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Supplemental Information

Determinants of Ion-Transporter Cancer Cell Death

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SUPPLEMENTARY INFORMATION

Synthetic ion transporter cancer cell death determinants

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TRANSPORT STUDIES IN LIPOSOMES

General procedure for ISE assay. Chloride concentrations in the transport experiments were determined using an Accumet chloride ion selective electrode (ISE) equipped with the Orion Star A211 benchtop meter. The electrode was calibrated against sodium chloride solutions of known concentrations prior to each experiment according to the supplier's manual. POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) was supplied by Avanti Polar Lipids and was stored at −20°C as a solution in chloroform (1 g POPC in 35 mL chloroform). A solution of Triton X-100 (11 w%) in H_2O:DMSO (7:1 v/v) was used as the detergent.

A lipid film of POPC was formed from a chloroform solution under reduced pressure and dried under vacuum for at least 8 hours. The lipid film was rehydrated by vortexing with the potassium chloride (300 mM) "internal" solution buffered to pH 7.2 with potassium phosphate salts (5 mM). The lipid suspension was then subjected to nine freeze-thaw cycles, where the suspension was alternatingly allowed to freeze in a liquid nitrogen bath, followed by thawing in a water bath. The lipid suspension was allowed to age for 30 min at room temperature and was subsequently extruded 25 times through a 200 nm polycarbonate membrane (Whatman Nucleopore) using an extruder set (Avanti Polar Lipids) to afford large unilamellar vesicles (LUVs) with a mean diameter of 200 nm. The extravesicular solution was exchanged with the potassium gluconate (300 mM) "external" solution buffered to pH 7.2 with potassium phosphate salts (5 mM) by passing through a Sephadex G-25 column using the external solution. The resulting suspension of LUVs was diluted to a standard volume (10 mL) with the external buffered solution to obtain the LUV stock solution with a known lipid concentration.

Typically, for each measurement, the LUV stock solution was diluted with an external buffered solution to a standard volume (5 mL) with a lipid concentration of 1 mM. A DMSO solution of the anionophore (10 μL) was added to start the experiment (t = 0 s) and the chloride efflux was monitored using the chloride-ISE. When a cationophore (valinomycin (Vln) or monensin (Mon)) was used, a DMSO solution of the cationophore (0.5 mM, 10 μL; 1 μM final concentration, 0.1 mol% carrier:lipid molar percent) was added to the LUV suspension 30 s prior to the addition of the test anionophore. At 5 min, the detergent solution (50 μL) was added to lyse the LUVs, and the total chloride reading was taken at 7 min. The initial value (t at 0) was set at 0% chloride efflux and the final chloride reading (t at 7 min) was set as 100% chloride efflux, to normalize the collected data from each measurement.

The initial rate of chloride efflux ($k_{initial}$) were obtained by nonlinear curve fitting analysis of the experimentally measured rate of normalised chloride efflux versus time (s), with the following single exponential function using *OriginPro 9.1*:

$$y = y_1 - a \cdot e^{-bx}$$

where y is the normalized chloride efflux and x is time (s). The initial rate of chloride efflux ($k_{initial}$) is then derived from $k_{initial}$ = a·b and is obtained in %·s^{-1}. The initial rate values are reported as mM·s^{-1} by multiplying 300 mM of total chloride concentration.

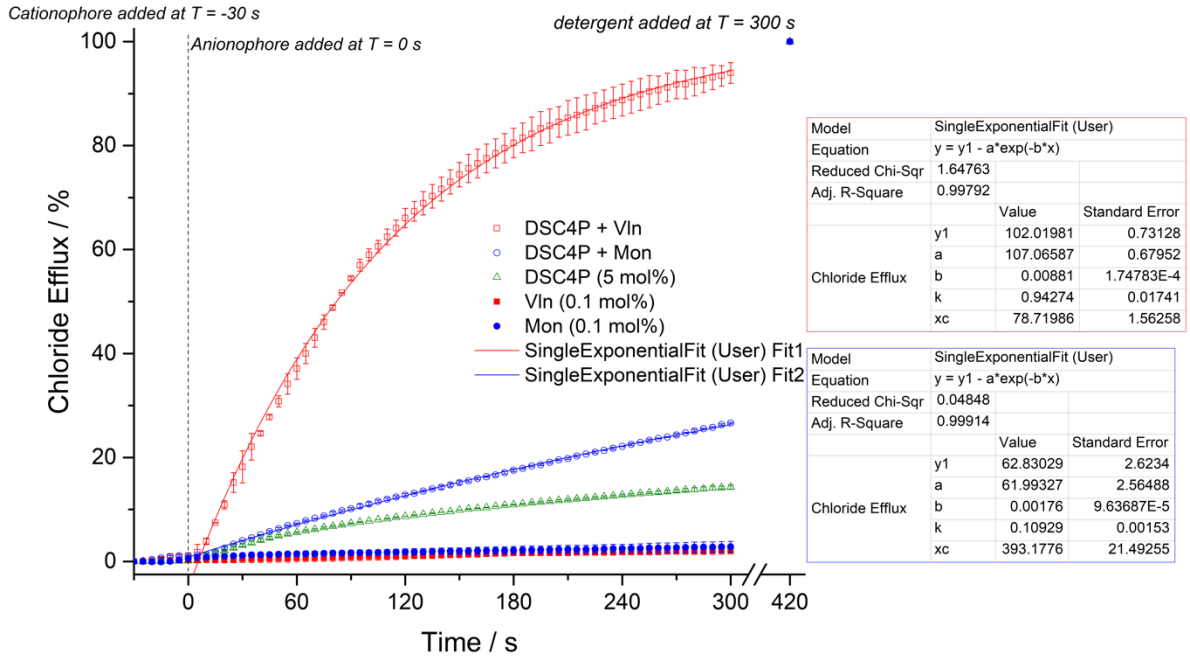


Figure S1. Cationophore-coupled KCl efflux assay of DSC4P-1. Cl^- efflux facilitated by DSC4P-1 (5 mol% carrier:lipid molar percent) in the absence or presence of cationophores (0.1 mol%, Vln or Mon) monitored over a period of 5 min. Detergent was added to lyse the LUVs at 300 s to obtain the 100% chloride concentration at 420 s. Solid lines are fitted curves using exponential function to calculate the initial rate ($k_{initial}$) of Cl^- efflux (%·s^{-1}). Error bars = SD from two replicate experiments.

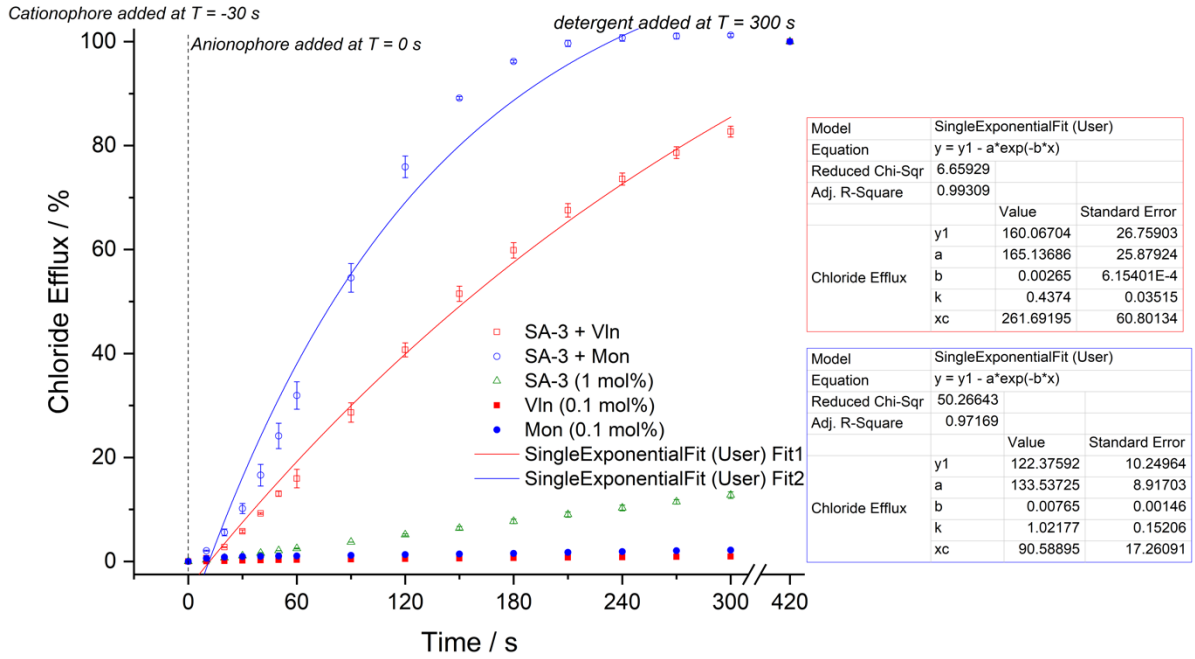


Figure S2. Cationophore-coupled KCl efflux assay of SA-3. Cl^- efflux facilitated by SA-1 (1 mol% carrier:lipid molar percent) in the absence or presence of cationophores (0.1 mol%, Vln or Mon) monitored over a period of 5 min. Detergent was added to lyse the LUVs at 300 s to obtain the 100% chloride concentration at 420 s. Solid lines are fitted curves using exponential function to calculate the initial rate ($k_{initial}$) of Cl^- efflux (%·s^{-1}). Error bars = SD from three replicate experiments.

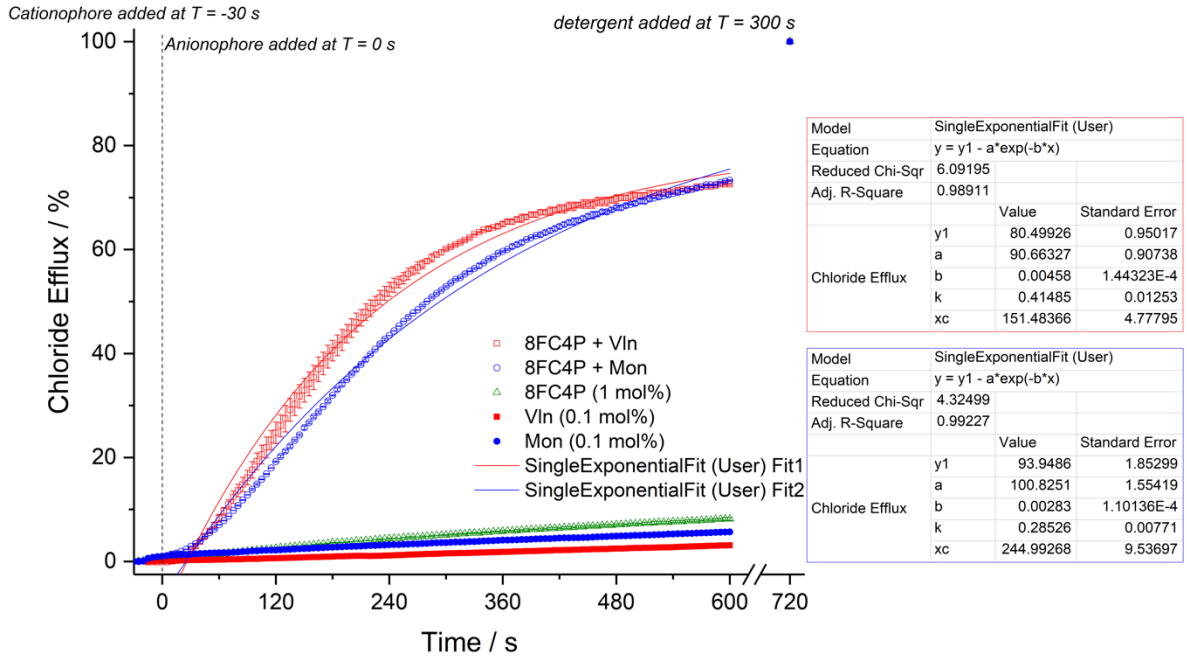


Figure S3. Cationophore-coupled KCl efflux assay of 8FC4P. Cl^- efflux facilitated by 8FC4P (1 mol% carrier:lipid molar percent) in the absence or presence of cationophores (0.1 mol%, Vln or Mon) monitored over a period of 5 min. Detergent was added to lyse the LUVs at 300 s to obtain the 100% chloride concentration at 420 s. Solid lines are fitted curves using exponential function to calculate the initial rate ($k_{initial}$) of Cl^- efflux (%·s^{-1}). Error bars = SD from two replicate experiments.

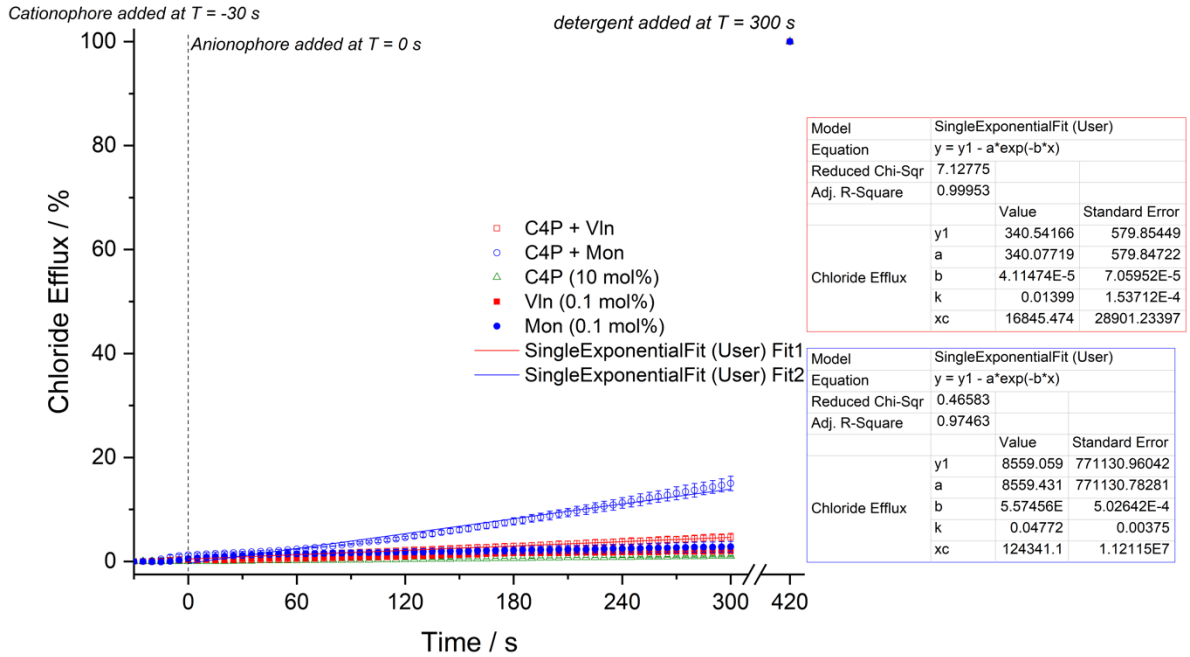


Figure S4. Cationophore-coupled KCl efflux assay of C4P. Cl^- efflux facilitated by C4P (10 mol% carrier:lipid molar percent) in the absence or presence of cationophores (0.1 mol%, Vln or Mon) monitored over a period of 5 min. Detergent was added to lyse the LUVs at 300 s to obtain the 100% chloride concentration at 420 s. Solid lines are fitted curves using exponential function to calculate the initial rate ($k_{initial}$) of Cl^- efflux (%·s^{-1}). Error bars = SD from two replicate experiments.

General procedure for HPTS assay. HPTS assays were conducted using POPC LUVs (mean diameter 200 nm) encapsulated with the membrane-impermeable pH-sensitive ratiometric fluorescent dye, 8-hydroxypyrene-1,3,6-trisulphonic acid (HPTS). The intra- and extravesicular solutions are identical salt solutions (100 mM) of potassium gluconate (K-Gluc) or *N*-methyl-D-glucamine chloride (NMDG-Cl) buffered to pH 7.0 with HEPES (10 mM); except for HPTS (1 mM) dissolved in the intravesicular solution. Fluorescence measurements for HPTS assay was conducted at 298 K on a Horiba FluoroMax-4 spectrofluorometer equipped with a 4-cuvettes multicell holder with stirring and temperature control.

