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A potent fluorescent transmembrane HCl transporter perturbs cellular pH and promotes cancer cell death

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A series of fluorescent coumarin bis-ureas **1**–**4** have been synthesised, and their anion transport properties studied. The compounds function as highly potent HCl co-transport agents in lipid bilayer membranes. Single crystal X-ray diffraction of compound **1** showed antiparallel stacking of the coumarin rings, stabilized by hydrogen bonds. Binding studies, using ^{1}H-NMR titration, showed moderate chloride binding in DMSO-d_6/0.5% with 1:1 binding mode (for transporter **1**) and 1:2 binding mode (host: guest, for transporters **2**–**4**). We examined the cytotoxicity of compounds **1**–**4** against three cancer cell lines, lung adenocarcinoma (A549), colon adenocarcinoma (SW620) and breast adenocarcinoma (MCF-7). The most lipophilic transporter **4** showed a cytotoxic effect against all three cancer cell lines. Cellular fluorescence studies showed compound **4** crossed the plasma membrane and localised in the cytoplasm after a short time. Interestingly, compound **4**, lacking any lysosome targeting groups, was co-localised with LysoTracker Red at 4 and 8 h in the lysosome. Cellular anion transport of compound **4** was assessed by measuring intracellular pH and showed a decrease in cellular pH, which may be due to the capacity of transporter **4** to co-transport HCl across biological membranes, as evidenced by the liposomal studies.

Introduction

The transport of ions across phospholipid bilayer membranes in biological systems is crucial in many biological processes, including cell migration and proliferation, maintaining cellular pH, membrane potential, and cellular secretions.[1, 2] Channelopathies are a group of diseases characterised by ion channel impairment, which include cystic fibrosis, epilepsy and cancer.[3-5] For example, the cystic fibrosis transmembrane conductance regulator (CFTR) is a channel in epithelial cell membranes responsible for facilitating the transport of chloride and bicarbonate. Dysfunctional CFTR channels with reduced anion transport cause cystic fibrosis which results in continual lung infections in patients, while impairment of sodium, potassium and T-type calcium channels is linked with epilepsy.[6] Channel replacement therapy has been proposed as a new approach to treating channelopathies in which the function of a faulty channel is replaced by an ionophore that can facilitate the flux of ions through a membrane by forming a lipophilic complex.[7]

A number of anionophores and anion exchangers show anti-cancer activity, including the natural product prodigiosin, squaramide derivatives and *o*-phenylenediamine-based bis-ureas.[8-12] Studies have shown that chloride transporters can trigger apoptosis in cells, whilst compounds that can co-transport H^+/Cl^- can also interfere with autophagy, presumably by deacidifying acidic organelles.[8, 13] However, our understanding of the action of anionophores within cells is still limited, with some studies showing particular classes of compounds exhibit toxicity whilst others show little toxicity but potent anion transport properties.[5, 8, 13, 14]

A previous study based on a series of fluorescent (thio)urea-based anionophores was conducted to further understand where anionophores localise in cells using fluorescence imaging techniques.[15] However, these mono-(thio)urea transporters showed modest anion binding and transport activities.[15, 16] We have conducted another study using squaramide-based

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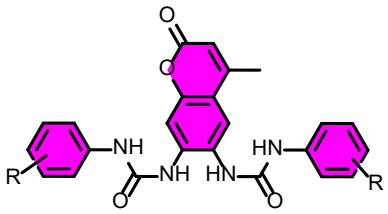


