Conformational control of HCl co-transporter: imidazole functionalised isophthalamide *vs.* 2,6-dicarboxamidopyridine†

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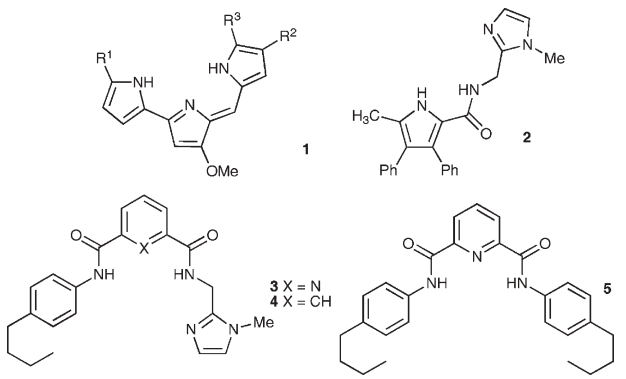
Received (in Austin, TX, USA) 5th March 2007, Accepted 27th March 2007
First published as an Advance Article on the web 4th April 2007
DOI: 10.1039/b703259e

Replacement of the central isophthalamide core in a synthetic HCl receptor, with a 2,6-dicarboxamidopyridine, leads to a more preorganised molecular structure that exhibits higher chloride affinity and membrane transport flux.

There is currently strong interest in the design of synthetic membrane transporters for anions.[1] The goal of our collaborative research programme is to develop HCl co-transport systems. These compounds are designed to act as functional mimics of the prodigiosin family of natural products (*e.g.*, **1**), which have been shown to promote the co-transport of H^+/Cl^- across bilayer membranes.[2] These natural products exhibit a range of potentially useful biological activities, including immunosuppression, induction of tumour cell apoptosis, and toxicity against bacteria, protozoa, fungi and the malaria parasite.[3] Recently, we demonstrated that compound **2** is a weak chloride receptor at neutral pH but when protonated shows a significantly enhanced chloride binding affinity.[4] Transport studies showed that **2** functions as an HCl co-transporter in vesicles, and that transport was accelerated by the presence of a pH gradient. More recently, Davis, Gale, Quesada and co-workers reported that an isophthalamide-derived carrier, with hydroxyl groups in the 4- and 6-positions, functions as a very efficient chloride transporter across vesicle membranes.[5] This functionalised system is more highly preorganised than a simple isophthalamide due to the presence of intramolecular hydrogen bonds. Furthermore, the internal hydrogen bonds decrease the molecular polarity. We decided to test the generality of this preorganisation concept and evaluate a series of putative HCl co-transporters **3** and **4** that contain either an isophthalamide or 2,6-dicarboxamidopyridine core and a pendant methylimidazole ring. As with **1** and **2**, compounds **3** and **4** contain two hydrogen bond donor groups and a basic site. However, the pyridyl analogue **3** was expected to possess a higher degree of preorganisation than the isophthalamide **4**, due to the well-known propensity of pyridine-2,6-dicarboxamides to adopt a *syn-syn* bis(amide) conformation that creates a convergent hydrogen bonding pocket.[6] We also prepared and evaluated the negative control compound **5**, which lacks the basic imidazole ring.

Compound **3** was synthesised by conversion of 6-(methoxycarbonyl)pyridine-2-carboxylic acid[7] to the acid chloride, followed by addition of (1-methyl-1*H*-imidazol-2-yl)methanamine,[8] subsequent saponification of the methyl ester, conversion to the acid chloride and finally addition of 4-butylaniline to afford compound **3** (47% yield). Compound **4** was obtained by addition of one equivalent of 4-butylaniline to N^1,N^3-bis(2-mercaptothiazolides)-isophthalamide[9] followed by the addition of (1-methyl-1*H*-imidazol-2-yl)methanamine[8] to afford **4** (yield 18%).



The anion binding affinities of receptors **3** and **4** were studied by standard ^{1}H NMR titration techniques with tetrabutylammonium chloride in DMSO-d_6, and the stability constants determined using the EQNMR computer program.[10] Chloride binding constants were found to be <10 M^{-1} for both compounds at 298 K (the titration data was fitted to a 1 : 1 binding model but the formation of a weak 1 : 2 receptor : anion complex cannot be ruled out). In other words, receptors **3** and **4** have a weak affinity for Cl^- under neutral conditions. The binding constants in DMSO-d_6 were also determined in the presence of one equivalent of HPF_6 at 298 K. Compound **4** also interacts weakly with chloride in the presence of HPF_6 (<10 M^{-1}), while compound **3** exhibits an enhanced binding affinity for chloride (59 M^{-1}) under these conditions. Such an enhancement was previously observed with compound **2**.‡ The influence of the imidazole in the structure of **3** was illustrated by comparison to control structure **5**. This compound was obtained in one step by addition of 4-butylaniline to pyridine-2,6-dicarbonyl dichloride. Binding studies showed that compound **5** has a weak affinity for chloride (<10 M^{-1}) in both the absence and presence of HPF_6, demonstrating the ability of the imidazole ring to enhance chloride affinity under acidic conditions.

