Prodigiosin is a chloride carrier that can function as an anion exchanger†

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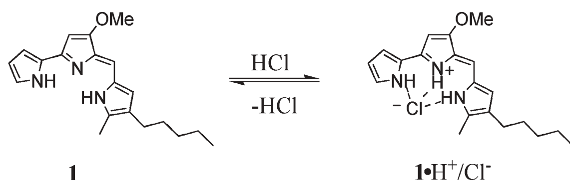
The natural product prodigiosin 1, often described as an H^+/Cl^- symport cotransporter, can transport Cl^- across lipid vesicles *via* an anion exchange (or antiport) mechanism.

As the field of anion recognition develops,[1] there has been a growing emphasis on identifying compounds that can transport anions across lipid membranes. In particular, synthetic chloride transporters have gained attention because of their potential to mediate crucial biological processes.[2] For example, Gokel and colleagues have shown that modified peptides form chloride ion channels.[3] The A.P. Davis and Smith groups have developed chloride carriers known as cholopads.[4,5] Our own contribution was the finding that a *1,3-alt*-calix[4]arene amide,[6] and some acyclic analogs,[7] could change the pH within vesicles in a Cl^- dependent fashion. The calix[4]arene amide, which we described as an H^+/Cl^- co-transporter, supported ion channel conductance across phospholipid membranes and cells.[6]

To better understand the mechanism of these calixarene-based transporters our attention was drawn to prodigiosin **1** (Scheme 1), the parent in a family of compounds isolated from *Serratia marcescens*.[8] These natural products and synthetic analogs have shown potent biological activities, particularly against human cancer cell lines.[9] Several mechanisms have been proposed to explain the prodigiosins' biological activity. Manderville and colleagues have shown that copper complexes of prodigiosins cleave duplex DNA.[10] Of interest to us, were reports that prodigiosins acidified cellular organelles and vesicles by transporting H^+Cl^- across membranes.[11,12] Other demonstrations that prodigiosin analogs can bind Cl^-,[13] coupled with findings from the Gale, Smith and Sessler labs that prodigiosin analogs transport Cl^- anions across phospholipid membranes,[14,15] strengthened our conviction that **1** might serve as a standard for gauging the potency and mechanism of other transmembrane chloride transporters.



Scheme 1 Prodigiosin **1** and its HCl bound ion pair.

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Thus, we planned to use prodigiosin **1** in two ways. First, we needed a carrier standard to compare and contrast to the calixarene analogs that we had found form ion channels.[6] Second, we also sought a H^+/Cl^- symporter (or A^-/Cl^- antiporter) to help establish whether these calixarenes truly functioned as H^+/Cl^- symporters. Below, we show that prodigiosin **1** acts as a chloride carrier in phospholipid vesicles. We also demonstrate that prodigiosin **1** is able to exchange Cl^- for NO_3^- anions during transmembrane transport, without any concomitant change in the internal pH of the liposomes. This finding is consistent with prodigiosin **1** functioning *via* an anion exchange (or antiport) mechanism.

Our first goal was to determine whether prodigiosin **1** functions as a mobile ion carrier or whether it might form a transmembrane channel. Although we expected prodigiosin **1** to act as an anion carrier, this key mechanistic aspect has not yet been reported to our knowledge. To distinguish carrier from channel function, we measured Cl^- influx into DPPC liposomes at temperatures above and below that lipid's gel-to-liquid crystalline phase transition temperature (41 °C). An ion carrier's efficiency, limited by diffusion through the membrane, is significantly crippled in a "frozen" gel-state.[4,16] We prepared DPPC liposomes (100 nm) that contained 1 mM of the chloride-dye lucigenin[5,17] and 100 mM $NaNO_3$ in 10 mM sodium phosphate (pH 6.4). The DPPC liposomes were diluted into a similar external solution (100 mM $NaNO_3$, 10 mM sodium phosphate, pH 6.4), and NaCl was added to establish a 25 mM chloride gradient across the membrane. After the NaCl pulse, we monitored the quenching of lucigenin's fluorescence (and thus the Cl^- influx into the DPPC liposomes) at both 43 °C and 37 °C.

