Bioorganic & Medicinal Chemistry Letters 26 (2016) 3665–3668

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Efficient transmembrane anion transport mediated by a bis(imidazolyl)-functionalized bis(choloyl) conjugate

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ARTICLE INFO

Article history:
Received 23 April 2016
Revised 29 May 2016
Accepted 31 May 2016
Available online 1 June 2016

Keywords:
Anion transporter
Cholic acid
Imidazole
Anionophoric activity

ABSTRACT

A bis(imidazolyl)-functionalized bis(choloyl) conjugate was synthesized and assessed for its transmembrane anionophoric activity by means of chloride ion selective electrode technique and pyranine assays. The results indicate that under the assay conditions, this conjugate was capable of mediating the symport of proton and anions, presumably via a channel mechanism. In addition, this compound was found to exhibit much higher anionophoric activity than the analogue without imidazolyl groups, revealing the significant role of the imidazolyl groups in the anion transport process.

Anions are ubiquitous in biology and critical for maintaining cellular functions.[1] Recognition, transport or transformation of anions is involved at some levels in almost every conceivable biochemical operation. In particular, transport of chloride across lipid bilayers, regulated in nature by transmembrane ion channels,[2] has attracted considerable attention. This is because malfunctioning of chloride transport is associated to various disorders, including nephrolithiasis and cystic fibrosis.[3] Particular interest is stimulated by the hypothesis that synthetic anion transporters may serve as therapeutic agents for these diseases, by replacing the action of defective channels that underlie the genetic conditions such as cystic fibrosis.[4]

Boosted by the aim to shine light on the factors that influence the transmembrane transport of anions, extensive studies have been carried out on the structure–activity relationship of synthetic anion transporters[4,5] and as a consequence lipophilicity,[6] configuration[7] and flexibility[8] have been identified as some of the most important factors influencing the anionophoric transport activity. Meanwhile, a strategy that is widely used to design an effective anion transporter is to deploy an array of hydrogen-bond donor groups onto a suitable scaffold, allowing multiple cooperative interactions with the anions that are to be transported.[9] High anion-transport activity has been achieved owing to the strong hydrogen-bonding interaction of anions with pre-organized donors.[10] In this regard, choloyl framework has proved highly effective, in part because its inward-directed hydroxyl groups are able to bind anions through hydrogen bonding.[11] Such hydrogen-bonding donor ability may be strengthened to generate more powerful anion receptors and transporters, for example by changing the hydroxyl groups into ammonium substituents,[12] or by appending stronger anion-binding functionality, such as guanidino[13] and poly(ammonium) groups.[14] On the other hand, imidazolyl groups are capable of forming complexes with anions largely through hydrogen bonding interactions.[15] Thus, imidazolyl groups have been incorporated in the structure of anion transport carriers,[16] and accordingly some imidazolyl derivatives and imidazolium salts have been found to be highly effective in transporting anions.[17] In addition, it is reported that imidazoles adopt self-organized structures mainly by forming hydrogen-bond chains,[17g] through which water and proton may be efficiently transported.[18]

Inspired by these findings, we hypothesize that incorporation of choloyl units with imidazolyl groups would lead to a conjugate, in which the cooperative interactions of both the hydroxyl and imidazolyl groups with anions and proton may be translated into enhanced transporting efficacy toward both chemical species. To test this hypothesis, we synthesized a bis(imidazolyl)-functionalized bis(choloyl) conjugate **1** (Fig. 1) and investigated its transmembrane anionophoric activity in detail by means of chloride ion selective electrode and pyranine assays. As discussed elsewhere, a bis(choloyl) conjugate is able to span the whole lipid bilayers in its fully expanded conformation.[13,14,19–21] This would enable the two imidazolyl groups in compound **1** to well reside within the membrane interior, and to form a certain level of hydrogen-bonding network with the hydroxyl groups to mediate the transmembrane transport of anions (vide infra). For comparison,

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http://dx.doi.org/10.1016/j.bmcl.2016.05.089
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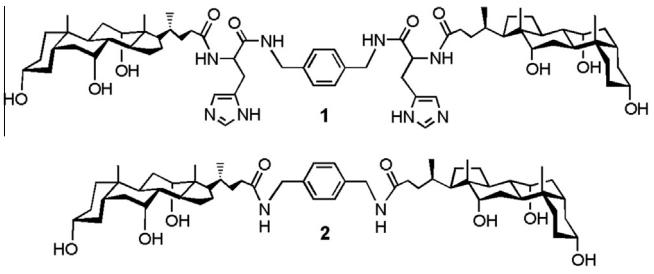


Figure 1. Structures of compounds **1** and **2** with and without imidazolyl groups, respectively.

we also prepared its analogue without imidazolyl groups, that is, compound **2**. Herein we report our findings.

Compound **1** was synthesized according to the approach shown in Scheme 1. Thus, activation with *N*-hydroxylsuccinimide (NHS) of compound **3** that was prepared from the reaction of cholic acid NHS ester with 1-(triphenylmethyl)-L-histidine, and subsequent reaction with *p*-bis(aminomethyl)benzene afforded compound **4**. Deprotection of the triphenylmethyl groups in compound **4** with trifluoroacetic acid (TFA) gave compound **1**. Compound **2** was prepared according to the procedures reported by us.[19] Compound **1** was fully characterized on the basis of NMR (^{1}H and ^{13}C) and ESI MS data (see SI).