The fluorescent probe encapsulated POPC LUVs were prepared as follows. A lipid film of POPC was formed from a chloroform solution under reduced pressure and dried under vacuum for at least 8 hours. The lipid film was hydrated by vortexing with the intravesicular solution of HPTS (1 mM) dissolved in a K-Gluc or NMDG-Cl (100 mM) solution buffered to pH 7.0 with HEPES (10 mM). The lipid suspension was then subjected to 9 freeze-thaw cycles, where the suspension was alternatingly allowed to freeze in a liquid nitrogen bath, followed by thawing in a water bath. The lipid suspension was allowed to age for 30 min at room temperature and was subsequently extruded 25 times through a 200 nm polycarbonate membrane (Whatman Nucleopore) using an extruder set (Avanti Polar Lipids). The unencapsulated fluorescent dye was removed by size exclusion chromatography on a Sephadex G-25 column using a dye-free extravesicular solution of K-Gluc or NMDG-Cl (100 mM) buffered to pH 7.4 with HEPES (10 mM). The resulting suspension of HPTS-encapsulated LUVs with a mean diameter of 200 nm was diluted to a standard volume (10 mL) with the extravesicular solution to obtain the LUV stock solution with a known lipid concentration.

Typically, for each measurement, the LUV stock solution was diluted with the dye-free extravesicular solution of K-Gluc or NMDG-Cl (100 mM) buffered to pH 7.0 with HEPES (10 mM) to a standard volume (2.5 mL) with a lipid concentration of 0.1 mM, in a disposable PMMA cuvette with a stirrer bar. The LUV suspension was allowed to equilibrate at 298 K for ~2 min in the cuvette cell holder of the spectrofluorometer.

In the K-Gluc assay, a DMSO solution of valinomycin (25 μM, 5 μL; 0.05 μM final concentration, 0.05 mol% carrier:lipid molar percent) was added to the LUV suspension, followed by a base pulse of KOH (0.5 M, 25 μL; 5 mM final concentration) to change the extravesicular solution to pH 8, hence generating a transmembrane pH gradient of $\Delta pH \approx 1$. Thereafter, a DMSO solution of the anionophore (5 μL) was added to start the experiment (t = 0 s); the fluorescence responses of the HPTS acid (λ_{ex} 403 nm | λ_{em} 510 nm) and the deprotonated HPTS (λ_{ex} 460 nm | λ_{em} 510 nm) were recorded simultaneously at 10 s time-intervals. At 210 s, a DMSO solution of monension (50 μM, 5 μL; 0.1 μM final concentration, 0.1 mol% carrier:lipid molar percent) was added to equilibrate the transmembrane pH gradient for calibration of the assay. In cases where oleic acid (OA) was added to saturate the LUVs with free fatty acid, a DMSO solution of OA (0.5 mM, 5 μL; 1 μM final concentration, 1 mol% carrier:lipid molar percent) was added prior to the addition of the anionophore.

In the NMDG-Cl assay, a base pulse of NMDG (0.5 M, 25 μL; 5 mM final concentration) was added to the LUV suspension to change the extravesicular solution to pH 8, hence generating a transmembrane pH gradient of ΔpH ≈ 1. Thereafter, a DMSO solution of the anionophore (5 μL) was added to start the experiment (t = 0 s); the fluorescence responses of the HPTS acid (λ_{ex} 403 nm | λ_{em} 510 nm) and the deprotonated HPTS (λ_{ex} 460 nm | λ_{em} 510 nm) were recorded simultaneously at 10 s time-interval. At 210 s, the detergent solution (25 μL) was added to lyse the LUVs to disperse the pH gradient for calibration of the assay. In cases where gramicidin D (GraD) was added as an assisting protonophore, a DMSO solution of GraD (0.05 mM, 5 μL; 0.1 μM final concentration, 0.1 mol% carrier:lipid molar percent) was added prior to the addition of the anionophore.

When free fatty acid (FFA) removal by BSA was required, fatty acid-free BSA was dissolved in the LUV stock solution to a final BSA concentration of 1 mol% (with respect to lipid). The BSA-containing LUV suspension was mixed gently for 30 min to afford FFA-removed LUVs before being used for fluorescence measurements.

The ratiometric fluorescence intensity of the deprotonated HPTS (λ_{ex} 460 nm | λ_{em} 510 nm) divided by the HPTS acid (λ_{ex} 403 nm | λ_{em} 510 nm) was normalized and reported as the fractional fluorescence intensity (I_f); calculated using the following equation, where R_t is the fluorescence ratio at each dependent variable time-point t, R_0 is the initial fluorescence ratio at t = 0 s, and R_1 is the averaged fluorescence ratio after the dissipation of pH gradient (t = 240–310 s).

$$I_f = \frac{R_t - R_0}{R_1 - R_0}$$

The fractional fluorescence intensity I_f at 200 s was plotted as a function of the anionophore concentration in mol% (carrier:lipid molar percent). Hill coefficients (n) and EC_{50} (200 s) values were calculated by fitting the curves to the following equation, where y is I_f (200 s) value with the anionophore loaded at concentration x, y_0 is the I_f (200 s) value obtained without the anionophore, y_1 is the maximum I_f value, n is the Hill coefficient, and k is the EC_{50} (200 s) value:

$$y = y_0 + (y_1 - y_0) \cdot \frac{x^n}{k^n + x^n}$$

The corrected EC_{50} value is derived from the following equation to obtain the explicit concentration x at 50% of y. This is a more accurate representation of the transport efficacy for carriers with a Hill plot profile of y_1 much lower than 1, see Figure S10.

$$\text{Correct } EC_{50} = \left[\frac{-k^{-n} \cdot (0.5 - y_1)}{0.5}\right]^{-\frac{1}{n}}$$

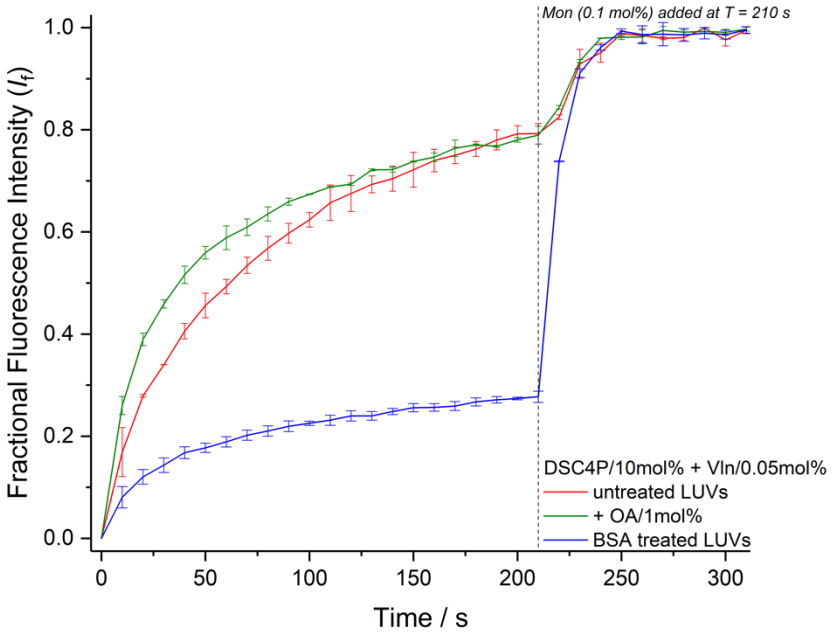


Figure S5. Overview of H^+/OH^- flux by DSC4P-1 in K-Gluc assay. Plot of H^+/OH^- flux facilitated by DSC4P-1 (10 mol% carrier:lipid molar percent) in the presence of Vln (0.05 mol%) monitored over a period of 210 s in three different LUV conditions: (1) Untreated LUVs, (2) in the presence of OA (1 mol%), and (3) BSA treated FFA-removed LUVs. Mon (0.1 mol%) was added at 210 s to equilibrate the transmembrane pH gradient. Error bars = SD from two replicate experiments.

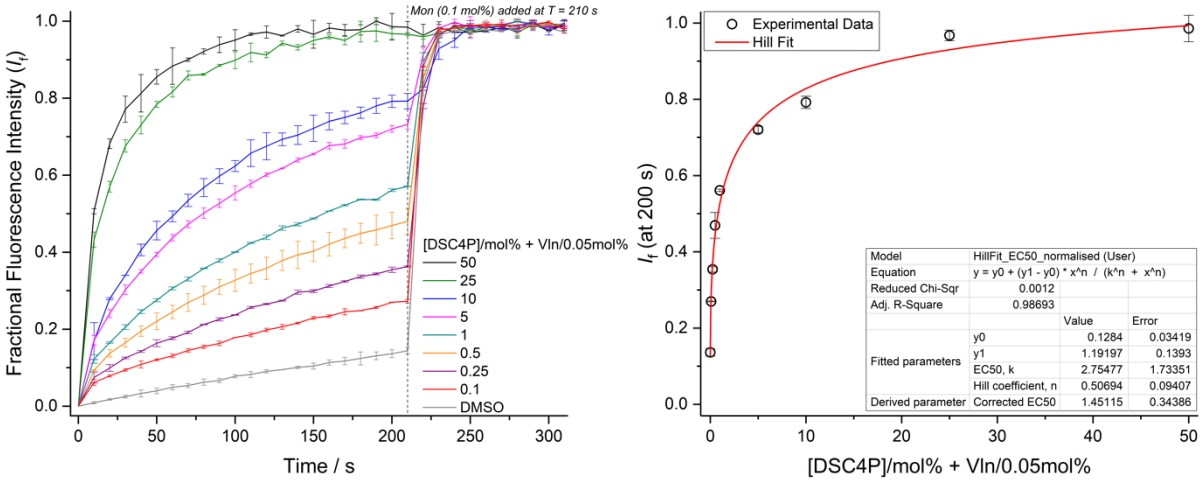


Figure S6. Hill analysis of DSC4P-1 in K-Gluc assay. Plots for Hill analysis of H^+/OH^- flux at 200 s facilitated by DSC4P-1 in the presence of Vln (0.05 mol%) monitored over a period of 210 s in untreated LUVs. Anionophore was added at 0 s, concentrations are shown as carrier:lipid molar percent. DMSO was used as a control and as the 0 mol% concentration data point in the Hill analysis. Mon (0.1 mol%) was added at 210 s to equilibrate the transmembrane pH gradient. Error bars = SD from two replicate experiments.

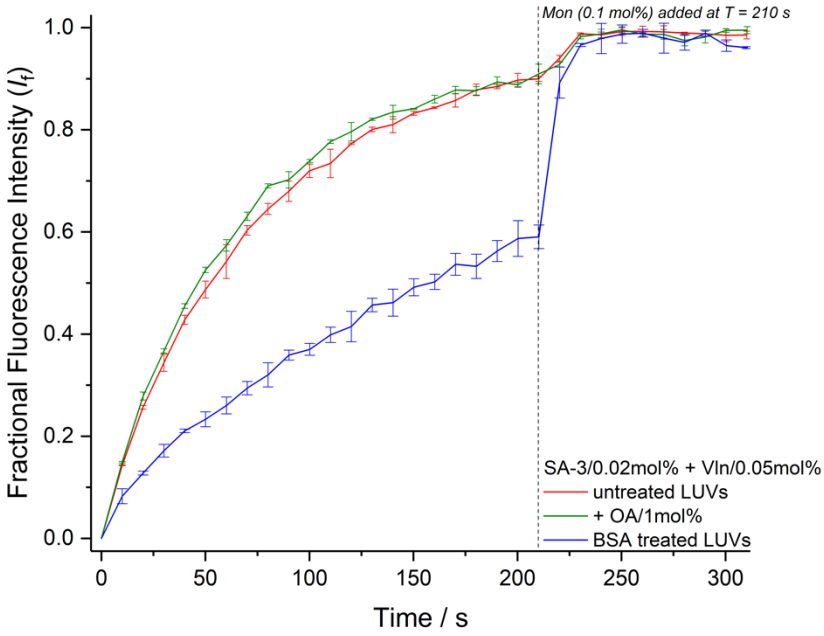


Figure S7. Overview of H^+/OH^- flux by SA-3 in K-Gluc assay. Plot of H^+/OH^- flux facilitated by SA-3 (0.02 mol% carrier:lipid molar percent) in the presence of Vln (0.05 mol%) monitored over a period of 210 s in three different LUV conditions: (1) Untreated LUVs, (2) in the presence of OA (1 mol%), and (3) BSA treated FFA-removed LUVs. Mon (0.1 mol%) was added at 210 s to equilibrate the transmembrane pH gradient. Error bars = SD from two replicate experiments.

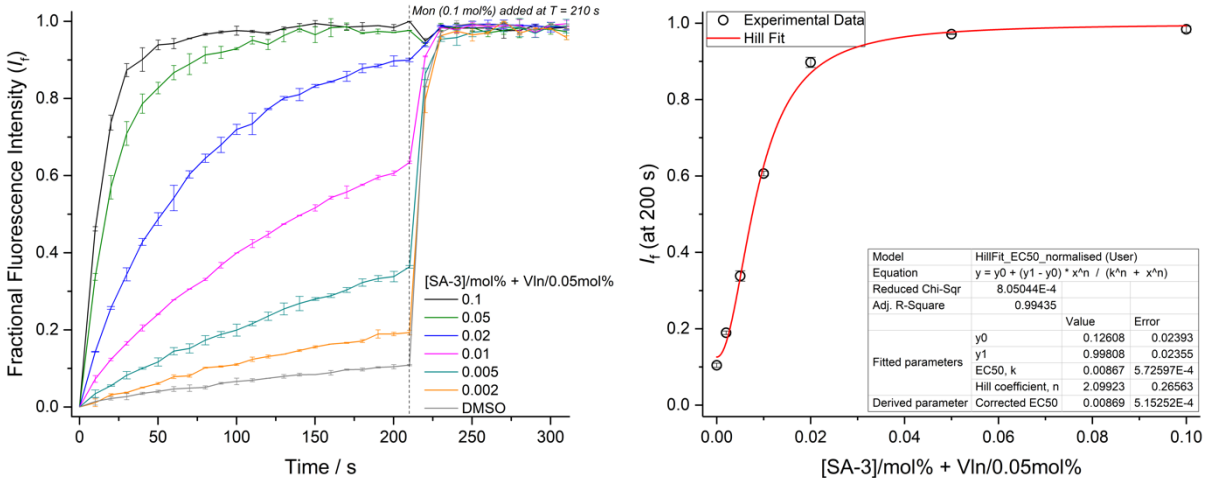


Figure S8. Hill analysis of SA-3 in K-Gluc assay. Plots for Hill analysis of H^+/OH^- flux at 200 s facilitated by SA-3 in the presence of Vln (0.05 mol%) monitored over a period of 210 s in untreated LUVs. Anionophore was added at 0 s, concentrations are shown as carrier:lipid molar percent. DMSO was used as a control and as the 0 mol% concentration data point in the Hill analysis. Mon (0.1 mol%) was added at 210 s to equilibrate the transmembrane pH gradient. Error bars = SD from two replicate experiments.

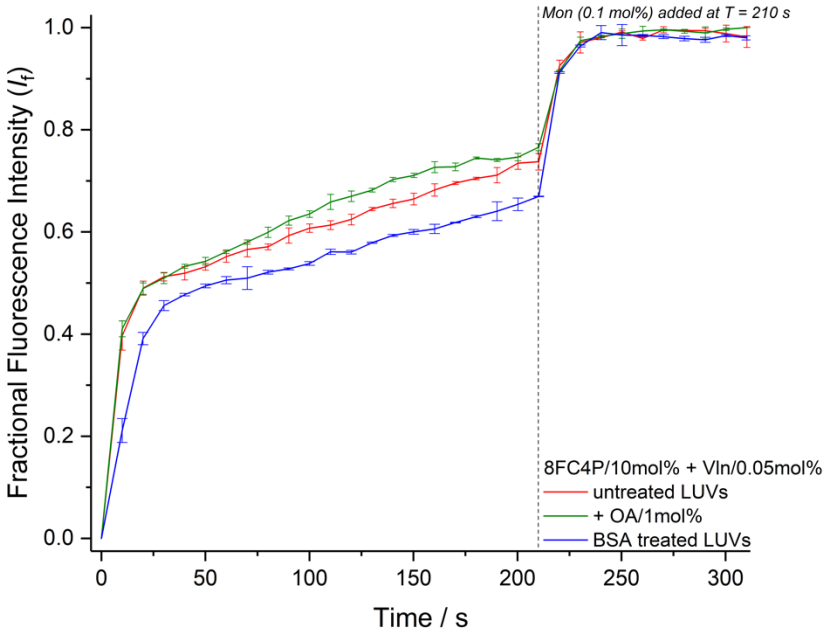


Figure S9. Overview of H^+/OH^- flux by 8FC4P in K-Gluc assay. Plot of H^+/OH^- flux facilitated by 8FC4P (10 mol% carrier:lipid molar percent) in the presence of Vln (0.05 mol%) monitored over a period of 210 s in three different LUV conditions: (1) Untreated LUVs, (2) in the presence of OA (1 mol%), and (3) BSA treated FFA-removed LUVs. Mon (0.1 mol%) was added at 210 s to equilibrate the transmembrane pH gradient. Error bars = SD from two replicate experiments.

