



Fluorescent squaramides as anion receptors and transmembrane anion transporters†

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A series of squaramide-based anion transporters functionalised with the 1,8-naphthalimide fluorophore has been developed for improved ionophoric activity and fluorescent imaging in cells.

The transport of chloride ions across cell membranes is an important physiological process, which regulates intra- and extracellular pH, membrane potential, and cellular volume that are crucial to many biological processes including cell migration, proliferation and differentiation.¹ Intracellular chloride concentrations are regulated by naturally occurring anion channels in biological systems. A number of diseases, including cystic fibrosis (CF), involve malfunctioning ion channels (these diseases are collectively known as channelopathies).² Development of small molecule synthetic anion transporters (anionophores)³ capable of restoring chloride permeability across lipid bilayers has been proposed as a new approach to treating this class of disease.⁴ In addition, anionophores have been reported to alter pH and ionic gradients between intracellular compartments, which may lead to cytotoxicity, affording the possibility of using these systems as anticancer agents.⁵

Our group have previously reported fluorescent anion transporters based on naphthalimide-(thio)urea conjugates and

demonstrated their potential in studying the partitioning, localisation and action of these fluorescent anionophores within living cells using fluorescence imaging techniques.⁶ Those compounds, however, show modest anion transport efficacy compared with our most active transporters.⁷ To improve the anion transport rate potentially allowing the use of fluorescent transporters at lower doses, we decided to use squaramide instead of (thio)urea as the anion binding site and functionalise it with a 1,8-naphthalimide fluorophore.⁸ Compared with the most extensively-utilised urea and thiourea groups for anion complexation, the squaramide group confers the advantage of enhanced anion-binding affinities⁹ which has been shown to benefit anion transport efficacy in artificial liposomes and cells.¹⁰ Herein we report the synthesis of four novel fluorescent squaramide-based anion transporters **1–4** (Fig. 1) and study of their anion binding and transmembrane transport properties as well as their live-cell imaging capabilities.

Compounds **1–4** were readily obtained by reaction of 4-(4-aminobutylamino)-1,8-naphthalimide with squarate monoesters (see ESI† for synthetic details and characterisation data). Single crystals of compound **4** suitable for X-ray diffraction analysis were obtained by slow evaporation of a DMSO solution of **4** at room temperature. The crystal structure (Fig. 2) showed

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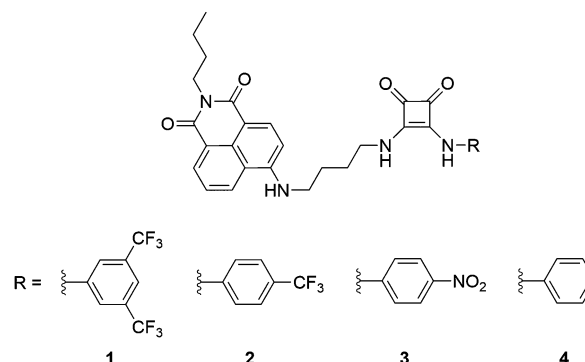


Fig. 1 Structures of compounds **1–4**.

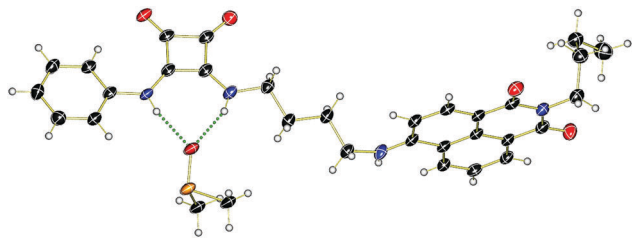


Fig. 2 Crystal structure of **4**.DMSO complex (CCDC 1530149).†

that compound **4** formed a 1:1 complex with DMSO *via* two squaramide NH...O hydrogen bonds (N...O distances of 2.834(2) and 2.821(3) Å and N–H...O bond angles of 163.3° and 155.1°, respectively). The 4-amino NH group of the naphthalimide moiety was not involved in the hydrogen bonding interaction with the DMSO solvate, despite the presence of a flexible 1,4-butylene linker between the naphthalimide and squaramide moieties.

