



# Preorganized Bis-Thioureas as Powerful Anion Carriers: Chloride Transport by Single Molecules in Large Unilamellar Vesicles

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Supporting Information

**ABSTRACT:** Transmembrane anion carriers (anionophores) have potential in biological research and medicine, provided high activities can be obtained. There is particular interest in treating cystic fibrosis (CF), a genetic illness caused by deficient anion transport. Previous work has found that anionophore designs featuring axial ureas on steroid and trans-decalin scaffolds can be especially effective. Here we show that replacement of ureas by thioureas yields substantial further enhancements. Six new bis-thioureas have been prepared and tested for Cl<sup>-</sup>/NO<sub>3</sub><sup>-</sup> exchange in 1-palmitoyl-2-oleoylphosphatidylcholine/cholesterol large unilamellar



vesicles (LUVs). The bis-thioureas are typically >10 times more effective than the corresponding ureas and are sufficiently active that transport by molecules acting singly in LUVs is readily detected. The highest activity is shown by decalin 9, which features N-(3,5-bis(trifluoromethyl)phenyl)thioureido and octyl ester substituents. A single molecule of transporter 9 in a 200 nm vesicle promotes  $Cl^{-}/NO_{3}^{-}$  exchange with a half-life of 45 s and an absolute rate of 850 chloride anions per second. Weightfor-weight, this carrier is only slightly less effective than CFTR, the natural anion channel associated with CF.

## INTRODUCTION

The promotion of transmembrane anion transport has become an important goal for supramolecular chemistry.<sup>1</sup> Although cation carriers (cationophores) are long established,<sup>2</sup> it is only recently that effective anionophores have become available. Such molecules have potential as tools for biophysical research and may reveal new modes of biological activity. In particular, there are several genetic disorders which involve defects in natural anion channels, the major example being cystic fibrosis (CF).<sup>4</sup> There is hope that anionophores may be used to treat these conditions via "channel replacement therapy".<sup>1d,5</sup>

If anionophores are to be applied in research or (especially) medicine, it is clearly desirable to maximize efficiency so that minimum quantities are required. We have described transporters based on "cholapod" (I),<sup>6</sup> trans-decalin (II),<sup>7</sup> and cyclohexane (III)<sup>8</sup> scaffolds (see Figure 1) which show very promising activities. These molecules promote measurable Cl<sup>-</sup>/  $NO_3^-$  exchange in synthetic vesicles, at levels down to transporter:lipid 1:250,000<sup>6b,c,7</sup> (and in one case 1:500,000).<sup>8</sup> The strategy which connects these systems involves the use of aliphatic scaffolds to deploy H-bond donor groups, most often ureas, with 1,5-diaxial relationships. The axial positioning of the polar groups restricts their conformational freedom, preorganizing them for anion binding.<sup>3d</sup> Counterproductive intramolecular H-bond formation is also prevented. The resulting binding sites show high affinities and are capable of extracting

anions from water. The lipophilic scaffolds promote solubility in membranes, allowing the molecules to act as anion carriers.

Although this work has focused largely on ureas as H-bond donor units, the corresponding thioureas have also been tested in some cases. In general the results have been positive. For example, in recent work on cyclohexanes III,<sup>8</sup> the tris-thiourea 10 (Figure 1) was roughly twice as active as its tris-ureido equivalent.9 However, to date this trend has not been systematically exploited to obtain optimal activities. We now report studies on six new bis-thioureas, derived from scaffolds I and II, which highlight the advantage of this motif for anion transport. We find that replacement of ureas by thioureas has variable effects, but often leads to a >10-fold increase in activity. The resulting systems can achieve rapid transport even when acting as single molecules in 200 nm vesicles and are up to 8 times more active than any previously reported from our laboratory.