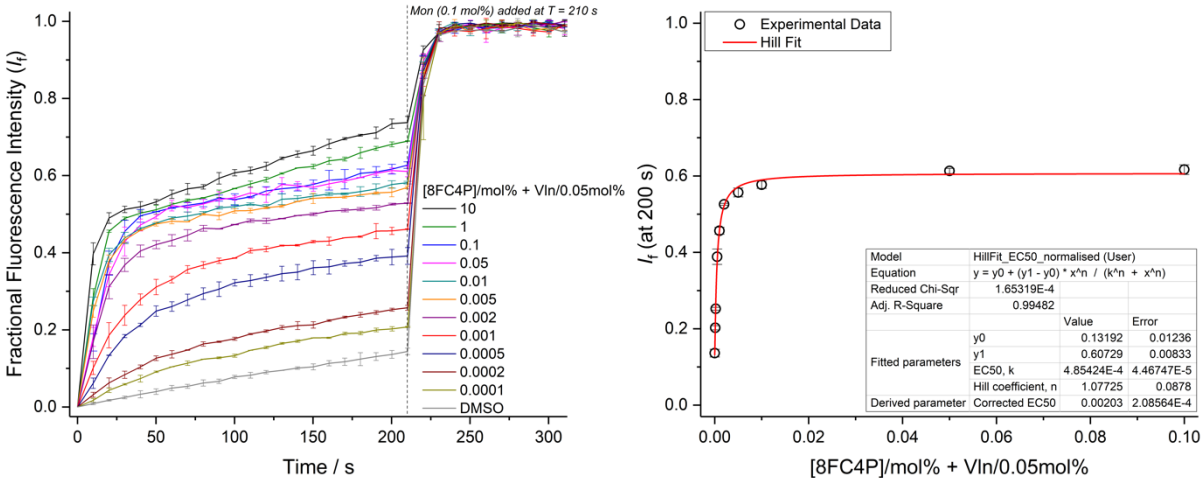


Figure S10. Hill analysis of 8FC4P in K-Gluc assay. Plots for Hill analysis of H^+/OH^- flux at 200 s facilitated by 8FC4P in the presence of Vln (0.05 mol%) monitored over a period of 210 s in untreated LUVs. Anionophore was added at 0 s, concentrations are shown as carrier:lipid molar percent. DMSO was used as a control and as the 0 mol% concentration data point in the Hill analysis. Mon (0.1 mol%) was added at 210 s to equilibrate the transmembrane pH gradient. Error bars = SD from two replicate experiments.

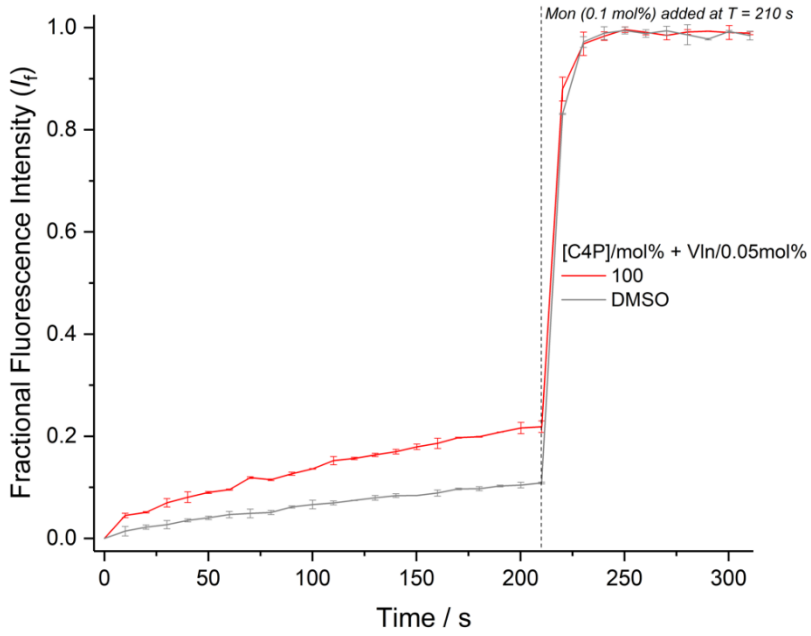


Figure S11. Overview of H^+/OH^- flux by C4P in K-Gluc assay. Plot of H^+/OH^- flux facilitated by C4P (100 mol% carrier:lipid molar percent) in the presence of Vln (0.05 mol%) monitored over a period of 210 s in untreated LUVs. DMSO was used as a control. Mon (0.1 mol%) was added at 210 s to equilibrate the transmembrane pH gradient. Error bars = SD from two replicate experiments.

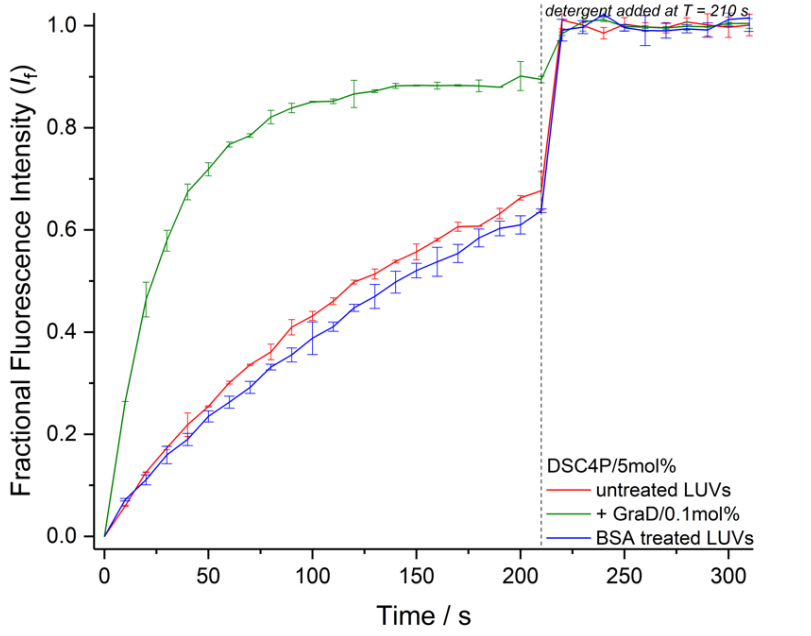


Figure S12. Overview of H^+/Cl^- cotransport by DSC4P-1 in NMDG-Cl assay. Plot of H^+/Cl^- cotransport facilitated by DSC4P-1 (5 mol% carrier:lipid molar percent) monitored over a period of 210 s in three different LUV conditions: (1) Untreated LUVs, (2) in the presence of GraD (0.1 mol%), and (3) BSA treated FFA-removed LUVs. Detergent was added at 210 s to lyse the LUVs to disperse the pH gradient. Error bars = SD from two replicate experiments.

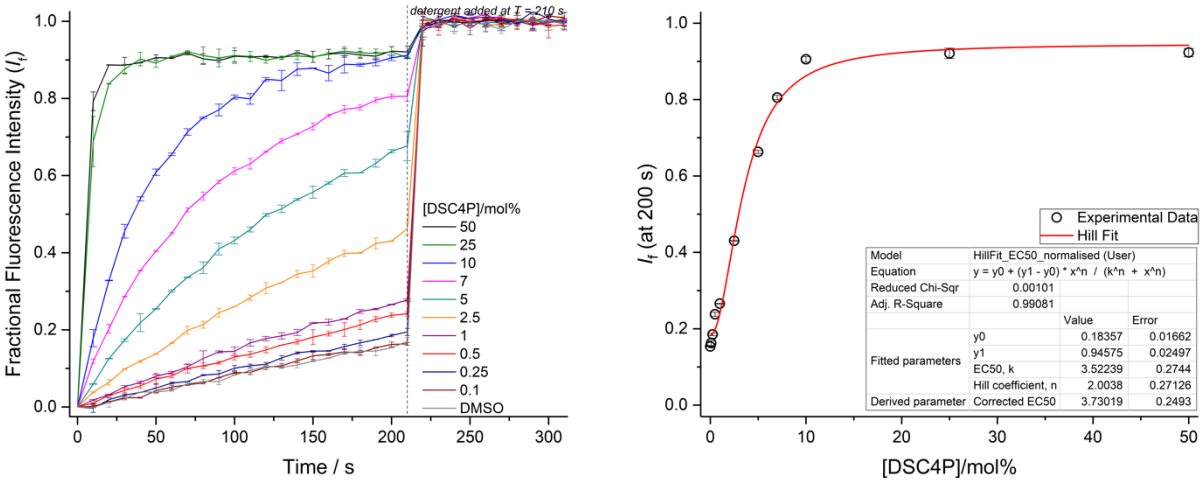


Figure S13. Hill analysis of DSC4P-1 in NMDG-Cl assay in untreated LUVs. Plots for Hill analysis of H^+/Cl^- cotransport at 200 s facilitated by DSC4P-1 monitored over a period of 210 s. Anionophore was added at 0 s, concentrations are shown as carrier:lipid molar percent. DMSO was used as a control and as the 0 mol% concentration data point in the Hill analysis. Detergent was added at 210 s to lyse the LUVs to disperse the pH gradient. Error bars = SD from two replicate experiments.

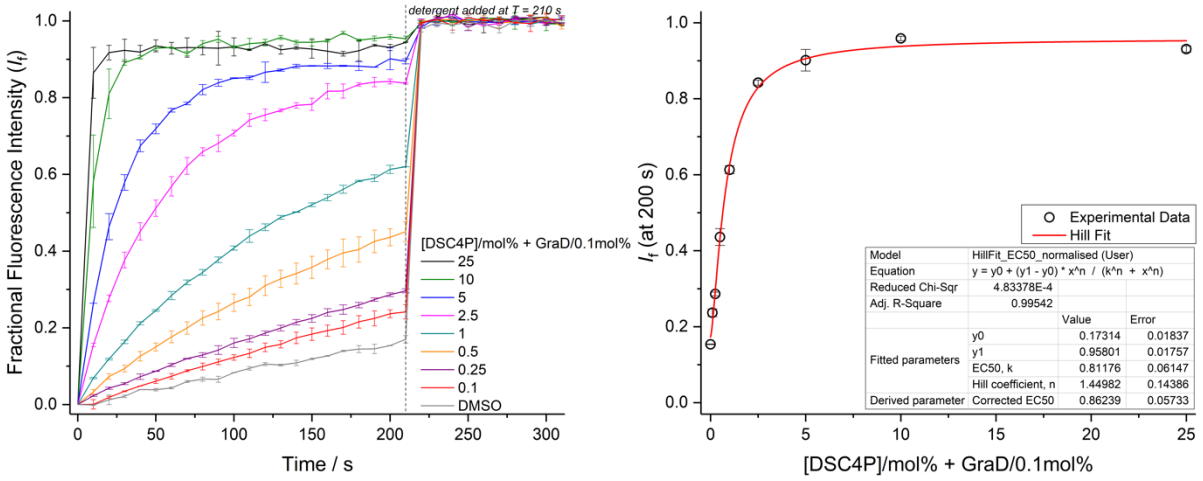


Figure S14. Hill analysis of DSC4P-1 in NMDG-Cl assay in the presence of GraD (0.1 mol%). Plots for Hill analysis of H^+/Cl^- cotransport at 200 s facilitated by DSC4P-1 monitored over a period of 210 s. Anionophore was added at 0 s, concentrations are shown as carrier:lipid molar percent. DMSO was used as a control and as the 0 mol% concentration data point in the Hill analysis. Detergent was added at 210 s to lyse the LUVs to disperse the pH gradient. Error bars = SD from two replicate experiments.

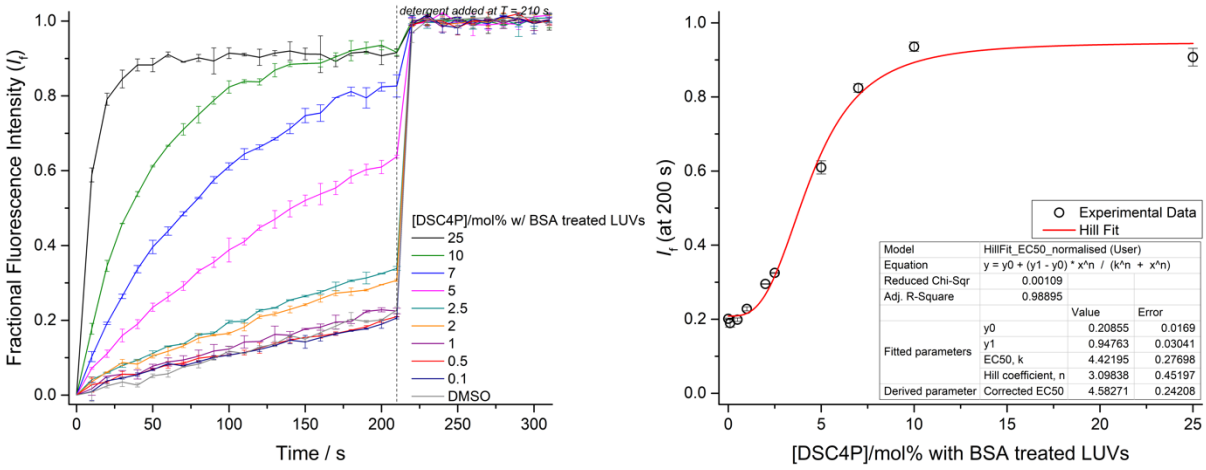


Figure S15. Hill analysis of DSC4P-1 in NMDG-Cl assay in BSA treated FFA-removed LUVs. Plots for Hill analysis of H^+/Cl^- cotransport at 200 s facilitated by DSC4P-1 monitored over a period of 210 s. Anionophore was added at 0 s, concentrations are shown as carrier:lipid molar percent. DMSO was used as a control and as the 0 mol% concentration data point in the Hill analysis. Detergent was added at 210 s to lyse the LUVs to disperse the pH gradient. Error bars = SD from two replicate experiments.

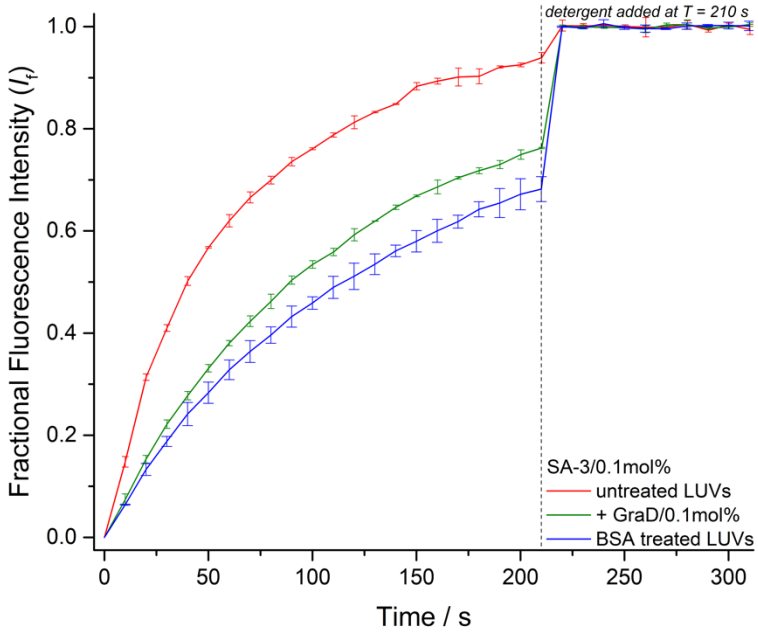


Figure S16. Overview of H^+/Cl^- cotransport by SA-3 in NMDG-Cl assay. Plot of H^+/Cl^- cotransport facilitated by SA-3 (0.1 mol% carrier:lipid molar percent) monitored over a period of 210 s in three different LUV conditions: (1) Untreated LUVs, (2) in the presence of GraD (0.1 mol%), and (3) BSA treated FFA-removed LUVs. Detergent was added at 210 s to lyse the LUVs to disperse the pH gradient. Error bars correspond to the SD from two repeats.