Crystals of the HCl complex of **3** were obtained by slow evaporation of a solution of **3** in a mixture of toluene–dichloromethane–methanol–HCl_{aq}(2 M)–isopropanol.§ The X-ray crystal structure is shown in Fig. 1 and reveals that in the solid state **3**·HCl forms a '2 + 2 dimer'. The chloride is bound by the two amide NH groups (N···Cl 3.11(3) Å, N···Cl 3.48(3) Å). These

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† Electronic supplementary information (ESI) available: Synthesis details and NMR titration curves. See DOI: 10.1039/b703259e

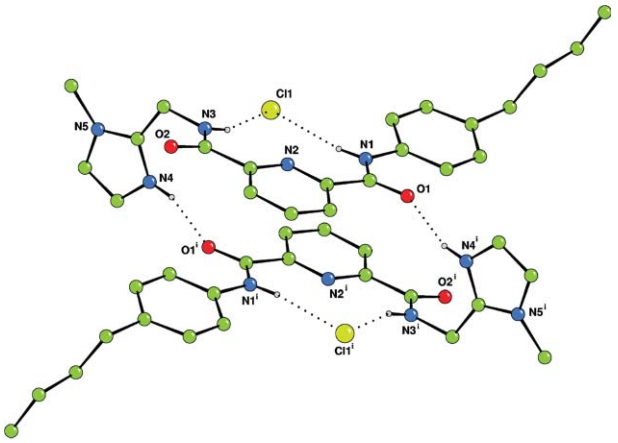

Fig. 1 Hydrogen bonded dimer of the HCl complex of **3** with selected atom labelling. Non-acidic hydrogen atoms are omitted for clarity. Symmetry operator (i) $-x + 1, -y + 2, -z$.

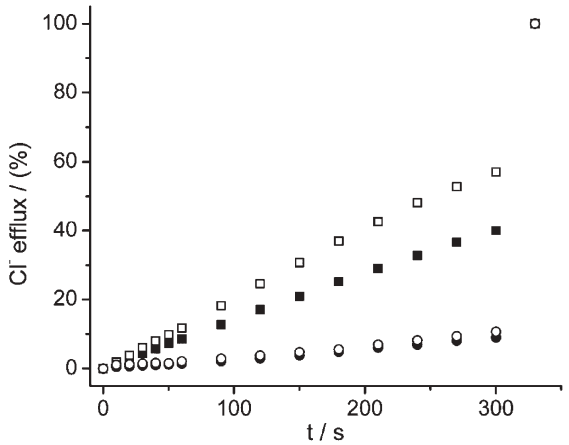

Fig. 2 Chloride efflux upon addition of **3** (■/□) or **4** (●/○) (8 μM in each case) to vesicles composed of POPC (empty symbols) or POPC–cholesterol (7 : 3 molar ratio) (filled symbols). The vesicles contained NaCl (500 mM) and were immersed in $NaNO_3$ (500 mM), pH 7.0 solution; at 300 s, they were lysed to obtain 100% chloride efflux.

