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# Phenylquinoxalinone CFTR activator as potential prosecretory therapy for constipation

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## Abstract

Constipation is a common condition for which current treatments can have limited efficacy. By high-throughput screening, we recently identified a phenylquinoxalinone activator of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel that stimulated intestinal fluid secretion and normalized stool output in a mouse model of opioid-induced constipation. Here, we report phenylquinoxalinone structure-activity analysis, mechanism of action, animal efficacy data in acute and chronic models of constipation, and functional data in ex vivo primary cultured human enterocytes. Structure-activity analysis was done on 175 phenylquinoxalinone analogs, including 15 synthesized compounds. The most potent compound, $CFTR_{act}$-J027, activated CFTR with $EC_{50}$ ~ 200 nM, with patch-clamp analysis showing a linear CFTR current-voltage relationship with direct CFTR activation. $CFTR_{act}$-J027 corrected reduced stool output and hydration in a mouse model of acute constipation produced by scopolamine and in a chronically constipated mouse strain (C3H/HeJ). Direct comparison with the approved prosecretory drugs lubiprostone and linaclotide showed substantially greater intestinal fluid secretion with $CFTR_{act}$-J027, as well as greater efficacy in a constipation model. As evidence to support efficacy in human constipation, $CFTR_{act}$-J027 increased transepithelial fluid transport in enteroids generated from normal human small intestine. Also, $CFTR_{act}$-J027 was rapidly metabolized in vitro in human hepatic microsomes, suggesting minimal systemic exposure upon oral administration. These data establish structure-activity and mechanistic data for phenylquinoxalinone CFTR activators, and support their potential efficacy in human constipation.

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## INTRODUCTION

Constipation is a common clinical problem affecting 15% of the U.S. population, with annual health-care costs estimated at ~7 billion dollars of which >800 million dollars is spent on laxatives.[1] The most frequent types of constipation include chronic idiopathic constipation (CIC), opioid-induced constipation (OIC), and constipation-predominant irritable bowel syndrome (IBS-C). Current treatment options include dietary modification and over-the-counter laxatives including agents that increase stool bulk, soften stool, create an osmotic load, or stimulate intestinal contraction.[2] There are 3 FDA-approved prescription drugs for treatment of various types of constipation: linaclotide, a peptide agonist of the guanylate cyclase C receptor that acts by inhibiting visceral pain, stimulating intestinal motility, and increasing intestinal secretion[3]; lubiprostone, a prostaglandin E analog that is thought to activate the enterocyte ClC-2 channel and perhaps CFTR[4,5]; and naloxegol, a peripherally acting $\mu$-opioid receptor antagonist.[6] These FDA-approved drugs generally showed efficacy in 40–50% of patients in different clinical trials albeit with a baseline 25–35% patient response to placebo.[7–9] Despite the wide range of therapeutic options, there is a continued need for safe and effective drugs to treat constipation.

We recently introduced the idea of prosecretory therapy for constipation by direct activation of the cystic fibrosis transmembrane conductance regulator (CFTR) $Cl^-$ channel, based on the idea that increasing intestinal fluid secretion would increase stool hydration and thereby accelerate intestinal transit. Intestinal fluid secretion involves active $Cl^-$ secretion across the enterocyte epithelium, which is controlled by apical membrane $Cl^-$ channels, including CFTR and perhaps $Ca^{2+}$ -activated $Cl^-$ channels. CFTR is a compelling target for constipation therapy as its over-activation by bacterial enterotoxins in cholera and traveler's diarrhea (enterotoxigenic *E. coli*) produces marked intestinal fluid secretion.[10,11] We recently reported that the phenylquinoxalinone $CFTR_{act}$-J027, a small-molecule CFTR activator identified by high-throughput screening, increased intestinal fluid secretion, and normalized stool output, stool water content, and intestinal transit in a mouse model of acute constipation produced by loperamide.[12] The compound had no effect in CFTR-deficient mice, showed no toxicity, and had minimal systemic exposure following oral administration because of rapid hepatic metabolism.

Here, for development of phenylquinoxalinones for therapy of constipation, we studied compound structure-activity relationships, mechanism of action by patch-clamp analysis, and animal efficacy in acute and chronic rodent models of constipation. In addition, functional studies were done in human enterocytes to support their potential utility for treatment of constipation in humans.

## MATERIALS AND METHODS

### General chemistry procedures

All chemicals were purchased from commercial suppliers and used without further purification. Commercial analogs were purchased from ChemDiv (San Diego, Calif). All other chemicals were purchased from Sigma-Aldrich (St. Louis, Mo) unless otherwise stated. Analytical thin layer chromatography was carried out on precoated plates (silica gel

60 F254, 250 μm thickness) and visualized with UV light. Flash chromatography was performed using 60 Å, 32–63 μm silica gel (Fisher Scientific, Waltham, Mass). Concentration *in vacuo* refers to rotary evaporation under reduced pressure. $^{1}$H NMR spectra were recorded at 400, 600, or 800 MHz at ambient temperature with acetone-d6, DMSO-d6, or $CDCl_3$ as solvents. $^{13}$C NMR spectra were recorded at 100, 150, or 200 MHz at ambient temperature. Chemical shifts are reported in parts per million (ppm) relative to the residual solvent peak. High-resolution mass spectra were acquired on an LTQ Orbitrap XL mass spectrometer equipped with an electrospray ionization source (ThermoFisher, San Jose, Calif), operating in the positive ion mode. Samples were introduced into the source via loop injection at a flow rate of 200 μL/min in a solvent system of 1:1 acetonitrile/water with 0.1% formic acid. Mass spectra were acquired using Xcalibur, version 2.0.7 SP1 (ThermoFinnigan, San Jose, Calif). The spectra were externally calibrated using the standard calibration mixture and then calibrated internally to <2 ppm with the lock mass tool. Analytical data are reported in Supplemental Data.

### Synthesis of $CFTR_{act}$-J102–J105, J109, J134–J141 (Path I). Path I/RXN 1: N-*benzyl-$R^1$-substituted-2-nitroanilines (1)*

A stirred solution of $R^1$-substituted-2-nitroaniline (4 mmol) and benzyl bromide (5 mmol) in water (8 mL) was sealed in a thick-wall glass tube (10 mL) and heated at 110°C overnight. The reaction mixture was cooled to room temperature, diluted with ethyl acetate, and solid sodium bicarbonate (4 mmol) was added. The resulting mixture was washed with water and the organic layer was dried over $MgSO_4$. After filtration, the organic layer was concentrated *in vacuo* and the final product was purified by silica gel column chromatography to yield brightly colored nitroaniline **1**.

### Path I/RXN 2: $N^1$-*benzyl-$R^1$-substituted-1,2-diaminobenzene (2)*

Nitroaniline **1** (5 mmol) was dissolved in ethanol (~100 mL; requires warming) and, after cooling to room temperature, Zn (50 mmol) and a 4 M HCl (4 mL) were added to the solution. The mixture was stirred until brightly colored **1** was consumed at which point the solution was filtered through a pad of Celite and the solvent was removed *in vacuo*. The residue was dissolved in ethyl acetate and the mixture was neutralized with 1 M NaOH. The ethyl acetate solution was washed with water, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The product was purified by silica gel column chromatography to yield **2** as a dark-colored viscous oil.

### Path I/RXN 3: N-(*2-(4-benzyl-$R^1$-substituted-3-oxo-3,4-dihydroquinoxalin-2-yl)-$R^3$-substituted-phenyl)acetamide (3)*

A solution of **2** (0.5 mmol) and 1-acetyl-($R^3$-substituted)indoline-2,3-dione (0.5 mmol) in glacial acetic acid (20 mL) was heated at 90°C overnight. Upon cooling to room temperature, the solvent was removed *in vacuo* and the product was washed with ethanol and filtered to yield **3**, which was used without further purification.