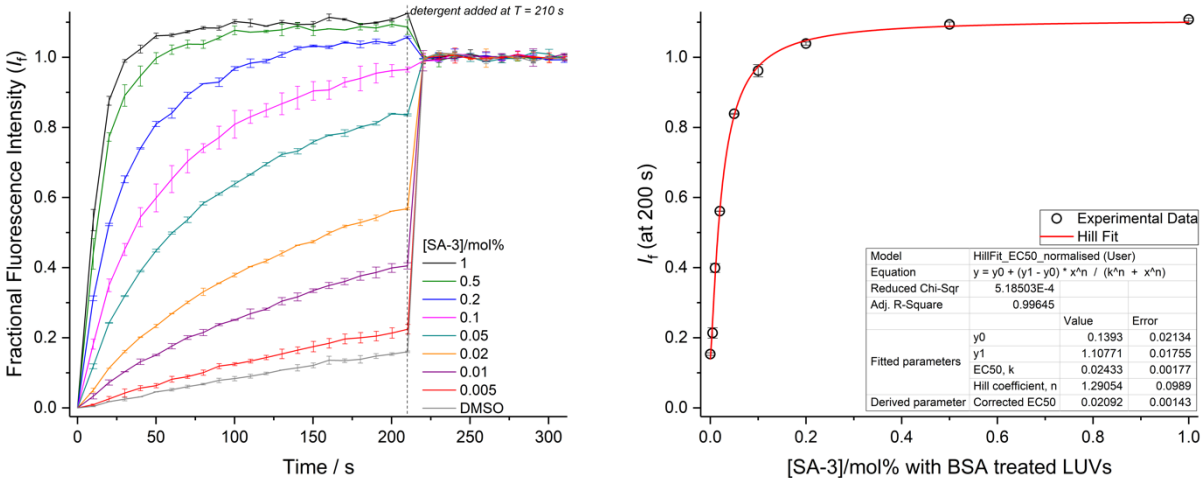


Figure S17. Hill analysis of SA-3 in NMDG-Cl assay in untreated LUVs. Plots for Hill analysis of H^+/Cl^- cotransport at 200 s facilitated by SA-3 monitored over a period of 210 s. Anionophore was added at 0 s, concentrations are shown as carrier:lipid molar percent. DMSO was used as a control and as the 0 mol% concentration data point in the Hill analysis. Detergent was added at 210 s to lyse the LUVs to disperse the pH gradient. Error bars = SD from two replicate experiments.

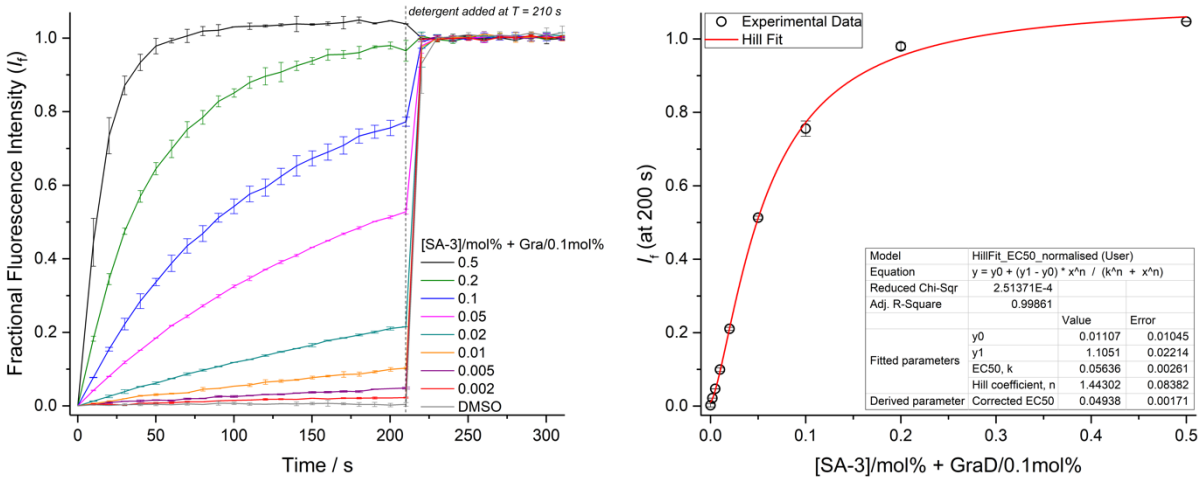


Figure S18. Hill analysis of SA-3 in NMDG-Cl assay in the presence of GraD (0.1 mol%). Plots for Hill analysis of H^+/Cl^- cotransport at 200 s facilitated by SA-3 monitored over a period of 210 s. Anionophore was added at 0 s, concentrations are shown as carrier:lipid molar percent. DMSO was used as a control and as the 0 mol% concentration data point in the Hill analysis. Detergent was added at 210 s to lyse the LUVs to disperse the pH gradient. Error bars = SD from two replicate experiments.

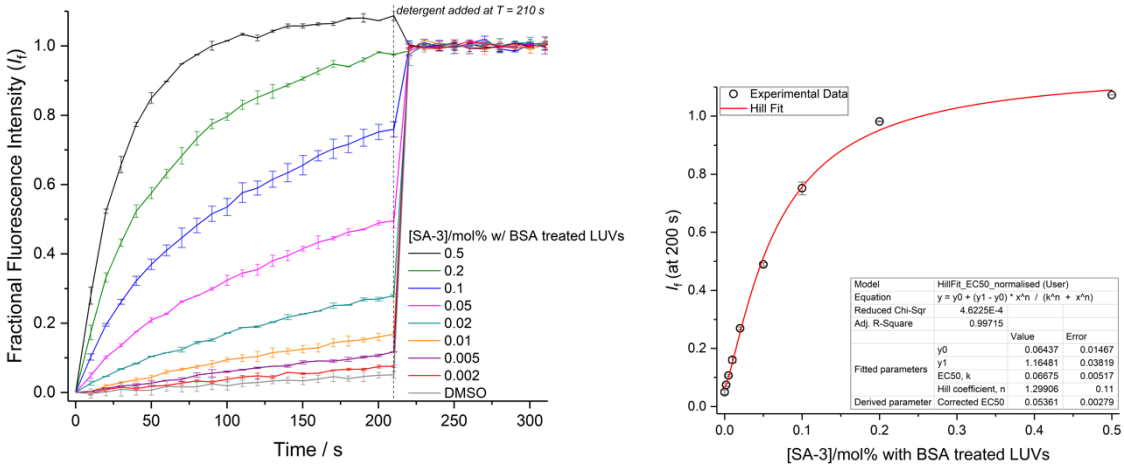


Figure S19. Hill analysis of SA-3 in NMDG-Cl assay in BSA treated FFA-removed LUVs. Plots for Hill analysis of H^+/Cl^- cotransport at 200 s facilitated by SA-3 monitored over a period of 210 s. Anionophore was added at 0 s, concentrations are shown as carrier:lipid molar percent. DMSO was used as a control and as the 0 mol% concentration data point in the Hill analysis. Detergent was added at 210 s to lyse the LUVs to disperse the pH gradient. Error bars = SD from two replicate experiments.

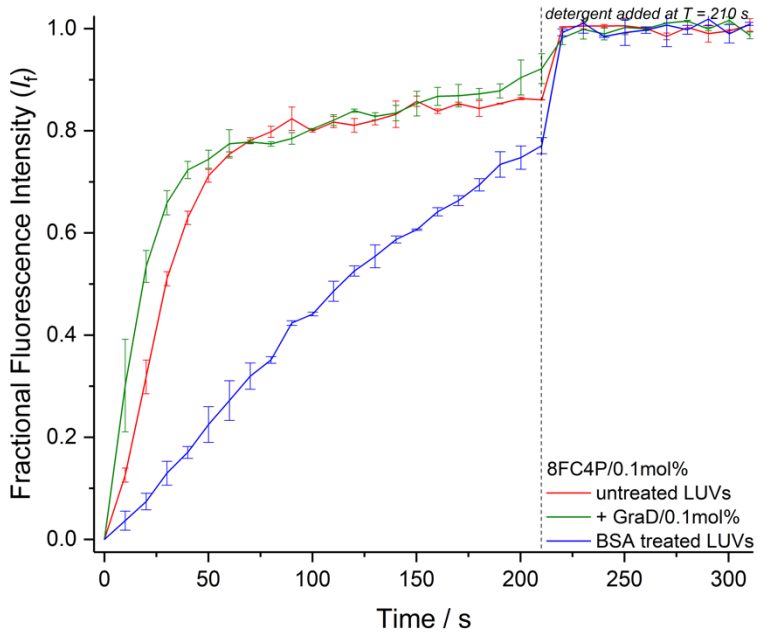


Figure S20. Overview of H^+/Cl^- cotransport by 8FC4P in NMDG-Cl assay. Plot of H^+/Cl^- cotransport facilitated by 8FC4P (0.1 mol% carrier:lipid molar percent) monitored over a period of 210 s in three different LUV conditions: (1) Untreated LUVs, (2) in the presence of GraD (0.1 mol%), and (3) BSA treated FFA-removed LUVs. Detergent was added at 210 s to lyse the LUVs to disperse the pH gradient. Error bars = SD from two replicate experiments.

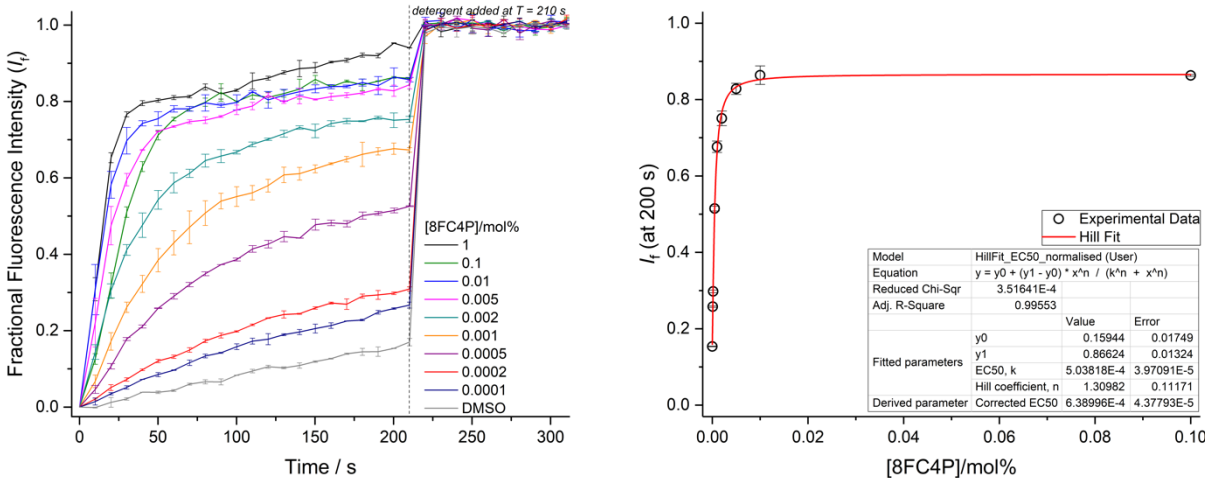


Figure S21. Hill analysis of 8FC4P in NMDG-Cl assay in untreated LUVs. Plots for Hill analysis of H^+/Cl^- cotransport at 200 s facilitated by 8FC4P monitored over a period of 210 s. Anionophore was added at 0 s, concentrations are shown as carrier:lipid molar percent. DMSO was used as a control and as the 0 mol% concentration data point in the Hill analysis. Detergent was added at 210 s to lyse the LUVs to disperse the pH gradient. Error bars = SD from two replicate experiments.

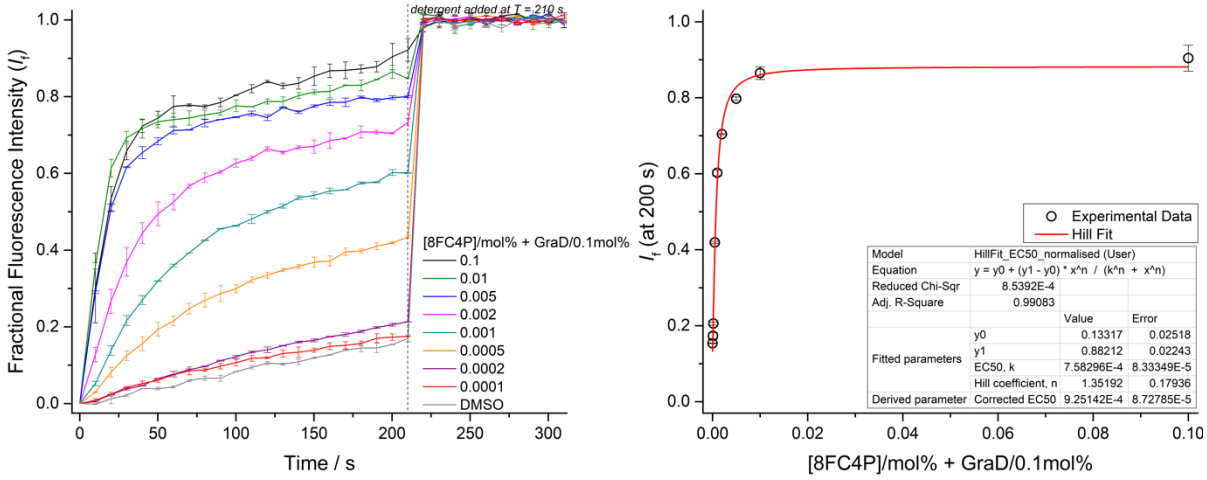


Figure S22. Hill analysis of 8FC4P in NMDG-Cl assay in the presence of GraD (0.1 mol%). Plots for Hill analysis of H^+/Cl^- cotransport at 200 s facilitated by 8FC4P monitored over a period of 210 s. Anionophore was added at 0 s, concentrations are shown as carrier:lipid molar percent. DMSO was used as a control and as the 0 mol% concentration data point in the Hill analysis. Detergent was added at 210 s to lyse the LUVs to disperse the pH gradient. Error bars = SD from two replicate experiments.

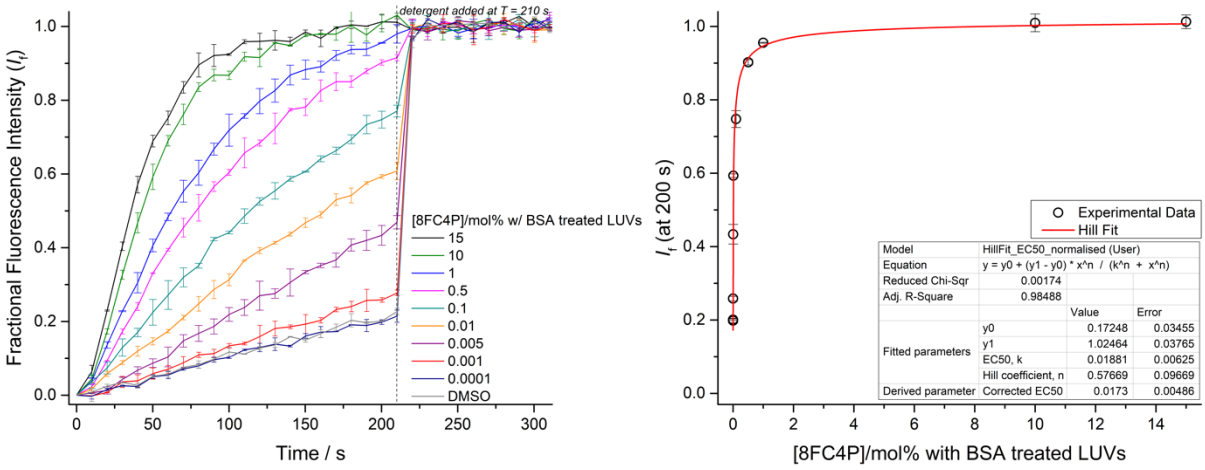


Figure S23. Hill analysis of 8FC4P in NMDG-Cl assay in BSA treated FFA-removed LUVs. Plots for Hill analysis of H^+/Cl^- cotransport at 200 s facilitated by 8FC4P monitored over a period of 210 s. Anionophore was added at 0 s, concentrations are shown as carrier:lipid molar percent. DMSO was used as a control and as the 0 mol% concentration data point in the Hill analysis. Detergent was added at 210 s to lyse the LUVs to disperse the pH gradient. Error bars = SD from two replicate experiments.

