Supporting Information

A Small Synthetic Molecule Functions as a Chloride-Bicarbonate Dual-Transporter and Induces Chloride Secretion in Cells

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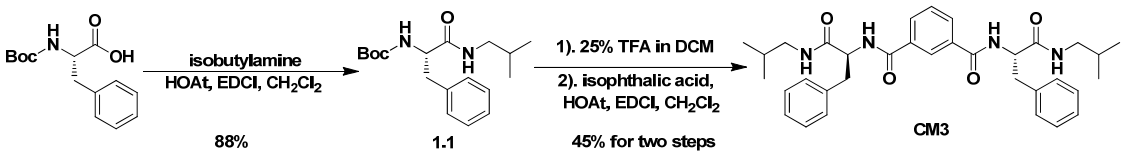
Synthesis of CM3

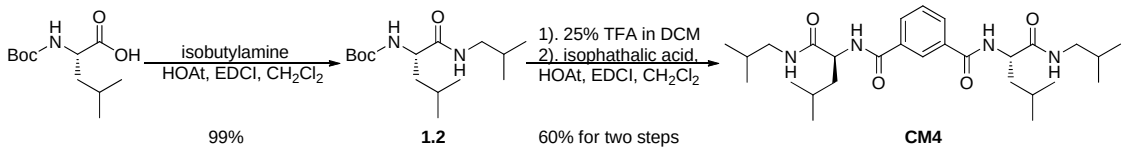


CM3 was prepared according to the above scheme. To a solution of Boc-L-Phenylalanine (2.66 g, 10 mmol) in CH_2Cl_2 (200 mL) were added HOAt (1.5 g, 12 mmol), isobutylamine (78 mg, 12 mmol) and EDCl (2.3g, 12 mmol) sequentially. The mixture was

stirred overnight. The organic solution was washed sequentially with saturated $NaHCO_3$ aqueous solution, 1 M HCl aqueous solution and brine. Then the organic layer was dried over anhydrous Na_2SO_4 and concentrated to afford compound **1.1** as a white solid (2.8 g, 87%).

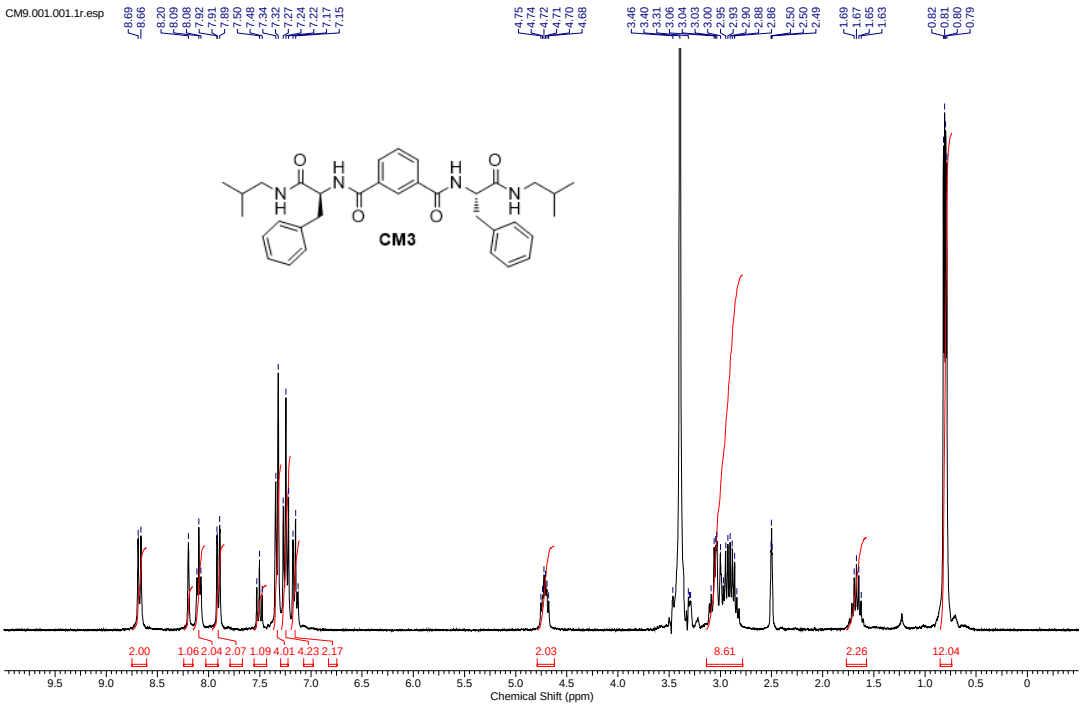
To a solution of **1.1** (2.8 g, 8.8 mmol) in CH_2Cl_2 (100 mL) was added TFA (30 mL). The mixture was stirred at room temperature for an hour. Then the reaction mixture was concentrated in vacuum and azeotroped with toluene 3 times to give the amine, which was used in next step without further purification. The oil collected (793 mg, 3.6 mmol) was suspended in CH_2Cl_2 (40 mL), followed by addition of HOAt (540 mg, 3.9 mmol), isophthalic acid (300 mg, 1.8 mmol), and finally EDCl (830 mg, 4.3 mmol). The mixture was stirred at room temperature overnight. The organic solution was sequentially washed with saturated $NaHCO_3$ solution, 1M HCl aqueous solution, and brine. Then the organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by flash column chromatography using CH_2Cl_2/MeOH to provide **CM3** as a white solid (472 mg, 45%). M.p. 264 – 266°C; $[\alpha]^{20}_D$ –27° (*c* = 0.1, $CHCl_3$); ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ 8.67 (d, *J* = 8.4 Hz, 2 H), 8.20 (s, 1 H), 8.09 (s, 2 H), 7.90 (d, *J* = 7.9, 1.3 Hz, 2 H), 7.50 (s, 1 H), 7.30 - 7.37 (m, 4 H), 7.24 (t, *J* = 7.4 Hz, 4 H), 7.16 (d, *J* = 7.1 Hz, 2 H), 4.64 - 4.79 (m, 2 H), 2.79 - 3.13 (m, 8 H), 1.67 (s, 2 H), 0.80 (dd, *J* = 6.6, 3.1 Hz, 11 H); ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ 171.2, 165.8, 138.3, 134.2, 130.1, 129.2, 128.1, 126.9, 126.3, 55.1, 46.1, 37.6, 28.1, 20.1; IR (KBr) 3294, 3089, 1644 cm^{-1}; LRMS (EI, 20 eV) *m/z* 570 (M^+, 2), 73 (100); HRMS (EI, 20 eV) for $C_{34}H_{42}O_4N_4$ (M^+): calcd 570.3206, found 570.3202.