### Path I/RXN 4: *3-(2-amino-$R^3$-substituted-phenyl)-1-benzyl-$R^1$-substituted-quinoxalin-2(1H)-one (4)*

To a solution of **3** (0.2 mmol) in methanol (125 mL) was added 4 M HCl (2.5 mL) and the resulting mixture was heated at 80°C overnight. Upon cooling to room temperature, the solvent was removed *in vacuo* and the reaction mixture was neutralized with 1 M NaOH solution. Product **4** was extracted with ethyl acetate or dichloromethane and purified by flash column chromatography.

### Synthesis of $CFTR_{act}$-J133, J142–144 (Path II). Path II/RXN 5: N-(*$R^3$-substituted-2-(3-oxo-3,4-dihydroquinoxalin-2-yl) phenyl)acetamide (5)*

A solution of 1-acetyl-($R^3$-substituted)indoline-2,3-dione (1 mmol) and *o*-phenylenediamine (1 mmol) in toluene (10 mL) was heated at 120°C overnight. The resulting tan precipitate of **5** was collected by filtration, washed sequentially with toluene and hexane, and then used in the next step without further purification.

### Path II/RXN 6: N-*(2-(4-$R^1$-substituted-benzyl-3-oxo-3,4-dihydroquinoxalin-2-yl)-$R^3$-substituted-phenyl)acetamide (6)*

A solution of **5** (0.5 mmol), $R^1$-substituted benzyl bromide (0.6 mmol) and $K_2CO_3$ (1 mmol) in DMF (20 mL) was stirred overnight at room temperature. The reaction mixture was diluted with water and extracted with ethyl acetate or dichloromethane. The organic layer was washed sequentially with water and brine and then dried over $MgSO_4$. Filtration and removal of solvent yielded **6** as a tan product that was washed with ethanol and used in the deacylation reaction described above without further purification.

### Cell culture

Fischer rat thyroid (FRT) cells stably co-expressing human wild-type CFTR and the halide-sensitive yellow fluorescent protein (YFP)-H148Q were as described.[13] Cells were cultured on plastic in Coon's-modified Ham's F12 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. For plate reader assays, cells were plated in black 96-well microplates (Corning-Costar Corp., New York, N.Y.) at a density of 20,000 cells per well and assayed 24–48 hours after plating.

### Plate reader assay of CFTR activity

CFTR activity was assayed as described.[13] Briefly, 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 μM) and a low concentration of forskolin (125 nM). Full concentration-dependence measurements were done for active compounds to determine $EC_{50}$ values. $I^-$ influx was measured in a plate reader by recording fluorescence continuously (200 ms per point) for 2 seconds (baseline) and then for 12 seconds after rapid (<1 s) addition of 165 μL of PBS in which 137 mM $Cl^-$ was replaced by $I^-$. The initial rate of $I^-$ influx was computed using exponential regression.

### Short-circuit current measurement

Short-circuit current was measured in FRT cells stably expressing human wild-type CFTR cultured on porous filters as described.[14] The basolateral solution contained (in mM): 120 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 25 $NaHCO_3$ and 5 HEPES (pH 7.4, 37°C). In the apical solution, 60 mM NaCl was replaced by Na gluconate, and $CaCl_2$ was increased to 2 mM, and the basolateral membrane was permeabilized with 250 $\mu$g/mL amphotericin B.

### Patch-clamp experiments

Whole-cell and inside-out membrane currents were recorded in FRT cells stably expressing human wild-type CFTR. For whole-cell experiments, the extracellular (bath) solution contained (in mM): 150 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 mannitol, 10 Na-HEPES (pH 7.4). The pipette (intracellular) solution contained (in mM): 120 CsCl, 10 TEA-Cl, 0.5 EGTA, 1 $MgCl_2$, 10 Cs-HEPES, 40 mannitol, 1 ATP (7.4). For inside-out patch-clamp experiments, the pipette solution contained (in mM): 150 N-methyl-D-glucamine chloride (NMDG-Cl), 3 $CaCl_2$, 2 $MgCl_2$, 10 Na-Hepes (pH 7.3). The bath solution contained (in mM): 150 NMDG-Cl, 2 $MgCl_2$, 10 EGTA, 10 Na-Hepes, 1 ATP (pH 7.3), and 125 nM catalytic subunit of protein kinase A (Promega, Sunnyvale, Calif). Pipette electrical resistance for both whole-cell and inside-out experiments was 3–5 M$\Omega$. The protocol for stimulation consisted of 600-ms voltage steps from −100 to +100 mV in 20 mV increments starting from a holding potential of −60 mV. The interval between steps was 4 s. Membrane currents were filtered at 1 kHz and digitized at 5 kHz. Data were analyzed using Igor software (Wavemetrics, Portland, Ore) with custom software kindly provided by Dr Oscar Moran.

### Cyclic nucleotide and cytoplasmic $Ca^{2+}$ measurements

For cAMP measurements, FRT cells were grown in 12-well plates and incubated with vehicle control or 10 $\mu$M $CFTR_{act}$-J027, with and without 90 nM foskolin, or 20 $\mu$M forskolin alone, for 10 minutes at 37°C. Cells were lysed by freeze-thaw, centrifuged to remove cell debris (600*g*, 10 min, 4°C), and assayed for cAMP using a parameter cAMP immunoassay kit (R&D Systems, Minneapolis, Minn). cGMP measurements were carried out similarly using a cGMP immunoassay kit (R&D Systems). For cytoplasmic calcium measurements, FRT cells in 96-well black-walled microplates were loaded with Fluo-4 NW at 48 hours after plating per the manufacturer's protocol (Invitrogen). Cells were pretreated for 2 minutes with 10 $\mu$M $CFTR_{act}$-J027 or vehicle control, with 100 $\mu$M ATP added as a calcium agonist. Fluo-4 fluorescence was measured with a M1000 fluorescence plate reader (TECAN, San Jose, Calif) equipped with syringe pumps and a monochromator (excitation/emission: 494/516 nm).

### In vitro gastric acid and metabolic stability

To study gastric acid stability, $CFTR_{act}$-J027 (10 $\mu$M) was dissolved in simulated gastric fluid (0.2 NaCl, 0.7% HCl, pH 2) and incubated for 3 hours at 37°C. To study in vitro metabolic stability, $CFTR_{act}$-J027 (5 mM) was incubated for specified times at 37°C with human liver microsomes (1 mg protein/mL; Sigma-Aldrich) in potassium phosphate buffer (100 mM) containing 1 mM NADPH. After specified incubation periods, the mixtures were

chilled on ice, and 0.5 ml of ice-cold ethyl acetate was added. Samples were centrifuged for 15 minutes at 3000 rpm, the supernatants evaporated to dryness, and the residues were dissolved in 100 μL mobile phase (acetonitrile:water, 3:1) and assayed by liquid chromatography–mass spectrometry (LC/MS). The solvent system consisted of a linear gradient from 5% to 95% acetonitrile over 16 minutes (0.2 mL/min flow). Mass spectra were acquired on a mass spectrometer (Waters 2695 and Micromass ZQ, Milford, Mass.) using electrospray (+) ionization, mass ranging from 100 to 1500 Da, cone voltage 40 V.

## Animals

Animal experiments were approved by UCSF Institutional Animal Care and Use Committee. Animals were housed in communal cages in a temperature- and humidity-controlled environment with 12 hour light/dark cycle and provided standard rodent chow and water *ad libitum*. Wild-type female CD1 mice were bred in the UCSF Laboratory Animal Resource Center. Female C3H/HeJ mice, which have a spontaneous mutation in *TLR4* (*Tlr4*$^{lps\text{-}d}$) and their control background C3H/HeOuJ mice were purchased from Jackson Laboratories (Bar Harbor, Maine).