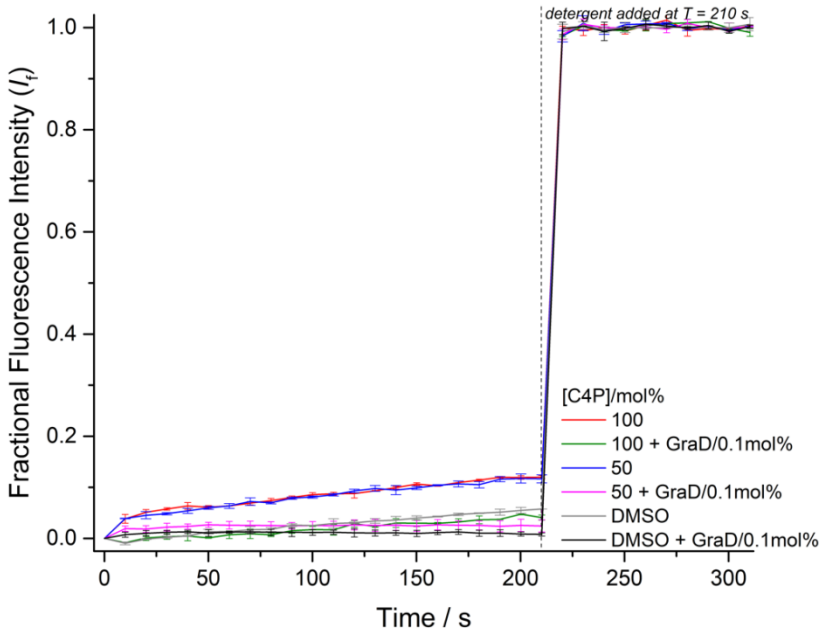


Figure S24. Overview of H^+/Cl^- cotransport by C4P in NMDG-Cl assay. Plot of H^+/Cl^- cotransport facilitated by C4P (50 mol% and 100 mol% carrier:lipid molar percent) monitored over a period of 210 s in two different LUV conditions: (1) Untreated LUVs and (2) in the presence of GraD (0.1 mol%). DMSO was used as a control. Detergent was added at 210 s to lyse the LUVs to disperse the pH gradient. Error bars = SD from two replicate experiments.

BIOLOGY

Cell culture and transfection. HeLa (human cervical cancer cells), A549 (human lung carcinoma epithelial cells), PLC/PRF/5 (human hepatoma cells), and HepG2 (human hepatocellular carcinoma cells) cells were cultured in RPMI 1640 (Invitrogen), DMEM (Invitrogen), or MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin and 50 units/mL streptomycin. FRT (Fischer rat thyroid epithelial) cells were cultured in Coon's modified F12 medium supplemented with 10% FBS, 50 units/mL penicillin and 50 units/mL streptomycin. HeLa cells were transfected with the tandem mRFP-EGFP-LC3 plasmid (Addgene) by using Lipofectamin 2000 (Invitrogen) and stably transfected cells were selected using 600 μg/mL G418 (Tocris) over 3 weeks. Cells were maintained at 37 ºC under a humidified atmosphere of 5% CO_2.

Measurement of intracellular Cl^- concentrations. Intracellular chloride concentrations were measured by using MQAE according to methods described previously.[1-3] HeLa, A549, and FRT cells were detached from the culture dish by treating with 2% trypsin-EDTA solutions in DPBS. After washing cells with DPBS, the number of live cells stained with 0.5% trypan blue was counted by using a hemacytometer. The suspended cells (5 x 10^3 cells) in culture media were seeded in triplicate onto 96-well plates. After 24 hours, the cells were washed with fresh culture media. One of 96-wells containing cells was treated with propidium iodide (PI) to detect dead cells. Mostly, no dead cells were observed. Determination of Stern-Volmer constants (K_{sv}) of MQAE in HeLa, A549 and FRT cells were initiated by treatment of the cultured cells with solutions containing 0–60 mM chloride ions, which were prepared by mixing 10 mM glucose, 5 mM HEPES and 150 mM KCl with 10 mM glucose, 5 mM HEPES and 150 mM potassium gluconate, followed by treatment with 10 μM tributyltin cyanide (a Cl^-/OH^- exchanger) and 5 μM nigericin (a K^+/H^+ exchanger). After 30 min incubation at 37 ºC, cells were washed with PBS. The cells were treated with 5 mM MQAE for 30 min at 37 ºC. After washing, the fluorescence intensity of MQAE in cells was measured using an Infinite® 200 PRO multimode microplate reader (TECAN, Austria) (λ_{ex} = 350 nm, λ_{em} = 460 nm). Stern-Volmer constants (K_{sv}) were calculated from a liner fit equation of $\frac{Fo}{F} = 1 + K_{sv}[Cl^-]$, where F_0 is the fluorescence intensity of MQAE at 0 mM Cl^- and F is the fluorescence intensity of MQAE at various concentrations of Cl^-. The Stern-Volmer constants were determined to be 39.6, 31.3 and 34.4 M^{-1} for FRT, A549 and HeLa cells, respectively (see Figure S25a).

To determine intracellular Cl^- concentrations, HeLa, A549 and FRT cells were treated with indicated concentrations of DSC4P-1, SA-3, 8FC4P or C4P in culture media for 2 hours at 37 ºC. After washing with PBS, cells were incubated with 5 mM MQAE for 30 min. After washing, the fluorescence intensity of MQAE in cells was measured using a microplate reader. By using Stern-Volmer plots, intracellular Cl^- concentrations were determined.

Determination of changes in Na^+ concentrations. HeLa, A549, and FRT cells were detached from the culture dish by treating with 2% trypsin-EDTA solutions in DPBS. After

washing cells with DPBS, the number of live cells stained with 0.5% trypan blue was counted by using a hemacytometer. The suspended cells (5 x 10^3 cells) in culture media were seeded in triplicate onto 96-well plates. After 24 hours, the cells were washed with fresh culture media. One of 96-wells containing cells was treated with propidium iodide (PI) to detect dead cells. Mostly, no dead cells were observed. The cells were treated with 10 μM SBFI-AM (ThermoFisher Sicentific) in culture media for 2 hours at 37 ºC. After washing with PBS to remove the remaining SBFI-AM, the cells were incubated with the indicated concentrations of DSC4P-1, SA-3, 8FC4P, or C4P in culture media for 2 hours at 37 ºC. The SBFI-AM fluorescence was measured using a microplate reader (λ_{ex} = 340 nm, λ_{em} = 500 nm).

Determination of changes in K^+ concentrations. HeLa, A549, and FRT cells were detached from the culture dish by treating with 2% trypsin-EDTA solutions in DPBS. After washing cells with DPBS, the number of live cells stained with 0.5% trypan blue was counted by using a hemacytometer. The suspended cells (5 x 10^3 cells) in culture media were seeded in triplicate onto 96-well plates. After 24 hours, the cells were washed with fresh culture media. One of 96-wells containing cells was treated with propidium iodide (PI) to detect dead cells. Mostly, no dead cells were observed. The cells were treated with 10 μM PBFI-AM (ThermoFisher Sicentific) in culture media for 1.5 hour at 37 ºC. After washing with PBS to remove the remaining PBFI-AM, solutions of HEPES buffer were added to the cells. Cells were incubated with DSC4P-1, SA-3, 8FC4P or C4P for 2 hours at 37 ºC. The PBFI fluorescence was measured using a microplate reader (λ_{ex} = 340 nm, λ_{em} = 500 nm).

Determination of changes in Ca^{2+} concentrations. HeLa, A549, and FRT cells were detached from the culture dish by treating with 2% trypsin-EDTA solutions in DPBS. After washing cells with DPBS, the number of live cells stained with 0.5% trypan blue was counted by using a hemacytometer. The suspended cells (5 x 10^3 cells) in culture media were seeded in triplicate onto 96-well plates. After 24 hours, the cells were washed with fresh culture media. One of 96-wells containing cells was treated with propidium iodide (PI) to detect dead cells. Mostly, no dead cells were observed. The cells were treated with 10 μM Fluo-4 NW (ThermoFisher Sicentific) in culture media for 1 hour at 37 ºC. After washing with PBS to remove the remaining Fluo-4 NW, solutions of HEPES buffer were added to each well. Cells were incubated with DSC4P-1, SA-3, 8FC4P or C4P for 2 hours at 37 ºC. Fluo-4 fluorescence was measured using a microplate reader (λ_{ex} = 485 nm, λ_{em} = 538 nm).

Measurement of cell death. Cancer cells were detached from the culture dish by treating with 2% trypsin-EDTA solutions in DPBS. After washing cells with DPBS, the number of live cells stained with 0.5% trypan blue was counted by using a hemacytometer. The suspended cells (5 x 10^3 cells) in culture media were seeded in triplicate onto 96-well plates. After 24 hours, the cells were washed with fresh culture media. One of 96-wells containing cells was treated with propidium iodide (PI) to detect dead cells. Mostly, no dead cells were observed. Cancer cells were incubated with various concentrations of DSC4P-1, SA-3, 8FC4P, or C4P in culture media. MTT assays were performed using standard procedures. The absorbance at 570 nm was measured by means of a microplate reader.

Sodium and chloride depletion study. HEPES-buffered solutions were prepared with the following compositions: 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose, 10 mM HEPES (pH 7.4), and 25 mM $NaHCO_3$. To prepare Na^+–free or Cl^-–free HEPES-buffered solutions, Na^+ or Cl^- ions in the buffer solutions were replaced with equimolar concentrations of either NMDG (N-methyl-D-glucamine) or gluconate salts, respectively. Cancer cells were incubated for 18 hours using the indicated concentrations of DSC4P-1, SA-3, 8FC4P, or C4P in normal, Na^+– free, or Cl^-–free HEPES buffer solutions at 37 °C under a humidified atmosphere of 5% CO_2. MTT assays were conducted using standard procedures.

Flow cytometry. For analysis of apoptosis, HeLa cells were incubated with DSC4P-1, SA-3, 8FC4P, or C4P for 24 hours at 37 °C. Untreated cells were used as a negative control. After washing with PBS twice, the cells were trypsinized with 0.5 mL of trypsin-EDTA (0.05% trypsin, 0.02% EDTA, Sigma-Aldrich) for 5-10 min at 37 °C and collected by centrifugation. The cells were stained with the FITC-annexin V apoptosis detection kit with propidium iodide (PI) according to the manufacturer's protocol. Flow cytometry was performed using a BD FACSVerse™ instrument (BD Biosciences) and the data were analyzed using the FlowJo™ software (BD Biosciences). For staining of the membrane potential-sensitive probe JC-1, cells treated with DSC4P-1, SA-3, 8FC4P, or C4P for 24 hours were incubated with JC-1 (final concentration = 2.5 μg/mL, AnaSpec) in PBS for 15 min at 37 °C. The red fluorescence signal was measured by monitoring the emission at 600 nm seen upon excitation at 550 nm. The green fluorescence was measured by monitoring the emission at 485 nm upon excitation at 535 nm.

Determination of cell size by flow cytometry. HeLa cells were incubated with DSC4P-1, SA-3, 8FC4P or C4P for 18 hours at 37 °C. Untreated cells were used as a negative control. Cell size was measured using a flow cytometer by exciting with a 488-nm argon laser and determining the distribution on a forward scatter versus side scatter dot plot. Light scattered in the forward direction is proportional to cell size, and light scattered at a 90° angle (side scatter) is proportional to cell density.

DNA fragmentation assays. HeLa cells were incubated with DSC4P-1, SA-3, 8FC4P, or C4P for 24 hours at 37 °C. The cells were lysed in a buffer containing 10 mM Tris, 1 mM EDTA, and 0.2% Triton X-100 at pH 8.0. Samples were incubated with 100 μg/mL RNase A for 0.5 h at 37 °C and then 100 μg/mL proteinase K for 10 min at 56 °C. The DNA was precipitated by adding 0.5 M NaCl-isopropyl alcohol. It was then washed with 70% ethanol. Samples were loaded on a 1.5% agarose gel and were subjected to electrophoresis at 100 V for 30 min in TBE (Tris/Borate/EDTA) buffer (0.5X). The resulting DNA was stained with RedSafe™ Nucleic Acid Staining Solution (Intron, Korea) and analyzed by a G:BOX Chemi Fluorescent & Chemiluminescent Imaging System (Syngene).

Measurements of changes in cell size by confocal microscopy. HeLa cells were incubated with Hoechest 33342 for 10 min. After washing with DPBS to remove the remaining

Hoechest 33342 stain, the cells were treated with DSC4P-1, SA-3, 8FC4P, or C4P in culture media. The change in cell size was monitored over a 1.5 hour period following initial incubation by means of confocal fluorescence microscopy (Zeiss LSM 800). Cell images were analyzed to measure changes in the cell size by drawing a region of interest (ROI) around the cell boundaries using the ImageJ software.

Isolation of cytosolic and mitochondrial fractions. HeLa cells were incubated with DSC4P-1, SA-3, 8FC4P, or C4P for 18 hours. Mitochondrial and cytosol fractions of the cells were prepared using a mitochondrial/cytosol fractionation kit (Biovision). Cells were harvested by centrifugation at 600 × g for 5 min and washed twice with cold PBS buffer. The cells were re-suspended in 250 μL extraction buffer containing the protease inhibitor mixture (Biovision) and dithiothreitol. After allowing to incubate on ice for 30 min, the cells were homogenized on ice and centrifuged at 700 × g for 10 min at 4 °C, and the supernatant collected. This supernatant was centrifuged again at 10,000 × g for 30 min at 4 °C. The resulting supernatant was harvested and used as the cytosolic fractions. The pellets were re-suspended and used as the mitochondrial fractions.

Western blot analysis. Proteins were separated by 6–12% SDS-PAGE. Rabbit caspase-3 (H277) polyclonal (1:1000, sc-7272, Santa Cruz Bio Technology), rabbit cleaved caspase-3 (Asp175) polyclonal (1:1000, #9661, Cell Signaling Technology), rabbit PARP polyclonal (1:1000, #9542, Cell Signaling Technology), mouse cytochrome c monoclonal (1:1000, k257, Biovision), rabbit anti-phospho-AMPK polyclonal (1:1000, #2531, Cell Signaling Technology), rabbit anti-phospho-beclin-1 polyclonal (1:1000, PA5-35394, ThermoFisher Scientific), rabbit anti-phospho-p38 polyclonal (1:1000, # 9211, Cell Signaling Technology), rabbit PKC polyclonal (1:1000, ab59411, Abcam), rabbit anti-LC3 monoclonal (1:2000, L8919, Sigma), mouse anti-p62 monoclonal (1:1000, sc-28359, Santa Cruz Bio Technology), mouse Bcl-2 monoclonal (1:1000, sc-7382, Santa Cruz Bio Technology), rabbit Bcl-xL monoclonal (1:1000, #2764, Cell Signaling Technology), rabbit Bid polyclonal (1:1000, #2002, Cell Signaling Technology), rabbit Bim monoclonal (1:1000, #2933, Cell Signaling Technology) and mouse anti-β-actin monoclonal (1:1000, sc-47778, Santa Cruz Bio Technology) antibodies were used as primary antibodies. Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000, sc-2357, Santa Cruz Bio Technology) and goat anti-mouse IgG (1:2000, sc-516102, Santa Cruz Bio Technology) were used as the secondary antibodies. The blots were developed by using a West-ZOL® plus Western Blot Detection System (Intron Biotechnology Inc., South Korea). Protein bands were analyzed using a G:BOX Chemi Fluorescent & Chemiluminescent Imaging System.

PLC activity assay. HeLa cells were incubated with the indicated concentrations of DSC4P-1, SA-3, 8FC4P, or C4P for 3 hours and the PLC activity was measured using an EnzChek direct PLC assay kit (Molecular Probes) according to the manufacturer's instruction.

Measurement of the level of ROS. HeLa cells were incubated with the indicated concentrations of DSC4P-1, SA-3, 8FC4P, or C4P over an 8 hour period. The cells were

incubated with 10 μM PF1 for 1 hour. The fluorescence intensity of PF1 was measured by using a microplate reader (λ_{ex} = 485 nm, λ_{em} = 535 nm).

