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Synthesis and biological effect of lysosome-targeting fluorescent anion transporters with enhanced anionophoric activity

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ABSTRACT

Two lysosome-targeting fluorescent anion transporters derived from coumarins, trifluoromethylated arylsquaramides and morpholines were synthesized, and their specificity and efficiency to target and alkalize lysosomes were investigated. They are able to target lysosomes specifically. Compared with the previous analogue without trifluoromethyl substituents, these two conjugates, in particular the one having a 3,5-bis(trifluoromethyl) substituent, exhibit significantly higher ability to facilitate the transport of chloride anions, alkalize lysosomes and reduce the activity of lysosomal Cathepsin B enzyme. The present finding suggests that improving the anionophoric activity of lysosome-targeting fluorescent anion transporters is favorable to the efficiency to alkalize lysosomes and deactivate lysosomal Cathepsin B enzyme.

During the past years, increasing interest has been attracted in identifying small-molecule anion transporters that are able to destroy the homeostasis of anions, in particular chloride anions [1-4]. Because the homeostasis of cellular anions provides the basis for cells to exert their function, synthetic anion transporters may be developed not only as model systems to study natural anion channels and transporters, but also as potential candidates for the discovery of chemotherapeutic agents for the treatment of cancers and infections [5-8]. Mechanistic study suggests that anion transporters may exert their biological effect by alkalizing acidic organelles, such as lysosomes [9-17]. For example, Gale *et al* have demonstrated that squaramide-based anion transporters are able to trigger cancer cell apoptosis by deacidifying lysosomes, inhibiting Cathepsin B enzyme activity and disturbing autophagy [18]. Quesada *et al* have shown that tambjamine analogues provoke an imbalance in the homeostasis of cellular anions and the disruption triggers lysosomal deacidification leading to a potent cytotoxic effect through necrosis in lung cancer cell lines and cancer stem cells [19]. We have also demonstrated that anion transport-active iridium complexes are able to alkalize lysosomes and deactivate lysosomal Cathepsin B enzyme, thus leading to an inhibition in the autophagic flux [20].

Inspired by these findings, we reasoned that an anion transporter equipped with a lysosome-targeting group might serve as a useful research tool to investigate the function of anion transporters within cells. However, few lysosome-targeting anion transporters have been explored to date [21]. In a previous study we have described a lysosome-targeting fluorescent ionophore, i.e., compound **1** (Fig. 1) [22]. This compound consists of a squaramido group for facilitating the transport of anions, a morpholinyl group for targeting lysosomes and a coumarinyl group for reporting fluorescence. We have demonstrated that compared with the analogue without a morpholinyl group (i.e., compound **2**, Fig. 1), compound **1** exhibits much higher ability to specifically target and efficiently alkalize lysosomes. This result suggests that molecular skeletons such as compound **1** have high potentials in constructing lysosome-targeting anion transporters.

However, the ionophoric activity of compound **1** was so weak that efficient lysosomal alkalization was observed only at relatively high concentrations. To ameliorate this, we designed two lysosome-targeting fluorescent anionophores based on trifluoromethylated arylsquaramides, that is, compounds **3** and **4** (Fig. 1). This design was grounded by previous findings that introducing electron-withdrawing substituents, such as trifluoromethyl groups into the squaramido subunits is favourable to the anion transport efficiency of squaramide-based anionophores [18, 23-28] and *N*-alkylmorpholine has been frequently used as a lysosome-targeting functional group [21].

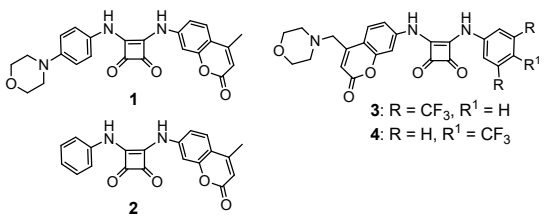
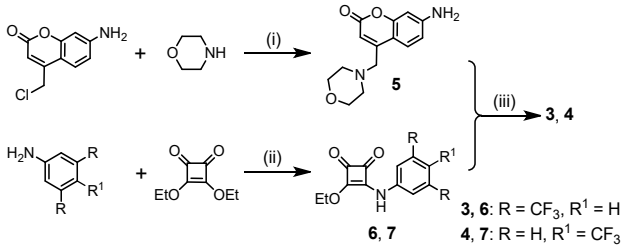


Fig. 1. Structures of compounds **1**-**4**.