## RESULTS AND DISCUSSION

Anionophore Design and Synthesis. The structures of the anion carriers discussed in this paper are shown in Figure 1. The new bis-thioureas are 4-6, based on the cholapod scaffold I,<sup>10</sup> and 7–9, based on the *trans*-decalin scaffold II.<sup>7</sup> The cholapods possess NHCOCF<sub>3</sub> in the steroidal  $3\alpha$  position, and

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**Figure 1.** Structures of the anionophores discussed in this paper. The compounds that have been reported previously are marked with an asterisk. On the logarithmic scale at the right these carriers are ranked from fastest (top) to slowest (bottom) based on their specific initial rate [I] for Cl<sup>-</sup>/NO<sub>3</sub><sup>-</sup> exchange. For the definition of [I] see the Analysis of Fluorescence Quenching Data section.

the electron-withdrawing N-aryl groups *p*-nitrophenyl, *p*-trifluoromethylphenyl, and 3,5-bis(trifluoromethyl)phenyl. These features were known to promote anion transport<sup>6,7</sup> but had not previously been used together with thioureas (as opposed to ureas). *Trans*-decalins 7–9 are the first to feature thioureido substituents, previous examples being bis-ureas.<sup>7</sup> *p*-Trifluoromethylphenyl and 3,5-bis(trifluoromethyl)phenyl N-aryl groups were also employed for this scaffold, while the ester side-chain was varied between ethyl (for 7 and 8) and octyl (for 9). The earlier study<sup>7</sup> had revealed an advantage for the octyl side-chain, and we were interested to discover whether this would translate from urea to thiourea. Also prepared for comparison purposes were two new cholapods bis-ureas, 1 and 2.



All new cholapod transporters were synthesized via the previously reported intermediate 13.<sup>11</sup> In the case of 1 and 2 the Boc groups were removed, the resulting amines were converted to ureas, and the trifluoroacetamido group was then

introduced by azide reduction and trifluoroacetylation. In the case of 4-6 the azide reduction—trifluoroacetylation was performed first, followed by Boc removal and introduction of the thioureas. The new decalin thioureas were synthesized via ethyl ester 14 (for 7 and 8) or octyl ester 15 (for 9). Ethyl ester 14 was prepared as previously reported, while octyl ester 15 was synthesized via a modification to the published route.<sup>7</sup> Details of all new synthetic procedures are given in the Supporting Information.

Transport and Binding Studies. The new compounds were assessed as Cl<sup>-</sup>/NO<sub>3</sub><sup>-</sup> antiporters using an assay based on the halide-sensitive fluorescent dye lucigenin, as previously described.<sup>6b</sup> Large unilamellar vesicles (LUVs) of ~200 nm diameter<sup>12</sup> were formed from 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and cholesterol in a 7:3 ratio with the transporter preincorporated in the lipid bilayer membrane. The vesicles contained sodium nitrate (225 mM) and lucigenin (0.8 mM) and were suspended in aqueous sodium nitrate (225 mM) such that the overall lipid concentration was 0.4 mM. Upon addition of sodium chloride (25 mM) to these vesicles exterior chloride and interior nitrate were exchanged, and the chloride influx was followed through quenching of the lucigenin fluorescence. The previously reported compounds  $3^{6c}_{,}$   $11^{7}_{,}$  and  $12^7$  were resynthesized and tested using this method to provide reliable comparison data, while the published results for cyclohexane  $10^8$  were reanalyzed (see below). Selected traces from experiments at transporter:lipid = 1:25,000 and 1:250,000 are shown in Figure 2. All the traces shown (and analyzed, see



Figure 2. Chloride transport by selected anionophores into 200 nm POPC/cholesterol (7:3) vesicles as followed by the lucigenin method (see text). Transporter:lipid = (a) 1:25,000 and (b) 1:250,000. Previously reported systems are marked with asterisks.

below) are the average of three to six fluorescence quenching experiments. Binding constants were measured to  $Et_4N^+Cl^-$  and  $Et_4N^+NO_3^-$  in chloroform, using an adaptation of Cram's extraction method,<sup>13</sup> and to  $Bu_4N^+Cl^-$  in DMSO- $d_6/H_2O$  (200:1) by <sup>1</sup>H NMR titration. Results are given in Table 1. Affinities for nitrate were generally somewhat lower than for chloride (up to 1 order of magnitude). The binding constants to chloride in wet DMSO were roughly 5 orders of magnitude lower than those in chloroform.

