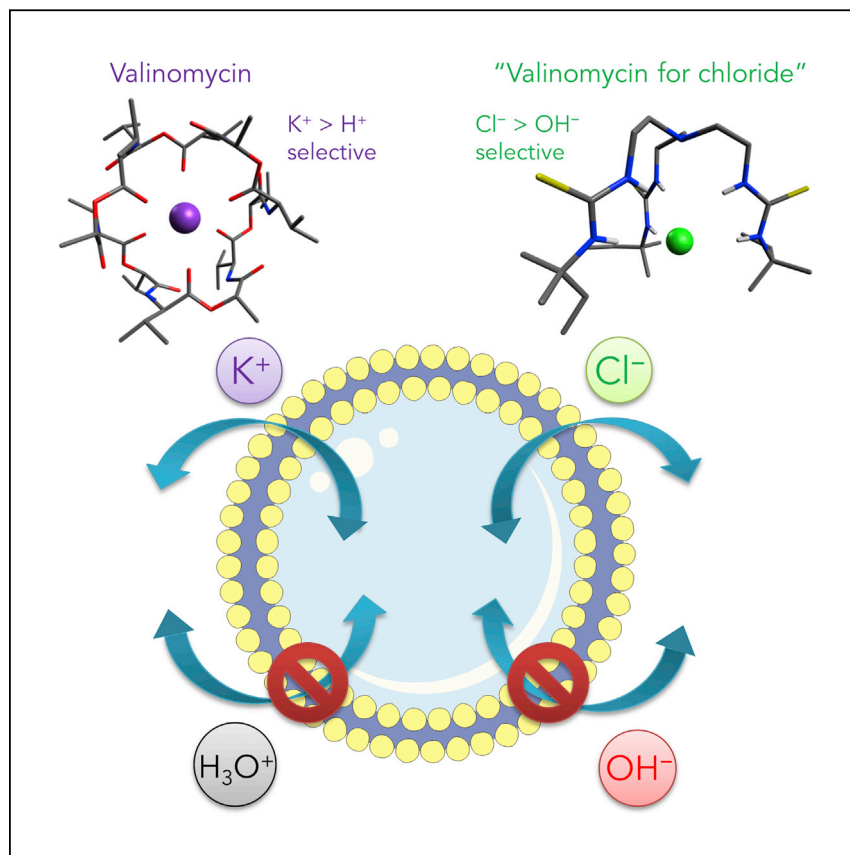


Article

Nonprotonophoric Electrogenic Cl^- Transport Mediated by Valinomycin-like Carriers

Synthetic anion transporters can facilitate H^+ transport via deprotonation, or OH^- transport via hydrogen bonding to OH^- , thus allowing dissipation of transmembrane pH gradients, an undesired side-effect for biomedical applications as Cl^- ionophores. To address this limitation, Gale and colleagues have developed two anionophores that show high $\text{Cl}^- > \text{H}^+/\text{OH}^-$ selectivity. Preliminary cellular studies support the biological relevance of the selectivity.

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HIGHLIGHTS

Anionophores can function via
electrogenic or electroneutral
mechanisms

Most synthetic anionophores also
function as protonophores

Proton and hydroxide transport
are two mechanisms for
protonophoric activity

Anion encapsulation confers
selectivity for chloride over
protons/hydroxide

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Article

Nonprotonophoric Electrogenic Cl⁻ Transport Mediated by Valinomycin-like Carriers

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SUMMARY

Synthetic transmembrane anion transporters (anionophores) have potential as tools for biomedical research and as therapeutic agents for diseases associated with anion-channel dysfunction. However, the possibility of H⁺ or OH⁻ transport by anionophores has received little attention, and an anionophore selective for Cl⁻ over H⁺/OH⁻ is currently unavailable. Here, we show that depending on anionophore acidity, many anionophores facilitate electrogenic H⁺ or OH⁻ transport, potentially leading to toxicity. Nevertheless, using several liposome-membrane-based assays, we identified two newly developed small molecules that promote electrogenic Cl⁻ transport without effectively dissipating the transmembrane pH gradient, essentially mimicking the electrogenic cationophore valinomycin. The Cl⁻ > H⁺/OH⁻ selectivity of anionophores showed a consistent positive correlation with the degree of Cl⁻ encapsulation and a negative correlation with the acidity of hydrogen-bond donors. Our study demonstrates that a valinomycin equivalent for Cl⁻-selective transport is achievable.

INTRODUCTION

Facilitation of transmembrane ion transport by small-molecule carriers that function by reversible binding of the transported ionic species is an important supramolecular process.^{1,2} The most notable example of a small-molecule carrier is the naturally occurring K⁺ carrier valinomycin (Figure 1), which has found many applications in the study of biological systems.³ Valinomycin facilitates transmembrane K⁺ transport by interfacial binding of K⁺, translocation of the cationic complex through the lipid membrane, release of K⁺ at the other interface of the membrane, and eventually back transport of the uncomplexed valinomycin to complete the cycle (Figure 2B, left).^{4,5} The action of valinomycin is electrogenic because there is a net transfer of charge across the membrane. Ionophores, such as valinomycin, that transfer charge across the membrane are termed “electrogenic” (i.e., they modify a membrane potential) or “electrophoretic” (i.e., they transport ions driven by a membrane potential). The more common term “electrogenic” is used throughout this article. By contrast, the action of some other cationophores is purely electro-neutral (i.e., there is no movement of net charge) because a metal ion (M⁺) is exchanged for H⁺ (Figure 2A, left). One example is the carboxylate-containing cationophore monensin,⁶ which carries a monovalent cation through the membrane as an overall neutral ion-pair complex (Figure 1) with no transmembrane movement of charged species.³ Valinomycin and monensin thus have fundamentally different ion-transport mechanisms controlled via membrane potential (valinomycin) or pH gradient (monensin).³

The Bigger Picture

The movement of ions and molecules through biological membranes is regulated by embedded protein channels, carriers, and pumps. The dysfunction of ion channels is the underlying cause of many diseases, notably cystic fibrosis, in which passive chloride ion transport is impaired. A number of groups are making small molecules that can carry chloride through lipid-bilayer membranes and so potentially replace the function of faulty channels, thereby ameliorating the symptoms of these types of diseases. However, many of these transporters exhibit a side effect of pH-gradient disruption, which can lead to cell death. Herein, we show the first examples of transporters with a high selectivity for chloride over proton and hydroxide, which represents a paradigm shift for transporter design. This is a significant step toward real biomedical application of anion transporters in the battle against cystic fibrosis and other diseases caused by faulty ion channels.

Although the transport properties of naturally occurring cationophores, many of which possess antibiotic properties, are well understood, natural or synthetic transmembrane anion transporters (anionophores) have only recently attracted significant attention.^{7,8} Anionophores are useful in complementing valinomycin and other cationophores in biomedical research^{9,10} and might be used to replace defective anion channels in treatments for genetic diseases such as cystic fibrosis^{11,12} or to induce cancer cell apoptosis by facilitating NaCl influx.¹³ The best known anionophore, the natural product prodigiosin (Figure 1), facilitates H⁺/Cl⁻ symport^{14,15} (cotransport; Figure 2A, right) and Cl⁻/NO₃⁻ antiport (exchange),¹⁶ but its non-electrogenic nature, reported by Sato et al.,¹⁷ is sometimes overlooked. A wide spectrum of synthetic anionophores that function by hydrogen bonding,^{7,8} halogen bonding,¹⁸ or metal coordination¹⁹ to anions have been developed. So far, research efforts have mainly focused on their efficacy in facilitating anion exchange, and little attention has been paid to anion-transport mechanisms, especially their ability to disrupt pH or proton gradients.

Transmembrane pH gradients are essential for cellular function.²⁰ It is therefore important to understand the role ionophores play in facilitating proton or hydroxide transport. (H⁺ transport produces the same effect as OH⁻ transport in the reverse direction. In this article, we use the term "H⁺/OH⁻ transport" to refer to the process of H⁺ and/or OH⁻ translocation through lipid bilayers. We also use "/" elsewhere to indicate a coupled process, e.g., "H⁺/Cl⁻ symport.") In this respect, valinomycin functions as a selective electrogenic K⁺ ionophore that does not facilitate H⁺/OH⁻ transport.²¹ By contrast, currently no anionophore has been identified with strong Cl⁻ > H⁺/OH⁻ selectivity.²² Indeed, prodigiosin,^{14,15} and some of the most powerful synthetic anionophores,^{23–27} have been shown to uncouple vacuolar type H⁺-ATPase (V-ATPase) and neutralize acidic cellular organelles (such as the Golgi apparatus, lysosomes, and endosomes) by facilitating H⁺/Cl⁻ symport or Cl⁻/OH⁻ antiport through organelle membranes. Monensin is also known to exhibit this neutralization effect by promoting Na⁺/H⁺ antiport through organelle membranes.⁶ Another possible yet unexamined action of synthetic anionophores is electrogenic H⁺ transport. Compounds known as "protonophores," such as carbonyl cyanide phenylhydrazones,²⁸ facilitate electrogenic H⁺ transport (Figure 2C, left), enabling them to uncouple oxidative phosphorylation by dissipating the proton gradient pumped by the electron transport chain.²⁹

Although prodigiosin and other functionally similar compounds are promising anti-cancer agents because of their ability to disrupt transmembrane pH gradients, electrogenic Cl⁻ carriers that do not facilitate H⁺/OH⁻ transport ("valinomycin-like" anionophores) are required for other applications. These include using anionophores for physiological research where H⁺/OH⁻ transport would complicate data interpretation. Examples include the identification of electrogenic H⁺-coupled metal-ion transport, such as H⁺/K⁺ symport (here the anionophore would dissipate the accumulation of a membrane potential like the use of valinomycin in the study of Cl⁻/H⁺ antiport by ClC transporters³⁰); and potential replacement of defective anion channels in genetic diseases where H⁺/OH⁻ transport would lead to toxicity (e.g., Best disease, Startle disease, Bartter syndrome, and most notably, cystic fibrosis).³¹