## Constipation models in mice

CD1 mice (age 8–10 weeks) were administered loperamide (0.3 mg/kg) or scopolamine (0.5 mg/kg) intraperitoneally (ip) to induce constipation. $CFTR_{act}$-J027 (10 mg/kg, in saline containing 5% DMSO and 10 % Kolliphor HS 15) was given ip or orally (po) either 1 hour before, at the same time, or 1 hour after loperamide/scopolamine in different experiments. Control mice were treated with vehicle only. Some mice were treated orally with lubiprostone (0.5 mg/kg) or linaclotide (0.5 mg/kg, Toronto Research Chemicals Inc, Toronto, Ontario, Canada). At designated time points, mice were placed individually in metabolic cages with food and water provided *ad libitum*. Stool samples were collected for 3 h, and total stool weight and number of fecal pellets were determined. Stool samples were dried at 80°C for 24 hours and stool water content was calculated as [wet weight − dry weight]/wet weight.

The efficacy of orally administered $CFTR_{act}$-J027 (10 mg/kg) was also tested in a genetically constipated mouse strain[15] (C3H/HeJ, age 8–11 weeks) and their wild-type counterparts (C3H/HeOuJ, age 8–11 weeks). Mice were placed in metabolic cages after $CFTR_{act}$-J027 or vehicle administration at zero time and stool was collected for 4 hours. Stool weight, pellet number, and water content were determined as described above. Whole-gut transit time was measured to assess gut motility, in which mice treated with $CFTR_{act}$-J027 (10 mg/kg, ip) or vehicle at zero time were given 100 μL blue marker (5% Evans blue, 5% gum Arabic) orally. The time of blue dye appearance in stool was determined. All experiments in C3H/HeJ and C3H/HeOuJ mice were done in paired animals to minimize variability.

## Closed-loop model of intestinal fluid secretion

Mice were given access to 5% dextrose water but not solid food for 24 hours before experiments. Mice were anesthetized with isoflurane and body temperature was maintained during surgery at 36°C–38°C using a heating pad. A small abdominal incision was made to

expose the small intestine, and closed mid-jejunal loops (length 2–3 cm) were isolated by sutures. Loops were injected with 100 μL vehicle alone or 100 μg $CFTR_{act}$-J027, lubiprostone or linaclotide in vehicle. The abdominal incision was closed with sutures, and mice were allowed to recover from anesthesia. Intestinal loops were removed at 90 minutes and loop length and weight were measured to quantify fluid secretion.

### Swelling measurements in human enteroids

Tissues from human subjects were obtained under approval of the Johns Hopkins University School of Medicine Institutional Review Board (protocol NA_00038329). Duodenal and jejunal biopsy specimens were obtained from adults during routine endoscopy at Johns Hopkins Hospital. Crypt isolation, enteroid propagation, and culture were as described.[16] For swelling measurements, enteroids were seeded in 35 mm dishes with bottom coverglass with 1.5 mL media. On the day of the experiment, the media was replaced with 3 mL Advanced DMEM/F12 and enteroids were incubated with 1 mM calcein green–acetoxymethyl ester for 1 hour at 37°C to label cytoplasm. Relative enteroid volume following addition of specified concentrations of forskolin was measured using a laser scanning confocal microscope (Fluoview FV10i-LIV; Olympus) at 37°C, 5% $CO_2$, and 95% relative humidity. In some studies, $CFTR_{act}$-J027 was added 10 minutes before forskolin. Images were acquired every 10 minutes and analyzed with MetaMorph version 7.7 software (Olympus) to quantify enteroid areas.

### Statistical analysis

Experiments with 2 groups were analyzed with Student's t-test; when there are 3 or more groups, analysis was done with one-way analysis of variance and post-hoc Newman-Keuls multiple comparisons test. $P < 0.05$ was taken as statistically significant.

## RESULTS

### Synthesis and structure-activity analysis of phenylquinoxalinone CFTR activators

Limited structure-activity relationship (SAR) information emerged from analysis of 160 commercially available phenylquinoxalinone analogs, as most had quite different structures or multiple substituent modifications in relation to $CFTR_{act}$-J027. Table I reports CFTR activity of selected commercial analogs most closely related to $CFTR_{act}$-J027 (J051–J062). The benzyl ($R^1$) substituent on the phenylquinoxalinone appeared to be important, as unsubstituted ($CFTR_{act}$-J051), methyl ($CFTR_{act}$-J052), and phenylacetyl ($CFTR_{act}$-J058) greatly reduced activity. Limited substitution ($R^1$ position) on the quinoxalinone ring showed that a nitro group ($CFTR_{act}$-J054) was tolerated. Substituent changes on the phenyl ring also strongly modulated activity; for example, changing $R^3$ and $R^4$ substituents to N-benzyl and bromo ($CFTR_{act}$-J056) or N-acetyl and unsubstituted ($CFTR_{act}$-J057) greatly reduced activity. Because of the limited information from commercial compounds, synthesis of targeted analogs was undertaken.

Two routes were developed to synthesize the phenylquinoxalinones analogs (Fig 1, A). The *Path I* route begins with *N*-benzylation of the appropriate 2-nitroaniline (→**1**) and subsequent nitro group reduction to give 1,2-diamino analog **2**. Condensation of this diamine

with the appropriate *N*-acylisatin in acetic acid delivers quinoxalin-2(1*H*)-one **3**; attempts to effect this transformation with nonacylated isatins usually produced the desired product in low yield. Finally, acid-catalyzed deacylation of **3** delivered the quinoxalin-2(1*H*)-one **4**. *Path II* allowed for late-stage introduction of the *N*-benzyl moiety, but requires the use of a symmetrical 1,2-diamine (here, 1,2-diaminobenzene) so as to avoid regioisomer formation in quinoxalin-2(1*H*)-one **5**. $N^1$-Benzylation with the appropriate benzyl bromide delivered quinoxalin-2(1*H*)-one **6** and deacylation gave the quinoxalin-2(1*H*)-one **4**.

CFTR activity was measured using a plate reader assay of iodide influx following extracellular addition of iodide in FRT cells stably expressing human wild-type CFTR and a yellow fluorescent protein fluorescent halide sensor. Fig 1, B shows the concentration-dependence data of selected compounds, with $EC_{50}$ values for all synthesized compounds given in Table I.

Fig 1, C summarizes structure-activity results for the synthesized compounds reported in Table I (compounds with J1## designations). In general, having a substitution group ($R^1$) on the benzyl group, especially in the *para*- or *ortho*-positions, reduced activity ($CFTR_{act}$-J102, -J103, -J104, -J143, and -J144). Acetylation of the amino group on $CFTR_{act}$-J027, giving $CFTR_{act}$-J109, also reduced activity. Analogs with halide substitution at the 6-position ($R^1$) of the quinoxalinone core ring ($CFTR_{act}$-J105, -J135, and -J136) gave similar or slightly reduced activity compared with $CFTR_{act}$-J027, and modifying $R^1$ to a methyl substituent was tolerated ($CFTR_{act}$-J141). Changing the 5-nitro on the phenyl ring of the phenylquinoxalinone to a 5-fluoro, 5-bromo, or 5-iodo slightly reduced activity (for example, compare $CFTR_{act}$-J134 to -J027), while shifting to the 6-position (compare $CFTR_{act}$-J134 to -J142) greatly reduced activity. $CFTR_{act}$-J135, the most potent synthesized analog, fully activated CFTR (Fig 1, D), as the cAMP agonist forskolin produced minimal further increase in current, with $EC_{50}$ of 308 nM, which was comparable to $CFTR_{act}$-J027. However, preliminary studies showed that $CFTR_{act}$-J135 was ineffective in the loperamide constipation model, in part because of its low solubility in an appropriate oral vehicle. As none of the commercial and synthesized analogs were significantly more potent than $CFTR_{act}$-J027, which was already shown to have favorable pharmacological properties and efficacy in mice,[12] subsequent studies were done with $CFTR_{act}$-J027.