Synthesis. Compounds **3** and **4** were synthesized according to the route shown in Scheme 1. Reflux of 7-amino-4-chloromethyl coumarin with morpholine in a mixture of acetonitrile and ethanol (to give compound **5**) and subsequent reflux with compound **6** or **7** in a mixture of DMF and toluene in the presence of zinc trifluoromethanesulfonate [$Zn(CF_3SO_3)_2$] afforded compounds **3** (80 %) and **4** (18 %), respectively. Compounds **6** and **7** were prepared

acco... ...nion compounds **3-5** were confirmed on basis of ESI MS (LR and HR) and NMR (^{1}H and ^{13}C) data (see experimental section and Fig. S1-S12 in the supporting information).



Scheme 1. Synthesis of compounds **3** and **4**. Reagents and conditions: (i) EtOH/CH_3CN (1/1, v/v), reflux; (ii) $Zn(CF_3SO_3)_2$, EtOH, rt to reflux; (iii) $Zn(CF_3SO_3)_2$, DMF/$C_6H_5CH_3$ (1/19, v/v), reflux.

Anion transport. To evaluate the anion transport efficiency of compounds **3** and **4** relative to compounds **1** and **2**, we measured their anionophoric activity by monitoring the efflux of chloride anions out of egg-yolk L-α-phosphatidylcholine (EYPC) vesicles (100 nm, extrusion) using chloride ion selective electrode techniques [31]. As shown in Fig. 2a and Fig. S13, upon the addition of 0.5 mol% of compounds **1**, **2** and **4**, almost no or slight increase in the concentration of extravesicular chloride anions was detected, suggesting that these compounds are inactive in facilitating the efflux of chloride anions out of the EYPC vesicles. In contrast, addition of compound **3** led to a significant efflux of chloride anions out of the EYPC vesicles, suggesting that it is very active in facilitating the transmembrane transport of chloride anions. This result indicates that compound **3** exhibits much higher anionophoric activity than compounds **1**, **2** and **4**. Quantitative characterization on the anion transport efficiency of these compounds based on pH discharge experiments revealed that the EC_{50} value is $(9.09\pm3.55)\times10^{-3}$ mol% for compound **3** and 0.11±0.01 mol% for compound **4**, respectively (Fig. S14). Because of the poor solubility (as well as the low activity), the EC_{50} value of compounds **1** and **2** could not be measured and was estimated to be significantly greater than 1.0 mol% [22]. This result suggests that compounds **3** and **4** exhibit at least 100- and 10-fold higher anionophoric activity than compound **1**, respectively. This enhanced activity is considered as a consequence of the increased lipophilicity induced by the trifluoromethyl substituents (*clogP* = 2.63 for compound **1**, 3.71 for compound **3** and 2.83 for compound **4**, respectively) [32-35].

transport properties by measuring the efflux of chloride anions in the presence of different alkali metal ions or anions (Fig. 2b-c). The observation that the anions rather than the alkali metal cations are able to regulate the efflux of chloride anions, suggests that compound **3** facilitates the transport of chloride anions *via* an anion-exchange process [17,31].

