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Brief Article

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Functional Characterization of Cholera Toxin Inhibitors Using Human Intestinal Organoids

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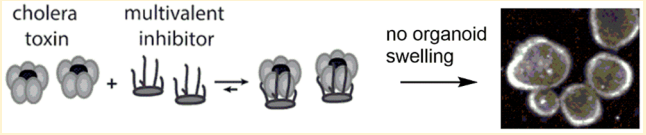
Supporting Information

ABSTRACT: Preclinical drug testing in primary human cell models that recapitulate disease can significantly reduce animal experimentation and time-to-the-clinic. We used intestinal organoids to quantitatively study the potency of multivalent cholera toxin inhibitors. The method enabled the determination of IC_{50} values over a wide range of potencies (15 pM to 9 mM). The results indicate for the first time that an organoid-based swelling assay is a useful preclinical method to evaluate inhibitor potencies of drugs that target pathogen-derived toxins.



INTRODUCTION

Cholera toxin induces secretory diarrhea, resulting in severe dehydration[1,2] and affecting 1.4−4.3 million individuals worldwide, of which 28000−143000 die each year.[3] Current protection consists of two vaccines, Shanchol and Dukoral, which are widely used but not very promising for children under six years of age, the most vulnerable group.[4] Inhibitory potency of bioactive compounds against cholera is typically evaluated in the rabbit ileal loop assay. However, this method is excessively stressful for the animals, time-consuming, and difficult to standardize.[5]

We recently described a simple and robust fluid secretion assay in a model of primary human intestinal organoids.[6,7] Chemical induction of cAMP induces a rapid accumulation of fluid in the central lumen, and the resulting organoid swelling can be simply quantified. Using unmodified cells, this assay offers a drug testing platform for disease conditions involving fluid secretion, such as cystic fibrosis[6] or secretory diarrhea. For the latter condition, this remains to be demonstrated.

Here we use the organoid swelling assay to characterize pharmacological inhibitors of cholera toxin. Cholera toxin consists of the core region part A and the outer pentameric part B that binds to the pentasaccharide part of GM1-gangliosides. The binding is required for cell internalization, where cholera toxin A_1 subunit is released into the cytosol (Figure 1).[1,2] A_1 triggers cAMP-dependent intestinal fluid hypersecretion via ion channels and transporters. The main channel is the cystic fibrosis transmembrane conductance regulator (CFTR), which secretes chloride and bicarbonate.[1,2,8] Hypersecretion is followed by paracellular hypersecretion of sodium and water.[1]

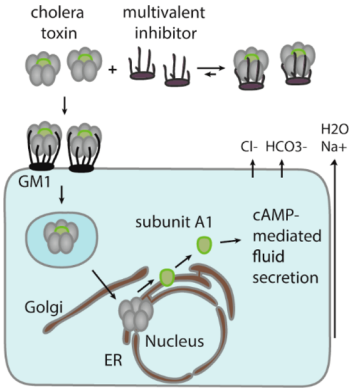


Figure 1. Proposed mechanism and effect of inhibitors on the pathway of cholera toxin.

Numerous inhibitors for cholera toxin have been developed over the years. Many of these are monovalent inhibitors derived from structure-based design,[9] however, high potency inhibitors so far were all based on multivalent designs[10−14] which contain several copies of the ligand attached to a single molecular scaffold such as a dendrimer.[15] Such inhibitors likely bridge several of the binding sites, which greatly enhances the potency, possibly in combination with aggregation mechanisms.[16] Cholera toxin inhibitors that are based on GM1os have shown the highest inhibitory potency so far.[10,15,17,18] Recently, pentavalent versus tetravalent GM1os-based inhibitors were synthesized, compared, and shown to be of similar

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subnanomolar potency in an ELISA assay, and they exhibited similar aggregation behavior of the toxin.[19]

Most studies have reported the potency of the compounds using the same ELISA assay involving immobilized GM1 and HRP-conjugated CTB_5. While this assay works well and is reproducible, a more biorelevant assay is needed. The mentioned rabbit ileal loop assay does function,[20] but practical and ethical issues make it less suitable. We hypothesized that the swelling assay using human intestinal organoids could functionally assess the potency of cholera toxin inhibitors, thereby providing an additive method to current animal models. We used a series of inhibitors with a large range of potencies to also evaluate the dynamic range of the assay.