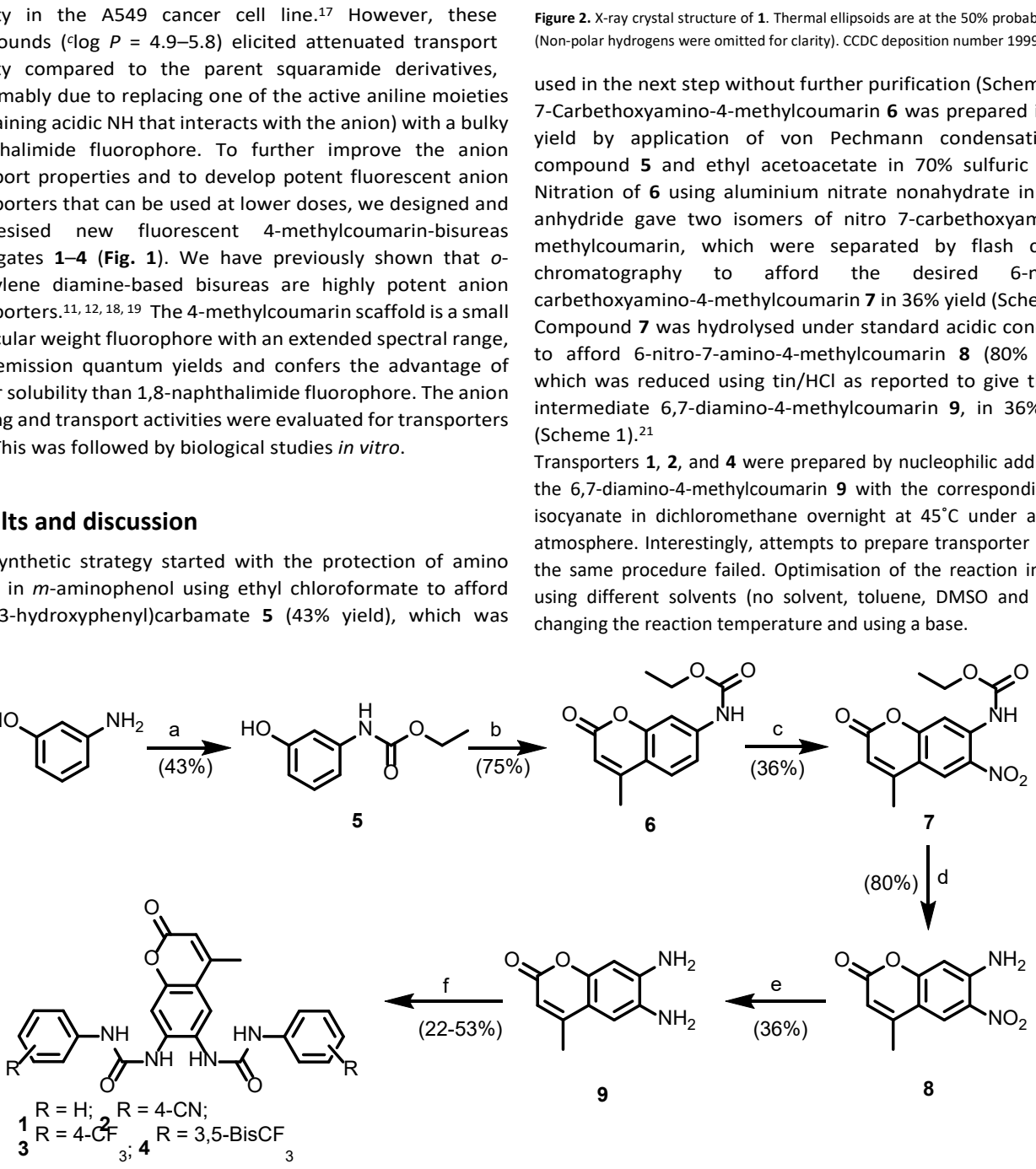
1, R = H
2, R = 4-CN
3, R = 4-CF_3
4, R = 3,5-BisCF_3

Figure 1: Structure of fluorescent transporters **1**–**4**.

fluorescent anionophores to investigate the anion transport activity in the A549 cancer cell line.[17] However, these compounds (^{c}log *P* = 4.9–5.8) elicited attenuated transport activity compared to the parent squaramide derivatives, presumably due to replacing one of the active aniline moieties (containing acidic NH that interacts with the anion) with a bulky naphthalimide fluorophore. To further improve the anion transport properties and to develop potent fluorescent anion transporters that can be used at lower doses, we designed and synthesised new fluorescent 4-methylcoumarin-bisureas conjugates **1**–**4** (**Fig. 1**). We have previously shown that *o*-phenylene diamine-based bisureas are highly potent anion transporters.[11, 12, 18, 19] The 4-methylcoumarin scaffold is a small molecular weight fluorophore with an extended spectral range, high emission quantum yields and confers the advantage of better solubility than 1,8-naphthalimide fluorophore. The anion binding and transport activities were evaluated for transporters **1**–**4**. This was followed by biological studies *in vitro*.