## Evidence for direct CFTR activation by $CFTR_{act}$-J027

$CFTR_{act}$-J027 at 10 $\mu$M did not cause activation of cell signaling mechanisms that could activate CFTR by phosphorylation or cross-talk mechanisms. Fig 2, A shows that $CFTR_{act}$-J027 did not elevate cellular cAMP when added alone or in the presence of a low concentration of forskolin (90 nM) (left). Also, $CFTR_{act}$-J027 did not elevate cellular cGMP (center), nor did it elevate cytoplasmic $Ca^{2+}$ when added alone or did it affect ATP-induced $Ca^{2+}$ signaling. These results support a direct activation mechanism $CFTR_{act}$-J027 action on CFTR.

Patch clamp was done to further investigate the mechanism of CFTR activation by $CFTR_{act}$-J027. In whole-cell recordings, CFTR was partially activated using a low concentration of forskolin (150 nM) to give ~25% maximal stimulation. Addition of 1 $\mu$M $CFTR_{act}$-J027 in the extracellular condition further increased CFTR activity by more than 4-fold (Fig 2*B–E*).

The currents activated by forskolin and $CFTR_{act}$-J027 changed linearly with applied voltage and were blocked by 10 $\mu$M $CFTR_{inh}$-172. CFTR activation was also measured under cell-free conditions using the inside-out patch-clamp configuration using large pipette tips to obtain macro-patches containing multiple CFTR channels. After inducing phosphorylation with submaximal ATP and the catalytic subunit of protein kinase A, $CFTR_{act}$-J027 strongly increased CFTR activity (Fig 2, E). $CFTR_{act}$-J027 thus activates CFTR by a direct binding mechanism, which is consistent with prior data showing that $CFTR_{act}$-J027 does not elevate cellular cAMP concentration.[12]

### $CFTR_{act}$-J027 efficacy in a scopolamine-induced mouse model of acute constipation

We previously showed $CFTR_{act}$-J027 efficacy in a loperamide-induced mouse model of constipation.[12] To show efficacy in an alternative, nonopioid mouse model of acute constipation, $CFTR_{act}$-J027 was administered orally 1 hour before scopolamine. Fig 3, A shows normalization of stool parameters by $CFTR_{act}$-J027 in the scopolamine-treated mice. $CFTR_{act}$-J027 was also effective when administered intraperitonally after development of constipation in both loperamide and scopolamine models (Fig 3, B). However, $CFTR_{act}$-J027 was not effective when given orally after development of constipation (Fig 3, C), which is not unexpected as both scopolamine and loperamide delay gastric emptying and intestinal transit, likely preventing $CFTR_{act}$-J027 delivery to its site of action.

### $CFTR_{act}$-J027 efficacy in a genetic mouse model of chronic constipation

$CFTR_{act}$-J027 was also tested in a genetic mouse model (C3H/HeJ) of chronic constipation. C3H/HeJ mice have a spontaneous mutation in Toll-like receptor 4 gene (*Tlr4*$^{lps\text{-}d}$), which causes impaired interactions between gut microbiota and enteric neurons resulting in reduced enteric neuronal survival with delayed intestinal transit and decreased stool output.[15] We found that C3H/HeJ mice have ~30% reduced stool output compared with control mice in a matched background (C3H/HeOuJ), with remarkably decreased stool water content and prolonged whole-gut transit time. Oral administration of 10 mg/kg $CFTR_{act}$-J027 increased 4-hour stool weight, pellet number, and water content in C3H/HeJ mice to the level of wild-type controls (Fig 4, A). $CFTR_{act}$-J027 normalized the increased whole-gut transit time in C3H/HeJ mice (Fig 4, B), but did not affect stool parameters or whole-gut transit time in the wild-type control mice.

### $CFTR_{act}$-J027 is stable in gastric acid and rapidly metabolized by human hepatic microsomes

Incubation of 10 $\mu$M $CFTR_{act}$-J027 in simulated gastric fluid (pH 2) at 37°C for 3 hour showed no compound degradation (Fig 5, A). In vitro metabolic stability measurements in human hepatic microsomes revealed rapid compound metabolism (elimination half-life ~35 min), with only 34% of the compound remaining at 60 minutes (Fig 5, B).

### Greater efficacy of $CFTR_{act}$-J027 compared with lubiprostone and linaclotide in increasing intestinal secretion and normalizing stool parameters in constipation

The efficacy of $CFTR_{act}$-J027 in increasing intestinal secretion was compared with that of the approved prosecretory drugs lubiprostone and linaclotide. In a first set of studies,

intestinal fluid accumulation was measured in a closed intestinal loop model. Closed mid-jejunal loops were injected with $CFTR_{act}$-J027 or test drug, and fluid accumulation was quantified after 90 minutes. Significantly greater intestinal fluid accumulation was produced by $CFTR_{act}$-J027 compared with equal doses of lubiprostone or linaclotide (Fig 6, A). In a second set of studies, drug efficacy was compared in a mouse model of scopolamine-induced constipation. $CFTR_{act}$-J027 was more effective in increasing stool output, pellet number, and water content compared with supramaximal (250–500 fold greater than human mg/kg dose) doses of lubiprostone and linaclotide (Fig 6, B).

### $CFTR_{act}$-J027 increases fluid secretion in enteroids generated from normal human duodenum and jejunum

To study $CFTR_{act}$-J027 efficacy in a model system of direct relevance to human intestine, swelling responses were measured in enteroids generated from normal human duodenum and jejunum. The enteroids comprise a sealed epithelial layer of enterocytes with CFTR expressed at the inner, luminal membrane, in which CFTR activation produces a swelling response over tens of minutes.[16,17] Fig 7, A shows representative confocal micrographs of calcein-stained enteroids before and at 40 minutes after exposure to $CFTR_{act}$-J027 or a low concentration of forskolin, individually or together. Little or no change was seen in enteroid size in the absence of activators or with $CFTR_{act}$-J027 alone, whereas forskolin alone produced a small increase in area that was greater when added with $CFTR_{act}$-J027.

As summarized in Fig 7, B, enteroids generated from human duodenum and jejunum showed a slow swelling response to 0.1 or 0.2 $\mu$M forskolin, which was increased by pretreatment for 10 minutes with $CFTR_{act}$-J027, with greatest effect seen for enteroids from jejunum. The response produced by $CFTR_{act}$-J027 was approximately 50% of that produced by maximal (5 $\mu$M) forskolin.

## DISCUSSION

$CFTR_{act}$-J027 showed efficacy in rodent models of constipation induced by opioid and nonopioid antimotility agents, as well as in chronically constipated C3H/HeJ mice. Opioid and nonopioid antimotility agents have been widely used to test laxatives in rodents, as these models are technically simple and informative in short-term studies, and the opioid models have high relevance to OIC in humans. However, they may have limited relevance to human CIC and IBS-C, which are chronic conditions. Genetic models of chronic constipation have more relevance to human CIC and IBS-C due to their chronic phenotype, and do not require pharmacological interventions targeting intestinal motility. Though there are genetically constipated models of Hirschprung's disease with spontaneous mutations in *endothelin receptor B* (*Ednrb*) and targeted mutation in Ednrb ligand *endothelin 3* that manifest aganglionic colon,[18–20] we used C3H/HeJ mice here because they manifest chronic constipation but with a milder phenotype and normal survival,[15] which make them more relevant to non-life-threatening CIC and IBS-C. We found that $CFTR_{act}$-J027 was effective in both pharmacological and genetic models of constipation, which supports its potential use in acute and various forms of chronic constipation in humans including CIC, IBS-C, and OIC.

