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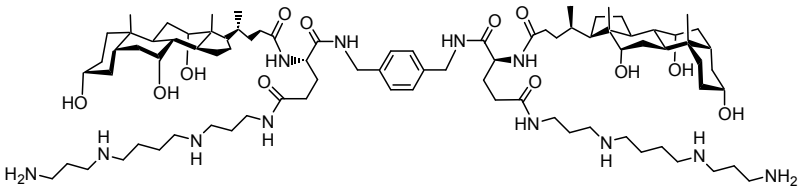
Graphical Abstract

Toward transmembrane anionophores based on rigid bis(choloyl) conjugates: reversal of the ion selectivity by appended polyamines

Yong-Ming Lu, Li-Qun Deng, and Wen-Hua Chen *

A bis(polyamine)-appending rigid linker-tethered bis(choloyl) conjugate was synthesized as a potent anionophore with high selectivity for iodide over the other monoanionic ions.



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ARTICLE TYPE

Toward transmembrane anionophores based on rigid bis(choloyl) conjugates: reversal of the ion selectivity by appended polyamines

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A bis(choloyl) conjugate tethered with a bis(polyamine)-appending rigid linker was synthesized and found to efficiently promote the exchange of anions across egg-yolk *L*-α-phosphatidylcholine-based liposomal membranes. This is in sharp contrast to a previous finding that its analog having two monoamino groups acted as a cation/proton antiporter.

Identification of small organic compounds that are capable of efficiently mediating the transport of anions, in particular chloride across cell membranes, has been attracting considerable interest from medicinal chemists. [1] This is primarily spurred by the high potentials of such compounds in the development of future treatments for channelopathies and cancers. [1] Thus, great efforts have been devoted to the creation of a wealth of non-peptidic synthetic anionophores, including (thio)ureas, [2] imidazolium salts, [3] calix[4]pyrroles, [4] prodigiosin analogs, [5] squaramides, [6] and others. [1] Such wide ranges of diverse structures may provide numerous options for future use, but on the other hand emphasize the challenges in search for sophisticated anionophores. To this end, one practical strategy may be to perturb the structures of a synthetic ionophore with established activity. For example, Gale *et al* have reported that the anion transport activity of bis-indolylurea-based transporters may be dramatically modulated by the length of the central alkyl chains. [7] Such small changes in structure make it possible to fine tune the anion transport activity and thereby lead to optimized transporters.

In a recent study we have shown that a rigid linker-tethered bis(choloyl) conjugate having two amino groups between the two choloyl subunits (compound **A**, Fig. 1), exhibits potent ionophoric activity across egg-yolk *L*-α-phosphatidylcholine (EYPC)-based liposomal membranes, whereas its analogue without additional amino groups is much less active. [8] This result suggests that the appending amino groups have a profound role in ameliorating the activity. However, compound **A**, though having (protonated) amino groups, was found to act as a cation/proton antiporter rather than an expected anionophore. Inspired by our recent findings that cholic acid-spermine conjugates, for example compound **B** (Fig. 1) are capable of mediating the transmembrane transport of anions, [9, 10] we reason that replacement of each of the monoamino groups in compound **A** with similar polyamines may afford an anionophore with promising transporting activity. Herein we report the synthesis of bis(choloyl) conjugate **1** having two polyamine chains between the two choloyl subunits (Scheme 1), and its potent anionophoric activity investigated by means of chromophoric and chloride ion selective electrode assays.

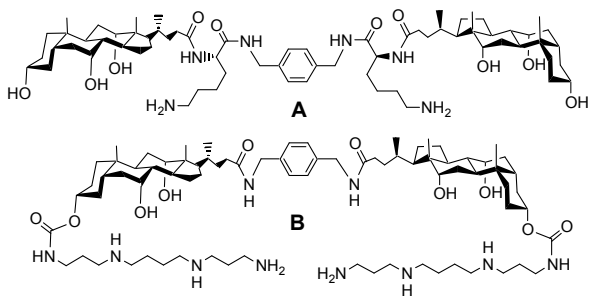

Figure 1. Structures of amino-modified bis(choloyl) conjugates **A** and **B**.

Compound **1** was synthesized according to the approach shown in Scheme 1. Thus, acylation of tri-Boc protected spermine [11] with compound **2** that was activated with *N*-hydroxylsuccinimide (NHS), followed by the deprotection of the Boc groups with trifluoroacetic acid (TFA), gave compound **1**. The structure of compound **1** was confirmed on the basis of NMR (^{1}H and ^{13}C), ESI MS (LR and HR) and IR data (see supporting information). The synthesis and ionophoric activity of compound **2** will be reported somewhere else.