The binding affinities of compounds **1–4** for chloride (as the tetra-*n*-butylammonium salt) were evaluated *via* ¹H NMR titrations in DMSO-*d*₆ (ESI†). For compound **1** upon the addition of Cl[−], significant downfield shifts were observed for the two

Table 1 Binding constants (M^{−1}) for the binding of compounds **1–4** to Cl[−], NO₃[−] and H₂PO₄[−] (tetrabutylammonium salts used for titrations) in DMSO-*d*₆ at 298 K as well as the corresponding Hammett constants (σ_p) of the substituents

Compound	K _a ^a (Cl [−])	K _a ^b (NO ₃ [−])	K _a (H ₂ PO ₄ [−])	σ _p
1	520	<10	>10 ^{4c}	0.85 ^e
2	240	<10	>10 ^{4c}	0.54 ^f
3	290	<10	>10 ^{4c}	0.78 ^f
4	160	<10	4400 ^d	0.00 ^f

^a The values of binding constant (K_a) were calculated by fitting the chemical shift changes of two squaramide NH resonances upon addition of TBACl assuming a 1:1 global fitting binding model. ^b All the proton signals were found to undergo negligible change in their chemical shifts during the titration process. ^c The binding constants were estimated based on the chemical shift changes of aromatic CH protons (adjacent to squaramide NH), due to the disappearance of NH signals during the titration process. ^d This value was determined by simultaneously fitting the chemical shift changes of two squaramide NHs and the naphthalimide 4-NH resonances upon addition of TBACl, using a 1:1 global fitting binding model. ^e The σ value for 3,5-bis(CF₃)-Ph substitution was obtained from the pK_a of 3,5-bis(trifluoromethyl)benzoic acid (*J. Org. Chem.*, 2016, **81**, 12116–12127). ^f Values obtained from ref. *Chem. Rev.*, 1991, **91**, 165–195.

squaramide NHs (Δδ > +1.3 ppm) as well as two aromatic CH protons from the 3,5-bisCF₃-Ph group (Δδ = +0.22 ppm). In contrast, the naphthalimide 4-NH only underwent a very slight downfield shift (Δδ = +0.05 ppm) over the course of the titration process, suggesting weak or no interaction of this proton with chloride. A global fitting analysis assuming a 1:1 binding model was employed to provide the binding constant (K_a), by simultaneous fitting of the chemical shift changes of two squaramide NHs as a function of added chloride.¹¹ As shown in Table 1, all compounds bound Cl[−] with affinities ranging from 160–520 M^{−1}. The relative strength of anion binding (**1** > **3** > **2** > **4**) correlated with the Hammett constants (σ_p) of their substituents. Much weaker binding was observed for NO₃[−] (K_a < 10 M^{−1}) and stronger binding for the more basic anion H₂PO₄[−] (4400 M^{−1} determined for **4**, and higher affinity of >10⁴ M^{−1} was found for more acidic compounds **1–3**). Different from Cl[−] binding, the complexation of tetrahedral H₂PO₄[−] by compound **4** seems to involve the naphthalimide 4-NH (Δδ = +1.24 ppm) in addition to two squaramide NHs.

