Synthetic ion transporters can induce apoptosis by facilitating chloride anion transport into cells

Sung-Kyun Ko[1,2†], Sung Kuk Kim[3†], Andrew Share[3], Vincent M. Lynch[3], Jinhong Park[4], Wan Namkung[4], Wim Van Rossom[5], Nathalie Busschaert[5], Philip A. Gale[5,6]*, Jonathan L. Sessler[1,3]* and Injae Shin[1]*

Anion transporters based on small molecules have received attention as therapeutic agents because of their potential to disrupt cellular ion homeostasis. However, a direct correlation between a change in cellular chloride anion concentration and cytotoxicity has not been established for synthetic ion carriers. Here we show that two pyridine diamide-strapped calix[4]pyrroles induce coupled chloride anion and sodium cation transport in both liposomal models and cells, and promote cell death by increasing intracellular chloride and sodium ion concentrations. Removing either ion from the extracellular media or blocking natural sodium channels with amiloride prevents this effect. Cell experiments show that the ion transporters induce the sodium chloride influx, which leads to an increased concentration of reactive oxygen species, release of cytochrome *c* from the mitochondria and apoptosis via caspase activation. However, they do not activate the caspase-independent apoptotic pathway associated with the apoptosis-inducing factor. Ion transporters, therefore, represent an attractive approach for regulating cellular processes that are normally controlled tightly by homeostasis.

Maintenance of the ion homeostasis of cells is essential to sustain life processes, including proliferation, differentiation and apoptosis. A considerable body of evidence provides support for the generally accepted notion that ion channels and transporters are involved in apoptosis (or programmed cell death)[1,2], which serves *inter alia* to remove unwanted or damaged cells[3]. Various cancer cells exhibit different patterns of ion channels that result in the dysregulation of ion concentrations and fluxes[4]. This dysregulation can manifest itself in operational terms as apoptotic resistance in cells[5], as well as an enhancement in proliferation[6] and the migration of cancer cells[7]. Taken to the extreme, however, the dysregulation of intracellular concentrations of ions, particularly of chloride[8,9], calcium[10] and potassium ions[11], has been shown to correlate closely with the onset of apoptosis[1]. The potency of channel blockers[4] and ionophores[12,13] as anticancer agents provides a strong incentive to explore new approaches to influence intracellular ion concentrations in a controlled fashion. Particularly attractive in this regard would be synthetic or semisynthetic ion carriers or transporters that are able to alter intracellular ion concentrations. Anion transporters based on small molecules have received particular attention because of their potential as therapeutic agents[14–17].

One of the most notable small-molecule-based natural anion transporters is prodigiosin. Compounds of this class are known to function as HCl receptors and transporters and have been studied extensively for, among other possible benefits, their anticancer effect[18–20]. It has been proposed that disruption of intracellular pH gradients by HCl transport[19] is one of the key mechanisms whereby prodigiosin promotes cancer cell death[21–23]. However, it is also possible that prodigiosin induces apoptosis by an alternative mechanism[22] that results in the depolarization of acidic compartments within cells. The effectiveness of prodigiosin against cancer cells has spurred the synthesis of many artificial receptors, so-called anionophores, that act as anion transporters[24–26]. Although various anionophores have been created, few have been tested in biological settings[15,27–29]. In one example, artificial tripodal anion transporters that contain urea and thiourea functionalities were found to inhibit cancer cell growth by acting as a presumed Cl^-/HCO_3^- anion exchanger (a so-called antiport agent)[15]. However, a direct correlation between a change in cellular chloride anion concentration and cytotoxicity has not been established in the case of synthetic carriers. Rather, it has been inferred based on transport studies of liposomal model membranes. As with prodigiosin, it is possible in these cases that apoptosis is induced by a different mechanism that results in perturbation of chemical gradients within the cancer cells. A key question is whether the carriers perturb chemical gradients, resulting in apoptosis, or trigger apoptosis, which causes a perturbation of chemical gradients. In this study, using time-dependent studies we show that changes in ionic gradients within A549 and HeLa cells precede, and therefore induce, apoptosis.

Normally, the extracellular chloride concentration (120 mM) is higher than the intracellular chloride concentration (4–60 mM). Nevertheless, in healthy cells no appreciable chloride influx takes place unless biological ion channels or transporters are activated[30]. Ion channels, such as the Na–K–Cl co-transporter, use the decrease in free energy that results from transporting Na^+ across the cellular membrane as the driving force to increase intracellular Cl^- concentrations[31]. A synthetic transporter that takes advantage of the Na^+ gradient and allows for the concomitant transport of Cl^- would be expected to enable the effective transport of Cl^- into cells.

[1]Department of Chemistry, Yonsei University, Seoul 120-749, Korea, [2]Chemical Biology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongwon-gun, Chungbuk 363-883, Korea, [3]Department of Chemistry & Biochemistry, University of Texas at Austin, Austin, Texas 78712-1224, USA, [4]College of Pharmacy, Yonsei Institute of Pharmaceutical Sciences, Yonsei University, Incheon 406-840, Korea, [5]Chemistry, University of Southampton, Southampton SO17 1BJ, UK, [6]Department of Chemistry, Faculty of Science, King Abdulaziz University, Jeddah 21589, Saudi Arabia, [†]These authors contributed equally to this work. *e-mail: sessler@cm.utexas.edu; injae@yonsei.ac.kr; philip.gale@soton.ac.uk
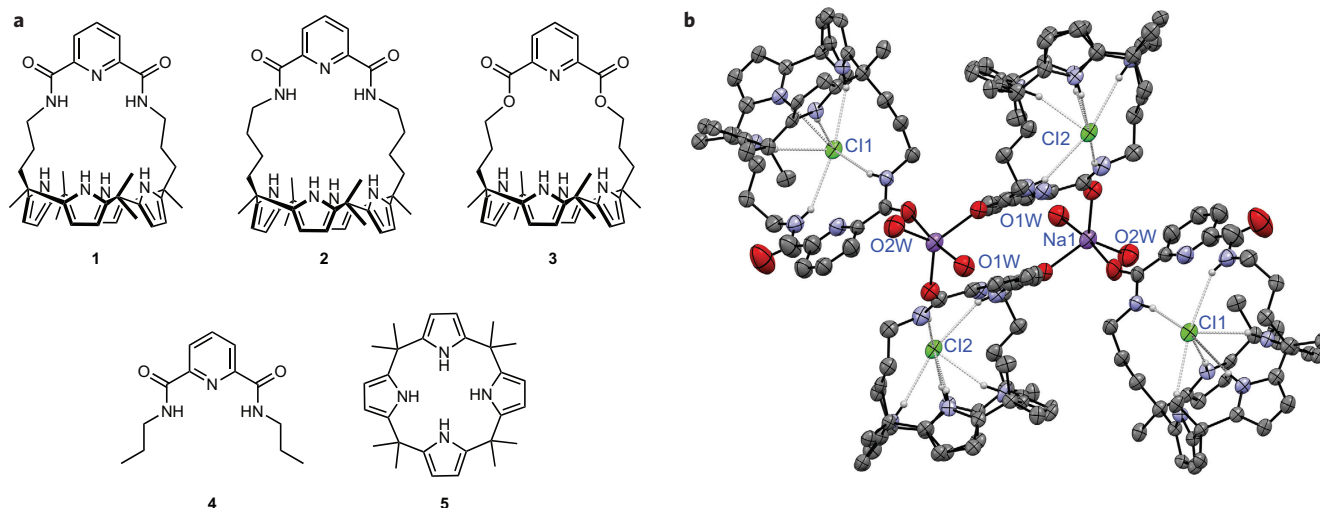
Figure 1 | Structures of compounds. a, Chemical structures used in this study. **b**, Partial view of the single-crystal structure of **1·NaCl**. Most hydrogen atoms, solvent molecules and two non-attributable tetramethylammonium cations are omitted for clarity.