To test whether compound **1** is capable of mediating anion transport across a bilayer membrane, we carried out chloride efflux experiments. [12] Thus, we prepared a series of large unilamellar EYPC vesicles (100 nm diameter, extrusion) loaded with sodium chloride and suspended them in an external $NaNO_3$ solution. A sample of compound **1** (of varying concentrations to lipid) was added as a DMSO solution. If compound **1** partitions into the phospholipid membranes and functions as a chloride transporter, chloride will be released from the vesicles and can be detected by using a chloride ion selective electrode. [12] After 300 s, the vesicles were lysed by addition of 5 wt% Triton X-100 and the final reading of the electrode was used to calibrate the 100% release of chloride. The results are shown in Fig. 2a and indicate that compound **1** was capable of releasing chloride under the measuring conditions and that the rate of chloride efflux was

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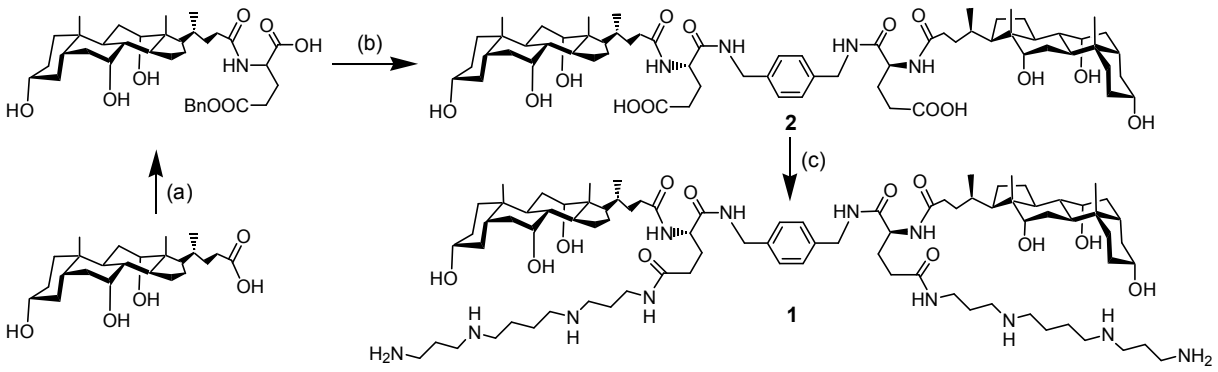
found to be concentration dependent. When the external solution was replaced with sodium sulfate, the chloride efflux activity was significantly inhibited (Fig. S6). This provides evidence that the observed chloride efflux is mediated *via* a Cl^-/NO_3^- exchange process as sulfate is strongly hydrated and is not readily transported. [7, 12]

To gain further insight into this as well as to investigate the ion selectivity among anions, we carried out pH discharge experiments with sodium salts of different anions (i.e., NO_3^-, Cl^-, Br^-, and I^-) and chloride salts of alkali metal ions (i.e., Li^+, Na^+, K^+, Rb^+ and Cs^+), respectively. For this purpose, a pH sensitive dye, pyranine (pK_a 7.2) was loaded within the vesicles and used as a fluorescence-responsive reporter of pH changes within the vesicle interior. If there is proton or hydroxide transport across the EYPC bilayers, this will lead to a variation in the internal pH of the vesicles and is detected by a change in the fluorescence intensity (FI) of pyranine. [10, 14] Thus, liposomes were prepared in a buffer containing an anion under study and then a pH gradient was applied. If the transport process is dependent of the anions, this suggests that anion transport is a dominant or rate-determining step and that compound **1** is anion-selective. Otherwise, if the rate is unaltered by the anions, this indicates that compound **1** has negligible selectivity among anions, or primarily is cation-selective. [9] Similarly, a cation-dependent transport process suggests that compound **1** is cation-selective. [8] As shown in Fig. 2b, addition of compound **1** to EYPC liposomal dispersions containing an internal aqueous phase of pH of 7.0 and an external aqueous phase of pH of 8.0, led to an increase in the fluorescence intensity of pyranine, indicating that compound **1** was capable of inducing pH discharge across the membrane. The changes in the fluorescence intensity of pyranine varied in the order of $I^- >> NO_3^- > Cl^- \approx Br^-$ indicate that compound **1** has high selectivity for iodide over the other three monoanions. No difference in the pH discharge activity was found when the chloride salts of different alkali metal ions were used (Fig. S7). These results strongly suggest that compound **1** was capable of promoting the exchange of anions, [15] which is in sharp contrast to a previous finding that compound **A** exhibited ionophoric activity *via* a cation/proton exchange process. [8]



Scheme 1. Synthesis of compound **1**. Reagents and conditions: (a) (i) NHS/DCC, THF; (ii) γ-benzyl-*L*-glutamate, Et_3N, THF-H_2O, room temperature; (b) (i) NHS/DCC, $CHCl_3$; (ii) *p*-bis(aminomethyl)benzene, $CHCl_3$; (iii) H_2-Pd/C, MeOH; (c) (i) NHS/DCC, DMF; (ii) Tri(Boc) spermine, DMF; (iii) TFA, CH_2Cl_2.