A modular approach was developed for efficient synthesis of phenylquinoxalinone analogs using substituted nitroanilines, benzyl bromides, and isatins. The most potent phenylquinoxalinones have favorable drug-like properties, including the presence of multiple hydrogen bond acceptors, average molecular weight of ~400 Da, aLogP of ~4.0, and topological polar surface areas of ~80 $Å^2$.[21,22]

Patch-clamp studies indicated direct activation of CFTR by $CFTR_{act}$-J027. CFTR activation by $CFTR_{act}$-J027 required a low level of phosphorylation, as produced by submaximal forskolin in whole-cell recordings and ATP/catalytic subunit of protein kinase A in inside-out patches, and by a low concentration of forskolin in enteroid swelling measurements. Basal CFTR phosphorylation is required as well for other CFTR activators, including the clinically approved compound VX-770, a potentiator of some mutant CFTRs causing cystic fibrosis.[23] The current-voltage relationship in cells stimulated with $CFTR_{act}$-J027 was linear, as expected for CFTR-mediated function. CFTR activation by $CFTR_{act}$-J027 in inside-out patches indicates a direct activation mechanism, likely at a site on the cytoplasmic domain of CFTR. Further studies are needed to define the precise binding site of $CFTR_{act}$-J027 on CFTR, which are likely to be quite challenging because of the large size of CFTR and its complex gating mechanism. Indeed, the sites of action of clinically approved potentiators and correctors of mutant CFTRs causing cystic fibrosis are not known.

We found greater effect of $CFTR_{act}$-J027 compared with lubiprostone or linaclotide in stimulating fluid secretion in mouse intestine, which translated to greater efficacy in increasing stool output and hydration in a scopolamine model of constipation. The greater efficacy of $CFTR_{act}$-J027 may be due to greater intestinal secretion. Lubiprostone and linaclotide are thought to act largely by inducing intestinal fluid secretion, though by different mechanisms. These compounds activate intestinal secretory pathways indirectly by increasing cellular cyclic nucleotide levels through their actions on prostanoid and guanylate cyclase C receptors, respectively.[3,5] The nonselective activation of cyclic nucleotide pathways may be responsible in part for the side effects of these drugs. Compared with lubiprostone and linaclotide, direct-acting CFTR activators have a defined mechanism of action targeting a single prosecretory ion transporter, with less likelihood of side effects due to absence of global cyclic nucleotide elevation.[24] Also, targeting CFTR directly rather than upstream receptor or signaling pathways is less likely to induce tachyphylaxis. Whether the greater therapeutic action of $CFTR_{act}$-J027 compared with lubiprostone or linaclotide seen here in mice will translate to humans must await clinical trial data.

The preclinical results here support the testing of $CFTR_{act}$-J027 or alternative CFTR-targeted activators in constipation in humans. We previously reported an $ED_{50}$ of 0.5 mg/kg for orally administered $CFTR_{act}$-J027 in a loperamide model in mice, which translates to a dose of 35 mg for a 70 kg human. The duration of effect for $CFTR_{act}$-J027 is at least 3–4 hours in mice, suggesting that a once-a-day administration may be adequate for treatment of constipation in humans. Pharmacology studies in mice[12] and the rapid metabolism found here with human hepatic microsomes ($t_{1/2}$ 35 min) predict minimal systemic exposure following oral administration of $CFTR_{act}$-J027 because of rapid hepatic metabolism probably by a first-pass mechanism, though formal pharmacokinetics measurements will be needed in humans. The minimal systemic exposure is expected to limit the extraintestinal

off-target effects of $CFTR_{act}$-J027, as evidenced by our prior toxicity studies in mice showing no effect of chronic oral high-dose $CFTR_{act}$-J027 on blood counts, serum chemistries, and lung water content.[12] A potential side effect of any laxative is diarrhea, which might occur with $CFTR_{act}$-J027 in a dose-dependent manner and require appropriate dose adjustment.

In conclusion, our results show that a CFTR-targeted small molecule increases intestinal fluid secretion and is efficacious orally in mouse models of acute and chronic constipation. $CFTR_{act}$-J027 induced enterocyte fluid secretion in human enteroids and showed rapid metabolism in human hepatic microsomes, supporting its utility for human constipation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

| | |
|---|---|
| **CFTR** | cystic fibrosis transmembrane conductance regulator |
| **CIC** | chronic idiopathic constipation |
| **OIC** | opioid-induced constipation |
| **IBS-C** | constipation-predominant irritable bowel syndrome |
| **ppm** | parts per million |
| **PBS** | phosphate-buffered saline |
| **LC/MS** | liquid chromatography mass spectrometry |
| **Ednrb** | endothelin receptor B |

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## AT A GLANCE COMMENTARY

**Cil O, et al**

### Background

Constipation is a common problem. Despite 2 FDA-approved drugs that promote intestinal fluid secretion, lubiprostone and linacotide, there remains an unmet need for drugs with better efficacy and side-effect profile. The cystic fibrosis transmembrane conductance regulator (CFTR) is a major prosecretory intestinal chloride channel, and hence a compelling target to promote stool hydration in constipation.

### Translational Significance

Our results support the application of a phenylquinoxalinone CFTR activator as first-in-class therapy of opioid and nonopioid constipation. The compound activates CFTR by direct binding, stimulates intestinal fluid secretion, and, when administered orally, normalizes stool output and hydration in experimental animal models of constipation.

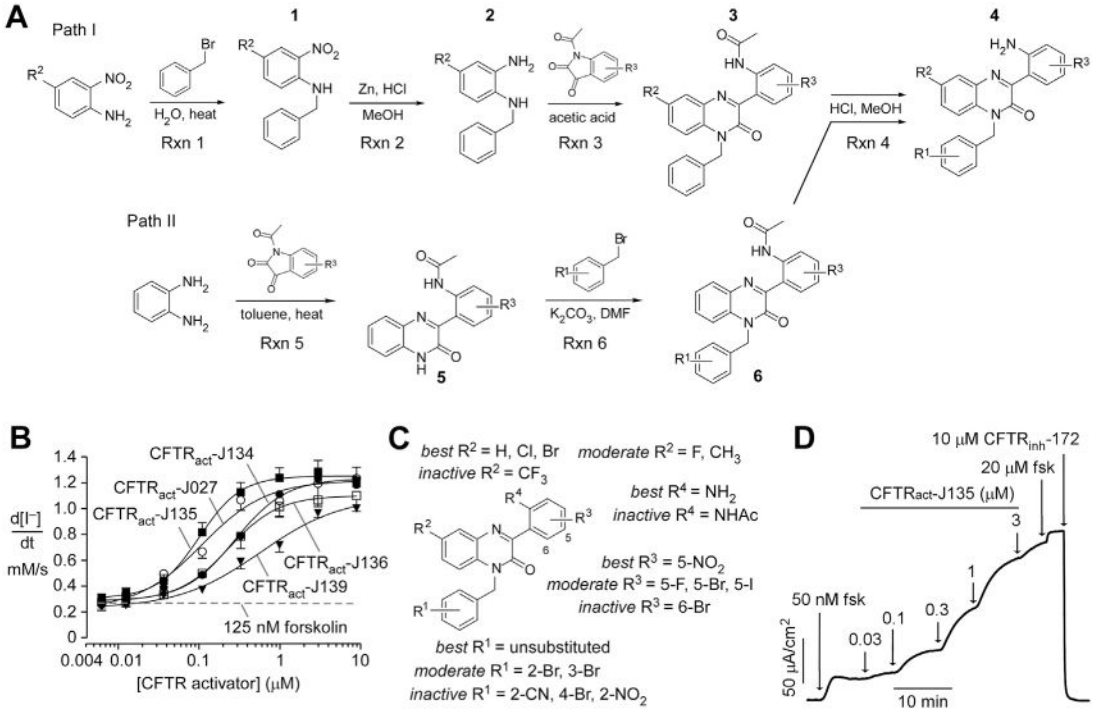



**Fig 1.**

Synthesis and structure-activity analysis of phenylquinoxalinone CFTR activators. **A**, General synthetic scheme. **B**, Concentration-dependent activation of CFTR by selected phenylquinoxalinones in FRT cells expressing wild-type CFTR (mean ± S.E.M., n =3). Dashed line indicates response to 125 nM forskolin. **C**, Structural determinants of phenylquinoxalinone activation of wild-type CFTR. **D**, Short-circuit current measurement in FRT cells expressing wild-type CFTR showing responses to indicated concentrations of forskolin (fsk), $CFTR_{act}$-J135, and $CFTR_{inh}$-172 (representative of 3 experiments).