Analysis of Fluorescence Quenching Data. Numerical analysis of the transport data was performed using a new procedure which, we believe, gives more relevant and reliable figures than that used previously. In earlier work we have plotted the decay of fluorescence F as the ratio  $F/F_0$  ( $F_0$  = initial fluorescence) and then analyzed the plots to give decay half-lives and initial rates. Here we continue to use  $F/F_0$  for illustrative purposes (see Figures 2 and 4) but employ the reciprocal  $F_0/F$  for quantification. According to the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + k_q \tau_0[Q]$$

it is the ratio  $F_0/F$  which is proportional to concentration of quencher Q (in this case chloride). Thus, plots of  $F_0/F$  are directly related to the increase in the concentration of chloride inside the vesicles, and the derivatives of these plots give the transport rates. The data were analyzed in two ways. First the first 500 s of the traces were fitted to a single exponential decay function:

$$\frac{F_0}{F} = y - a \cdot e^{-bt}$$

to give an approximate half-life  $t_{1/2} = \ln(2)/b$ . Though not perfect, the fits were of reasonable quality and better than those previously obtained for  $F/F_0$  (see, for example, Figures S38 and S39 in the Supporting Information). Second the curves were modeled accurately as a double exponential function:

$$\frac{F_0}{F} = y - a \cdot e^{-bt} - c \cdot e^{-dt}$$

Table 1. Transport and Binding Data for bis-Ureas/Thioureas 1–12

structure		transport		binding		
	scaffold	$t_{1/2}$ at 1:25,000 (s) <sup>a</sup>	specific initial rate $[I]$ $(s^{-1})^b$	$K_{\rm a}$ to Et <sub>4</sub> N <sup>+</sup> Cl <sup>-</sup> in CHCl <sub>3</sub> $[M^{-1}]^c$	$K_{\rm a}$ to ${\rm Et_4N^+NO_3^-}$ in ${\rm CHCl_3}$ ${\left[ {{{\rm M}^{ - 1}}}  ight]^c}$	$K_{\mathrm{a}}$ to $\mathrm{Bu}_{4}\mathrm{N}^{+}\mathrm{Cl}^{-}$ in DMSO $[\mathrm{M}^{-1}]^{d}$
1	I	220	150	$2.5 \times 10^{9}$	n.d.	n.d.
2	Ι	64	450	$4.5 \times 10^{9}$	n.d.	n.d.
3* <sup>6c</sup>	Ι	79	340	$2.0 \times 10^{9}$	$2.5 \times 10^{8}$	n.d.
4	Ι	15	1800	$2.3 \times 10^{9}$	$2.1 \times 10^{8}$	$1.7 \times 10^{4}$
5	I	28	1300	$8.0 \times 10^{9}$	$1.1 \times 10^{9}$	$3.1 \times 10^{4}$
6	I	26	1500	$1.6 \times 10^{10}$	$1.4 \times 10^{9}$	$1.8 \times 10^{4}$
7	II	158	200	$1.2 \times 10^{8}$	$2.9 \times 10^{7}$	$1.5 \times 10^{3}$
8	II	16	2600	$4.7 \times 10^{8}$	$2.2 \times 10^{8}$	$2.4 \times 10^{3}$
9	II	9	3800	$5.0 \times 10^{8}$	$2.6 \times 10^{8}$	$2.6 \times 10^{3}$
10* <sup>8</sup>	III	68	460	$3.0 \times 10^{7}$	$2.5 \times 10^{6}$	$6.7 \times 10^{2}$
11* <sup>7</sup>	II	192	90	n.d. <sup>e</sup>	n.d. <sup>e</sup>	$8.8 \times 10^{2}$
12* <sup>7</sup>	II	88	340	$6.2 \times 10^{8}$	$4.5 \times 10^{8}$	n.d.

