

A synthetic ion transporter that disrupts autophagy and induces apoptosis by perturbing cellular chloride concentrations

Nathalie Busschaert,^{a,b+} Seong-Hyun Park,^{c+} Kyung-Hwa Baek,^c Yoon Pyo Choi,^c Jinhong Park,^d Ethan N. W. Howe,^{a,e} Jennifer R. Hiscock,^{a,f} Louise E. Karagiannidis,^a Igor Marques,^g Vítor Félix,^g Wan Namkung,^d Jonathan L. Sessler,^{*h} Philip A. Gale,^{*a,e} Injae Shin^{*c}

^aChemistry, University of Southampton, Southampton, SO17 1BJ, UK.

^b*Current address:* Silver Center for Arts and Science, New York University, Department of Chemistry, 100 Washington Square East, New York, NY 10003, USA.

^cDepartment of Chemistry, Yonsei University, 03722 Seoul, Korea. Corresponding author: injae@yonsei.ac.kr

^dCollege of Pharmacy, Yonsei Institute of Pharmaceutical Sciences, Yonsei University, 21983 Incheon, Korea.

^e*Current address:* School of Chemistry, University of Sydney, NSW 2006, Australia. Corresponding author: philip.gale@sydney.edu.au

^f*Current address:* School of Physical Sciences, University of Kent, Park Wood Road, Canterbury, Kent, CT2 7NH, UK.

^gDepartment of Chemistry, CICECO – Aveiro Institute of Materials, Department of Medical Sciences, iBiMED – Institute of Biomedicine, University of Aveiro, 3810-193, Aveiro, Portugal.

^hDepartment of Chemistry, University of Texas at Austin, 78712-1224 Austin, Texas, USA. Corresponding author: sessler@cm.utexas.edu

⁺These two authors contributed equally to this work.

Keywords: squaramide, supramolecular chemistry, apoptosis, ion transport, autophagy

Abstract

Perturbations in cellular chloride concentrations can affect cellular pH, autophagy and lead to the onset of apoptosis. With this in mind synthetic ion transporters have been used to disturb cellular ion homeostasis and thereby induce cell death; however, it is not clear whether synthetic ion transporters can also be used to disrupt autophagy. Here we show that squaramide-based ion transporters enhance the transport of chloride anions in liposomal models and promote sodium chloride influx into the cytosol. Liposomal and cellular transport activity of the squaramides is shown to correlate with cell death activity, which is attributed to caspase-dependent apoptosis. One ion transporter was also shown to cause additional changes in the lysosomal pH which leads to impairment of lysosomal enzyme activity and disruption of autophagic processes. This disruption is independent of the initiation of apoptosis by the ion transporter. This study provides the first experimental evidence that synthetic ion transporters can disrupt both autophagy and induce apoptosis.

Introduction

Autophagy is a self-eating process that contributes to cell survival under starvation conditions¹, and the discoveries of mechanisms for autophagy were recognized by this year's Nobel Prize in Physiology or Medicine.^{2,3} During autophagy, subcellular organelles and other cell components are sequestered in double-membrane vesicles termed autophagosomes, and they are delivered to lysosomes for digestion and recycling. It is known that levels of autophagy in cancer cells are generally higher than those in normal cells⁴, leading to suggestions that autophagy-disrupting agents may be potentially useful in treatment of cancers^{5,6}.

Apoptosis or programmed cell death is an intrinsic self-killing process that is necessary for maintaining tissue homeostasis⁶. Multiple previous studies have served to demonstrate that tumor cells are resistant to apoptotic cell death via multiple anti-apoptotic processes⁷. Thus, much effort has been made to discover potent apoptosis inducers that can be utilized as anticancer agents^{8,9}. One approach that we have explored recently involves the use of ion transporters to disrupt cellular ion homeostasis¹⁰.

Normally, the extracellular chloride concentration (~120 mM) is much higher than the cytosolic chloride concentration (5–40 mM)¹¹. These concentrations are maintained through the action of transmembrane ion transporters and channels¹². It is known that the dysregulation of intracellular concentrations of ions, particularly chloride anions, correlates closely with the onset of apoptosis¹³. Physiological perturbations in cellular chloride concentrations can also affect pH and autophagy¹⁴. Lysosomal pH values are typically maintained at < 5.0 by sustaining a *ca.* 80 mM lysosomal chloride concentration through the action of the lysosomal chloride transporters¹¹. When the lysosomal chloride concentration is decreased to the level where the lysosomal pH exceeds 5.0^{11,15}, the activity of lysosomal enzymes required for the digestion of biomolecules is reduced leading to autophagy disruption. These observations lead us to suggest that synthetic ion transporters, which modulate cytosolic and lysosomal chloride concentrations and pH and consequently induce apoptosis and disrupt autophagy, may have a role to play as antitumor pharmaceuticals.

The effect of ion transporters on autophagy has not been investigated, however several carrier systems have been reported that induce apoptosis. For instance, prodigiosin, a naturally occurring small molecule-based ion transporter, and its analogues are known to induce cancer

cell death by altering intracellular pH gradients through HCl transport^{16,17,18}. Recently, we found that a synthetic diamide-strapped calix[4]pyrrole **1** (Fig. 1a), which promotes an influx of Cl⁻ and Na⁺ into cells, enhances cell death via caspase-dependent apoptosis¹⁰. Interestingly, bis-(*p*-nitrophenyl)ureidodecalin **2** (Fig. 1a) has been shown to function as an anion transporter without affecting cell viability¹⁹.

In an effort to determine the effects of perturbing ion homeostasis on organelle and cell function, we have carried out a comparative study involving the known chloride transporting mono-squaramide **3**²⁰ and a series of related mono-, bis- and tris-squaramides **4-8** (Fig. 1b). Tripodal squaramide systems analogous to **7** and **8** were reported to possess interesting anion binding properties²¹, while urea and thiourea analogues of **4-8** were found to be highly active anion transporters with cytotoxic activity^{22,23,24}. These compounds were therefore expected to function as potent anion transporters with biological activity. As detailed below, several transporters promote apoptosis, whereas squaramide **3** additionally serves to increase the lysosomal pH, leading to autophagy disruption. To our knowledge, this represents the first time that a synthetic ion transporter has been shown to affect the function of a subcellular organelle.

Results and Discussion

Ion transport activity of synthetic squaramides in a liposome model. To test whether compounds **3-8** act as effective ion transporters, unilamellar 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles containing 489 mM NaCl with 5 mM sodium phosphate (pH 7.2) were prepared according to standard procedures²⁰. The vesicles were suspended in a 489 mM external NaNO₃ solution with 5 mM sodium phosphate (pH 7.2) and chloride efflux induced by the addition of a DMSO solution of **3-8** (1 mol% w.r.t. lipid) was monitored using a chloride selective electrode. As shown in Fig. 2a, compound **3** transports all of the chloride anions from the vesicles within *ca.* 120 sec, while **7** and **8** were inactive. Measurements of the initial rate of chloride transport (k_{ini} , Fig. 1b) revealed a transport activity order of **3** > **5** > **6** > **4** >> **7** ~ **8** (or **3** >

that the squaramides are capable of $\text{Cl}^-/\text{NO}_3^-$ transport (Fig. 2a, Fig. S49), $\text{Cl}^-/\text{HCO}_3^-$ transport (Supplementary Figs. S26 and S49) and to a lesser extent $\text{Cl}^-/\text{SO}_4^{2-}$ transport (Supplementary Figs. S32-S42 and S49). Assays using the pH-sensitive dye HPTS^{25,26} revealed that squaramides **3-6** can dissipate a pH gradient, presumably through H^+/Cl^- symport or Cl^-/OH^- antiport (Supplementary Figs. S44-S47). In contrast, a ^{23}Na NMR spectroscopic study revealed that the squaramides are unable to transport Na^+ ions effectively across POPC bilayers at pH 7.2 (Supplementary Figs. S33-S35). Tripodal compounds **7** and **8** were found to be inactive transporters.