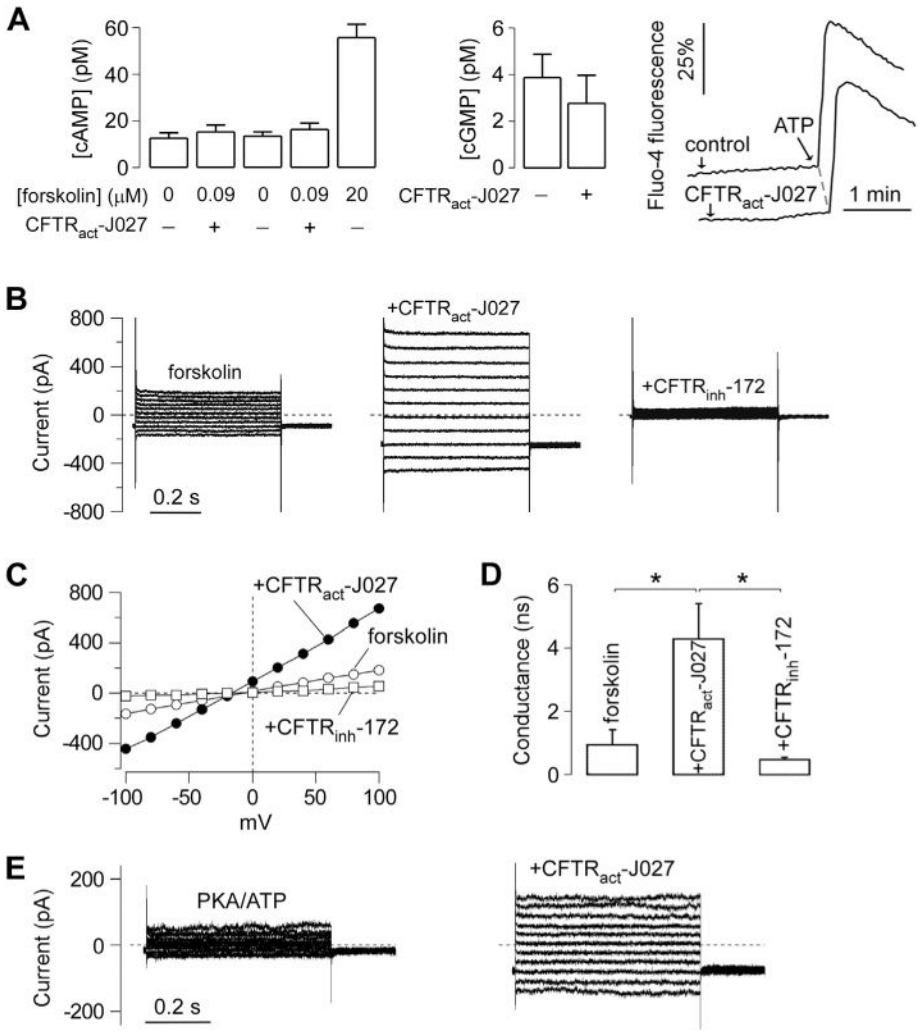



**Fig 2.**

Patch-clamp analysis of CFTR activation by $CFTR_{act}$-J027. **A**, $CFTR_{act}$-J027 does not cause cAMP, cGMP, or $Ca^{2+}$ signaling. Studies done as described in Methods with 10 $\mu$M $CFTR_{act}$-J027. *Left*: cAMP content in FRT cells at 10 minutes after the addition of $CFTR_{act}$-J027 without and with 90 nM forskolin. *Center*: cGMP content in FRT cells at 10 minutes after the addition of $CFTR_{act}$-J027. *Right*: cytoplasmic calcium measured by Fluo-4 fluorescence. Cells were treated for 2 minutes with 10 $\mu$M $CFTR_{act}$-J027 or vehicle control, before 100 $\mu$M ATP added as a calcium agonist. **B**, Representative whole-cell patch-clamp in FRT cells expressing human wild-type CFTR. Each panel shows superimposed membrane currents elicited at voltages between −100 and +100 mV (with 20 mV steps). Cells were exposed to a submaximal concentration of forskolin (fsk, 150 nM) with and then to $CFTR_{act}$-J027 (1 $\mu$M) followed by $CFTR_{inh}$-172 (10 $\mu$M). **C**, Current-voltage relationships from the experiment in **B**. **D**, Membrane conductance deduced from experiments as in **B** (mean =S.E.M., 4 experiments). *$P < 0.05$. **E**, Currents measured in inside-out patch-clamp experiment. CFTR was activated by submaximal ATP and catalytic subunit of protein kinase A (PKA), followed by $CFTR_{act}$-J027 (1 $\mu$M). The voltage stimulation protocol was the same used for whole-cell experiments. Data representative of 3 sets of experiments.