Herein we describe the design and synthesis of two diamide-strapped calix[4]pyrroles (C4Ps) that facilitate chloride transport in liposomal model membranes and in mammalian cells. In liposomal models we show that the strapped calixpyrroles facilitate chloride transport and that chloride transport is enhanced significantly in the presence of cation transporters[32]. In the cells we show that the compounds facilitate chloride influx and concomitantly sodium entry mainly via sodium channels. As detailed below, our results serve to demonstrate that synthetic transporters can be used to induce an influx of Cl⁻ as well as of Na⁺, and that this leads to an increased level of reactive oxygen species (ROS), the release of cyto-chrome c from the mitochondria and induction of apoptotic cell death via the caspase-dependent pathway.

Results and discussion

Synthesis and ion-binding studies of diamide-strapped calixpyrroles. For this study two new pyridine diamide-strapped C4Ps (compounds **1** and **2**; Fig. 1a) were prepared. The diamide functionality present in these targets was expected to provide effective ion-pair recognition and through-membrane transport. To allow for an appropriate testing of this hypothesis, the diester control system **3** was prepared and tested, as were compounds **4** and **5**.

Receptors **1–3** were prepared according to the general method first introduced by Lee and co-workers[33,34]. Full synthetic details are included in the Supplementary Information. All new compounds were characterized fully by standard spectroscopic means, with receptors **1** and **3** further characterized via single-crystal X-ray diffraction analyses (Supplementary Figs 1 and 2). ¹H NMR studies were performed to probe the interaction of these macro-cycles with alkali metal chloride salts. On the addition of NaCl, KCl and CsCl (5.0 equiv.) in 10% D₂O in DMSO-d_6 to the receptors, the NH peaks of both pyrroles and amides underwent significant downfield shifts (Supplementary Figs 3 and 4), consistent with the fact that both the pyrrole and the amide NH protons participate in binding the chloride anion via hydrogen-bonding interactions[35]. On subjecting compounds **1** and **2** to titration with NaCl in 10% D₂O in DMSO-d_6, two sets of distinguishable resonances were seen for all observable proton signals in the ¹H NMR spectra recorded before saturation (at 1.0 equiv.) was achieved (Supplementary Figs 5 and 6). Such findings are consistent with the suggestion that receptors **1** and **2** bind chloride strongly and with a 1:1 binding stoichiometry. Downfield shifts of CH proton res-onances associated with the strap, especially the aromatic pyridine

ring and aliphatic Hₑ signals, were observed on treatment with NaCl. This is taken as evidence that the sodium cation interacts with the carbonyl oxygen atoms of the amide groups. Support for this conclusion comes from single-crystal X-ray diffraction analyses, which revealed the sodium counter-cation bound via an outside binding mode (Fig. 1b and Supplementary Fig. 7). This stands in contrast with the 'within-the-bowl' binding mode seen for larger cations, such as the caesium and tetraethylammonium cations (Supplementary Figs 8–10).

In contrast to **1** and **2**, compounds **3–5**, which lack either an amide group or a C4P subunit, proved less effective as receptors for chloride. Association constants (K_a) of 617 and 198 M⁻¹, respectively, were calculated for compounds **3** and **5** with chloride; these values are significantly lower than that observed for com-pound **1** under identical conditions ($K_a = 4.55 \times 10^4$ M⁻¹).

Ion-transport activity of diamide-strapped calixpyrroles in a liposome model. The ability of compounds **1–5** to transport chloride and sodium across model membranes that consisted of 1-palmitoyl-2-oleoylphosphatidyl choline (POPC) phospholipid bilayers was assessed through a series of unilamellar vesicle assays monitored by ion-selective electrodes, fluorescence or ²³Na NMR spectrometry using methods described previously[36]. These model membrane studies can allow for limiting mechanisms of transport (including anion and cation co-transport ('symport'), anion-for-anion exchange ('antiport') and 'dual host', wherein the anion and cation are carried by separate transporters) to be identified for synthetic carrier systems.

On the basis of these transport studies, detailed in the Supplementary Information, we conclude that the strapped C4Ps **1** and **2** function as moderately effective chloride anion transporters in the liposomal phospholipid bilayer (via a chloride/nitrate antiport process in the presence of intra- and extravesicular sodium cations), whereas compounds **3**, **4** and **5** have a much-reduced chloride-transport activity relative to the amide-strapped calixpyrroles (Fig. 2a). In the presence of monensin, a known sodium ionophore and carrier, the efflux of chloride mediated by compounds **1** and **2** was greatly promoted as monitored at 300 s. In this case transport presumably occurs via coupled sodium and chloride co-transport by the monensin and calixpyrrole[32]. On this basis, we propose that over the course of the much longer timescale (about 18 hours of incubation) associated with cellular incubation studies, com-pounds **1** and **2** will be capable of mediating the transport of Cl⁻ coupled to biological Na⁺ transport processes. However, it is
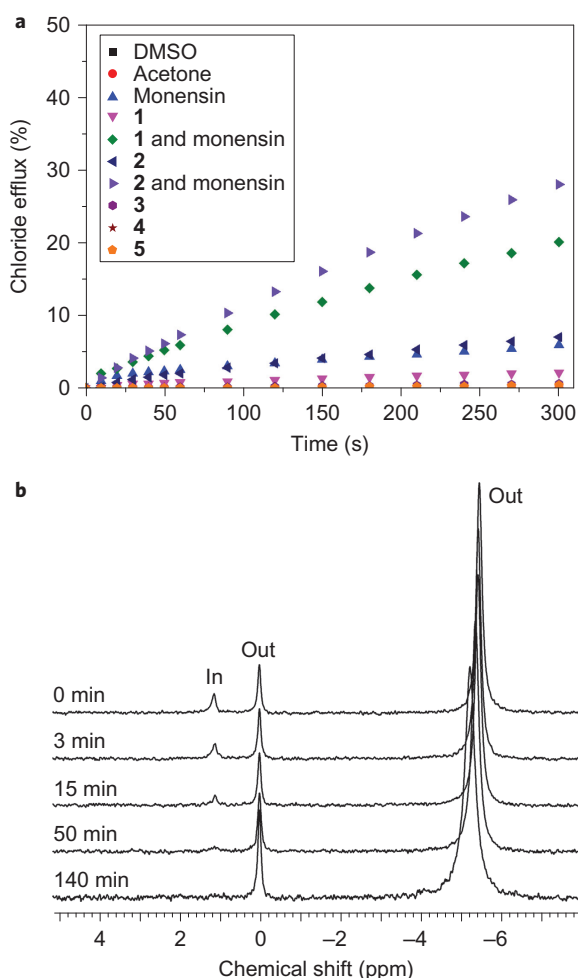
Figure 2 | Transport studies using liposomal model membranes. a, Change in extravesicular chloride concentration over time seen for unilamellar vesicles that contained 489 mM NaCl suspended in a 167 mM K_2SO_4 solution, buffered to pH 7.2 with 20 mM sodium phosphate salts, on the addition of a DMSO solution of receptors **1–5** (2 mol%), an acetone solution of monensin (2 mol%) or a DMSO/acetone solution of either receptor **1** or **2** and monensin (2 mol% each). **b,** ^{23}Na NMR spectrum before the addition and three minutes, 15 minutes, 50 minutes and 140 minutes after the addition of **2** (2 mol%) to vesicles that contained 489 mM NaCl dispersed in 167 mM K_2SO_4 solution, buffered to pH 7.2 with 20 mM potassium phosphate salts, with the addition of adequate $Na_7Dy(PPPi)_2$ (sodium bis (tripolyphosphate)dysprosium(III)).

