Using "small" molecules to facilitate exchange of bicarbonate and chloride anions across liposomal membranes.

Jeffery T. Davis,[1] Philip A. Gale,[2] Oluyomi A. Okunola,[1] Pilar Prados,[3] Jose Carlos Iglesias-Sánchez,[3] Tomás Torroba,[4] and Roberto Quesada[4]

[1]Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742, USA. [2]School of Chemistry, University of Southampton, Southampton, SO17 1BJ, United Kingdom. [3]Departamento de Química Orgánica, Universidad Autónoma de Madrid, 28049 Madrid, Spain. [4]Departamento de Química, Universidad de Burgos, 09001 Burgos, Spain. Correspondence should be addressed to J.T.D. (jdavis@umd.edu), P.A.G. (philip.gale@soton.ac.uk) or R.Q. (rquesada@ubu.es)

Bicarbonate is involved in a wide range of biological processes including respiration, regulation of intracellular pH and fertilization. In this study we use a combination of NMR and ion-selective electrode techniques to show that the natural product prodigiosin, a tripyrrolic molecule produced by microorganisms such as *Streptomyces* and *Serratia*, facilitates chloride/bicarbonate exchange (antiport) across liposomal membranes. Higher concentrations of simple synthetic molecules based on a 4,6-dihydroxyisophthalamide-core are also shown to facilitate this antiport process. While it is well known that proteins regulate Cl^-/HCO_3^- exchange in cells, these results suggest that small molecules may also be able to regulate the concentration of these anions in biological systems.

Bicarbonate is an important anion. It is a substrate in photosynthesis,[1] it regulates intra- and extracellular pH,[2] it is generated during cellular respiration from CO_2,[3] and it acts as a cellular signal to activate sperm for fertilization.[4] Under physiological conditions most dissolved inorganic carbon exists as HCO_3^-. Bicarbonate cannot diffuse freely across lipid membranes,[5] and bicarbonate transport is facilitated *in vivo* by membrane-bound proteins that function *via* Cl^-/HCO_3^- exchange or Na^+/HCO_3^- co-transport mechanisms.[6] Dysregulation of bicarbonate transport can lead to conditions such as cystic fibrosis, heart disease and infertility.[7-9] The lack of structural data for these proteins means that little is known regarding the anion binding sites that modulate their affinity and selectivity.[8,9] Despite the importance of transmembrane bicarbonate transport, there have been no studies that focus attention on the use of "small" molecules to promote the *efficient* transport of bicarbonate anion across lipid membranes in contradistinction to the growing body of work on transmembrane chloride transport.[10-17]. Previously in a study that focused on chloride transport, a

synthetic steroid-based receptor was reported to support detectable Cl^- efflux from liposomes upon addition of extravesicular HCO_3^-.[10] The challenge of achieving bicarbonate transport was eloquently expressed by A. P. Davis, Sheppard and Smith in 2007: "*A specific goal would be a mimic of chloride/bicarbonate exchangers that play important roles in red blood cells and epithelial tissues. The design challenge here is to produce a transporter that can extract the very hydrophilic bicarbonate anion into the lipophilic interior of a bilayer membrane.*"[11]

Prodigiosin **1**,[18] is a natural product produced by microorganisms such as *Streptomyces* and *Serratia*.[19,20] This tripyrrolic metabolite has potent inmunosuppressive and anticancer activities. Prodigiosin causes selective apoptosis of cancer cells,[21] and its structural analogue obatoclax is in clinical trials for treatment of cancer.[22] The origin of prodigiosin's biological activity has yet to be unambiguously established, although it facilitates co-transport of HCl,[23-28] and anion exchange of chloride across lipid bilayers.[29,30] Indeed, the antibiotic activity of prodigiosin-like compounds has been related to their activity as transmembrane Cl^- carriers.[28] Due to the presence of hydrogen bond donors and acceptors in prodigiosin **1**, we reasoned that it might function as a receptor and a membrane transport agent for bicarbonate (Figure 1).

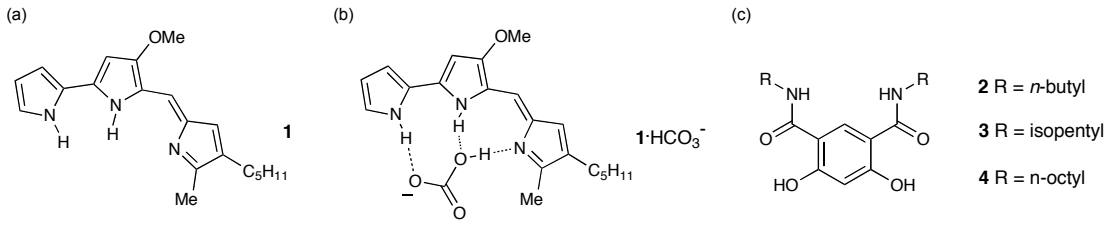

Figure 1. The structures of the bicarbonate transporters studied in this work: prodigiosin **1** (a), the putative prodigiosin bicarbonate complex (b) and the structures of synthetic transporters **2** – **4** (c).

