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Efficient, non-toxic anion transport by synthetic carriers in cells and epithelia

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Transmembrane anion transporters (anionophores) have potential for new modes of biological activity, including therapeutic applications. In particular they might replace the activity of defective anion channels in conditions such as cystic fibrosis. However, data on the biological effects of anionophores is scarce, and it remains uncertain whether such molecules are fundamentally toxic. Here we report a biological study of an extensive series of powerful anion carriers. Fifteen anionophores were assayed in single cells by monitoring anion transport in real time through fluorescence emission from halide-sensitive yellow fluorescent protein. A bis-(p-nitrophenyl)ureidodecalin showed especially promising activity, including deliverability, potency and persistence. Electrophysiological tests showed strong effects in epithelia, close to those of natural anion channels. Toxicity assays yielded negative results in three cell lines, suggesting that promotion of anion transport may

not be deleterious to cells. We therefore conclude that synthetic anion carriers are realistic candidates for further investigation as treatments for cystic fibrosis.

The transport of anions across cell membranes has become an important goal for supramolecular chemistry¹⁻⁴. It is well-known that cation carriers (cationophores) can serve as antibiotics and toxins^{5,6}, and it seems likely that anion carriers might also show useful biological activity. However, suitable anionophores have only recently become available⁷⁻¹⁰, and the nature of their biological effects is still in question. A particular hope is that anionophores might be used to replace the activity of endogenous anion channels which are missing or defective^{11,2}. Such deficiencies underlie a number of medical conditions including Best disease, Startle disease, Bartter's syndrome and, most notably, the widespread life-shortening genetic disease cystic fibrosis (CF)^{12,13}.

If anionophores are to be used to treat these "channelopathies", it must be shown that they can be delivered to cells in sufficient quantities to produce substantial effects, of the same order of magnitude as endogenous anion channels, and that these quantities are not toxic to the cells. However, while a wide variety of anionophores have been studied in synthetic membranes (principally large unilamellar vesicles, or LUVs), the range of systems subjected to biological investigations is still quite limited. Moreover, from the point of view of CF treatment, the results thus far have been mixed. On the positive side, a few systems have been tested in whole cells, epithelia or (in one case) genetically modified mice¹⁴, using electrophysiological methods such as patch-clamp and Ussing chamber, and shown to induce anion conduction without obvious toxic effects. These anionophores include synthetic

peptides conceptually related to natural anion channels^{11,15-17}, as well as the steroid-based system **1** from the authors' laboratory¹⁸, and other small organic molecules^{14,19,20}.

Less encouragingly, a number of molecules showing anion transport in LUVs have tested positive for anti-cancer cytotoxicity (potentially valuable, but incompatible with CF treatment)^{8,21-25}. Many of these systems, including the well-studied prodigiosin **2**²⁶, transport protons as well as anions^{8,21-23}. Intracellular acidification can lead to apoptosis²⁶, so in these cases proton transport could underlie toxicity. On the other hand, a recent study on calixpyrroles **3** suggested that anion transport *as such* could be cytotoxic²⁵. As the transport activity of **3** was only weak, this raises the concern that powerful anionophores might be highly toxic and thus unsuitable for CF treatment.

We have developed a range of anionophores based on powerful anion binding sites created from hydrogen bond donor groups mounted on alicyclic scaffolds (see for example Figure

1)^{10,27}. Scaffolds include steroids^{18,28,29}, trans-decalins^{30,31} and substituted cyclohexanes³², and the common design motif is the positioning of axially-directed H-bond donors in 1,5-relationships. Conformational factors promote the convergence of NH groups to create binding sites with high affinities for inorganic anions^{10,32,29}. Several lines of evidence suggest that the compounds act as anion carriers as opposed to self-assembled channels^{10,28}. They are also electrogenic, transporting only anions and therefore mediating transmembrane flow of electrical current. Some of the compounds have shown outstanding activities for anion transport when pre-loaded into LUVs, promoting rapid chloride-nitrate exchange at transporter:lipid ratios as low as 1:1,000,000³¹.

Thus far the only biological testing of these “1,5-diaxial” systems has been the early study on **1**, referred to above¹⁸. The assay involved the application of **1** to the apical membrane of oriented Madin Darby canine kidney cell (MDCK) epithelia, mounted in an Ussing chamber, and measurement of the resulting electrical current caused by Cl⁻ transport. The methodology is well-established, but not so convenient for screening large numbers of compounds. Moreover, for secure interpretation, it should be complemented by studies using an alternative technique, ideally at the single cell level. We now report a new fluorescence-based protocol for testing anionophores in cells, and its application to 15 of the 1,5-diaxial family of anionophores. In particular, we identify one decalin-based compound, the *p*-nitrophenylurea **13**, which shows special promise. We confirm that **13** is also highly active in the Ussing chamber assay, giving currents which compare well with natural levels of anion transport. Finally, we show that **13** exhibits no or minimal toxicity towards three epithelial cell lines, encouraging the hope that anionophores might indeed be used as treatments for channelopathies such as CF.

