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Anion transport by ortho-phenylene bis-ureas across cell and vesicle membranes†

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Ortho-phenylene bis-ureas serve as anionophores in cells expressing halide-sensitive yellow fluorescent protein, as well as in synthetic vesicles. Activities can reach high levels, and are strongly dependent on the deliverability of the transporters.

The development of synthetic anion transporters (anionophores) has become a major theme of supramolecular chemistry.1 An important motivation is the potential for anionophores to be applied therapeutically, for example in the treatment of cancer and channelopathies such as cystic fibrosis (CF).2 Much of the work focuses on using small molecule anion carriers, which can bind an anion on one side of the membrane, traverse the membrane and then release the anion on the other side.2

While the 1,5-diaxial anionophores are known to be exceptionally powerful,7 other families have also shown good activity in synthetic vesicle assays. In particular, ortho-phenylene bis-ureas (OPBUs) of general structure 4 have proven effective for Cl−/NO3−, Cl−/HCO3−, and Cl−/carboxylate antiport, in some cases at very low transporter:lipid ratios.8,9 These carriers are also especially easy to prepare, being accessible from commercially available starting materials in one or two steps. They thus provide a valuable opportunity for the study of structure–activity relationships. Here, we report that OPBUs are active in cells, at levels comparable to the 1,5-diaxial anionophores. We also explore the effects of substituents which modify lipophilicity and electronic characteristics, and the correlation between results in synthetic vesicles and data from the biological assay. The results highlight the importance of deliverability in the development of anionophores for biological applications.

The OPBU anionophores employed in this work are shown in Fig. 1. To ensure high activities (and thus ease of measurement), the urea units were uniformly terminated with 3,5-bis-phenylene bis-ureas (OPBUs) of general structure 4 for the OPBU carriers studied in the present work.

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**Fig. 1** 1,5-Diaxial anionophores 1–3, previously shown to be active in YFP-FRT cells and the general structure 4 for the OPBU carriers studied in the present work.
function as anion receptors by performing NMR titrations in Org. Biomol. Chem. This journal is © The Royal Society of Chemistry 2017

Calculated log $p_a$ values are often the most powerful derivatives in their selves and others have shown that anionophores bearing this (trifluoromethyl)phenyl groups. Studies conducted by ourselves and others have shown that anionophores bearing this substituent are often the most powerful derivatives in their respective series. Bis-urea 4a was known, and had previously been shown to possess good transport activity in large unilamellar vesicles (LUVs). The new compounds 4b–g were synthesised by refluxing the appropriate diaminobenzene with 3,5-bis(trifluoromethyl)phenyl isocyanate in DCM. The preparation of esters 4f and 4g required prior synthesis of the corresponding alkyl diaminobenzoates from 3,4-diaminobenzoic acid. Full experimental details are given in the ESI.

To characterise 4a–g, we first investigated their ability to function as anion receptors by performing NMR titrations in DMSO-d$_6$/0.5% H$_2$O with tetrabutylammonium chloride as guest. In all experiments the most significant movements were observed for the urea NH signals, indicating that they are all involved in chloride binding. Residual distribution analysis indicated that all receptors bind the anion in both 1:1 and 1:2 (host:guest) stoichiometries (see ESI† for further discussion) and titration data were subsequently fitted to this model. The second binding event was found to be very weak in all cases and 1:1 binding is the predominant process. The results are given in Table 1. In line with previous work, $K_{1:1}$ values are rather moderate and not especially sensitive to the type of substituent present on the central aromatic ring. For comparison, diureidodecalin 2 bound Bu$_4$N$^+$Cl$^-$ with $K_a = 680$ M$^{-1}$ under the same conditions.

To assess the transport properties of these molecules in synthetic membranes, we preferred a method which could distinguish between intrinsic activity (the ability to transport anions once in the membrane) and overall practical effectiveness (which also depends on the ability to find and enter the membrane). In the previous studies on OPBUs, transport activity in LUVs was assayed using an ion selective electrode (ISE) to detect chloride anions leaving the vesicles. This method requires that the test anionophore be externally added to the vesicles after they have been prepared; this is because preincorporation leaves the vesicles permeable to anions at all stages of the experiment, so that chloride efflux can occur before the measurement is initiated. The test thus gives a measure of practical effectiveness, but cannot be adapted to show intrinsic transport ability. For the present work, we employed an alternative assay which relies on a halide-sensitive fluorophore (lucigenin) which can be trapped inside the vesicles. This detects chlorine influx, allowing the experiment to be started by addition of chloride to the external solution. Anionophores can therefore be preincorporated in the vesicles (to measure intrinsic activity) or added externally (to assess practical effectiveness).

