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Thiourea isosteres as anion receptors and transmembrane transporters†

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Compounds containing cyanoguanidine and 3-amino-1,2,4-benzothiadiazine-1,1-dioxide have been studied as anion receptors and transporters. Significant affinity for oxo-anions was observed in organic solution and the receptors were found to function as transmembrane chloride/nitrate antiporters with transport rates enhanced in the presence of valinomycin – K^+ complex.

The transport of anions across lipid bilayers is vital to a number of important biological processes. Dysregulation of passive chloride transport causes a number of diseases including cystic fibrosis. There is therefore intense current interest in developing synthetic transmembrane transporters to act as potential future therapeutic substitutes for naturally occurring ion channels (and as tools for the study of anion transport processes in cells).[1] Recent work within our group has highlighted the anion transport capabilities of a variety of molecules[2] including a series of simple thioureas.[3,4] However, some thiocarbamates and thioureas have been found to be toxic,[5] with the mechanism of toxicity not fully understood.[6] Non-classical isosteres such as substituted guanidine ligands **1** and **2**, offer a promising non-toxic alternative to thioureas. In particular cyanoguanidine has comparable physical properties to thiourea (e.g. pK_a: 15 (thiourea) and 14 (cyanoguanidine); hydrophobic partition P: 0.09 (thiourea) and 0.07 (cyanoguanidine); C-N bond lengths: 1.34 Å both thiourea and cyanoguanidine; N-C-N angle: 119° (thiourea) and 124° (cyanoguanidine), respectively) and is present in the drug cimetidine.[7] However, *N,N',N''*-trisubstituted guanidine groups often adopt a *trans* orientation of the two hydrogen atoms of the guanidine subunit and hence are not preorganised to bind anionic guests (Fig. 1).[8] Alternative conformationally locked systems containing parallel hydrogen bond donors based upon 3-amino-1,2,4-benzothiadiazine-1,1-dioxide (e.g. **3**). These groups have been synthesised previously[9] but have not yet applied to anion recognition. As both cyanoguanidines and derivatives of 3-amino-1,2,4-benzothiadiazine-1,1-dioxide are known to be compatible with biological systems[7,9-11] we decided to synthesise some potential anion receptors **1**-**3** containing these groups and study their lipid bilayer transport properties compared to simple thiourea **4**.

The receptors **1**-**4** were synthesised by modifications to literature procedures (see ESI†).[4,11,12] Crystal structures of the both cyanoguanidine receptors **1** and **2** show the compounds adopt the *trans* orientation of the two hydrogen atoms of the guanidine subunit in the solid state (Fig. 1a and Fig. 1b). Strong intermolecular alternating N-H···N hydrogen bonds (N···N distances of 2.932(4) and 2.950(4) Å in **1** and 2.857(3) Å in **2**, respectively, Table S1 ESI†) between the cyanoguanidine C≡N-group and the *cis*-orientated N-H group of two ligands support the present arrangement. In addition weaker N-H···N hydrogen bonds (N···N distance of 3.122(3) and 3.127(3) Å in **1** and 3.013(3) Å in **2**, respectively) are formed between the cyanoguanidine C≡N- group and the *trans*-orientated N-H group to link the dimer. This results in 2-D network of hydrogen bonds in the crystal. In contradistinction to these results, the *cis*-orientation is observed for the thiourea group in the crystal structure of **4** (Fig. S1 ESI†).

