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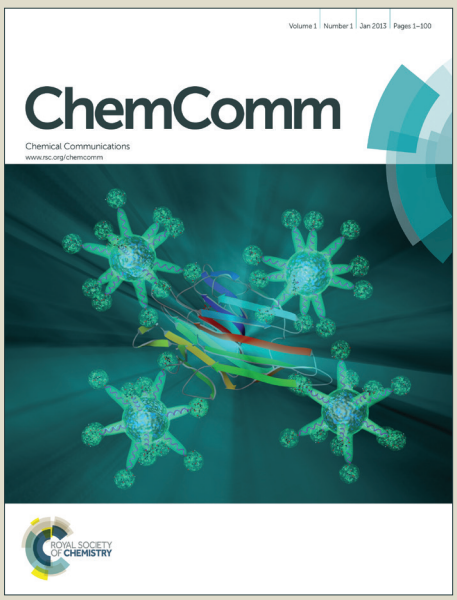

ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

Azobenzene-based chloride transporters with light-controllable activities†

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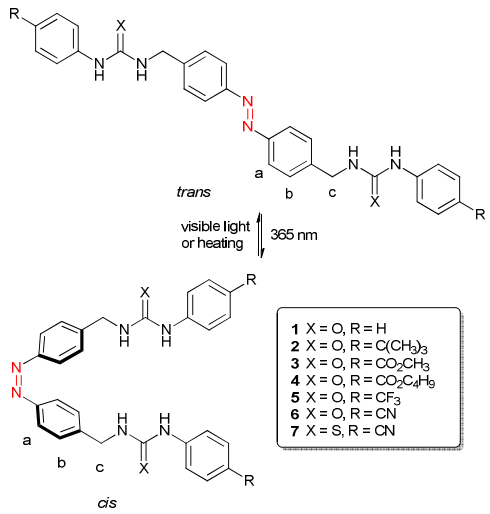
Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Synthetic chloride transporters containing two urea groups linked through a diazobenzene spacer have been prepared and the *trans*-to-*cis* isomerization by light stimulation results in dramatic changes in the chloride transport activities across lipid and cell membranes.

A variety of synthetic molecules that facilitate chloride transport across lipid membranes by carrier or channel mechanism have been reported in recent years.[1,2] In particular, small carrier molecules are attractive due to the therapeutic advantages in the treatment of diseases related to defective chloride transport.[3] A unique feature of natural ion channels or transporters is that their action can be precisely controlled by biological stimuli. However, synthetic anion transporters that show stimuli-responsive transport activities are extremely rare.[4,5] Herein, we describe for the first time synthetic transporters that exhibit the photoresponsive activities of transporting chloride ions across lipid and plasma membranes. The transporters consist of two urea binding sites linked through a diazobenzene spacer that can adopt either *trans* or *cis* conformation in response to light stimulation. The *cis* isomers are proven to be active mobile carriers for chloride ions while the *trans* isomers are all ineffective.

As a light-responsible unit, diazobenzene scaffold[6] is chosen because the *trans*-to-*cis* isomerization gives rise to dramatic changes in the binding site and molecular shape of the transporters. For this study, a series of diazobenzene-based diurea compounds **1**-**7** have been first prepared and synthetic details are described in the Supporting Information. Under ambient conditions, all of the azo compounds prepared here existed in the thermally stable *trans* isomers (> 98%) in the dark. Irradiation with ultraviolet light (365 nm, 5 min) led to the corresponding *cis* isomers as major components (90-96%) based on the ^{1}H NMR (400 MHz) integrations in DMSO-d_6 (Table S1). Upon *trans*-to-*cis* isomerization, ^{1}H NMR signals for CH protons on the internal diazophenyl ring were characteristically shifted upfield ($\Delta\delta$ = 0.60 ~ 0.72) due to the ring current of adjacent diazophenyl rings while the CH signals on the terminal phenyl rings remain nearly constant (Fig. S1-9†). In addition, upon UV irradiation (30 sec) of azo compounds, a π-π* absorption band around 340 nm was reduced significantly but an n-π* band around 440 nm increased (Fig. S1-9†) as a result of the *trans*-to-*cis* isomerization.