(FIGURE 1)

Results and Discussion

Anionophore synthesis and studies in synthetic membranes. The anionophores employed in this study are shown in Figure 1, grouped according to scaffold (steroid-based cholapod, trans-decalin or cyclohexane) and principal H-bond donor group (urea, thiourea and squaramide). Compounds **4-6**, **8-12**, **17** and **18** have been reported previously (for references see Table 1). The new compounds **7** and **13-16** were prepared according to known methodology as discussed in the supplementary information. As part of this work an X-ray crystal structure was obtained for **13** co-crystallised with $\text{Me}_4\text{N}^+\text{Cl}^-$, showing the chloride anion held through 4 $\text{NH}\cdots\text{Cl}^-$ hydrogen bonds (Fig. 1 inset). This is the first crystallographic structure of an anion complexed to an uncharged 1,5-diaxial receptor/transporter.

The new receptors underwent standard testing for affinity to chloride in CHCl_3 and dimethyl sulfoxide (DMSO), and for anion transport (chloride/nitrate exchange) when preloaded into 200 nm LUVs. The latter assay was performed using the well-established “lucigenin method”³³ in which the influx of chloride into the vesicles is followed through quenching of fluorescence from the halide-sensitive fluorophore lucigenin. The data are listed in Table 1, along with corresponding values for the known compounds and other relevant parameters (molecular weight and cLogP – see later). The transport activity is given as the specific initial rate [*I*], a

quantity defined in previous work to allow comparison of anionophores with widely differing activities³¹.

Also listed in Table 1 are the results of a new test for “deliverability”. This was motivated by concern that some anionophores might perform much better when preloaded into LUV membranes than when delivered externally (i.e. added to LUV suspensions). The test involved addition of the anionophores as solutions in methanol to transporter-free vesicle suspensions, using a standard protocol involving rapid expulsion from a microsyringe. Fluorescence decay curves were acquired after addition of chloride, and the results were compared to experiments using preincorporated transporter. The results were not perfectly reproducible but showed a reasonable level of consistency. Delivery was then graded on a four-point scale (A-D) as discussed further in the supplementary information.

(TABLE 1)

Anion transport in live cells – a fluorescence-based assay. We sought a method which would allow the real-time monitoring of anion transport into individual cells while subjecting the cells to minimal disruption and stress. For the latter reason, we preferred to avoid loading the cells with externally-added indicators³⁵, but instead to use a halide-sensitive fluorophore which could be genetically encoded and produced within the cells. Among the variations of green fluorescent protein (GFP) there are yellow versions (YFPs) which are known to be sensitive to halide concentrations. Verkman and coworkers have shown that these can be introduced into cell lines by transfection, then used in assays for chloride channel modulators³⁶. A variant YFP-

H148Q/I152L is especially sensitive for iodide vs. chloride, which allows it to be used to follow halide exchange³⁷.

Our method for anionophore testing employed Fischer rat thyroid (FRT) cells expressing YFP-H148Q/I152L (hereafter termed YFP-FRT cells). The experiment is outlined in Figure 2a. YFP-FRT cells are first exposed to phosphate buffered saline (PBS) solution containing a potential anionophore (1 μ M) and the lipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, 10 μ M) for 10 minutes at 37 °C (Fig. 2a/1-2). The POPC is employed to aid delivery of lipophilic agents to the cell membranes. The anionophore/POPC mixture is removed by perfusion with PBS, and the cells are observed in a fluorescence microscope. NaI (10 mM) is then added to the extracellular PBS solution (Fig. 2a/3). If no transporter is present, iodide cannot enter the cell because a balancing flow of chloride is not possible. FRT cells are relatively impermeable to anions because they lack key epithelial ion channels (including cystic fibrosis transmembrane conductance regulator (CFTR), the channel defective in CF³⁸). However, in the presence of an anionophore, iodide influx occurs leading to YFP fluorescence quenching (Fig. 2a/4). In a final step (not shown in Figure 2a), the iodide can be removed from the external solution by perfusion with PBS, leading to a reversal of the transport process and recovery of fluorescence.