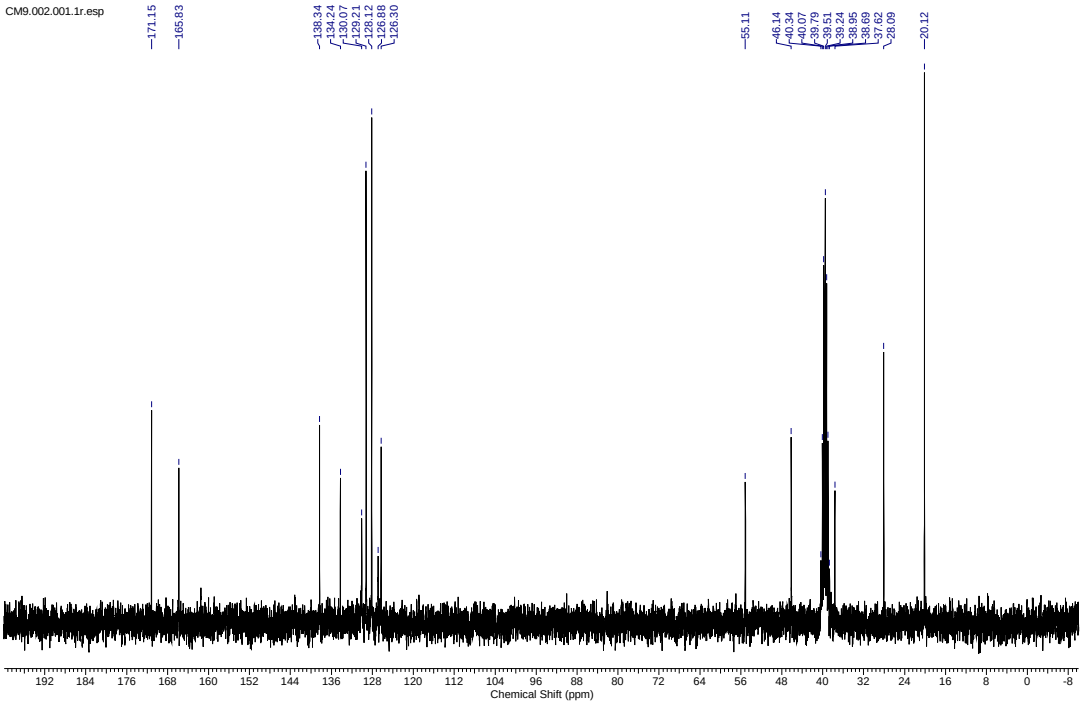
Synthesis of CM4

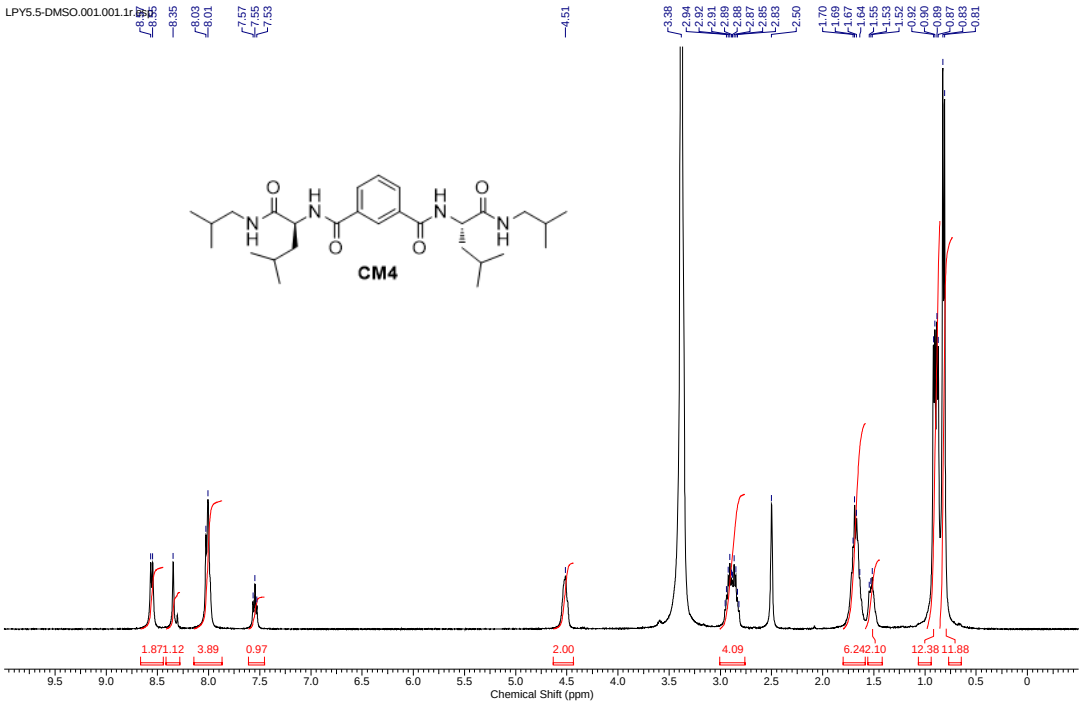


Following the procedure and stoichiometric ratio described in the synthesis of compound **1.1**, compound **1.2** was obtained as a white solid (4.63 g, 99%) from Boc-L-Leucine from (4 g, 16.1 mmol). To a solution of **1.2** (500 mg, 1.75 mmol) in CH_2Cl_2 (5 mL) was added TFA (5 mL) After stirred at for 3 hours, Then the reaction mixture was concentrated in vacuum and azeotroped with toluene 3 times to give the amine, which was used in next step without further purification. The oil collected was suspended in CH_2Cl_2, followed by the addition of HOAt (340 mg, 2.5 mmol), isophthalic acid (138 mg, 0.83 mmol), triethylamine (0.7 mL, 5.0 mmol), and finally EDCI (868 mg, 2.92 mmol). The mixture was stirred at room temperature overnight. The organic solution was sequentially washed with saturated $NaHCO_3$ solution, 1M HCl aqueous solution, and brine. Then the organic layer was dried over anhydrous Na_2SO_4 and concentrated. The crude oil was purified by flash column chromatography to afford **CM4** (280 mg, 60%) as a white solid. M.p. 240−242ºC; $[\alpha]^{20}_D$ –32.1º (*c* = 1.00, $CHCl_3$); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.56 (d, *J* = 7.8 Hz, 2 H), 8.35 (s, 1 H), 8.02 (d, *J* = 7.4 Hz, 4 H), 7.55 (t, *J* = 7.6 Hz, 1 H), 4.42 - 4.65 (m, 2 H), 2.82 – 2.95 (m, 4 H), 1.58 - 1.82 (m, 6 H), 1.44 - 1.58 (m, 2 H), 0.89 (dd, *J* = 13.0, 6.5 Hz, 12 H), 0.82 (d, *J* = 6.5 Hz, 12 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.1, 165.9, 134.3, 130.2, 128.1, 126.8, 52.1, 46.0, 40.8, 28.1, 24.5, 23.0, 21.6, 20.1; IR ($CHCl_3$) 3400, 1653 cm^{-1}; LRMS (EI, 20 eV) m/z 503 (M^+, 1), 329 (100); HRMS (EI, 20 eV) for C28H46N4O4 (M^+): calcd 502.3519, found 502.3486.