The association constants (K_a's) between azo compounds and chloride ion were determined by ^{1}H NMR titrations at 24 ± 1 °C in 10% (v/v) DMSO-d_6/$CDCl_3$ saturated with water (<0.1%).[7] The association constants were calculated by nonlinear squares fitting[8] of the titration curves plotting the downfield shifts of two urea NH signals against the concentration of added tetrabutylammonium chloride (Fig. S10-23†). The results are summarized in Table 1. Trends are apparent. First, the *cis* isomers show larger association constants than the corresponding *trans* isomers by approximately one order of magnitude. This is anticipated because four convergent NH protons in the *cis* isomers can participate in the hydrogen bonding with a chloride ion in a chelative manner but such a binding mode is not possible in the *trans* isomers. According to Job's plots, the *cis* isomers formed 1:1 complexes with chloride ion (Fig. S10-23†).[9] Second, electron-withdrawing substituents (CN, CF_3, CO_2R) at the *para* positions of terminal phenyl rings increase the binding affinities as anticipated based on the enhanced hydrogen bond donor ability of the aromatic urea NH proton. In addition, the association constant of thiourea **7** was slightly larger than that of the corresponding urea **6**. Third, relative affinities of some anions were revealed using *cis*-**2** as a representative compound and the association constants (K_a's) were determined to increase in the order of HSO_4^- (410 M^{-1}) > Cl^- (310 M^{-1}) > Br^- (170 M^{-1}) > NO_3^- (76 M^{-1}) > I^- (40 M^{-1}) (Fig. S24†).



Scheme 1. Molecular structures of compounds **1**-**7**.

Table 1. Association constants (K_a's, M^{-1})[a] between compounds **1-9** and tetrabutylammonium chloride, EC_{50} values (mol% to lipid)[b] at 300 sec and Hill coefficients (n, mol% to lipid)[b] for compounds **1-9**.

Compound	*trans*	*cis*		
	K_a	K_a	EC_{50}	n
1	–[c]	–[c]	5.34	1.59
2	34	310	–[d]	–[d]
3	–[c]	–[c]	4.62	1.24
4	140	1000	–[d]	–[d]
5	260	1300	2.35	1.08
6	410	2800	1.13	1.07
7	610	5900	0.19	1.48
8	570	8400	0.38	0.90
9	820	$> 10^4$	0.15	1.22

[a] Association constants K_a (M^{-1}) were measured in 10% (v/v) DMSO-d_6/$CDCl_3$ saturated with water (< 0.1%) at 24 ± 1 °C and errors were within 15%. [b] Determined using Origin 8.0 (Fig. S27-33†). [c] Not determined due to low solubility. [d] Not determined due to low activities.

The chloride transport activities of *trans*- and *cis*-azo compounds **1-7** were measured using unilamellar 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) vesicles containing sodium chloride (500 mM in 5 mM phosphate buffer at pH = 7.2). The vesicles were suspended in an isotonic sodium nitrate solution and the chloride efflux mediated by each compound (2 mol% to lipid) was monitored using a chloride selective electrode. As shown in Fig. 1a and 1b, the transport activities of *trans* isomers are all negligible but the *cis* isomers exhibit moderate to high activities of transporting chloride ions across a POPC membrane. As mentioned, the *cis* isomers bind chloride ion more strongly than the *trans* isomers, which is in part responsible for differences in the transport activities between *trans* and *cis* isomers. In addition, the *trans* isomer has a planar, extended structure while the *cis* isomer adopts a nonplanar, concave conformation with a relatively large dipole moment (~ 3D) around the diazo bond.[10] These structural features could affect the partitioning and shuttling rate of the isomers, both of which are important variables to determine the transport activity. Furthermore, chloride ion can be entrapped in the middle of the binding site surrounded by four phenyl planes in the *cis* isomer. This may decrease the contact area of the bound chloride ion with the lipid surface, which may speed up the shuttling movement of the complex in the lipid bilayer. It was demonstrated by Davis and Judd that a cholaphane with a more enclosed binding site showed the increased activity of chloride transport across a lipid membrane compared to acyclic analogues.[11]