\*Compounds reported previously. n.d. = Not determined. <sup>*a*</sup>Transporter:lipid =1:25,000. Obtained from fits (0–500 s) of  $F_0/F$  to a single exponential function. <sup>*b*</sup>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. <sup>*c*</sup>Obtained by extraction of Et<sub>4</sub>N<sup>+</sup>Cl<sup>-</sup> or Et<sub>4</sub>N<sup>+</sup>NO<sub>3</sub><sup>-</sup> from water into chloroform at 303 K, as described in ref 13c. <sup>*d*</sup>Obtained from <sup>1</sup>H NMR titrations with Bu<sub>4</sub>N<sup>+</sup>Cl<sup>-</sup> in DMSO- $d_6/H_2O$  (200:1) at 298 K. <sup>*e*</sup>Not determined due to low solubility of **11** in chloroform.

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then differentiated at t = 0 to obtain the initial rate  $I = a \cdot b + c \cdot d$ . Experiments at different transporter loadings showed that I varied linearly with concentration (see below), so the values were divided by the transporter:lipid ratio and averaged to give specific initial rates [I]. These values for [I] allow the quantitative comparison of transporters over a wide range of activities. As the specific initial rate is independent of the transporter:lipid ratio, each carrier can be studied at loadings that are appropriate for the particular case. The values for the specific initial rates [I] are plotted on the scale in Figure 1. They are also listed in Table 1, together with the values for  $t_{1/2}$  at transporter:lipid =1:25,000.

Trends in Transport Rates. The data in Table 1 show that mutation from bis-urea to bis-thiourea does indeed lead to an increase in transport activity. Although this was expected, the differences are remarkable. In most cases where the comparison can be made (e.g., 4 vs 1, 8 vs 11, 9 vs 12), the increase in activity is at least 1 order of magnitude. In Figure 2, the scale of the effect can be appreciated by comparing traces for 12 (purple line) and 9 (red line). The net result is that five of the compounds (4-6, 8, 9) are more active than cyclohexanebased system 10, the most powerful previously reported from our laboratory. The most active is the trans-decalin 9 with an octyl side-chain and 3,5-bis(trifluoromethyl)phenyl thiourea binding groups. This molecule is eight times more effective than published "champion" 10. Relating this carrier to transporters from other laboratories is more difficult, but comparison with published data suggests that 9 is substantially more active than other exceptional systems.<sup>9c,14</sup> We therefore believe that 9 is the most effective anionophore yet reported in the literature.

The superior performance of the thioureas does not seem to be due to improved anion affinities. In contrast to transport, the change from urea to thiourea does not have a large, or even a consistent, effect on anion binding.<sup>15</sup> Thus, while thioureidocholapod 5 binds chloride in chloroform roughly twice as strongly as bis-urea 2, the trend is reversed for decalins 9 and 12 (see Table 1). Instead we suspect that the thiourea's advantage derives from the relative lipophilicities of the externally directed =O vs =S groups. We have previously shown that transport by anionophores is favored by "lipophilic balance", i.e., the even distribution of lipophilicity around the binding site.<sup>16</sup> By extension, one might propose that the ideal anionophore design presents an unbroken shield of lipophilic surface to the membrane interior. In bis-ureas the polar urea oxygens compromise this shield. Urea oxygens are good Hbond acceptors and are further improved on binding to anions (Figure 3a). The oxygens should thus bind quite strongly to water molecules, encouraging the complex to remain at the membrane-water interface. By contrast the thiourea sulfurs possess lower surface charge densities and are poorer H-bond acceptors. The complex is thus better able to separate from the interface and transfer through the membrane. Calculations of surface potentials provide a means of illustrating the difference (Figure 3b). The urea oxygens in 11·Cl<sup>-</sup> represent foci of negative charge nearly as intense as the bound chloride (e.g., -392 kJ mol<sup>-1</sup> for urea O, -420 kJ mol<sup>-1</sup> for Cl<sup>-</sup>). The thiourea sulphurs in  $8 \cdot Cl^-$  stand out much less strongly, ranging from  $-238 \text{ kJ mol}^{-1}$  at the farthest extent from the center (represented by green patches, see Figure 3b) to -345kJ mol<sup>-1</sup> on the C=S  $\pi$ -face (yellow surface, see Figure 3b).