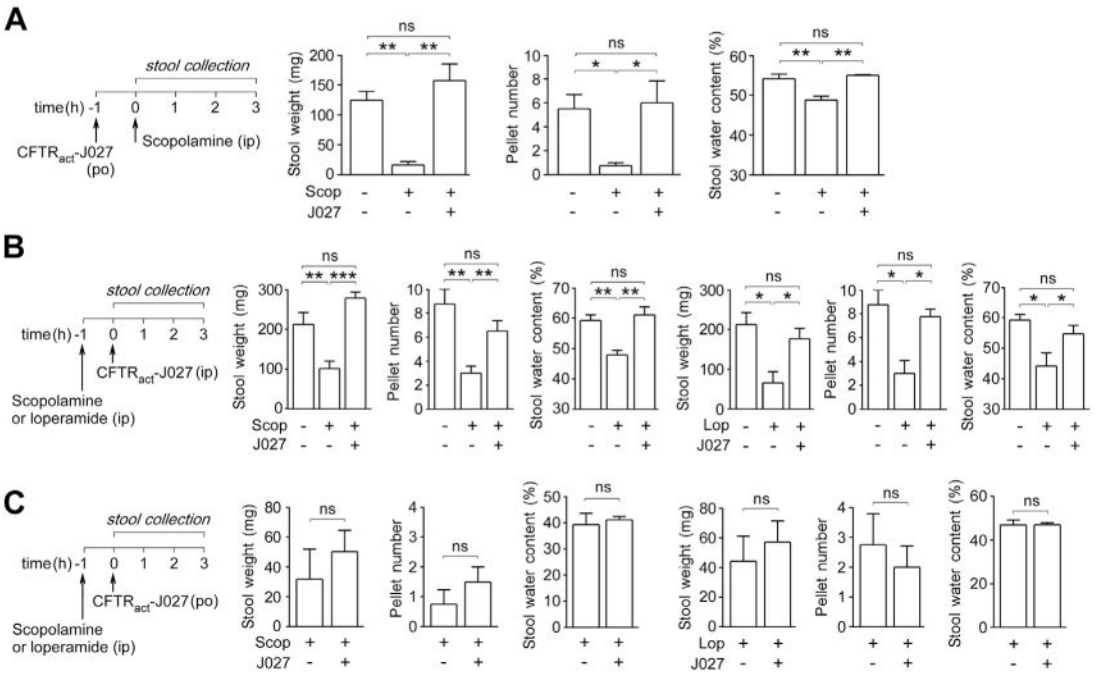



**Fig 3.**

CFTR$_{act}$-J027 efficacy in mouse models of acute constipation. **A**, Experimental protocol (left) and 3-hour stool weight, pellet number, and water content in mice treated with CFTR$_{act}$-J027 (10 mg/kg, po) or vehicle 1 hour before scopolamine (0.5 mg/kg, ip) (mean ± S.E.M., 4 mice per group). **B**, Experimental protocol (left) and 3-hour stool weight, pellet number, and water content in mice treated with CFTR$_{act}$-J027 (10 mg/kg, ip) or vehicle 1 hour after scopolamine (0.5 mg/kg, ip) or loperamide (0.3 mg/kg, ip) (mean ± S.E.M., 4 mice per group). **C**, Experimental protocol (left) and 3-hour stool weight, pellet number, and water content in mice treated with CFTR$_{act}$-J027 (10 mg/kg, po) or vehicle 1 hour after scopolamine (0.5 mg/kg, ip) or loperamide (0.3 mg/kg, ip) (mean ± S.E.M., 4 mice per group). One-way analysis of variance was used for **A** and **B**, Student's t-test was used for **C**, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ns: not significant.

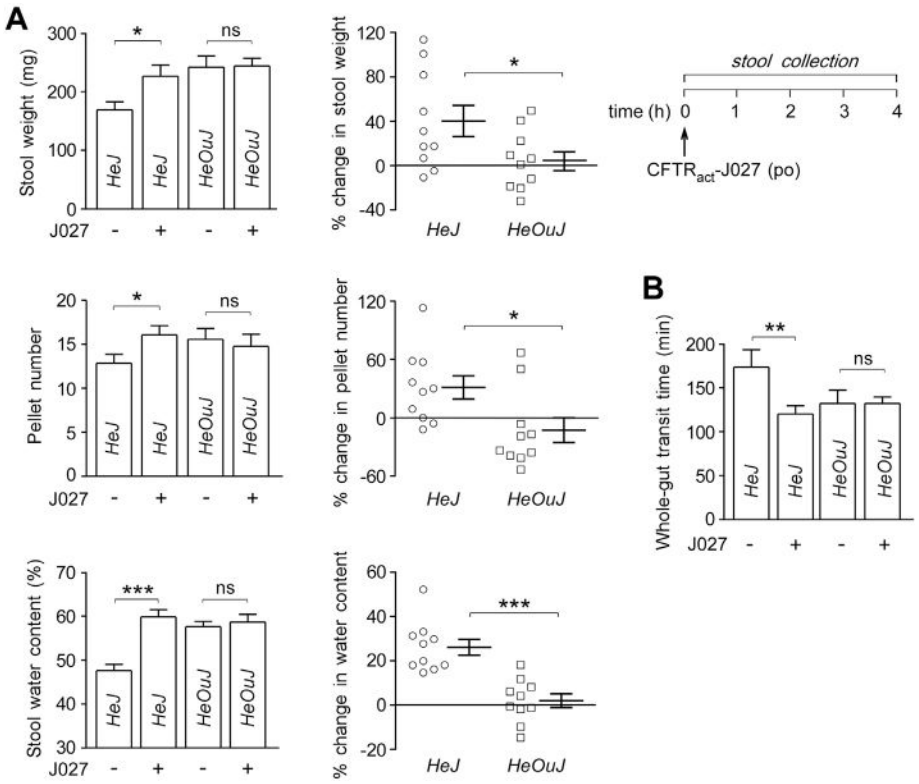



**Fig 4.**

$CFTR_{act}$-J027 reverses constipation in chronically constipated C3H/HeJ mice. **A**, Four-hour stool weight, pellet number, and water content (left) and percent change in these parameters after $CFTR_{act}$-J027 (10 mg/kg, po) or vehicle treatment (center) in C3H/HeJ and C3H/HeOuJ mice (mean ± S.E.M., 10 mice per group). Experimental protocol is on top right. **B**, Whole-gut transit time in C3H/HeJ and C3H/HeOuJ mice treated with $CFTR_{act}$-J027 (10 mg/kg, ip) or vehicle at zero time (mean ± S.E.M., 5 mice per group). Student's t-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ns: not significant. All experiments were done in paired animals.

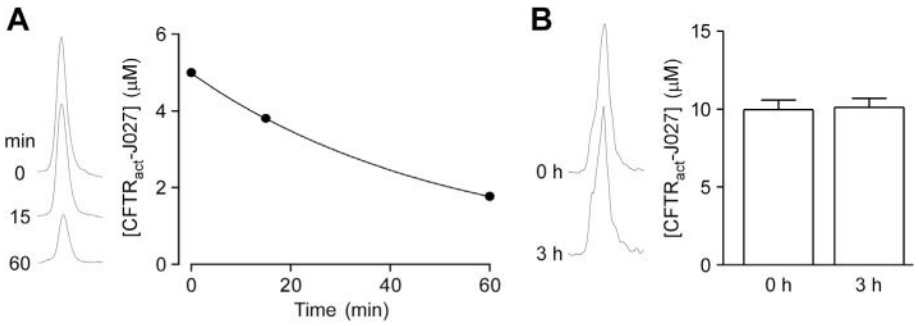



**Fig 5.**

$CFTR_{act}$-J027 is rapidly metabolized by human liver microsomes and is gastric acid-stable. **A**, In vitro metabolic stability of $CFTR_{act}$-J027 assayed in human liver microsomes in the presence of NADPH after incubation for specified times, with representative chromatograms on the left. **B**, Stability of $CFTR_{act}$-J027 in simulated gastric fluid (pH 2) after 0 or 3 hour incubations, with representative chromatograms on the left (mean ± S.E.M., n =3).

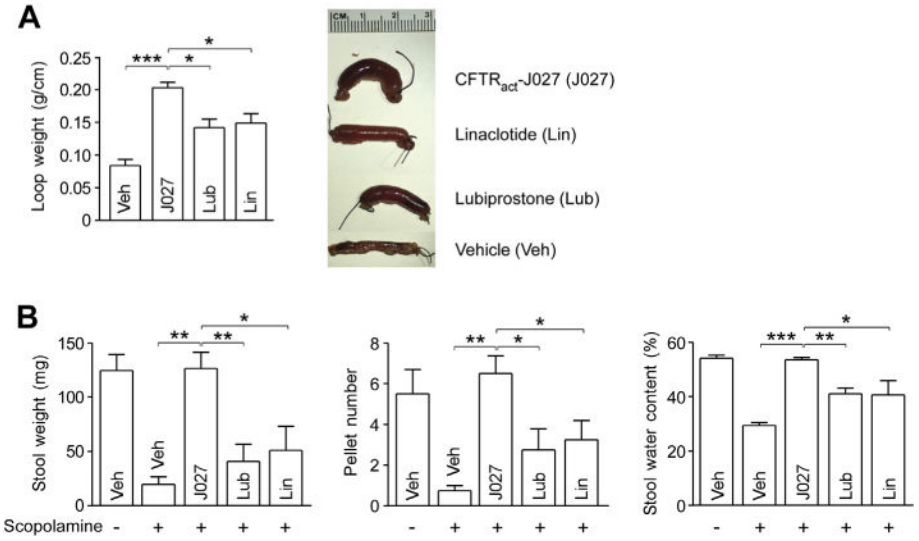



**Fig 6.**

Relative efficacy of $CFTR_{act}$-J027 with lubiprostone and linaclotide in increasing intestinal fluid secretion and stool output. **A**, Intestinal fluid secretion was measured in closed midjejunal loops in mice. Loops were injected with 100 $\mu$L vehicle or 100 mg $CFTR_{act}$-J027, lubiprostone or linaclotide. Loop weight/length was measured at 90 minutes (mean ± S.E.M., 4–8 loops per group, representative photos on right). **B**, Three-hour stool weight, pellet number, and water content in mice orally treated with $CFTR_{act}$-J027 (10 mg/kg), lubiprostone (0.5 mg/kg), linaclotide (0.5 mg/kg), or vehicle in a scopolamine model of constipation as done in Fig 3, A (mean ± S.E.M., 4 mice per group). One-way analysis of variance, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