The relative transport activities of *cis* isomers have been quantitatively compared by Hill analyses based on the EC_{50} values that correspond to the concentrations needed to achieve 50% chloride transport in 300 sec. As summarized in Table 1, the activities of the *cis* isomers strongly depend on the nature of substituents at the *para* positions of the terminal phenyl rings. For example, the EC_{50} values (mol% to lipid) decrease in the order of R = H (5.34) > CO_2CH_3 (4.62) > CF_3 (2.35) > urea-CN (1.13) > thiourea-CN (0.19), which nicely correlates to the trend of

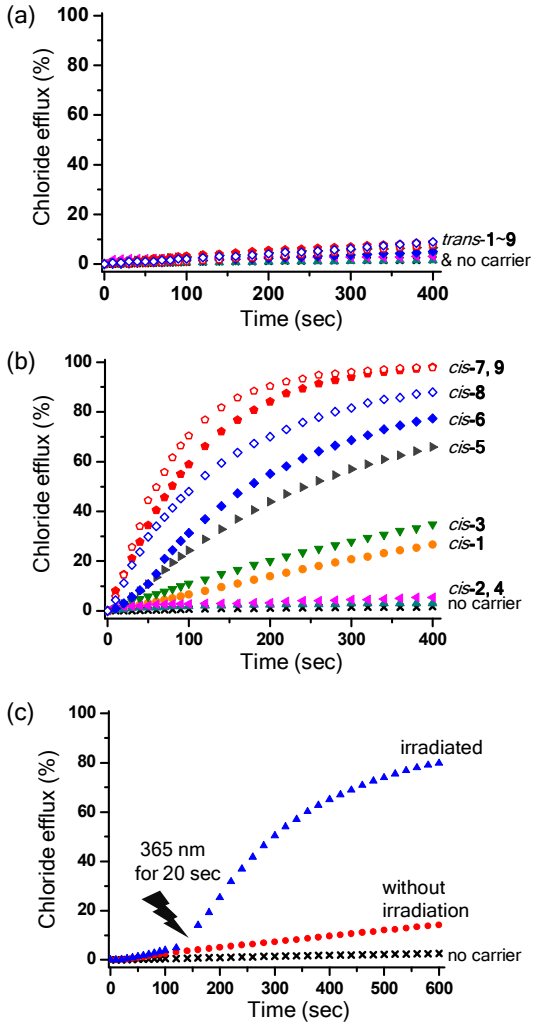
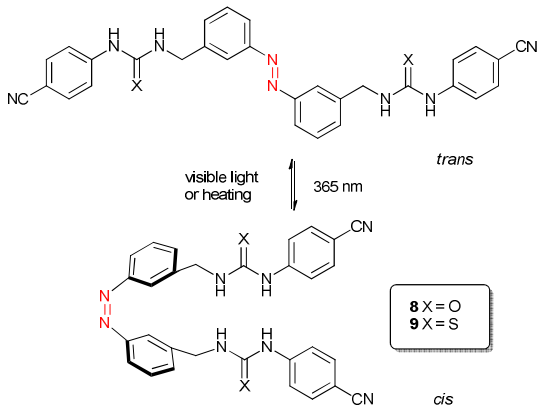


Fig. 1 Plots of chloride efflux against time (sec) across a POPC membrane. (a) Chloride effluxes facilitated by *trans* isomers **1-9** (2 mol% to lipid). (b) Chloride effluxes facilitated by *cis* isomers **1-9** (2 mol% to lipid). (c) The *in situ* activation of chloride efflux by UV irradiation (365 nm, 20 sec) of *trans*-**7** after 2 min transport. Chloride effluxes from vesicles containing NaCl (500 mM in 5 mM phosphate buffer at pH = 7.2) into a $NaNO_3$ (500 mM in 5 mM phosphate buffer at pH = 7.2) solution were measured using a chloride selective electrode.