The poor transport ability of **7** and **8** is surprising given the potent anion transport activity of the corresponding urea/thiourea analogues²³. Lipophilicity, which determines the ability of a compound to partition into the membrane, has been linked to transmembrane anion transport activity^{22,27}, however, no clear correlation²⁸ between lipophilicity and anion transport with these compounds could be found (Supplementary Fig. S97a). We thus sought to find a relationship between the anion transport and anion affinity of the squaramides. In $\text{DMSO-}d_6/0.5\%$ water (Supplementary Table S8 and Figs. S68-S95), the bis- and tris-squaramides **5-8** displayed 1:2 receptor-anion binding stoichiometries. This results in highly charged complexes that would be unable to diffuse through a non-polar lipid bilayer.

Given the above, we calculated the most positive values of the surface electrostatic potential of **3-8** ($V_{s,max}$, Fig. 2b), a descriptor that has been shown to correlate strongly with hydrogen bond donating ability²⁹ and anion binding²². Fig 2b shows a plot of the initial rate of transport (k_{ini}) as a function of $V_{s,max}$. The bell-shaped plot, with **3** at the maximum, leads us to suggest that some compounds interact too weakly with anions to allow efficient chloride transport (low $V_{s,max}$, compound **4**), while others bind anions too strongly (high $V_{s,max}$, compounds **7-8**). Anion transport requires both the uptake of the anion on one side of the membrane and release of the anion on the other side of the membrane. A ‘Goldilocks’ effect in terms of binding and transport has thus often been suggested for anion transporters^{30,31}. Transporter **3** appears to embody this effect.

electroneutral processes no net transfer of charge occurs and the transport of the two ions (one cation and one anion, or two different anions) cannot be separated. Using a recently developed method,^{32,33} and exploiting both valinomycin (an electrogenic K^+ transporter)³⁴ and monensin (an electroneutral M^+/H^+ transporter),³⁵ we conclude that **3** is capable of both electrogenic Cl^- transport and electroneutral H^+/Cl^- (OH^-/Cl^-) transport (Fig. 2c and Fig. 2d). Bis-squaramides **5** and **6** appeared to be significantly more efficient in mediating electroneutral H^+/Cl^- (OH^-/Cl^-) transport than electrogenic Cl^- transport (Supplementary Figs. S50 and S51). No selectivity for the transport of Cl^- over H^+ (or OH^-) is seen for **3**, **5** or **6** (Supplementary Table S7).

Cellular ion transport and cell death activities of synthetic squaramides. As a first test of whether the synthetic squaramides **3-8** could act as ion transporters in cells, their ability to promote changes in cytosolic chloride concentrations was assessed in analogy to what was done previously¹⁰. Briefly, Fischer rat thyroid epithelial (FRT) cells, stably expressing a mutant yellow fluorescent protein (YFP-F46L/H148Q/I152L) whose fluorescence is quenched sensitively by chloride ions, were incubated with each compound. Based on the extent of fluorescence quenching, the relative chloride transport activity in cells (cf. Fig. 3a and Supplementary Fig. S98a) was found to mirror what was found in the liposomal studies, namely **3** > **5** > **4** >> **7** for *para*-CF₃ compounds and **6** > **8** for *meta*-CF₃ compounds (Fig. 1b). Since FRT cells lack endogenous chloride channels/transporters, we can exclude the possibility that the chloride ion influx is the result of natural chloride channels/transporters.

We then determined which cations, if any, entered the cells treated with the synthetic squaramides **3-8**. In an initial study, changes in intracellular sodium concentrations were measured by treating FRT cells with the sodium fluorescent probe SBFI-AM (sodium-binding benzofuran isophthalate acetoxymethyl ester) followed by treatment with **3-8**¹⁰. It was found that while **3**, **5**

concentrations remain unchanged upon incubation, as inferred from experiments using potassium (PBFI-AM; potassium-binding benzofuran isophthalate acetoxymethyl ester) and calcium (Fluo-4) fluorescent probes (Supplementary Fig. S99)¹⁰. Such findings are consistent with the relatively low extracellular concentrations of potassium (4 mM vs. 145 mM for Na⁺) and calcium (1.5 mM) and the unfavourable gradients that might be expected in the case of K⁺ (intracellular concentration = 150 mM)¹³. These findings provide support for the notion that transporters **3**, **5** and **6** function as chloride anion transporters in conjunction with channel-mediated sodium cation co-transport (cf. Supplementary Fig. S100).

An increase in cytosolic chloride concentrations is known to induce cell death^{10,36}. We thus investigated the effect of squaramides **3-8** on cell viability. HeLa (human cervical cancer cells) and A549 cells (human lung carcinoma epithelial cells) were incubated with each compound at various concentrations. An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was employed to measure cell viabilities. It was found that **3**, **5** and **6** with good ion transport activity in cells effectively induce cell death, but **4**, **7** and **8** with little or no ion transport activity in cells display very low effect on cell death (Supplementary Fig. S101). The half maximum inhibitory concentration (IC₅₀) values of **3**, **5** and **6** were determined in several cancer cell lines (HeLa, A549, PLC/PRF/5 (hepatocellular carcinoma cells) and HepG2 (hepatoblastoma cells)) and found to vary from 2 to 6 μ M (Fig.1b and Supplementary Fig. S102).

To examine whether the observed synthetic transporter-induced cell death results from increased concentrations of chloride and sodium ions in cells, the effect of extracellular Cl⁻ and Na⁺ on cell death induced by **3**, **5** and **6** was determined^{10,37}. HeLa and A549 cells were incubated with various concentrations of each compound in buffers containing both chloride and sodium ions (HEPES-buffered solutions) or in analogous buffers depleted in either chloride anions (Cl⁻-free solutions) or sodium cations (Na⁺-free solutions). As expected for an ion transport-based effect, the cell death activity of **3**, **5** and **6** was reduced in both the Cl⁻-free and Na⁺-free media (Supplementary Fig

Synthetic transporters promote apoptosis. Multiple previous studies have served to show that dysregulation of ion homeostasis, particularly via chloride influx, can induce apoptosis^{10,36,38}. To test if the present synthetic transporters accelerate apoptosis, HeLa and A549 cells were separately exposed to **3**, **5** and **6**, as well as FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone), a control apoptosis inducer that depolarizes mitochondrial membranes³⁹. The cells were then treated with a mixture of fluorescein-labeled annexin V and propidium iodide (PI). The results of flow cytometry analysis showed that cells treated with **3**, **5** or **6**, as well as FCCP, display positive annexin V binding and PI uptake (Fig. 3c and Supplementary Figs. S105a and S106a). The finding indicates that **3**, **5** and **6** have apoptosis-inducing activity. Analysis of cell size by flow cytometry revealed that cells treated with **3**, **5**, **6** as well as FCCP exhibit a considerable degree of cell shrinkage (Supplementary Figs. S105b, S106b and S107). This finding also serves to rule out cell death via necrosis as a dominant mechanism because necrosis normally leads to cell swelling. The loss of mitochondrial membrane potential, a hallmark of apoptosis, was further investigated using a membrane potential sensitive probe JC-1⁸. The results of fluorescence activated cell sorting (FACS) analysis revealed an increase in JC-1 green fluorescence and a decrease in red fluorescence in cells treated with **3**, **5**, **6** or FCCP, as would be expected for apoptosis (Fig. 3d and Supplementary Figs. S105c and S106c)⁴⁰. An increase in DNA fragmentation was also observed in cells treated with **3**, **5**, **6** or FCCP (Supplementary Figs. S106d and S108). Finally, cells incubated with **3** were subjected to gene expression profiling analysis of mRNAs using human DNA chips. Analysis of DNA chip data shows that a number of positive regulators of apoptosis are upregulated by more than twofold compared to an untreated control, whereas expression levels of negative regulators of apoptosis are downregulated (Supplementary Table S10). Taken together, these results provide clear evidence that cells treated with synthetic transporters **3**, **5** and **6** undergo apoptosis but not necrosis.

Synthetic transporters induce caspase-dependent apoptosis. To test whether the transporters **3**, **5** and **6** induce caspase-dependent apoptosis⁴¹, HeLa and A549 cells were incubated with each compound at various concentrations. The caspase activities of the treated cell lysates were then determined by using a colorimetric peptide substrate for caspases, Ac-DEVD-pNA (pNA, *p*-

nitroaniline). They were found to increase in cells treated with **3**, **5** or **6** (Fig. 4a and Supplementary Fig. S109a). On the other hand, almost no caspase activity was seen when Ac-DEAD-CHO, a known inhibitor of caspases, was added to the lysates of cells treated with **3**, **5** or **6**.