NMR spectrum of CM3



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CM4
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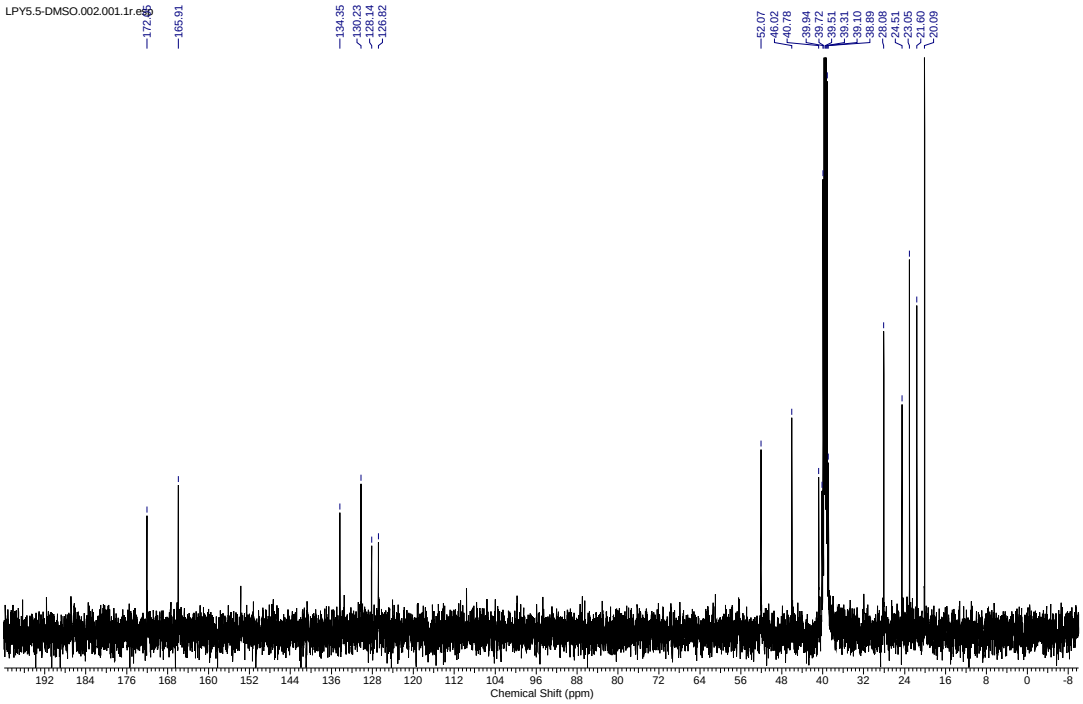
Chemical Shift (ppm)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Chemical Shift (ppm)

Liposome based HPTS base pulse assay

Egg yolk L-*α*-phosphatidylcholine (EYPC, 91 mg, 120 μmol) was dissolved in a $CHCl_3$/MeOH mixture, the solution was evaporated under reduced pressure and the resulting thin film was dried under high vacuum for 3 h. The lipid film was hydrated in 1.2 mL of Buffer A, containing 0.1 mM HPTS, 10 mM HEPES, pH = 6.8, 100 mM NaCl (or 75 mM Na_2SO_4), for 2 h. During hydration, the suspension was submitted to 5 freeze-thaw cycles (liquid nitrogen, water at room temperature). The large multilamellar liposome suspension (1 mL) was submitted to high pressure extrusion at room temperature (25 extrusions through a 0.1 μm polycarbonate membrane afforded a suspension of LUVs with an average diameter of 100 nm). The LUV suspension was separated from extravesicular fluorescent dye by size exclusion chromatography (SEC) (stationary phase: Sephadex G-50, mobile phase: Buffer B containing 10 mM HEPES, pH = 6.8, 100 mM NaCl or 75 mM Na_2SO_4), and diluted with the Buffer B to give a stock solution with a lipid concentration of 10 mM (assuming 100% of lipid was incorporated into liposomes).

Typically, 100 μL of HPTS-loaded liposomes (stock solution) were suspended in 1.9 mL of the isotonic corresponding buffer and placed into a fluorometric cell. HPTS emission at 510 nm was monitored with excitation wavelengths at 403 nm and 460 nm simultaneously. During the experiment, 20 μL of DMSO (negative control) or 1 mM DMSO stock solution of testing compound was added through an injection port, followed by injection of aqueous NaOH solution (20 μL, 0.5 M). Addition of the NaOH base pulse caused a pH increase of approximately 1 pH unit in the extravesicular buffer. Maximal changes in dye emission were obtained at the end of each experiment by lysis of the

liposomes with detergent (40 μL, 5% aqueous Triton X-100). The final transport trace was obtained as a ratio of the emission intensities monitored at 460 and 403 nm and normalized to 100% of transport (I_{rel} = $[(I_{460}/I_{403})_t$-$(I_{460}/I_{403})_{initial}]/[(I_{460}/I_{403})_{initial}$-$(I_{460}/I_{403})_{final}]$). As shown in Figure S1, **CM3** induced continuous HPTS fluorescence change in NaCl solution, but no obvious fluorescent change in Na_2SO_4 solution, suggesting the possibility of Cl^- transport instead of Na^+ transport.

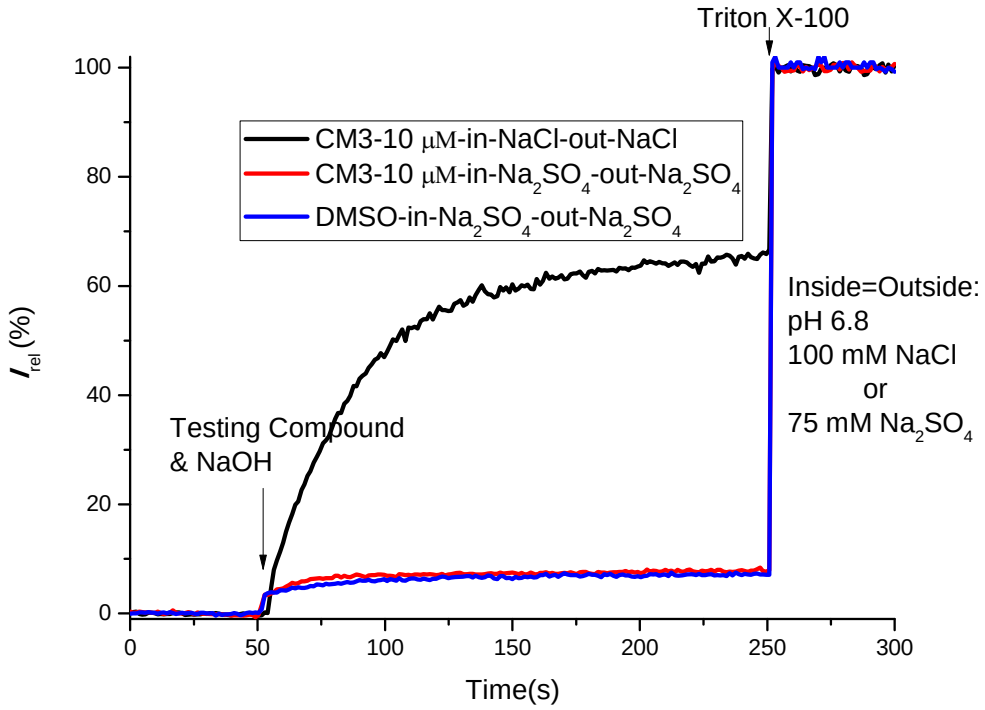


Figure S1. HPTS base pulse assays of **CM3**. In all experiments, HPTS-filled liposomes were suspended in isotonic salt solutions. For the curve shown in black (**CM3**-in-NaCl-out-NaCl), both intra- and extravesicular solutions contained 10 mM HEPES (pH 6.8) and 100 mM NaCl. For curves shown in red and blue (**CM3**- and DMSO-in-$NaSO_4$-out-Na_2SO_4), both intra- and extravesicular solutions contained 10 mM HEPES (pH 6.8) and

75 mM Na_2SO_4. At t = 50 s, DMSO solution of **CM3** (20 μL, 10 μM final concentration) or DMSO (20 μL, negative control) was added to the extravesicular solutions, followed by addition of a NaOH solution (20 μL, 0.5 M). At t = 250 s, 40 μL of 5% triton X-100 was added to lyse the liposomes.