Immunocytochemistry. HeLa cells were incubated with the indicated concentrations of DSC4P-1, SA-3, 8FC4P, or C4P for 12 hours at 37 ºC. The cells were incubated with MitoTracker Red (ThermoFisher Scientific) for 30 min followed by fixation with 4% formaldehyde in PBS buffer for 15 min. The cells were treated with mouse active monomeric Bax monoclonal antibody (1:200, Enzo Life Science) for 1 hour at room temperature followed by incubation with Alexa-Fluor 488 conjugated mouse IgG (1:200, Invitrogen, Molecular Probes) for 1 hour at room temperature and mounted with DAPI (Invitrogen- S86 Molecular Probes). The cells were imaged by means of confocal fluorescence microscopy. Cell images were analyzed using the ZEN 2011 software.

Measurement of caspase activity. HeLa cells were incubated with various concentrations of DSC4P-1, SA-3, 8FC4P, or C4P for 18 hours at 37 ºC. The cells were lysed in a buffer containing 50 mM HEPES at pH 7.4, 5 mM CHAPS, and 5 mM DTT. Cell lysates were placed into the appropriate wells of 96-well plate. Assay buffer containing 20 mM HEPES at pH 7.4, 0.01% CHAPS, 5 mM DTT and 2 mM EDTA was added to each of the wells. A caspase inhibitor Ac-DEVD-CHO (20 μM) was added to the appropriate wells. Caspase activity was determined by adding 200 μM Ac-DEVD-pNA (Sigma-Aldrich, USA) to each of the wells. The enzyme-catalyzed release of pNA was monitored at 405 nm using a microplate reader.

Measurement of lysosomal Cl^- concentrations. HeLa cells were incubated with various concentration of DSC4P-1, SA-3, 8FC4P, or C4P for 4 hours at 37 ºC. The cells were incubated with 5 mM MQAE-MP for 30 min. The cells were imaged by means of confocal fluorescence microscopy. Cell images were analyzed using the ZEN 2011 software.

Determination of cathepsin B and L activities in cells. HeLa cells were incubated with the indicated concentrations of DSC4P-1, SA-3, 8FC4P, or C4P in culture media for 6 hours at 37 °C. The cells were treated with 1 μL MR-$(RR)_2$ or 1 μL MR-$(FR)_2$ (ThermoFisher Scientific) in culture media for an additional 4 h at 37 ºC. After washing twice with 400 μL Dulbecco's phosphate buffered saline (DPBS, without calcium and magnesium) to remove remaining fluorogenic substrates, the cells were fixed in 2.5% formaldehyde for 10 min at 37 ºC and were imaged by means of confocal fluorescence microscopy. The images obtained in this way were analyzed using the ZEN2011 software.

Fluorescence cell imaging. HeLa cells, stably expressing mRFP-EGFP-LC3, were incubated with the indicated concentrations of DSC4P-1, SA-3, 8FC4P, C4P, torin-1 or bafilomycin A1. The cells were fixed in 2.5% formaldehyde for 10 min at 37 ºC. The cells were imaged by using confocal fluorescence microscopy. Cell images were analyzed by means of the ZEN 2011 software.

Supplementary References

(1) Verkman, A.S., Sellers, M.C., Chao, A.C., and Leung, T. (1989). Synthesis and characterization of improved chloride-sensitive fluorescent indicators for biological applications. Anal. Biochem. 178, 355-361.

(2) Rocha-González, H.I., Mao, S., and Alvarez-Leefmans, F.J. (2008). Na^+, K^+, $2Cl^-$ cotransport and intracellular chloride regulation in rat primary sensory neurons: thermodynamic and kinetic aspects. J. Neurophysiol. 100, 169-184.

(3) Koncz, C., and Daugirdas J.T. (1994). Use of MQAE for measurement of intracellular [Cl^-] in cultured aortic smooth muscle cells. Am. J. Physiol. 267, H2114-2123.

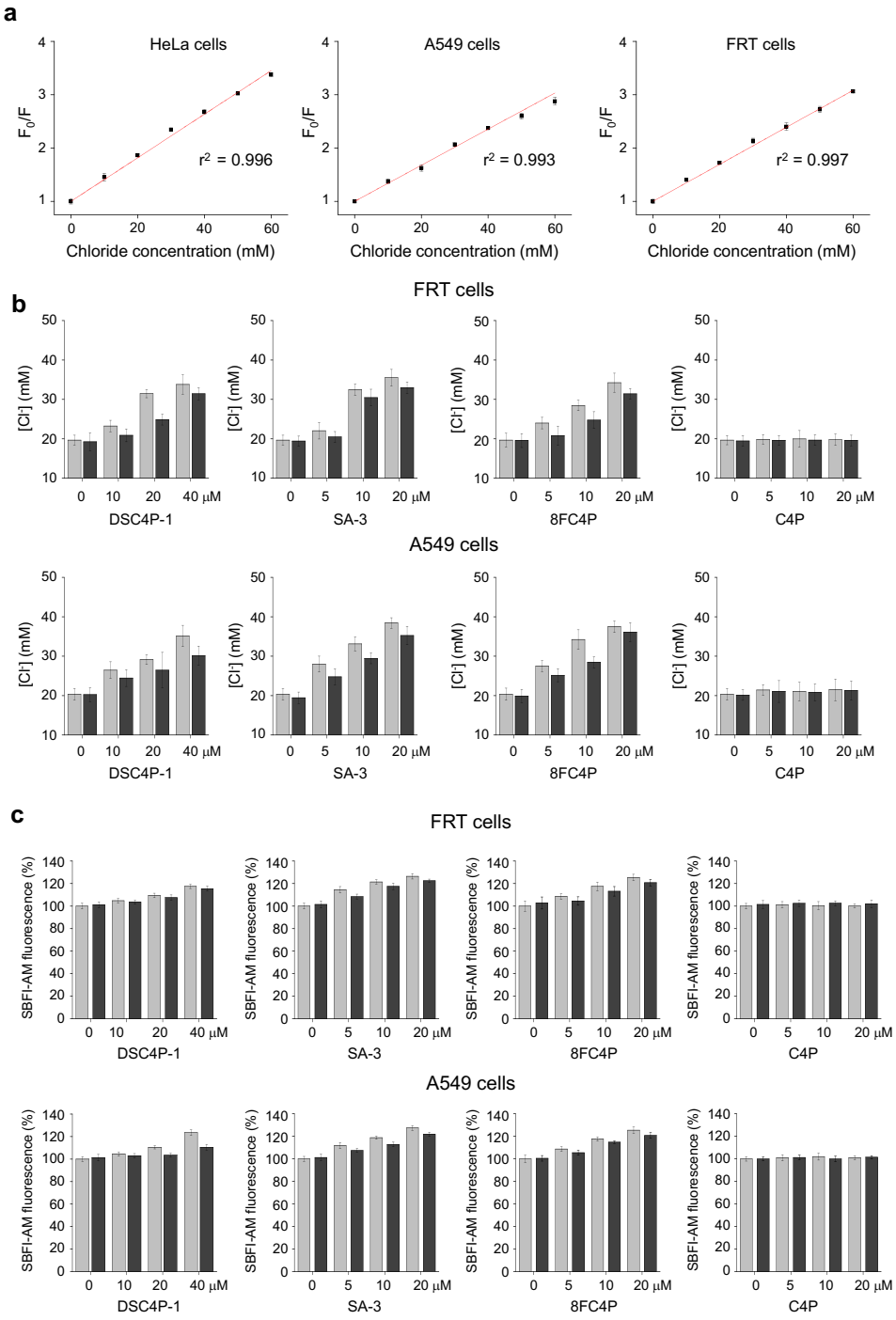


Figure S25. Effect of synthetic ion transporters on intracellular Cl^- and Na^+ concentrations. (a) Stern-Volmer plots for quenching of MQAE by Cl^- (F_0: fluorescence intensity in the absence of Cl^-, F: fluorescence in the presence of Cl^-) (mean ± s.d., n = 3). The Stern-Volmer constants were determined to be 39.6, 31.3 and 34.4 M^{-1} for FRT, A549 and HeLa cells respectively. (b) FRT and A549 cells were treated for 2 hr with various concentrations of the indicated compounds in the absence (grey bars) and presence (black bars) of 1 mM amiloride. After washing with DPBS three times, the cells were incubated with 5 mM MQAE for 0.5 hr. Intracellular chloride ion concentrations were determined using Stern-Volmer plots shown in (a) (mean ± s.d., n = 3). (c) FRT and A549 cells were treated with

10 μM SBFI-AM for 2 hr and then the incubated with various concentrations of indicated compounds for 2 hr in the absence (grey bars) and presence (black bars) of 1 mM amiloride. The SBFI-AM fluorescence was monitored to determine changes in the intracellular sodium ion concentrations (mean ± s.d., n = 3).

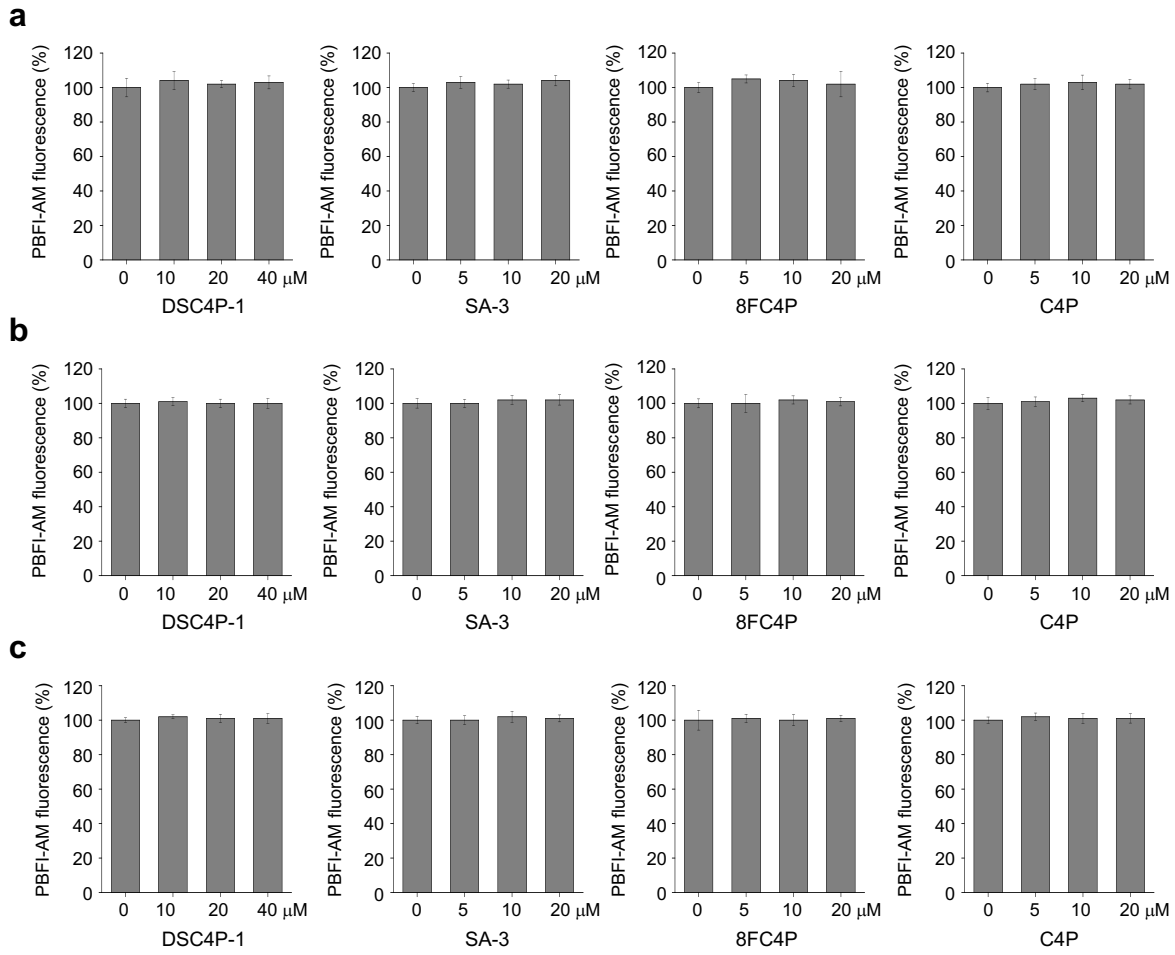


Figure S26. Effect of synthetic ion transporters on intracellular K^+ concentrations. (a) FRT, (b) HeLa and (c) A549 cells were treated with 10 μM PBFI-AM for 2 hr and then incubated with various concentrations of the indicated compounds for 2 hr. The PBFI fluorescence intensity was monitored to determine changes in the intracellular potassium ion concentrations (mean ± s.d., n = 3).

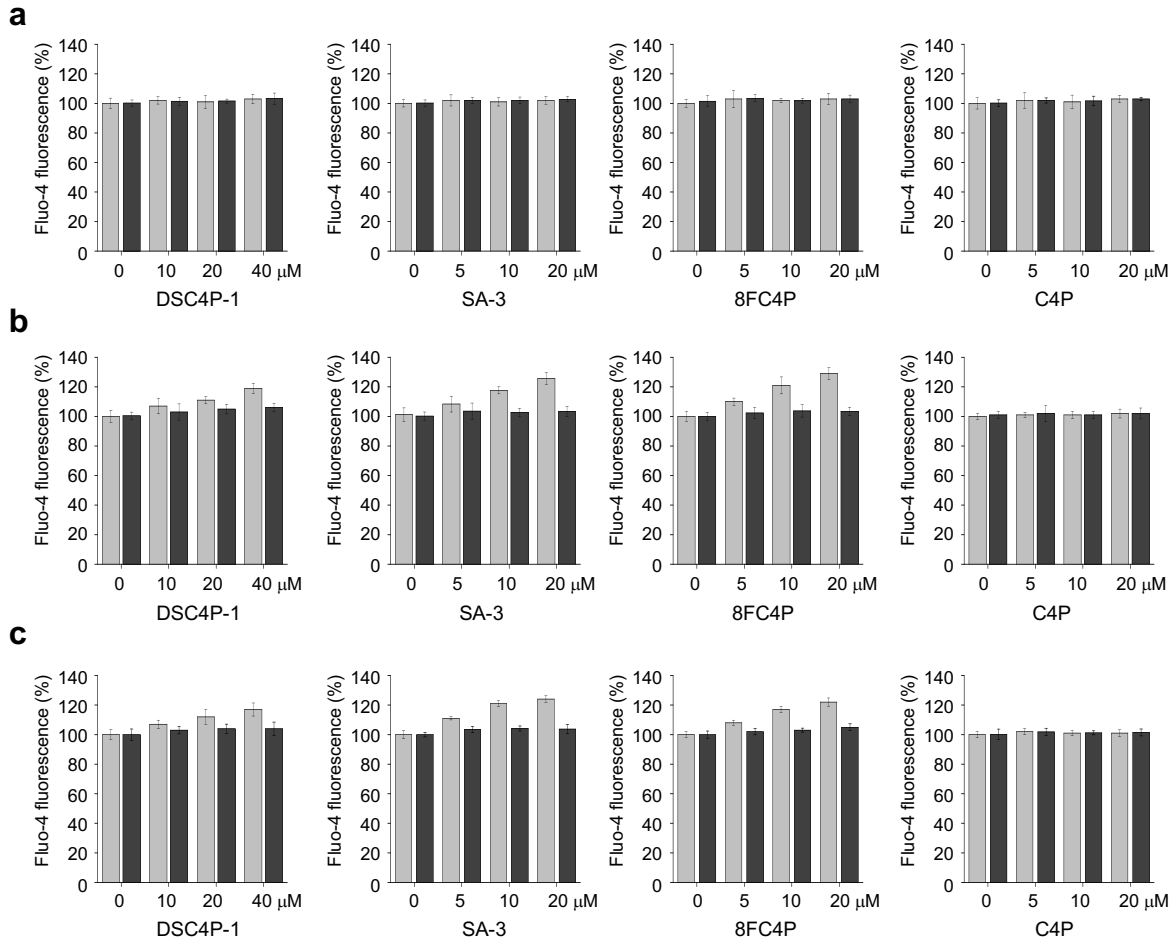
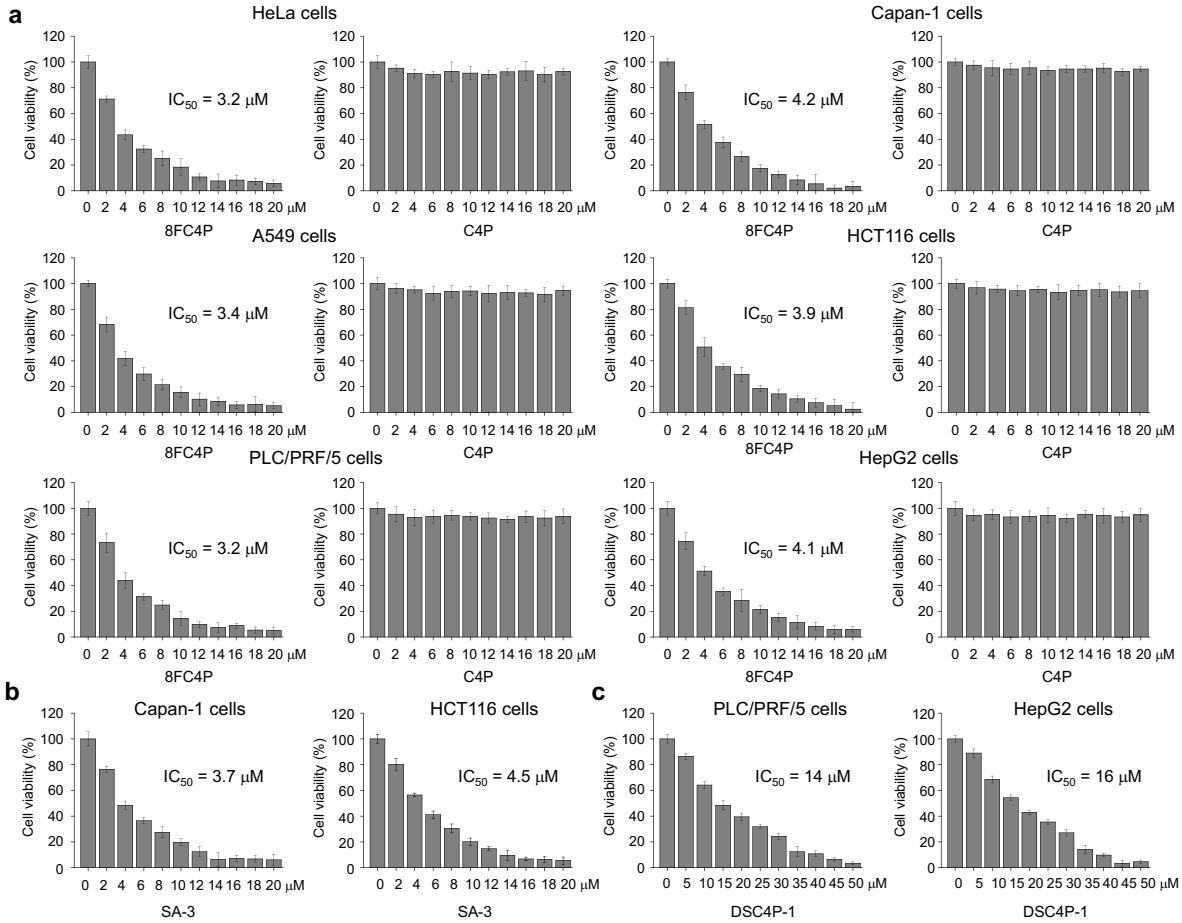