During caspase-dependent apoptosis, cytochrome c is released from the mitochondria into the cytosol where it associates with Apaf-1 to form the apoptosome⁴². The apoptosome complex activates caspase-9, which subsequently leads to caspase-3 activation through the cleavage of procaspase-3⁴². In tests carried out with HeLa and A549 cells cytochrome c release into the cytosol and generation of cleaved caspase-3 were seen after incubation with **3**, **5** or **6** (Fig. 4b and Supplementary Fig. S109b). Additional experiments, aimed at evaluating cleavage of a cellular caspase substrate, poly(ADP-ribose) polymerase (PARP), revealed that the cleaved product of PARP is generated in cells treated with **3**, **5** or **6**.

The apoptosis inducing factor (AIF) is typically translocated into the nucleus during caspase-independent apoptosis⁴³. The level of AIF in the nucleus was thus examined after incubating HeLa and A549 cells with **3**, **5** or **6**. No appreciable transport of AIF translocation into the nucleus was observed (Supplementary Fig. S110). These results support the conclusion that transporters **3**, **5** and **6** promote caspase activation but not AIF-associated, caspase-independent apoptosis.

To evaluate whether ion influx promoted by transporters **3**, **5** and **6** is a cause or a consequence of apoptosis, HeLa cells were incubated with each compound at different time periods and then treated with the sodium fluorescent probe SBFI-AM, the Cl⁻-quenching fluorescent probe MQAE (*N*-(ethoxycarbonylmethyl)-6-methoxy-quinolinium bromide)^{10,44}, the membrane potential sensitive probe JC-1, and fluorescein-annexin V. These studies revealed that sodium chloride transport into cells takes place shortly after incubation with **3**, **5** or **6** (Fig. 4c-d). However, increases in the JC-1 green fluorescence and annexin V positive cells (indicators of apoptosis⁴⁵) occur at later times (Fig. 4e and Supplementary Fig. S111). We thus conclude that ion influx is the likely cause of apoptosis, rather than apoptosis leading to changes in ion flux, as might be expected if **3**, **5** and **6** were operating via a non-transport mechanism.

Synthetic transporter 3 increases the lysosomal pH and impairs lysosomal enzyme activity.

Dysregulation of intracellular chloride concentrations can lead to suppression of cell

proliferation *via* autophagy disruption by altering the lysosomal pH and impairing lysosomal enzyme activity^{14,46}. Autophagy starts with engulfment of cytoplasmic constituents by the phagophore, which is then expanded to form the double-membrane autophagosome (Supplementary Fig. S112). The autophagosome subsequently fuses with the lysosome to generate the autolysosome. Degradation requires lysosomal enzymes, whose activities are maximal at low pH ($\text{pH} \leq 5$)⁴⁷. The lysosomal pH is known to be correlated with chloride anion concentrations, which in turn are regulated by lysosomal chloride transporters.^{14,48} Higher pH values are correlated with relatively lower lysosomal chloride anion concentrations. Since the lysosomal chloride concentrations are higher (*ca.* 80 mM) than cytosolic chloride concentrations (5–40 mM)¹¹, a lysosomal membrane permeable chloride ion transporter might be expected to disrupt the autophagic process by promoting the transfer of chloride anions and therefore H^+ out of the lysosome.

To examine whether synthetic transporters **3**, **5** and **6** affect the lysosomal pH, HeLa cells, pretreated with fluorescein-tetramethylrhodamine-tagged dextran (a ratiometric lysosomal pH indicator⁴⁹), were separately incubated with **3**, **5** and **6**, along with a control **8**. Treatment with **3** leads to an increase in the lysosomal pH to *ca.* 7.0. In contrast, a lysosomal pH of less than 5.0 was measured in cells treated with **5**, **6** or **8** (Fig. 5a and Supplementary Figs. S113 and S114). Although we could not determine the lysosomal chloride concentration directly owing to the lack of appropriate probes, the pH-modulation results suggest that **3** decreases the lysosomal chloride anion concentrations under conditions where the other systems **5**, **6** and **8** do not.

Because the lysosomal pH increases when cells were treated with **3**, we anticipated that this transporter would suppress lysosomal enzyme activities. To test this, we determined the activity of cathepsins B and L, which are critical enzymes that degrade proteins in lysosomes during autophagy, by using cell-permeable fluorogenic peptide substrates, MR-(RR)₂ and MR-(FR)₂, respectively⁵⁰. The intact probes are nonfluorescent, but once the respective peptide moiety attached to the probes is cleaved by action of lysosomal cathepsins B and L, a red fluorescent dye accumulates in the cytoplasm. HeLa cells treated with **3**, **5** or **6** were thus separately incubated with MR-(RR)₂ or MR-(FR)₂. As a control the cells were also treated with leupeptin, an inhibitor of serine and cysteine proteases, followed by incubation of MR-(RR)₂ or MR-(FR)₂. Confocal microscopy image analysis revealed that while cells treated with **5** or **6** exhibit strong red fluorescence signals in analogy to untreated cells, cells treated with **3** or leupeptin were

characterized by a significantly attenuated red fluorescence (Fig. 5b). We thus conclude that **3** increases the lysosomal pH, leading to reduction in cathepsin B and L activity.

Transporter 3 disrupts the autophagic process. Since cathepsin B and L activity was disrupted by **3**, we anticipated that **3** (but not **5** or **6**) would impair the autophagic process in cells. The microtubule-associated protein 1 light chain 3-II (LC3-II) and p62 are often used to monitor autophagy⁵. Typically, increased levels of LC3-II and decreased levels of p62 are observed during autophagy induction. In contrast, the levels of both LC3-II and p62 are increased when autophagic flux is disrupted. HeLa cells were thus incubated with **3**, along with **5** and **6**. Torin-1 and bafilomycin A1 (BfA1) were used as controls for autophagy induction and inhibition, respectively. Increased levels of both LC3-II and p62 were seen in cells treated with **3** compared to untreated cells (Fig. 5c and Supplementary Fig. S115a). Such an increase was also observed in cells treated with the autophagy inhibitor BfA1. In contrast, LC3-II and p62 levels in cells treated with **5** and **6** were similar to those in untreated cells. As expected, cells treated with torin-1 were characterized by an increase in the LC3-II level and a decrease in the p62 level.

HeLa cells, stably expressing a tandem mRFP-EGFP-LC3 fusion protein (mRFP, monomeric red fluorescence protein; EGFP, enhanced green fluorescence protein), were also incubated with **3**, **5**, **6**, as well as torin-1 and BfA1. It is known that both RFP and GFP fluorescence signals are observed in the autophagosome. Typically, RFP but not GFP signals are seen in the acidic autolysosome⁵¹. Confocal microscopy image analysis of HeLa cells treated with **3** revealed a conspicuous increase in yellow vesicles, reflecting a merger of the GFP and RFP fluorescence emission signals (Fig. 5d and Supplementary Fig. S115b). This phenomenon was also observed in BfA1 treated cells. In contrast, cells treated with torin-1 display mainly red fluorescent vesicles, a finding that is indicative of autolysosome formation. Cells treated with **5** and **6** displayed fluorescence patterns similar to those of untreated cells (Supplementary Fig. S116). On this basis, we conclude that autolysosomes are not being formed in cells treated with **3**. Rather, we suggest that transporter **3** increases the lysosomal pH, disrupts lysosomal cathepsin activity in cells, and as a consequence impairs the autophagic process.

Transporters **3**, **5** and **6** all display good chloride ion transport activity at the lysosomal pH (≤ 5.0), as inferred from liposomal studies carried out at pH 7.2 and 4.0 (Supplementary Fig. S48). We thus believe that the different bioactivity of these three transporters reflects the fact

that **3** moves to the lysosome efficiently. In contrast, **5** and **6** are transported into cells but are unable to leave the cell plasma membranes and reach the lysosomes due to their high lipophilicity (Supplementary Table S9) or their potential to bind strongly to the phospholipid headgroups (high $V_{s,max}$, Supplementary Table S9). This is a very specific manifestation of the Goldilocks effect noted above.