relative binding affinities towards chloride ion.[12] On the other hand, *cis*-**3** with methyl ester substituents has moderate transport activity but *cis*-**4** with longer butyl esters is inactive despite the same binding affinity (Fig. 1b). Lipophilic groups were in general known to improve the transport activity of carriers[13] but negative effects were observed when the carriers became too lipophilic,[14] possibly due to the poor deliverability of the carriers into the lipid bilayer and/or the reduced shuttling rate in the membrane. It was also suggested that the transport activity depends on the relative position and balance of lipophilic appendages in the carriers.[15]

Using compound **7** with the highest chloride transport activity, it was examined that the chloride transport could be switched on by the *in situ* irradiation without pretreatment of the carrier into the *cis* isomer (Fig. 1c). The transport activity of thermally stable *trans*-**7** was negligible prior to irradiation. When the solution was



Scheme 2. Molecular structures of compounds **8** and **9**.

irradiated for 20 sec by UV (365 nm) light during the transport experiment in 120 sec, the chloride transport was suddenly activated as a result of the *in situ* isomerization of *trans*-**7** to *cis*-**7**.

To improve further the chloride transport activity based on the modeling studies, we prepared two more azo compounds **8** and **9** wherein the urea groups were located at the *meta* position to the diazo unit. Compared with the corresponding *para*-linked analogues **6** and **7**, compounds **8** and **9** were determined to form more stable complexes with chloride ion (Table 1). As a consequence, *cis*-**8** and *cis*-**9** showed better transport activities with the EC_{50} values of 0.38 and 0.15 mol% to lipid, respectively. The activities of the *trans* isomers are however negligible.

As a representative example, *cis*-**6** was chosen to reveal whether azo compounds act as mobile carriers or channels. The transport experiment was repeated in vesicles composed of 7:3 POPC/cholesterol molar ratio. Cholesterols with a rigid molecular framework presumably decrease the fluidity of the membrane to slow down the shuttling rate of a mobile carrier,[16] which results in reducing the transport activity. Indeed, the transport rate of *cis*-**6** was slightly reduced in a 7:3 POPC/cholesterol membrane (Fig. S25†), supporting the carrier mechanism. In addition, Hill coefficients on the chloride transport of **1**-**9** were in the range of n = 0.9 to 1.6 (Table 1), suggesting also that azo compounds function as monomeric transporters.[17]

Next, the chloride efflux from vesicles to a sodium nitrate solution may occur via Cl^-/NO_3^- antiport and/or Cl^-/Na^+ symport. To differentiate them, the transport experiment was carried out in a sodium sulfate solution (166 mM in 5 mM phosphate buffer at pH = 7.2). Here, possible Cl^-/SO_4^{2-} antiport is negligible because sulfate ion is too hydrophilic to be transported by small carriers although synthetic molecules capable of transporting the sulfate ions were reported recently by Gale and co-workers.[18] The chloride efflux mediated by *cis*-**6** was very efficient in a sodium nitrate solution but was extremely sluggish in a sodium sulfate solution (Fig. S26†), confirming that the chloride transport occurs mostly via Cl^-/NO_3^- antiport.

To investigate whether effective POPC chloride transporters **6**-**9** are able to transport chloride ions across the plasma membrane of mammalian cells, we examined changes in intracellular chloride concentration in Fischer rat thyroid epithelial (FRT) cells. The FRT cells were chosen because of their low basal halide transport activity, rapid growth on uncoated plastic plates, and stable expression of transfected cDNAs. The intracellular concentration of chloride was measured using a halide sensor YFP-