To measure intrinsic activity, we prepared LUVs, (∼200 nm diameter) from 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC) and cholesterol (7:3 ratio) with the putative transporter preincorporated in the membrane. The interior of the LUVs contained aqueous NaNO$_3$ (225 mM) and lucigenin. The vesicles were suspended in aqueous NaNO$_3$ (225 mM) and the experiment commenced by the addition of a pulse of NaCl

Table 1 Binding and transport data for OPBUs 4a–g

| Compound | $\text{clog} P_a$ | $K_a:1:1$ (M$^{-1})$ | $K_a:1:2$ (M$^{-1})$ | $F_0/F$ (s$^{-1})$ | Transport in LUVs (initial slope $I$ of $F_0/F$) (s$^{-1})$ | Transport in YFP-FRT cells (|dF/dt|) (s$^{-1})$ |
|----------|-----------------|------------------|------------------|-------------------|----------------------|-------------------|
| 4a       | 9.9             | 136              | 1                | 0.073             | 0.041                | 0.66              |
| 4b       | 10.4            | 146              | 1                | 0.073             | 0.027                | 0.37              |
| 4c       | 10.1            | 157              | 1                | 0.042             | 0.018                | 0.43              |
| 4d       | 8.8             | 166              | 3                | 0.0073            | 0.012                | 1.61              |
| 4e       | 9.2             | 146              | 2                | 0.011             | 0.013                | 1.17              |
| 4f       | 10.8            | 138              | 3                | 0.017             | 0.011                | 0.66              |
| 4g       | 11.6            | 134              | 1                | 0.019             | 0.0029               | 0.15              |

$^a$ Calculated log $P$, an estimate of lipophilicity where $P$ is the partition coefficient between $n$-octanol and water. Values were calculated using TorchLite (available as freeware from http://www.cresset-group.com). $^b$ Determined by $^1$H NMR titration in DMSO-d$_6$/0.5% H$_2$O at 298 K. Data were analysed using a 1:1 + 1:2 (host:guest) binding model. $^c$ Values are proportional to rates of Cl$^-$/NO$_3^-$ exchange. Experiments were conducted in 200 nm POPC/cholesterol (7:3) LUVs with transporter: lipid = 1:25 000. I was obtained by fitting $F/F_0$ versus time (0–500 s) to a double exponential decay function, for further details see ref. 7 and ESI. $^d$ Ratio of $I$ from experiments employing external addition/preincorporation of transporter (see discussion in text). $^e$ I/K$^+$ exchange mediated by the transporter (50 µM) in YFP-FRT cells. Obtained by fitting the decay in YFP fluorescence to a first order exponential function and subtracting fluorescence quenching of the vehicle (0.5–1% v/v DMSO).
(25 mM). The decay in lucigenin fluorescence emission corresponds to an increase in intravesicular chloride concentration. To obtain values for comparison, the data were expressed as $F_o/F$ (which is proportional to intravesicular chloride concentrations) then initial slopes $I$ were obtained using a curve fitting procedure. Decay curves ($F/F_o$) for a series of experiments with transporter : lipid = 1 : 25 000 are shown in Fig. 3, and corresponding values for $I$ are listed in Table 1.

The experiments revealed that all compounds in this series possess high intrinsic anionophore activity, clearly measurable at a very low transporter loading (0.004 mol%). The most powerful were bis-ureas 4a and 4b, based on halogenated central scaffolds. In the case of 4a, a dose–response study was performed and showed activity down to transporter : lipid = 1 : 1000k (Fig. S38†).

When comparing the activities of esters 4d–g, there is clearly a positive correlation between initial rate and the length of the ester alkyl chain/lipophilicity (Table 1 and Fig. 4, red circles). In principle this effect could arise due to different amounts of transporter in the membrane; less lipophilic esters (shorter chains) might be present in lower concentrations due to leaching from the membrane into the aqueous phase. To examine this possibility, we conducted a leaching study on methyl ester 4d, the least lipophilic member in this series and thus the most likely to partition into water. Briefly, LUVs with 4d preincorporated in the membrane were prepared as previously described. The vesicles were then successively diluted with aqueous NaNO$_3$ which should result in a loss of transporter from the lipid bilayers if it were capable of leaching. However, these studies indicated that 4d resides exclusively within the membrane and is not lost to the aqueous phase (see ESI† for further details). It thus seems that the longer alkyl chains promote intrinsic transport activity, possibly by positioning the transporter or complex more favourably within the membrane.