Autophagy disruption and apoptosis induction by **3 take place via independent pathways.**

To determine whether the autophagy disruption and apoptosis induced by **3** are linked, HeLa cells were incubated with **3** in the absence and presence of a cell-permeable pan-caspase inhibitor ZVAD-FMK (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone).⁵² Caspase activation promoted by **3** was almost completely suppressed in the presence of ZVAD-FMK (Supplementary Fig. S117). However, ZVAD-FMK itself has no influence on the autophagic process (Fig. 6a and 6b). Cells co-treated with **3** and ZVAD-FMK displayed similar levels of LC3-II and p62 when compared to those in cells treated with **3** alone. HeLa cells stably expressing mRFP-EGFP-LC3 were also treated with either **3** alone or co-treated with **3** and ZVAD-FMK. Similar mRFP puncta co-localized with EGFP were seen in both cases (Fig. 6b and supplementary Fig. S118). Further studies were carried out using BfA1. This substance is an inhibitor of vacuolar H⁺ ATPase that blocks autophagy by impairing lysosomal acidification and fusion of autophagosomes with lysosomes⁵³. In contrast to **3**, treatment with BfA1 alone does not promote caspase-dependent apoptosis. On the other hand, HeLa cells co-treated with **3** and BfA1 undergo apoptosis to a similar degree when compared with cells treated with **3** alone (Fig. 6c-e). Finally, we examined the time-dependence of the apoptosis induction and autophagy disruption produced by **3**. An increase in the levels of LC3 and p62, as well as the appearance of co-localized puncta of mRFP with EGFP, were observed at early times after treatment of cells with **3** (Supplementary Fig. S119) On the other hand, apoptosis promoted by **3** took place at later times (Fig. 4e). Collectively, the findings indicate that the autophagy disruption induced by **3** precedes apoptosis and occurs independently.

Conclusions

On the basis of the findings presented here we propose that transporter **3** (but not **5** or **6**) mediates its observed autophagy impairment function by promoting chloride transport. This

leads to an increase in lysosomal pH, which results in impairment of activity of lysosomal enzymes critical for the degradation of biomolecules during autophagy. This disrupts the autophagic process (cf. Supplementary Fig. S120). Independent of its effect on the lysosomes, the increase in cytosolic sodium cations and chloride anions mediated by **3** (as well as **5** and **6**) results in cytochrome c release from the mitochondria into the cytosol, promoting caspase-dependent apoptosis. As a consequence, transporter **3** promotes induction of apoptosis as well as disrupting autophagy. We thus suggest that synthetic ion transporters, such as **3**, which promote apoptosis and inhibit autophagy may in due course find a role as anticancer agents.

Data availability statement

Data collected in Southampton has been made available online at <http://dx.doi.org/10.5258/SOTON/402335> to comply with the EPSRC open data policy. Other data is available from the authors.

Acknowledgements

This study was supported financially by the National Creative Research Initiative (grant no. 2010-0018272 to I.S.) and Basic Science Research programs (grant no. NRF-2012R1A1A1040142 to W.N.) in Korea. P.A.G thanks the EPSRC for postdoctoral fellowships (NB and ENWH) (EP/J009687/1) and the Royal Society and the Wolfson Foundation for a Research Merit Award. The work in Austin was supported by the National Institutes of Health (grant GM103790 to J.L.S.). The theoretical studies were supported by projects P2020-PTDC/QEQ-SUP/4283/2014, CICECO – Aveiro Institute of Materials (UID/CTM/50011/2013) and iBiMED – Institute of Biomedicine (UID/BIM/04501/2013), financed by National Funds through the FCT/MEC and, co-financed by QREN-FEDER through COMPETE, under the PT2020 Partnership Agreement. I.M. thanks the FCT for the PhD scholarship SFRH/BD/87520/2012. We thank Harriet J. Clarke for resynthesizing compound **5**.

Author contributions

I.S. and N.B. designed the study and wrote the manuscript. J.L.S. and P.A.G. helped with writing the manuscript. I.S., J.L.S and P.A.G. supervised the project. S.-H.P., K.-H.B. and Y.P.C. performed biological studies. N.B. designed, synthesized and characterized the compounds and

performed the ion-transport studies in liposomes and the anion binding studies. E.N.W.H., J.R.H. and L.E.K. helped with some of the liposomal studies. I.M. and V.F. carried out DFT calculations and $V_{s,max}$ calculations. J.P. and W.N. carried out ion-transport activity studies in cells. Correspondence and request for materials should be directed to P.A.G.

Additional information

Supplementary information is available in the online version of the paper. Details of synthesis and characterization of compounds **3-8**. Crystallographic data for **5** and **8**. Experimental for liposomal transport studies. Experimental for anion binding studies and $V_{s,max}$ calculations. Experimental for cell studies and measurements of intracellular ion concentrations.

Competing financial interests

The authors declare no competing financial interests.

References

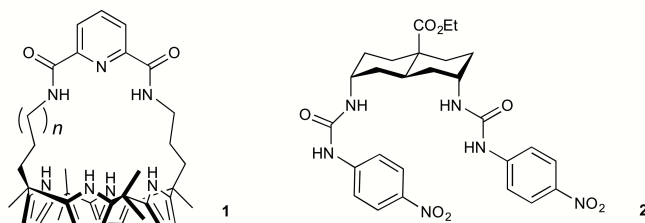
1. Mizushima, N. & Komatsu, M. Autophagy: renovation of cells and tissues. *Cell* **147**, 728-741 (2011).
2. Ohsumi, Y. Molecular dissection of autophagy: two ubiquitin-like systems. *Nat. Rev. Mol. Cell. Biol.* **2**, 211-216 (2001).
3. Kuma, A. *et al.* The role of autophagy during the early neonatal starvation period. *Nature* **432**, 1032-1036, (2004).
4. White, E. Deconvoluting the context-dependent role for autophagy in cancer. *Nat. Rev. Cancer* **12**, 401-410 (2012).
5. Baek, K.-H., Park, J. & Shin, I. Autophagy-regulating small molecules and their therapeutic applications. *Chem. Soc. Rev.* **41**, 3245-3263 (2012).
6. Newmeyer, D. D. & Ferguson-Miller, S. Mitochondria: releasing power for life and unleashing the machineries of death. *Cell* **112**, 481-490 (2003).
7. Igney, F.H. & Krammer, P.H. Death and anti-death: tumour resistance to apoptosis. *Nat. Rev. Cancer* **2**, 277-288 (2002).
8. Williams, D. R., Ko, S.-K., Park, S., Lee, M.-R. & Shin, I. An apoptosis-inducing small molecule that binds to heat shock protein 70. *Angew. Chem. Int. Ed.* **47**, 7466-7469 (2008).
9. Ko, S.-K. *et al.* A small molecule inhibitor of ATPase activity of HSP70 induces apoptosis and has antitumor activities. *Chem. Biol.* **22**, 391-403 (2015).
10. Ko, S.-K. *et al.* Synthetic ion transporters can induce apoptosis by facilitating chloride anion transport into cells. *Nat. Chem.* **6**, 885-892 (2014).
11. Stauber, T. & Jentsch, T. J. Chloride in vesicular trafficking and function. *Annu. Rev. Physiol.* **75**, 453-477 (2013).
12. Gould, D. Human physiology. From cells to systems, 3rd edition. *J. Adv. Nurs.* **28**, 680-682 (1998).
13. Yu, S. P., Canzoniero, L. M. T. & Choi, D. W. Ion homeostasis and apoptosis. *Curr. Opin. Cell Biol.* **13**, 405-411 (2001).
14. Hosogi, S., Kusuzaki, K., Inui, T., Wang, X. & Marunaka, Y. Cytosolic chloride ion is a key factor in lysosomal acidification and function of autophagy in human gastric cancer cell. *J. Cell. Mol. Med.* **18**, 1124-1133 (2014).
15. Anke, D. *et al.* CFTR regulates phagosome acidification in macrophages and alters bactericidal activity. *Nat. Cell Biol.* **8**, 933-944 (2006).
16. Sessler, J. L. *et al.* Synthesis, anion-binding properties, and in vitro anticancer activity of prodigiosin analogues. *Angew. Chem. Int. Ed.* **117**, 6143-6146 (2005).
17. Ohkuma, S. *et al.* Prodigiosins uncouple lysosomal vacuolar-type ATPase through promotion of H⁺/Cl⁻ symport. *Biochem. J.* **334**, 731-741 (1998).
18. Melvin, M. S. *et al.* Double-strand DNA cleavage by copper-Prodigiosin. *J. Am. Chem. Soc.* **122**, 6333-6334 (2000).
19. Li, H. *et al.* Efficient, non-toxic anion transport by synthetic carriers in cells and epithelia. *Nat. Chem.* **8**, 24-32 (2016).
20. Busschaert, N. *et al.* Squaramides as potent transmembrane anion transporters. *Angew. Chem. Int. Ed.* **51**, 4426-4430 (2012).
21. Jin, C. *et al.* Squaramide-based tripodal receptors for selective recognition of sulfate anion. *Chem. Commun.* **49**, 2025-2027 (2013).
22. Busschaert, N. *et al.* Towards predictable transmembrane transport: QSAR analysis of anion