RESULTS AND DISCUSSION

For comparison with the organoid assay, ELISA data were used (Table 1). We used the data from the recently reported

Table 1. Inhibitory Potency of Cholera Toxin Inhibitors as IC_{50} (nM)

compd	ELISA assay[a]	organoid assay[b]
1	0.160 (ref 19)	0.034
2	0.260 (ref 19)	0.015
3	110	424
4	8	18
5 (galactose)	2.4×10^8 (ref 24)	9.1×10^6

[a]Inhibition of CTB_5-HRP (40 ng/mL) binding to GM1 coated plates. [b]Inhibition of the swelling of intestinal organoids due to the action of cholera toxin (10 ng/mL).

tetravalent and pentavalent GM1os compounds **1** and **2** (Figure 2),[19] which are potent inhibitors in the picomolar range. Subsequently, the hydrophilic monovalent GM1os derivative **3** was used, which showed an IC_{50} of 110 nM. It is relevant to mention the word hydrophilic GM1 derivative. This derivative does not aggregate and behaves like a single molecule. This in contrast to GM1 derivatives with lipophilic tails that form aggregates and can vary widely in their ability to inhibit cholera toxin. To mention two examples, GM1 with two lipophilic tails (i.e., native GM1 ganglioside), which forms large micelles,[21] inhibits in the low nanomolar range (IC_{50} 2.5 nM measured by us using the ELISA, and similar values are obtained by others[22]). GM1 with one lipohilic tail leads to relatively poor micromolar inhibition through an apparent counterproductive aggregation. It seems the polar derivative **3** is in between the two.[15,17] Next, the nonspanning[23] GM1os dimer **4** with a short spacer was included in the panel. Its IC_{50} of 8 nM was ca. 1 order of magnitude better than the monovalent **3**, which may be due to statistical rebinding effects, secondary binding effects of the second ligand, or even the bridging of pentamers. Finally, free galactose (**5**) was included as a weak millimolar inhibitor.

To develop the organoid assay, intestinal organoids were stimulated dose dependently with cholera toxin to select a nonsaturating concentration for inhibitor testing while retaining maximal assay sensitivity (Figure 3). Stimulating organoids for 4 h with 10 ng/mL cholera toxin induced similar levels of swelling as saturating doses but with suboptimal kinetics. A lag phase of approximately 60−100 min was observed before cholera toxin induced swelling, consistent with the uptake and processing of the toxin (Figure 3). For comparison, amounts of

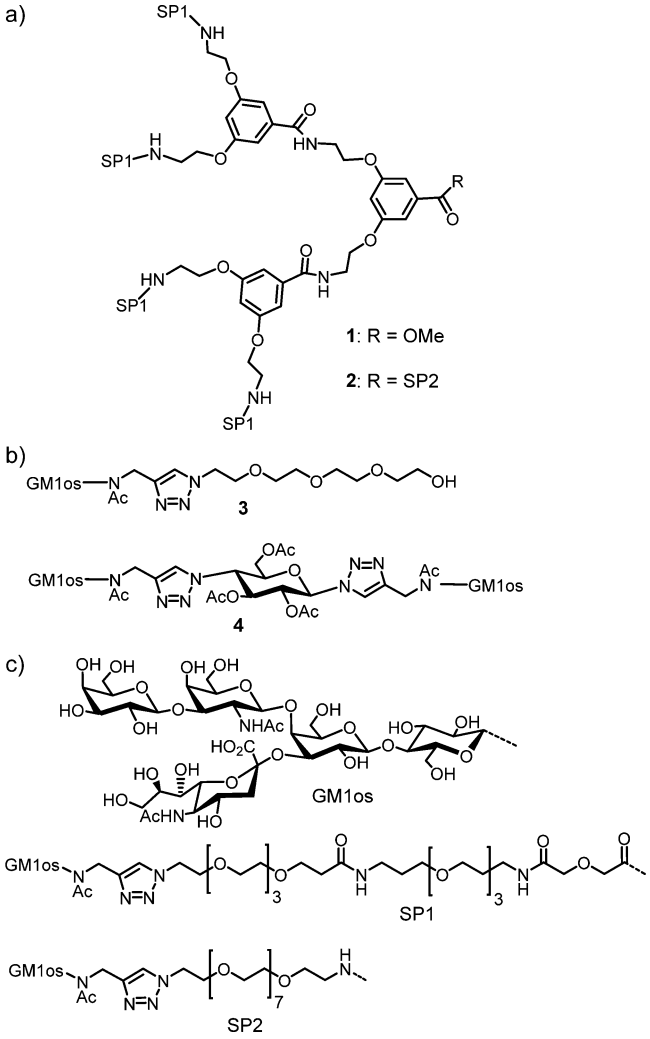

Figure 2. Structures of (multivalent) cholera toxin inhibitors. (a) Structures of cholera toxin inhibitors **1** and **2**. (b) Structures of inhibitors **3**−**4**. (c) Additional structure elements GM1os, SP1, and SP2.