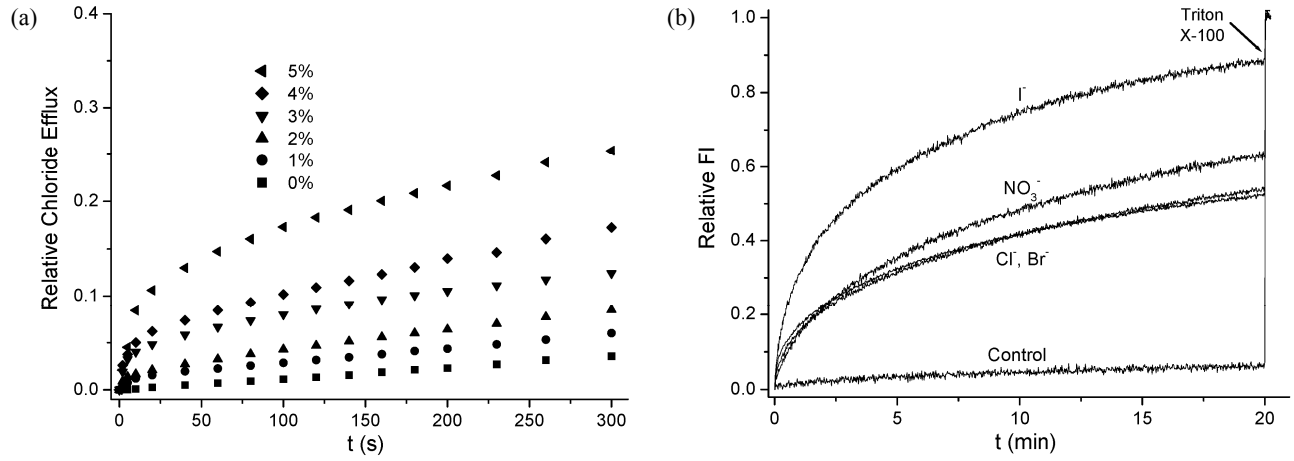


Figure 2. (a) Chloride efflux promoted by compound **1** of varying concentrations in EYPC vesicles (1.78 mM phospholipid) loaded with 500 mM NaCl buffered to pH 7.0 with 25 mM HEPES. The vesicles were dispersed in 500 mM $NaNO_3$ buffered to pH 7.0 with 25 mM HEPES. DMSO was used as a blank. (b) Discharge of a pH gradient across EYPC-based liposomal membranes (1.33 mM phospholipid), in the presence of compound **1** of 3.0 mol%. Intravesicular conditions: 0.1 mM pyranine in 25 mM HEPES (pH 7.0, 50 mM salt); and extravesicular conditions: 25 mM HEPES (pH 8.0, 50 mM salt). Ex 460 nm; em 510 nm. The salts used were $NaNO_3$, NaCl, NaBr and NaI. The experiment that was conducted in NaCl media and in the absence of compound **1** was used as a control.

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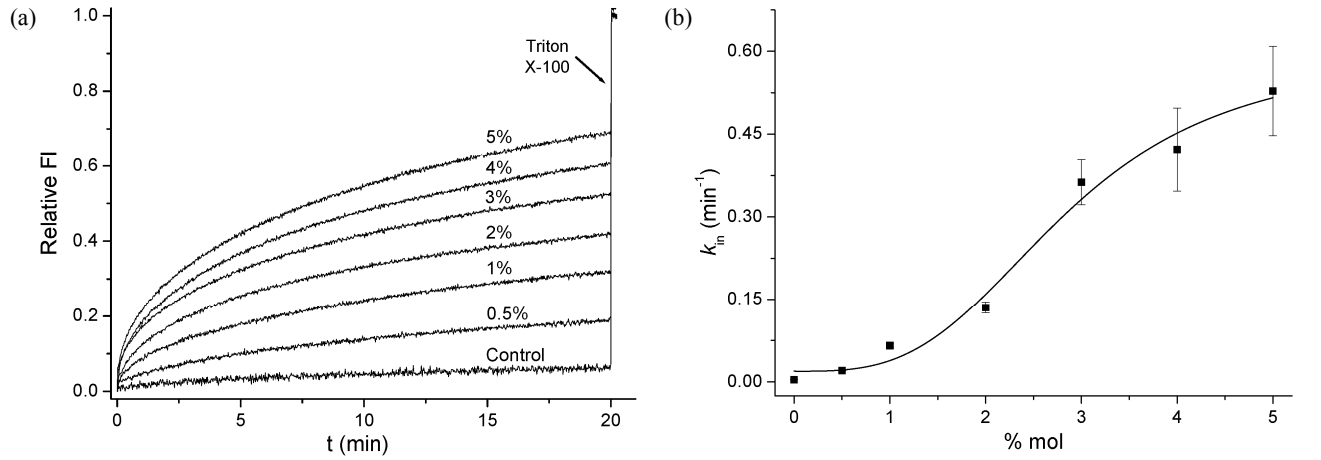