Lysosome-targeting specificity. The lysosome-targeting specificity of compounds **3** and **4** was studied by comparing their co-localization with that of a specific lysosome-targeting agent, such as commercially avaible LysoTracker® [36]. Because compounds **3** and **4** emit strong blue fluorescence at around 480 nm (Fig. S15) [37], we chose LysoTracker® Deep Red with the maximum excitation/emission wavelengths at 577/590 nm, to study the specificity of compounds **3** and **4** to target lysosomes. It can be seen from Fig. 3 that the blue fluorescence of compounds **3** and **4** overlaps well with the red fluorescence of LysoTracker® Deep Red. The Pearson's co-localization coefficient Rr is 0.84 for compound **3** and 0.89 for compound **4**, respectively, very similar with that of compound **1** (Rr = 0.86) [22]. This result clearly suggests that both compounds **3** and **4** exhibit high specificity to target lysosomes. Given the fact that compound **2** exhibits much lower ability to localize in lysosomes (Rr = 0.49) [22], the high lysosome-targeting specificity of compounds **1**, **3** and **4** may be ascribed to the presence of a morpholinyl group in the structures.

Alkalization of acidic organelles. As compounds **3** and **4** exhibit high lysosome-targeting ability and anionophoric activity, we conducted an acridine orange (AO) co-staining to explore their ability to alkalize acidic organelles in HeLa cells. It is reported that AO emits orange fluorescence in acidic organelles and green fluorescence in alkaline environments [38]. As shown in Fig. 4, treatment of the HeLa cells with compound **1**, **3** or **4** led to concentration-dependent disappearance in the orange fluorescence of AO and an increase in the green fluorescence, suggesting that compounds **1**, **3** and **4** are able to increase the pH of intracellular acidic organelles. Quantitative analysis based on the pixel intensity ratio of the red fluorescence of AO to the green fluorescence indicates that their efficiency is quite different (Fig. S16). For example, at the concentration of 5.0 μM, this ratio relative to the background was 0.69 for compound **1**, 0.08 for compound **3** and 0.52 for compound **4**, respectively, which reveals their ability to alkalize acidic organelles in the order of compound **3** > **4** > **1**. This is consistent with their anionophoric activity on the liposome models.

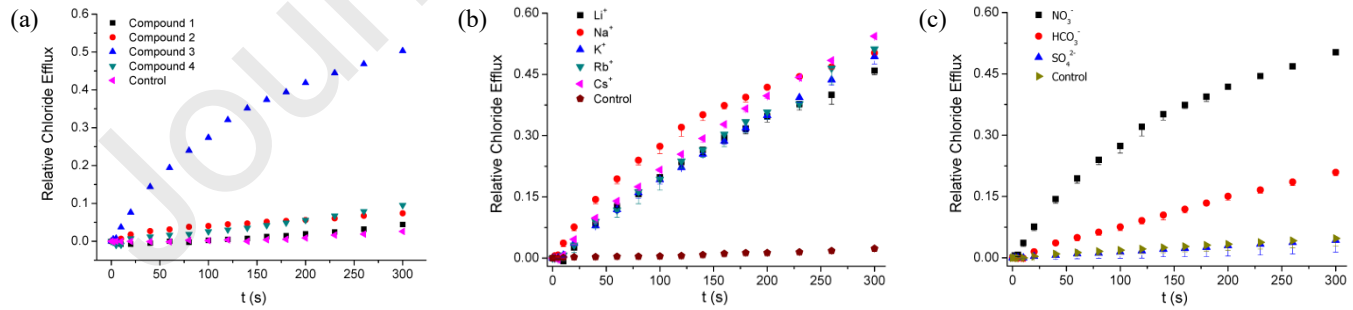


Fig. 2. (a) Relative chloride efflux out of EYPC liposomes enhanced by 0.5 mol% of compounds **1**-**4**, under the conditions of an intravesicular 500 mM NaCl solution (25 mM HEPES buffer, pH 7.0) and extravesicular 500 mM $NaNO_3$ solution (25 mM HEPES buffer, pH 7.0). (b, c) Relative chloride efflux out of EYPC liposomes enhanced by compound **3** (0.5 mol%), under the conditions for (b): an intravesicular 500 mM MCl solution (25 mM HEPES buffer, pH 7.0) and extravesicular 500 mM MNO_3 solution (25 mM HEPES buffer, pH 7.0) (M = Li, Na, K, Rb or Cs); for (c): an intravesicular 500 mM NaCl solution (25 mM HEPES buffer, pH 7.0) and extravesicular 500 mM $NaNO_3$, 500 mM $NaHCO_3$ or 250 mM Na_2SO_4 solution (25 mM HEPES buffer, pH 7.0).