Results and discussion

The synthetic strategy started with the protection of amino group in *m*-aminophenol using ethyl chloroformate to afford ethyl(3-hydroxyphenyl)carbamate **5** (43% yield), which was used in the next step without further purification (Scheme 1).[20] 7-Carbethoxyamino-4-methylcoumarin **6** was prepared in 75% yield by application of von Pechmann condensation of compound **5** and ethyl acetoacetate in 70% sulfuric acid.[20] Nitration of **6** using aluminium nitrate nonahydrate in acetic anhydride gave two isomers of nitro 7-carbethoxyamino-4-methylcoumarin, which were separated by flash column chromatography to afford the desired 6-nitro-7-carbethoxyamino-4-methylcoumarin **7** in 36% yield (Scheme 1). Compound **7** was hydrolysed under standard acidic conditions to afford 6-nitro-7-amino-4-methylcoumarin **8** (80% yield), which was reduced using tin/HCl as reported to give the key intermediate 6,7-diamino-4-methylcoumarin **9**, in 36% yield (Scheme 1).[21]



Transporters **1**, **2**, and **4** were prepared by nucleophilic addition of the 6,7-diamino-4-methylcoumarin **9** with the corresponding aryl isocyanate in dichloromethane overnight at 45˚C under an inert atmosphere. Interestingly, attempts to prepare transporter **3** using the same procedure failed. Optimisation of the reaction involved using different solvents (no solvent, toluene, DMSO and $CHCl_3$), changing the reaction temperature and using a base.

Scheme 1: Ethyl chloroformate/DEE; b) Ethyl acetoacetate, 70% H_2SO_4; c) $Al(NO_3)_3.9\ H2O$, acetic anhydride.; d) Conc H_2SO_4, Glacial acetic acid, reflux.; e) Sn/HCl, reflux.; f) Suitable isocyanate, DCM, 45 °C, or Suitable isocyanate, 45 °C.

Figure 2. X-ray crystal structure of **1**. Thermal ellipsoids are at the 50% probability level. (Non-polar hydrogens were omitted for clarity). CCDC deposition number 1999498.

Table 1. Transport and chloride binding properties of compounds **1**–**4**.

		1	**2**	**3**	**4**
Binding properties	**^{c}Log *P* [a]**	3.67	2.77	4.80	5.96
	1:1 (K_a), DMSO-d_6/0.5% H_2O	81	-	-	-
	covfit [b]	4.6×10^{-4}	-	-	-
	1:2 (K_a), DMSO-d_6/0.5% H_2O	-	K_{11}: 186; K_{12}: 2	K_{11}: 239; K_{12}: 9	K_{11}: 178; K_{12}: 5
	β_{21}[c]	-	372	2.2×10^{3}	890
	Covfit[b]	-	1.8×10^{-4}	2.0×10^{-4}	3.2×10^{-4}
Transport properties	**Cl^-/NO_3^- (EC_{50}, mol%) [d]**	0.43	0.0070	0.0074	0.015
	n[e]	1.2	1.2	1.2	0.93
	KCl (EC_{50}, mol%) [f]	0.038	0.00051	0.00063	0.00097
	n[e]	0.99	1.2	1.2	1.1

[a] ^{c}log *P* values calculated using VCCLab. [b] The covariance of the fit (covfit) is calculated by dividing the covariance of the residual (experimental data – calculated data) with the covariance of the experimental data. [c] The association constant (β_{21}) for the 2:1 and 1:2 host:guest complex calculated by multiplying K_{11} and K_{12} and K_{11} and K_{21}, respectively. [d] EC_{50} from the Cl^-/NO_3^- exchange assay. [e] Hill coefficient (n) as an indicator of the stoichiometry the complex mediating transport. [f] EC_{50} from the KCl assay measuring H^+/Cl^- symport.

The optimal conditions were found by using no solvent and adding an excess of the 4-trifluoromethylphenyl isocyanate under an inert atmosphere. By applying these conditions, transporter **3** was obtained, however, in a low yield (22%).