We also investigated the bicarbonate transport ability of **2**-**4** in order to ascertain whether these compounds function as chloride/bicarbonate antiporters, especially in comparison to prodigiosin **1**. We recently reported the transmembrane chloride transport activity of 4,6-dihydroxyisophthalamide **2**.[31] Isophthalamides have convergent amide NH groups that can form hydrogen bonds with anions.[32,33] In the case of **2**, conformational control of the anion binding cleft by intramolecular hydrogen bonds between the 4,6-dihydroxy units and the neighbouring amide carbonyls resulted in an improved anion affinity and in optimal activity for transmembrane transport of chloride anion. In fact an analogue of compound **2** lacking the OH groups was shown not to function as a membrane transport agent for chloride.[31] For this study, we synthesized related isophthalamides **3** and **4**, functionalized with different alkyl substituents, as we reasoned that membrane activity might be attenuated by the identity of the lipophilic tails attached to the isophthalamide (Figure 1).

Phospholipid vesicles have been extensively used as models for biological membranes. Unilamellar vesicles of a defined size are easily produced with control of the entrapped solution. These liposomes can be suspended in an external medium of different composition and the transporter-facilitated release of encapsulated substrates or the influx of substances from the external milieu to the interior of the vesicles can be monitored by fluorescence, NMR or ion selective electrode techniques. Below, we report 1) the first demonstration that transmembrane chloride/bicarbonate exchange is facilitated by prodigiosin **1** and synthetic receptors **2**-**4** and 2) NMR methods to directly monitor the transport of bicarbonate into lipid vesicles. These NMR studies, in combination with the use of chloride-selective electrodes, allow the flux of both components of an antiport process to be monitored. We hope that these studies will set the stage for further development of selective transporters for bicarbonate and perhaps, in the longer term, lead to new approaches for treating diseases caused by defective bicarbonate transport.

RESULTS

Prodigiosin and its analogues bind chloride in the solid-state and in solution.[28,34] Furthermore, the use of chloride-selective dyes and electrodes revealed that prodigiosin **1** transports Cl^- anions across lipid membranes.[28,29] Although it was reported 50 years ago that prodigiosin reacts with carbonic acid to give a protonated adduct,[35] there has been no direct evidence presented that prodigiosin **1** can bind bicarbonate. We studied the anion complexation properties of prodigiosin **1** by ^{1}H NMR titration methods in CD_2Cl_2. These NMR studies showed that bicarbonate binds to **1**, causing shifts of proton resonances in prodigiosin upon addition of tetraethyl-ammonium bicarbonate. The NMR signals in **1** that were most affected by bicarbonate addition were the H2 proton on the A ring and the methyl group on the C ring (see Supporting Information). These would be the carbon-bound protons that would be expected to be most influenced by anion binding, as they are closest to prodigiosin's putative anion-binding cleft (Figure 1) (the pyrrole NH protons are not visible in the ^{1}H NMR of prodigiosin in the free base form in CD_2Cl_2). The changes in these chemical shifts upon addition of tetraethylammonium bicarbonate were greater than those for the same protons in **1** upon addition of tetrabutylammonium chloride or nitrate (K_a= 7.8 and 7.0 M^{-1}, respectively), presumably reflecting the higher basicity of the bicarbonate anion. We could not calculate a stability constant for the prodigiosin-bicarbonate complex because, in addition to changes in chemical shifts for **1**, a second set of NMR peaks emerged during bicarbonate titration. This slow exchange process may be due to higher-order complex formation with bicarbonate or to an HCO_3^- triggered interconversion of rotamers.[36] Experiments were repeated with the protonated form of prodigiosin as the methanesulfonate salt. Under the same experimental conditions, addition of bicarbonate resulted in deprotonation of $\mathbf{1}\cdot H^+$ as evidenced by loss of the pyrrole NH resonances in the ^{1}H NMR spectrum (see supplementary information), whilst addition of chloride causes a downfield shift of the NH resonances indicating hydrogen bond formation to the halide anion. At pH 7.2

both protonated and free base forms of prodigiosin are present and it may be that any putative antiport process involves both forms. Whilst a stability constant for bicarbonate complexation was not obtained, these NMR titrations demonstrated that the free base form of prodigiosin **1** binds bicarbonate in solution. Electrospray mass spectrometry in negative mode on a solution of prodigiosin **1** in acetonitrile revealed the presence of both chloride and bicarbonate adducts (see ESI), providing more evidence for complex formation between prodigiosin **1** and bicarbonate.