- binding and transport. *Chem. Sci.* **4**, 3036-3045 (2013).
23. Busschaert, N. *et al.* Structure-activity relationships in tripodal transmembrane anion transporters: the effect of fluorination. *J. Am. Chem. Soc.* **133**, 14136-14148 (2011).
 24. Moore, S.J. *et al.* Chloride, carboxylate and carbonate transport by ortho-phenylenediamine-based bisureas. *Chem. Sci.* **4**, 103-117 (2013).
 25. Clement, N. R. & Gould, J. M. Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate) as a probe of internal aqueous hydrogen ion concentration in phospholipid vesicles. *Biochemistry* **20**, 1534-1538 (1981).
 26. Busschaert, N. *et al.* Thiosquaramides: pH switchable anion transporters. *Chem. Sci.* **5**, 3617-3626 (2014).
 27. Valkenier, H., Haynes, C. J. E., Herniman, J., Gale, P. A. & Davis, A. P. Lipophilic balance - a new design principle for transmembrane anion carriers. *Chem. Sci.* **5**, 1128-1134 (2014).
 28. Li, Z., Deng, L.-Q., Chen J.-X., Zhou, C.-Q. & Chen W.-H. Does lipophilicity affect the effectiveness of a transmembrane anion transporter? Insight from squaramido-functionalized bis(choloyl) conjugates. *Org. Biomol. Chem.* **13**, 11761-11769 (2015).
 29. Hagelin, H., Murray, J. S., Politzer, P., Brinck, T. & Berthelot, M. Family-independent relationships between computed molecular surface quantities and solute hydrogen bond acidity/basicity and solute-induced methanol O-H infrared frequency shifts. *Can. J. Chem.* **73**, 483-488 (1995).
 30. Behr, J. P., Kirch, M. & Lehn, J. M. Carrier-mediated transport through bulk liquid membranes: dependence of transport rates and selectivity on carrier properties in a diffusion-limited process. *J. Am. Chem. Soc.* **107**, 241-246 (1985).
 31. Vargas Jentzsch, A. *et al.* Ditopic ion transport systems: anion- π interactions and halogen bonds at work. *Angew. Chem. Int. Ed.* **50**, 11675-11678 (2011).
 32. Wu, X. *et al.* Nonprotonophoric electrogenic chloride transport mediated by valinomycin-like carriers. *Chem* **1**, 127-146 (2016).
 33. Howe, E. N. W. *et al.* pH-Regulated Nonelectrogenic Anion Transport by Phenylthiosemicarbazones. *J. Am. Chem. Soc.* **138**, 8301-8308 (2016).
 34. Läuger, P. Mechanisms of biological ion transport — carriers, channels, and pumps in artificial lipid membranes. *Angew. Chem. Int. Ed.* **24**, 905-923 (1985).
 35. Mollenhauer, H. H., James Morré, D. & Rowe, L. D. Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochim. Biophys. Acta.* **1031**, 225-246 (1990).
 36. Tsukimoto, M., Harada, H., Ikari, A. & Takagi, K. Involvement of chloride in apoptotic cell death induced by activation of ATP-sensitive P2X7 purinoceptor. *J. Biol. Chem.* **280**, 2653-2658 (2005).
 37. Bortner, C. D. & Cidlowski, J. A. Uncoupling cell shrinkage from apoptosis reveals that Na⁺ influx is required for volume loss during programmed cell death. *J. Biol. Chem.* **278**, 39176-39184 (2003).
 38. Saha, T., Hossain, M. S., Saha, D., Lahiri, M. & Talukdar, P. Chloride-mediated apoptosis-inducing activity of bis(sulfonamide) anionophores. *J. Am. Chem. Soc.* **138**, 7558-7567 (2016).
 39. Waterhouse, N. J. *et al.* Cytochrome c maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process. *J. Cell Biol.* **153**, 319-328 (2001).
 40. Tzung, S.-P. *et al.* Antimycin a mimics a cell-death-inducing Bcl-2 homology domain 3.

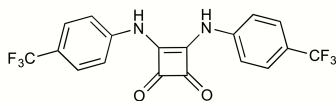
- Nat. Cell Biol.* **3**, 183-191 (2001).
41. Elmore, S. Apoptosis: a review of programmed cell death. *Cell Death. Toxicol. Pathol.* **35**, 495-516 (2007).
 42. Li, P. *et al.* Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479-489 (1997).
 43. Joza, N. *et al.* Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature* **410**, 549-554 (2001).
 44. Cho, H. J. *et al.* A small molecule that binds to an ATPase domain of Hsc70 promotes membrane trafficking of mutant cystic fibrosis transmembrane conductance regulator. *J. Am. Chem. Soc.* **133**, 20267-20276 (2011).
 45. Perelman, A. *et al.* JC-1: alternative excitation wavelengths facilitate mitochondrial membrane potential cytometry. *Cell Death Dis* **3**, e430 (2012).
 46. Yang, Y.-P. *et al.* Application and interpretation of current autophagy inhibitors and activators. *Acta Pharmacol. Sin.* **34**, 625-635 (2013).
 47. Xie, Z. & Klionsky, D. J. Autophagosome formation: core machinery and adaptations. *Nat. Cell Biol.* **9**, 1102-1109 (2007).
 48. Graves, A. R., Curran, P. K., Smith, C. L. & Mindell, J. A. The Cl^-/H^+ antiporter ClC-7 is the primary chloride permeation pathway in lysosomes. *Nature* **453**, 788-792 (2008).
 49. DiCiccio, J. E. & Steinberg, B. E. Lysosomal pH and analysis of the counter ion pathways that support acidification. *J. Gen. Physiol.* **137**, 385-390 (2011).
 50. Creasy, B. M., Hartmann, C. B., White, F. K. H. & McCoy, K. L. New assay using fluorogenic substrates and immunofluorescence staining to measure cysteine cathepsin activity in live cell subpopulations. *Cytometry Part A* **71A**, 114-123 (2007).
 51. Kimura, S., Noda, T. & Yoshimori, T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* **3**, 452-460 (2007).
 52. Chen, Y., McMillan-Ward, E., Kong, J., Israels, S. J. & Gibson, S. B. Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. *Cell Death Differ* **15**, 171-182 (2007).
 53. Jahreiss, L., Menzies, F.M. & Rubinsztein, D. C. The itinerary of autophagosomes: from peripheral formation to kiss-and-run fusion with lysosomes. *Traffic* **9**, 574-587 (2008).

Figures and Figure Legends

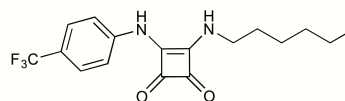
a Previously reported anion transporters with biological activity



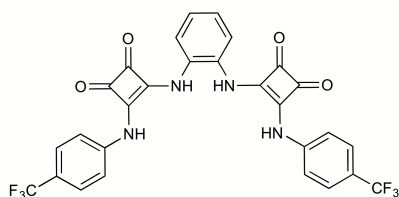
b Squaramide-based anion transporters



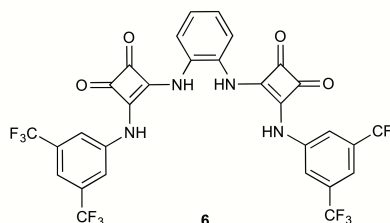
$k_{ini}(\text{transport}) = 2.14\ \%s^{-1}$
 $V_{s,max} = 88.32\ \text{kcal}\cdot\text{mol}^{-1}$
 $IC_{50}(\text{HeLa}) = 3.4\ \mu\text{M}$