The chloride–nitrate transport activities of compounds **1–4** across lipid bilayers were evaluated using liposome-based techniques based on a chloride ion-selective electrode (ISE, Fig. 3).¹² Briefly, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) unilamellar vesicles (mean diameter 200 nm) were loaded with NaCl (489 mM, buffered to pH 7.2 with 5 mM sodium phosphate), and then suspended in a NaNO₃ solution (489 mM, buffered to pH 7.2 with 5 mM sodium phosphate). The Cl[−]/NO₃[−] transport process was initiated by the addition of DMSO solution of compounds **1–4** to these vesicles, and the resultant chloride efflux was monitored using an ISE. Hill analysis¹³ was conducted by following chloride efflux at different concentrations of transporters (in mol% with respect to lipid concentration) to obtain EC₅₀ value (the concentration of transporter needed to achieve 50% chloride efflux in 270 s) and Hill coefficient *n* (indicating the stoichiometry of the formed complex during the transmembrane transport process¹⁴). As shown in Table 2, compound **1** with two CF₃ substituents possessed the best transport activity with a substantially lower EC₅₀ value (0.15 mol%) than previously reported naphthalimide-(thio)ureas (>0.4 mol%).⁶ The anion transport activity of the compounds (**1** > **3** > **2** > **4**) followed the same trend as their anion binding affinities. The Hill coefficient values of ~1 is consistent with a mobile carrier mechanism *via* formation of a 1:1 receptor–anion complex (Fig. 4).

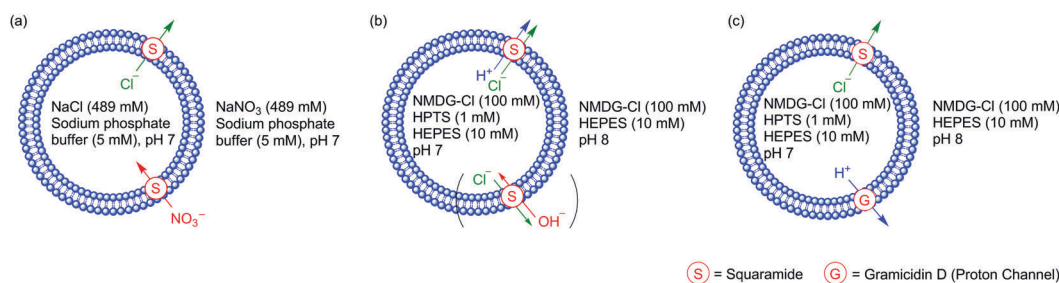


Fig. 3 Schematic illustration of the assays utilised for measuring the Cl[−]/NO₃[−] antiport using the ISE (a); the H⁺/Cl[−] symport or OH[−]/Cl[−] antiport using the NMDG-Cl assay in the absence (b) and presence (c) of Gramicidin D (proton channel).

Table 2 Summary of anion transport parameters (k_{ini} , EC_{50} and n) and calculated lipophilicity values ($c \log P$) for compounds **1–4**

Comp.	k_{ini}^a	$EC_{50,270s}^b$ (Cl^-/NO_3^-)	n^c	$EC_{50,200s}^d$ (Cl^-H^+/OH^-)	n^c	$c \log P^e$
1	1.2	0.15	0.9	0.018	1.5	5.8
2	0.57	0.63	0.8	0.055	1.3	5.4
3	1.0	0.26	1.0	0.041	1.4	4.9
4	0.33	2.07	0.9	0.27	1.8	5.1

^a Values determined by fitting the plot of relative chloride release (y) versus time (x) for 2 mol% transporter to lipid to an asymptotic function $y = a - b \times c^x$. The initial rate of chloride release (k_{ini} in $\% s^{-1}$) was obtained by $-b \ln(c)$. ^b Molar percentage with respect to lipid needed to obtain 50% chloride efflux at 270 s from the Cl^-/NO_3^- exchange assay. ^c Hill coefficient from Hill analysis. ^d Molar percentage with respect to lipid needed to obtain 50% chloride efflux at 200 s from the HPTS assay. ^e The $\log P$ values were calculated *via* VCLLab.