The structure of compound **1** was confirmed by single crystal X-ray diffraction (**Fig. 2**, see ESI). Single crystals were obtained by slow evaporation of a DMF/ethanol solution of **1** at room temperature. The crystal structure (**Fig. 2**) showed that coumarin rings are stacked on top of each other in an antiparallel manner and stabilised by the intermolecular hydrogen bonds (see ESI (fig. S9) for more details).

The anion binding abilities of putative transporters **1**–**4** in solution were investigated using ^{1}H-NMR titration studies in DMSO-d_6/0.5% H_2O with tetrabutylammonium chloride. The change in chemical shift of the four urea NHs against the equivalents of anion added was fitted globally to 1:1 (for transporter **1**) and 1:2 (host: guest, for transporters **2**–**4**) binding modes using BindFit.[22] The results demonstrate moderate chloride affinity in the range of 81–239 M^{-1} in this highly competitive media, similar to the previously reported *o*-phenylenebisureas. Apparent strong binding affinities were observed for transporters **1**-**4** with tetrabutylammonium dihydrogenphosphate, using 1H NMR titration techniques, however stability constants could not be determined due to peak broadening.

Receptors **1**–**4** were investigated for their chloride transport properties across lipid bilayer *via* liposome-based techniques using a chloride ion selective electrode (ISE) (**Fig. 3a**, **Table 1**). Briefly, unilamellar POPC vesicles with a diameter 200 nm were prepared as reported and loaded with 489 mM KCl, buffered to pH = 7.2 and suspended in 489 Mm KNO_3 solution buffered to pH = 7.2. The chloride efflux, as an indication of Cl^-/NO_3^- exchange process, was measured using ISE upon the addition of DMSO solution of transporters **1**–**4** to the prepared liposomes.[16] Hill plots were performed by monitoring chloride efflux at different concentrations of tested compounds (expressed as mol% with respect to lipid concentration) to calculate EC_{50} (defined as the concentration required to achieve 50% of the chloride efflux at t = 270 s) and Hill coefficient. The EC_{50} is used as a measure of anion transporter potency, while the Hill coefficient has been linked to the stoichiometry of the formed complex during the transport across the lipid bilayer.[23] As illustrated in **Table 1** and **Fig. 3**, the unsubstituted coumarin-bisureas hybrid **1** emerged as the least active transporter with EC_{50} = 0.43 mol%, followed by transporter **4** (EC_{50} = 0.015 mol%) (**Fig. 3d**, **Table 1**). Transporters **2** and **3**, with *p*-CN and *p*-CF_3 substituents, respectively, emerged as the most active transporters with low EC_{50} values of 0.0070 and 0.0074 mol%, respectively. Transporters **2**–**4** (EC_{50} = 0.015 – 0.0074 mol%) (**Fig. 3d**, **Table 1**) showed superior transport activity across the lipid bilayer than the previously reported fluorescent naphthalimide-(thio)ureas with at least 10 times lower EC_{50} values.[15, 17]

By utilising the cationophore coupled-KCl assay,[16] valinomycin (Vln) or monensin (Mon) were used to investigate the mechanism of anion transport of the fluorescent transporters **1**–**4** (**Fig. 3b**–**c**, see ESI). This assay was used to determine whether the anionophore transports only chloride in a uniport process resulting in a net flow of charge across the membrane (an electrogenic transporter), if the anionophore couples to valinomycin, or if the anionophore couples to monensin it shows it is functioning as an H^+/Cl^- co-transporter resulting in an electroneutral transport process (no net flow of charge). If the anionophore couples to both cationophores, it is unselective and can facilitate either chloride uniport or HCl symport (**Fig. 3**).