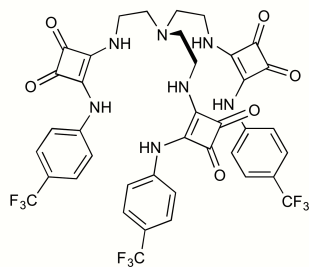
$k_{ini}(\text{transport}) = 0.38\ \%s^{-1}$
 $V_{s,max} = 78.22\ \text{kcal}\cdot\text{mol}^{-1}$
 $IC_{50}(\text{HeLa}) > 20\ \mu\text{M}$



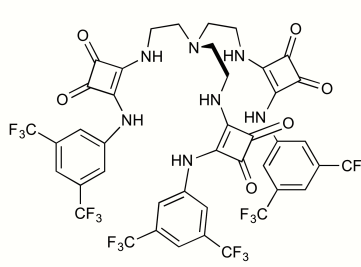
$k_{ini}(\text{transport}) = 0.98\ \%s^{-1}$
 $V_{s,max} = 107.30\ \text{kcal}\cdot\text{mol}^{-1}$
 $IC_{50}(\text{HeLa}) = 5.8\ \mu\text{M}$



$k_{ini}(\text{transport}) = 0.65\ \%s^{-1}$
 $V_{s,max} = 110.49\ \text{kcal}\cdot\text{mol}^{-1}$
 $IC_{50}(\text{HeLa}) = 5.7\ \mu\text{M}$



$k_{ini}(\text{transport}) = 0.002\ \%s^{-1}$
 $V_{s,max} = 110.46\ \text{kcal}\cdot\text{mol}^{-1}$
 $IC_{50}(\text{HeLa}) = \text{inactive}$



$k_{ini}(\text{transport}) = 0.004\ \%s^{-1}$
 $V_{s,max} = 114.93\ \text{kcal}\cdot\text{mol}^{-1}$
 $IC_{50}(\text{HeLa}) = \text{inactive}$

Ion transporter activity in liposomes: $3 > 5 \sim 6 > 4 >> 7 \sim 8$ (or $3 > 5 > 4 >> 7$ (*p*-CF₃) and $6 > 8$ (*m*-CF₃))
 Ion transporter activity in cells: $3 \sim 6 > 5 > 4 >> 7 \sim 8$ (or $3 > 5 > 4 >> 7$ (*p*-CF₃) and $6 > 8$ (*m*-CF₃))
 Cancer cell death activity: $3 \sim 6 \sim 5 > 4 > 7 > 8$ (or $3 \sim 5 > 4 > 7$ (*p*-CF₃) and $6 > 8$ (*m*-CF₃))

Figure 1. Structure of anion transporters and their activity. **a**, Structures of previously reported anion transporters **1** and **2**. **b**, Structures of squaramide-based anion transporters **3-8** and the initial rate of chloride transport mediated by 1 mol% transporter from liposomes filled with a buffered NaCl solution and submerged in a buffered NaNO₃ solution (k_{ini}), calculated $V_{s,max}$ values and IC_{50} values towards HeLa cells. The inset shows the relative activity (from high to

low) for the various transporters in different assays.

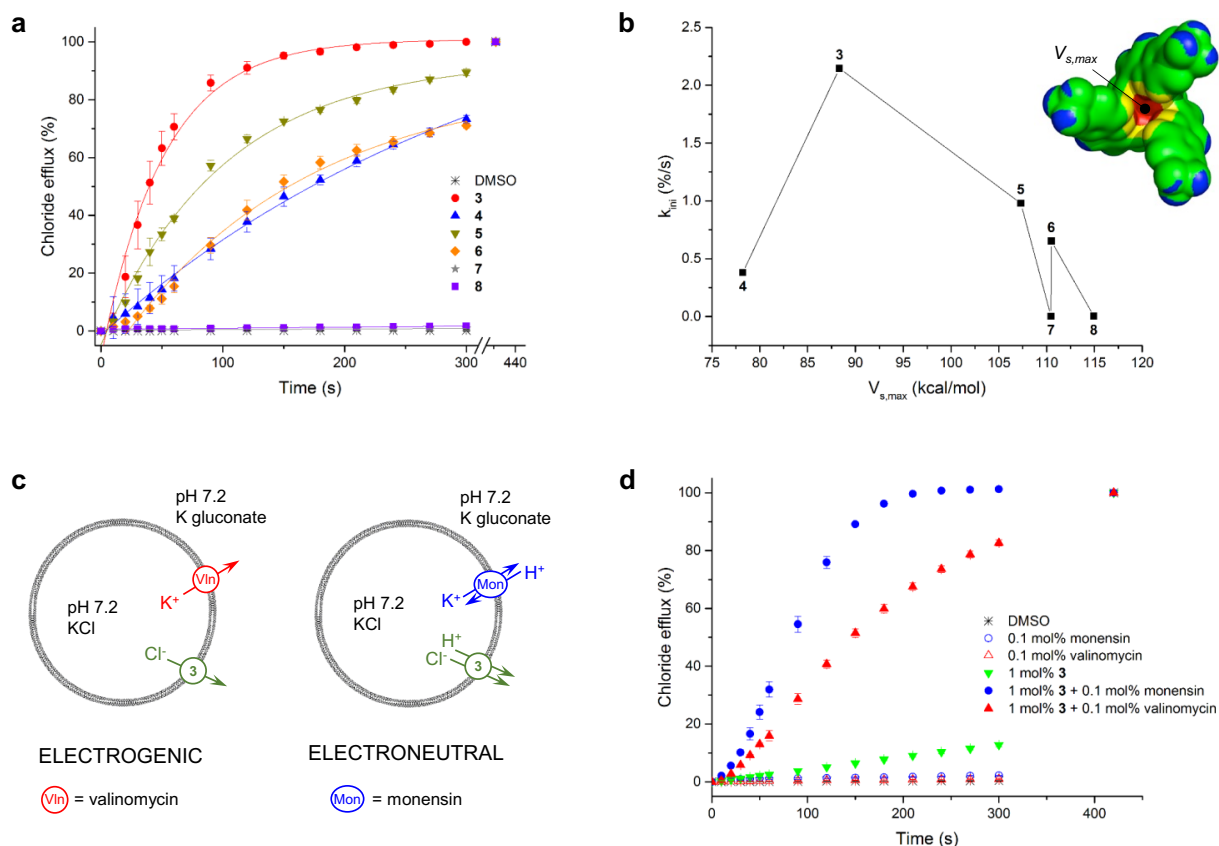


Figure 2. Ion transport studies using liposomal model membranes. **a**, Cl^-/NO_3^- antiport mediated by **3-8** (added as DMSO solutions, final concentration 1 mol% w.r.t. lipid) from POPC vesicles loaded with 489 mM NaCl with 5 mM phosphate salts (pH 7.2) and suspended in a 489 mM $NaNO_3$ solution with 5 mM phosphate salts (pH 7.2). Error bars represent standard deviations and lines correspond to the initial rate of transport (k_{ini}) calculations. **b**, Plot representing the correlation between the initial rate of transport (k_{ini}) and $V_{s,max}$. Inset shows the surface electrostatic potential with $V_{s,max}$ of compound **7** (colour scales ranges from blue to red, in $kcal\ mol^{-1}$, as follows: blue = lower than -7.5 ; green = between -7.5 and 40.0 ; yellow = between 40.0 and 87.5 ; and red = greater than 87.5). **c**, Experimental setup to determine electrogenic or electroneutral anion transport. POPC vesicles are loaded with 300 mM KCl with 5 mM phosphate salts (pH 7.2) and suspended in a 300 mM potassium gluconate solution with 5 mM phosphate salts (pH 7.2). Electrogenic K^+ transport by valinomycin can only occur if it is balanced by electrogenic Cl^- transport by **3**. Electroneutral K^+/H^+ antiport by monensin can only occur if the pH gradient is dissipated by electroneutral H^+/Cl^- transport by **3**. **d**, Results of the experiment described in **c**, showing both electrogenic and electroneutral anion transport by squaramide **3**. Transporters are added as DMSO solutions to start the experiments (resulting in a final concentration of 0.1 mol% w.r.t. lipid for valinomycin and monensin, and 1 mol% w.r.t.

lipid for squaramide **3**). Error bars represent standard deviations.