An interesting feature of Table 1 is the relatively low activity of cholapod 5. As a bis-thiourea with the strongly electron-





Figure 3. Ureas vs thioureas as anion carriers. (a) In bis-ureas 11 and 12 the urea oxygens are strong H-bond acceptors and become even stronger on binding to chloride (due to transfer of electron density from Cl<sup>-</sup> through HNC to O). They are thus attractive to water molecules which may slow passage through the membrane, e.g., by impeding departure from the membrane–water interface. In thioureas 8 and 9 the sulfur atoms are less polar so the effect is much reduced. (b) Calculated surface potentials for 11·Cl<sup>-</sup> (left) and 8·Cl<sup>-</sup> (right). Computations were performed using Spartan '06, Hartree–Fock method, 6-31G\* basis set. The colors represent the energy required to bring a point positive charge to the surface of each molecule and are plotted on the same scale: red = -420 kJ mol<sup>-1</sup> (i.e., strongly negative surface).

withdrawing 3,5-bis(trifluoromethyl)phenyl substituent, further supported by a  $3\alpha$ -NHCOCF<sub>3</sub> group, this might have been expected to outperform all competition. However, it is only 3 times more powerful than the corresponding bis-urea 2 and, uniquely, is less effective than the *p*-trifluoromethylphenylsubstituted analogue 4. The 3,5-bis(trifluoromethyl)phenyl substituent consistently confers higher affinities than *p*trifluoromethylphenyl, and usually this is associated with improved transport activity. However, in this case, with the chloride affinity in CHCl<sub>3</sub> approaching  $10^{10}$  M<sup>-1</sup>, it seems possible that binding is too strong and decomplexation has become rate-limiting.<sup>3a,17</sup> Transporter 6 may be similarly affected, but in this case the relevant comparisons are not available.

Transport at Low Loadings: The Transition to One Carrier Molecule per LUV. As transporters increase in activity, it becomes convenient to study them at decreasing loadings. As mentioned earlier, in previous work we have employed transporter:lipid ratios as low as 1:500,000. One result of this trend is that, for commonly sized LUVs, the distribution of transporter molecules between vesicles becomes highly uneven. For example, a rough calculation suggests that 200 nm vesicles should be composed of ~500,000 lipid molecules (see Supporting Information for details). Thus, at transporter:lipid ~1:250,000 and lower, a significant proportion of vesicles should contain no transporter at all.<sup>18</sup> As loadings decrease further, one approaches a situation where essentially all vesicles contain 1 or 0 transporter molecules, and all observed transport is due to anionophores acting as single molecules.

This has interesting consequences for the fluorescence decay profiles. The initial rate *I* should depend linearly on transporter



**Figure 4.** Chloride transport by bis-thioureas at low loadings. (a,b) Traces for  $F/F_0$  in 200 nm vesicles mediated by **9** and **6**. (c,e,g,j) Plots of initial rates *I* vs transporter:lipid ratio for **9**, **6**, and **8** in 200 nm vesicles and **6** in 100 nm vesicles. (d,f,h,j) Corresponding results from half-life  $t_{1/2}$  calculations. For details on measurements and analyses see text and Supporting Infomation.