Typical fluorescence traces and controls are shown in Figure 2b. If neither anionophore nor NaI were added, a steady decay was observed (blue trace; presumably due to photobleaching). If the cells were treated with just POPC, and then iodide was added, the decay profile was almost unchanged (red trace). However, several of the compounds from Figure 1 gave a clear response. For example, after treatment of the cells with **12** or **13**,

addition of iodide caused a pronounced increase in fluorescence decay rates (green and purple traces). Washing with PBS was initiated 5 min after addition of iodide, and caused substantial recovery. A set of images showing the initial fluorescence decay for cells treated with **13** is shown in Figure 2c.

(FIGURE 2)

To quantify anion transport by different anionophores, we fitted first order exponential functions to the first 2 minutes of the I^- -induced fluorescence decay to measure the initial slope, averaging the values over multiple cells (typically 10-65 from at least three independent experiments). The results are shown graphically in Figure 2d, and are also listed in Table 1 (right hand column) after correction for background decay. Representative traces for each compound tested are shown in Figure 2e. Three of the agents (**12-14**) were also tested using a variation of the protocol in which the POPC was omitted and compounds were added to the cells as solutions in DMSO. The results are given in Table 1 (right hand column, numbers in brackets) and Supplementary Fig. S28. The data in Table 1/Figure 2d reveal large differences in activity. There seems to be little correlation between transport rates measured in vesicles and activity in cells. Various explanations are possible, but delivery to the cell membranes is presumably a major issue. The steroidal cholapods are surprisingly inactive. Only the bis-thiourea **10**, which is extremely effective in LUVs, shows substantial activity in the YFP-FRT cells. The contrast between **7** and **13** is especially striking – in LUVs their performance is almost identical, yet in the cells they are at opposite ends of the spectrum. One might speculate that the “naturalness” of the steroidal framework is a disadvantage, perhaps leading

to specific inhibitory interactions with biomolecules. Alternatively, the delivery method may be especially inefficient for the steroids.

Decalins **12-16** and cyclohexanes **17/18** are generally more active, and among these compounds it is the bis-*p*-nitrophenylurea **13** which stands out. Interestingly, this is the *least* active of the non-steroidal agents when preincorporated into LUVs. However it scores highest in the LUV deliverability test, and this suggests that deliverability may be the dominant factor among these compounds. In support of this conclusion, the advantage of **13** vs. **12** and **14** is even greater when DMSO is used as delivery vehicle (i.e. in the absence of POPC). Presumably POPC can compensate to some extent for poor intrinsic deliverability, by forming water-soluble aggregates with lipophilic anionophores. However, when DMSO is used as the vehicle the compounds will precipitate after addition, and must then locate and enter the cell membranes without assistance. It may be significant that **13** is the least lipophilic of all the compounds tested (cLogP = 4.5), so is presumably the best able to migrate through aqueous solution. It is also the smallest (MW = 569), and comes closest to satisfying Lipinski's "rule of 5" for drug-like molecules³⁹.

Concentration dependence and persistence of transport by 13. The exceptional activity of **13** prompted a more detailed study of this anionophore. Figures 3a and b show the relationship between the concentration of **13** and anion transport. At all concentrations tested, the initial slope determined in the presence of the anionophore was greater than that of the vehicle DMSO ($P < 0.01$). As expected, increasing the concentration of **13** led to progressive increases in fluorescence decay rates. Previous studies of decalin anionophores in LUVs have shown roughly linear relationships between concentration and activity^{30,31}.

We also performed measurements over a longer timescale to investigate the persistence of anion transport by **13**. In typical experiments, YFP-FRT cells were first treated with **13** (10 μ M) in DMSO (0.1% v/v) for 10 minutes at 37 °C before being exposed to NaI (10 mM) for 5 minutes and finally washed with iodide-free PBS for 25 minutes. Subsequently, the cells were subjected to repeated cycles of NaI followed by PBS. Figure 3c shows the time course of fluorescence in one of the cells which underwent five cycles of NaI followed by PBS. All NaI/washing cycles produced dips in fluorescence which were superimposed on the background decay. Comparison of initial slope values for the first and fifth exposures to I^- revealed little change in the rate of iodide entry into the cells; although a small difference in slope was observed, it was not statistically significant (Fig. 3d). These data are consistent with transport studies in vesicles, which show that **13** does not leach from the vesicle membrane into the aqueous phase (see supplementary information). Thus, we interpret these results to suggest that the decalin bis-urea **13** remains at the cell membrane for at least 2 hours, where it retains substantial anion transport activity.