important to appreciate that in the absence of monensin and in the absence of a hydrophobic extravesicular anion, such as nitrate, compound **2** will co-transport sodium and presumably chloride (symport mechanism), as observed using ^{23}Na NMR techniques over longer timescales (see Fig. 2b). Thus, even in the absence of fully functioning biological sodium transport mechanisms, residual biological activity is predicted for carriers **1** and **2**. Evidence in support of this proposition is provided below.

Ion transporters induce an increase in intracellular chloride and sodium concentrations. Previous studies have served to show that a stimulated influx of chloride ions into cells induces cell death[8,9]. To examine the effect of the present ion transporters on cell viability, several cancer and normal cell lines (A549, lung adenocarcinoma epithelial cells; Capan-1, pancreas adenocarcinoma cells; HCT116, colorectal carcinoma cells; HeLa, cervical cancer cells; MRC-5, human fetal lung fibroblast cells; NRK, normal rat kidney cells;

267B1, human fetal prostate epithelial cells) were treated with various concentrations (0–80 μM) of **1–5** for 18 hours. Cell viabilities were then measured using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In the cases of **1** and **2**, but not of **3–5**, the number of viable cells was found to decrease in a dose-dependent manner with half-maximum inhibitory concentration (IC_{50}) values that ranged from 10 to 15 μM, irrespective of cell type (Supplementary Fig. 79).

We then investigated changes in the concentrations of several intracellular ions after treatment of cells with **1** and **2**, as well as a negative control, **3**. Alterations in intracellular chloride concentrations were evaluated by incubating Fischer rat thyroid epithelial (FRT) cells with each compound for two hours. These cells express a mutant yellow fluorescent protein (YFP-F46L/H148Q/I152L) whose fluorescence is sensitively quenched by chloride ions in the cytosol[37]. The results obtained show that **1** and **2** induce significantly increased intracellular chloride concentrations based on the decreased fluorescence of a mutant YFP in the cells (Fig. 3a). However, the intracellular chloride concentrations were not altered in cells treated with control **3**. As FRT cells lack chloride channels, we can rule out the possibility that the chloride ion influx observed in **1** and **2** takes place via chloride channels. Rather, we suggest that it is the result of active synthetic transporter-induced chloride transport.

To provide support for the proposed mechanism, for compounds **1** and **2** we next investigated which cations, if any, entered the cells along with the chloride ions. Initially, changes in intracellular sodium concentrations were measured by incubating A549 and HeLa cells with the sodium fluorescent probe SBFI-AM (sodium-binding benzofuran isophthalate acetoxymethyl ester) for 1.5 hours followed by treatment with 15 μM solutions of **1** and **2**, as well as of control compound **3**, for two hours in each case[38]. It was found that carriers **1** and **2** proved effective in increasing the intracellular sodium concentration (Fig. 3b and Supplementary Fig. 80b). To check whether sodium ions enter cells directly as a result of the transport by carriers **1** and **2** or via endogenous sodium channels, we examined the effect of amiloride on the sodium influx elicited by synthetic transporters. Amiloride is an inhibitor that directly blocks sodium channels. In its presence, the sodium influx induced by **1** and **2** was attenuated substantially, although not eliminated completely (Supplementary Fig. 80c,d).

The above findings lead us to conclude that (1) receptors **1** and **2** act predominantly as chloride anion transporters and (2) the sodium counter ions enter cells predominantly through cellular sodium channels during the course of the transporter-induced chloride influx. Consistent with this hypothesis is the finding that the chloride entry induced by **1** and **2** is reduced in the presence of amiloride (Supplementary Fig. 80e).

In contrast to what proved true for sodium, neither the intracellular potassium nor calcium concentrations were altered in cells treated with **1** and **2**, as inferred from experiments that involved preincubation of the cells with the potassium and calcium fluorescent probes, PBFI-AM (potassium-binding benzofuran isophthalate acetoxymethyl ester) and Fluo-4, respectively (Supplementary Fig. 80f,g)[39,40]. As the extracellular sodium concentration (145 mM) is normally much higher than its intracellular concentration (12 mM) and the extracellular potassium concentration (4 mM) is much lower than its intracellular concentration (150 mM)[1], transporters **1** and **2** induce sodium influx but not potassium influx.