The data in Fig. 1 show that, in the presence of 0.004 mol% prodigiosin (relative to lipid concentration), Cl^- influx was relatively rapid at 43 °C, a temperature above the DPPC phase transition. In contrast, little Cl^- influx above background is observed upon addition of the same amount of prodigiosin at 37 °C, a temperature where the DPPC is in its gel state. These results are consistent with prodigiosin **1** acting as a chloride anion carrier. As a positive control, the same assay using a calix[4]arene tetrabutylamide, a compound that we believe forms a self-assembled channel, showed that chloride transport was only partially diminished at 37 °C (Electronic supplementary information†). Chloride gradient assays using EYPC liposomes at 25 °C (where the lipid is in its liquid crystalline state) showed that the pseudo-first order rate constant for Cl^- influx was linear with respect to prodigiosin concentration.† These latter data are further evidence that prodigiosin **1** operates as a chloride anion carrier.[18]

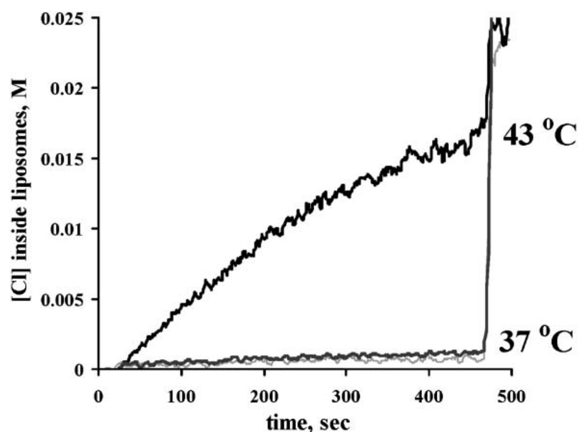
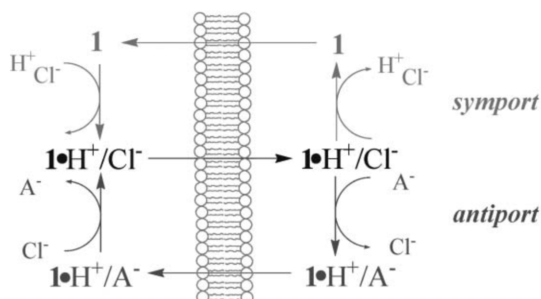


Fig. 1 Chloride influx into DPPC liposomes at 43 °C and 37 °C. The data at each temperature is the average of 3 runs using 0.004 mol% prodigiosin **1**. The trace shown in grey represents a DMSO blank at 37 °C.

We next investigated the ability of prodigiosin **1** to exchange other anions for the transported Cl^- anion. Transport processes mediated by prodigiosins have been primarily studied using pH sensitive dyes in both liposome and cell-based assays; the ability to acidify cells and vesicles has led to the view that prodigiosins act as H^+/Cl^- symporters.[11,12] In their original reports, Ohkuma and coworkers specifically noted that H^+/Cl^- symport and OH^-/Cl^- antiport are functionally equivalent.[11] Over the years, however, consideration of the OH^-/Cl^- antiport mechanism seems to have faded, and the prodigiosins are typically described as H^+/Cl^- symporters.[19]

As depicted in Scheme 2, the symport pathway involves release of bound HCl from $\mathbf{1{\cdot}H^+/A^-}$; the neutral carrier **1** then diffuses across the membrane to bind another HCl at the lipid–water boundary. The antiport pathway requires that prodigiosin **1** remains protonated as $\mathbf{1{\cdot}H^+}$; after anion exchange the carrier moves back across the membrane as a lipophilic ion pair $\mathbf{1{\cdot}H^+/A^-}$. Prodigiosin **1** should exist primarily in its protonated form at acidic or neutral pH, given that the pK_a of $\mathbf{1{\cdot}H^+}$ is between 7 and 8.[20]