Liposome-Based SPQ assay

Preparation of SPQ-Loaded EYPC Liposomes

Egg yolk L-*α*-phosphatidylcholine (EYPC, 91 mg, 120 μmol) was dissolved in a $CHCl_3$/MeOH mixture, the solution was evaporated under reduced pressure and the resulting thin film was dried under high vacuum for 3 h. The lipid film was hydrated in 1.2 mL of the solution containing 0.5 mM SPQ, 10 mM HEPES, pH 7.0, and 200 mM $NaNO_3$ (or 0.5 mM SPQ, pH 9.0, 100 mM $NaHCO_3$,) for 2 h. During hydration, the suspension was submitted to 5 freeze-thaw cycles (liquid nitrogen, water at room temperature). The large multilamellar liposome suspension (1 mL) was submitted to high pressure extrusion at room temperature (25 extrusions through a 0.1 μm polycarbonate membrane afforded a suspension of large unilamellar vesicles (LUVs) with an average diameter of 100 nm). The LUV suspension was separated from extravesicular fluorescent dye by size exclusion chromatography (SEC) (stationary phase: Sephadex G-50, mobile phase: 10 mM HEPES, pH 7.0, 200 mM $NaNO_3$ solution or pH 9.0, 100 mM $NaHCO_3$) and diluted with the 200 mM $NaNO_3$ (or 100 mM $NaHCO_3$) solution to give a stock solution with a lipid concentration of 10 mM (assuming 100% of lipid was incorporated into liposomes).

SPQ Chloride Transport Assay in Liposomes

Typically, 100 μL of $NaNO_3$ and SPQ-loaded liposome (stock solution, pH 7.0) was suspended in 1.9 mL of the solution containing 10 mM HEPES and 200 mM NaCl, pH 7.0. The mixture was placed into a fluorometric cell. SPQ emission at 430 nm was monitored with excitation wavelengths at 360 nm. At t = 100 s, THF (20 μL, negative control) or THF solution of **CM1** or **CM3** (20 μL, given concentration) was added through an injection port.

SPQ Bicarbonate Transport Assay in Liposomes

Typically, 100 μL of $NaHCO_3$ and SPQ-loaded liposome (stock solution, pH 9.0) was suspended in 1.9 mL of the solution containing 5 mM Tris and 100 mM NaCl, pH 9.0. The mixture was placed into a fluorometric cell. SPQ emission at 430 nm was monitored with excitation wavelengths at 360 nm. At t = 50 s, THF (20 μL, negative control) or THF solution of **CM1** or **CM3** (20 μL, 2 mM) was added through an injection port.

Single-Channel Recording on Giant Liposomes

Giant liposomes were prepared for single-channel recording. 16 mg of POPC and 4 mg of PS were dissolved in 2 mL of distilled water. The mixture was intermittently stirred with a Vortex mixer for 20 min, and then sonicated under nitrogen protection for 10 min. The mixture was centrifuged at 160 000 g for an hour, and then the pellet was re-suspended in 200 μL of 10 mM MOPS buffer (pH 7.2, containing 5% (w/w) ethylene glycol. The re-suspended mixture was deposited on a clean glass slide in 15 μL aliquot and submitted to

partial dehydration (3 – 6 h) at 4 ºC. Before use, the sample was rehydrated for 10 h at 4ºC by using 15 µL of bath solution. For patch-clamp measurements of giant liposomes, 1–3 µL of hydrated liposome suspension was dropped on a Petri dish and diluted with bath solutions. Single-channel currents through giant liposome membranes in presence of **CM3** were measured with cell-attached patch configuration of the patch-clamp technique. Patch pipettes (resistance, 7–10 MΩ) were filled with internal pipette solution. When the seal resistance reached up to 10 GΩ, single channel currents were recorded with EPC 9 patch clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) in voltage-clamp mode. Pipette and membrane capacitance were electronically compensated. Ramp protocol was applied with Pulse (HEKA) software. Single channel currents were digitalized at 0.15 ms sampling interval, filtered at 0.5 kHz.

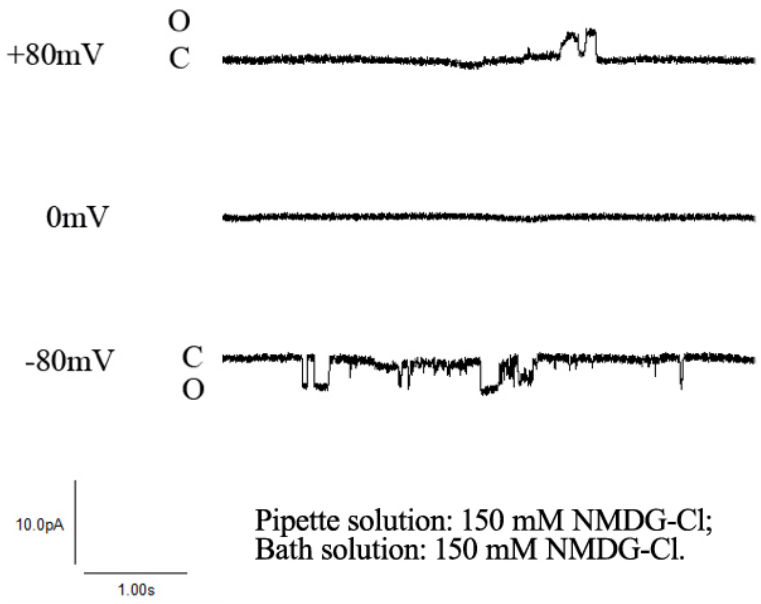


Figure S2. Typical single channel currents of self-assembled channels were recorded in the presence of **CM3** at 10 µM, when both intra- and extravesicular solutions were symmetric 150 mM NMDG-Cl.

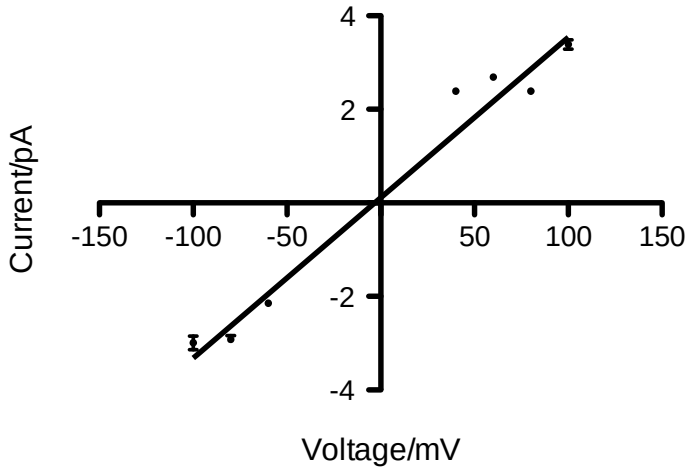


Figure S3. The relative ion selectivity test for **CM3** at 10 μM. 150 mM NaCl and 10 mM HEPES were applied as bath solution while 135 mM NMDG-Cl, 15 mM NaCl and 10 mM HEPES were applied as pipette solution. The reversal potential was – 3.8 ± 1.2 mV. The calculated relative permeability of chloride ion against sodium ion was 7.4 ± 2.1.