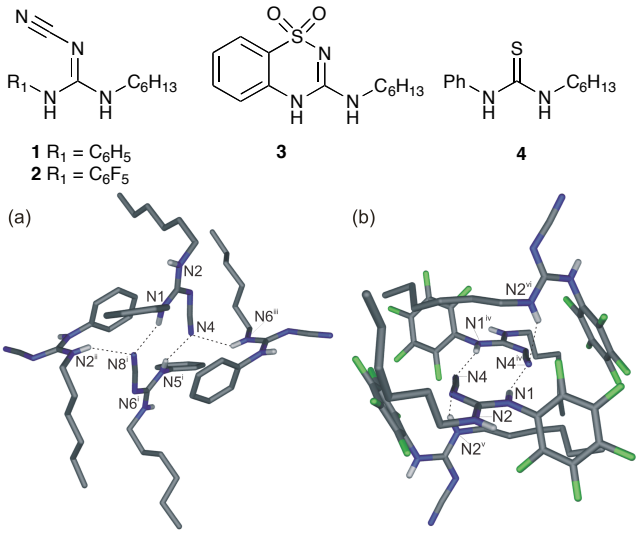

Fig. 1 Schematic draw of the crystal structure (a) of **1** and (b) of **2**, selected atom labels, dotted lines represent hydrogen bonds, non-interacting hydrogen atoms omitted for clarity, for **2** major occupied species only, Symmetry codes: i = 1+x,y,z; ii = 1/2+x,3/2-y,-z; iii = -1/2+x,1/2-y,-z; iv = 2-x,1-y,1-z; v = -2-x,-1/2+y,1/2-z; vi = 2-x,-1/2+y,1/2-z.

Proton NMR titration techniques were used to measure the stability of the receptor : anion complexes. Job plot analyses indicate a 1:1 binding mode for all the receptors (see ESI†). Stability constants for the formed complexes were determined using the EQNMR computer program.[13] Proton NMR titrations were conducted in DMSO-d_6/0.5 % water solutions with tetrabutylammonium anion salts. The results show that in all cases the receptors bind sulfate strongly with *log* K_a of 3.59 and

3.62 for receptor **1** and **4** and *log* K_a, > 4 for receptors **2** and **3**. Stability constants with chloride, dihydrogen phosphate, benzoate and acetate were also determined (Table 1). Addition of tetrabutylammonium nitrate under these conditions did not cause a shift of the receptors' proton resonances.

Detailed examinations of the titration spectra of receptor **1** show a significant difference in the NH-proton shift upon the titration with sulfate and chloride (Fig. 2). The stack plot shows the simultaneous shift of the NH resonances by 3.3 and 3.5 ppm upon the addition of sulfate. This is evidence that leads us to suggest that the receptor is conformationally flexible in solution and in the presence of sulfate both NH groups are oriented towards the anion. On the other hand, the weaker binding of chloride results in a significant shift of only one proton signal by 0.3 ppm in the presence of excess chloride, evidence to suggest that anion interacts with only a single NH group in this case.

Table 1 Stability constants *log K* of 1:1 complexes in DMSO-d_6/H_2O 0.5 %, 25 °C. Errors are estimated to be < 15 %.

anion	**1**	**2**	**3**	**4**
SO_4^{2-}	3.59	> 4	> 4	3.61[a)]
$H_2PO_4^-$	2.37	-[b)]	3.74	2.42
Cl^-	0.56[a)]	-[c)]	1.56	-[c)]
$PhCOO^-$	2.03	-[b)]	3.70	2.32
CH_3COO^-	2.51	3.23	3.91	2.64

a) error 20 % b) broadened NMR resonances, c) small shifts – could not be fitted to a 1:1 or 1:2 binding model

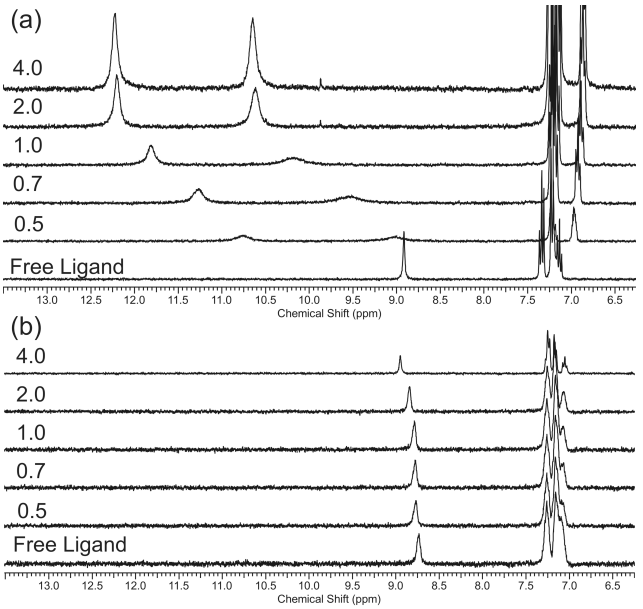

Fig. 2 NMR titrations of compound **1** with a) $TBA_2{\cdot}SO_4$ (top) and b) TBA·Cl (bottom).