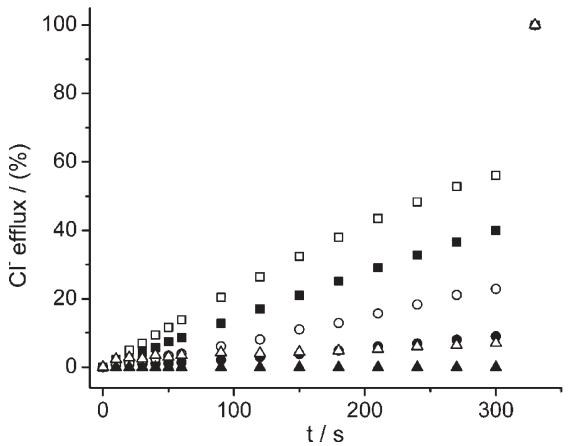

Fig. 3 Chloride efflux upon addition of **3** (■/□), **4** (●/○), or **5** (▲/△) (8 μM in each case) to vesicles composed of POPC–cholesterol (7 : 3 molar ratio) under neutral (filled symbols) and pH gradient (empty symbols) conditions. The vesicles contained: neutral, NaCl (500 mM), pH 7.0, or pH gradient, NaCl (500 mM), citric acid (5 mM), pH 4.0 solutions, and were immersed in $NaNO_3$ (500 mM), pH 7.0, or $NaNO_3$ (500 mM), sodium phosphate (5 mM), pH 7.0 solutions, respectively. At 300 s, they were lysed to obtain 100% chloride efflux.

nitrogen atoms also form hydrogen bonds with the nitrogen of the pyridine ring ($N\cdots N_{py}$ 2.770(3) Å, $N\cdots N_{py}$ 2.727(3) Å). The protonated imidazole does not directly hydrogen bond with the bound chloride, but rather is involved in an intermolecular hydrogen bond with the amide carbonyl of the adjacent molecule ($N\cdots O$ 2.67(7) Å). Hydrogen bonding to the amide carbonyl oxygens can enhance chloride transport in two ways. First, it increases chloride affinity by increasing the acidity of the amide NH residues.[11] Second, the lipophilicity of the molecule is raised because the amide carbonyl oxygens cannot readily interact with water.

Initially, compounds **3**, **4**, and **5** were tested for their abilities to transport Cl^- across unilamellar vesicles (200 nm diameter) composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or POPC–cholesterol (7 : 3 molar ratio) under neutral and pH gradient conditions. The neutral vesicles contained NaCl (500 mM), pH 7.0, and were immersed in $NaNO_3$ (500 mM), pH 7.0; the pH gradient vesicles contained NaCl (500 mM), citric acid (5 mM), pH 4, and were immersed in $NaNO_3$ (500 mM), sodium phosphate (5 mM), pH 7.0. A chloride selective electrode was used to detect chloride release from the vesicles.[4] Under neutral conditions, only compound **3** was an effective chloride transporter, with efflux rate slightly inhibited by the presence of cholesterol (Fig. 2). Structurally, **3** and **4** only vary by a single nitrogen atom but their transport activities are quite different, lending support to the concept of enhanced transport by a receptor (**3**) that has a more preorganised and convergent binding pocket. In the presence of a pH gradient, the rates of chloride efflux were enhanced (Fig. 3); furthermore, compounds **3** and **4** (but not **5**) are able to deacidify the acidic interior of the vesicles (Fig. 4).¶ These results are consistent with a transport mechanism that involves simultaneous co-transport of HCl by the carrier. With the data in hand we cannot state if the kinetically active transport complex (under pH gradient conditions) is a 1 : 1 ion pair of **3**·HCl, or the "2 + 2' dimer shown in Fig. 1. The fact that compound **5** shows little or no H^+ or Cl^- transport activity indicates that the pyridine nitrogen (in compounds **3** and **5**) is not protonated under the transport conditions.

To gain additional mechanistic insight, the transport mediated by **3** under neutral conditions was examined in more detail. A transporter concentration study (Fig. 5) showed that chloride efflux increases linearly with increasing concentration of **3**, supporting a mobile carrier mechanism (*i.e.*, the carrier diffuses across the bilayer membrane).[1a,12] The observation that transport rate is diminished by the presence of cholesterol (Fig. 2), is also consistent with a carrier diffusion process, assuming no change in carrier partitioning.[13] Finally, we find that the chloride efflux promoted by **3** under neutral conditions is strongly dependent on the identity of the anion in the vesicle external solution. Specifically, substitution of $NaNO_3$ in the external solution for Na_2SO_4 leads to almost complete loss of chloride efflux. This is evidence for an anion exchange mechanism that only allows chloride efflux if there is a corresponding influx of external anion (*i.e.*, A^-/Cl^- antiport). Thus, there appears to be a change in the