$H^{13}CO_3^-$-NMR Spectroscopy for Bicarbonate Transport

Egg yolk L-*α*-phosphatidylcholine (EYPC, 50 mg, 71 μM) was dissolved in a $CHCl_3$/MeOH mixture, the solution was evaporated under reduced pressure and the resulting thin film was dried under high vacuum for 3 h. The lipid film was hydrated with 0.5 mL buffer solution containing, which was prepared in a 9:1 H_2O/D_2O mixture, containing 450 mM NaCl, 20 mM HEPES, pH 7.3. After five freeze-thaw cycles, the large multilamellar liposome suspension was submitted to high pressure extrusion at room

temperature (41 extrusions through a 1 μm polycarbonate membrane afforded a suspension of giant liposomes). The NaCl-loaded giant liposome suspension obtained after extrusion was used in the ^{13}C-NMR transport assays without further purification. The lipid concentration of stock liposome solution was 132 mM (assuming 100% lipid retention after extrusion).

260 μL of stock (132 mM) liposome solution (NaCl 450 mM, pH 7.3) was mixed with 340 μL of solution containing 150 mM Na_2SO_4, 88 mM $NaH^{13}CO_3$, and 20 mM HEPES at pH 7.3. The final concentration for the liposome and $NaH^{13}CO_3$ was 58 mM and 50 mM, respectively. The ^{13}C-NMR of the liposome mixture (NaCl inside, Na_2SO_4 and $NaH^{13}CO_3$ outside) was then taken. After data acquisition, a solution of $MnCl_2$ was added at a final Mn^{2+} concentration of 0.5 mM ($[Mn^{2+}]/[H^{13}CO_3^-] = 1/100$), followed by another set of data acquisition. A final set of ^{13}C-NMR spectroscopic data was collected after the addition of a DMSO solution of **CM3** (3 μL, 100 mM, 0.87 mol% ligand-to-lipid ratio) or DMSO (3 μL, negative control) to the mixture.

pKa determination of CM1

The pKa value of compound **CM1** was calculated according to reported method[1], by monitoring absorbance changes in the UV-Vis spectra as a function of variations pH of phosphate buffer. The UV-Vis spectra were recorded in a CARY 50 bio UV-Visible spectrophotometer at 25 °C. Standard 10 mm quartz glass cells were used. Stock solutions of compounds were prepared in MeOH (10 mM). A 30 μM solution of the compound was prepared with different pH (from 4.94 to 11.21). The pKa values were determined from a

plot of log (ionization ratio) vs pH (Eq.1).

$A \rightarrow$ Absorbance at each pH

$A_{A^-} \rightarrow$ Neutral form absorbance

$A_{AH} \rightarrow$ Protonated form absorbance

$$\mathbf{Log\ [(A-A_{A^-})/(A_{AH}-A)] = -pH + pK_a}$$

Equation 1. Log absorbance vs pH

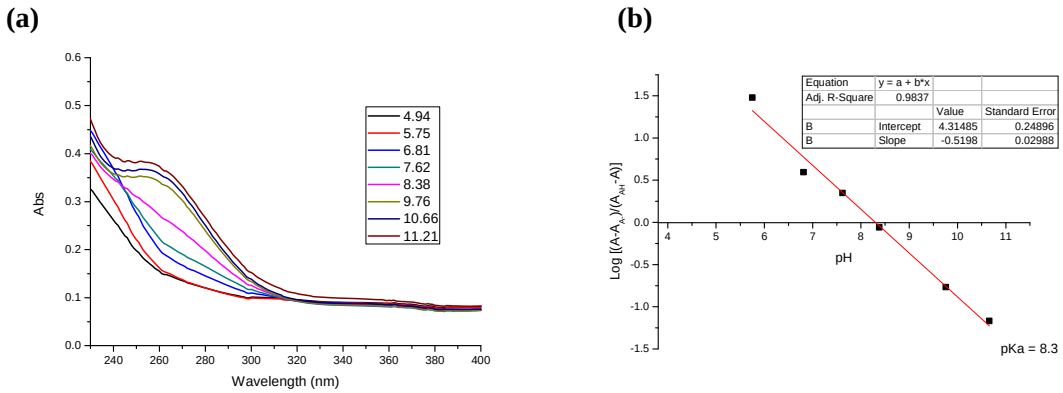


Figure S4. a). UV-vis absorbance spectra for **CM1** as a function of pH in 0.1 M phosphate buffer at 25 °C. b) Log (ionization ratio) vs. pH for **CM1,** pKa = 8.3.

Ussing Chamber-Based Short-Circuit Current Measurement

Statistical Analysis of Data

Changes in I_{sc} (I_{sc}) were quantified by subtracting the current obtained after stimulation with that measured immediately prior to the stimulation. Data were presented as mean ± standard error (SE), and *n* indicated the number of experiments in each group.

Statistical analysis was performed by using the two-tailed unpaired t-test or the one-way ANOVA as appropriate. All statistical procedures were computed by using Prism version 6.0 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

Preparation for the Short-Circuit Current Measurement in Calu-3 Monolayer

Cell Culture and Seeding

Calu-3 cells were ordered from ATCC (American Type Culture Collection, Manassas, VA, USA). They were maintained in Dulbecco's Modified Eagle Medium (D-MEM, containing 4500 mg/L D-glucose and 2 mM L-Glutamine) supplemented with 10% fetal bovine serum, 100 I.U./mL penicillin, and 100 mg/mL streptomycin. Cells were cultured in plastic flasks and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. For short-circuit current (I_{sc}) measurement, cells were seeded onto Millipore filter membranes (Millipore, 0.45 μm pore size) with a culture area of about 0.45 cm^2. The medium was changed every other day and I_{sc} measurement was carried out after 17–21 days of culture.

Chemicals

Forskolin, $CFTR_{inh}$-172, bumetanide, acetazolamide and nystatin were obtained from Sigma-Aldrich (St. Louis, MO, USA). They were dissolved in DMSO to make stock solutions, respectively. **CM3** was synthesized in our lab, and was dissolved in DMSO to make a 100 mM stock solution. All cell culture reagents, including D-MEM, fetal bovine

serum, trypsin, penicillin, and streptomycin, were obtained from Invitrogen (Grand Island, NY, USA). All other general laboratory reagents were purchased from Sigma-Aldrich.

Preparation of Bathed Solutions

The Krebs-Henseleit (K-H) solution contained NaCl (117 mM), $NaHCO_3$ (25 mM), KCl (4.7 mM), $MgSO_4$ (1.2 mM), KH_2PO_4 (1.2 mM), $CaCl_2$ (2.5 mM), and D-glucose (11 mM), and attained a pH of 7.4 when bubbled with CO_2 (5% in O_2). The low Cl^- solution (10 mM) was prepared by iso-osmotically replacing NaCl, KCl, and $CaCl_2$ with sodium gluconate, potassium gluconate and calcium gluconate, respectively. The Cl^--free solution was prepared by iso-osmotically replacing NaCl and KCl with sodium gluconate and potassium gluconate, respectively, while $CaCl_2$ was replaced with 11 mM calcium gluconate to compensate for the Ca^{2+}-buffering capacity of gluconate ion. The HCO_3^--free solution was composed of NaCl (117 mM), KCl (4.7 mM), $MgCl_2$ (1.2 mM), $CaCl_2$ (2.56 mM), HEPES (10 mM), Tris (5.6 mM) and glucose (11.1 mM). This solution was gassed with 99.99% O_2.