As a process functionally equivalent to H⁺ transport,³² OH⁻ transport, at first glance, seems an unlikely process for synthetic anionophores to facilitate, considering the high energy penalty required to dehydrate OH⁻ ($\Delta G_{\text{hydration}}(\text{OH}^-) = -430 \text{ kJ mol}^{-1}$ compared with $\Delta G_{\text{hydration}}(\text{Cl}^-) = -340 \text{ kJ mol}^{-1}$).³³ However, OH⁻

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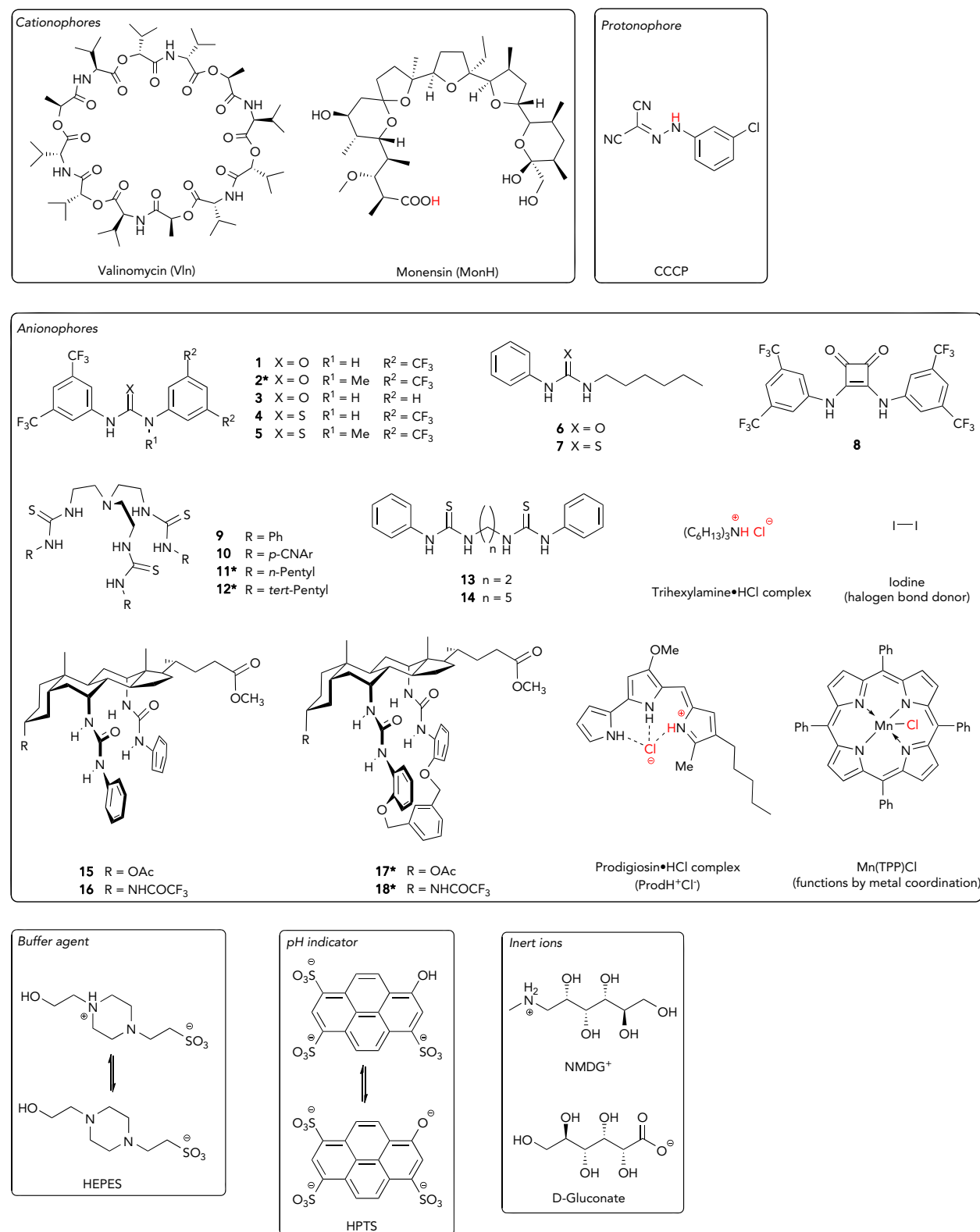
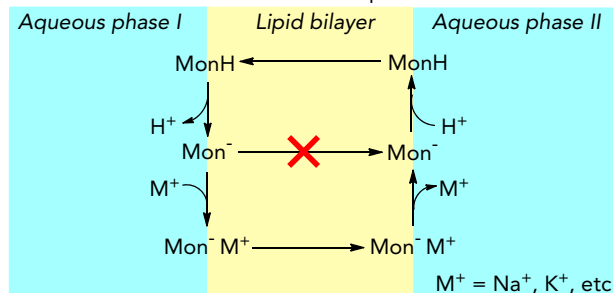


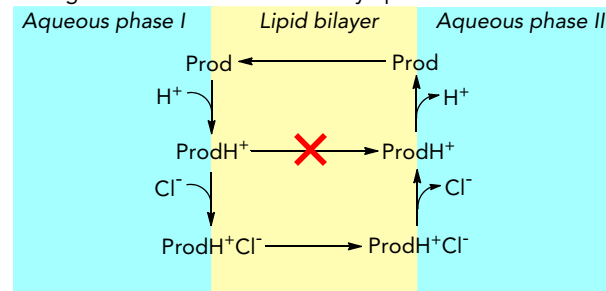
Figure 1. Structures of Anionophores and Other Reagents Used

Novel anionophores are marked with an asterisk. For trihexylamine, prodigiosin, and MnTPP, their Cl⁻ complexed forms are shown.

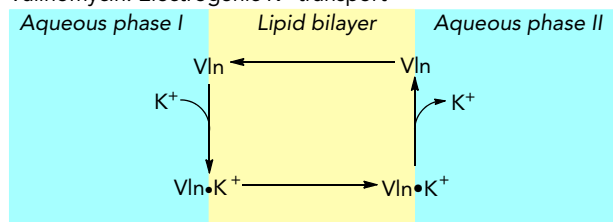
A Monensin: Electroneutral M^+/H^+ antiport



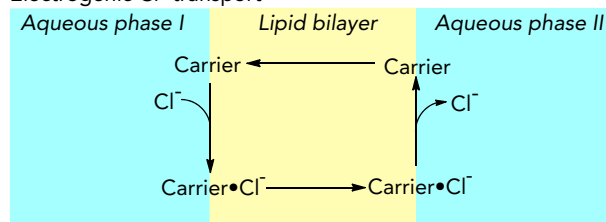
Prodigiosin: Electroneutral H^+/Cl^- symport



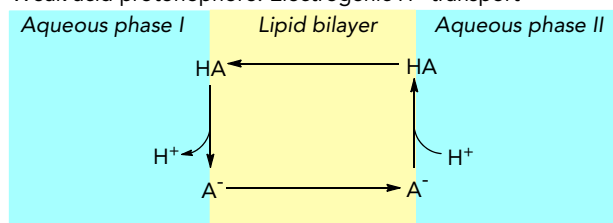
B Valinomycin: Electrogenic K^+ transport



Electrogenic Cl^- transport



C Weak acid protonophore: Electrogenic H^+ transport



Electrogenic OH^- transport

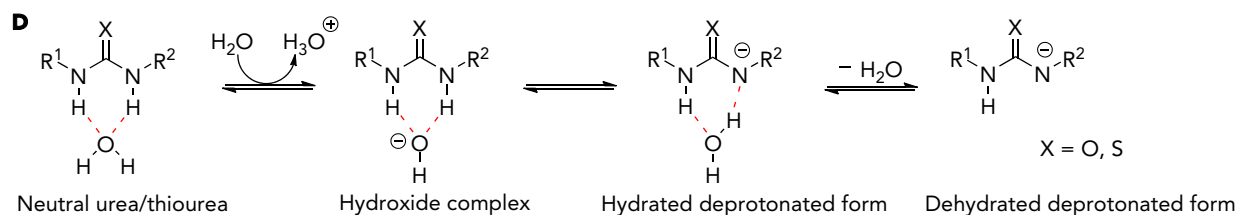
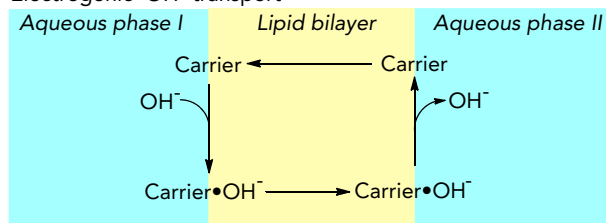


Figure 2. Schematic Representations of Ion-Transport Mechanisms

(A) Electroneutral M^+/H^+ antiport facilitated by monensin (Mon, left) and electroneutral H^+/Cl^- symport facilitated by prodigiosin (Prod, right).

(B) Electrogenic K^+ transport facilitated by valinomycin (Vln, left) and electrogenic Cl^- transport facilitated by an electrogenic anion carrier (right).

(C) Electrogenic H^+ transport facilitated by a weak acid protonophore (HA, left) and electrogenic OH^- transport facilitated by an electrogenic anion carrier (right). Note that these two processes are functionally equivalent.

(D) Equilibria involving hydrogen bonding to OH^- and deprotonation of ureas and thioureas. Hydrogen bonds are represented by red dashed lines.

is an extremely strongly coordinating anion, and previous studies have demonstrated that synthetic anionophores, including halogen-bond-based transporters that do not possess H^+ binding groups, can dissipate transmembrane pH gradients in which OH^- transport coupled with Cl^- transport appears to be the only plausible mechanism.¹⁸ In fact, the routine use of the 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) assay, which measures the rate of pH-gradient dissipation, to evaluate

anion-transport activity suggests that designing a Cl^- -selective anionophore that does not facilitate OH^- (or H^+) transport is likely to be challenging.