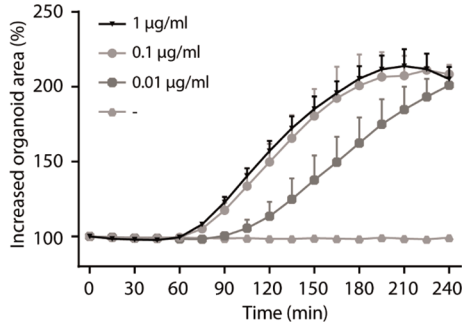

Figure 3. Organoids were stimulated with cholera toxin as indicated (μg/mL), and organoid swelling was measured by relative area increase in time (t = 0 min: 100%), n = 1 triplicate ± SD.

cholera toxin detected in diarrhea has been detected as high as 1 μg/mL.[25]

We next assessed dose-dependent inhibition of cholera toxin-mediated swelling of the four GM1os-based structures that differ in valency for binding cholera toxin B subunit (Figure 2). Free galactose (**5**) was again measured as a weak reference inhibitor. Organoids were stimulated with cholera toxin, with or

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without inhibitors. We found that tetravalent and pentavalent GM1os compounds **1** and **2** were most potent in inhibiting cholera toxin-induced swelling, with IC_{50} values in the picomolar range (IC_{50} of 34 and 15 pM, respectively) (Figure 4a, Table 1). Monovalent and bivalent GM1os compounds **3** and **4** were less potent (IC_{50} of 424 and 18 nM, respectively) but still effective. The monovalent compound **3** cannot take advantage of bridging binding sites within a toxin, while the bivalency of **4** seems to help here by a factor of ~20-fold. Monovalent galactose was the least potent (IC_{50} 9.1 mM), as expected.[10] Also, for the highest dosages of inhibitors tested, organoids were additionally stimulated with forskolin, an adenyl cyclase activator, to control for toxicity of the inhibitors on the CFTR channel. Forskolin-stimulated organoid swelling was observed for all the conditions tested, indicating that the cholera toxin inhibitors do not inhibit CFTR chloride secretion by acting on CFTR directly (Figure 4b). Representative images are depicted in Figure 5.

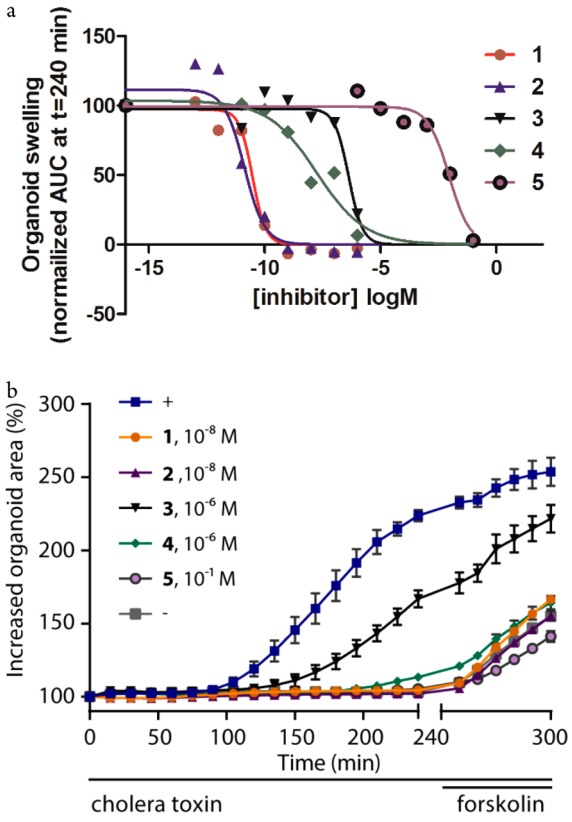

Figure 4. Inhibitory potency of cholera toxin inhibitors indicated by intestinal organoid swelling. (a) Dose-dependent inhibition of cholera toxin-induced swelling, monitored for 4 h. Data represent three independent experiments (conditions in triplicate). (b) Organoid swelling curves after stimulation with cholera toxin (0.01 μg/mL), preincubated with the most effective dose of cholera toxin inhibitors (concentrations as indicated) and subsequent stimulation with 0.1 μM forskolin, n = 1 triplicate ± SD.