(FIGURE 3)

Chloride transport by anionophores in epithelia - Ussing chamber experiments. The application of anionophores in CF treatment requires that they should be effective in epithelia (for example the bronchial epithelia in the lungs of CF patients).¹³ As a further test of their potential, we investigated whether the anionophores could mediate Cl^- currents in epithelia using the Ussing chamber technique¹⁸. YFP-FRT epithelial cells were grown on permeable

filter supports to form polarised epithelia with a high electrical resistance. Prior to mounting in Ussing chambers, the epithelia were treated with test anionophores (2 μ M) in the presence of POPC (20 μ M) for 10 minutes. To study anionophore-mediated Cl^- currents in the apical membrane under identical conditions to those used to study CFTR, we permeabilised the basolateral membrane with nystatin and imposed a large Cl^- concentration gradient across epithelia. Representative recordings of apical membrane Cl^- current ($I_{\text{apical}}^{\text{Cl}}$) are shown in Figure 4a. For comparison, the cyclic adenosine monophosphate (cAMP)-stimulated $I_{\text{apical}}^{\text{Cl}}$ generated by type I MDCK epithelia, which endogenously express CFTR, is shown in Figure 4b. The maximum currents from each recording are represented quantitatively in Figure 4c.

In YFP-FRT epithelia treated with the vehicle POPC, there was little or no increase in $I_{\text{apical}}^{\text{Cl}}$. However, in epithelia pretreated with the decalin bis-urea **13**, a robust increase in $I_{\text{apical}}^{\text{Cl}}$ was observed. With some decline in current magnitude, this $I_{\text{apical}}^{\text{Cl}}$ was sustained until the Cl^- concentration gradient across the epithelium was abolished (Fig. 4a). Notably, the maximum current was roughly half that measured for the MDCK epithelia (Fig. 4b,c). Thus, even at this low level, **13** comes close to reproducing the anion flows generated by natural CFTR channels. With some differences in time course, pretreatment of epithelia with cholapod bis-thiourea **10** and decalin bis-urea **12** also led to significant $I_{\text{apical}}^{\text{Cl}}$ (Figure 4a,c).

(FIGURE 4)

Toxicity studies. Finally, the toxicity of the most effective anionophore **13** and a second decalin **12** were tested in three different epithelial cell lines (MDCK, FRT and HeLa) using two methods. MDCK and FRT cells are model systems used to investigate epithelial ion transport and cell biology. FRT is used especially for CFTR research because it forms an epithelium which lacks endogenous CFTR expression.³⁸ HeLa cells are widely used for cell biology studies, and were employed for toxicity testing on calixpyrrole **3**²⁵. First the cells were treated with single doses of anionophore (1 – 30 μ M) in DMSO, allowed to stand for 18 hours, then tested for viability using sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium) (XTT). The results are summarised in Figure 5a. Decalin **12** showed some signs of cytotoxicity, albeit not statistically significant, towards FRT and HeLa cells at the highest levels used (10 and 30 μ M), but **13** gave negative results throughout. It should be noted that **13** showed high transport activity in the YFP-FRT assay at the lowest of the concentrations tested (1 μ M) using the same method of delivery (DMSO). Second, the cells were allowed to proliferate over 3 days in the presence of anionophore (1 μ M) then assayed using XTT. The results from this method are shown in Figure 5b. In this case the decalin **12** showed quite strong inhibition for HeLa cells, but the effect of **13** was only minor. As **13** is a stronger anionophore than **12**, it seems likely that the toxic effects of the latter are not purely due to anion transport.

It is interesting to compare these results to those obtained by Sessler and co-workers for calixpyrroles **3**²⁵. Although the latter are weak anionophores in synthetic vesicles, with apparent activities >1000 times lower than **13**, they were found to be toxic towards several cell lines including HeLa (IC_{50} = 10 μ M). By contrast **13** is a highly effective anionophore in cells yet shows no toxicity towards HeLa, at levels up to 30 μ M. Taken together, the results for **3**,

12 and **13** imply that anionophore toxicity is compound specific, and that anion transport *by itself* is insufficient to cause cell death. Further investigations will be needed to unravel the effects involved, but the implication that some powerful anionophores are non-toxic augurs well for applications in CF treatment.