In this article, we address H^+/OH^- transport facilitated by anionophores. We provide unambiguous evidence that (1) prodigiosin cannot facilitate electrogenic transport of Cl^- , H^+ , or OH^- and (2) many synthetic anionophores can facilitate electrogenic H^+/OH^- transport. We propose that the actual transport process responsible for the observed electrogenic transport of H^+ or OH^- likely depends on the acidity of hydrogen-bond donors in the anionophore. Most importantly, we have developed two “valinomycin-like” electrogenic anionophores that show outstanding $\text{Cl}^- > \text{H}^+/\text{OH}^-$ selectivity. One of these compounds was identified as an active anionophore functioning in live cells. Distinct from other active anionophores reported, this selective anionophore did not effectively neutralize lysosomal pH.

RESULTS AND DISCUSSION

Evidence of Electrogenic H^+/OH^- Transport

Previously, Busschaert et al.³⁴ and Vargas Jentzsch et al.¹⁸ have demonstrated that anionophores dissipate a transmembrane pH gradient in the presence of Cl^- in vesicle-based experiments employing the intravesicular pH indicator HPTS (Figure 1).³⁵ Provided that the anionophores do not cause membrane defects or HPTS leakage, these data indicate that anionophores facilitate H^+/OH^- transport (accompanied by Cl^- transport as the counterion pathway to maintain electroneutrality). However, an alternative mechanism of H^+ transport via simple (unassisted) diffusion coupled with Cl^- transport facilitated by anionophores has been proposed,³⁶ and in theory pH-buffer transport is also possible. Assuming H^+/Cl^- symport, such a process might be an obligatorily coupled electroneutral process (Figure 2A, right). In this case, because H^+ transport cannot be separated from Cl^- transport, the anionophore is unable to facilitate electrogenic H^+ transport (Figure 2C). Alternatively, H^+/Cl^- symport or Cl^-/OH^- antiport can be an indirectly coupled process with separate pathways for electrogenic H^+ (or OH^-) transport (Figure 2C) and electrogenic Cl^- transport (Figure 2B, right). To the best of our knowledge, this possibility has never been examined experimentally in the literature.

To identify the mechanism of H^+/OH^- transport by anionophores, a new HPTS assay (here termed the tetrabutylammonium hydroxide [TBAOH] assay; Figure 3A) was devised for testing purely electrogenic H^+/OH^- transport (in other words, protonophoric activity), in which ionophore-induced dissipation of a pH gradient across large unilamellar vesicle (LUV) membranes (induced by adding 5 mM TBAOH to a vesicle suspension) in a lightly buffered sodium D-gluconate (100 mM) medium was monitored. In this assay, gluconate (Figure 1) transport is negligible because of its large size and hydrophilicity. The presence of TBA^+ , a membrane-permeable cation,³⁷ provides a counterion pathway for electrogenic H^+/OH^- transport (driven by the pH gradient), which would otherwise build up an opposing membrane potential preventing bulk pH change. This leads to overall TBA^+/OH^- symport or TBA^+/H^+ antiport in the presence of an electrogenic H^+/OH^- ionophore, dissipating the pH gradient. Under our experimental conditions, the low H^+ permeability of intact lipid bilayers does not allow for observable pH-gradient dissipation over the timescale of several minutes (see DMSO control in Figure S23).

Results from the TBAOH assay were compared with data from another HPTS assay (termed the *N*-methyl-D-glucamine chloride [NMDG-Cl] assay; Figure 3B, left) set

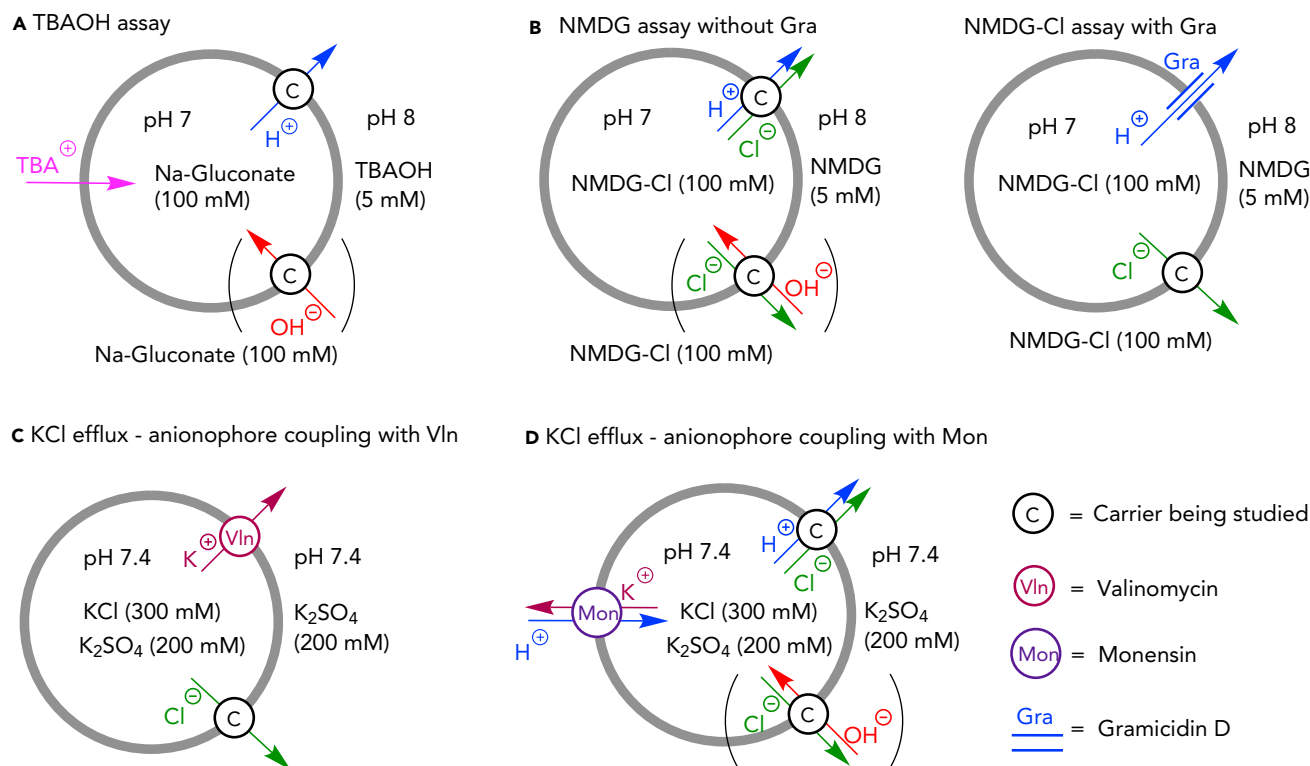


Figure 3. LUV-Based Assays Used in This Study

(A) TBAOH assay. Electrogenic H^+/OH^- transport facilitated by the test ionophore couples with simple diffusion of tetrabutylammonium ion (TBA^+), leading to pH-gradient dissipation (measured by intravesicular pH indicator HPTS) by TBA^+/H^+ antiport or TBA^+/OH^- symport (shown in brackets).

(B) NMDG-Cl assay in the absence (left) and presence (right) of proton channel gramicidin D (Gra). In the absence of gramicidin, the test anionophore facilitates H^+/Cl^- symport or Cl^-/OH^- antiport (shown in brackets) to dissipate the pH gradient (measured by HPTS). In the presence of gramicidin, which facilitates H^+ transport, the test anionophore is only required to facilitate Cl^- transport to dissipate the pH gradient. If H^+/OH^- transport is rate limiting with the test anionophore alone (i.e., if the test anionophore shows $\text{Cl}^- > \text{H}^+/\text{OH}^-$ selectivity), gramicidin will improve the rate-limiting step and thereby accelerate the rate of pH-gradient dissipation.

(C) Anionophore-facilitated electrogenic Cl^- transport coupling with valinomycin (Vln) facilitated electrogenic K^+ transport, leading to net KCl efflux (measured using a Cl^- -selective electrode).

(D) Anionophore-facilitated H^+/Cl^- symport (or Cl^-/OH^- antiport shown in brackets) coupling with monensin (Mon) facilitated K^+/H^+ antiport, leading to net KCl efflux (measured by a Cl^- -selective electrode). The buffering agent HEPES is omitted for clarity.

up for H^+/Cl^- symport or Cl^-/OH^- antiport, in which NMDG-Cl (100 mM) was used in place of sodium gluconate (100 mM), and NMDG (5 mM) was used in place of TBAOH (5 mM) as the base added to create a pH gradient. The conditions used for the NMDG-Cl assay were similar to those of the HPTS assays employed by Vargas Jentzsch et al.,¹⁸ but NMDG-Cl was used instead of NaCl for the sake of another selectivity assay described below. Control experiments were conducted to ensure that the HPTS response represented transport of Cl^- and H^+/OH^- and not other processes such as Na^+/H^+ antiport, gluconate/ OH^- antiport, or HPTS leakage from vesicles (Figures S31, S32, and S57–S60). After test ionophores were studied at different concentrations, Hill plot analyses were performed to obtain a Hill coefficient, which indicated the stoichiometry of the unstable³⁸ active species mediating ion transport, and the effective concentration to reach 50% of maximum transport at 200 s (EC_{50} value, low EC_{50} values indicate high activity) to quantify ion-transport activity.³⁵

Table 1. Summary of Acidity and Membrane-Transport Data of Ureas/Thioureas 1–6, Prodigiosin, and CCCP

Compound	pK _a	EPN ^a (E _h)	TBAOH Assay ^b		NMDG-Cl Assay ^c	
			n	EC ₅₀ (mol %)	n	EC ₅₀ (mol %)
1	13.8 ^d	−18.2786	0.9	0.065	1.4	0.046
2	ND ^e	−18.2798	1.9	0.83	2.4	0.61
3	16.1 ^d	−18.2947	1.5	0.26	2.3	0.16
4	8.5 ^d , 8.9 ^f	−18.2723	0.9	0.014	1.2	0.015
5	10.7 ^f	−18.2774	1.1	0.013	1.9	0.26
6	ND ^e	−18.3453	2.9	12	3.2	20
Prodigiosin	ND ^e	ND ^e	– ^g	>1 ^g	1.4	0.000061
CCCP	6.0 ^h	ND ^d	1.0	0.0054	– ⁱ	>100 ⁱ

^aElectrostatic potential at the nitrogen nucleus is expressed in atomic units. The structures were optimized using the PM6 semi-empirical method, assuming the *syn* conformer. The EPN values were calculated at the B3LYP/6-311++G(d,p) level of theory with the SMD water-solvation model.

