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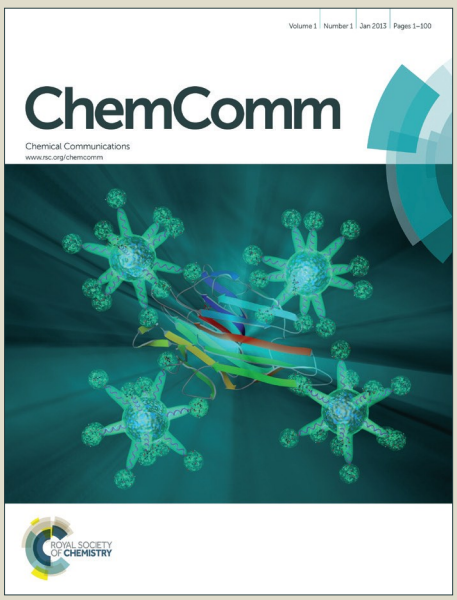

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Communication

Synthetic anion transporters that bear a terminal ethynyl group

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Cl^- transporters that bear a terminal ethynyl group were synthesized; they consist of non-pyrrolic hydrogen bond motifs such as phenolic OH, amide NH, and triazole CH. The ethynyl group of these non-pyrrolic analogs plays an important role in chloride efflux and they exhibit no significant cytotoxic activity.

Chloride anions are vital to maintain homeostasis in living organisms, and their concentration is precisely regulated by chloride-selective ion channels and related mechanisms. Therefore, malfunctioning chloride ion channels cause serious diseases, including cystic fibrosis,[1] Bartter's syndrome, and some forms of myotonia.[2] A synthetic channel or transporter can be a potential remedy for these diseases and a valuable research target. Many synthetic molecules capable of functioning as anion transporters have been developed based on a variety of molecular scaffolds, including polyamides (or ureas),[3] electron-deficient aryls,[4] steroids,[5] pyrroles,[6] and others.[7] Among them, prodigiosins, a family of naturally occurring pyrrole alkaloids, are known to promote the cotransport of H^+/Cl^- across bilayer membranes, which can lead to tumor cell apoptosis *via* increased intracellular pH.[8] In addition, they possess a wide range of potentially useful biological activities such as immunosuppression and toxicity against bacteria, protozoa, fungi, and malaria parasites.[9] However, there is no conclusive evidence that cancer activity is directly related to the co-transport of H^+/Cl^-. It is also known that prodigiosins are involved in mitogen-activated kinase signaling cascades,[10] and promote double-strand DNA cleavages in the presence of Cu(II) and O_2.[11] Moreover, their potential utility as drug candidates for many indications has not been clearly proven due to their high toxicity. Nonetheless, prodigiosin is a well-studied compound and provides design suggestions for synthetic transporters. Thus, a few structural analogs of prodigiosin have been reported, and their core structures are mostly preserved.[12] Herein, we report the first non-pyrrolic prodigiosin analogs that effectively transport chloride anions across membranes. In a direct comparison with synthetic prodigiosins, our congeners transport chloride anions *via* an antiport mechanism with comparable Cl^- efflux activity[13, 14] and exhibit no significant cytotoxic activity even at concentrations as high as 500 μM in carcinoma cell lines such as HT-29 and DLD-1, while the cytotoxicity of the synthetic prodigiosin is in the range of 2–3 μM (IC_{50}) in A549 human lung cancer cells[12c] and transport of H^+/Cl^- occurs *via* a symport mechanism. This non-toxic nature of the present compounds coupled with Cl^- efflux activity is unprecedented. Thus, we believe that these findings put us one step forward in the journey to understand and find a remedy for biological diseases related to Cl^- malfunction.

