders in children. Therefore, it is important that researchers understand the pathogenesis of infectious secretory diarrhea.

4.1. ETEC diarrhea

ETEC secretes specific adhesin and enterotoxins (heat-labile and heat-stable) that boost the levels of intracellular cyclic nucleotides, resulting in activation of apical CFTR Cl− channels and, hence, Cl− secretion (10, 49). After ETEC enters the intestine, secretory adhesin binds to intestinal epithelial cell-specific receptors and then colonizes the intestinal lumen. Enterotoxins are direct pathogenic factors for secretory diarrhea, activating the membrane adenylate cyclase by binding to the intestinal epithelial cell receptor (50). After cAMP activates cAMP-dependent protein kinase, it phosphorylates the CFTR Cl− channel. That phosphorylated channel remains open and secretes excess Cl− to cause secretory diarrhea (51).

4.2. Rotaviral diarrhea

Rotavirus infection leads to fluid secretion as well as morphological changes in the intestinal epithelium, resulting in age-related secretory diarrhea. The non-structural protein NSP4 is produced on the basal side of rotavirus-infected intestinal cells and is encoded by the rotavirus gene. This protein is thought to act as an enterotoxin that elevates the concentration of cytoplasmic Ca2+ by binding to a membrane receptor (integrin alpha1 beta2) or the neuropeptide galanin, and/or by activating enteric nerves (52). When the concentration of cytoplasmic Ca2+ increases, the activated CaCC in intestinal epithelial cells releases chloride ions to produce secretory diarrhea (53).

5. DISCOVERY AND DEVELOPMENT OF CFTR AND CaCC INHIBITORS

Secretory diarrhea is caused by the activation of cyclic nucleotide and/or Ca2+ signaling pathways in intestinal epithelial cells, which increase the
Peptide inhibitors of chloride channels for treating secretory diarrhea

conductance of Cl\(^-\) channels at the apical membrane. Being able to inhibit those Cl\(^-\) channels presents an attractive strategy for treatment via anti-secretory drugs. Screening of small molecule collections and natural peptides has identified several classes of Cl\(^-\) channel inhibitors that show efficacy in diarrhea models but must still be tested clinically.

5.1. Small-molecule inhibitors

Three chemical types of small molecules that show CFTR inhibition have been identified from the screening of synthetic small-molecule libraries (14). The prototype of CFTR inhibitors includes the thiazolidinone CFTRinh-172, which prevents CFTR Cl\(^-\) conductance by binding at or near arginine-347 on the cytoplasmic side of CFTR and then stabilizing the channel in its closed state (54). In mouse diarrhea models of heat-stable enterotoxin-induced intestinal fluid secretion, CFTRinh-172 has an anti-secretory effect. A second type of absorbable CFTR inhibitor that targets the cytoplasmic surface is the PPQ/BPO compound. Such compounds are effective in polycystic kidney disease models, but have not been tested in models of diarrhea (55). A third chemical type of small-molecule CFTR inhibitors (glycine hydrazides) binds to the extracellular CFTR surface in the channel pore itself, as demonstrated by patch clamp analysis (56, 57). However, by locating an external site on the intestine, the underlying disorder is the accessing of CFTR in the deep intestinal crypts that are resistant to a strong convective rinse in secretory diarrhea.

The initial phenotype-based screen was performed with human colonic cell line HT-29. Those efforts identified several small-molecule CaCC inhibitors, e.g., CaCCinh-A01, which fully inhibits CaCC-dependent halide flux in different intestinal cell lines. Since then, this inhibitor has been shown to prevent secretory diarrhea in a neonatal mouse model of rotavirus (58).

5.2. Peptide inhibitors

Protein-protein interactions are the basis of cellular functions. Most of these interactions involve short peptide motifs, and interest is increasing in the use of peptide-based, targeted therapeutics (59). The advantages of peptides are their specificity, relative safety, ease of production, and their ability to be modified through chemical synthesis and molecular biology techniques (60, 61). Screening the natural peptide inhibitors of Cl\(^-\) channels represents an attractive source of antidiarrheal therapeutics because they are generally inexpensive and have the potential for rapid translation to the clinic. Various leaf teas, especially green and black, manifest a wide range of CaCC inhibitory activities. Our laboratory has utilized a peptide inhibitor screen with a patch clamp recording technique to demonstrate strong CaCC inhibition by black tea, which contains numerous natural peptides. Because CaCC activation is involved in rotaviral diarrhea, we found that the black tea extract prevented rotaviral diarrhea in neonatal mice but had no effect on the rotaviral infection. Control experiments showed that a tea extract with minimal in vitro CaCC activity did not prevent rotaviral diarrhea. However, the use of black tea extracts for CaCC-dependent diarrhea requires more in-depth study. Natural products, with a defined mechanism of action, represent a possibly inexpensive and readily available therapy for secretory diarrhea.
6. SUMMARY AND PERSPECTIVE

Anti-secretory drug therapy has considerable potential to reduce the morbidity and mortality that are associated with infectious diarrhea. The identification and validation of small molecules and effective natural products, as well as repurposed drugs, that can block infectious diarrhea through Cl− channels is of great importance. Precise regulation of specific protein–peptide interactions is a promising new therapeutic strategy. The use of peptides as a means of modulation represents an exciting path toward this goal. Several different technologies have been introduced to aid in the discovery of peptide binders that will be suitable for use as drug frameworks. Although the development of anti-secretory drugs still presents multiple challenges, the future for peptide drugs is becoming brighter as more candidates are now approved or in clinical trials. The next decade may bring the prospect of new drugs against secretory diarrhea.

7. ACKNOWLEDGEMENTS

This research was supported by the Scientific Research Foundation of Hunan Provincial Education Department (No. 80162).

8. REFERENCES

1. W. H. Organization: GHO | Global Health Observatory Data Repository (African Region) | Life tables by country - Côte d'Ivoire


11. A. M. Pattison, E. S. Blomain, D. J. Merlino, F. Wang, M. A. Crissey, C. L. Kraft, J. A. Rappaport, A. E. Snook, J. P. Lynch and
Peptide inhibitors of chloride channels for treating secretory diarrhea


**Abbreviations**: ETEC: enterotoxic *Escherichia coli*; CFTR: cystic fibrosis transmembrane conductance regulator; CaCC: Ca2+-activated Cl- channel; NSP4: non-structural rotaviral protein
4; ABC: ATP binding cassette; NBD: nucleotide binding domain; TMEM16A: transmembrane protein 16A

Key Words: Secretory diarrhea, Peptides, CFTR, CaCC, ETEC, Rotavirus, Review

Send correspondence to: Pengfei Huang, 36 Lushan Road, Changsha 410081, Tel: 86-0731-88872358, Fax: 86-0731-88872358, E-mail: perfehuang@foxmail.com