The data indicate for the first time that multivalent GM1os-based compounds are highly effective in limiting cholera toxin-induced fluid secretion in human intestinal primary cells (Figure 4a). The organoid assay exhibited a very large dynamic range by clearly showing inhibition from the pico- to the millimolar range. The values correlated well with those obtained from the GM1-based ELISA type assay. Nevertheless,

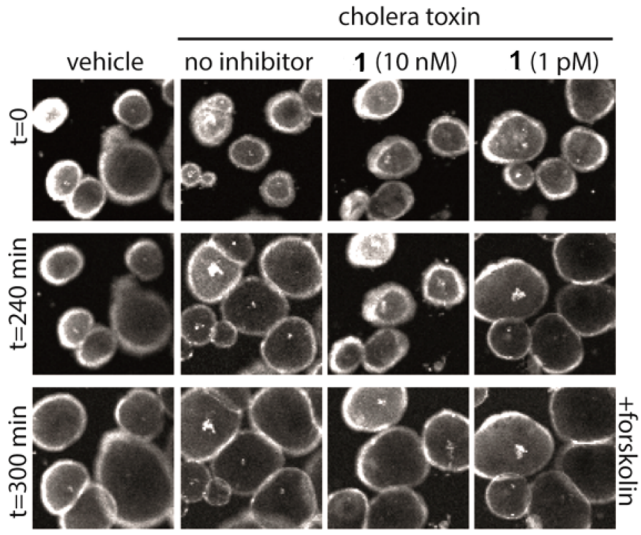

Figure 5. Representative images of organoids upon cholera toxin stimulation, with or without inhibitor **1**, conditions and time points as indicated.

the best inhibitors **1** and **2** showed even lower values with the organoids. This can be explained by the fact that the organoid assay requires less cholera toxin (i.e., 10 ng/mL) to give a clearly observable phenomenon than the ELISA assay. Because we are talking IC_{50} values, 50% of the present CT molecules represents ca. 58 pM, a value close to the observed IC_{50} values for **1** and **2**. The inhibition seems to be almost one to one in terms of stoichiometry, irrespective of whether at these concentrations aggregation phenomena[16] play a role, which is remarkable.

The organoid assay now makes it possible to do a biorelevant assay while avoiding animal experiments using rabbit intestines and while quantitatively showing the functional impact of inhibitors with large differences in potency (Figure 4a). However, with the use of organoids, the toxins are likely to bind basolateral to the epithelial cells instead of luminal, which could have resulted in an overestimation of the potencies. Furthermore, we did not take into account the adhesion of the bacterium itself.

The data suggest that the cholera toxin inhibitors could be used as prophylactic or acute drugs to inhibit cholera toxins to bind to cells. When the toxins are already bound to epithelial cells and internalized, other approaches are needed such as reducing Cl^- hypersecretion or stimulating Na^+ absorption via cyclase inhibitors or NHE3 activators, respectively.[8,26]

CONCLUSION

The human primary organoid culture model provides a simple, robust, and functional intestinal assay for compound inhibitory potency testing. The total organoid swelling easily quantitates fluid transport across the intestinal epithelium, mimicking the in vivo tissue. This assay platform will be important for preclinical development of drugs targeting pathogen-induced secretory diarrhea as shown here for compounds targeting cholera toxin.

EXPERIMENTAL SECTION

Human Rectal Biopsies. Rectal biopsies were collected from a human subject after informed consent and approved by the local Ethics Committee.

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Generating and Culturing Organoids. Organoids were generated, and biobanked, slightly differently as previously described.[6] After washing with PBS, crypts were isolated from the biopsies, via incubation in 10 mM EDTA for 60−90 min at 4 °C at a rocking platform. Crypts were collected, centrifuged, and supernatant removed. Crypts pellet was taken up in 40% Matrigel (Corning, diluted in culture medium), and droplets of crypts suspension were plated onto 24-well plates. After solidification of the Matrigel (±10 min, 37 °C), droplets were immersed in culture medium (advanced DMEM/F12 supplemented with HEPES, GlutaMAX, penicillin, streptomycin, N-2, B-27, mEGF (Life Technologies), *N*-acetylcysteine, nicotinamide, SB202190 (Sigma), A83-01 (Tocris), and 50% Wnt3a-, 20% Rspo-1-, and 10% Noggin-conditioned media). In 7 days, crypts grew out into full grown organoids, which were passaged via mechanical disruption weekly. Medium was refreshed every 2−3 days, and organoids were passaged at least two times before assays were performed.