The anion-transport activity and selectivity of compound **1** across egg-yolk phosphatidylcholine (EYPC)-based liposomal membranes was studied by means of chloride ion selective electrode technique and pH discharge assays.[14,22]

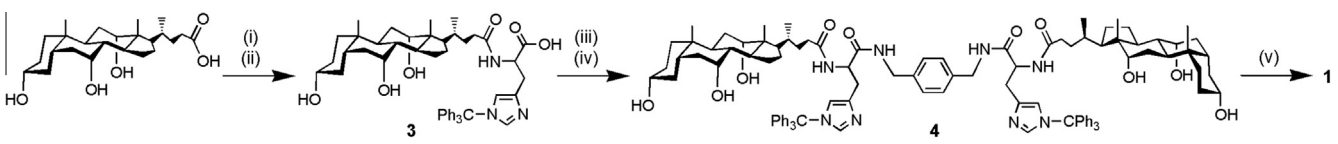
First, to assess whether compound **1** is capable of mediating anion transport, we carried out chloride efflux experiments. Thus, we prepared a series of large unilamellar EYPC vesicles (100 nm diameter, extrusion) loaded with sodium chloride and suspended them in an external isotonic $NaNO_3$ solution. Upon the addition of a sample of compound **1** (of varying concentrations in molar percent relative to lipid) as a DMSO solution, the efflux of chloride anions from the vesicles was detected by using a chloride ion selective electrode. 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 anions. The results indicate that compound **1** is capable of efficiently releasing chloride anions and the rate of chloride efflux is concentration dependent (Fig. 2a). The finding that compound **1** is much more active than compound **2** (Figs. 2b and S9), reveals the significant role of the imidazolyl groups in the ion transport process. Analysis of the relationship between the relative chloride efflux at 260 s and time afforded the Hill coefficient n that reveals the stoichiometry of the transport process, and the parameter k_2/K_{diss} that measures the effectiveness of a transporter (Fig. S10). Here, k_2 and K_{diss} stand for the intrinsic rate constant and the dissociation constant of the self-association process, respectively.[17h,23] The n value of 1.71 ± 0.12 for compound **1** indicates that, on average, two molecules are assembled into the transport-active species. Interestingly, the k_2/K_{diss} value is $1.45 \times 10^{-2}\ s^{-1} \cdot mol\%^{-2}$, suggesting that compound **1** is more active than guanidino-functionalized bis(choloyl) conjugate ($k_2/K_{diss} = 7.36 \times 10^{-3}\ s^{-1}\ mol\%^{-2}$) previously reported by us,[13] and benzimidazoliums ($k_2/K_{diss} = 1.18 \times 10^{-3}\ s^{-1}\ mol\%^{-2}$) reported in literature.[17g]

To gain insight into the probable mechanism of action of compound **1**, we repeated the chloride efflux experiments in the presence of the chloride salts of group I alkali metal ions (Li^+, Na^+, K^+, Rb^+ and Cs^+).[14] The data are shown in Figure 3a, and indicate that the chloride efflux activity of compound **1** is essentially independent of those metal ions, excluding their role in the permeation process. In addition, replacement of the external nitrate with strongly hydrated sulfate has no inhibitory effect on the chloride efflux (Fig. S11), ruling out the possibility that anion exchange is a predominant process. These results suggest that the chloride efflux was due to a process of proton/chloride symport for compound **1**.[24]

Then, in order to investigate the ion selectivity of compound **1** among anions, we carried out pH discharge experiments in the presence of sodium salts of different anions (i.e., NO_3^-, Cl^-, Br^-, and I^-).[25] In this test, a pH gradient is established across the liposomal membranes and the increase in the fluorescence of the entrapped pyranine indicates the transmembrane transport of proton or hydroxide. The results are shown in Figure 3b and indicate that addition of compound **1** to EYPC liposomal dispersions containing an internal pH of 7.0 and an external aqueous phase of pH of 8.0, led to an increase in the pyranine fluorescence. This suggests that compound **1** is capable of inducing pH discharge across the membrane. The activity varies with the used anions, strongly suggesting that anions are involved in the transporting process probably via a symport process with proton.

In addition, to clarify whether compound **1** functions as a mobile carrier or as a channel/pore-forming agent, we conducted pH discharge experiments using vesicles derived from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol. Because the condensing and ordering effects of cholesterol on lipid membranes retard the diffusion of an ion across a rigid membrane, cholesterol assays have been frequently used as evidence for a mobile carrier mechanism in anion transport.[21,26] As shown in Figure 4, the pH discharge activity of compound **1** is essentially unaffected across lipid membranes derived from POPC with 30% cholesterol. This implies that compound **1** functions probably via a channel mechanism.

Taken together, the above-mentioned results suggest that compound **1** is capable of mediating the transmembrane symport of proton and chloride. Notably, compound **1** exhibits higher activity than compound **2**, though the greater lipophilicity of the latter ($c\log P = 5.27$) relative to the former ($c\log P = 3.34$) makes the partitioning of compound **2** into the phospholipid bilayers favorable, which should have an advantageous effect on the ion transport.[27] This clearly reveals the significant role of the two imidazolyl groups in the ion transport process. The chloride transport activity may be ascribed to the binding ability of the hydroxyl and imidazolyl groups with chloride anions, evidenced from our recent report that benzimidazolyl-based receptors are capable of binding and transporting anions through the formation of hydrogen-bonding interactions.[17a] The ability to transport proton implies that the functional groups in compound **1** may form a certain level of



Scheme 1. Synthesis of compound **1**. Reagents and conditions: (i) NHS, DCC, THF; (ii) 1-(triphenylmethyl)-L-histidine, THF-H_2O; (iii) NHS, DCC, THF; (iv) *p*-bis(aminomethyl) benzene, THF; (v) TFA, CH_2Cl_2.