Compounds **1**–**4** were found to be highly efficient electroneutral H^+/Cl^- co-transporters (**Fig. 3e**, see ESI), while transporter **1** is less selective and can also function as a chloride uniporter. The selectivity for HCl co-transport may be the result of the high affinity of the bis-urea motif for phosphate resulting in strong phospholipid headgroup interactions (which is presumably lower for transporter **1**, which lacks electron-withdrawing substituents).[24]

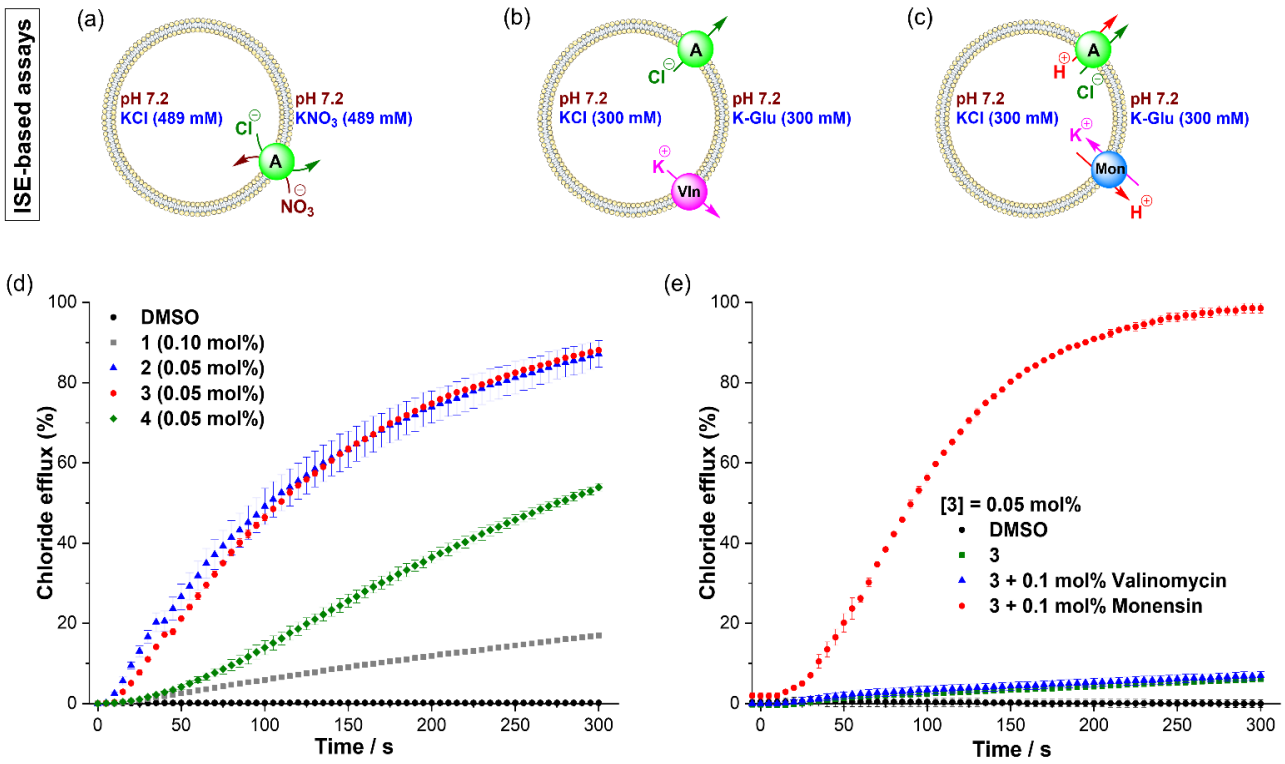


Figure 3. (a–c) Schematic representation of ISE-based assays used to investigate the mechanism of anion transport of receptors **1**–**4** (a) Cl^-/NO_3^- antiport, (b) cationophore coupled-KCl, valinomycin, and (c) monensin to measure the Cl^- uniport and M^+/Cl^- transport, respectively. (d) Chloride efflux achieved by transporters **1**–**4** (0.10 mol% for transporter **1** and 0.05 mol% for transporters **2**–**4**) from unilamellar POPC vesicles containing 489 mM KCl buffered to pH 7.2 with 5 mM potassium phosphate salts, suspended in 489 mM KNO_3 buffered to pH 7.2 with 5 mM phosphate salts. At the endpoint of each experiment (t = 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 at least two trials. DMSO was used as a control experiment (e) Chloride efflux achieved by transporter **3** at 0.05 mol% (rtl) in the absence or presence of cationophores (monensin or valinomycin) monitored over a period of 5 min (see ESI for more further details).