concentration, even at the very low loadings. The rate of decrease in fluorescence output is the average of the rates from all vesicles, and provided the anion concentration gradients are identical, the distribution of transporters between vesicles is unimportant. A higher rate of decrease from transporter-rich vesicles is balanced by lower rates from transporter-poor or transporter-free vesicles (for further discussion see Supporting Information page S57). By contrast, the behavior of  $t_{1/2}$  with transporter concentration is more complex. At high loadings the distribution between vesicles should be relatively even, so that the decay profiles from all vesicles will be similar and observed  $t_{1/2}$  will vary linearly with transporter concentration. However, once one approaches the "digital regime" (0 or 1 molecules per vesicle), the shape of the curves should change. Vesicles with no transporter should provide nearly constant fluorescence output, decaying slowly due to background unmediated anion transport. For vesicles with one transporter, output should decrease according to a standard pattern with a characteristic half-life. The net effect on observed fluorescence traces is that (a) the terminal plateau should rise, due to the population of vesicles with no transporter and (b) the measured  $t_{1/2}$  should tend to the constant value which is characteristic of the single-molecule-per-vesicle system.

The high activities of the new anionophores provided an opportunity to demonstrate this behavior. Figure 4a,b show data from *trans*-decalin 9 and cholapod 6 respectively, obtained in 200 nm vesicles at transporter:lipid ratios down to  $1:10^6$ . In both cases there is clear evidence for transporter-free vesicles at the lower loadings (1:100,000 or less). The traces appear to be consistent with two processes, a roughly exponential decay and a much slower decrease, approximately linear with time. The

latter is presumably due partly to slow unmediated anion transport in carrier-free vesicles, as observed for the blank experiment with no transporter. However, the rates are slightly higher than observed for the blanks, suggesting an unknown supplementary process. After subtraction of this linear component, the data were analyzed to give values for  $t_{1/2}$  as depicted in Figure 4d,f. The uncorrected data were analyzed to give values for I as depicted in Figure 4c.e. Similar analyses were performed for data from 8 in 200 nm vesicles (Figures 4g,h) and 6 in 100 nm vesicles (Figures 4i,j). In all cases the initial rate I was proportional to concentration throughout the range. However, as expected the values for  $t_{1/2}$  stabilized as the loading decreased. Moreover this occurred at an earlier stage for the smaller 100 nm vesicles, again as expected. The limiting  $t_{1/2}$ values refer to Cl<sup>-</sup>/NO<sub>3</sub><sup>-</sup> exchange mediated by a single transporter molecule, and serve to highlight the activity of these anionophores. For trans-decalin 9 in 200 nm vesicles, this single-molecule  $t_{1/2}$  is just ~46 s.<sup>19</sup>

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Finally the potential for exploiting anionophores to treat channelopathies depends on the rates of transport achievable, compared to the missing/defective channels. It is therefore relevant to quantify the activities in absolute terms (i.e., anion flux per transporter per second). Estimates may be made from the single-molecule  $t_{1/2}$ , as discussed in the Supporting Information. For *trans*-decalin **9** the  $t_{1/2}$  of 46 s in 200 nm vesicles translates to a rate of 850 Cl<sup>-</sup>/s for a single transporter at a chloride concentration gradient of 25 mM.<sup>20</sup> For comparison, the rate of chloride transfer through the channel associated with CF (the cystic fibrosis transmembrane conductance regulator, CFTR) was measured using the patch-clamp technique.<sup>21,6a</sup> At a concentration gradient of

137 mM, it was found that CFTR could support a flow of ~1.2  $\times$  10<sup>6</sup> Cl<sup>-</sup>/s. Assuming that rates scale linearly with Cl<sup>-</sup>,<sup>22</sup> 9 would be expected to transport 4.7  $\times$  10<sup>3</sup> Cl<sup>-</sup>/s under these conditions. Although the channel is thus ~250 times more effective, it is also ~200 times larger than 9.<sup>23</sup> On a weight-forweight basis, the synthetic carrier thus comes remarkably close to matching the protein.

# CONCLUSION

In conclusion we have shown that bis-thioureido-cholapods and -decalins can serve as anionophores with remarkable activities. With optimal substituents they can effect a quite rapid chloridenitrate exchange in LUVs, even when operating as single molecules. Their activity has been quantified and compares well with that of natural anion channels, considering the difference in molecular size. Introducing these highly hydrophobic molecules into cells may be challenging and will be a major focus of future work. However, if the delivery problem can be solved, the intrinsic efficiencies of these anionophores augur well for applications in biophysics and perhaps as treatments for channelopathies such as CF.