(FIGURE 5)

Therapeutic potential of anionophores in CF

Cystic fibrosis is a complex disease caused by 2,000 mutations in the CFTR gene, which affects multiple organ systems in the body⁴⁰. While symptomatic therapy remains the bedrock of patient care, rational treatments which target the root cause of the disease are beginning to appear. Approaches include gene therapy, which has recently achieved modest success in a clinical trial⁴¹; the CFTR potentiator Ivacaftor⁴², which has proved very effective for a small subset of patients with specific CFTR mutations; the CFTR corrector Lumacaftor⁴³, which has been used with Ivacaftor for the largest subset of patients (though only with moderate results); and bypass therapy, which aims to provide alternative pathways for anion transport. Bypass therapy has previously involved the activation of chloride channels other than CFTR⁴⁴, but anionophores could provide a novel variant acting independently of endogenous proteins. Key advantages would be the use of small drug-like molecules (unlike the large biomolecules required for gene therapy), and applicability to all CFTR mutations (unlike Ivacaftor and Lumacaftor).

We anticipate that anionophores would be delivered to the lungs of CF patients, the major site of disease, by aerosolisation as for inhaled antibiotics and gene therapy vectors. Adjunct

therapeutic agents (e.g. mucolytics, DNase and anticholinergics)⁴⁵ might be necessary to optimise anionophore delivery to respiratory epithelia, while modulation of basolateral membrane K⁺ channels might be used to regulate their activity¹¹. Clinical trials of anionophores would also need to optimise dosing regimens to ensure stable expression of anionophores at the apical membrane of respiratory epithelia. The delivery of anionophores to affected epithelia beyond the lungs is a more difficult challenge, and may require cell-type-specific targeting. As a preliminary step, we have established that the presence of serum proteins does not affect the delivery of **13** to YFP-FRT cell membranes. The fluorescence-based transport assay was performed as described earlier, except that cell culture medium containing fetal bovine serum (FBS, 10%) was present during the delivery of **13** to YFP-FRT cells. After adding **13**, the response of the cells to iodide was not significantly different from experiments performed in the absence of FBS (see Supplementary Fig. S29).

Conclusions

In conclusion, we describe a method which allows real-time monitoring of anion transport across cell membranes, induced by synthetic anion carriers, without the requirement for specialist electrophysiological apparatus. The method has been used to show that certain anionophores are capable of substantial effects over short timescales (~5 min). The bis-ureiododecalin **13** is especially potent and persistent, and is readily delivered to cells without special adjuvants. The activity of **13** was confirmed by electrophysiological studies in epithelia, where its effect at low dosage (2 μ M) is close to that of naturally expressed CFTR channels. Importantly, when delivered in amounts which cause high levels of anion transport, **13**

showed no cytotoxicity. This encourages us to believe that **13** and related anionophores have realistic potential as treatments for diseases such as cystic fibrosis.

Methods

Cells and cell culture. FRT cells stably expressing the halide sensor YFP-H148Q/I152L (YFP-FRT cells)^{36,46} were a generous gift of AS Verkman (University of California, San Francisco). YFP-FRT cells were cultured as described previously for FRT cells³⁸ except that media contained 10% fetal bovine serum, 2 mM glutamine and the selection agent G418 (0.5 mg/mL). For fluorescence microscopy studies, YFP-FRT cells were plated onto glass coverslips and used 4 – 5 days later. For Ussing chamber studies, they were seeded onto permeable filter supports and cultured for 6 – 10 days to form polarised epithelia³⁸; when transepithelial resistance (R_t) exceeded 6 k Ω cm², they were used for experiments.

Reagents. Anionophores were synthesized as described in the supplementary information (**7**, **13-16**) or the specified references. POPC was purchased from Avanti Polar Lipids, Inc. (Alabama, AL). All other chemicals were of reagent grade and supplied by the Sigma-Aldrich Company Ltd. (Gillingham, UK).

Addition of anionophore/POPC mixtures to cells. POPC (10 mM) and anionophore (1.0 mM) stock solutions were prepared in deacidified chloroform or methanol. To achieve the final anionophore concentration of 1 μ M [*i.e.*, anionophore (1 μ M) : POPC (10 μ M)], 10 μ L aliquots of each stock solution were added to dry deacidified chloroform (500 μ L), the mixture stirred

briefly and the solvent evaporated under a stream of nitrogen and then under high vacuum for 1 h. The resulting lipid film was hydrated with PBS (500 μ L) (for composition, see below *Fluorescence microscopy*), sonicated for 30 seconds, and stirred for 1 h, after which the mixture was frozen and thawed 10x. The resulting vesicle solution was diluted with PBS to 10 mL, giving final concentrations of 1 μ M anionophore and 10 μ M POPC. Alternative concentrations were obtained by adjusting the dilution. Control solutions containing POPC only were prepared in an identical manner, but without added anionophore.