^bA fluorescence assay for electrogenic H⁺/OH[−] transport. Large unilamellar vesicles (LUVs) of POPC (mean diameter = 200 nm) were loaded with HPTS (1 mM) and sodium gluconate (100 mM) buffered at pH 7.0 with 10 mM HEPES. The vesicles were suspended in an external solution of sodium gluconate (100 mM) buffered at pH 7.0 with 10 mM HEPES. At the beginning of the experiment, a base pulse of TBAOH (5 mM) was added to create a pH gradient, and the dissipation of the pH gradient induced by the test ionophore (added as a DMSO solution) was monitored by HPTS fluorescence. Lipid concentration for fluorescence measurement was 0.10 mM. Dose-dependent Hill plot analysis was performed to obtain a Hill coefficient (n) and an effective concentration to reach 50% of maximum transport (EC₅₀) at 200 s for each ionophore. Ionophore concentrations are shown as ionophore-to-lipid molar ratios. See Figures S23–S29 for Hill plots.

^cA fluorescence assay for H⁺/Cl[−] symport or Cl[−]/OH[−] antiport. The internal and external medium used was NMDG-Cl (100 mM) buffered at pH 7.0 with 10 mM HEPES, and the base pulse used was NMDG (5 mM). The other conditions are the same as in the TBAOH assay. See Figures S34–S55 for Hill plots.

^dIn DMSO, reported by Jakab et al.⁴¹

^eNot determined.

^fIn acetonitrile-water (9:1 v/v with 0.1 M TBAPF₆), determined by potentiometric titrations.

^gNo transport at 1 mol % concentration. Higher concentrations were not tested because of interference with HPTS fluorescence. See Figure S30.

^hIn water, reported by Šturdík et al.⁴³

ⁱNo transport even at 100 mol % concentration. See Figure S56.

Several simple ureas/thioureas 1–6, prodigiosin, and a known protonophore carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP; Figure 1) were tested, and the results are presented in Table 1.

Although prodigiosin was extremely active in the NMDG-Cl assay, corresponding to H⁺/Cl[−] symport or Cl[−]/OH[−] antiport, it could not dissipate the pH gradient in the TBAOH assay (Table 1), consistent with its inability to facilitate electrogenic transport processes.¹⁷ This conclusion is consistent with the lack of protonophoric activity or alteration of cellular ATP levels reported for prodigiosins.^{14,15} A likely interpretation of these data is that the charged, protonated form of prodigiosin is unable to move through the membrane in the absence of a transportable anion (Figure 2A, right). Consistent with this idea, prodigiosin failed to facilitate electrogenic Cl[−] transport (see the section [Coupling between Cationophores and Anionophores: Direct Evidence of “Valinomycin-likeness”](#)). CCCP transports H⁺, but not Cl[−], and therefore was active in the TBAOH assay and silent in the NMDG-Cl assay (Table 1). By contrast, the neutral hydrogen-bond donors 1–6 responded in both assays (Table 1). Transport rates were essentially unaffected by switching the external buffer from HEPES to phosphate (Figure S33), indicating that transport of the buffer is unlikely. Further evidence for H⁺/OH[−] transport that completely rules out buffer

transport is afforded by the observation of H⁺/OH⁻ transport under buffer-free conditions (Supplemental Information, Section 11.1). Thus, like the protonophore CCCP, ureas and thioureas 1–6 facilitated electrogenic H⁺/OH⁻ transport.

The molecular mechanism for H⁺ conductance induced by weak acid protonophores such as CCCP involves the transmembrane movement of both neutral and deprotonated anionic forms of the protonophore (Figure 2C, left).³⁹ Anionophores acting as hydrogen-bond donors might adopt a CCCP-like deprotonation mechanism. This process, coupled with electrogenic Cl⁻ transport, would lead to overall H⁺/Cl⁻ symport in the NMDG-Cl assay. Alternatively, anionophores might produce the same effect as protonophores by reversibly binding and releasing OH⁻ or hydrated OH⁻ (Figure 2C, right). To identify the mechanism responsible for the protonophoric activity, ureas/thioureas and their mono-N-methylated analogs were compared in both assays. N-Methylation is expected to dramatically weaken anion (Cl⁻ or OH⁻) binding and transport because only a single NH hydrogen-bond donor is present.⁴⁰

In the NMDG-Cl assay, N-methylation dramatically decreased the activities of both urea 1 and thiourea 4, consistent with weakened Cl⁻ binding by N-methylation. However, results from the TBAOH assay were more complex; urea 1 was still far more active than N-methyl urea 2, but thiourea 4 and its N-methyl analog 5 showed similar activities (Table 1). To rationalize these effects, pK_a values of some compounds were determined by potentiometric titrations in acetonitrile-water (9:1 v/v), and literature pK_a values⁴¹ (in DMSO) were tabulated (Table 1). Electrostatic potential values at the nitrogen nucleus (EPN)⁴² obtained with density functional theory calculations (see Supplemental Information, Section 7.1) were used to compare acidity when the pK_a values were too high for determination by potentiometric titration.

Because of its high acidity, a significant proportion of compound 4 was deprotonated under the experimental conditions (external pH ~8 after base pulse), likely allowing H⁺ transport in the same manner as CCCP.⁴³ Despite its weaker acidity, compound 5 also existed in equilibrium with its deprotonated form in water, and the following lines of evidence lead us to suggest that 5 transported H⁺ via a CCCP-like mechanism: (1) 5 was much better at transporting H⁺/OH⁻ than Cl⁻ (Table 1, compare EC₅₀ values in the two assays), and (2) 5 showed a Hill coefficient of ~2 in the NMDG-Cl assay, suggesting Cl⁻ transport via a 2:1 (5·Cl⁻) complex. However, the different Hill coefficient of ~1 in the TBAOH assay suggests that an alternative process from anion binding was occurring. We attribute this process to a CCCP-like deprotonation mechanism. These observations for 5 are in sharp contrast to other ureas/thioureas, which show EC₅₀ values of the same order of magnitude and similar Hill coefficients in the two assays (Table 1). When comparing 4 and 5, the disadvantage of the weaker acidity of 5 is likely compensated by its higher lipophilicity (it has been shown that carrier lipophilicity is favorable for membrane transport²⁵) and weaker binding to the lipid phosphate head group, leading to similar activities of 4 and 5 in H⁺ transport. It is also possible that 4 transports OH⁻ as a result of stronger anion binding, different from the H⁺ transport mechanism of 5.

Ureas 1–3 have pK_a values higher than 13 (Table 1; although we were unable to determine the pK_a of 2 by potentiometric titration, 2 is less acidic than 1, as indicated by their EPN values). Therefore, H⁺ transport by a deprotonation mechanism at physiological pH is less likely for ureas 1–3 than thioureas 4 and 5. The higher activity of 1 than of the N-methyl urea 2 in the TBAOH assay seems to support the hypothesis that 2 and possibly also 1 facilitated OH⁻ transport by hydrogen bonding to OH⁻.

However, because *N*-methylation weakens acidity, comparison between **2** and **3** is more conclusive. When compared with **3**, compound **2** is more acidic on the basis of their EPN values (Table 1), and it is also more lipophilic. Therefore, if both **2** and **3** facilitated H⁺ transport via deprotonation, then **2** would be more active than **3** in the TBAOH assay. However, the TBAOH assay revealed that **2** had a lower activity than **3**, highlighting the importance of two NH hydrogen-bond donors, which supports the hypothesis of OH⁻ transport. This is also supported by the similarly high Hill coefficients in the two assays, in the cases of **2**, **3**, and **6**, indicating the complexation of both Cl⁻ and OH⁻ by more than one receptor molecule. Although **3** and **6** contain two NH hydrogen-bond donors, they showed high Hill coefficients, presumably because of the less acidic nature of the NH hydrogen-bond donors (less electron-deficient anion binding site) than of those in **1** and **4**, and therefore they needed more carrier molecules to transport Cl⁻ and OH⁻. This is supported by the report that relatively weak halogen-bonding anionophores show high Hill coefficients in anion transport.¹⁸ The above-mentioned results demonstrate that OH⁻ (or hydrated OH⁻) transport is the more likely pathway for less acidic anionophores to facilitate H⁺/OH⁻ transport, while the more acidic **5** can deprotonate in water and thereby transport H⁺ in the same way as CCCP.

It should be noted that the OH⁻ complex (1:1) and the hydrated deprotonated form of a urea/thiourea are structurally similar, differing only by small changes in the position of the proton (Figure 2D). This difference would disappear in the case of a single-well [N⋯H⋯OH]⁻ hydrogen bond (similar to the [F⋯H⋯F]⁻ ion).⁴⁴ This highlights the fact that, when direct deprotonation of a receptor cannot take place, OH⁻ can nonetheless form a strong complex with a less acidic receptor. This might help to explain the surprising observation that synthetic anionophores may transport OH⁻ as efficiently as Cl⁻, despite the hydrophilicity of OH⁻. We next show, however, that by rational design of the anionophore structure, we can reduce OH⁻ or H⁺ transport in relation to Cl⁻ transport.