Taken together, the above results provide support for the conclusion that compounds **1** and **2** act as into-cell Cl^- transporters under conditions in which control systems, such as **3**, do not. The chloride influx induced by transporters **1** and **2** is coupled to sodium entry mediated largely through the action of natural
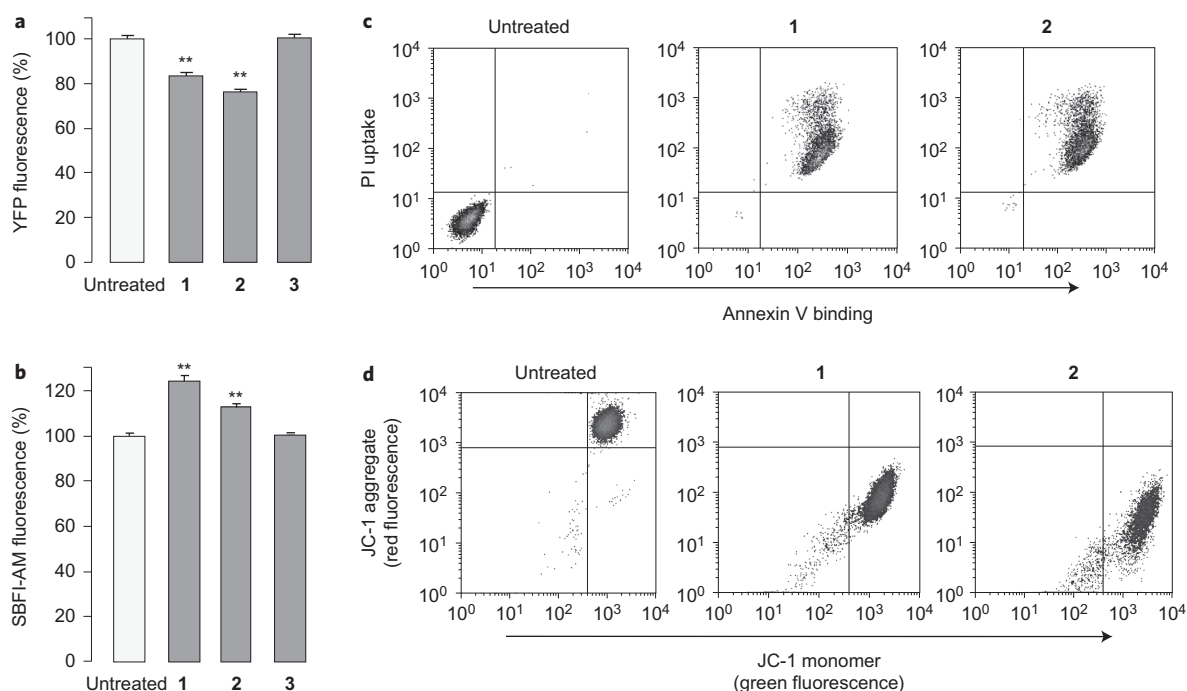
Figure 3 | Ion transporters induce apoptosis. a, FRT cells were incubated with 15 µM of **1–3** for two hours and YFP fluorescence was measured to deduce changes in the respective intracellular chloride ion concentrations (mean ± s.e.m., $n = 10$). **b**, A549 cells pretreated with 10 µM SBFI-AM for 1.5 hours were incubated with 15 µM of **1–3**. The SBFI-AM fluorescence was then measured to examine changes in the intracellular sodium ion concentrations (mean ± s.e.m., $n = 6$). **$**P < 0.001$. **c**, Flow cytometry of A549 cells treated with 15 µM of **1** or **2** 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 A549 cells treated with 15 µM of **1** or **2** for 18 hours and stained with JC-1. Untreated cells are shown as a negative control. Also shown is a dot plot of red fluorescence (FL2, JC-1 aggregate) versus green fluorescence (FL1, JC-1 monomer).

sodium ion channels. The coupled transfer of both chloride anions and sodium cations is the result of charge considerations and is required to maintain ion balance within the cells.

Transporters induce apoptotic cell death. A number of studies have served to demonstrate that dysregulation of ion homeostasis, particularly via chloride influx, can induce cell shrinkage and lead to apoptosis[8,9]. To investigate whether synthetic transporters could lead to apoptosis, HeLa and A549 cells were incubated with 15 µM of **1** or **2** for 18 hours and then treated with a mixture of fluorescein-annexin V and propidium iodide (PI). The results of flow cytometry analysis revealed that **1** and **2** induced apoptosis, as inferred from the observation of positive annexin V binding and PI uptake (Fig. 3c and Supplementary Fig. 81a)[41]. In addition, the loss of mitochondrial membrane potential, a hallmark of apoptosis, was also examined using a JC-1 probe that is sensitive to membrane potential[42]. The intensity of dye-derived red fluorescence in cells treated with 15 µM of **1** or **2** for 18 hours decreased significantly, as would be expected for apoptotic cell death (Fig. 3d and Supplementary Fig. 81b). An increase in DNA fragmentation was also observed in cells treated with **1** or **2** for 18 hours (Supplementary Fig. 82). Cells treated with 15 µM of **1** for six hours were also subjected to a gene-expression profiling analysis of mRNA. It was found that a number of genes involved in apoptosis were dysregulated by more than twofold compared to controls (Supplementary Table 8). Taken together, these results provide clear evidence that transporters **1** and **2** have apoptosis-inducing activity.

Transporters induce caspase activation but do not activate the apoptotic pathway associated with the apoptosis inducing

factor (AIF). Apoptotic cell death takes place mainly via caspase-dependent and independent mechanisms[43]. As an initial test of whether the present ion transporters induce caspase-dependent apoptosis, HeLa cells were incubated with **1** or **2** and the proteolytic activities associated with caspases were examined. The activities of caspases in lysates of cells treated with 10 and 15 µM of **1** or **2** for 18 hours were measured using a colorimetric peptide substrate, Ac-DEVD-pNA (pNA, *p*-nitroaniline), for caspases. Increases in caspase activity were observed for both **1** and **2** (Fig. 4a). However, when Ac-DEAD-CHO, a known inhibitor of caspases, was added to the lysates of cells treated with **1** or **2**, caspase activities were attenuated.

Cytochrome *c* and caspase-3 play an important role in caspase-dependent apoptosis. Cytochrome *c*, which is located in the space between the inner and outer mitochondrial membranes, is released from the mitochondria into the cytosol during the caspase-dependent apoptosis. The released cytochrome *c* binds to Apaf-1 to form the apoptosome[44]. The cytochrome *c*/Apaf-1 complex activates caspase-9, which subsequently induces activation of caspase-3 through the cleavage of procaspase-3[44]. Thus, we examined the release of cytochrome *c* from the mitochondria and caspase-3 activation by treating cells with **1** or **2**. Immunoblot analysis of HeLa and A549 cells treated with both 10 and 15 µM of **1** or **2** for 18 hours revealed that the cytochrome *c* was released from the mitochondria into the cytosol and procaspase-3 was proteolytically cleaved to produce caspase-3 (Fig. 4b). Additional experiments aimed at evaluating cleavage of an endogenous caspase substrate, PARP, by western blot analysis revealed that the cleaved product of PARP was generated in the treated cells.