For cell fluorescence measurements, YFP-FRT cells plated on glass coverslips (confluency, 80%) were washed 3x with PBS before transfer to a perfusion chamber mounted on the stage of a Leica DM IRB microscope. YFP-FRT cells were treated with anionophores by incubation with anionophore (1 μ M) and POPC (10 μ M), unless otherwise indicated, in 1 mL PBS for 10 min at 37 °C. Any anionophore not incorporated into YFP-FRT cell membranes was removed from the chamber by perfusion with PBS.

For Ussing chamber recordings, the apical membrane of YFP-FRT epithelia was washed 3x with PBS before treatment with anionophores [anionophore (2 μ M) and POPC (20 μ M) in 0.25 mL PBS for 10 min at 37 °C]. Any unincorporated anionophore was washed away when YFP-FRT epithelia were mounted in Ussing chambers.

Fluorescence microscopy. Anionophore-mediated anion transport by YFP-FRT cells was quantified by measuring I^- -induced quenching of YFP fluorescence. At the end of the anionophore incubation period, a field of view with bright YFP-FRT cells was selected for study and cells were perfused with phosphate buffered saline (PBS) (composition (mM): 137 NaCl,

2.7 KCl, 8.1 Na₂HPO₄, 1.5 KH₂PO₄, 1 CaCl₂ and 0.5 MgCl₂, pH 7.40) for 5 minutes. Then, YFP-FRT cells were perfused with (i) PBS containing I⁻ (10 mM) (made by preparing PBS with 127 mM NaCl and 10 mM NaI to maintain osmolarity) for 5 min and finally (ii) PBS for 20 – 30 min to remove thoroughly I⁻ from the perfusion chamber. In some experiments, if fluorescence had recovered sufficiently, anionophore-treated YFP-FRT cells were repeatedly perfused with PBS containing I⁻ (10 mM) for 5 min before cells were washed with PBS only for 20 – 30 min. In these prolonged experiments, no data were collected between NaI/washing cycles. The rate of solution perfusion during all interventions was 8 – 10 mL min⁻¹; temperature was 37 °C.

For cell fluorescence measurements, we used the Volocity (Improvision) data acquisition and analysis system and a cooled CCD camera (Hamamatsu ORCA ER Firewall) with the Leica DM IRB inverted epifluorescence microscope equipped with an oil objective (x63, numerical aperture 1.32), excitation filter wheel and multiple band dichroic and emission filters (YFP: excitation, 500 ± 10 nm; emission, 545 ± 25 nm). Cell fluorescence data were sampled at 6 s time intervals.

We analysed fluorescence data from 4 – 6 cells per coverslip with at least 3 coverslips tested per intervention. Cell fluorescence values (F) are expressed relative to the fluorescence value immediately before iodide (10 mM) addition. By fitting first order exponential functions to the first two minutes of the fluorescence decay following I⁻ (10 mM) perfusion, we determined the initial slope to quantify anion transport by test anionophores.

Ussing chamber studies. Anionophore-mediated Cl^- currents in YFP-FRT epithelia were recorded using similar conditions to those employed to study CFTR^{38,47}. See supplementary information for details.

Cytotoxicity and cell proliferation studies. See supplementary information for details.

Statistics. Results are expressed as means $\pm$ SEM of n observations. To compare sets of data, we used Student's t -test. Differences were considered statistically significant when $P < 0.05$. All tests were performed using SigmaStatTM (Systat Software Inc., San Jose, CA).

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AUTHOR CONTRIBUTIONS

A.P.D., D.N.S. and H.L. conceived and designed the experiments. H.L., H.V., L.W.J., P.R.B., S.H., J.A.C., O.J. and H.A.S. performed the research. A.P.D., D.N.S, H.L., and H.V. analysed the data and co-wrote the manuscript. A.P.D. and D.N.S. directed the study.

ADDITIONAL INFORMATION

Supplementary information and chemical compound information are available in the online version of the paper. Reprints and permissions information is available online at

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

FIGURE CAPTIONS

Figure 1 | Structures of anionophores. Formulae for compounds **4-18** and X-ray crystal structure of the complex of **13** with $\text{Me}_4\text{N}^+\text{Cl}^-$ (shown from two angles). The chloride anion and $\text{NH}\cdots\text{Cl}^-$ hydrogen bonds are shown in green, the Me_4N^+ cation is coloured pink. The crystallographic data have been deposited with the Cambridge Crystallographic Data Centre, CCDC number 1420195.