Cl⁻ > H⁺/OH⁻ Selectivity

We investigated whether it is possible for anionophores to possess selectivity for electrogenic Cl⁻ transport over H⁺/OH⁻ (including electrogenic and non-electrogenic pathways) transport, which we refer to as Cl⁻ > H⁺/OH⁻ selectivity. Because of its perfect K⁺ > H⁺/OH⁻ selectivity, low concentrations of valinomycin do not facilitate K⁺/H⁺ antiport unless a protonophore is present.⁴⁵ On the basis of this rationale, an HPTS assay for Cl⁻ > H⁺/OH⁻ selectivity was devised and employed (Figure 3B). In this modified NMDG-Cl assay, we studied the effects of the proton channel gramicidin D on the rate of pH-gradient dissipation (indicating H⁺/Cl⁻ symport or Cl⁻/OH⁻ antiport) induced by anionophores. If H⁺/OH⁻ transport was rate limiting (i.e., electrogenic Cl⁻ transport was faster than H⁺/OH⁻ transport, namely Cl⁻ > H⁺/OH⁻ selectivity), pH-gradient dissipation would be significantly accelerated by gramicidin, which facilitates electrogenic H⁺ transport. Conversely, if Cl⁻ transport was rate limiting (or if the anionophore did not facilitate electrogenic Cl⁻ transport faster than H⁺/Cl⁻ symport or Cl⁻/OH⁻ antiport), the rate of pH-gradient dissipation would be unaffected by gramicidin. The neutral proton channel gramicidin was employed to prevent potential intermolecular interaction between anionophores and proton carriers such as carbonyl cyanide phenylhydrazones. The use of inert NMDG⁺ (Figure 1) as the cation component precluded gramicidin itself from dissipating the pH gradient by facilitating M⁺/H⁺ antiport. The ratio between EC₅₀ values obtained in the absence and presence of gramicidin (selectivity [S] value shown in Table 2) was used to quantify Cl⁻ > H⁺/OH⁻ selectivity. It is important to note that the selectivity value obtained is a relative value dependent on the

Table 2. Summary of Cl[−] Transport Activity and Cl[−] > H⁺/OH[−] Selectivity

Anionophore	Without Gra		With Gra (0.1 mol %)		Selectivity (S) ^b
	n	EC ₅₀ (mol %)	n	EC ₅₀ (mol %) ^a	
1	1.4 ^c	0.046 ^c	1.4	0.043	1.1
2	2.4 ^c	0.61 ^c	1.9	0.29	2.1
3	2.3 ^c	0.16 ^c	2.0	0.11	1.4
4	1.2 ^c	0.015 ^c	1.2	0.015	1.0
5	1.9 ^c	0.26 ^c	1.8	0.33	0.8
6	3.2 ^c	20 ^c	3.1	13	1.5
7	2.5	7.9	2.7	5.9	1.3
8	1.3	0.00074	1.3	0.00085	0.9
9	1.3	0.042	1.1	0.0030	14
10	1.2	0.0011	1.2	0.0012	0.9
11	1.3	0.11	1.3	0.0028	39
12	1.2	0.067	1.4	0.00086	78
13	–	too inactive	–	too inactive	– ^d
14	1.2	9.5	1.0	1.3	7.4
15	1.5	0.25	1.2	0.077	3.3
16	1.3	0.016	1.4	0.017	0.9
17	–	too inactive	0.9	0.089	– ^e
18	1.4	0.18	1.2	0.0017	100
Trihexylamine	1.1	0.026	1.1	0.023	1.1
I ₂	1.6	6.9	1.5	6.1	1.1
Prodigiosin	1.4 ^c	0.000061 ^c	1.3	0.000059	1.0
Mn(TPP)Cl	1.1	0.0051	1.2	0.0045	1.1

Hill plot analysis of anionophores 1–18, trihexylamine (Hex₃N), iodine (I₂), prodigiosin (Prod), and Mn(TPP) Cl in the NMDG-Cl assay in the absence and presence of the proton channel gramicidin D (Gra). The NMDG-Cl assay is a fluorescence assay for H⁺/Cl[−] symport or Cl[−]/OH[−] antiport. Large unilamellar vesicles (LUVs) of POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; mean diameter, 200 nm) were loaded with HPTS (1 mM) and NMDG-Cl (100 mM), and buffered at pH 7.0 with 10 mM HEPES. The vesicles were suspended in an external solution of NMDG-Cl (100 mM) buffered at pH 7.0 with 10 mM HEPES. At the beginning of the experiment, a base pulse of NMDG (5 mM) was added to create a pH gradient, and the dissipation of the pH gradient induced by the test ionophores (added as a DMSO solution, or ethanol and acetonitrile solutions in some cases) was monitored by HPTS fluorescence. Lipid concentration for fluorescence measurement was 0.10 mM. Dose-dependent Hill plot analysis was performed to obtain a Hill coefficient (n) and an effective concentration to reach 50% of maximum transport (EC₅₀) at 200 s for each ionophore. Ionophore concentrations are shown as ionophore-to-lipid molar ratios. See Figures S34–S55 for Hill plots.

^aEC₅₀ in the presence of Gra indicates Cl[−] transport activity, since without Gra, H⁺/OH[−] transport may be rate limiting. Note that the “activity” here is the “total activity” of electroneutral H⁺/Cl[−] symport (or Cl[−]/OH[−] antiport) and electrogenic Cl[−] transport. The Gra concentration used (0.1 mol %) has been optimized to ensure maximum acceleration of pH-gradient dissipation for a Cl[−] > H⁺/OH[−]-selective anionophore.

^bCl[−] > H⁺/OH[−] selectivity (S) is quantified by the EC₅₀ in the absence of Gra divided by EC₅₀ in the presence of Gra; an S value of 1 indicates H⁺/OH[−] transport faster than Cl[−] transport, i.e., no selectivity.

^cThe same data as shown in Table 1.

^dToo inactive for Hill plot analysis. A single-concentration comparison showed no selectivity (Figure S46).

^eNo S value is given because, without gramicidin, 17 was too inactive for Hill plot analysis. This indicates a very high Cl[−] > H⁺/OH[−] selectivity that cannot be quantified by this method.

Cl[−] concentration employed. It should not be regarded as the absolute ratio between the permeability values of Cl[−] and H⁺/OH[−]. Table 2 presents selectivity data for the 22 anionophores shown in Figure 1 (refer to Section 3 in the

Supplemental Information for synthesis of new compounds), the majority of which are hydrogen-bond donors.

Prodigiosin showed no selectivity, consistent with its reported non-electrogenic nature (Table 2).¹⁷ The commercially available Cl[−] ionophore Mn(TPP)Cl¹⁹ was also unselective, likely because the Lewis acid metal center facilitated efficient OH[−] transport. Simple monopodal ureas/thioureas 1–7 and a squaramide 8 showed rather weak to no selectivity.

While testing the Cl[−] > H⁺/OH[−] selectivity of anionophores, we serendipitously identified that the tripodal thiourea 9,⁴⁶ but not its fluorinated analogs,²⁵ was both relatively highly selective (14-fold) and comparatively active (Table 2). New tripodal thioureas were therefore synthesized in an attempt to improve both activity and selectivity. Interestingly, adding electron-withdrawing cyano substituents to 9 (compound 10) led to loss of selectivity (pK_a of 10 was 10.5 in 9:1 acetonitrile-water; Figure S21). Conversely, changing the phenyl group to an *n*-pentyl group (compound 11) noticeably improved selectivity to 39-fold (Table 2). This result suggests that selectivity is sensitive to the acidity of the NH groups. By changing the linear *n*-pentyl group to the bulky *tert*-pentyl group, both activity and selectivity further improved from 11 to 12 (Table 2). Of note, 12 showed a remarkable selectivity of 78-fold, whereas its Cl[−] transport activity matched that of squaramide 8,⁴⁷ one of our most active anionophores reported to date. Despite the presence of a basic tertiary amine group and therefore the possibility of Cl[−] transport as a protonated ion-pair complex for the tripodal thioureas (Figure S63), pH-dependent anion-transport studies (Figure S65) demonstrated that the tripodal thioureas actually transported Cl[−] in their neutral forms (forming anionic complexes), unlike the “HCl receptors” prodigiosin and trihexylamine. Consistent with this idea, the crystal structure of the 12·tetraethylammonium chloride (TEACl) complex (Figure 4A) demonstrates the ability of the neutral form of 12 to encapsulate Cl[−] with six NH⋯Cl[−] hydrogen bonds (see Figure S22 for a ball-and-stick model).

Two effects arising from the tripodal anionophores might be key to their superior Cl[−] > H⁺/OH[−] selectivity in comparison to that of monopodal anionophores: (1) enhanced affinity for Cl[−] due to the chelate effect⁴⁸ (e.g., compare the Cl[−] affinity of 7 [*K* = 14 M^{−1}]⁴⁹ and 9 [*K* = 190 M^{−1}]⁴⁶ in DMSO-*d*₆/0.5% H₂O) and (2) anion encapsulation enforcing a high degree of anion desolvation. Although the Hill coefficient of ~2.6 in the case of 7 (Table 2) suggests that Cl[−] is likely sequestered by three thiourea molecules, like binding of Cl[−] within the tripodal thiourea cage 9, the alkyl spacer in 9 enforces a higher degree of Cl[−] desolvation than does the 3:1 7·Cl[−] complex, where the three thiourea moieties are not interconnected. To rationalize these effects, the selectivity of bithioureas 13 and 14 was examined. Both compounds can bind Cl[−] via four NH⋯Cl[−] hydrogen bonds, but the long alkyl spacer in 14 enforces a higher degree of anion desolvation (compare Figures 4B and 4C). Indeed, appending an additional thiourea moiety led to enhancement of Cl[−] affinities of both 13 (*K*₁ = 150 M^{−1}, *K*₂ = 3.4 M^{−1}; Figure S16) and 14 (*K*₁ = 530 M^{−1}, *K*₂ = 6.2 M^{−1}; Figure S18) in DMSO-*d*₆/0.5% H₂O in relation to that of 7 (*K* = 14 M^{−1}).⁴⁹ But, improvement of selectivity (relative to 7, 1.3-fold selectivity) was only observed for 14 (7-fold selectivity), whereas 13 demonstrated no selectivity (Figure S46), underscoring the importance of anion encapsulation. This idea is also supported by the higher selectivity of 12 than that of 11, given that the bulky alkyl substituents in 12 make Cl[−] less solvent accessible than linear alkyl substituents in 11. The exact reason behind the benefit of anion encapsulation for selectivity is unclear at the moment. We propose that this could be related to the higher hydration enthalpy of OH[−] than of Cl[−].⁵⁰ When the dehydration is caused by the part of the receptor that does not