We next compared the transmembrane anion transport activity of the natural product **1** *vs.* synthetic Cl^- transporters **2**-**4**. The transmembrane anion transport abilities of **1**-**4** were evaluated by monitoring chloride efflux from unilamellar POPC (1-palmitolyl-2-oleoyl-*sn*-glycero-3-phosphocholine) vesicles using a chloride selective electrode.[10] These studies were conducted using nitrate in the extravesicular solution. Nitrate is more hydrophobic than bicarbonate and is frequently used to assess chloride transport efficiency. Liposomes were loaded with a sodium chloride solution and suspended in a sodium nitrate solution. The transporters **1-4**, dissolved in a small amount (10 μL) of DMSO, were added to the extravesicular solution and chloride efflux was monitored over 300 s. At the end of the experiment the vesicles were lysed by the addition of detergent and the final value was normalized to equal complete chloride efflux. Prodigiosin **1** proved to be a potent chloride transporter using this assay. A 0.005% molar carrier to lipid concentration of prodigiosin **1** showed similar transport activity to the 0.1% molar carrier to lipid concentration for synthetic compounds **2-4** (Figure 2). These carrier loadings were capable of complete chloride efflux within 300 s, with the isopentyl-substituted isophthalamide **3** being the most active synthetic transporter under these conditions.