Figure S27. Effect of synthetic ion transporters on intracellular Ca^{2+} concentrations. (a) FRT, (b) HeLa and (c) A549 cells were treated with 10 μM Fluo-4 NW for 2 hr and then incubated with various concentrations of the indicated compounds for 2 hr in the absence (grey bars) and presence (black bars) of an IP3 receptor inhibitor, 2-APB (10 μM). The Fluo-4 NW fluorescence intensity was monitored to determine changes in the intracellular calcium ion concentrations (mean ± s.d., n = 3).



d

	8FC4P (μM)[a]	SA-3 (μM)[b]	DSC4P-1 (μM)[c]
HeLa	3.2 ± 0.27	3.4 ± 0.19	10 ± 0.48
A549	3.4 ± 0.34	3.1 ± 0.21	15 ± 0.26
PLC	3.2 ± 0.35	4.2 ± 0.31	14 ± 0.42[a]
HepG2	4.2 ± 0.16	2.6 ± 0.29	16 ± 0.32[a]
Capan-1	3.9 ± 0.29	3.7 ± 0.24[a]	15 ± 0.32
HCT116	4.1 ± 0.32	4.5 ± 0.37[a]	15 ± 0.24

[a]This study
[b]*Nat. Chem.* **9**, 667–675 (2017).
[c]*Nat. Chem.* **6**, 885–892 (2014)

Figure S28. Determination of IC_{50} values of synthetic ion transporters. (a) HeLa, A549, PLC, HepG2, Capan-1, and HCT116 cells were treated with various concentrations of the indicated substances for 24 hr. Cell death was measured by means of an MTT assay (mean ± s.d., n = 3). (b) Capan-1 and HCT116 cells were treated with various concentrations of SA-3 for 24 hr. Cell death was measured by means of an MTT assay (mean ± s.d., n = 3). (c) PLC and HepG2 cells were treated with various concentrations of DSC4P-1 for 24 hr. Cell death was measured by means of an MTT assay (mean ± s.d., n = 3). (d) IC_{50} values (μM) for the synthetic ion transporters of this study in various cancer cell lines (mean ± s.d., n = 3).

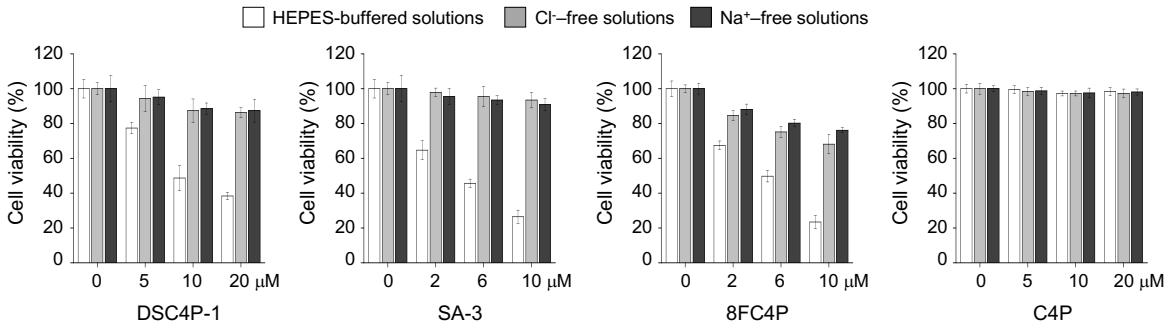


Figure S29. **Effect of ions on synthetic ion transporter-induced cell death.** A549 cells were incubated for 18 hr with various concentrations of the synthetic ion transporters of this study in normal HEPES-buffered solutions, Cl^--free HEPES-buffered solutions, or Na^+-free HEPES-buffered solutions. Cell death was measured using MTT assays (mean ± s.d., n = 3).

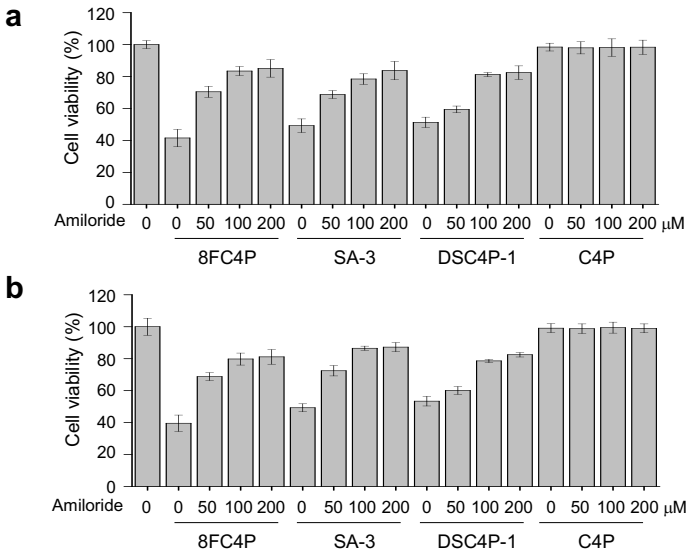


Figure S30. **Effect of amiloride on cell death induced by synthetic ion transporters.** (a) HeLa and (b) A549 cells were incubated for 24 hr with 5 μM 8FC4P, 5 μM SA-3, 20 μM DSC4P-1, or 20 μM C4P in the presence of various concentrations of amiloride. Cell death was measured by means of an MTT assay (mean ± s.d., n = 3).

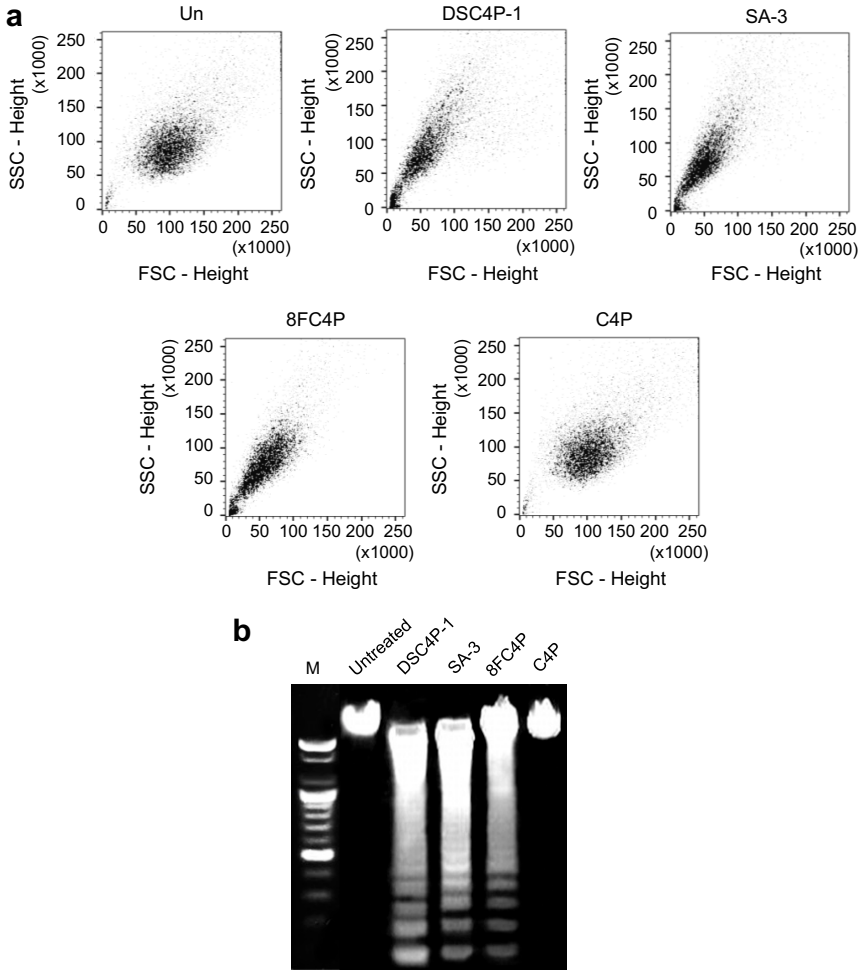


Figure S31. Synthetic ion transporters induce apoptosis. (a) HeLa cells were treated with 40 μM DSC4P-1, 10 μM SA-3, 10 μM 8FC4P, or 40 μM C4P for 24 hr and the cell size was determined by means of flow cytometry (FSC; forward scatter, SSC; side scatter). Untreated cells are shown as a negative control. (b) HeLa cells were treated with 40 μM DSC4P-1, 10 μM SA-3, 10 μM 8FC4P, or 40 μM C4P for 24 hr. The DNA fragments were visualized by staining with the RedSafe™ nucleic acid staining solution.

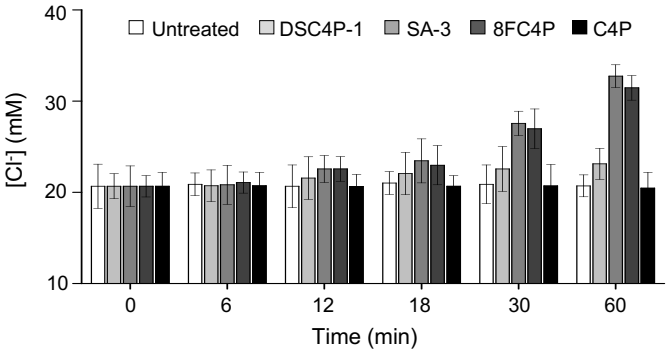


Figure S32. Synthetic ion transporters increase intracellular Cl^- concentrations at early times. HeLa cells, pretreated with 5 mM MQAE for 0.5 hr, were incubated with 20 μM DSC4P-1, 10 μM SA-3, 10 μM 8FC4P, or 20 μM C4P for the indicated time period. Intracellular chloride ion concentrations were determined using Stern-Volmer plots shown in Figure S25a (mean ± s.d., n = 3).

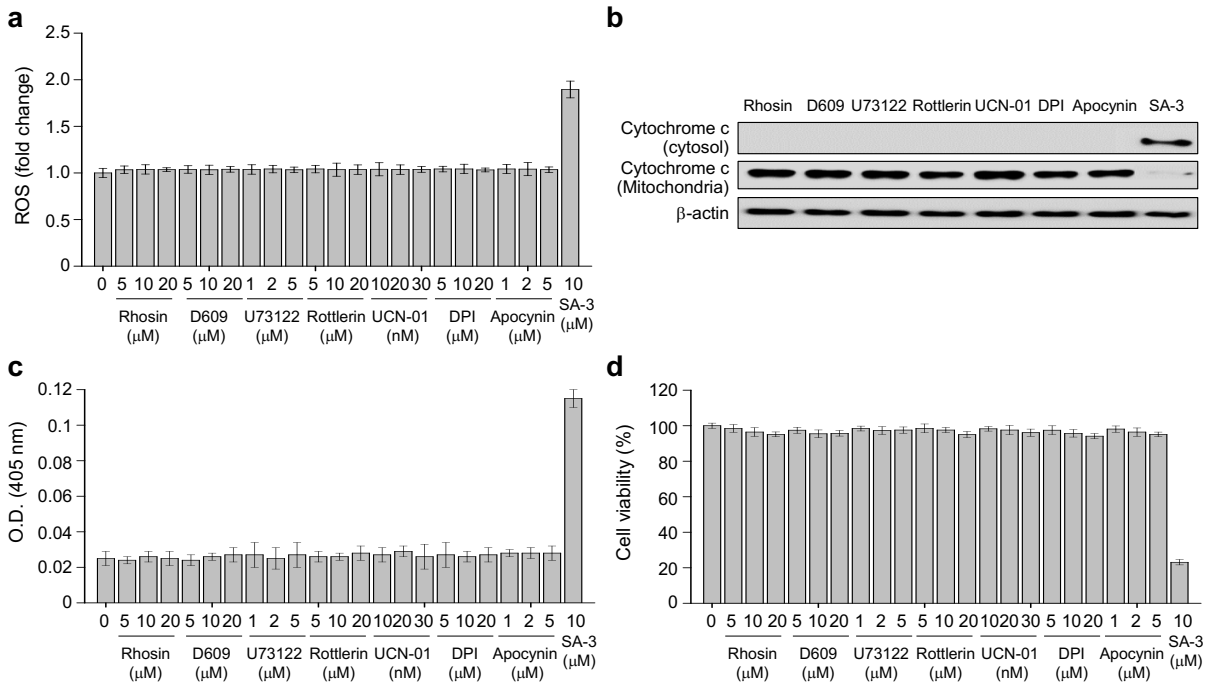


Figure S33. Inhibitors do not affect apoptotic cell death. (a) HeLa cells were individually incubated for 8 hr with indicated concentrations of each inhibitor. Treated cells were incubated with 10 μM PF1 for 1 hr. The fluorescence intensity of PF1 was measured using a microplate reader (mean ± s.d., n = 3). (b) HeLa cells were individually treated for 18 hr with 20 μM rhosin, 10 μM D609, 5 μM U73122, 10 μM rottlerin, 20 nM UCN-01, 10 μM DPI and 5 μM apocynin. The treated cells were subjected to western blotting with the corresponding antibodies. SA-3 (10 μM) was used as a positive control. (c) HeLa cells were separately treated for 18 hr with indicated concentrations of each inhibitor. The caspase activities of the cell lysates were determined using 200 μM Ac-DEVD-pNA (mean ± s.d., n = 3). (d) HeLa cells were treated for 24 hr with indicated concentrations of each inhibitor. Cell death was measured by means of an MTT assay (mean ± s.d., n = 3).