Previous reports of chloride transport mediated by prodigiosins have typically employed assays that indirectly detect chloride transport by following the dissipation of a pH gradient across the liposomal membrane.[11,21–22] As mentioned above, the H^+/Cl^- symport process cannot be distinguished from an OH^-/Cl^- antiport mechanism using such base–pulse assays. We hoped to overcome such mechanistic ambiguities by monitoring both Cl^- influx and internal liposomal pH as a function of internal anion using separate batches of liposomes that were prepared and manipulated under conditions that were as similar as possible. In this way, we hoped to simultaneously track Cl^- and H^+ (or OH^-) transport.



Scheme 2 H^+/Cl^- symport (light grey, top part of image) *vs.* A^-/Cl^- anion antiport (darker grey, lower part of image) pathways.

EYPC liposomes (100 nm) containing 1 mM of the Cl^- sensitive lucigenin dye,[5,17] in an internal solution of 10 mM sodium phosphate (pH 6.4), 75 mM Na_2SO_4 were suspended in a similar solution (10 mM sodium phosphate, pH 6.4, with 75 mM Na_2SO_4). This liposome suspension was then subjected to a 25 mM sodium chloride gradient. Fig. 2A shows that in the presence of 0.02 mol% prodigiosin, Cl^- influx occurred as soon as the NaCl pulse was applied. However, under these conditions anion influx stopped at an internal Cl^- concentration (5 mM) that was only about 20% of its equilibrium value, as determined after rupture of the liposomes with Triton-X (Fig. 2A). In complimentary experiments using similar EYPC liposomes in sulfate solution (Fig. 2B), but containing the pH sensitive dye HPTS,[21] addition of 25 mM NaCl in the presence of 0.02 mol% prodigiosin resulted in acidification within the liposomes (Δ pH = 0.25). These results indicate that either a H^+ influx or OH^- efflux occurs along with Cl^- transport into the liposomes. The results in Fig. 2A and B are consistent with either the H^+/Cl^- symport or the OH^-/Cl^- antiport mechanism.

As portrayed in Fig. 2C–D, we conducted a similar set of transport assays using solutions that contain nitrate (ΔG_{hyd} = -300 kJ mol^{-1}), an anion that is more lipophilic than sulfate (ΔG_{hyd} = -1080 kJ mol^{-1}), hydroxide (ΔG_{hyd} = -430 kJ mol^{-1}) and chloride (ΔG_{hyd} = -340 kJ mol^{-1}).[23] Two populations of EYPC liposomes were made containing 10 mM sodium phosphate (pH 6.4), 100 mM $NaNO_3$. Again, one set of EYPC liposomes contained 1 mM of lucigenin dye to monitor Cl^- influx, and the other set contained the HPTS dye to monitor internal pH. As shown in Fig. 2C, Cl^- influx was again rapid, with an initial rate similar to that shown for the sulfate-containing liposomes in Fig. 2A. In contrast with the data in Fig. 2A, the chloride transport in these nitrate-containing liposomes went nearly to equilibrium (internal [Cl^-] = 20 mM) in a few seconds. Furthermore, the corresponding liposomes containing the HPTS dye showed that the liposomal pH did not change under these experimental conditions with nitrate as the predominant anion (Fig. 2D). The constant pH in a system that is experiencing a significant change in Cl^- concentration indicates that prodigiosin **1** must promote anion exchange. In contrast, a symport mechanism would predict a decrease in internal pH as H^+/Cl^- is co-transported across the membrane by **1**.

Although generally accepted as an H^+/Cl^- symporter,[8,11] our results show that prodigiosin **1** can facilitate anion exchange (antiport) with lipophilic anions such as nitrate. Of course, it is possible that prodigiosin's mechanism of Cl^- transport may change from antiport to symport,[24] depending on the assay conditions. Finally, the results described in this communication may have implications for those interested in developing prodigiosin analogs as therapeutics. These studies should also help establish prodigiosins, or analogs, as standards in Cl^- transport assays. We hope that our findings will prompt others to further probe the ion transport mechanism and utility of these interesting natural products.