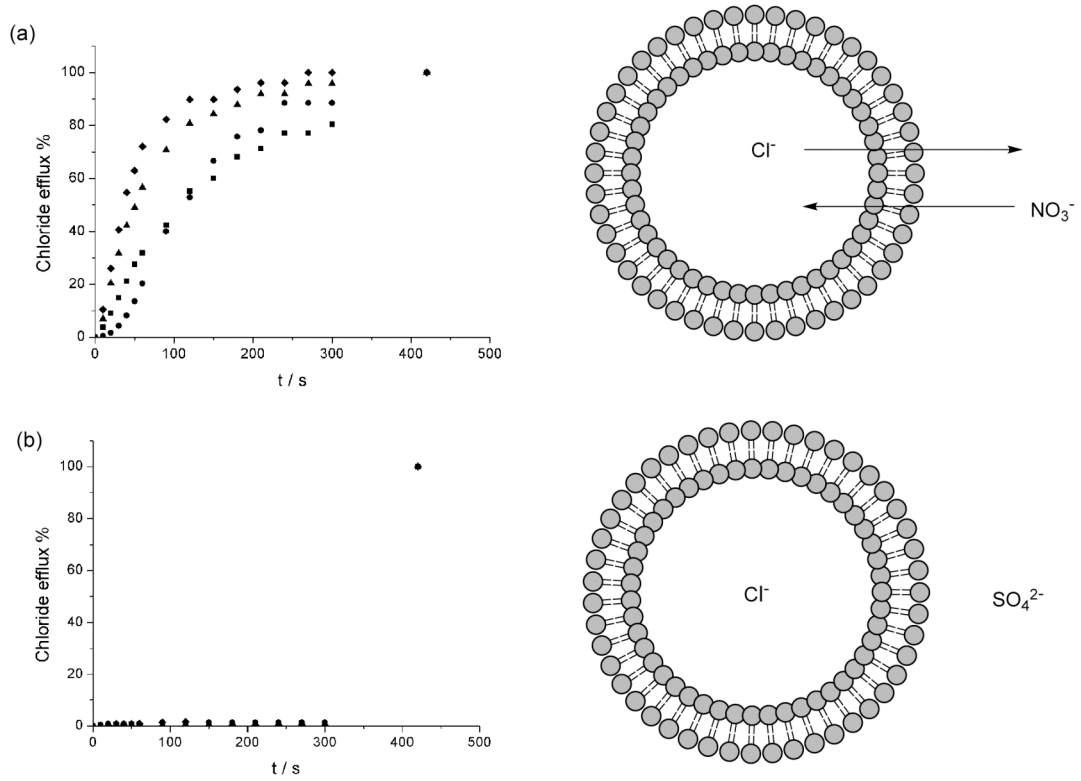

Figure 2. A comparison of chloride efflux, measured using a Cl^- selective electrode, from synthetic vesicles mediated by compounds **1** – **4** in nitrate and sulfate solution. (a) Chloride efflux promoted upon addition of **1** (♦) (0.005 % molar carrier to lipid) and **2** (■), **3** (▲), **4** (●) (0.1 % molar carrier to lipid) to unilamellar POPC vesicles loaded with 488 mM NaCl 5 mM phosphate buffer pH 7.2 dispersed in 488 mM $NaNO_3$ 5 mM phosphate buffer pH 7.2. At t = 300 s the vesicles were lysed by addition of detergent and the final reading at t = 420 s was considered to equal 100% chloride efflux. (b) Chloride efflux studies upon addition of compounds **1** (0.04 % molar carrier to lipid) and **2**-**4** (0.5 % molar carrier to lipid) to vesicles composed of POPC. The vesicles contained NaCl (488 mM) and were immersed in Na_2SO_4 (166 mM), pH 7.2 solution; at 300 s the vesicles were lysed to obtain 100% chloride efflux.

In the assay depicted in Figure 2a, the anion transport activity can occur either *via* H^+/Cl^- or Na^+/Cl^- co-transport or by Cl^-/NO_3^- exchange. To distinguish between these alternative mechanisms, we carried out the Cl^- electrode transport assay while varying the anion in the external medium. If transport occurs by an anion exchange mechanism, changing the external anion should impact the transport rate, whilst a H^+/Cl^- or Na^+/Cl^- co-transport mechanism should not be affected by the external anion. As depicted in Figure 2b, the transport assay was repeated by suspending the chloride-loaded vesicles in a sulfate-containing external medium. As the sulfate dianion carries a higher charge and is significantly more hydrophilic than nitrate, transport activity by compounds **1**-**4** should be reduced if an anion exchange mechanism is operative. Indeed, with sulfate as the external anion, no chloride efflux from the liposomes was detected upon addition of **1**-**4**, supporting a chloride/nitrate exchange (antiport) mechanism for mediating anion transport across the vesicle bilayer.

While both nitrate and bicarbonate have similar sizes and shapes, bicarbonate is significantly more hydrated than nitrate and, as stressed by A. P. Davis *et al,*[11] it is more challenging to transport bicarbonate than nitrate across a lipid bilayer.[37,38] Prompted by the ability of prodigiosin **1** to bind bicarbonate and by the Cl^-/NO_3^- anion exchange activity shown by **1**-**4**, we designed an experiment to determine whether these compounds could facilitate transmembrane bicarbonate/chloride exchange. Chloride-loaded vesicles were suspended in a sulfate-containing medium. After 2 minutes, a solution of bicarbonate was added and chloride efflux was monitored over an additional 5 minutes. At the end of the experiment the vesicles were lysed to calibrate the experimental data to 100% chloride release. The results shown in Figure 3 confirmed that negligible chloride efflux was detected in the presence of sulfate as the external anion. Addition of bicarbonate to the extravesicular solution switched on chloride efflux in the presence of **1**-**4**, indicating that these compounds enable chloride/bicarbonate antiport across liposomal membranes. As was observed for Cl^-/NO_3^- exchange, prodigiosin **1** (at 0.04 % molar carrier to lipid) was more efficient than synthetic carriers **2**-**4** (1 % molar carrier to lipid) in catalyzing Cl^-/HCO_3^- transmembrane exchange.