To evaluate whether the pan-caspase inhibitor ZVAD-FMK (benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone),
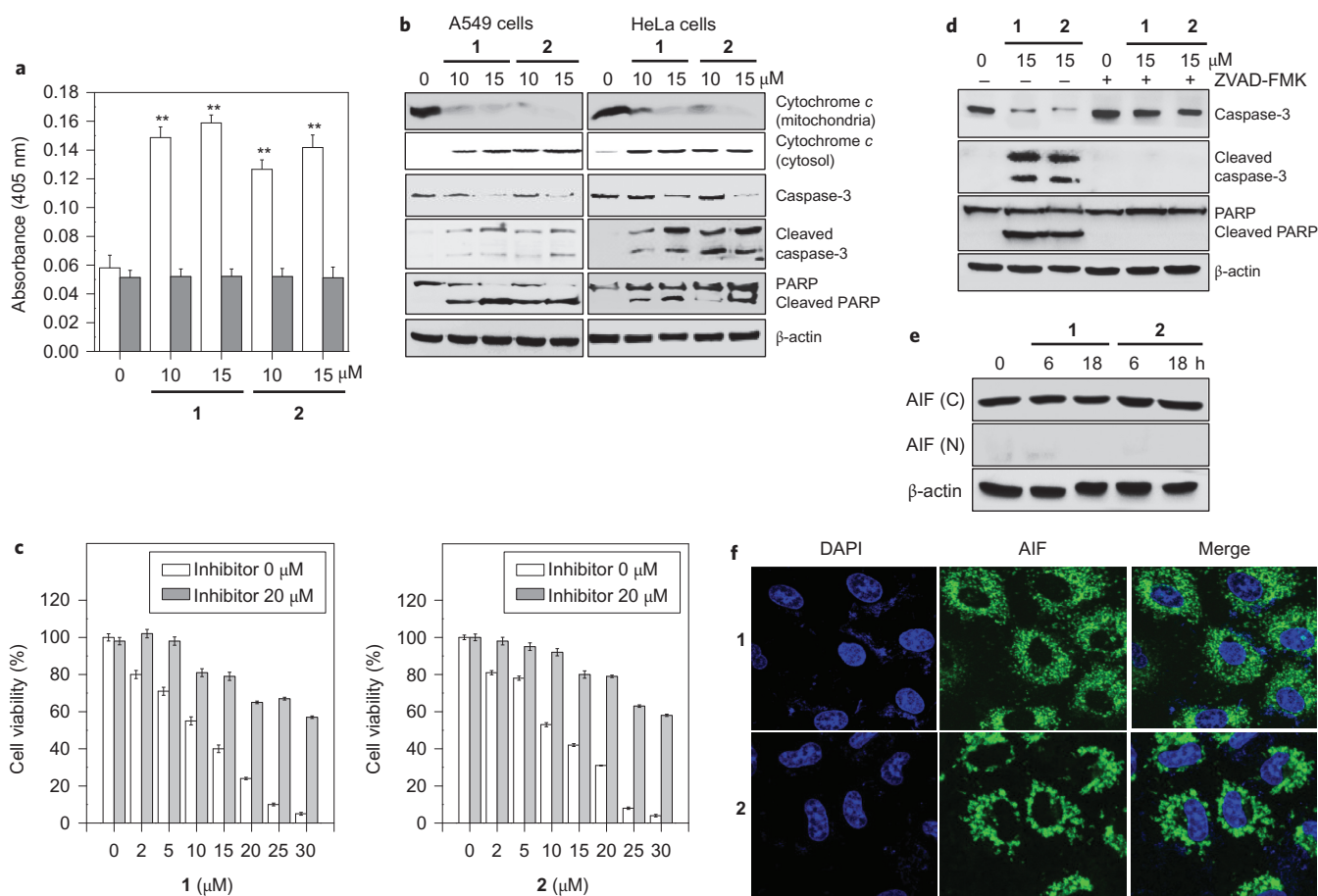
Figure 4 | Ion transporters induce caspase activation but do not activate the AIF-associated apoptotic pathway. a, The caspase activities of lysates of HeLa cells treated with 10 and 15 μM of either **1** or **2** for 18 hours were measured using acetyl-DEVD-pNA in the absence (white bar) or presence (grey bar) of 200 μM Ac-DEVD-CHO (mean ± s.d., **$P < 0.001$). **b**, A549 and HeLa cells were treated with 10 and 15 μM of **1** or **2** for 18 hours and the indicated proteins were immunoblotted using the appropriate corresponding antibodies. **c**, HeLa cells were preincubated with 20 μM of the caspase inhibitor ZVAD-FMK for three hours, and then treated with various concentrations of **1** or **2** for 18 hours. The effect of this caspase inhibitor on cell survival was determined using a MTT assay (mean ± s.d.). **d**, HeLa cells preincubated with 20 μM ZVAD-FMK were treated with 15 μM of **1** or **2** for 18 hours and the indicated proteins were immunoblotted using the corresponding antibodies. **e**, HeLa cells were treated with 10 μM of **1** or **2** for six and 18 hours. Western blot was performed with AIF antibody. **f**, HeLa cells, treated first with 10 μM of **1** or **2** for 18 hours, were immunostained using an anti-AIF antibody. The nuclei were stained with DAPI.

which is a cell-permeable, irreversible caspase inhibitor with broad specificity, would protect cells against the effect of transporters **1** and **2**, HeLa cells preincubated with 20 μM ZVAD-FMK for three hours were treated with 15 μM of either **1** or **2** for 18 hours. Under these conditions the cells were largely protected from apoptotic cell death (Fig. 4c). The results of the western blot analysis provided further confirmation that caspase activation induced by **1** and **2** was suppressed by treatment with 20 μM of the inhibitor (Fig. 4d). These results provide support for the proposal that compounds **1** and **2** induce apoptosis via a caspase-dependent pathway.

We then evaluated whether **1** and **2** induced cell death via AIF-mediated caspase-independent apoptosis. AIF is translocated into the nucleus during the caspase-independent apoptosis[45]. Therefore, a comparison of the AIF levels in the cytosolic and nuclear fractions would provide a measure of apoptosis that occurs in a caspase-independent manner. Thus, HeLa cells were treated with 10 μM of **1** or **2** for either six or 18 hours. It was found that treatment with **1** or **2** did not induce AIF translocation into the nucleus (Fig. 4e,f). On this basis, we conclude that transporters **1** and **2** induce caspase activation but do not activate the AIF-associated apoptotic pathway.

Effect of chloride and sodium ions on transporter-induced cell death. To determine whether cell death is correlated with enhanced levels of chloride or sodium influx, we evaluated the effect of extracellular Cl^- and Na^+ on cells treated with **1** and **2**[9,46]. Here, HeLa and A549 cells were incubated for four hours with various concentrations (0–25 μM) of **1** or **2** in buffers with both chloride and sodium ions (Hank's balanced salt solution (HBSS)) or in analogous buffers free of either chloride anion (Cl^--free HBSS) or sodium cation (Na^+-free HBSS). When added to cells in the Cl^--free or Na^+-free buffers, transporters **1** and **2** proved less potent; that is, they produced a remarkably lower decrease in cell viability compared to those seen in buffers containing both ions (Fig. 5a and Supplementary Figs 83–85).