## ASSOCIATED CONTENT

#### **S** Supporting Information

Details of anionophore synthesis and characterization, binding studies, anion transport experiments and analysis, and comparison of anionophores to CFTR. This material is available free of charge via the Internet at http://pubs.acs.org.

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#### Notes

The authors declare no competing financial interest.

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## **REFERENCES**

(1) (a) Sessler, J. L.; Allen, W. E. CHEMTECH 1999, 29, 16-24.
(b) Davis, A. P.; Sheppard, D. N.; Smith, B. D. Chem. Soc. Rev. 2007, 36, 348. (c) Davis, J. T.; Okunola, O.; Quesada, R. Chem. Soc. Rev. 2010, 39, 3843. (d) Busschaert, N.; Gale, P. A. Angew. Chem., Int. Ed. 2013, 52, 1374.

(2) Pressman, B. C. Annu. Rev. Biochem. **1976**, 45, 501–530. Lehn, J.-M. Angew. Chem., Int. Ed. Engl. **1988**, 27, 89–112. Fyles, T. M. Inclusion Aspects of Membrane Chemistry; Osa, T. , Atwood, J., Ed.; Kluwer: Dordrecht, 1991; Vol. 2, pp 59–110.

(3) For example see: (a) Vargas Jentzsch, A.; Hennig, A.; Mareda, J.; Matile, S. Acc. Chem. Res. **2013**, 46, 2791. (b) Gale, P. A.; Pérez-Tomás, R.; Quesada, R. Acc. Chem. Res. **2013**, 46, 2801. (c) Gokel, G. W.; Negin, S. Acc. Chem. Res. **2013**, 46, 2824–2833. (d) Valkenier, H.; Davis, A. P. Acc. Chem. Res. **2013**, 46, 2898.

(4) Ashcroft, F. M. Ion Channels and Disease; Academic Press: London, 2000.

(5) Broughman, J. R.; Shank, L. P.; Takeguchi, W.; Schultz, B. D.; Iwamoto, T.; Mitchell, K. E.; Tomich, J. M. *Biochemistry* **2002**, *41*, 7350–7358.

(6) (a) Koulov, A. V.; Lambert, T. N.; Shukla, R.; Jain, M.; Boon, J. M.; Smith, B. D.; Li, H.; Sheppard, D. N.; Joos, J.-B.; Clare, J. P.; Davis,

A. P. Angew. Chem., Int. Ed. 2003, 42, 4931. (b) McNally, B. A.;
Koulov, A. V.; Smith, B. D.; Joos, J.-B.; Davis, A. P. Chem. Commun.
2005, 1087. (c) McNally, B. A.; Koulov, A. V.; Lambert, T. N.; Smith,
B. D.; Joos, J.-B.; Sisson, A. L.; Clare, J. P.; Sgarlata, V.; Judd, L. W.;
Magro, G.; Davis, A. P. Chem.—Eur. J. 2008, 14, 9599.

(7) Hussain, S.; Brotherhood, P. R.; Judd, L. W.; Davis, A. P. J. Am. Chem. Soc. 2011, 133, 1614.

(8) Cooper, J. A.; Street, S. T. G.; Davis, A. P. Angew. Chem., Int. Ed. 2014, 53, 5609.