Figure 2 | Evaluation of anionophore-mediated anion transport in YFP-FRT cells by fluorescence microscopy. **a**, Schematic of the experiment (cations omitted for clarity). For details, see the text. **b**, Representative time courses of cell fluorescence in untreated YFP-FRT cells (not perfused with I^-) and YFP-FRT cells treated with POPC alone or together with **12** or **13** (POPC, 10 μM ; anionophores, 1 μM). During the indicated period, cells were perfused with PBS containing NaI (10 mM). Cell fluorescence values were normalized to the fluorescence intensity before NaI perfusion. **c**, Images of cells + **13** + POPC at time points 1-4 from **b** above. **d**, Anion transport activity of anionophores determined from the initial slope of the fluorescence decay. YFP-FRT cells were treated with test anionophores (1 μM) in POPC (10 μM) prior to measurement of cell fluorescence. The vertical dashed line indicates the initial slope of the fluorescence decay for control cells treated with POPC (10 μM) only. Data are means $\pm$ SEM ($n = 10 - 65$ cells from at least three independent experiments); *, $P < 0.05$ vs. POPC; **, $P < 0.01$ vs. POPC. **e**, Representative time courses of cell fluorescence. Values of cell fluorescence were normalized to the fluorescence intensity before addition of NaI. During the 5 min period indicated by yellow shading, cells were perfused with PBS containing NaI (10 mM).

Figure 3 | Anion transport by the decalin bis-urea **13 is concentration-dependent and persistent.** **a**, Representative time courses of cell fluorescence from YFP-FRT cells treated with increasing concentrations of **13** in DMSO (0.1% v/v) and DMSO (0.1% v/v) only. During the indicated period, cells were perfused with PBS containing Nal (10 mM). Cell fluorescence values were normalized to the fluorescence intensity before Nal perfusion (F_0). **b**, Relationship between concentration of **13** and anionophore activity determined from the initial slope of the fluorescence decay. **c**, Representative long duration time course of cell fluorescence from YFP-FRT cells treated with **13** (10 μ M) in DMSO (0.1% v/v) taken through five cycles of iodide addition-removal; no data were collected during the breaks in the recording. **d**, Persistence of anionophore activity. Values represent normalized initial rate of fluorescence decay during the first and fifth exposures to Nal (10 mM). In panels b and d, data are means $\pm$ SEM (b, n = 25 – 65 cells from at least three independent experiments; d, compound **13**, n = 37 and DMSO (0.1% v/v), n = 26 cells from at least three independent experiments); **, $P < 0.01$ vs. control. Other details as in Figure 2.

Figure 4 | Anionophores generate persistent apical membrane Cl^- currents in YFP-FRT epithelia **a**, Representative recordings of apical membrane Cl^- current ($I_{\text{apical}}^{\text{Cl}}$) in YFP-FRT epithelia treated with POPC alone or together with the indicated anionophores (POPC, 20 μ M; anionophores, 2 μ M). The basolateral membrane was permeabilised with nystatin (0.36 mg ml^{-1}) and a Cl^- concentration gradient imposed across the epithelium (basolateral $[\text{Cl}^-]$, 149 mM; apical $[\text{Cl}^-]$, 14.8 mM); transepithelial voltage was clamped at 0 mV. For **13**, the Cl^- concentration gradient was abolished after ~ 120 min (see arrow) by Cl^- addition to the apical solution. **b**, Representative cAMP-stimulated $I_{\text{apical}}^{\text{Cl}}$ in type I MDCK epithelia expressing native

CFTR recorded under identical conditions to those used to study anionophore-generated $I_{\text{apical}}^{\text{Cl}}$. In addition to nystatin, forskolin (FSK, 10 μM) was introduced to the apical solution to activate CFTR. **c**, Magnitude of anionophore-generated $I_{\text{apical}}^{\text{Cl}}$ in YFP-FRT epithelia. The change in $I_{\text{apical}}^{\text{Cl}}$ produced by anionophores (difference between baseline after nystatin addition and maximum current) is compared with the CFTR-mediated $I_{\text{apical}}^{\text{Cl}}$ activated by forskolin (10 μM) in type I MDCK epithelia. Data are means $\pm$ SEM (anionophores, $n = 6 - 13$; cAMP-stimulated $I_{\text{apical}}^{\text{Cl}}$, $n = 69$); *, $P < 0.05$ and **, $P < 0.01$ vs. POPC.