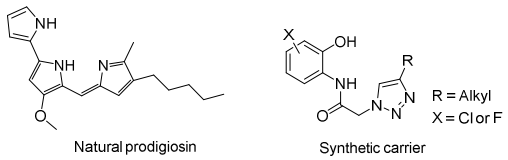

Fig. 1 Natural prodigiosin (flat) and rationally designed non-flat structure of synthetic anion transporter based on prodigiosin's hydrogen-bonding motifs using computational calculations (Fig S13 in ESI).

To obtain the target compounds shown in Scheme 1, ethyl 2-azidoacetate (**1**) was reacted with hepta-1,6-diyne, which afforded triazole-substituted ester **2**. Then, **2** was hydrolyzed and then subjected to amide coupling reactions with various anilines, which resulted in one of our target compounds (**4b**). Compound **4g** was partly reduced from **4b,** while **4a** was alternatively synthesized by completely reducing **4b**.

The abilities of **4a**–**4g** to transport Cl^- were compared by monitoring efflux across large unilamellar vesicles (LUVs) prepared with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). The LUVs were loaded with NaCl (488 mM) and suspended in a phosphate buffer solution (pH = 7.2) containing $NaNO_3$ (488 mM). The efflux of chloride ions was monitored by adding **4a**–**4g** (4 mol% relative to POPC) dissolved in DMSO as a function of time using a glass-bodied chloride-selective electrode. After 600 s, Triton-X was added to rupture the vesicles and the chloride concentration was normalized to the final concentration of $[Cl^-]$, which was considered 100%. The results are shown in Fig. 2. The observed chloride efflux of **4a** was 5.1%, which was slightly higher than that of our negative control (DMSO). Although the terminal alkyne group of **4b** was

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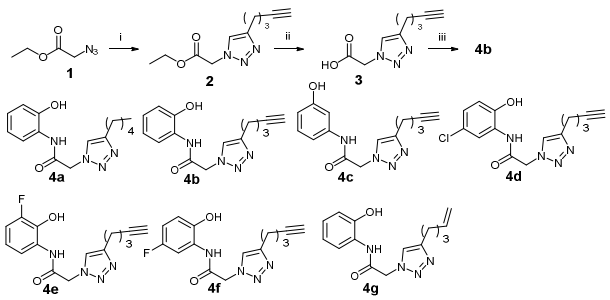
introduced to dimerize **4a** and increase the chloride binding constant, the obtained **4b** bearing an ethynyl group showed five-fold higher activity than **4a**. The *meta*-regioisomer **4c** had slightly higher efflux activity than its congener **4b**. These unexpected results led us to synthesize vinyl derivative **4g** from the partial reduction of **4b**. The chloride efflux of **4g** was similar to that of **4a**. These results indicate that the ethynyl groups are pivotal for the observed activities. The efflux activity was further optimized by introducing halogen groups to the benzene ring of **4b**. Similar modifications have been successful in other transporters.[3b] When F or Cl groups were introduced onto the phenyl group, **4b**, **4f,** and **4e** showed two-fold increases in chloride efflux compared to **4c** and **4b**. In general, the F group showed higher activity than the Cl group and thus, **4f** was the best compound.



Scheme 1. Synthetic scheme of chloride transporters: (i) Sodium-L-ascorbate, $CuSO_4$, 1,6-hepadiyne (or hept-1-yne), water/DMSO (v/v = 3:1) 60°C; (ii) NaOH, MeOH; (iii) Aminophenol, PyBop, DIEA, CH_2Cl_2.

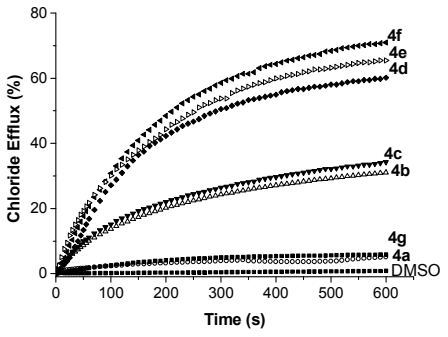


Fig. 2 Chloride efflux upon addition of **4a**–**4g** (4 mol% relative to POPC) to vesicles composed of POPC. The vesicles contained NaCl (488 mM) and were immersed in $NaNO_3$ (488 mM), pH 7.0 solution; at 600 s, they were lysed to obtain 100% chloride efflux.