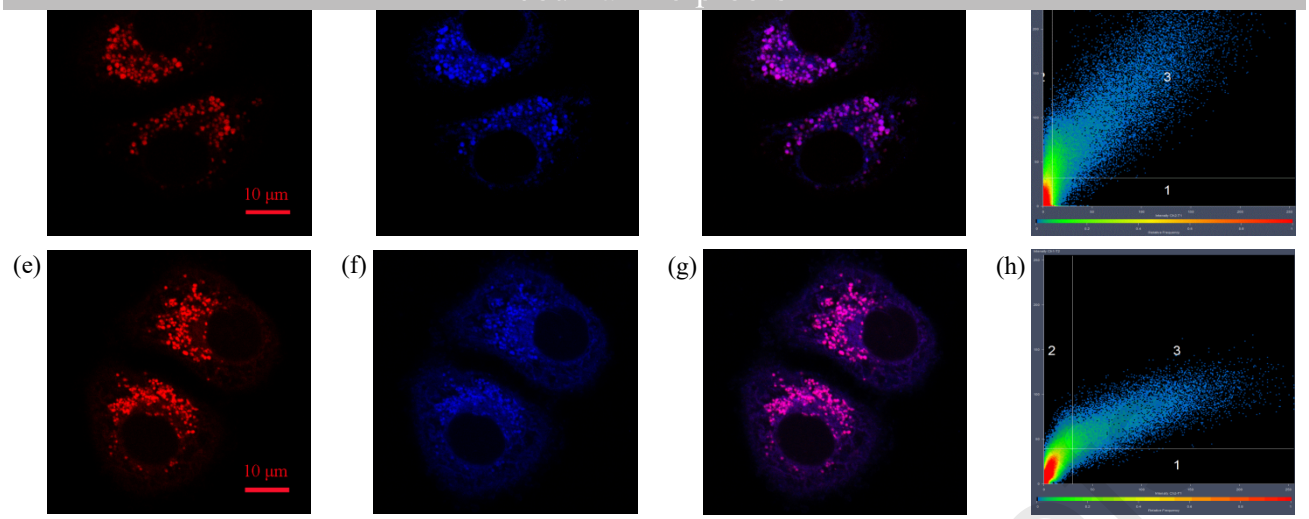


Fig. 3. Confocal imaging of HeLa cells by (a, e) LysoTracker™ Deep Red (1.0 μM) and (b, f) 20 μM of compound **3** (b) or **4** (f) for 6 h. (c) and (g) were merged from (a)/(b) and (e)/(f), respectively; (d, h) Fluorescent inetensity correlation plots. The Pearson's coefficient Rr is 0.84 for compound **3** and 0.89 for compound **4**, respectively. Blue fluorescence: λ_{ex} 405 nm, λ_{em} BP 425-470 nm; Red fluorescence: λ_{ex} 561 nm, λ_{em} BP 575-640 nm.