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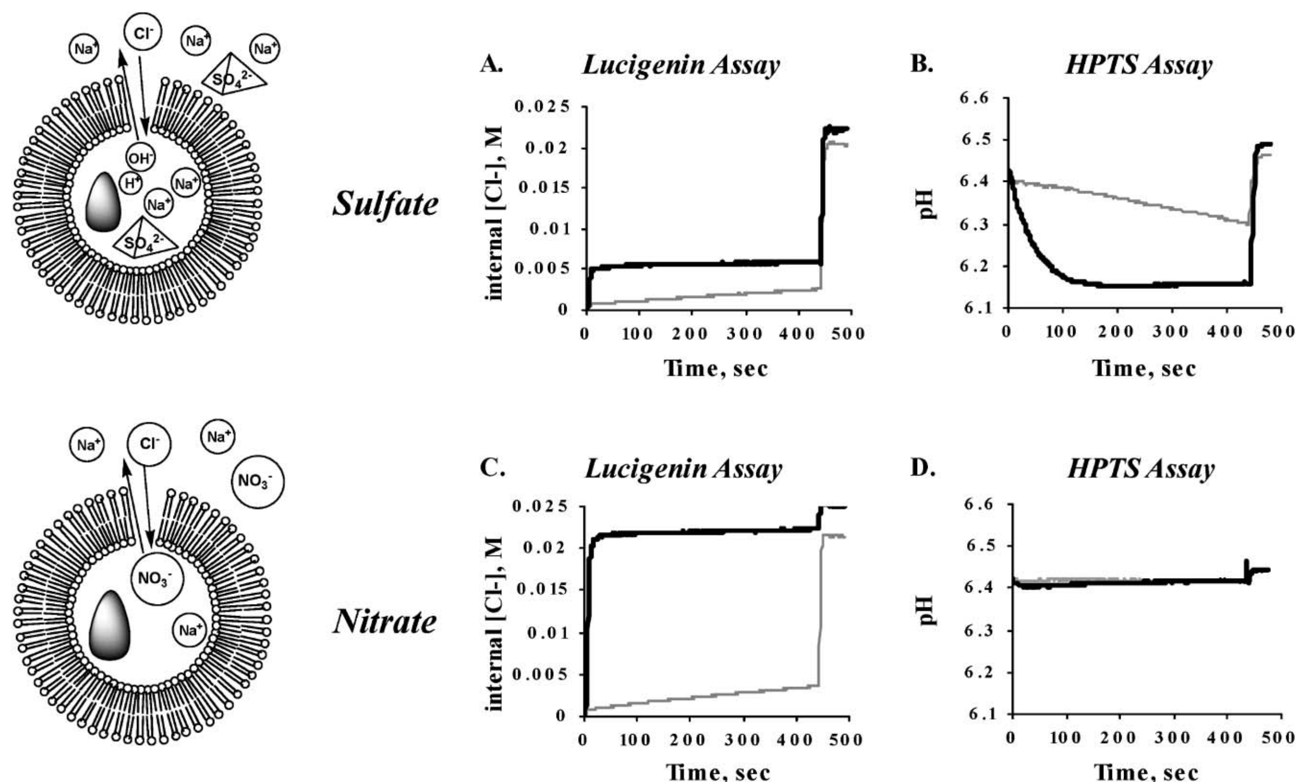

Fig. 2 Chloride gradient assays on 100 nm EYPC liposomes in 10 mM sodium phosphate (pH 6.4) containing either 75 mM Na_2SO_4 (A and B) or 100 mM $NaNO_3$ (C and D). The chloride gradient was initiated by adding NaCl to give an external concentration of 25 mM. Chloride concentration inside the vesicles (panels A and C) was calculated from the fluorescence of entrapped lucigenin dye. The pH (panels B and D) is calculated from the fluorescence ratio of HPTS dye emitted at 510 nm when excited at 403 and 460 nm in a dual wavelength assay. The trace shown in grey represents DMSO blanks.

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