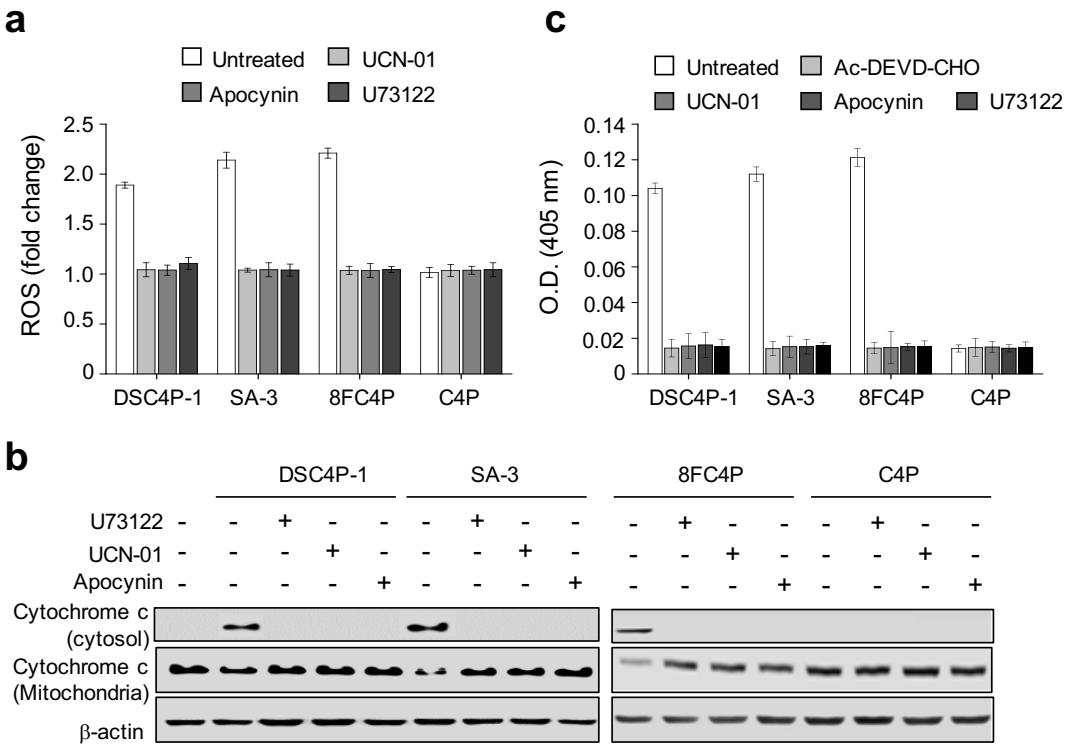


Figure S34. Co-treatment of the synthetic ion transporters with inhibitors blocks caspase activation. (a) HeLa cells were individually incubated for 8 hr with 20 μM DSC4P-1, 10 μM SA-3, 10 μM 8FC4P and 20 μM C4P in the absence and presence of each of indicated inhibitors (20 nM UCN-01, 5 μM apocynin and 5 μM U73122). Treated cells were incubated with 10 μM PF1 for 1 h. The fluorescence intensity of PF1 was measured using a microplate reader (mean ± s.d., n = 3). (b) HeLa cells, treated with 10 μM 8FC4P, 10 μM SA-3, 40 μM DSC4P-1 or 40 μM in the absence and presence of each of indicated inhibitors (20 nM UCN-01, 5 μM apocynin and 5 μM U73122) for 18 hr, were subjected to western blotting with the corresponding antibodies. (c) HeLa cells were separately incubated for 18 hr with 20 μM DSC4P-1, 10 μM SA-3, 10 μM 8FC4P and 20 μM C4P. The caspase activities of the cell lysates were determined using 200 μM Ac-DEVD-pNA in the absence and presence of 20 μM Ac-DEVD-CHO (mean ± s.d., n = 3). In addition, HeLa cells were separately incubated for 18 hr with 20 μM DSC4P-1, 10 μM SA-3, 10 μM 8FC4P and 20 μM C4P in the presence of each of indicated inhibitors (20 nM UCN-01, 5 μM apocynin, 5 μM U73122). The caspase activities of the cell lysates were determined using Ac-DEVD-pNA (200 μM).

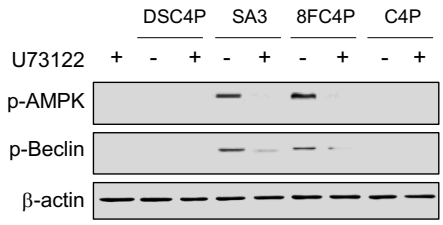


Figure S35. Co-treatment of synthetic ion transporters with U73122 blocks autophagy induction. HeLa cells, treated with 20 μM DSC4P-1, 10 μM SA-3, 10 μM 8FC4P or 20 μM C4P in the absence and presence of 5 μM U73122 for 8 hr, were subjected to immunoblotting with the corresponding antibodies.

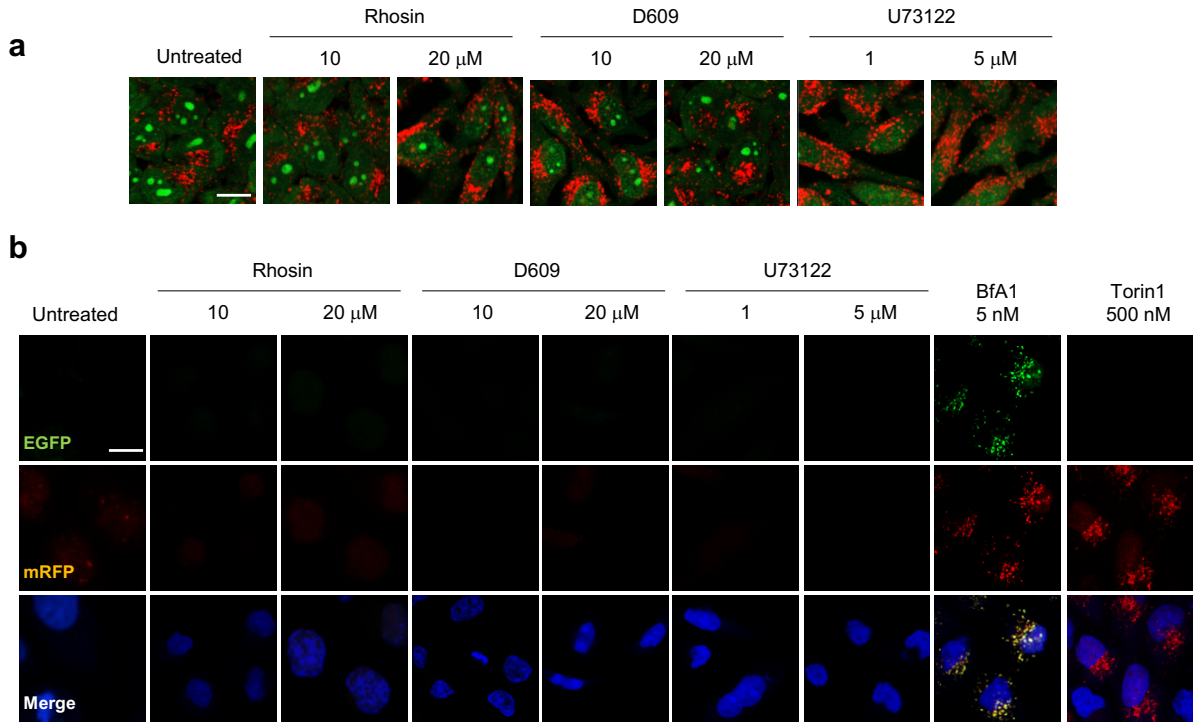


Figure S36. Rhosin, D609 and U73122 do not affect autophagy. (a) HeLa cells, treated with indicated concentrations of rhosin, D609 or U73122 for 4 hr, were incubated with 100 nM acridine orange for 30 min. Cell images were obtained by means of confocal microscopy (scale bar: 10 μm). (b) HeLa cells stably expressing mRFP-EGFP-LC3 fusion protein were treated with indicated concentrations of rhosin, D609 or U7312 for 12 hr. Cell images were obtained by means of confocal microscopy (scale bar: 10 μm). BfA1 and torin-1 were used as controls for autophagy inhibition and induction, respectively.

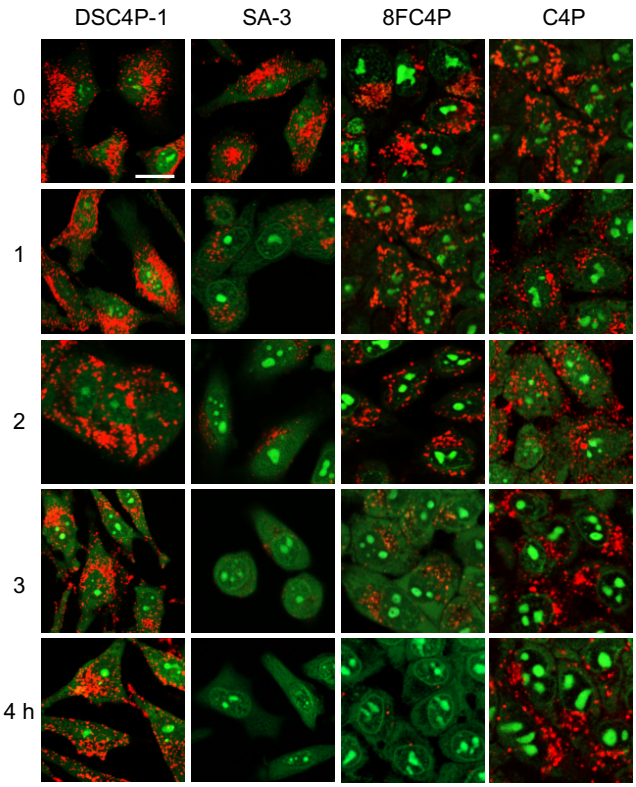


Figure S37. Synthetic ion transporters increase the lysosomal pH. HeLa cells were treated with 20 μM DSC4P-1, 10 μM SA-3, 10 μM 8FC4P, or 20 μM C4P for 4 hr. The treated cells were incubated with 100 nM acridine orange for 30 min. Cell images were obtained by means of confocal microscopy (scale bar: 10 μm).

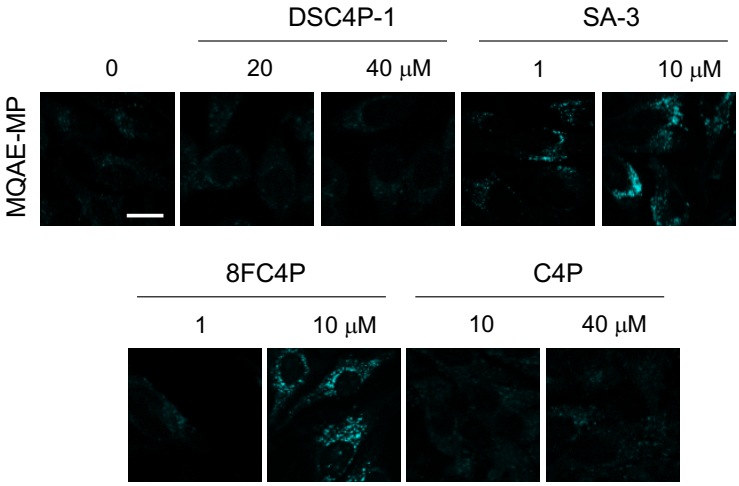


Figure S38. Treatment of synthetic ion transporters leads to changes in the lysosomal chloride ion concentrations. HeLa cells were treated with the indicated concentrations of 8FC4P, SA-3, DSC4P-1, or C4P for 4 hr. The treated cells were incubated with 5 mM MQAE-MP for 30 min and cell images were obtained by means of confocal microscopy (scale bar: 10 μm).

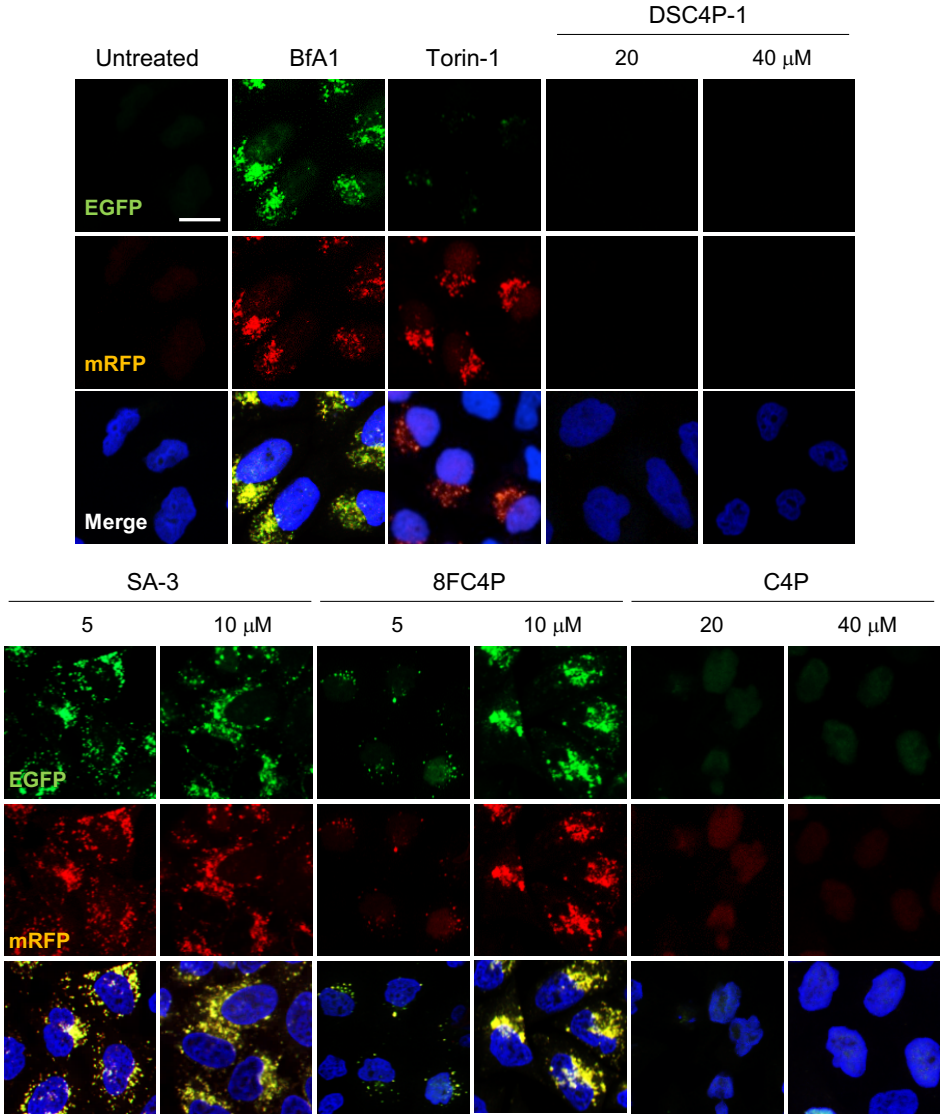


Figure S39. Effects of synthetic transporters on autophagy. (a) HeLa cells stably expressing the mRFP-EGFP-LC3 fusion protein were individually treated with the indicated concentrations of 8FC4P, C4P, DSC4P-1, and SA-3 for 12 hr. BfA1 (5 nM) and torin-1 (500 nM) were used as autophagy inhibitor and autophagy inducer controls, respectively. Cell images were obtained by means of confocal fluorescence microscopy (scale bar: 10 μm).

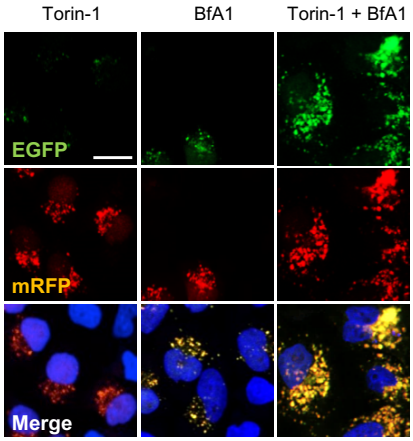


Figure S40. Effect of co-treatment of an early autophagy inducer (torin-1) and an autophagy inhibitor (BfA1) on autophagy. HeLa cells stably expressing mRFP-EGFP-LC3 were incubated with 500 nM torin-1, 5 nM BfA1, or a combination of 500 nM torin-1 with 5 nM BfA1 for 12 hr. Cell images were obtained by means of confocal fluorescence microscopy (scale bar: 10 μm).