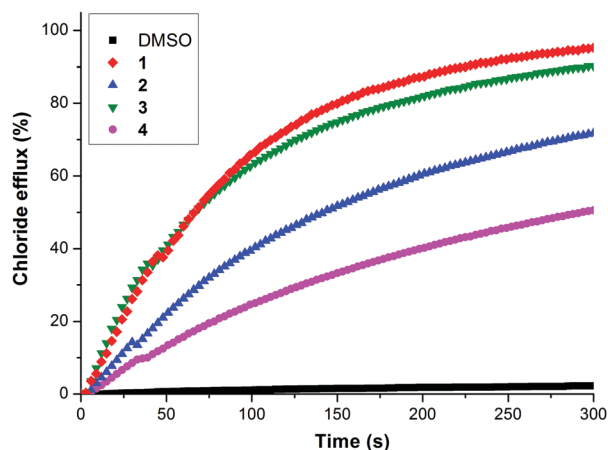


Fig. 4 Chloride efflux promoted by compounds **1–4** (2 mol% compound to lipid) from unilamellar POPC vesicles containing 489 mM NaCl buffered to pH 7.2 with 5 mM phosphate salts, suspended in 489 mM $NaNO_3$ buffered to pH 7.2 with 5 mM phosphate salts. At the endpoint of each experiment (300 s), the detergent (Triton X-100) was added to lyse the vesicles and calibrate the electrode to 100% chloride efflux. Each point represents the average of three trials. Pure DMSO was utilised as a control.

Previous studies have shown that anion transporters could dissipate the pH gradient across lipid bilayer in vesicle-based experiments, *via* H^+/Cl^- symport or OH^-/Cl^- antiport mechanism.¹⁵ In order to explore this possibility, a series of assays were performed using fluorescent dye HPTS (8-hydroxypyrene-1,3,6-trisulfonic acid) as pH indicator and *N*-methyl-D-glucamine (NMDG) chloride as a chloride ion source.¹⁶ Briefly, POPC liposomes were firstly prepared containing 1 mM HPTS and 100 mM NMDG-Cl buffered to pH 7.0, and suspended in an external solution of 100 mM NMDG-Cl buffered to pH 7.0. Then, NMDG (5 mM) was added as a base pulse to create a transmembrane pH gradient ($\Delta pH = 1.0$) and then the transporter was immediately added into the above suspension to trigger ion transport process and the resultant dissipation of pH gradient (Fig. 3b). As shown in Fig. 5, the H^+/Cl^- cotransport (or OH^-/Cl^- antiport) took place. To quantitatively evaluate the Cl^-H^+/OH^- transport activity of compounds **1–4**, Hill analysis was also carried out to give the corresponding values of EC_{50} and Hill coefficient (n).

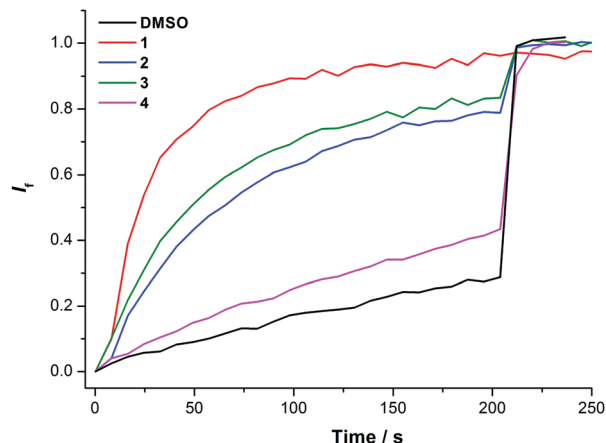


Fig. 5 H^+/Cl^- symport or OH^-/Cl^- antiport facilitated by compounds **1–4** (0.2 mol% compound to lipid) from unilamellar POPC vesicles containing both 1 mM HPTS and 100 mM NMDG-Cl buffered to pH 7.0 with 10 mM HEPES buffer and suspended in an external solution of 100 mM NMDG-Cl buffered to pH 7.0 with 10 mM HEPES buffer. At the endpoint of each experiment (200 s), the detergent was added to lyse the vesicles and collapse the pH gradient for calibration of HPTS fluorescence. Pure DMSO was utilised as a control experiment.