The extent of cell shrinkage, a further marker of apoptosis, was also measured for A549 cells treated with **1** or **2** for two hours in the presence and absence of Cl^-. Analysis of cell size by flow cytometry revealed that, whereas cells treated with **1** and **2** in HBSS exhibited a large degree of cell shrinkage, almost no change in cell size was observed for cells treated in Cl^--free HBSS (Fig. 5b). This is taken as additional evidence that the tested cells undergo apoptosis. On the same basis, cell death via necrosis is ruled out because such a process induces cell swelling.
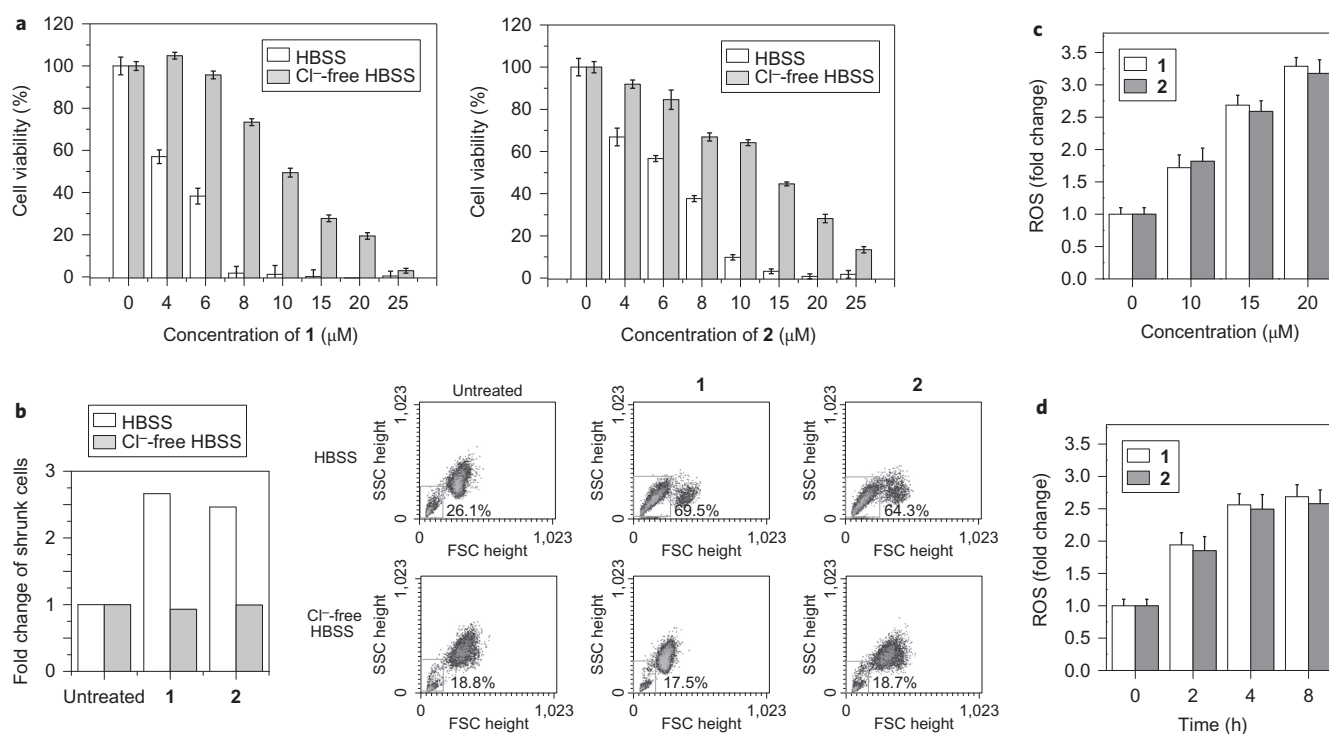
Figure 5 | Effect of ions on transporter-induced cell death. a, A549 cells were incubated with various concentrations (0–25 μM) of **1** or **2** in HBSS or Cl⁻-free HBSS for four hours. Cell death was measured using a MTT assay (mean ± s.d.). **b**, A549 cells were incubated with 15 μM of **1** or **2** for two hours in HBSS or Cl⁻-free HBSS, and the percentage of shrunk cells within the total cell population was determined using flow cytometry. **c,d**, HeLa cells were treated with 10, 15 and 20 μM concentrations of the two ion transporters considered in this study (white bar, **1**; grey bar, **2**) for eight hours (**c**) or with 15 μM of the same ion transporters (white bar: **1**, grey bar: **2**) for various indicated incubation times (**d**). The cells were then treated with 10 μM DCFH-DA to monitor the ROS production (mean ± s.d.). FSC, forward scatter; SSC, side scatter.

We also examined if amiloride, which blocks activities of sodium channels in cells but rarely induces cell death on its own, affected cell death induced by **1** and **2**. In these studies, HeLa and A549 cells were incubated for 18 hours with 15 μM of **1** or **2** in the presence of various concentrations (0–400 μM) of amiloride. The results showed that treatment with amiloride reduced the extent of cell death mediated by transporters **1** and **2** in a dose-dependent manner. This finding is fully consistent with the suggestion that the inhibitor indirectly suppresses carrier-mediated chloride influx for charge-balance reasons, as discussed above (Supplementary Fig. 86). Taken together, we conclude that the transporter-mediated apoptosis induced by transporters **1** and **2** is dependent on the presence of both extracellular chloride and sodium ions and requires active sodium channels to occur at a high level.

Transporters induce ROS generation in cells but do not modulate intracellular pH. It has previously been shown that a number of therapeutic agents induce cell death via promoted chloride influx or via increased levels of intracellular ROS[8]. Therefore, we examined whether transporters **1** and **2** promote ROS generation in cells. For these studies, HeLa cells were treated with either **1** or **2** at different concentrations and for varying incubation periods. The levels of intracellular ROS were then measured by treating the cells with 10 μM dichlorofluorescein diacetate (DCFH-DA)[47]. After treatment with **1** and **2**, an increase in the fluorescence intensity was observed (Fig. 5c,d), which indicates ROS production.

We also tested whether transporters **1** and **2** induced changes in intracellular pH. In this study, FRT cells were incubated with 15 μM of **1**–**3** for 1.5 hours and then treated for 30 minutes with the pH-sensitive fluorescent probe BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein)[48]. For all three compounds,

intracellular pH was almost unchanged under these incubation conditions (Supplementary Fig. 87). This leads us to conclude that a decrease (or increase) in cytosolic pH is not implicated in the early events that result in the apoptosis induced by **1** and **2**.

Finally, we conducted experiments to study the temporal relationship between changes in the intracellular concentrations of sodium chloride, ROS production and apoptosis. In this study, A549 cells, which were incubated with 15 μM of **1** or **2** at different time periods, were treated with the sodium fluorescent probe SBFI (sodium-binding benzofuran isophthalate), the Cl⁻-quenching fluorescent probe MQAE (N-(ethoxycarbonylmethyl)-6-methoxy-quinolinium bromide)[49], the ROS probe DCFH-DA (dichlorodihydrofluorescein diacetate) and FITC (fluorescein isothiocyanate)–annexin V. The results indicate that annexin V-positive cells appear approximately four hours after incubation with synthetic transporters, but the sodium chloride influx and ROS increase take place earlier (Supplementary Fig. 88). On this basis, we conclude that ion influx induced by the present ion transporters is likely to be a cause of apoptosis, rather than apoptosis leading to changes in ion flux, as might be expected if compounds **1** and **2** were operating via a non-transport mechanism.

Taken together, all the results show that transporters **1** and **2** cause an increase in intracellular concentrations of sodium chloride and ROS levels. The increased level of ROS mediated by **1** and **2** induces the release of cytochrome *c* from the mitochondria, as proved true in other instances[50], and this, in turn, leads to caspase-dependent apoptosis.

Conclusions
In summary, the pyridine-strapped diamides **1** and **2** mediate the transport of Cl⁻ and concomitantly the Na⁺ influx in cellular milieux. These systems, but not **3**–**5**, induce apoptosis via a

caspase-dependent pathway. The transporter-mediated cellular response was found to be strongly dependent on extracellular sodium and chloride ions. We also show that these transporters increase intracellular sodium chloride concentrations, enhance cellular ROS levels, cause the release of cytochrome c from the mitochondria and induce caspase activation. On this basis we propose that receptors **1** and **2** could emerge as useful molecular tools that may be used to induce apoptosis via modes of action that differ from the approaches normally used to trigger so-called programmed cell death. This, in turn, is expected to increase our understanding of how ion fluxes modulated through the application of external agents or induced through disease states give rise to physiological outcomes at the cellular and ultimately organismal levels.

