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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

CFTR Activator Increases Intestinal Fluid Secretion and Normalizes Stool Output in a Mouse Model of Constipation

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SUMMARY

Activation of the cystic fibrosis transmembrane conductance regulator chloride channel drives fluid secretion in the intestine. High-throughput screening identified a small-molecule cystic fibrosis transmembrane conductance regulator activator that increases intestinal fluid secretion. Oral administration of the activator corrected constipation in a mouse model.

BACKGROUND & AIMS: Constipation is a common clinical problem that negatively impacts quality of life and is associated with significant health care costs. Activation of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel is the primary pathway that drives fluid secretion in the intestine, which maintains lubrication of luminal contents. We hypothesized that direct activation of CFTR would cause fluid secretion and reverse the excessive dehydration of stool found in constipation.

METHODS: A cell-based, high-throughput screen was performed for 120,000 drug-like, synthetic small molecules. Active compounds were characterized for mechanism of action and one lead compound was tested in a loperamide-induced constipation model in mice.

RESULTS: Several classes of novel CFTR activators were identified, one of which, the phenylquinoxalinone $CFTR_{act}$-J027, fully activated CFTR chloride conductance with an half-maximal effective concentration (EC_{50}) of approximately 200 nmol/L, without causing an increase of cytoplasmic cyclic adenosine monophosphate. Orally administered $CFTR_{act}$-J027 normalized stool output and water content in a loperamide-induced mouse model of constipation with a 50% effective dose of approximately 0.5 mg/kg; $CFTR_{act}$-J027 was without effect in cystic fibrosis mice lacking functional CFTR. Short-circuit current, fluid secretion, and motility measurements in mouse intestine indicated a prosecretory action of $CFTR_{act}$-J027 without direct stimulation of intestinal motility. Oral administration of 10 mg/kg $CFTR_{act}$-J027 showed minimal bioavailability, rapid hepatic metabolism, and blood levels less than 200 nmol/L, and without apparent toxicity after chronic administration.

CONCLUSIONS: $CFTR_{act}$-J027 or alternative small-molecule CFTR-targeted activators may be efficacious for the treatment of constipation. *(Cell Mol Gastroenterol Hepatol 2016;2:317–327; http://dx.doi.org/10.1016/j.jcmgh.2015.12.010)*

Keywords: CFTR; Constipation; High-Throughput Screening; Loperamide.

Constipation is a common clinical complaint in adults and children that negatively impacts quality of life. The prevalence of chronic constipation has been estimated to be 15% in the US population, with annual health care costs estimated at approximately 7 billion dollars, with more than 800 million dollars spent on laxatives.[1,2] The mainstay of constipation therapy includes laxatives that increase stool bulk, such as soluble fiber; creation of an osmotic load, such as polyethylene glycol; or stimulation of intestinal contraction, such as the diphenylmethanes. There also are surface laxatives that soften stool such as docusate sodium and probiotics such as *Lactobacillus paracasei*.[3] The Food and Drug Administration–approved drug linaclotide, a peptide agonist of the guanylate cyclase C receptor, acts by inhibiting visceral pain, stimulating intestinal motility, and increasing intestinal secretion.[4,5] A second approved drug, lubiprostone, a prostaglandin E analog, is thought to activate a putative enterocyte ClC-2 channel,[6] although the mechanistic data are less clear. Despite the wide range of therapeutic options, there is a continued need for safe and effective drugs to treat constipation.

Intestinal fluid secretion involves active Cl^- secretion across the enterocyte epithelium through the basolateral membrane Na^+/ K^+/ $2Cl^-$ cotransporter, the luminal membrane cystic fibrosis transmembrane conductance regulator

Abbreviations used in this paper: cAMP, cyclic adenosine monophosphate; CFTR, cystic fibrosis transmembrane conductance regulator; CSBM, complete spontaneous bowel movement; DMSO, dimethyl sulfoxide; EC_{50}, half-maximal effective concentration; FRT, Fischer rat thyroid; IP, intraperitoneal; PBS, phosphate-buffered saline; YFP, yellow fluorescent protein.

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(CFTR) Cl^- channel, and the Ca^{2+}-activated Cl^- channel. The electrochemical and osmotic forces created by Cl^- secretion drive Na^+ and water secretion.[7] In cholera and traveler's diarrhea, CFTR is activated strongly by bacterial enterotoxins through increase of intracellular cyclic nucleotides.[8,9] CFTR is an attractive target to increase intestinal fluid secretion in constipation because it is expressed robustly throughout the intestine and its activation can strongly increase intestinal fluid secretion. An activator targeting CFTR directly is unlikely to produce the massive, uncontrolled intestinal fluid secretion seen in cholera because the enterotoxins in cholera act irreversibly to produce a sustained increase of cytoplasmic cyclic adenosine monophosphate (cAMP), which not only activates CFTR but also basolateral K^+ channels, which increase the electrochemical driving force for Cl^- secretion; cholera enterotoxins also inhibit the luminal NHE3 Na^+/H^+ exchanger involved in intestinal fluid absorption.[10,11]

Motivated by these considerations and the continuing need for safe and effective drug therapy of constipation, we report the identification and characterization of a nanomolar potency, CFTR-targeted, small-molecule activator, and provide proof of concept for its prosecretory action in the intestine and efficacy in constipation.