Cholera Toxin and Inhibitors. Cholera toxin from *Vibrio cholerae* (Sigma) was used to stimulate organoid fluid secretion. Cholera toxin inhibitors were preincubated for 4 h with cholera toxin prior to organoid stimulation. The (multivalent) structures of the inhibitors are based on GM1os, and monovalent free galactose serves as a reference compound.[19]

Monovalent Inhibitor 3. A solution of GM1os-β-NAc-propargyl (Elicityl, Grenoble) (8.3 mg, 7.7 μmol), 11-azido-3,6,9-trioxaundecanol (3.4 mg, 15.5 μmol), $CuSO_4{\cdot}5H_2O$ (1.9 mg, 7.7 μmol), and sodium ascorbate (3.1 mg, 15.5 μmol) in H_2O/DMF (1/1, v/v, 2 mL) was heated under microwave irradiation at 80 °C for 20 min. The reaction mixture was treated with ion-exchange resin Cuprisorb, concentrated, and subjected to preparative HPLC purification (gradient of 5% MeCN and 0.1% TFA in H_2O to 5% H_2O and 0.1% TFA in MeCN). The product was obtained after lyophilization as a colorless glass (4 mg, 40%). Purity >95% determined by HPLC. Selected ^{1}H NMR (500 MHz, D_2O) δ: 7.93 (s, 1H, CHtriazole), 5.14 (d, J = 8 Hz, 1H, H-1, Glc), 4.78 (H-1, GalNAc), 4.64 (m, 2H-NCH_2Ctriazole), 4.58 (m, 2H-NtriazoleCH_2), 4.56−4.50 (m, 2H, H-1, Gal(V), H-1, Gal(II)), 4.18−4.09 (m, 3H), 4.03 (m, 1H, H-2, GalNAc), 3.86 (m, 1H, H-9a, NeuAc), 3.37 (m, 1H), 2.65 (m, 1H, H-3eq, NeuAc), 2.25 (s, 3H, Glc-NC(O)CH_3), 2.03 (s, 3H, NHC(O)-CH_3), 2.00 (s, 3H, NHC(O)CH_3), 1.92 (t, 1H, J = 12 Hz, H-3ax, NeuAc). ^{13}C NMR (126 MHz, D_2O) δ (obtained from HSQC spectrum): 125.47 (CHtriazole), 105.37 (C-1, Gal(II)), 103.20 (C-1, Gal(V)), 103.14 (C-1, GalNAc), 87.50 (C-1, Glc), 80.97, 78.62, 77.79, 77.45, 75.52, 75.22, 75.00, 74.98, 74.84, 73.72, 73.17, 72.93, 72.34 (CH_2CH_2OH), 71.31, 70.66, 70.44, 70.23 (OCH_2CH_2O), 69.42 (NCH_2CH_2O), 69.34, 69.24, 68.67, 68.55, 63.46 (C-9, NeuAc), 63.46 (C-9, NeuAc), 61.13 (C-CH_2CH_2OH), 61.60, 60.87, 60.71 (4 × C-6, GalNac, Gal, Glc), 52.26 (C-5, NeuAc), 51.82 (C-2, GalNAc), 50.68 (NtriazoleCH$_2$), 37.60 (C-3, NeuAc), 37.00 (NCH_2Ctriazole), 23.26 (NHC(O)CH_3), 22.73 (NHC(O)CH_3), 21.86 (Glc-NC(O)CH_3). HRMS (Q-TOF) m/z calcd for $[M - H]^-$ 1295.5001, found 1295.4981.