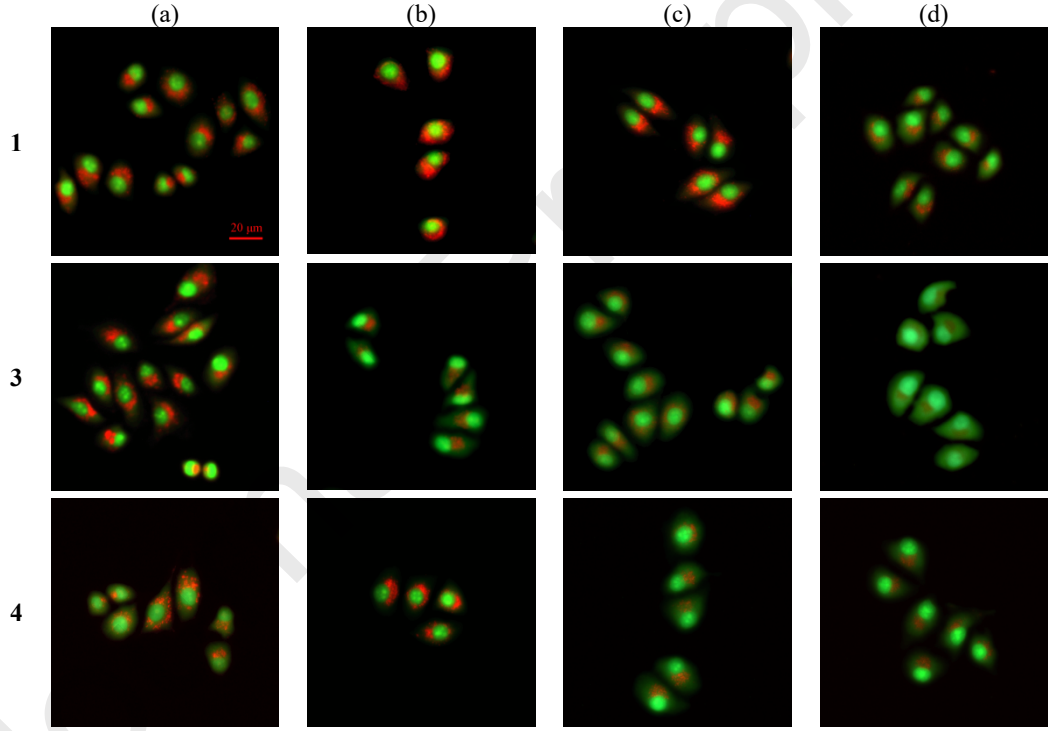


Fig. 4. AO staining of (a) untreated HeLa cells and (b,c,d) HeLa cells treated with compounds **1**, **3** and **4** of 1.25 μM (b), 2.5 μM (c) and 5.0 μM (d) for 4 h, respectively. Green fluorescence: λ_{ex} BP 470/40 nm, λ_{em} BP 525/50 nm; Red fluorescence: λ_{ex} BP 546/12 nm, λ_{em} BP 575-640 nm.

Alkalization of lysosomes. The above-mentioned findings that compounds **3** and **4** exhibit high ability to target lysosomes specifically and alkalize acidic organelles efficiently, make us reasoning that they should be able to alkalinize lysosomes. To evaluate this, we carried out the co-staining experiments of compounds **3** and **4** with commercially available LysoSensor Green DND-189, a pH-sensitive fluorescent probe that specifically targets lysosomes [39]. As showed in Fig. 5 and Fig. S16-S17, treatment of the HeLa cells with compound **1**, **3** or **4** led to a concentration-dependent reduction in the green fluorescence, indicative of an increase in the lysosomal pH. This result indicates that compounds **1**, **3** and **4** are able to alkalize lysosomes. Quantitative analysis of the green fluorescent intensity relative to the control reveals the reduction of the green fluorescence in the order of compound **3** > **4** > **1** suggest that compound **3** exhibits the strongest ability to alkalize lysosomes and we believe is ascribed to its highest anionophoric activity.

Effect on the activity of Cathepsin B enzyme. It is known that lysosomes contain a variety of degrading enzymes that are able to break down carbohydrates, nucleic acids, lipids and proteins. [21] The enzymes in lysosomes, such as Cathepsin B enzyme, exert the highest biological activity in an acidic environment (pH 4.5-5), and lysosomal alkalization leads to a decrease or deactivation of the

activ... This result suggests that the activity of Cathepsin B enzyme decreases and compounds **1**, **3** and **4** are able to change the lysosomal pH. Quantitative analysis of the red fluorescent intensity relative to the control indicates that at the tested concentration (5 μM), compound **3** weakened the red fluorescence more significantly than compounds **1** and **4**. This result suggests that compound **3** is the strongest in deactivating Cathepsin B enzyme.