Methods

Synthesis. All the synthetic methods are described in the Supplementary Information. Full characterization data are also provided, including crystallographic data for **1**, **3**, **1·NaCl**, **1·CsCl**, **2·Et₄N⁺Cl⁻**, **2·NaCl** and **2·CsCl**, as are the experimental details for the liposomal transport studies and for the cell studies and measurements of intracellular ion concentrations.

Membrane transport. Typical membrane transport tests were carried out using POPC-based model membranes as detailed in the Supplementary Information. Chloride concentrations during the transport experiments were determined using an Accumet chloride-selective electrode calibrated against sodium chloride solutions of known concentrations prior to each experiment, or based on lucigenin fluorescence intensities measured using a Varian Cary Eclipse Fluorescence Spectrophotometer. A lipid film of POPC was formed from a chloroform solution under reduced pressure and dried under vacuum for at least four hours. The lipid film was rehydrated by vortexing with the internal solution. The lipid suspension was then subjected to nine freeze–thaw cycles, in which the suspension was alternatively allowed to freeze in a liquid-nitrogen bath, followed by thawing in a water bath. The lipid suspension was allowed to age for 30 minutes at room temperature and subsequently extruded 25 times through a 200 nm polycarbonate membrane (Nucleopore) using a LiposoFast-Basic extruder set (Avestin, Inc.). The resulting unilamellar vesicles were dialysed (Spectra/Por 2 Membrane, molecular weight cut-off 12–14 kD) against the external medium to remove unencapsulated internal salts or passed through a Sephadex (G-50) column to remove unencapsulated dye. The unilamellar POPC vesicles were then suspended in the external solution so that the lipid concentration per sample was 1 mM. A DMSO or acetone solution of the carrier molecule was added to start the experiment and the chloride or Na⁺ efflux was monitored using a chloride-sensitive electrode, lucigenin fluorescence or ²³Na NMR spectroscopy. At the end of the experiment the vesicles were lysed with a solution of polyoxyethylene(8)lauryl ether. Other transport experiments designed to distinguish antiport from symport mechanisms are described in detail in the Supplementary Information.

Biological studies. Cell-culture studies were carried out with A549, Capan-1, HCT116, HeLa, MRC-5, NRK, 267B1 and Calu-3 cells. FRT cells were transfected stably with the plasmid that contained the halide sensor yellow fluorescent protein (YFP) gene, a mutant YFP (YFP-F46L/H148Q/I152L) that is quenched sensitively by chloride ions in the cytosol. Measurements of cell death were made by plating 0.1 ml of cells in triplicate in 96-well plates for 24 hours and incubating with various concentrations of the compounds subject to analysis in culture media, HBSS, Cl⁻-free or Na⁺-free HBSS. Measurements of intracellular Cl⁻ concentrations were made using FRT cells that expressed the mutant YFP. The cells were incubated with each compound (15 μM) for two hours at 37 °C. The YFP fluorescence was measured using a fluorescence microplate reader (M200 pro, TECAN) ($\lambda_{excitation}$ (λ_{ex}) = 480 nm, $\lambda_{emission}$ (λ_{em}) = 530 nm). For the measurements of the time-dependent influx of Cl⁻ in A539 cells, the cells were incubated with culture media that contained 10 mM MQAE for one hour at 37 °C. After washing with PBS, the cells were incubated with culture media that contained each compound (15 μM) for the indicated times at 37 °C. The MQAE fluorescence was measured using a fluorescence microplate reader (λ_{ex} = 350 nm, λ_{em} = 460 nm). Intracellular Na⁺ concentrations were measured using A549 and HeLa cells incubated with each compound (15 μM) for the indicated times at 37 °C. The SBFI-AM fluorescence was measured using a fluorescence microplate reader (λ_{ex} = 340 nm, λ_{em} = 500 nm). Intracellular K⁺ concentrations were measured using Calu-3 cells incubated with each compound (15 μM) for two hours at 37 °C. The PBFI fluorescence was measured using a fluorescence microplate reader (λ_{ex} = 344 nm, λ_{em} = 500 nm). Western blot analyses were carried out in accord with normal procedures, as detailed in the Supplementary Information. Caspase activity was determined using acetyl-DEVD-pNA, a preferred substrate for caspase-3 and -7. The enzyme-catalysed release of pNA was monitored at 405 nm in an ultraviolet microplate reader. For *in vivo* caspase-inhibition studies, the pancaspase inhibitor ZVAD-FMK was used. HeLa cells were pretreated with 20 μM ZVAD-FMK for three hours and then incubated with various concentrations of each compound for 18 hours. Cells were analysed by MTT assay and western blot as described above. The

generation of ROS was studied using HeLa and A549 cells treated with each compound for eight hours. In addition, the cells were incubated with 15 μM of each compound for two, four and eight hours. The treated cells were incubated with 10 μM of the fluorescent probe DCFH-DA and monitored using fluorescence microscopy (λ_{ex} = 485 nm, λ_{em} = 535 nm). Full details of these cell studies and other analyses are provided in the Supplementary Information.