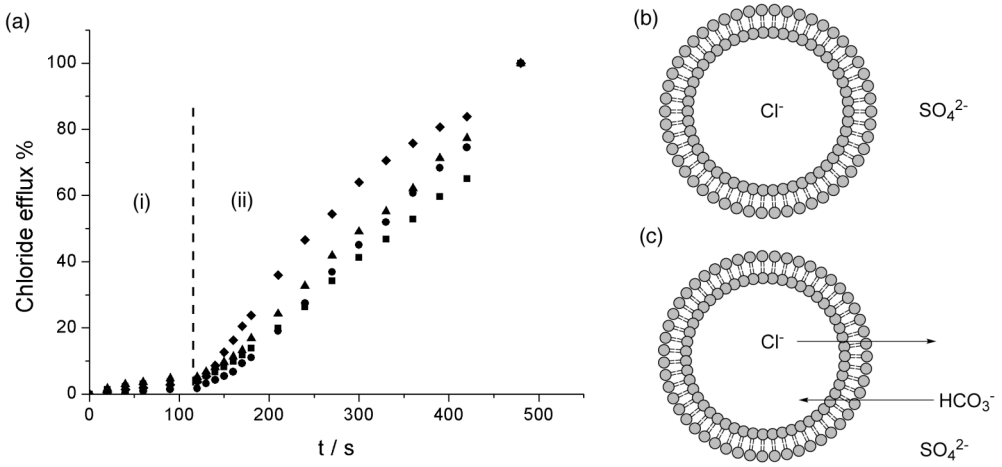

Figure 3. An experiment showing chloride transport measured using a Cl^- selective electrode commencing upon addition of a bicarbonate pulse to the external solution. (a) i) Chloride efflux promoted upon addition of **1** (♦) (0.04 % molar carrier to lipid) and **2** (■), **3** (▲), **4** (●) (1 % molar carrier to lipid) to unilamellar POPC vesicles loaded with 451 mM NaCl and 20 mM phosphate buffer pH 7.2 dispersed in 150 mM Na_2SO_4 20 mM phosphate buffer pH 7.2. ii) At t = 120 s a solution of $NaHCO_3$ was added to give a 40 mM external concentration. At t = 420 s the vesicles were lysed by addition of detergent and the final reading at t = 540 s was considered to equal 100% chloride efflux. (b) In the presence of the carrier compounds **1**-**4** chloride was not released from the vesicles when suspended in a sulfate solution. (c) Upon introduction of bicarbonate to the solution, chloride efflux began as one component of the chloride/bicarbonate antiport mechanism.

Under the above assay conditions, addition of bicarbonate induced small changes (~0.2 units) in the pH of the external medium. We carried out control experiments in the presence of compounds **1-4** to rule out the possibility that chloride efflux was driven by a pH gradient. Addition of NaOH to the external medium resulted in no significant chloride efflux. Furthermore, addition of bicarbonate solutions to a suspension of vesicles without the presence of transporters **1-4** resulted in no chloride efflux.

The experiments depicted in Fig. 3 provided strong, yet indirect, evidence that transporters **1-4** move bicarbonate across lipid membranes. We next used ^{13}C NMR spectroscopy to verify that transporters **1-4** facilitate transmembrane HCO_3^-/Cl^- exchange. We developed experiments that use paramagnetic Mn^{2+} to bleach the ^{13}C NMR signal for extravesicular $H^{13}CO_3^-$, allowing for discrimination of extravesicular and intravesicular $H^{13}CO_3^-$. We based these paramagnetic NMR protocols on previous experiments that 1) monitored transmembrane chloride transport in liposomes by ^{35}Cl NMR,[39,40] and 2) showed that intracellular and extracellular $H^{13}CO_3^-$ could be distinguished in plant cells.[41,42] Figure 4 shows data from the first set of NMR experiments conducted to illustrate transporter-mediated HCO_3^-/Cl^- exchange. These NMR experiments were done under similar conditions as described for the Cl^- electrode experiments in Figure 3. Thus, EYPC liposomes (5 μm) filled with 450 mM NaCl were suspended in a sulfate solution and 50 mM $H^{13}CO_3^-$ was added to the NMR sample. A sharp ^{13}C NMR signal for extravesicular $H^{13}CO_3^-$ was observed at δ 161 ppm. Upon addition of 0.5 mM Mn^{2+}, this signal was broadened into the baseline as the paramagnetic cation interacted with extravesicular bicarbonate. After addition of transporters (prodigiosin **1** in Figure 4b and isophthalamide **4** in Figure 4c), a sharp ^{13}C NMR signal for $H^{13}CO_3^-$ (δ ~161 ppm) was restored. This renewed ^{13}C NMR signal must be caused by ligand-mediated transport of HCO_3^- into the liposome since the paramagnetic Mn^{2+} is impermeable to the phospholipid bilayer. Importantly, the control experiment in which DMSO was added without transporter, did not result in any restoration of ^{13}C NMR signal (Figure 4d).