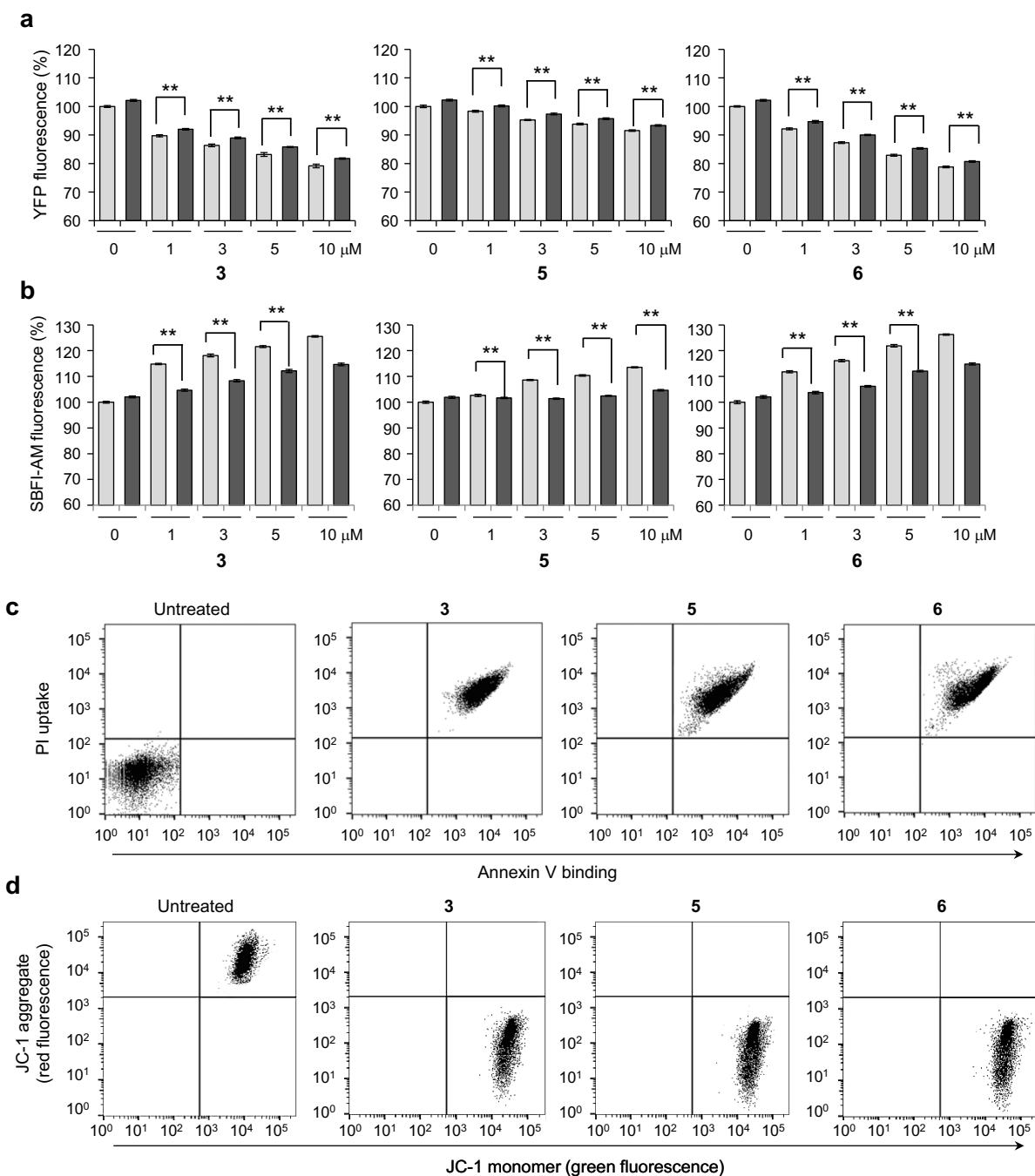


Figure 3. Synthetic transporters induce apoptosis. **a**, FRT cells stably transfected with a mutant YFP gene were incubated with various concentrations of indicated compounds for 2 hours in the absence (grey bar) and presence (black bar) of 1 mM amiloride. The YFP fluorescence was then measured to examine changes in intracellular chloride ion concentrations (mean \pm s.d., $n = 3$, ** indicates p -value < 0.001 with the Student's t test). **b**, FRT cells pretreated with 10 μM SBFI-AM for 1.5 hours were incubated with various concentrations of indicated compounds for 2 hours in the absence (grey bar) and presence (black bar) of 1 mM amiloride. The SBFI-AM fluorescence was then measured to examine changes in intracellular sodium ion

concentrations (mean \pm s.d., $n = 3$, ** indicates p -value < 0.001 with the Student's t test). **c**, Flow cytometry of HeLa cells treated with 10 μ M of indicated compounds for 18 hours and stained with fluorescein-annexin V and PI (annexin V binding versus PI uptake). Untreated cells are shown as a negative control. **d**, Flow cytometry of HeLa cells treated with 10 μ M of indicated compounds for 18 hours and stained with JC-1 (red fluorescence (FL2, JC-1 aggregate) versus green fluorescence (FL1, JC-1 monomer)). Untreated cells are shown as a negative control.

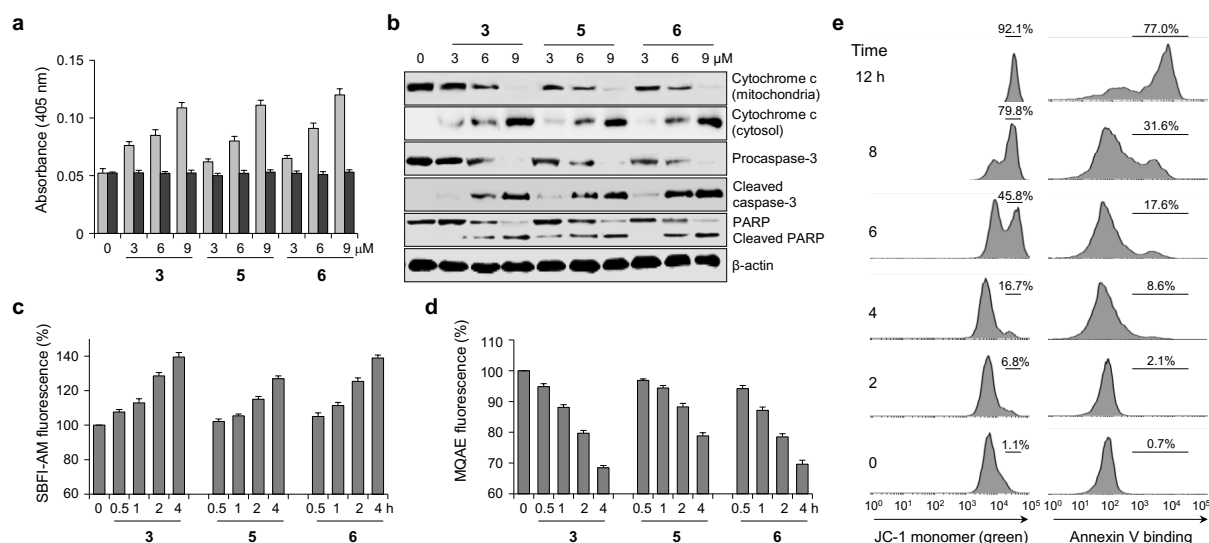


Figure 4. Synthetic transporters induce caspase-dependent apoptosis. **a**, Caspase activities of lysates of HeLa cells treated with the indicated compounds for 18 hours were measured by using 200 μM acetyl-DEVD-pNA in the absence (grey bar) and presence (black bar) of 20 μM Ac-DEVD-CHO (mean ± s.d., n = 3). **b**, HeLa cells were treated with the indicated compounds for 18 hours. Immunoblotting was conducted using the corresponding antibodies. β-Actin was used as a loading control. **c**, HeLa cells pretreated with 10 μM SBFI-AM were incubated with 10 μM of the indicated compounds for the indicated times. The SBFI-AM fluorescence was then measured to probe changes in the intracellular sodium ion concentration (mean ± s.d., n = 3). **d**, HeLa cells pretreated with 10 mM MQAE for 1 hour were incubated with 10 μM of each compound for the indicated times. The MQAE fluorescence was then measured to determine changes in the intracellular chloride ion concentration (mean ± s.d., n = 3). **e**, Flow cytometry of HeLa cells treated with 10 μM **3** for the indicated time and then stained with (left) JC-1 or (right) fluorescein-annexin V.