Short-Circuit Current (I_{sc}) Measurement

The confluent monolayers were clamped vertically between the two halves of an Ussing chamber. Monolayers were short-circuited by using a voltage-clamp amplifier. The current required (I_{sc}) to nullify the transepithelial potential difference was displayed on-line on a pen recorder. The effect of various ion transport inhibitors on **CM3**-evoked I_{sc} was tested by pretreating the epithelia with inhibitors for at least 5 minutes before the addition of synthetic compounds. The inhibitors were maintained in the bathing solution

throughout the experiments. In most cases, positive I_{sc} means anion efflux from apical membranes or cation influx across apical membranes. The viability of the cell monolayers was monitored by measuring the transepithelial resistance: a transepithelial potential difference of 1 mV was applied to the monolayers periodically and the resultant change in current was used to calculate the transepithelial resistance according to the Ohm's law.

Experimental Results in Calu-3 Monolayers

Short-Circuit Current (I_{sc}) Measurement in Calu-3 Monolayers (Low Basal-Current)

In all these short-circuit current measurements, monolayers were mounted in Ussing chambers and bathed bilaterally with the K-H solution. The ionic nature of the I_{sc} increase induced by **CM3** in Calu-3 cells was investigated by using different kinds of channel inhibitors. As shown in Figure S5, CFTR is the main anion secreting ion channel on the apical. CFTR inhibitor CFTR$_{inh}$-172, can reversibly and selectively inhibit CFTR conductance. The Na-K-Cl cotransporter (NKCC) aids in the active transport of sodium, potassium, and chloride into and out of cells, which is responsible for the main cytosolic Cl^- intake pathway from the basolateral side. Bumetanide can block Cl^- uptake by inhibition of NKCC1. Intracellular bicarbonate is either generated from the hydration of CO_2 catalyzed by carbonic anhydrase or absorbed through the basolateral sodium-bicarbonate cotransporter (NBC1). Acetazolamide and DIDS inhibit the carbonic anhydrase and the basolateral membrane channel NBC1, respectively.

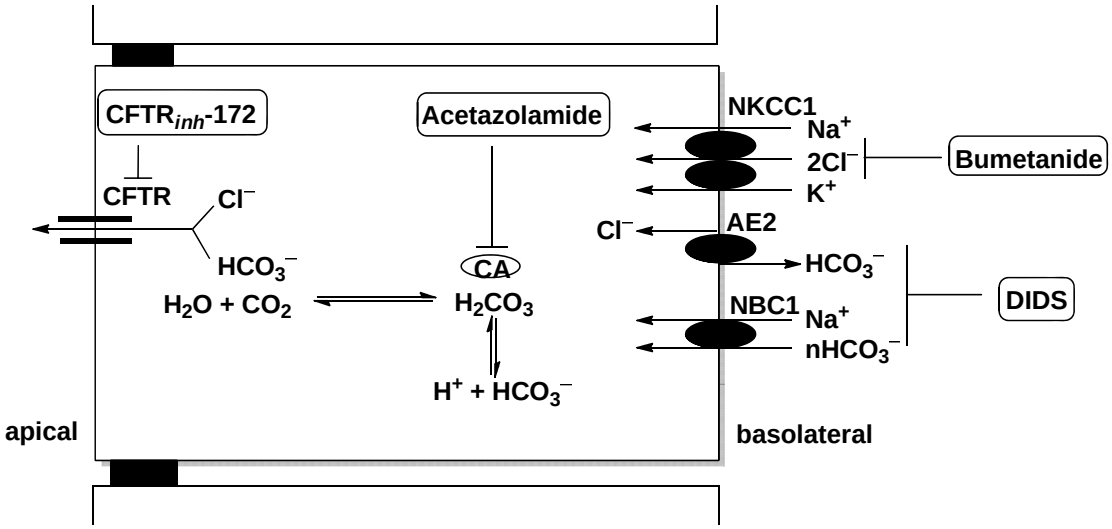
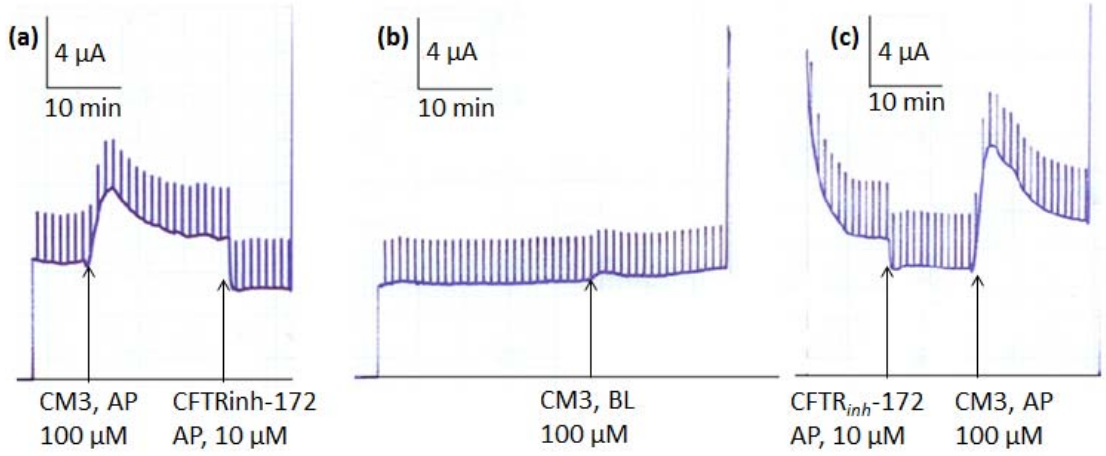


Figure S5. A simplified model of channel inhibitors for Calu-3 anion secretion.