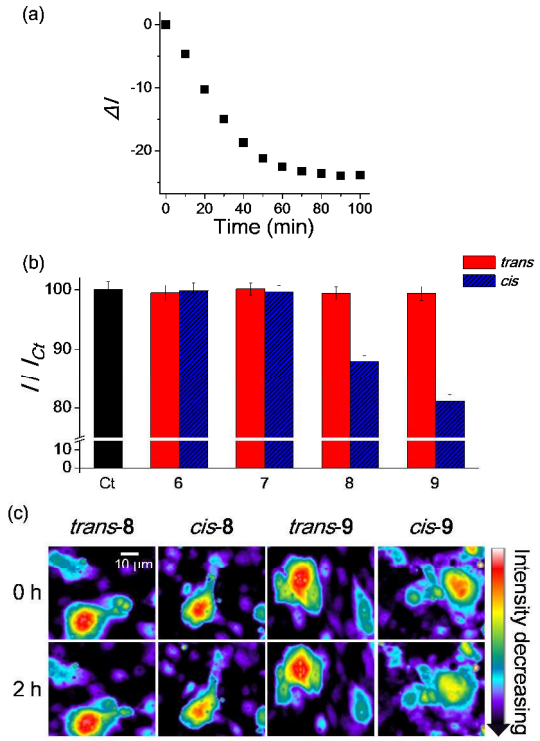


Fig. 2 Fluorescence changes of YFP transfected in FRT cells. (a) Time dependent fluorescence changes of YFP in FRT cells treated *cis*-**9** (30 μM). $\Delta I = I_t - I_0$ when I_0 is initial fluorescence intensity and I_t is fluorescence intensity at time. (b) Proportional fluorescence changes of YFP after 2 h exposure to each isomer of **6**-**9**. The control (Ct) fluorescence intensities calibrated to 100%. (c) Pseudo-color images of YFP fluorescence at 0 and 2 h after addition of each isomer of **8** and **9**.

F46L/H148Q/I152L, a mutant EYFP.[19] The FRT cells were stably transfected with the halide sensor YFP and the effects of **6**-**9** on the intracellular chloride concentration were monitored by the fluorescence change of the YFP.[20] First, the cells were exposed to the most efficient transporter *cis*-**9** (30 μM) and the YFP fluorescence changes were monitored every 10 min. The fluorescence intensity was gradually decreased and reached a plateau in 90 min (Fig. 2a) as a result of the facilitated influx of extracellular chloride ions into the FRT cells. Based on this observation, cells were treated with each of *trans* and *cis* isomers of compounds **6**-**9** (30 μM), and the YFP fluorescence intensities were measured and compared after 2 h (Fig. 2b, 2c and Fig. S34†). The YFP fluorescence in FRT cells was greatly reduced on exposure to *cis*-**8** or *cis*-**9** with respect to control (Ct), while no change was observed with all the *trans* isomers, *cis*-**6** and *cis*-**7**. These results clearly indicate that *cis*-**8** and *cis*-**9** induce chloride influx in living cells. An important difference was realised between the chloride transport experiments on live cells and on POPC vesicles. In vesicle experiments, *para*-linked analogues *cis*-**6** and *cis*-**7** were very efficient in the chloride transport, but no activity was observed in live cells. Only *meta*-linked analogues *cis*-**8** and *cis*-**9** showed chloride transport activities in *in vitro* experiment regardless of functional groups, thiourea and urea. This is possibly attributed to the intrinsic difference in the permeability of these molecules between POPC and plasma membranes.[21]

In conclusion, we have demonstrated for the first time that

synthetic mobile carriers are able to exhibit the photoswitchable activity of facilitating chloride transport across lipid and plasma membranes. Thermally stable *trans*-azo compounds are practically inactive but their activities can be switched on by the UV irradiation which results in isomerising them into the corresponding *cis* compounds. This study provides us with an opportunity to develop synthetic molecules that can transport chloride ions to a specific target in living systems through spatiotemporal control using light.

This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (NRF-2013R1A2A2A05005796). G.C.K. and H.-G.J. acknowledge the fellowship of the BK 21-plus program and Y.R.C. acknowledges the fellowship of the National Junior Research (NRF-2013H1A8A1003806).

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† Electronic Supplementary Information (ESI) available: Synthetic procedures, characterization, ^{1}H NMR and UV/visble spectra of all new compounds, experimental details for titrations, transports in vesicles and live cells. See DOI: 10.1039/b000000x/

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