ability of compounds **1**, **3** and **4** to increase the lysosomal pH, we tested the effect of these conjugates on the activity of Cathepsin B enzyme using Magic Red Cathepsin assays. In this assay, when the Magic Red® MR-$(RR)_2$ Cathepsin B substrate, a cell-permeable, non-fluorescent probe, is hydrolyzed by lysosomal Cathepsin B enzymes, the two R-R peptide sequences are cleaved from the Magic Red molecule and as a consequence red fluorescence emits [20]. As shown in Fig. 6 and Fig. S20-21, treatment of the HeLa cells with

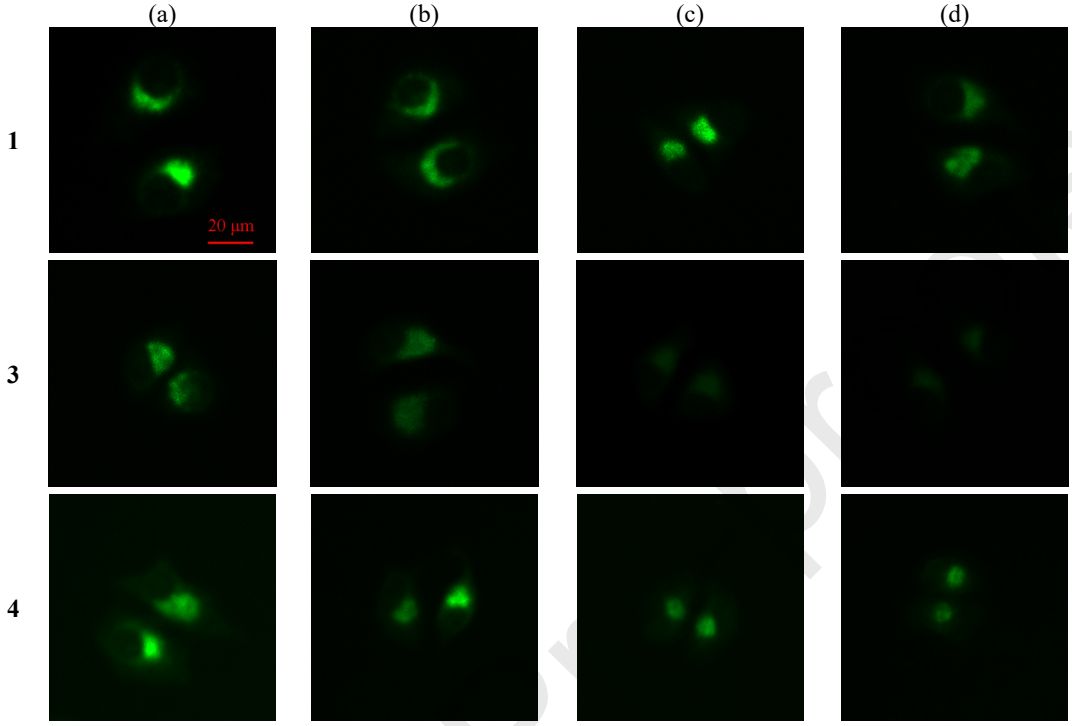


Fig. 5. LysoSensor Green DND-189 (1.0 μM, 1 h) staining of (a) untreated HeLa cells and (b,c,d) HeLa cells treated with compounds **1**, **3** and **4** of 1.25 μM (b), 2.5 μM (c) and 5.0 μM (d) for 4 h, respectively. Green fluorescence: λ_{ex} BP 470/40 nm, λ_{em} BP 525/50 nm.