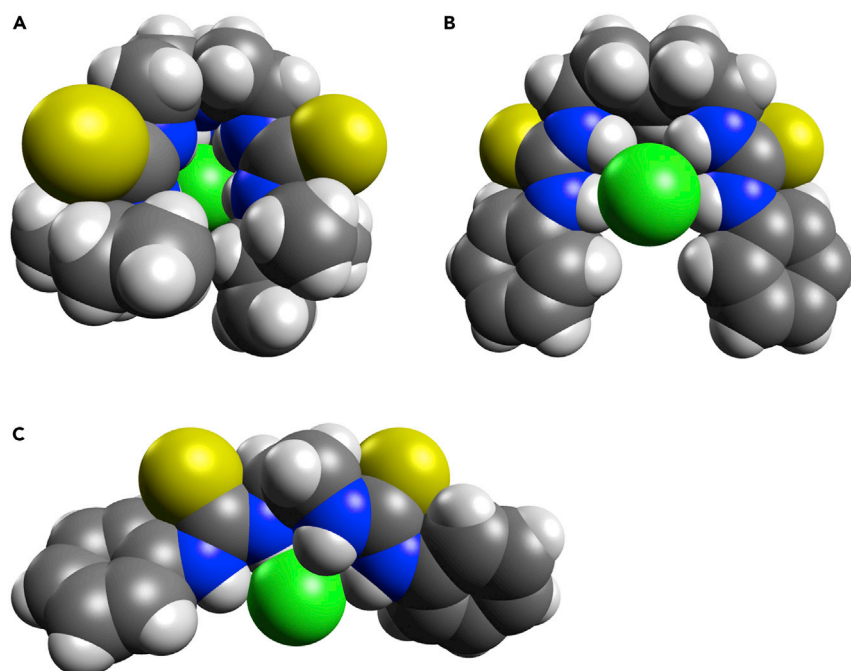


Figure 4. Space-Filling Representations of Crystallographic or Optimized Structures of Cl^- Complexes

(A) X-ray crystal structure of the **12**·TEACl complex (CCDC: 1431251; see [Section 8 in the Supplemental Information](#) for details).

(B and C) Molecular models of Cl^- complexes of (B) **14** and (C) **13**, optimized using the semi-empirical PM6 method.

Atom colors are as follows: gray, C; white, H; blue, N; yellow, S; and green, Cl.

significantly contribute enthalpically to binding anions, such as a spacer group, the anion-binding dehydration enthalpic cost for receptors that enforce a higher degree of dehydration will be higher than for those enforcing a lower degree of dehydration. This may confer greater selectivity on anionophores that encapsulate anions to a higher degree.

Although, in general, more acidic hydrogen-bond donors are favorable for anion binding and transport (in terms of activity),^{49,51} [Table 2](#) demonstrates that $\text{Cl}^- > \text{H}^+ / \text{OH}^-$ selectivity is severely compromised when more acidic hydrogen-bond donors are present. For example, the selectivity sequence of **11** > **9** \gg **10** illustrates this effect. An obvious disadvantage of higher-acidity anionophores is the possibility of H^+ transport via a CCCP-like deprotonation mechanism ([Figure 2C](#), left). Deprotonation is, however, not the only reason for the observed negative correlation between selectivity and acidity, given that even the halogen-bonding-based anionophore iodine showed no selectivity despite its inability to transport H^+ by deprotonation of the receptor ([Table 2](#)). It seems that more acidic (thus more electron-deficient) anionophores favor the transport of the more charge-dense OH^- over the less charge-dense Cl^- . This idea is consistent with the previous report that a CH hydrogen-bond-based anionophore, which is much less acidic than NH hydrogen-bond receptors, showed a high selectivity for Cl^- over the more charge-dense HCO_3^- .⁵² In contrast, a highly acidic bisurea anionophore lacked the $\text{Cl}^- > \text{HCO}_3^-$ selectivity.⁵²

The steroid scaffold is known to pre-organize two or three anion-binding motifs, resulting in powerful Cl^- ionophores (“cholapods”).^{53–55} We subjected several

reported and new cholapod-based urea anionophores to the selectivity test. Compound **15**⁵³ showed modest selectivity of 3-fold, which was eliminated when a trifluoroacetamide group,⁵⁴ as an additional hydrogen-bond donor, was introduced (compound **16**), likely because of its high acidity (Table 2). However, remarkable selectivity was achieved by **17** and **18**, which feature binding-site enclosure (Table 2).⁵⁶ Compound **17** was extremely selective to the extent that H⁺/OH⁻ transport was too inactive for Hill plot analysis. However, compound **17** was not a very active chloride carrier (Table 2). Despite containing an acidic trifluoroacetamide group, the more active **18** demonstrated a surprising 100-fold selectivity (Table 2). Presumably, the advantages of binding-site enclosure, possibly also from a decrease in urea NH acidity due to electron-donating alkoxy substituents, outweigh the disadvantage of an acidic trifluoroacetamide group. It is of interest to note that the Cl⁻ affinities of **17** ($K = 1.2 \times 10^5 \text{ M}^{-1}$) and **18** ($K = 3.6 \times 10^5 \text{ M}^{-1}$) were dramatically lower than those of **15** ($K = 1.5 \times 10^7 \text{ M}^{-1}$)⁵³ and **16** ($K = 2.8 \times 10^8 \text{ M}^{-1}$)⁵⁴ in water-saturated chloroform (for binding-constant determination, see Supplemental Information, Section 5). The higher Cl⁻ > H⁺/OH⁻ selectivity observed with the weakly binding but more encapsulating receptors **17** and **18** than with **15** and **16** again supports our proposition that anion encapsulation to a high degree, but not high-affinity anion binding, benefits selectivity.

Further evidence of the Cl⁻ > H⁺/OH⁻ selectivity of **12** and **18** was demonstrated by their coupling with the proton carrier CCCP to facilitate H⁺/Cl⁻ symport or Cl⁻/OH⁻ antiport in HPTS (Figure S62), ion-selective electrode (ISE; Figure S67), and osmotic-response assays (Figure S70). Thus, **12** and **18** couple with H⁺ transporters irrespective of the H⁺ transport mechanism (channel or carrier). This also suggests that H⁺ transport and Cl⁻ transport are independent processes in this case, ruling out possible intermolecular interactions between a proton transporter and **12** or **18**. As a complementary test, an HPTS assay conducted using KCl as the medium demonstrated that the activities of **12** and **18** were unaffected by the presence of the K⁺ ionophore valinomycin (Figure S62), which can replace potentially rate-limiting Cl⁻ transport with faster K⁺ transport as the counterion pathway. This result indicates that Cl⁻ transport is not rate limiting for the pH change⁵⁷ (for further explanation, see Supplemental Information, Section 9.4), which further supports the Cl⁻ > H⁺/OH⁻ selectivity of **12** and **18**.

Coupling between Cationophores and Anionophores: Direct Evidence of “Valinomycin-likeness”

Because valinomycin (an electrogenic cationophore) and monensin (a non-electrogenic cationophore) function via fundamentally different mechanisms, we used these compounds to investigate the anion-transport mechanisms of representative anionophores. Ionophore-induced Cl⁻ efflux was measured by ISE from KCl-loaded vesicles suspended in an inert external K₂SO₄ solution. Here, Cl⁻ transport was mainly driven by the large Cl⁻ concentration gradient (300 mM inside and ~0 mM outside), but no measurable Cl⁻ efflux could occur in the presence of an anionophore alone because of the buildup of a membrane potential (if the test anionophore was an electrogenic Cl⁻ carrier) or a pH gradient (if the test anionophore was a H⁺/Cl⁻ symporter or a Cl⁻/OH⁻ antiporter). Valinomycin dissipated the membrane potential from electrogenic Cl⁻ transport⁵⁸ (the K⁺ gradient also provided a small additional driving force for Cl⁻ transport), allowing electrically coupled K⁺/Cl⁻ flux in the presence of an electrogenic Cl⁻ transporter (Figure 3C).⁵⁹ By contrast, monensin dissipated the pH gradient accumulated by Cl⁻/OH⁻ antiport (or H⁺/Cl⁻ symport) through K⁺/H⁺ antiport, leading to formal KCl flux in the presence of a H⁺/Cl⁻ symporter or Cl⁻/OH⁻ antiporter (Figure 3D).