Bivalent Inhibitor 4. A solution of GM1os-β-NAc-propargyl (Elicityl, Grenoble) (8.2 mg, 7.6 μmol), 1,4-diazido-1,4-deoxy-2,3,6-tri-*O*-acetyl glucose[27] (1 mg, 2.8 μmol), $CuSO_4{\cdot}5H_2O$ (1.9 mg, 7.6 μmol), and sodium ascorbate (3 mg, 15.2 μmol) in H_2O/DMF (1/1, v/v, 2 mL) was heated under microwave irradiation at 80 °C for 20 min. The reaction mixture was treated with ion-exchange resin Cuprisorb, concentrated, and subjected to preparative HPLC purification (gradient of 5% MeCN and 0.1% TFA in H_2O to 5% H_2O and 0.1% TFA in MeCN). The product was obtained after lyophilization as a white fluffy compound (3.2 mg, 45%). Purity >95% determined by HPLC. Selected ^{1}H NMR (500 MHz, D_2O) δ: 8.19 (s, 1H, CHtriazole), 8.08 (s, 1H, CHtriazole), 6.34 (d, J = 10 Hz, 1H, H-1, Glc′), 5.95 (m, 1H, H-3, Glc′), 5.72 (m, 1H, H-2, Glc′), 5.26 (m, 1H, H-4, Glc′), 5.14 (m, 2H, H-1, Glc), 4.58−4.51 (m, 4H, H-1, Gal(V), H-1, Gal(II)), 4.04 (m, 2H, H-2, GalNAc), 3.38 (m, 2H), 2.66 (m, 2H, H-3eq, NeuAc), 2.25 (s, 6H, Glc-NC(O)CH_3), 2.06 (s, 3H, OC(O)CH_3), 2.02 (s, 6H, NHC(O)CH_3), 2.01 (s, 6H, NHC(O)-CH_3), 1.94−1.89 (m, 8H, H-3ax, NeuAc, 2 × OC(O)CH_3). ^{13}C NMR (126 MHz, D_2O) δ (obtained from HSQC spectrum): 125.78 (CHtriazole), 124.51 (CHtriazole), 105.37 (C-1, Gal(II)), 103.24 (C-1, Gal(V)), 103.10 (C-1, GalNAc), 87.41 (C-1, Glc), 85.61 (C-1, Glc′), 80.90, 78.65, 77.59, 77.46, 75.52, 75.38, 75.06, 75.02, 74.81, 74.79 (C-5, Glc′), 73.74, 73.15, 72.79 (C-3, Glc′), 72.75, 71.32, 71.31 (C-2, Glc′), 70.63, 70.61, 69.23, 69.17, 68.68, 68.54, 63.50 (C-9, NeuAc), 62.50 (C-6, Glc′), 61.64, 60.94, 60.77 (4 × C-6, GalNac, Gal, Glc), 59.90 (C-4, Glc′), 52.27 (C-5, NeuAc), 51.86 (C-2, GalNAc), 37.71 (C-3, NeuAc), 36.89 (NCH_2Ctriazole), 36.88 (NCH_2Ctriazole), 23.22 (NHC(O)CH_3), 22.74 (NHC(O)CH_3), 21.80 (Glc-NC(O)-CH_3), 20.64, 20.19 (3 × C-OC(O)CH_3). HRMS (Q-TOF) m/z calcd for $[M - 2H]^{2-}$ 1254.4322, found 1254.4335.

Organoid Swelling Assay. Measuring CFTR function in intestinal organoids was performed slightly different as previously described.[6] Organoids cultured for 7 days were mechanically disrupted and reseeded into flat-bottom 96-well plates in 40% Matrigel and culture medium. Plates were incubated overnight at 37 °C, 5% CO_2. The next day, cholera toxin was incubated with cholera toxin inhibitors for 4 h at rt prior to the organoid stimulation. Organoids were stained with calcein AM (Invitrogen), 1 h prior to the stimulation. Organoids were stimulated with the mix of cholera toxin (0.01 μg/mL) and inhibitors (titration) for 4 h and additionally with forskolin (0.1 μM, Sigma) for some conditions for another hour. Swelling of organoids was monitored in time using the Zeiss LSM 710 confocal microscope (images every 15 min during cholera stimulation, every 10 min during forskolin stimulation). Organoid area increase was analyzed using Volocity, and calculations were done with GraphPad Prism 6.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b00770.

Synthetic scheme and NMR spectra of compounds **3** and **4** (PDF)

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Notes

The authors declare the following competing financial interest(s): J.M.B. is inventor on a patent application related to these findings (PCT/IB2012/057497).

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■ ABBREVIATIONS USED

CFTR, cystic fibrosis transmembrane conductance regulator; GM1os, GM1 oligosaccharides

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