To examine the unprecedented effect of the ethynyl group, binding constants for the compounds were examined by ^{1}H NMR titrations with chloride anions in the form of TBA salt. The changes of chemical shift (OH, NH, and CH) and hydrogen bond length trends of the analogs were not the same as those of prodigiosin (Fig S13 in ESI). In addition, the ethynyl-CH of **4** was not changed upon the addition of chloride ions and the observed binding constants were all similar (11 to 23 M^{-1}), regardless of the presence or absence of the ethynyl group (Table S1).[16] Increased lipophilicity has been also considered as one of the important factors for an effective Cl^- transporter,[3b] and lipophilicity was predicted based on the log P value. The log P values of **4a**, **4b**, and **4e** were obtained by standard pH-metric method. The experimental values indicated that the introduced ethynyl group in **4b** and **4e** reduced the overall lipophilicity of **4a** (**4e** = 2.48, **4b** = 1.63, and **4a** = 2.84). The log P values and binding constants (indicating possible interactions) for chloride, nitrate, and phosphate anions in DMSO (ESI), and POPC in CD_2Cl_2/DMSO (Fig. S1 and S2 in ESI) could not account for the ethynyl group effect. However, previous studies have shown that the ethynyl group can more easily form hydrogen bonds with water[15a] in the hydrated membrane[15b] than alkane and alkene (more π-C and C_{sp}-H). Thus, we propose that the compounds containing the ethynyl group could be more effectively localized in the hydrated membrane than other compounds. Although these possibilities require further studies, the ethynyl group effect is crucial for the Cl^- efflux in our repeated experiments.

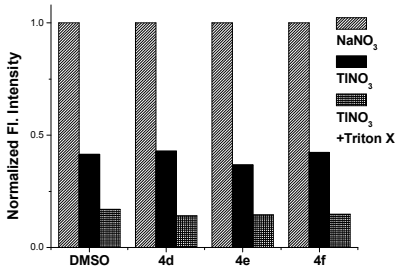


Fig. 3 Observed fluorescence changes upon the addition of $NaNO_3$, $TlNO_3$, or $TlNO_3$ + Triton X to vesicles composed of POPC where 16 μL of each transporter (10 mM in DMSO, 4 mol% relative to POPC) or DMSO was added in advance.

We also conducted a Tl^+/ANTS assay (ANTS: 8-amino-1,3,6-naphthalenetrisulfonic acid disodium salt for Tl^+ as a quencher, excitation and emission at 355 nm and 512 nm, respectively) to exclude the possibility that synthesized molecules could rupture (or damage) POPC vesicles, enabling chloride ions to leak out of the vesicles during the chloride transport assay. In the assay, vesicles were prepared with POPC and ANTS, and extravesicular ANTS was removed using a PD-10 desalting column while ANTS within POPC vesicles remained. However, the normalized fluorescence was reduced to 60% due to some remaining ANTS outside of the vesicles in the presence of DMSO as a negative control upon the addition of $TlNO_3$ to the solution, as shown in Fig. 3. Further fluorescence reduction was seen in the presence of $TlNO_3$ when Triton X was added. Under these conditions, ANTS that leaked from the vesicles was further quenched with Tl^+. As shown in Fig. 3, **4d**, **4e**, **4f**, and DMSO showed similar fluorescence intensities in the presence of Tl^+ with or without Triton X. Thus, these experiments proved that **4d**, **4e**, and **4f** do not induce the rupture (or damage) of the vesicles (Fig. S5 in ESI). However, this possibility has not been thoroughly examined in recent studies. If a compound plays a similar role to Triton X or detergents, Cl^- leaks out of the membrane. Therefore, we could mistake Cl^- efflux for Cl^- leakage and observe significant cytotoxicity of the compound in cancer cell lines as well.[13]