In order to study the chloride transport properties of compounds **1**–**3** and the control compound **4** we prepared a series of unilamellar 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) vesicles loaded with KCl (489 mM) and suspended them in an external KNO_3 (489 mM) solution using previously reported procedures.[6,7] A sample of receptor **1**-**4** (2 % molar carrier to lipid) was added as a DMSO solution and the resultant Cl^- efflux monitored using a chloride selective electrode. After 300 s, the vesicles were lysed by addition of detergent and the final reading of the electrode was used to calibrate 100 % release of chloride. The results are shown in Fig. 3 and reveal chloride transport of compounds **1** and **3** (both 5 % after 270 s). The pentafluorophenyl substituted receptor **2** was found to be more active and approaching 29 % release of chloride over the timescale of the experiment. This is comparable to the transport ability of the thiourea receptor **4**. The latter shows a chloride efflux of approximately 30 %, which is similar to the activity of a previously reported phenyl thiourea receptor.[4]

There are a number of potential mechanisms that could be responsible for releasing chloride under the conditions of the experiment. We repeated the transport experiments with sodium rather than potassium salts and found no change in the chloride transport rate (evidence against a K^+/Cl^- co-transport mechanism). The transport experiments were repeated under similar conditions but with sulfate as the extravesicular anion. Under these conditions no chloride transport was observed. This is evidence that leads us to suggest that Cl^-/NO_3^- antiport is the predominant mechanism responsible for the release of chloride from the vesicles. Further studies in POPC/cholesterol 70 : 30 vesicles showed a reduction in chloride transport rate consistent with the compounds **1**-**4** functioning as discrete molecular carriers and not as channels (Fig. S2 – S5 ESI†).

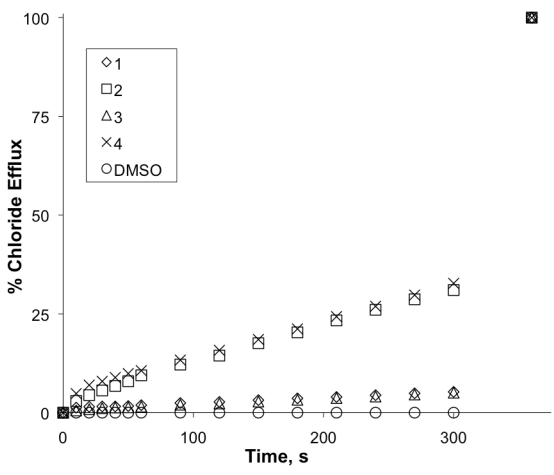

Fig. 3 Chloride efflux promoted by 0.02 molar equiv. of receptors **1**-**4** from unilamellar POPC vesicles loaded with 489 mM KCl buffered to pH 7.2 with sodium phosphate salts. The vesicles were dispersed in 489 mM KNO_3 buffered to pH 7.2 with 5 mM sodium phosphate salts. At the end of the experiment, detergent was added to lyse the vesicles and calibrate the ISE to 100 % chloride release. Each point represents the average of three trials.

We then repeated these experiments in the presence of the potassium ionophore valinomycin. The same experimental conditions were used, namely KCl (489 mM) loaded vesicles suspended in an external KNO_3 (489 mM) solution. The results are shown in Fig. 4 and reveal an enhancement in chloride transport when both valinomycin and one of the receptors **1**-**4** is present. For example a total chloride efflux of 58 % was observed for **2** and valinomycin present together compared to 29 % and 5 % in the presence of either receptor alone in the previous experiment. A similar result was obtained with compound **4**, namely an enhancement from 30 % in absence of valinomycin to 56 % in the present of both, **4** and valinomycin. The chloride transport for **1** and **3** is increased from 5 % to 23 % if

valinomycin is added in an equimolar amount. The chloride efflux for the receptors **1**-**4** at 270 s in the absence and presence of valinomycin are given in Table 2. As no potassium gradient is present, the enhancement observed may be caused by the presence of valinomycin bound potassium cations in the membrane possibly either enhancing extraction of the anions from the aqueous phase into the membrane or stabilizing the receptor bound anion complexes in the lipid bilayer.