Materials and Methods

Materials

High-throughput screening was performed using a diverse collection of 120,000 drug-like synthetic compounds obtained from ChemDiv, Inc (San Diego, CA) and Asinex (Winston-Salem, NC). For structure-activity analysis, 600 commercially available analogs (ChemDiv, Inc) of active compounds identified in the primary screen were tested. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise.

$CFTR_{act}$-J027 Synthesis

Potassium carbonate (2.5 g, 18.4 mmol) and benzyl bromide (0.73 mL, 6.2 mmol) were added to a solution of *o*-phenylenediamine (1 g, 9.24 mmol) in dimethylformamide (DMF) (30 mL), and then stirred overnight at ambient temperature. The reaction mixture was diluted with CH_2Cl_2, washed with water, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash chromatography to yield the intermediate N^1-benzylbenzene-1,2-diamine as a brown liquid. ^1H NMR (300 MHz, $CDCl_3$): δ 7.45–7.31 (m, 5H), 6.86–6.69 (m, 4H), 4.35 (s, 2H), 3.50 (br, 3H); MS: m/z 199 $(M+H)^+$. Then, a solution of the intermediate (400 mg, 2 mmol) and 5-nitroisatin (380 mg, 2 mmol) in acetic acid (5 mL) was refluxed for 2 hours. The reaction mixture was cooled to room temperature and solvent was removed under reduced pressure. The residue was dissolved with methanol to crystallize 3-(2-amino-5-nitrophenyl)-1-benzylquinoxalin-2(1H)-one ($CFTR_{act}$-J027) as a yellow solid with more than 99% purity. ^1H NMR (300 MHz, [DMSO] *d6*): δ 9.15 (d, 1H, J = 2.8 Hz), 8.07 (dd, 1H, J = 2.7, 9.2 Hz), 7.97 (dd, 1H, J = 1.2, 7.9 Hz), 7.82 (br, 2H), 7.60-7.27 (m, 7H), 6.92 (d, 1H, J = 9.2 Hz), 5.59 (br, 2H); ^{13}C NMR (75 MHz, DMSO-*d6*): δ 155.0, 154.6, 153.3, 136.3, 135.3, 132.8, 132.2, 131.0, 130.0, 129.5, 129.1, 127.7, 127.3, 126.8, 124.1, 116.1, 115.9, 115.4, 45.9; MS: m/z 373 $(M+H)^+$.

Cell Culture

Fischer rat thyroid (FRT) cells stably co-expressing human wild-type CFTR and the halide-sensitive yellow fluorescent protein (YFP)-H148Q were generated as previously described.[12] Cells were cultured on plastic in Coon's-modified Ham's F12 medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. For high-throughput screening, cells were plated in black 96-well microplates (Corning-Costar Corp, Corning, NY) at a density of 20,000 cells per well. Screening was performed 24–48 hours after plating.

High-Throughput Screening

Screening was performed using a Beckman Coulter (Brea, CA) integrated system equipped with a liquid handling system and 2 FLUOstar fluorescence plate readers (BMG Labtechnologies, Durham, NC), each equipped with dual syringe pumps and 500 $\pm$ 10 nm excitation and 535 $\pm$ 15 nm emission filters (see Galietta et al[12] for more detail). CFTR- and YFP-expressing FRT cells were grown at 37°C/5% CO_2 for 24–48 hours after plating. At the time of assay, cells were washed 3 times with phosphate-buffered saline (PBS) and then incubated for 10 minutes with 60 μL of PBS containing test compounds (at 10 μmol/L) and a low concentration of forskolin (125 nmol/L). Each well was assayed individually for I^- influx in a plate reader by recording fluorescence continuously (200 ms/point) for 2 seconds (baseline) and then for 12 seconds after rapid (<1 s) addition of 165 μL of PBS in which 137 mmol/L Cl^- was replaced by I^-. The initial rate of I^- influx was computed by determining exponential regression. All compound plates contained negative controls (DMSO vehicle) and positive controls (20 μmol/L forskolin).

Short-Circuit Current Measurement

Short-circuit current was measured in FRT cells stably expressing wild-type human CFTR cultured on porous filters as described.[12] The basolateral solution contained (in mmol/L): 130 NaCl, 2.7 KCl, 1.5 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$, 10 glucose, and 5 Na-HEPES (pH 7.3, 37°C). In the apical solution, 65 mmol/L NaCl was replaced by Na gluconate, and $CaCl_2$ was increased to 2 mmol/L, and the basolateral membrane was permeabilized with 250 μg/mL amphotericin B. Short-circuit current was measured in freshly harvested adult mouse colon at 37°C using symmetric Krebs-bicarbonate buffer (pH 7.4, in mmol/L: 117 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11.1 D-glucose, 1.2 KH_2PO_4, and 24.8 $NaHCO_3$).

cAMP Assay

Intracellular cAMP activity was measured using a GloSensor luminescence assay (Promega Corp, Madison, WI). FRT null cells were stably transfected with the pGloSensor

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