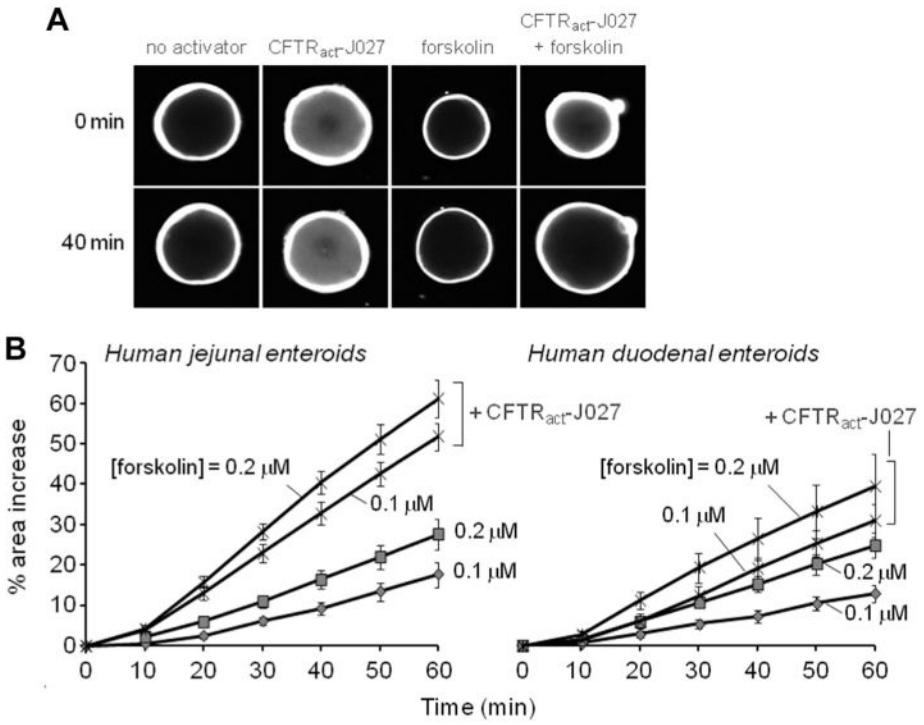
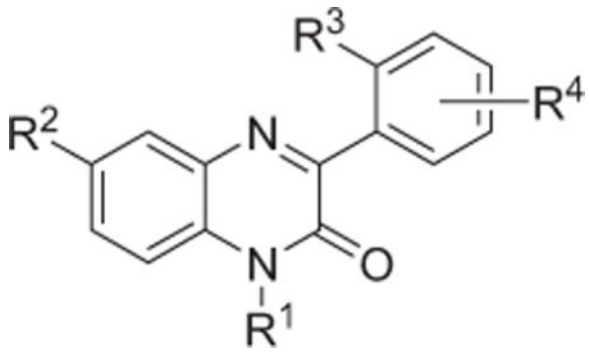



**Fig 7.**

$CFTR_{act}$-J027 produces swelling in enteroids generated from human small intestine. **A**, Representative confocal fluorescence micrographs of calcein-stained human jejunal enteroids before (0 min) and at 40 minutes after exposure to $CFTR_{act}$-J027 (5 $\mu$M) and forskolin (0.3 $\mu$M), added individually or together. **B**, Summary of percentage increase in enteroid area, as deduced by confocal fluorescence microscopy of calcein-stained enteroids, relative to initial area. Data shown for enteroids from human jejunum (left) and duodenum (right). Mean ± S.E.M., n ≥ 10 enteroids for each condition.

**Table I**

CFTR activation by phenylquinoxalinone analogs




| Compound | $R^1$ | $R^1$ | $R^3$ | $R^4$ | $EC_{50}$ ($\mu$M) |
|---|---|---|---|---|---|
| $CFTR_{act}$-J027 | Bn | H | $NH_2$ | 5-$NO_2$ | 0.2 |
| $CFTR_{act}$-J051 | H | H | $NH_2$ | 5-$NO_2$ | >25 |
| $CFTR_{act}$-J052 | $CH_3$ | H | $NH_2$ | 5-$NO_2$ | ~10 |
| $CFTR_{act}$-J053 | $CH_3$ | H | $NH_2$ | H | >25 |
| $CFTR_{act}$-J054 | $CH_3$ | $NO_2$ | $NH_2$ | 5-$NO_2$ | ~10 |
| $CFTR_{act}$-J055 | $CH_3$ | $NO_2$ | NHMe | 5-$NO_2$ | >25 |
| $CFTR_{act}$-J056 | Bn | H | NHBn | 5-Br | ~15 |
| CFTR-J057 act | Bn | H | NHAc | H | ~15 |
| CFTR-J058 act | phenylacetyl | H | NHAc | H | ~20 |
| CFTR-J059 act | 3-BrBn | H | NHAc | H | ~19 |
| $CFTR_{act}$-J060 | Bn | H | NHAc | 5-$CH_3$ | >25 |
| $CFTR_{act}$-J061 | phenethyl | H | NHAc | H | ~20 |
| $CFTR_{act}$-J062 | *n*-propyl | H | H | 5-NHAc | >25 |
| $CFTR_{act}$-J102 | 4-BrBn | H | $NH_2$ | 5-$NO_2$ | >25 |
| $CFTR_{act}$-J103 | 3-BrBn | H | $NH_2$ | 5-$NO_2$ | 1.2 |
| $CFTR_{act}$-J104 | 2-BrBn | H | $NH_2$ | 5-$NO_2$ | 7.3 |
| $CFTR_{act}$-J109 | Bn | H | NHAc | 5-$NO_2$ | 1.5 |

| Compound | $R^1$ | $R^1$ | $R^3$ | $R^4$ | $EC_{50}$ ($\mu M$) |
|---|---|---|---|---|---|
| $CFTR_{act}$-J105 | Bn | F | $NH_2$ | 5-$NO_2$ | 0.53 |
| $CFTR_{act}$-J135 | Bn | Cl | $NH_2$ | 5-$NO_2$ | 0.11 |
| $CFTR_{act}$-J136 | Bn | Br | $NH_2$ | 5-$NO_2$ | 0.26 |
| $CFTR_{act}$-J133 | Bn | H | $NH_2$ | 5-F | 0.79 |
| $CFTR_{act}$-J134 | Bn | H | $NH_2$ | 5-Br | 0.31 |
| $CFTR_{act}$-J140 | Bn | $CH_3$ | $NH_2$ | 5-F | 0.70 |
| $CFTR_{act}$-J141 | Bn | $CH_3$ | $NH_2$ | 5-I | 0.47 |
| $CFTR_{act}$-J143 | 2-$NO_2$Bn | H | $NH_2$ | 5-I | >25 |
| $CFTR_{act}$-J144 | 2-CNBn | H | $NH_2$ | 5-I | >25 |
| $CFTR_{act}$-J142 | Bn | H | $NH_2$ | 6-Br | 1.5 |
| $CFTR_{act}$-J139 | Bn | $CH_3$ | $NH_2$ | 6-Br | 0.65 |

Approximate $EC_{50}$ of commercial analogs ($CFTR_{act}$-J051–J062) deduced from two-concentration point analysis. $EC_{50}$ of synthesized compounds measured from full dose-response study.