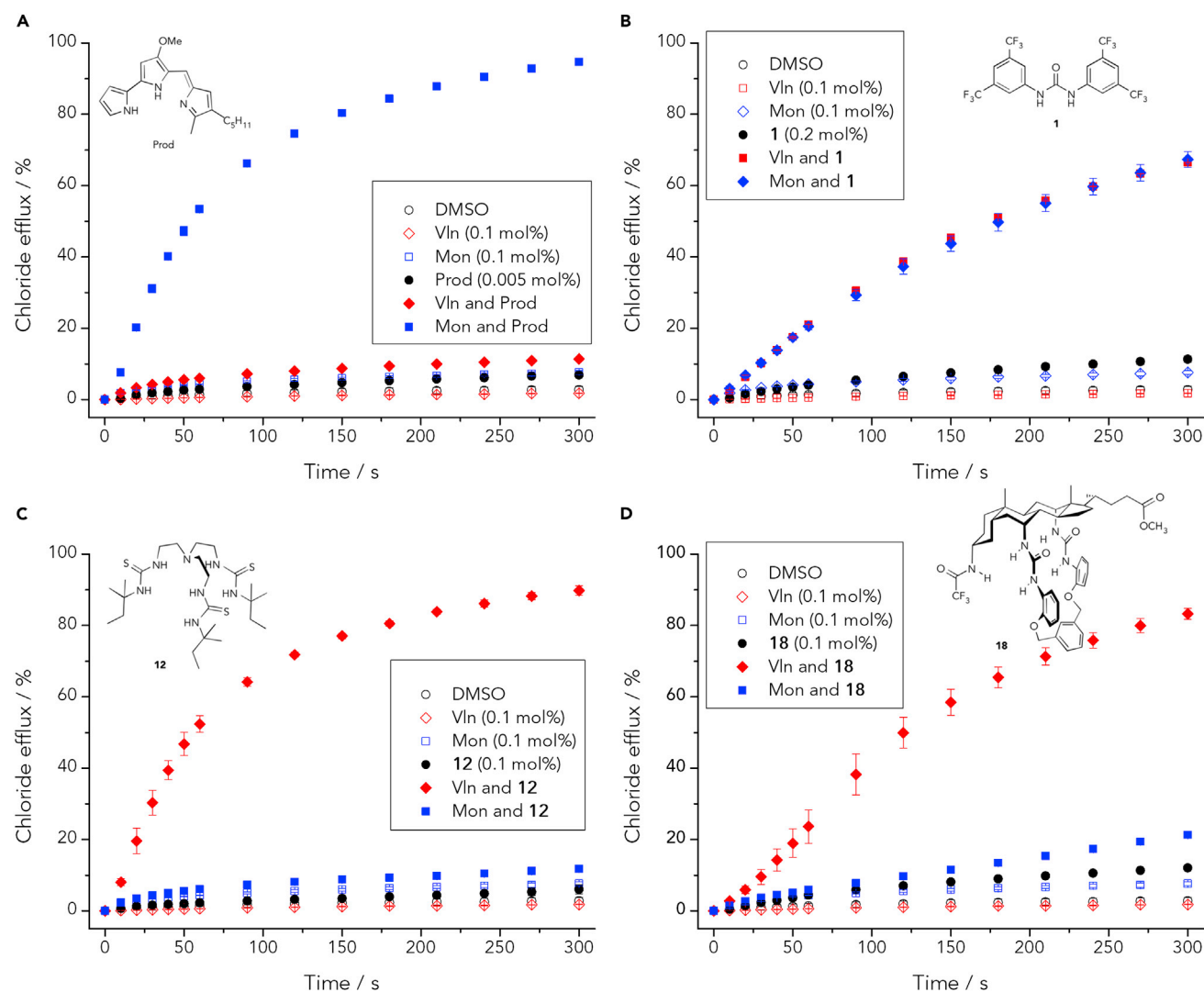


Figure 5. Coupling between Cationophores and Anionophores to Facilitate Net KCl Flux

(A–D) POPC LUVs (mean diameter = 200 nm) were loaded with KCl (300 mM) and K_2SO_4 (200 mM) buffered at pH 7.4 with 5 mM HEPES. The vesicles were suspended in an external solution of K_2SO_4 (200 mM) buffered at pH 7.4 with 5 mM HEPES. Cl^- efflux induced by (A) prodigiosin (Prod), (B) 1, (C) 12, or (D) 18 in the absence and presence of valinomycin (Vln, 0.1 mol % with respect to lipid) or monensin (Mon, 0.1 mol %) was monitored using a Cl^- -selective electrode. All ionophores were added to the vesicle suspensions as DMSO solutions. Detergent was added at 5 min to release all Cl^- and calibrate Cl^- efflux to 100%. Lipid concentration was 1.0 mM. Ionophore concentrations are shown as ionophore-to-lipid molar ratios. Error bars represent standard deviations from two repeats. The same DMSO, Vln, and Mon controls were used in all figures. See Figures 3C and 3D for schematic illustrations.

Figure 5A shows that prodigiosin coupled with monensin, but not valinomycin, demonstrating that prodigiosin cannot facilitate electrogenic Cl^- transport and is thus the anionophore equivalent of the non-electrogenic cationophore monensin. The unselective anionophore 1 coupled with both valinomycin and monensin (Figure 5B), demonstrating that 1 can facilitate both electrogenic Cl^- transport and electroneutral Cl^-/OH^- antiport (or H^+/Cl^- symport) at the same concentration. The same transport rates observed in the presence of valinomycin and monensin suggest that Cl^- transport is the same rate-limiting process, also ruling out potential

transport acceleration via ion pairing between the valinomycin·K⁺ complex and the 1·Cl⁻ complex. Taking into account that **1** also facilitated electrogenic H⁺/OH⁻ transport (Table 1), **1** can be regarded as a charge-inversed equivalent of “valinomycin + CCCP” (but with a much lower activity). Importantly, the selective anionophores **12** and **18** coupled with valinomycin, but not monensin (Figures 5C and 5D), indicating that they can facilitate electrogenic Cl⁻ transport with little H⁺/OH⁻ transport at the same concentration. Potential transport acceleration via ion pairing in the case of **12** and **18** coupling with valinomycin is unlikely given that valinomycin did not accelerate **12** and **18** in the HPTS assay (Figure S62). The “valinomycin-likeness” of selective anionophores **12** and **18** is thereby firmly established. Thus, the results reveal that prodigiosin, **1**, and **12** (**18**) are representative examples of three types of anionophores with different functions (for further details, see Supplemental Information, Sections 9.4, 12, and 14).

Anion-Transport and pH-Perturbation Studies in Cells

To begin to investigate the action of anionophores in cells, we used model systems to study the effects of anionophores on anion transport, pH-gradient disruption, and cytotoxicity. For these experiments, we used Fischer rat thyroid (FRT) cells, a cell line used to investigate epithelial ion transport,⁶⁰ and A549 cells, a cell line employed for pH-gradient disruption and cytotoxicity studies.²⁶ To measure anionophore-mediated Cl⁻ transport in FRT cells, we used FRT cells engineered to express the halide-sensitive yellow-fluorescent protein, YFP-H148Q/I152L.^{11,61,62} Facilitated Cl⁻ transport through the plasma membrane was measured indirectly by I⁻ entry into the cells coupled with the exit of intracellular Cl⁻, leading to quenching of YFP fluorescence by I⁻.¹¹ Compound **18** was inactive in this assay (data not shown), likely because of low deliverability.¹¹ By contrast, Figure 6A demonstrates that compound **12** mediated anion transport with high activity (similar to the active anionophores reported previously).¹¹

We performed cytotoxicity studies in human lung adenocarcinoma (A549) cells. Compound **12** exhibited modest toxicity to A549 cells with a half-maximum inhibitory concentration (IC₅₀, determined after treatment for 24 hr) of 43 ± 4 μM. Interestingly, analog **10** was significantly less toxic with IC₅₀ > 100 μM (Supplemental Information, Section 13). Possibly related to this result, we found that **10** was unable to facilitate electrogenic Cl⁻ transport in LUV assays (Figure S73A).

In lysosomes, an acidic luminal pH is established by H⁺ pumping into lysosomes by V-ATPase and a counterion pathway, which is likely Cl⁻/H⁺ antiport via CIC-7.^{63,64} Prodigiosin is known to neutralize lysosome pH by electroneutral H⁺/Cl⁻ symport out of lysosomes, which is a possible cause of its toxicity.^{14,15} Compound **12** was tested for its ability to neutralize lysosome pH in A549 cells using the pH-sensitive fluorescent dye acridine orange (AO) to stain lysosomes and endosomes. After treating A549 cells with 50 μM **12**, a concentration close to its IC₅₀, only a small proportion of the orange fluorescence inside the cells disappeared compared with the DMSO control (Figure 6B), suggesting only slight neutralization of lysosomal pH. This result contrasts with recently reported tambjamine-derived anionophores that led to complete disappearance of AO fluorescence in the same cell line at concentrations close to their IC₅₀ (~10 μM).²⁶ Similarly, analogs of **9** with electron-withdrawing fluorine or trifluoromethyl substituents,²⁵ other anionophores with highly acidic hydrogen-bond donors,^{23,24} and other tambjamine-derived “HCl receptors”²⁷ exhibited potent lysosomal pH neutralization, although they were tested in different cell lines. Anionophores bearing highly acidic hydrogen-bond donors