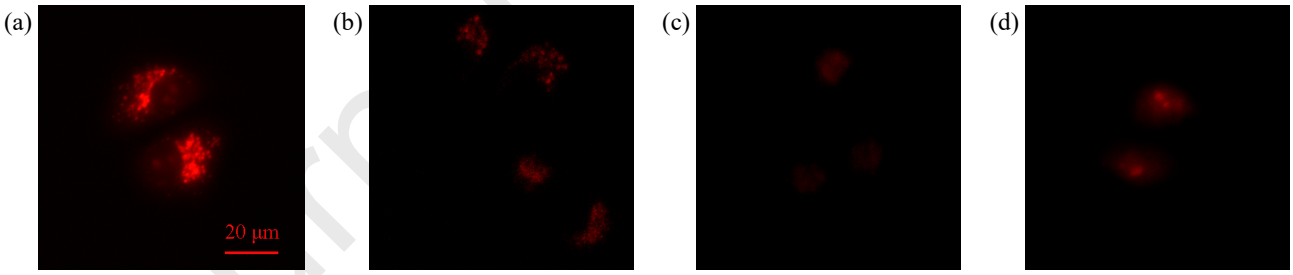


Fig. 6. HeLa cells pretreated with 5 μM of compounds **1** (b), **3** (c) and **4** (d) for 4 h, respectively, and then incubated with MR-$(RR)_2$ for 1 h. (a) Untreated HeLa cells. Red fluorescence: λ_{ex} BP 546/12 nm, λ_{em} BP 575-640 nm.

In summary, we have successfully synthesized two lysosome-targeting fluorescent anion transporters derived from coumarins, trifluoromethylated arylsquaramides and morpholines, and investigated their specificity and efficiency to target and alkalize lysosomes. The results have shown that these two conjugates, in particular the one having 3,5-bis(trifluoromethyl)phenyl substituents, exhibit highly enhanced anion transport activity and are able to specifically target lysosomes, efficiently alkalize lysosomes and significantly deactivate Cathepsin B enzyme. The present finding strongly suggests that enhancing the anion transport activity of a lysosome-targeting anionophore is favourable to the efficiency to alkalize lysosomes and deactivate Cathepsin B enzyme. Further efforts aimed at exploring the potential biological activity are under progress. The outcome will be reported in due course.

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Supplementary Material

Supplementary data (experimental procedures and data for the synthesis, anion recognition, anion transport and biological activity of each compound) associated with this article can be found, in the online version, at http:// dx.doi.org/….

Graphical Abstract

Synthesis and biological effect of lysosome-targeting fluorescent anion transporters with enhanced anionophoric activity

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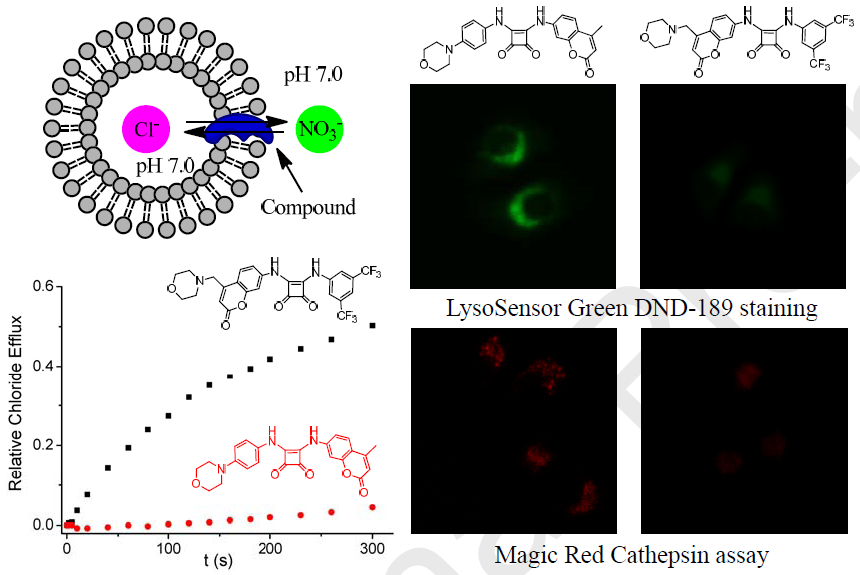

Trifluoromethylated lysosome-targeting fluorescent anion transporters exhibited high specificity and efficiency to target and alkalize lysosomes.