(9) Other workers have also observed this effect. See, for example: (a) Busschaert, N.; Gale, P. A.; Haynes, C. J. E.; Light, M. E.; Moore, S. J.; Tong, C. C.; Davis, J. T.; William A. Harrell, J. Chem. Commun. **2010**, 46, 6252. (b) Andrews, N. J.; Haynes, C. J. E.; Light, M. E.; Moore, S. J.; Tong, C. C.; Davis, J. T.; W, A. H., Jr; Gale, P. A. Chem. Sci. **2011**, 2, 256. (c) Busschaert, N.; Wenzel, M.; Light, M. E.; Iglesias-Hernández, P.; Pérez-Tomás, R.; Gale, P. A. J. Am. Chem. Soc. **2011**, 133, 14136. (d) Busschaert, N.; Kirby, I. L.; Young, S.; Coles, S. J.; Horton, P. N.; Light, M. E.; Gale, P. A. Angew. Chem., Int. Ed. **2012**, 51, 4426.

(10) Brotherhood, P. R.; Davis, A. P. Chem. Soc. Rev. 2010, 39, 3633.
(11) Del Amo, V.; Siracusa, L.; Markidis, T.; Baragaña, B.; Bhattarai, K. M.; Galobardes, M.; Naredo, G.; Pérez-Payán, M. N.; Davis, A. P. Org. Biomol. Chem. 2004, 2, 3320.

(12) Average diameter = 193 nm by dynamic light scattering. For details see Supporting Information.

(13) (a) Timko, J. M.; Moore, S. S.; Walba, D. M.; Hiberty, P. C.; Cram, D. J. J. Am. Chem. Soc. 1977, 99, 4207–4219. (b) Ayling, A. J.; Broderick, S.; Clare, J. P.; Davis, A. P.; Pérez-Payán, M. N.; Lahtinen, M.; Nissinen, M. J.; Rissanen, K. Chem.—Eur. J. 2002, 8, 2197. (c) Clare, J. P.; Ayling, A. J.; Joos, J.-B.; Sisson, A. L.; Magro, G.; Pérez-Payán, M. N.; Lambert, T. N.; Shukla, R.; Smith, B. D.; Davis, A. P. J. Am. Chem. Soc. 2005, 127, 10739.

(14) Moore, S. J.; Haynes, C. J. E.; Gonzalez, J.; Sutton, J. L.; Brooks, S. J.; Light, M. E.; Herniman, J.; Langley, G. J.; Soto-Cerrato, V.; Perez-Tomas, R.; Marques, I.; Costa, P. J.; Felix, V.; Gale, P. A. *Chem. Sci.* **2013**, *4*, 103–117.

(15) Similar observations have been made previously. For examples see refs 8 and 9a–d. The inconsistencies may be partially due to conformational differences between ureas and thioureas. See: Bryantsev, V. S.; Hay, B. P. J. Phys. Chem. A **2006**, 110, 4678.

(16) Valkenier, H.; Haynes, C. J. E.; Herniman, J.; Gale, P. A.; Davis, A. P. *Chem. Sci.* **2014**, *5*, 1128–1134.

(17) Behr, J. P.; Kirch, M.; Lehn, J. M. J. Am. Chem. Soc. 1985, 107, 241.

(18) Occupancy should follow a Poisson distribution. For transporter:lipid 1:250,000 and 500,000 lipid molecules per vesicle (i.e., 2 transporters/vesicle), the predicted occupancies are: 0, 14%; 1, 27%, 2, 27%, 3, 18%, etc. Note that this calculation presumes a homogeneous population of perfectly spherical vesicles. The results herein suggest that in practice the number of transporter-free vesicles is somewhat larger than predicted. For further discussion see Supporting Information.

(19) Based on the average of measurements at 1:500,000 and 1:1,000,000.

(20) Absolute rates by transporters 6 and 8 were determined by this same method and found to be 660 and 730  $Cl^{-}/s$ , respectively.

(21) Cai, Z. W.; Taddei, A.; Sheppard, D. N. J. Biol. Chem. 2006, 281, 1970.

(22) Experiments suggest this assumption is justified in the range  $[Cl^-] = 0-32$  mM. See page S61 of the Supporting Information. Unfortunately higher concentrations cannot be tested using our methodology because of the effect of osmotic stress on the vesicles.

(23) The molecular weights of CFTR and 9 are  $\sim$ 180,000 and 867, respectively.