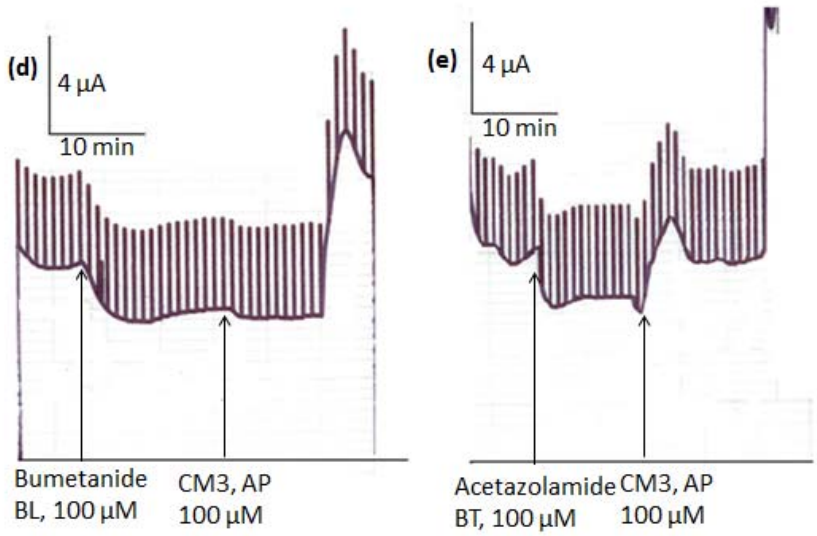


Figure S6. The effect of ΔI_{sc} elicited by **CM3** in Calu-3 cell monolayers. Monolayers were mounted in Ussing chambers and were bathed bilaterally with normal K-H solution. (a) **CM3** was added to the apical side of cell monolayers (100 μM final concentration). (b) **CM3** (100 μM) was added to the basolateral side of cell monolayers. (c) Cell monolayers were pretreated with CFTR$_{inh}$-172 before the addition of **CM3**. CFTR$_{inh}$-172 (10 μM) was added to the apical side of cell monolayers, followed by apical application of **CM3** (100 μM). (d) Cell monolayers were pretreated with bumetanide before the addition of **CM3**. Bumetanide (100 μM) was added to the basolateral side, followed by apical addition of **CM3** (100 μM). (e) Cell monolayers were pretreated with acetazolamide before the addition of **CM3**. Acetazolamide was added bilaterally to both sides of cell monolayers (100 μM), followed by apical addition of **CM3** (100 μM). Cumulative data from identical experiments to those shown in (a) to (e) was shown in Figure 6a of the text. The black horizontal lines in (a) to (e) represented the zero current levels.

Short-Circuit Current (I_{sc}) Measurement in Calu-3 Monolayers (High Basal-Current).

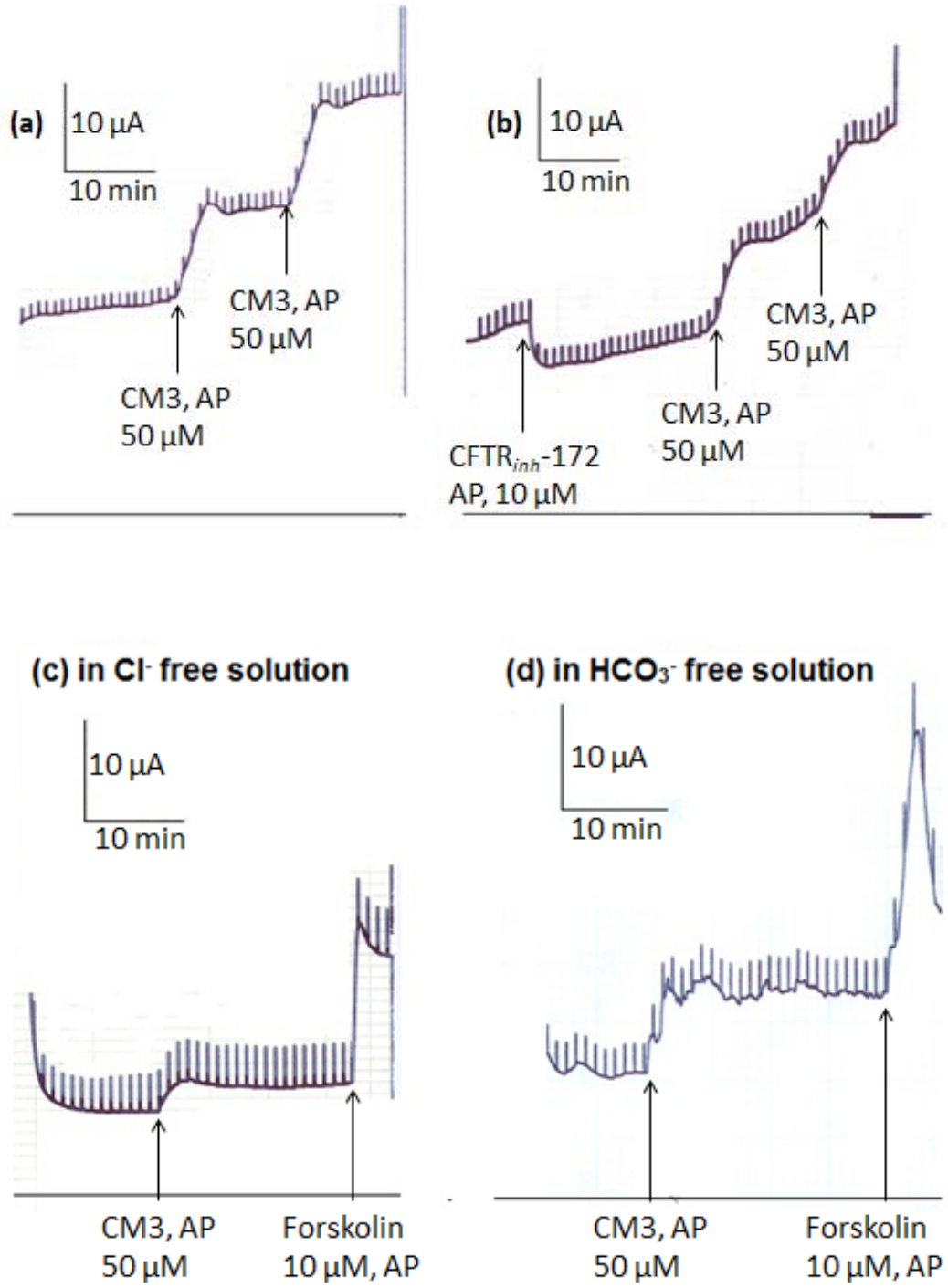


Figure S7. The effect of ΔI_{sc} elicited by **CM3** in Calu-3 cell monolayers with high basal current (19.82 ± 1.28 μA/cm^2, n = 32). For (a) and (b), monolayers were mounted in Ussing chambers and were bathed bilaterally with the K-H solution. In (a), **CM3** was added from the apical side with 50 μM concentration increase after each addition. In (b), CFTR$_{inh}$-172 (10 μM) was added to the apical side of the monolayers, followed by apical application of **CM3** with 50 μM concentration increase after each addition. (c) Monolayers were bathed bilaterally with the chloride-free solution. **CM3** was added to the apical side at 50 μM. (d) Monolayers were bathed bilaterally with the bicarbonate-free solution and **CM3** was added to the apical side at 50 μM. In (c) and (d), after **CM3** application, the monolayers were stimulated with an adenylete cyclase activator, forskolin. Cumulative data from identical experiments to those shown in (a) to (d) was shown in Figure 6b of the text. The black horizontal lines in (a) to (d) represented the zero current levels.

Preparation for Short-Circuit Current Measurement in CFBE41o- Cell Monolayer

Cell Culture and Seeding

CFBE41o- cells were obtained from Dr. Dieter Gruenert (UCSF School of Medicine, CA, USA). CFBE41o- cells were maintained in Minimum Essential Medium with Earle's salt (MEM) supplemented with 10% (vol/vol) fetal bovine serum, 1% (vol/vol) L-glutamine, 100 I.U./mL penicillin, and 100 mg/mL streptomycin. Cells were cultured in plastic flasks coated with fibronectin and collagen (BD Biosciences, Bedford, MA) and were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. For short-circuit

current (I_{sc}) measurement, cells were seeded onto Transwell-COL membranes (Costar, Cambridge, MA) with 0.4 mm pore diameter (culture area 0.2 cm^2). Cells reached confluence after about 10 days of culture, with transmembrane resistance greater than 150 $\Omega \cdot cm^2$.