Patch clamp experiments were also conducted with **4d** and the results were compared with those obtained using gramicidin A (gA) as a positive control for pore formation (ion channel). Although channel activity of **4d** was not observed at concentrations as high as 2 mM, the addition of gA to the solution (0.2 nM) resulted in ion channel activity (Fig. S12 in ESI). After excluding vesicle damage and ion channel

mechanisms, the transport experiment was carried out to determine anti-port mechanisms under the two different pH conditions using **4e** and **4d** (Fig. S10 in ESI):[12b] (a) inside vesicle, pH 4.0 and outside vesicle, pH 6.7; and (b) inside and outside vesicle, pH 7.0. Without a pH gradient (b condition), similar values (%) of Cl^- efflux were observed for **4b** and **4e** (compared in Fig. 2), while the efflux values of both **4b** and **4e** were similarly reduced to less than half of the values from (b) experiment under the conditions of (a) (i.e., more favorable conditions for symport).[14] These studies conclusively indicate that our compounds rely on an antiport mechanism rather than a symport mechanism, shown in Fig. 4.

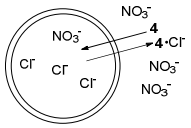


Fig.4 Schematic representation of the transport model of **4**.

To determine the cytotoxicity of the compounds, *in vitro* cytotoxic activity was tested using a live/dead assay in HT29 and DLD-1 cell lines. Initially, a single point assay (50 μM) revealed that there was no apparent cytotoxicity for all compounds. Further assays were conducted using up to 500 μM of **4c** and **4e** and no apparent cytotoxicity was observed, even at such a high concentration (Fig. S11 in ESI). Although further biological studies are warranted, these properties coupled with Cl^- efflux activity could be beneficial for biological applications.

In summary, the obtained analogs contain non-pyrrolic hydrogen bond motifs such as phenolic OH, amide NH, and triazole CH. Based on structure–activity relationship studies, the ethynyl groups are important for efflux activity. Unlike other prodigiosin analogs, our non-pyrrolic analogs rely on an antiport mechanism; this inference was supported by Tl^+/ANTS, pH variable Cl^- efflux assays, and patch clamp experiments. Considering the non-toxicity and Cl^- activity of our compounds, they could be useful for the study and understanding of Cl^- efflux-related biological events and diseases. These biological applications are currently pursued in our laboratories.

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13. The Cl^- efflux activity of **4f** (71%, measured at pH = 7.0) could be comparable to the activity of synthetic prodigiosins (81%, measured at pH = 7.4).[12c, 14]
14. The driving force of symport (H^+/Cl^-) is the proton egress to outside the vesicle in Cl^- efflux. Therefore, a higher pH outside the vesicle is highly desired for effective H^+/Cl^- transportation.[12c]
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16. UV-Vis titrations were also carried out to obtain the binding constants for the transporters. The obtained binding constants for chloride anions of **4a**, **4b**, and **4c** were 2500 to 4900 M^{-1}, and are shown in Table S1. The reliable binding constants of **4f**, **4e**, and **4d** could not be calculated due to the large errors associated with small UV-Vis changes during the titration. These observed binding constant differences have been observed in previous studies owing primarily to differences in host concentrations (possible aggregation) and methods (intrinsic limitation). Although our compounds strictly follows the Beer–Lambert law using absorbance changes (Fig. S3 in ESI), absorbance changes resulting from aggregation are not as sensitive as fluorescence changes (note: all compounds are not fluorescent). Although we were not able to identify possible aggregation at the range of NMR concentrations, these results still cannot rule out possible aggregation of our compounds at the below NMR concentrations. The following reference discusses these differences: M. Albrecht, Triyanti, S. Schiffers, O. Osetska, G. Raabe, T. Wieland, L. Russo and K. Rissanen, *Eur. J. Org. Chem*, **2007**, 2850.
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