Figure 3. (a) Discharge of a pH gradient across EYPC-based liposomal membranes (1.33 mM phospholipid) at room temperature, in the presence of compound **1** of varying concentrations. Intravesicular conditions: 0.1 mM pyranine in 25 mM HEPES (pH 7.0, 50 mM NaCl); Extravesicular conditions: 25 mM HEPES (pH 8.0, 50 mM NaCl). Ex 460 nm; em 510 nm. (b) A Hill plot of k_{in} versus the mol% concentrations of compound **1** in EYPC-based liposomal membranes at room temperature. The solid line is nonlinear least-squares fit of the data according to the Eq. $k_{in} = k_0 + k_{max}\times[\text{compound } \mathbf{1}]^n/([\text{compound } \mathbf{1}]^n + [EC_{50}]^n)$, where n = 3.2±1.2.

To quantitatively characterize the ion-transporting efficiency of compound **1**, we repeated the pH discharge experiments by varying the concentrations, and then analyzed the relationship between the initial rate constants (k_{in}'s) and the concentrations by using a Hill equation $k_{in} = k_0 + k_{max}\times[\text{compound } \mathbf{1}]^n/([\text{compound } \mathbf{1}]^n + [EC_{50}]^n)$. [16] Here n is the Hill coefficient that reveals the stoichiometry of the transport process, EC_{50} is defined as the "effective" monomer concentration (mol%) of compound **1** that is needed to reach 50% of the maximum activity (k_{max}) and k_0 is the rate constant for the background, respectively. It is clear from Fig. 3a that the k_{in}'s had strong dependence on the concentrations of compound **1** in the membrane. Hill analysis (Fig. 3b) afforded the Hill coefficient n of 3.2±1.2, suggesting that two to four molecules of compound **1** are assembled to form the transport-active species. The EC_{50} value was calculated to be 2.8±0.5 mol% (relative to the EYPC concentration). This value is comparable to that obtained for phenylthioureas (3.1 mol%) and compound **B** (2.0 mol%) and greater than that obtained for imidazolium salts (5.7 mol%). [2, 3, 9] Thus, compound **1** is considered to be a very effective class of anionophores.

The anion transporting properties of compound **1** may be rationalized by taking its structure into account. The rigid and planar phenyl linker is expected to enable compound **1** to span the entire lipid bilayers in its extended configuration. Such an orientation would drive the two polyamino chains into the interior of the membrane. According to the report that N_1-acetylspermine has pK_a values of 10.1, 8.6 and 7.3, [17] compound **1** is partially protonated and thereby is multiply positively charged under the conditions of chloride efflux and pH discharge. Thus, compound **1** is able to provide a positively charged pathway for anions that are to be transported, while the anions may be stabilized by forming electrostatic and hydrogen-bonding interactions with the (partially) protonated polyamino groups and the inward-directed hydroxyl groups of the sterols. [18] The slightly lower activity of compound **1** relative to compound **B**, is a likely consequence of the lower lipophilicity of compound **1** that makes the partitioning within the phospholipid bilayers unfavorable. [19, 20]

In conclusion, a bis(choloyl) conjugate tethered with a bis(polyamine)-appending rigid linker has been successfully synthesized and fully characterized. Pyranine assay and chloride ion selective electrode technique have indicated that this conjugate is capable of efficiently promoting the exchange of anions with high selectivity for iodide over the other monoanionic ions. The present findings raise the possibility that the ion selectivity of amino-functionalized bis(choloyl) conjugates may be modulated by the appended (poly)amino groups. Further efforts aimed at creating more effective anionophores are currently in progress in our laboratory.

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Notes and references

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