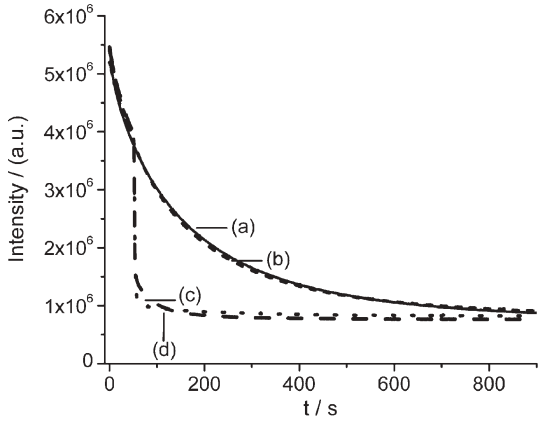

Fig. 4 Proton efflux as measured by the change in Lysosensor Blue™ fluorescence intensity. (a) Background efflux. Addition (8 μM) of: (b) **5**; (c) **4**; or (d) **3**, at t = 50 s to vesicles containing Lysosensor Blue™ (1.3 μM), NaCl (500 mM), citric acid (5 mM), pH 4.0. The vesicles were dispersed in an external solution of $NaNO_3$ (500 mM), sodium phosphate (5 mM), pH 7.0.

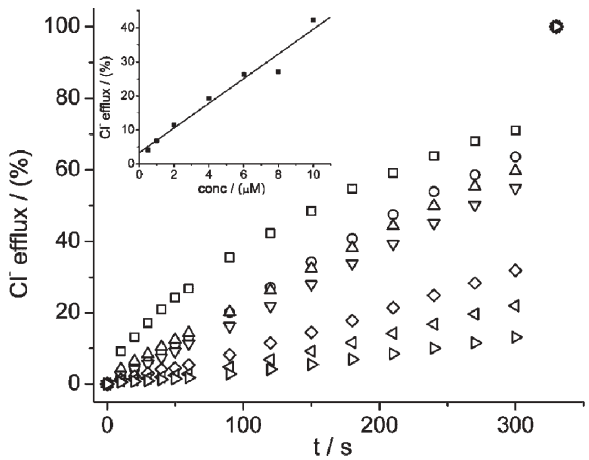

Fig. 5 Chloride efflux upon addition of **3** at increasing concentration (0.5 (▷), 1(◁), 2(◇), 4(▽), 6(△), 8(○) and 10 μM (□)) to POPC vesicles. The vesicles contained NaCl (500 mM) and were immersed in $NaNO_3$ solution (500 mM), pH 7.0; they were lysed at 300 s to obtain 100% chloride release. Inset shows the linear correlation (R^2 = 0.98) between concentration of **3** and chloride efflux.

transport mechanism with assay conditions, from H^+/Cl^- co-transport under pH gradient conditions to A^-/Cl^- antiport under neutral conditions, a feature exhibited by prodigiosin.[2c] Further work is needed to elucidate these finer mechanistic details.

The results of this study contribute to an emerging picture in the design of membrane transport carriers.[5] Subtle changes in the conformation of a transport carrier can produce large changes in binding affinity and carrier lipophilicity and, as a consequence, enhancements in transport flux.

PAG thanks the EPSRC for a post-doctoral fellowship (JG) and the EPSRC together with Professor Mike Hursthouse for access to the crystallographic facilities at the University of Southampton. BDS acknowledges funding support from the NIH (USA).

Notes and references

‡ With **2** in acetonitrile-d_3, chloride association constants increased from 60 M^{-1} to 397 M^{-1} in the presence of HPF_6. When these studies were repeated in DMSO-d_6, it was found that the affinity for chloride was enhanced from <10 M^{-1} to 40 M^{-1}. Solubility problems prevented titration studies with compounds **3** and **4** in acetonitrile-d_3.

§ Crystal data for the HCl complex of **3**: $C_{29}H_{34}ClN_5O_2$, M_r = 520.06, T = 12(2) K, triclinic, space group $P\bar{1}$, a = 7.3811(3), b = 12.1320(4), c = 17.4317(7) Å, α = 76.000(2), β = 85.986(2), γ = 85.254(2)°, V = 1507.39(10) Å^3, ρ_{calc} = 1.146 g cm^{-3}, μ = 0.159 mm^{-1}, Z = 2, reflections collected: 20076, independent reflections: 5309 (R_{int} = 0.0515), final R indices [$I > 2\sigma I$]: $R1$ = 0.0680, w$R2$ = 0.1570, R indices (all data): $R1$ = 0.0862. w$R2$ = 0.1678. CCDC 638924. For crystallographic data in CIF format see DOI: 10.1039/b703259e

¶ The acidity inside the vesicles was measured using the encapsulated fluorescent pH indicator Lysosensor Blue™ (Molecular Probes Inc.), whose emission intensity decreases as pH increases. The data in Fig. 4 shows that the background proton efflux rate is quite substantial due to the large pH gradient. This feature has been observed previously and is discussed in ref. 4.

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