Chemicals

DIDS and amiloride were obtained from Sigma-Aldrich (St. Louis, MO, USA), and were dissolved in DMSO to make stock solutions, respectively. **CM3** was dissolved in DMSO to make 100 mM stock solutions. All cell culture reagents, including MEM, fetal bovine serum, trypsin, penicillin, and streptomycin, were obtained from Invitrogen (Grand Island, NY, USA). All other general laboratory reagents were purchased from Sigma-Aldrich.

Preparation of Bath Solutions

The bicarbonate-buffered K-H solution contained NaCl (117 mM), $NaHCO_3$ (25 mM), KCl (4.7 mM), $MgSO_4$ (1.2 mM), KH_2PO_4 (1.2 mM), $CaCl_2$ (2.5 mM) and D-glucose (11 mM), and attained a pH value of 7.4 when bubbled with CO_2 (5% in O_2). The low Cl^- solution (10 mM) was prepared by iso-osmotically replacing NaCl, KCl, and $CaCl_2$ with sodium gluconate, potassium gluconate and calcium gluconate, respectively.

Statistical Analysis of Data

I_{sc} was quantified by subtracting the current obtained after stimulation with that measured immediately prior to the stimulation. Data were presented as mean ± standard error (SE), and *n* indicated the number of experiments in each group. Statistical analysis was performed by using the Student's *t*-test or the one-way ANOVA as appropriate. All statistical procedures were computed by using Prism version 6.0 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

Short-Circuit Current (I_{sc}) Measurement in CFBE41o- Monolayers

The I_{sc} measurement in CFBE41o- cell monolayers was similar to that in Calu-3 monolayers. In most experiments, the CFBE41o- monolayers were initially bathed bilaterally with K-H solution. To generate a basolateral-to-apical Cl^--gradient favorable for apical Cl^- secretion, the apical K-H solution was changed to the low chloride solution. The effect of various ion transport inhibitors on synthetic compound-evoked ΔI_{sc} was tested by pretreating the epithelia with inhibitors for at least 5 minutes before the addition of **CM3**. The inhibitors were maintained in the bathing solution throughout the experiments. In most cases, positive ΔI_{sc} meant anion efflux from apical membranes or cation influx across apical membranes. The viability of the cell monolayers was monitored by measuring the transepithelial resistance: a transepithelial potential difference of 1 mV was applied to the

monolayers periodically and the resultant change in current was used to calculate the transepithelial resistance according to the Ohm's law.

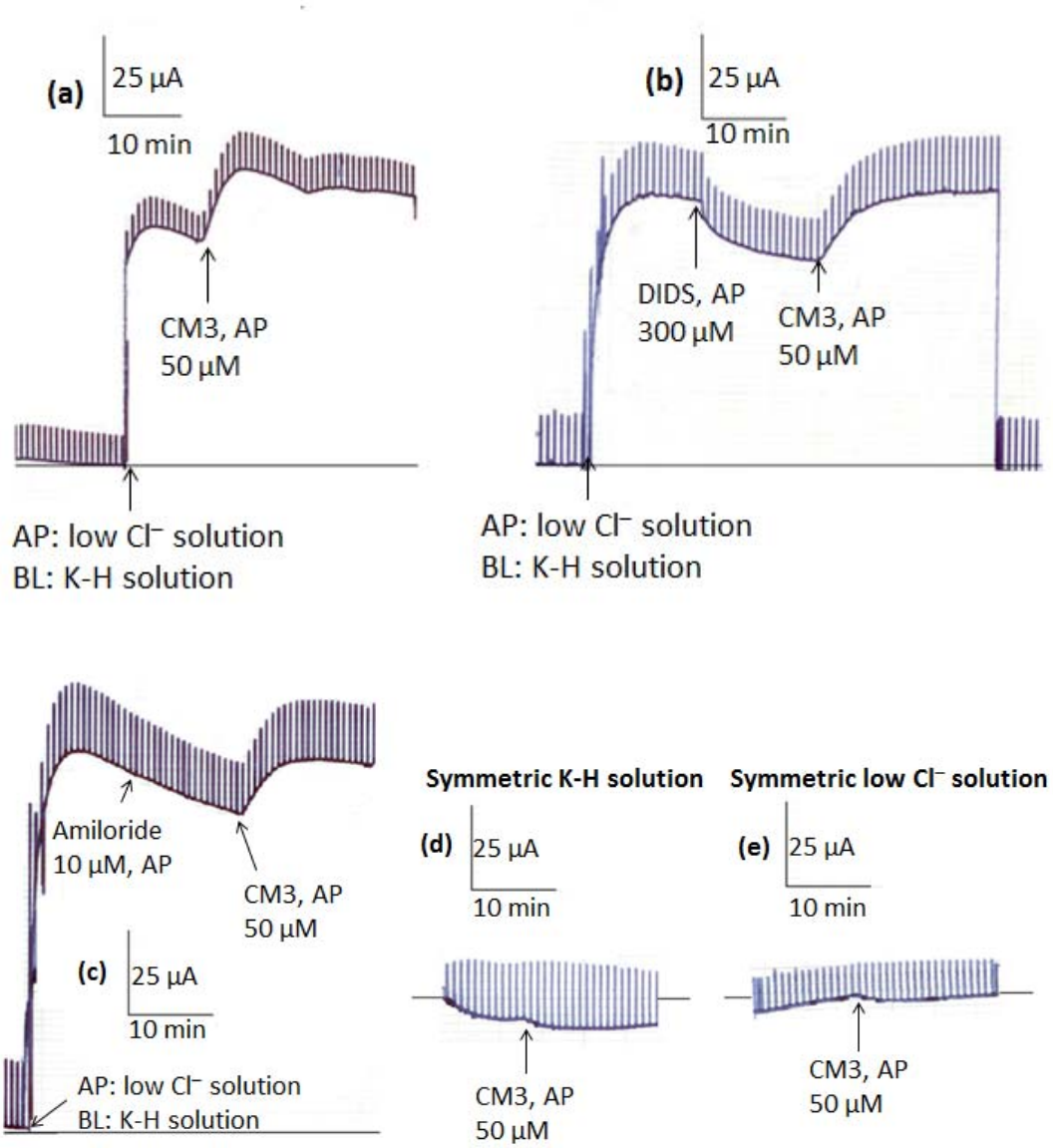


Figure S8. ΔI_{sc} elicited by **CM3** in CFBE41o- epithelial monolayers. In (a) to (c), CFBE41o- monolayers were initially bathed with K-H solution. Then a basolateral-to-apical Cl^- gradient was imposed across the monolayers by changing the apical K-H solution to the low Cl^- solution to facilitate apical Cl^- secretion. (a) **CM3** was added to the apical

side of the CFBE41o- monolayers at 50 μM. (b) DIDS (300 μM) was added to the apical side of the monolayers before apical application of CM3 (50 μM). (c) Amiloride (10 μM) was added to the apical side of the monolayers before apical application of CM3 (50 μM). (d) CFBE41o- monolayers were bathed bilaterally with the K-H solution. CM3 was added to the apical side at 50 μM. (e) CFBE41o- monolayers were bathed bilaterally with the low chloride solution. **CM3** was added to the apical side at 50 μM. Cumulative data from identical experiments to those shown in (a) to (e) was shown in Figure 7 of the text. The black horizontal lines in (a) to (e) represented the zero current line.

Reference

1. G. S. Patterson, *J. Chem. Educ.* 1999**, 76**, 395.