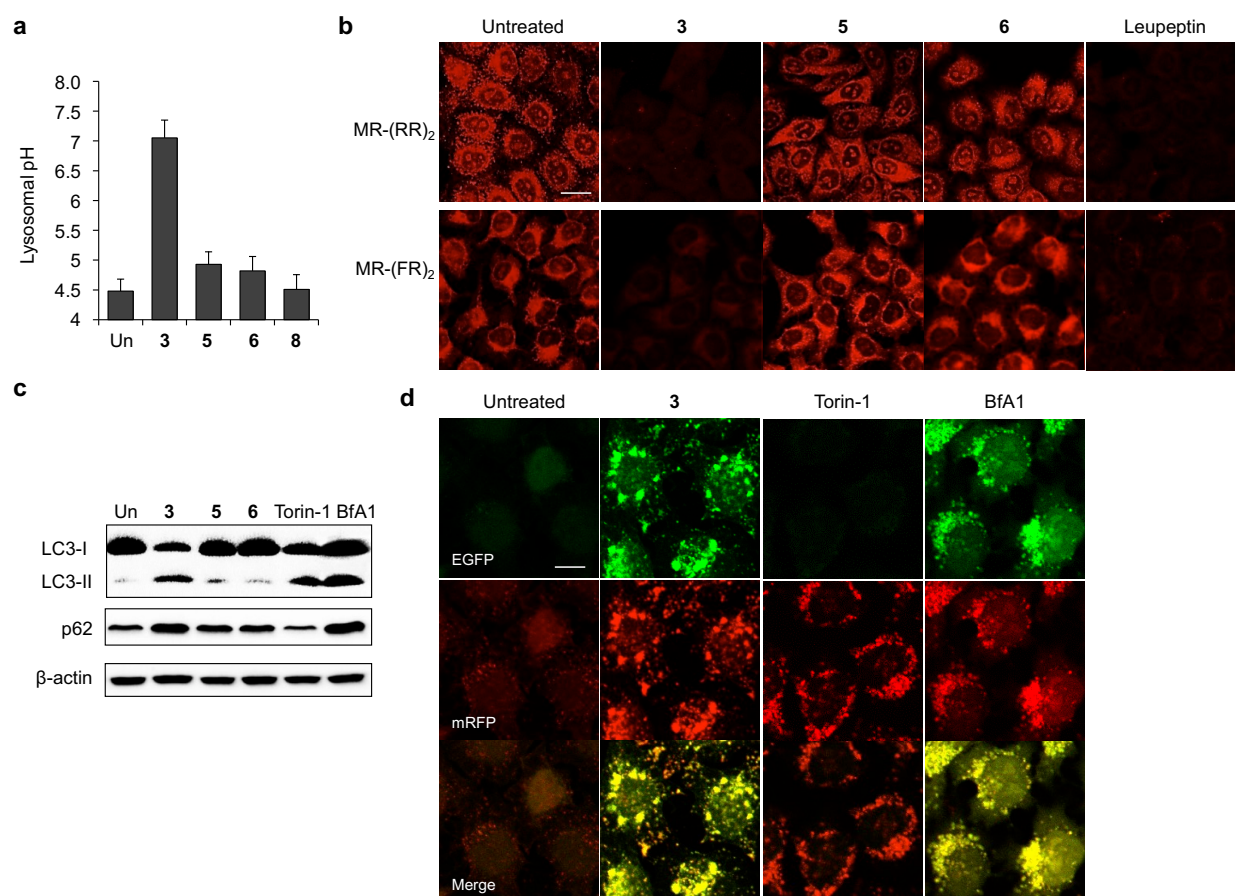


Figure 5. Effect of synthetic transporters on autophagy. **a**, HeLa cells pretreated with fluorescein-tetramethylrhodamine-tagged dextran for 12 hours were incubated with 10 μ M of indicated compounds for 12 hours. The lysosomal pHs were calculated by using a pH titration curve shown in Fig. S109 (mean \pm s.d., $n = 3$). **b**, HeLa cells pretreated with **3** (4 μ M), **5** (4 μ M), **6** (4 μ M) or leupeptin (5 μ M) for 6 hours were incubated with MR-(RR)₂ or MR-(FR)₂ for 4 hours. Cell images were obtained using confocal fluorescence microscopy (scale bar = 20 μ m). **c**, HeLa cells were treated with **3** (4 μ M), **5** (4 μ M), **6** (4 μ M), torin-1 (1 μ M) or BfA1 (5 nM) for 24 hours. Expression levels of LC3 and p62 were examined using western blots. Torin-1 and BfA1 were used as controls for autophagy induction and inhibition, respectively. **d**, HeLa cells stably expressing mRFP-EGFP-LC3 were treated with **3** (4 μ M), torin-1 (1 μ M) or BfA1 (5 nM) for 24 hours. Cell images were obtained using confocal fluorescence microscopy (scale bar = 10 μ m).

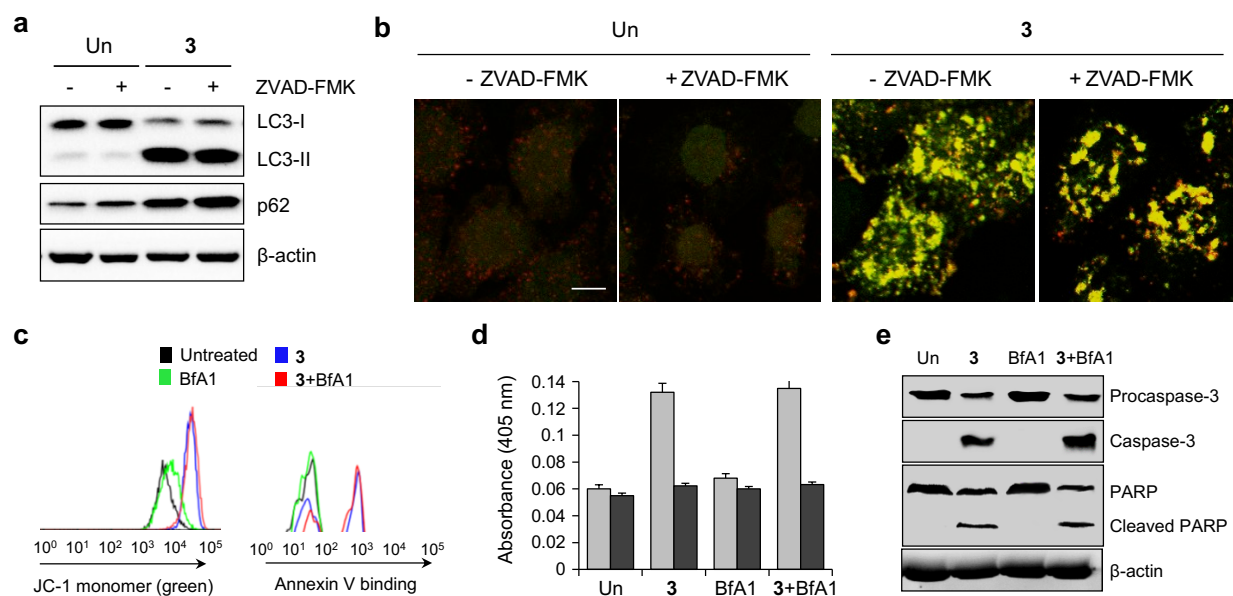


Figure 6. Effect of apoptosis induction promoted by 3 is independent of its ability to disrupt autophagy. **a**, HeLa cells were treated for 12 hours with 10 μ M **3** in the absence and presence of 40 μ M ZVAD-FMK. The indicated proteins were immunoblotted using the corresponding antibodies. ‘Un’ indicates no treatment of cells with **3**. **b**, HeLa cells stably expressing mRFP-EGFP-LC3 were treated for 24 hours with 10 μ M **3** in the absence and presence of 40 μ M ZVAD-FMK. Cell images of EGFP and mRFP, obtained using confocal fluorescence microscopy, were merged (scale bar = 10 μ m). ‘Un’ indicates no treatment of cells with

Table of contents summary

In this paper we show that a squaramide-based anion transporter can cause changes in the lysosomal pH which leads to impairment of lysosomal enzyme activity and disruption of autophagic processes. This study provides the first experimental evidence that synthetic ion transporters can disrupt both autophagy and induce apoptosis.