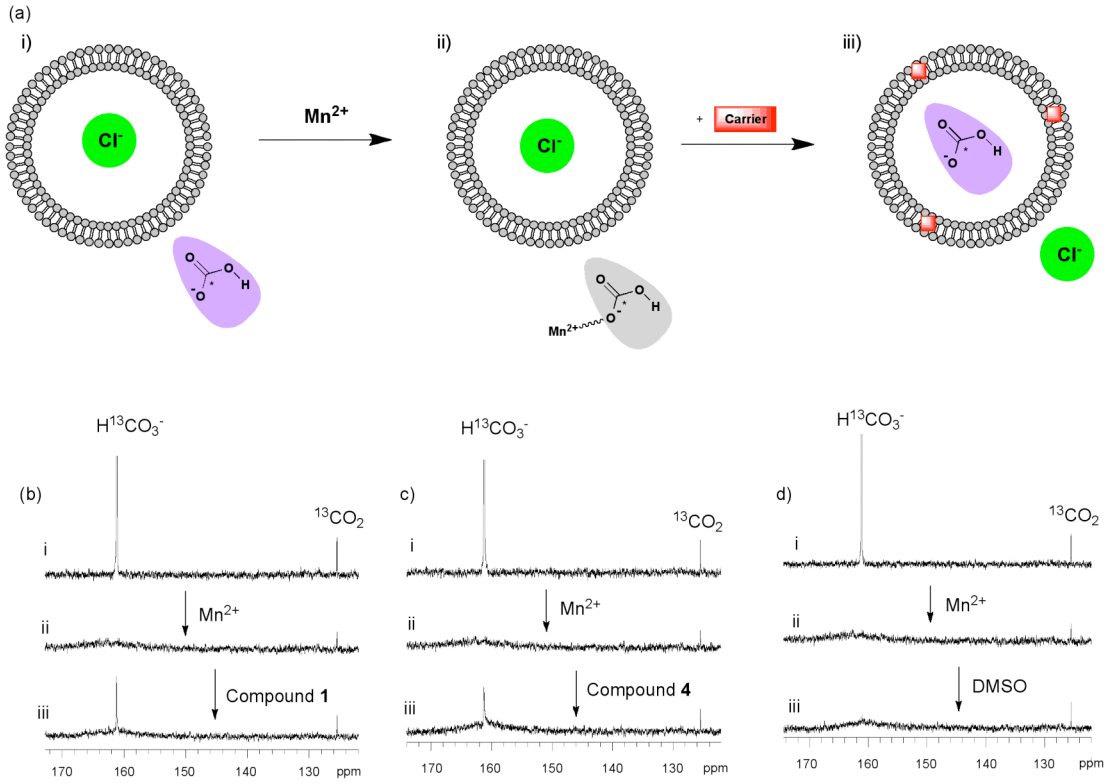

Figure 4. Carbon-13 NMR experiments demonstrate that both natural products and synthetic receptors are capable of chloride/bicarbonate antiport by allowing bicarbonate to enter the vesicles as shown by the HCO_3^- ^{13}C NMR resonance reappearing in the presence of paramagnetic $MnCl_2$ in the external solution. (a) A representation of titration sequence and ^{13}C NMR data (b-d) for monitoring transmembrane transport of HCO_3^- into Cl^--loaded EYPC liposomes by **1** and **4**: **i**) a $NaH^{13}CO_3$ pulse (50 mM) was added to EYPC vesicles loaded with 450 mM NaCl, 20 mM HEPES (pH 7.3) and dispersed in 150 mM Na_2SO_4, 20 mM HEPES (pH 7.3); **ii**) NMR spectra after addition of 0.5 mM Mn^{2+} (1:100 $Mn^{2+}/H^{13}CO_3^-$ ratio); **iii**) NMR spectra after addition of transporter or DMSO (**1** – 0.1 mol %, **4** – 1 mol % relative to lipid, or DMSO – 403 mol%).

Figure 5 shows data from another NMR experiment designed to verify transporter-mediated HCO_3^-/Cl^- exchange. In these experiments we monitored bicarbonate efflux from vesicles loaded with $H^{13}CO_3^-$ upon addition of transporters **1** or **4**. Thus, EYPC vesicles filled with $H^{13}CO_3^-$ and suspended in Na_2SO_4 solution were aged overnight at 4 ºC. Two ^{13}C NMR signals separated by 1 ppm (δ ~162 and ~161 ppm) were observed, corresponding to separate signals for intravesicular and extravesicular $H^{13}CO_3^-$ (Figure 5b-d). No leakage of $H^{13}CO_3^-$ from these vesicles occurred after addition of 50 mM NaCl. A DMSO solution of the transporters was then added to give ligand-to-lipid ratios of 0.1 mol % for **1** or 1 mol % for **4**. These transporters promote $Cl^-/H^{13}CO_3^-$ exchange, as confirmed by observation of only the NMR signal for extravesicular $H^{13}CO_3^-$ (Figure 5b/c). After addition of 0.5 mM Mn^{2+} (1:100 Mn^{2+}/

$H^{13}CO_3^-$), this $H^{13}CO_3^-$ signal was broadened into the baseline, confirming that all of the intravesicular $H^{13}CO_3^-$ ions had been exchanged into the extravesicular milieu (Figure 5b/c). A control experiment confirmed this interpretation (Figure 5d). Thus, after addition of DMSO, the separate signals for intravesicular and extravesicular $H^{13}CO_3$ remained unchanged. Addition of Mn^{2+} to this control sample erased the extravesicular $H^{13}CO_3^-$ signal, whereas the intravesicular $H^{13}CO_3^-$ signal remained intact since Mn^{2+} cannot cross the lipid membrane.