Figure 5 | Evaluation of anionophore toxicity using MDCK, FRT and HeLa cells. **a**, Viability of MDCK, FRT and HeLa cells in the absence or presence of the anionophores **13** or **12**. Cells were treated with the indicated concentrations of **13** or **12** or the vehicle (DMSO, 0.1% v/v) for 18 h before cell viability was measured using XTT. Cell viability is presented as specific absorbance values (means $\pm$ SEM; $n = 4$ with 3 – 4 replicates per treatment). There was no significant difference between the vehicle and individual treatments for each of the three cell lines ($P > 0.05$ vs. 0.1% DMSO). **b**, Time course of cell proliferation in the continuous presence of either **13** or **12** (1 μM) or the vehicle (DMSO, 0.1% v/v). Anionophore- or vehicle-containing medium was changed every other day and cell viability measured each day using XTT. Cell proliferation is presented as values of specific absorbance (means $\pm$ SEM; $n = 3 - 6$ with 4 – 8 replicates per time point and treatment); **, $P < 0.05$ vs. 0.1% DMSO at the same time point.

Table 1 | Anionophore properties and binding/transport data.

Compound			Binding		Transport in LUVs		Transport in YFP-FRT cells
	MW (g/mol)	cLogP ^[a]	K_a to Cl ⁻ in CHCl ₃ (M ⁻¹) ^[b]	K_a to Cl ⁻ in DMSO-d ₆ (M ⁻¹) ^[c]	Specific Initial rate [<i>I</i>] (s ⁻¹) ^[d]	Deliverability ^[e]	Corrected Absolute Initial Slope dF/dT (× 10 ³) (min ⁻¹) ^[f]
4 ^{18,33}	701	7.8	1.5×10 ⁷	<i>n.d.</i>	1	<i>n.d.</i>	6
5 ³⁰	837	9.8	1.8×10 ⁸	<i>n.d.</i>	18	A	1
6 ²⁸	754	7.9	2.8×10 ⁸	<i>n.d.</i>	4	A	~0
7 ^[g]	832	8.8	2.8×10 ⁹	<i>n.d.</i>	23	A	~0
8 ³¹	890	9.9	2.5×10 ⁹	<i>n.d.</i>	150	B	7
9 ³¹	1026	12.0	4.5×10 ⁹	<i>n.d.</i>	450	D	~0
10 ³¹	922	10.2	2.3×10 ⁹	1.7×10 ⁴	1760	C	20
11 ³¹	876	7.8	1.6×10 ¹⁰	1.8×10 ⁴	1500	C	8
12 ³⁰	751	9.0	~6×10 ⁸ ^[h]	8.8×10 ²	91	B	29 (18) ^[i]
13 ^[g]	569	4.5	<i>n.d.</i> ^[j]	6.8×10 ²	22	A	39 (39) ^[i]
14 ^[g]	653	6.8	<i>n.d.</i> ^[j]	6.9×10 ²	45	C	26 (5) ^[i]
15 ^[g]	719	5.4	<i>n.d.</i> ^[j]	1.3×10 ⁴	100	B	36
16 ^[g]	855	7.5	<i>n.d.</i> ^[j]	1.5×10 ⁴	113	C	26
17 ³²	865	11.4	<i>n.d.</i> ^[j]	4.0×10 ²	89	B	14
18 ³²	1069	14.4	3×10 ⁷	6.7×10 ²	465	D	22

^[a] Calculated using TorchV10lite. ^[b] Obtained by extraction of Et₄N⁺Cl⁻ from water into chloroform at 303 K ³⁴. ^[c] Obtained from ¹H NMR titrations with Bu₄N⁺Cl⁻ in DMSO-d₆/H₂O (200:1) at 298 K. ^[d] Transporter preincorporated in vesicles. Specific initial rate [*I*] = Initial slope of *F*₀/*F* vs. time *t*, divided by the transporter/lipid ratio in the vesicle bilayers and averaged over a range of experiments at different ratios. Vesicles (200 nm) are composed of 70% POPC + 30% cholesterol + transporter. Anion transport is induced by a NaCl concentration gradient of 25 mM, while 225 mM NaNO₃ is present both inside and outside the vesicles. ^[e] A = excellent deliverability: Cl⁻/NO₃⁻ exchange rates seem unaffected by transporter delivery method (preincorporation into LUVs or addition after LUV preparation). B,C,D: external delivery is less effective than preincorporation, to increasing extents (see supplementary information). ^[f] The slope of measurements from cells exposed to vehicle only was subtracted from that of POPC (10 μM) / transporter (1 μM) mixtures. Unless indicated, the vehicle was POPC (10 μM). ^[g] Compound not reported previously. ^[h] Measured for the corresponding octyl ester to enhance solubility in chloroform. ^[i] Vehicle = DMSO (0.1% v/v). ^[j] Not determined due to low solubility in chloroform.

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5