From Table 2, the H^+/Cl^- cotransport (or OH^-/Cl^- antiport) activity of **1–4** followed the same order as that of the Cl^-/NO_3^- exchange. The presence of proton channel gramicidin D did not affect the rate of pH gradient dissipation of all compounds (ESI^+), suggesting that H^+ or OH^- transport is not rate-limiting in this assay (acceleration of H^+ transport by gramicidin D did not improve the overall transport rate).¹⁶ Note that for all compounds the EC_{50} values in the ISE Cl^-/NO_3^- exchange assay is about 10 times the values in the HPTS assay, which is consistent with the fact that the ISE assay requires a higher ion flux (~ 500 mM of Cl^- and NO_3^-) than the HPTS assay (5 mM of Cl^- and H^+ or OH^-) for the transport process to reach thermodynamic equilibrium.¹⁷

The presence of the 1,8-naphthalimide fluorophore within compounds **1–4** allows them to be used for live cell imaging. This was explored using the human lung carcinoma A549 cell line. Fluorescence micrographs were collected after incubation of the cells with compounds **1–4** (1.0 μM) for 24 hours and then free compound was washed away with PBS buffer. Interestingly, the most active transporter **1** exhibited bright green fluorescence within the cytoplasm of A549 cells (Fig. 6), confirming its ability to fluorescently stain the cells. Importantly, compound **1** up to 100 μM exhibited no toxicity to A549 cells as determined using a CCK-8 assay (ESI^+). In comparison, compound **2** showed a poor fluorescent staining of A549 cells even at 10 μM . The second most active transporter **3** demonstrated a marginal fluorescence in A549 cells, which became stronger with increasing concentration; however, a deliverability issue at 10 μM impeded its ability to stain cells at high concentrations, under which conditions precipitation was observed. The least active transporter **4** did not show any detectable cell staining at all concentrations (ESI^+).

In summary, a series of squaramide-based fluorescent anion receptors were synthesized, bearing a 1,8-naphthalimide moiety

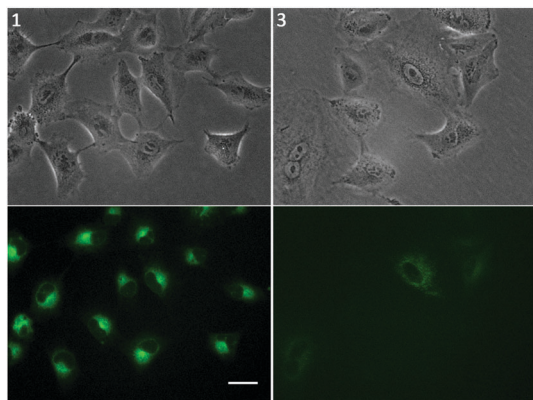


Fig. 6 Fluorescent micrographs of A549 cells after incubation with compounds **1** (1.0 μM , left) and **3** (1.0 μM , right) for 24 hours. The bright-field and fluorescent images were displayed in the upper and lower row, respectively. Scale bar: 25 μm .

as the fluorophore. Their anion binding and transmembrane transport properties towards biologically important chloride ions were investigated. The results indicated that these compounds have moderate to relatively strong affinities towards Cl^- in $\text{DMSO}-d_6$, with the highest affinity of 520 M^{-1} observed for the most acidic compound **1**. Compounds **1–4** facilitate chloride transport into the vesicles *via* $\text{Cl}^-/\text{NO}_3^-$ antiport and Cl^-/H^+ symport (or functionally equivalent Cl^-/OH^- antiport) mechanisms. The relative transmembrane transport activities of these compounds were mainly dictated by their chloride affinities. The most active transporter **1** was found to have the highest efficiency in fluorescent staining of living A549 cells among the squaramides while being nontoxic and possessing substantially higher anion transport activity than previously reported fluorescent anion transporters.⁶ These results represent an important step forward in developing highly active fluorescent transmembrane anion transporters that are useful for studying the sub-cellular localisation and pharmacokinetics of anion transporters in biological systems.

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Conflicts of interest

There are no conflicts to declare.

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