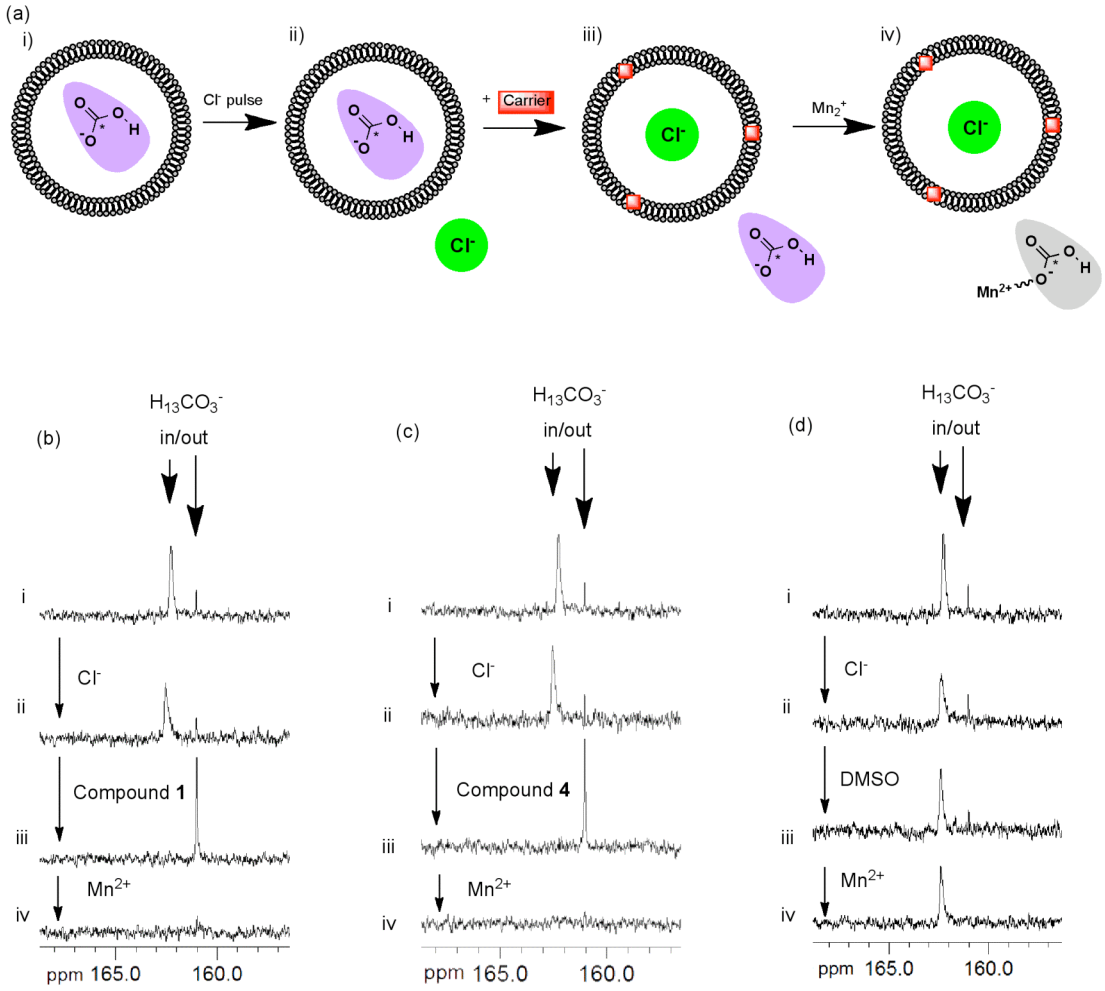

Figure 5. Carbon-13 NMR experiments demonstrate that both natural products and synthetic receptors are capable of chloride/bicarbonate antiport by releasing encapsulated bicarbonate as evidenced by the HCO_3^- ^{13}C resonance disappearing in the presence of paramagnetic $MnCl_2$ in the external solution. Representation of the titration sequence (a) and NMR stack plots (b-d) for monitoring the transmembrane transport of HCO_3^- ions in $H^{13}CO_3^-$-loaded EYPC liposomes by **1** and **4**. A 50 mM NaCl pulse was added to EYPC vesicles loaded with 100 mM $NaH^{13}CO_3$, 20 mM HEPES buffer (pH 7.5) and dispersed in 75 mM Na_2SO_4, 20 mM HEPES buffer (pH

7.3), and ^{13}C-NMR data was acquired before (**i**) and after (**ii**) the Cl^- pulse. NMR spectra were also collected after the addition of transporter or DMSO (**1** – 0.1 mol%, **4** – 1 mol%, or DMSO – 870 mol% (10 μL); **iii**), followed by addition of 0.5 mM Mn^{2+} (1:100 Mn^{2+}/Cl^- ratio; **iv**).