Broadening of the NH resonances of the bis-ureas occurred upon addition of tetrabutylammonium dihydrogenphosphate, which precluded the determination of stability constants in DMSO-d_6/0.5% H_2O solution. However, the titrations appear to saturate upon addition of one equivalent of the added anion consistent with strong complex formation.

The unsubstituted bisurea-based fluorescent receptor **1** was found to have the lowest activity in HPTS-KCl (HCl symport, **Fig. S23**) with EC_{50} `= 0.038 mol%. Appending electron-withdrawing groups increased the ability to dissipate the pH gradient, with EC_{50} = 0.00051 - 0.00097 mol% (**Table 1**). The addition of the K^+ transporter valinomycin or proton transporter CCCP did not significantly affect the transport rates of **1**–**4** in the HPTS assay, consistent with the inability of these compounds to facilitate uniport processes (**Fig. 4**).

The transport of fatty acid carboxylates across lipid bilayer membranes by anionophores can result in pH dissipation as the transported carboxylate protonates and then diffuses back across the bilayer and deprotonates. We used oleic acid (1.0 mol%) as a source of fatty acid and BSA (bovine serum albumin) to sequester fatty acids from liposomes in HPTS-KCl (HCl symport) assay to investigate whether the fatty acids could play a role in the pH dissipation facilitated by receptors **1**–**4**. The addition of fatty acid lowered the transport activity of tested compounds **1**–**4**, presumably due to their ability to competitively bind to the carboxylate headgroups, as previously reported[11], thus lowering the chloride transport activity. BSA-

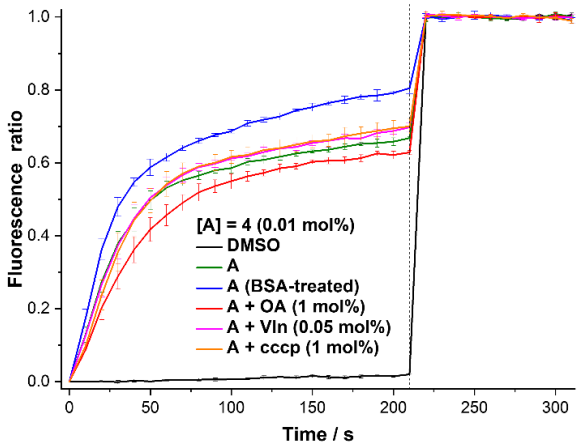


Figure 4. Using KCl-KOH assay from POPC vesicles loaded with KCl (100 mM), buffered to pH 7.0 with HEPES (10 mM), different conditions were applied including using BSA-treated lipid, addition of oleic acid at 1.0 mol%, addition of the protonophore CCCP at 1.0 mol%, or addition of valinomycin at 0.05 mol%, on the rate of chloride transport of receptor 4 (0.01 mol%).