After measuring the intrinsic activities of OPBUs 4a–g we next sought to establish how readily they could be delivered by external addition to membranes (the second component of practical effectiveness). LUVs were prepared in a manner analogous to that previously described, except that the anionophore was not added to the initial lipid mixture. Instead a solution of the anionophore in acetone was externally added to rapidly stirred preformed vesicles to give a notional transporter to lipid loading of 1 : 25 000. After 5 minutes, NaCl (25 mM) was added and the decay in fluorescence analysed as before (Table 1 and ESI†). Deliverability ($D$) was calculated by dividing the initial rate of transport from this experiment to that observed for preincorporated anionophore. If a transporter is perfectly deliverable, values for $D$ are found to be ≥1 and this was observed for esters 4d and 4e. However, all other OPBUs tested displayed inferior activity when externally added, indicating that they are not perfectly deliverable ($D < 1$). Within the ester series it is evident that deliverability decreases as a function of lipophilicity (Table 1 and Fig. 4). This has a substantial effect on the activity of 4g; whereas it was the most powerful transporter in the ester series when preincorporated inside vesicles, it is the least active when added externally. Octyl ester 4g would thus be an ideal candidate for further investigation in coiled-coil driven membrane fusion studies. As demon-
strated previously, this is a highly efficient method of delivering lipophilic anionophores to membranes.

Following characterisation of their transport properties in synthetic membranes, OPBUs 4a–g were tested in cells. For these studies, we used Fischer rat thyroid cells expressing the halide sensor YFP-H148Q/I152L (YFP-FRT cells). YFP-FRT cells were seeded in a 96-well plate and cultured for 3 to 4 days until 90% confluence. The cells were washed twice with phosphate buffered saline (PBS) solution before incubating with a PBS solution containing the test anionophore (50 μM) for 10 min. The experiment was commenced by the addition of NaI (100 mM) and the rate of chloride/iodide exchange measured by fitting the decay in YFP fluorescence to a first order exponential function (see ESI† for full experimental details).

All compounds were found to mediate chloride/iodide exchange in cells leading to a decay in YFP fluorescence (Fig. 5, Fig. S45† and Table 1). The most active by some margin was bis-urea 4a, based on the difluorooromatic scaffold. Reassuringly, transporter 4a was also the most active when externally delivered to LUVs, and joint first when preincorporated. This adds to our confidence that the YFP-FRT assay does indeed measure anion transport, rather than some other phenomenon. Anionophore 4a is somewhat more active than 2, which gave |dF/dt| = 0.013 s⁻¹ in the present assay. The overall relationship between activity in cells and vesicles is shown in Fig. 6. Provided the vesicle measurements involve external addition of anionophore, a rough correlation emerges (Fig. 6a). There is, however, considerable scatter; the poor performance of dichloride 4b in the cells, despite its good activity in LUVs, is especially noticeable. This is difficult to explain, but may result from a specific interaction between 4b and the natural membrane. The results imply that, while experiments in LUVs can give some guidance, these synthetic assemblies are not ideal surrogates for cells and cannot substitute for biological testing. When results in cells are compared to those in vesicles with preincorporated OPBU, the correlation is very poor (Fig. 6b). This highlights the importance of considering deliverability as well as intrinsic activity when developing biologically active anionophores.

In conclusion, we have shown that a series of ortho-phenylene bis-urea (OPBU) anionophores are active in cells as well as synthetic vesicles. The most effective in all assays is bis-urea 4a, based on a difluorooriented central scaffold. Activities depend both on electronic factors and lipophilicity. In particular, the sequence 4d–g illustrates the role of lipophilicity in promoting intrinsic activity, but the contrary effect on deliverability and thus on effectiveness in cells. These bis-ureas are readily prepared, so that many other variations are potentially accessible. They therefore hold promise as tools for obtaining further insight into anionophore biological activity, and perhaps for the development of medical applications.

**Conflicts of interest**

There are no conflicts to declare.
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Notes and references


11 For modelling and crystallography of OPBU-Cl− complexes, see ref. 8 and 9 respectively. Both methods support the participation of all 4 NH groups as H-bond donors.


15 With some anionophores, external addition to LUVs gives higher rates than preincorporation, resulting in values of D > 1. We presume that this observation is due to the actual concentration of lipid being lower than the notional 0.4 mM. Vesicle preparation involves some loss of lipid, so that subsequent addition of anionophore (external addition) results in a transporter to lipid loading that is higher than intended. In contrast, when the transporter is preincorporated in the membrane, the lipid and transporter are lost together during vesicle preparation so that the intended transporter to lipid ratio is maintained.