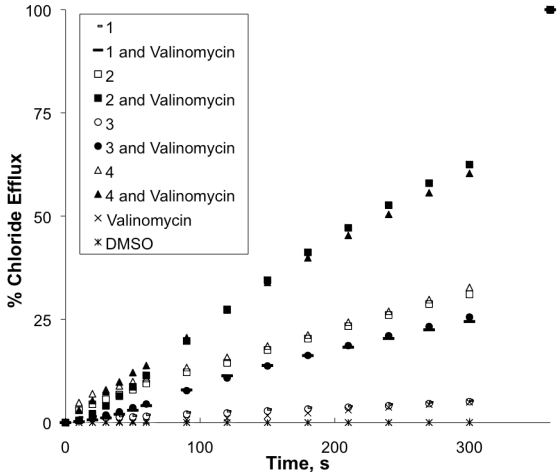

Fig. 4 Chloride efflux promoted by 0.02 molar equiv. of receptors **1**-**4** in the presence and absence of valinomycin (0.02 molar equiv.) from unilamellar POPC vesicles loaded with 489 mM KCl buffered to pH 7.2 with sodium phosphate salts. The vesicles were dispersed in 489 mM KNO_3 buffered to pH 7.2 with 5 mM sodium phosphate salts. At the end of the experiment, detergent was added to lyse the vesicles and calibrate the ISE to 100 % chloride release. Each point represents the average of three trials.

Table 2 Chloride efflux (%) promoted by 0.02 molar equiv. of receptors **1**–**4** in the absence and presence of valinomycin (0.02 molar equiv.) from unilamellar POPC vesicles loaded with 489 mM KCl buffered to pH 7.2 with sodium phosphate salts after 270 s. The vesicles were dispersed in 489mM KNO_3 buffered to pH 7.2 with 5 mM sodium phosphate salts.

	Chloride efflux at 270 s (%)			
	1	**2**	**3**	**4**
Absence of valinomycin	5	29	5	30
Presence of valinomycin	23	58	23	56

Conclusions

This work has demonstrated that thiourea isosteres such as functionalised cyanoguanidines and the hexyl derivative of 3-amino-1,2,4-benzothiadiazine-1,1-dioxide are capable of binding and transporting anions. Compound **2** shows transport activity which is similar to thiourea derivative **4** whilst the transport properties of all the compounds are enhanced in the presence of valinomycin-K^+. These initial studies with simple systems suggest that these hydrogen-bonding motifs may be optimised for anion complexation and transport. We are currently working to incorporate these new anion transport hydrogen bonding motifs into more efficient transporters for chloride and other anions.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Ligand synthesis, additional crystallogrphy, transport and NMR studies. CCDC 821666 - 821668. See DOI: 10.1039/b000000x/

‡ Crystal data for **1**: $C_{14}H_{20}N_4$, M = 244.34, orthorhombic, a = 10.2071(2) Å, b = 11.2105(3) Å, c = 24.2602(6) Å, V = 2776.01(11) Å^3, T = 120 K, $P2_12_12_1$ (no. 19), Z = 8, 27 023 reflections measured, 3585 unique (R_{int} = 0.0775) of which 3585 were used in the calculations, R_1 = 0.0546 (2806 with F > 2σ(F)), for this light atom structure Friedel opposites were merged and delta f'' value for all elements set to zero.

2: Cystal data for $C_{14}H_{15}N_4F_5$, M = 334.30, monoclinic, a = 13.6862(9) Å, b = 10.6669(7) Å, c = 11.3955(7) Å, β = 109.57(4), V = 1567.52(17) Å^3, T = 120 K, P21/c (no. 14), Z = 4, 25 328 reflections measured, 3614 unique (R_{int} = 0.0916) of which 3614 were used in the calculations, R_1 = 0.0706 (2773 with F > 2σ(F)), carbon 12 and 13 of the alkyl chain in **2** are disordered over 2 positions with an occupancy of 0.5 each.

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