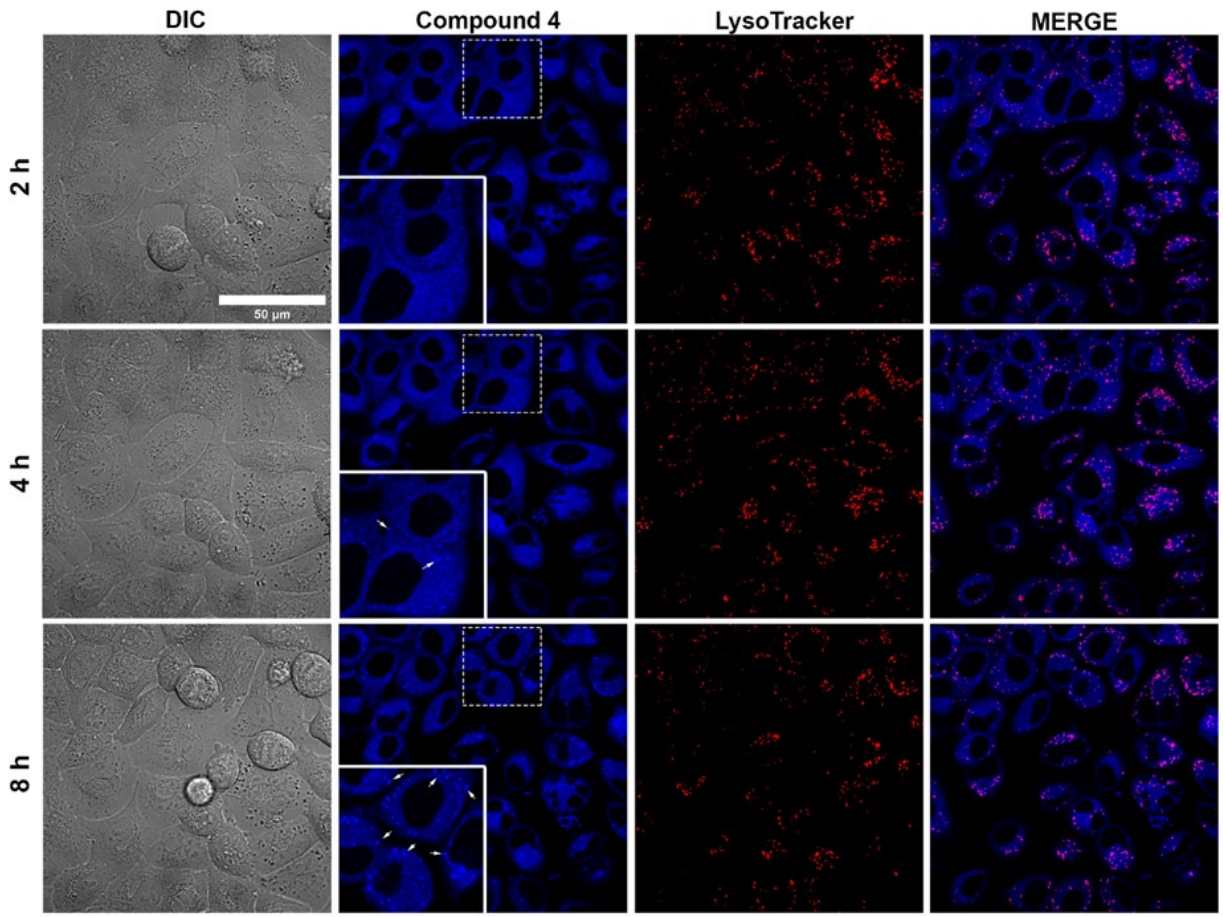


Figure 5. Intracellular localisation of compound **4** (in blue) in the A549 cell line. Different images were taken every 30 min during the whole experiment and at the same position. Compound **4** (IC_{75}) and LysoTracker Red (50 nM) (in red) localisation were evaluated up to 8 h of incubation. Inserts (1.75X zoom) show in detail the accumulation of compound **4** in dots resembling lysosomes. In merge images, we can confirm that compound **4** is co-localised with LysoTracker Red. DIC = differential interference contrast.

treated liposomes were used to remove all fatty acids from the HPTS-KCl liposomes. The transport activity of the fluorescent receptors **1**–**3** was not affected by fatty acid removal, suggesting that these transporters independently could transport HCl or protons. However, transporter **4** showed an increase in the pH dissipation, which might indicate that the activity of this particular receptor is greatly compromised by the presence of fatty acids.

The cell viability effects of these compounds were assessed in three human cancer cell lines: lung adenocarcinoma (A549), colon adenocarcinoma (SW620) and breast adenocarcinoma (MCF-7) using the MTT assay. Of the four compounds assayed surprisingly, only the most lipophilic, compound **4**, showed significant cytotoxic activity against all tested cell lines. Thus, the inhibitory concentrations at 50% (IC_{50}) observed were: the lowest for SW620 cells (IC_{50} = 0.51 ± 0.07 µM) followed by A549 cells (IC_{50} = 1.86 ± 0.39 µM) and MCF-7 cells (IC_{50} = 17.92 ± 4.25 µM) (ESI **Table S1**). This study showed for the first time coumarin-bisurea compounds may possess cytotoxic effects. Then, to evaluate the subcellular localisation of this compound, firstly a lambda scan analysis was performed, showing that compound **4** exhibited the highest excitation after irradiation with a 405 nm laser, obtaining the highest emission at around 520 nm. There was no excitation with the other lasers (488, 561 and 633 nm) (ESI **Figure S33**). Secondly, confocal microscopy images showed that compound **4** was predominantly present in the cytoplasm early in the experiment but after 4 h of incubation slight accumulations (small and more bright dots) started to occur. To evaluate if these accumulations were localised in lysosomes, a Lysotracker Red staining was used. This tracker has a selective localisation with lysosomes based on their pH. These accumulations were co-localised with LysoTracker Red at 4 and 8 h (**Fig. 5**), confirming that this compound accumulates in lysosomes.