References

1. Yu, S. P., Canzoniero, L. M. T. & Choi, D. W. Ion homeostasis and apoptosis. *Curr. Opin. Cell Biol.* **13**, 405–411 (2001).
2. Okada, Y. *et al.* Volume-sensitive chloride channels involved in apoptotic volume decrease and cell death. *J. Membr. Biol.* **209**, 21–29 (2006).
3. Newmeyer, D. D. & Ferguson-Miller, S. Mitochondria: releasing power for life and unleashing the machineries of death. *Cell* **112**, 481–490 (2003).
4. Arcangeli, A. *et al.* Targeting ion channels in cancer: a novel frontier in antineoplastic therapy. *Curr. Med. Chem.* **16**, 66–93 (2009).
5. Lehen'kyi, V., Shapovalov, G., Skryma, R. & Prevarskaya, N. Ion channels and transporters in cancer. 5. Ion channels in control of cancer and cell apoptosis. *Am. J. Physiol. Cell Physiol.* **301**, C1281–C1289 (2011).
6. Becchetti, A. Ion channels and transporters in cancer. 1. Ion channels and cell proliferation in cancer. *Am. J. Physiol. Cell Physiol.* **301**, C255–C265 (2011).
7. Cuddapah, V. A. & Sontheimer, H. Ion channels and transporters in cancer. 2. Ion channels and the control of cancer cell migration. *Am. J. Physiol. Cell Physiol.* **301**, C541–C549 (2011).
8. Yu, L. *et al.* A protective mechanism against antibiotic-induced ototoxicity: role of prestin. *PLoS ONE* **6**, e17322 (2011).
9. 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).
10. Lee, J. M., Davis, F. M., Roberts-Thomson S. J. & Monteith, G. R. Ion channels and transporters in cancer. 4. Remodeling of Ca²⁺ signaling in tumorigenesis: role of Ca²⁺ transport. *Am. J. Physiol. Cell Physiol.* **301**, C969–C976 (2011).
11. Remillard, C. V. & Yuan, J. X-J. Activation of K⁺ channels: an essential pathway in programmed cell death. *Am. J. Physiol. Lung Cell Mol. Physiol.* **286**, L49–L67 (2004).
12. Gupta, P. B. *et al.* Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell* **138**, 645–659 (2009).
13. Ding, W-Q., Liu, B., Vaught, J. L., Yamauchi, H. & Lind, S. E. Anticancer activity of the antibiotic clioquinol. *Cancer Res.* **65**, 3389–3395 (2005).
14. Fürstner, A. Chemistry and biology of roseophilin and the prodigiosin alkaloids: a survey of the last 2500 years. *Angew. Chem. Int. Ed.* **42**, 3582–3603 (2003).
15. Busschaert, N. *et al.* Structure–activity relationships in tripodal transmembrane anion transporters: the effect of fluorination. *J. Am. Chem. Soc.* **133**, 14136–14148 (2011).
16. Busschaert, N. *et al.* Towards predictable transmembrane transport: QSAR analysis of anion binding and transport. *Chem. Sci.* **4**, 3036–3045 (2013).
17. Shen, B., Li, X., Wang, F., Yao, X. & Yang, D. A synthetic chloride channel restores chloride conductance in human cystic fibrosis epithelial cells. *PLoS ONE* **7**, e34694 (2012).
18. Sato, T. *et al.* Prodigiosins as a new group of H⁺/Cl⁻ symporters that uncouple proton translocators. *J. Biol. Chem.* **273**, 21455–21462 (1998).
19. Sessler, J. L. *et al.* Synthesis, anion-binding properties, and *in vitro* anticancer activity of prodigiosin analogues. *Angew. Chem. Int. Ed.* **44**, 5989–5992 (2005).
20. Gale, P. A. *et al.* Co-transport of H⁺/Cl⁻ by a synthetic prodigiosin mimic. *Chem. Commun.* **30**, 3773–3775 (2005).
21. Pérez-Tomás, R., Montaner, B., Llagostera, E. & Soto-Cerrato, V. The prodigiosins, proapoptotic drugs with anticancer properties. *Biochem. Pharmacol.* **66**, 1447–1452 (2003).
22. Melvin, M. S. *et al.* Double-strand DNA cleavage by copper prodigiosin. *J. Am. Chem. Soc.* **122**, 6333–6334 (2000).
23. Soto-Cerrato, V., Viñals, F., Lambert, J. R. & Pérez-Tomás, R. The anticancer agent prodigiosin induces 21(WAF1/C1P1) expression via transforming growth factor-beta receptor pathway. *Biochem. Pharmacol.* **74**, 1340–1349 (2007).
24. Davis, A. P., Sheppard, D. N. & Smith, B. D. Development of synthetic membrane transporters for anions. *Chem. Soc. Rev.* **36**, 348–357 (2007).
25. Davis, J. T., Okunola, O. & Quesada, R. Recent advances in the transmembrane transport of anions. *Chem. Soc. Rev.* **39**, 3843–3862 (2010).
26. Gale, P. A. From anion receptors to transporters. *Acc. Chem. Res.* **44**, 216–226 (2011).
27. Díaz de Greñu, B. *et al.* Synthetic prodigiosin obatoclax (GX15-070) and related analogues: anion binding, transmembrane transport, and cytotoxicity properties. *Chem. Eur. J.* **17**, 14074–14083 (2011).
28. Moore, S. J. *et al.* Towards 'drug-like' indole-based transmembrane anion transporters. *Chem. Sci.* **3**, 2501–2509 (2012).

29. Li, X., Shen, B., Yao, X-Q. & Yang, D. Synthetic chloride channel regulates cell membrane potentials and voltage-gated calcium channels. *J. Am. Chem. Soc.* **131,** 13676–13680 (2009).
30. Gould, D. Human physiology. From cells to systems, 3rd edition. *J. Adv. Nurs.* **28,** 680–682 (1998).
31. Russell, J. M. Sodium–potassium–chloride cotransport. *Physiol. Rev.* **80,** 211–276 (2000).
32. Moore, S. J., Fisher, M. G., Yano, M., Tong, C. C. & Gale, P. A. A dual host approach to transmembrane transport of salts. *Chem. Commun.* **47,** 689–691 (2011).
33. Yoon, D-W., Hwang, H. & Lee, C-H. Synthesis of a strapped calix[4]pyrrole: structure and anion binding properties. *Angew. Chem. Int. Ed.* **41,** 1757–1759 (2002).
34. Lee, C-H., Miyaji, H., Yoon, D-W. & Sessler, J. L. Strapped and other topographically nonplanar calixpyrrole analogues. Improved anion receptors. *Chem. Commun.* 24–34 (2008).
35. Custelcean, R. *et al.* Calix[4]pyrrole: an old yet new ion-pair receptor. *Angew. Chem. Int. Ed.* **44,** 2537–2542 (2005).
36. Delort, A-M., Gaudet, G. & Forano E. *Environmental Microbiology: Methods and Protocols* (eds Spencer, J. F. T. & Ragout de Spencer, A. L.) 389–405 (Humana Press, 2004).
37. Jayaraman, S., Haggie, P., Wachter, R. M., Remington, S. J. & Verkman, A. S. Mechanism and cellular applications of a green fluorescent protein-based halide sensor. *J. Biol. Chem.* **275,** 6047–6050 (2000).
38. Minta A. & Tsien R. Y. Fluorescent indicators for cytosolic sodium. *J. Biol. Chem.* **264,** 19449–19457 (1989).
39. Meuwis, K., Boens, N., De Schryver, F. C., Gallay, J. & Vincent, M. Photophysics of the fluorescent K^+ indicator PBFI. *Biophys. J.* **68,** 2469–2473 (1995).
40. Gee, K. R. *et al.* Chemical and physiological characterization of fluo-4 Ca^{2+}-indicator dyes. *Cell Calcium* **27,** 97–106 (2000).
41. 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).
42. Salvioli, S., Ardizzoni, A., Franceschi, C. & Cossarizza, A. JC-1, but not DiOC6 (3) or rhodamine 123, is a reliable fluorescent probe to assess ΔΨ changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. *FEBS Lett.* **411,** 77–82 (1997).
43. Elmore, S. Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* **25,** 495–516 (2007).
44. 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).
45. Joza, N. *et al.* Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature* **410,** 549–554 (2001).
46. 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).
47. Ko, S-K. & Shin, I. Cardiosulfa induces heart deformation in zebrafish through the AhR-mediated, CYP1A-independent pathway. *ChemBioChem* **13,** 1483–1489 (2012).
48. Han, J. & Burgess, K. Fluorescent indicators for intracellular pH. *Chem. Rev.* **110,** 2709–2728 (2010).
49. 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).
50. Circu, M. L. & Aw, T. Y. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic. Biol. Med.* **48,** 749–762 (2010).

Author contributions

J.L.S, I.S. and P.A.G. designed the study and supervised the work. S-K.K. performed biological studies. S.K.K. designed and synthesized compounds and performed ion-binding studies in solution. S.K.K. and V.M.L. carried out the X-ray single-crystal structure analysis. P.A.G., W.VR., N.B. and A.S. designed and performed the ion-transport studies in liposomes. J.P. and W.N. carried out ion-transport activity studies in cells.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to P.A.G., J.L.S and I.S.

Competing financial interests

The authors declare no competing financial interests.