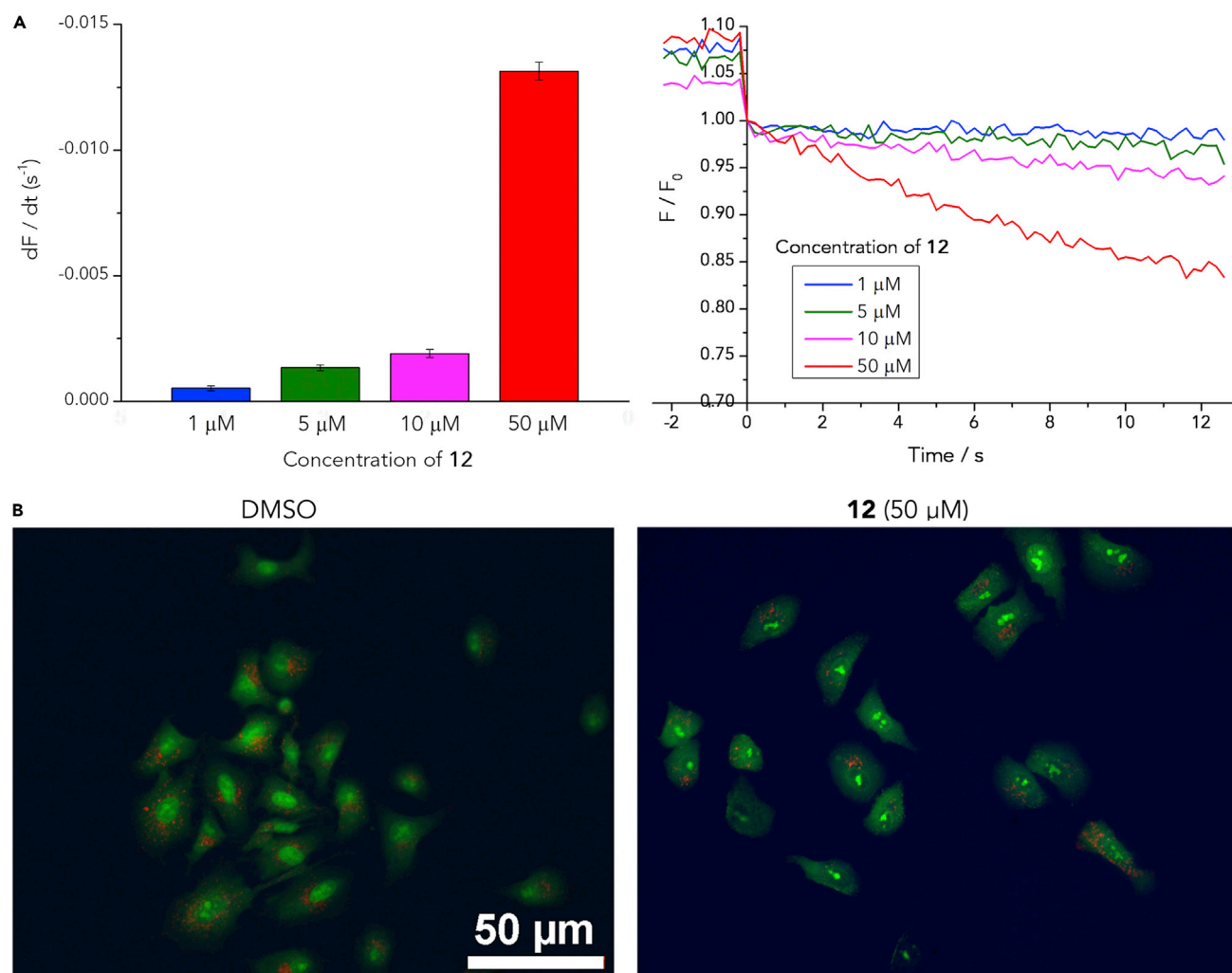


Figure 6. Compound 12 Mediates Anion Transport without Effectively Neutralizing Lysosomal pH in Cells

(A) (Left) Anion transport by FRT cells expressing the halide sensor YFP-H148Q/I152L at the indicated concentrations of compound 12 was determined from the fit of first-order exponential functions to the fluorescence decay elicited by NaI (100 mM). Fluorescence quenching by the anionophore vehicle (1–10 μM , 0.1% v/v DMSO; 50 μM , 0.5% v/v DMSO) was subtracted from each test measurement to determine the transport activity of compound 12. Data are shown as means \pm SEM ($n = 35$ –40 from at least three independent experiments). (Right) Representative time courses of cell fluorescence. Values of cell fluorescence were normalized to the fluorescence intensity at $t = 0$ s.

(B) Acridine orange staining of human lung adenocarcinoma (A549) cells treated with DMSO control (0.5% v/v) or compound 12 (50 μM , added in DMSO). The acridine orange staining was performed four times in duplicate, and similar results were obtained.

(e.g., 8 and 10) or functioning as “HCl receptors” (e.g., prodigiosin and trihexylamine) are unfavorable for selectivity, as demonstrated in our examples. The results reported here are consistent with the Cl⁻ > H⁺/OH⁻ selectivity of 12, indicative of low activity in facilitating H⁺/Cl⁻ symport or Cl⁻/OH⁻ antiport, processes required for lysosomal pH neutralization. The selectivity, however, is not as perfect as the K⁺ > H⁺/OH⁻ selectivity of valinomycin (Figure S61A); this possibly explains the minor lysosomal pH neutralization observed.

The change in intracellular (cytosolic) pH (pH_i) of A549 cells was also measured using the intracellular pH indicator SNARF-1 after anionophore treatment. Compound **12** induced a decrease in pH_i of 0.42 ± 0.04 pH units in this cancerous cell line. The exact cause of the decrease in pH_i is unclear at this moment. One possibility is $\text{Cl}^-/\text{HCO}_3^-$ antiport⁴⁶ facilitated by **12**. The decrease in pH_i might also be an indirect consequence of electrogenic Cl^- transport facilitated by **12**, which could trigger or couple with other cellular ion transporters involved in pH_i regulation under the experimental conditions.

Conclusions

The application of synthetic anionophores to biological systems is still at an early stage. However, in this article, we report a significant step forward by first developing assays to measure the selectivity of anionophores for Cl^- over H^+/OH^- and second developing electrogenic anionophores with high selectivity. Specifically, we have demonstrated that many small-molecule anionophores are capable of facilitating electrogenic H^+/OH^- transport. By comparing a series of ureas/thioureas and mono-N-methylated ureas/thioureas, we provide evidence that receptors acting through hydrogen bonding can transport H^+ by a CCCP-like deprotonation mechanism or OH^- by hydrogen bonding; the latter pathway is more likely for less acidic hydrogen-bond donors. To emulate the highly selective electrogenic K^+ carrier valinomycin, we developed the synthetic small-molecule electrogenic Cl^- carriers **12** and **18**, for which H^+/OH^- transport is suppressed. The remarkable $\text{Cl}^- > \text{H}^+/\text{OH}^-$ selectivity of these systems is confirmed by (1) coupling with both proton channel gramicidin and proton carrier CCCP to facilitate H^+/Cl^- symport (or Cl^-/OH^- antiport), as shown by fluorescence, ISE, and osmotic response assays; and (2) coupling with valinomycin, but not with monensin, to facilitate net KCl flux. Our results provide guidelines to identify different anion-transport mechanisms (e.g., electroneutral, electrogenic, and pH/proton -gradient dissipation) and develop anionophores with desired functions by rational design. For example, the ability to encapsulate Cl^- and the absence of highly acidic hydrogen-bond donors seem to be crucial characteristics of electrogenic $\text{Cl}^- > \text{H}^+/\text{OH}^-$ anionophores. As one of the first proven examples of “valinomycin-like” anionophores, **12** was found to facilitate Cl^- transport in cells without effectively neutralizing lysosome pH, unlike other anionophores that achieve high anion-transport activity by using highly acidic hydrogen-bond donors or functioning as prodigiosin-like “HCl receptors.” We propose that compounds of this type can play an important role in biomedical research and are potentially more suitable for treating “channelopathies,” such as cystic fibrosis, than other unselective anionophores.

EXPERIMENTAL PROCEDURES

HPTS assays based on pH-gradient dissipation¹⁸ were conducted using 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) LUVs (mean diameter = 200 nm) loaded with the pH-sensitive fluorescence dye HPTS (1 mM), with the unencapsulated HPTS removed by size-exclusion chromatography. The internal and external solutions used were identical salt solutions (Na^+ -gluconate, NMDG-Cl, $(\text{NMDG})_2\text{SO}_4$, K^+ -gluconate, or KCl, depending on the assay) buffered with 10 mM HEPES (pH 7.0). For each measurement, a 2 mL sample containing 0.1 mM lipid was used. To this vesicle suspension was added a base pulse (TBAOH, NaOH, KOH, or NMDG, depending on the assay; final base concentration = 5 mM) to generate a transmembrane pH gradient. At the beginning of each measurement, a DMSO solution (in some cases ethanol and acetonitrile solutions were used) of the test ionophore was added, and the ratiometric fluorescence of

HPTS ($\lambda_{\text{ex}} = 460 \text{ nm}$, $\lambda_{\text{em}} = 510 \text{ nm}$, base form divided by $\lambda_{\text{ex}} = 403 \text{ nm}$, $\lambda_{\text{em}} = 510 \text{ nm}$, acid form) was recorded. In cases where an assisting ionophore (e.g., gramicidin D) was used, the assisting ionophore was added to the vesicle suspension after the addition of the base pulse and prior to the addition of the test ionophore. At 200 s, a detergent was added to destroy the pH gradient and calibrate fluorescence. The fluorescence ratio was normalized to a fractional value. Dose-dependent Hill plot analyses were performed to obtain Hill coefficients (n) and EC_{50} (200 s) values.

ISE assays were conducted using POPC LUVs (mean diameter = 200 nm) loaded with NH_4Cl , KCl , or NaCl (300 mM for salt-efflux experiments and 500 mM for anion-exchange experiments) and suspended in a Cl^- -free external solution containing Na_2SO_4 , K_2SO_4 , or NaNO_3 and a buffer. The unencapsulated Cl^- was removed by dialysis. For each measurement, a 5 mL sample containing 1 mM lipid was used. At the beginning of each measurement, a DMSO solution (or ethanol solution for trihexylamine) of the test anionophore was added to the vesicle suspension, and Cl^- efflux was monitored by a Cl^- -selective electrode. In cases where an assisting ionophore (CCCP, valinomycin, or monensin) was used, the assisting ionophore was added to the vesicle suspension prior to the addition of the test anionophore. At 5 min, a detergent was added to lyse the vesicles and release all Cl^- to calibrate Cl^- efflux to 100%.

For further information about the different HPTS and ISE assays employed, see [Sections 9 and 10 in the Supplemental Information](#).

For details of the biological studies, including anionophore-mediated anion transport, cytotoxicity, and intracellular pH measurements, see [Section 13 in the Supplemental Information](#).

ACCESSION NUMBERS

The accession number for the 12·TEACl complex reported in this article is CCDC: 1431251.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedure, 76 figures, and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.chempr.2016.04.002>.

AUTHOR CONTRIBUTIONS

Conceptualization, X.W. and P.A.G.; Methodology, X.W., P.A.G., A.P.D., V.S.-C., R.P.-T., H.L., and D.N.S.; Investigation, X.W., L.W.J., E.N.W.H., A.M.W., V.S.-C., H.L., N.B., and H.V.; Writing – Original Draft, X.W., L.W.J., P.A.G., E.N.W.H., and D.N.S.; Writing – Review & Editing, X.W., P.A.G., E.N.W.H., D.N.S., H.L., A.P.D., H.V., and V.S.-C.; Funding Acquisition, P.A.G., A.P.D., R.P.-T., D.N.S., and Y.-B.J.; Supervision, P.A.G., A.P.D., R.P.-T., D.N.S., and Y.-B.J.

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