Finally, to demonstrate the capacity of compound **4** to act as an anionophore/ionophore across the lysosomal membrane, anion transport within cells was assessed by measuring intracellular pH using pH Rodo Red AM Staining kit. Upon treatment with compound **4** (IC_{75}) intracellular pH dropped significantly. A decrease in Δ= −0.2 and Δ= −0.35 pH units were observed after 1 and 8 h of treatment, respectively (**Fig. 6a**). These results were confirmed by confocal microscopy, observing an enhanced red fluorescence after 1 h and, and a more significantly enhanced fluorescence after 8 h of treatment, indicating a critical decrease in intracellular pH (**Fig. 6b**). This intracellular

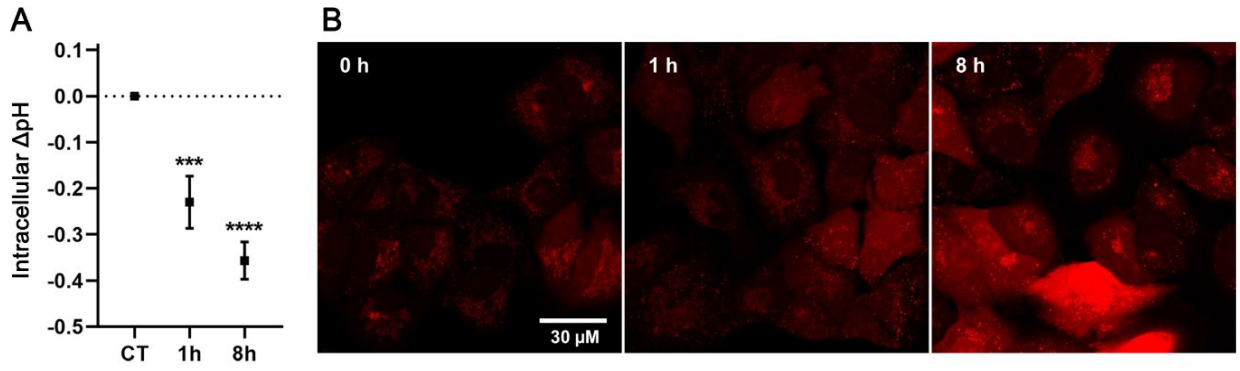


Figure 6. Intracellular pH modifications produced by compound **4**. (A). Intracellular pH quantification after treatment with IC_{75} of compound **4** for 1 and 8 h in A549 cells. pH Rodo Red AM staining kit was used. **Figure 6** shows mean ± SEM. Statistical differences against control non-treated group (CT) are shown as ***p < 0.001 and ****p < 0.0001. (B). After treatment with IC_{75} of compound **4** for 1 h and 8 h, cells were stained with pH Rodo Red AM staining kit and red fluorescence was visualized by confocal microscopy. Acidification of intracellular pH could be identified by the enhanced fluorescence intensity. Images were captured from Carl Zeiss LSM 880 spectral confocal laser scanning microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and data were managed with ZEN 2 Blue edition software (Zeiss).

acidification is in accordance with previous reports using other anion transporter compounds (**7**).[14]

Conclusions

In this study we have prepared fluorescent analogues of *o*-phenylene bis-urea anion transporters containing 4-methyl coumarin. As with the non-fluorescent parent compounds, these bis-ureas are capable of binding and transporting chloride across lipid bilayer membranes at low concentrations. Studies in cancer cell lines shows that the bis-ureas facilitate similar organelle deacidification processes as the parent compounds resulting in a reduction in intracellular pH.[5d] Interesting intracellular localisation studies show that compound **4** localises in lysosomes. Targeting anionophores to specific subcellular compartments may offer an additional degree of control of the effects of these compounds on cells. We are currently exploring this aspect of the chemistry of synthetic anion transporters.

Conflicts of interest

There are no conflicts to declare.

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