DISCUSSION

This work identifies "small" molecules that function as transmembrane bicarbonate carriers. The ion selective electrode assays showed that both prodigiosin **1** and synthetic isophthalamides **2-4** facilitate the release of encapsulated chloride from POPC phospholipid liposomes in the presence of trigonal planar oxoanions such as nitrate and bicarbonate. This efflux is produced *via* an exchange mechanism with external nitrate or bicarbonate anions.[29,31] Replacing nitrate with sulfate in the external medium resulted in no anion transport activity, as an anion exchange mechanism is not possible due to sulfate's hydrophilicity. An assay in which bicarbonate was added to the external sulfate medium showed that chloride transport mediated by **1-4** was restored, evidence that these compounds can extract the hydrophilic bicarbonate from aqueous solution into the interior of the phospholipid membrane, thus facilitating transmembrane HCO_3^-/Cl^- exchange. The ^{13}C NMR assays provided direct evidence for transmembrane bicarbonate transport, as the intra- and extra-vesicular ^{13}C-labelled bicarbonate populations could be distinguished. This NMR data, when combined with results from the Cl^--selective electrode experiments, firmly establish that compounds **1-4** enable the transmembrane exchange of Cl^-/HCO_3^- anions.

In conclusion, we have demonstrated that "small" molecules, including the natural product prodigiosin **1** and synthetic transporters **2-4**, facilitate the chloride/bicarbonate exchange process that is typically mediated by membrane proteins. This is the first report that prodigiosin **1** can catalyze chloride/bicarbonate antiport. This discovery may well present an alternative mechanism by which prodigiosin **1** can influence biological systems. Furthermore, prodigiosin **1** is more efficient at catalyzing Cl^-/HCO_3^- exchange than are synthetic isophthalamides **2-4**, suggesting that the tripyrrole unit with its array of hydrogen bonding donor and acceptor groups may be an excellent motif on which to base the synthesis of potent bicarbonate receptors and transporters. Such synthetic bicarbonate/chloride antiporters may prove to be useful tools for biomembrane research. Efforts aimed at producing improved and selective bicarbonate transporters and the investigation of their biological activity are underway in our laboratories.

METHODS

Preparation of Phospholipid Vesicles. A chloroform solution of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (20 mg/mL) (Genzyme) was evaporated *in vacuo* using a rotary evaporator and the lipid film obtained was dried under high vacuum for at least 2 hours. The lipid film was rehydrated by addition of a sodium

chloride solution (488 mM NaCl and 5 mM phosphate buffer, pH 7.2 or 451 mM NaCl and 20 mM phosphate buffer, pH 7.2) and followed by careful vortexing. The lipid suspension was then subjected to 9 freeze-thaw cycles and 29 extrusions through a 200 nm polycarbonate Nucleopore membrane using a LiposoFast Basic extruder (Avestin, Inc.) to obtain unilamellar vesicles. The vesicles were dialyzed against a $NaNO_3$ solution (488 mM $NaNO_3$ and 5 mM phosphate buffer, pH 7.2) or Na_2SO_4 solution (150 mM Na_2SO_4 and 20 mM phosphate buffer, pH 7.2) to remove unencapsulated NaCl.

ISE Transport Assays. Unilamellar vesicles (200 nm mean diameter) composed of POPC containing an encapsulated solution of 488 mM NaCl and 5 mM phosphate buffer pH 7.2 or 451 mM NaCl and 20 mM phosphate buffer pH 7.2, were suspended in a solution of 488 mM $NaNO_3$ and 5 mM phosphate buffer pH 7.2, or 150 mM Na_2SO_4 and 20 m M phosphate buffer pH 7.2, for a final lipid concentration of 1 mM. A DMSO solution of the carrier molecule, typically 10 μL to avoid influence of the solvent molecules in the assay, was added and the chloride release from vesicles was monitored using an Accumet chloride selective electrode for 7 minutes. At a time (t) = 5 min the vesicles were lysed with detergent (polyoxyethylene (8) lauryl ether) to release all chloride ions; the resulting value was considered to represent 100% release and used as such.

For the anion exchange assays in the vesicles suspended in a Na_2SO_4 solution a solution of $NaHCO_3$ or $NaNO_3$ was added for a final concentration of 40 mM at t = 2 min. The chloride efflux was monitored for another 5 minutes and at a time (t) = 7 min the vesicles were lysed with detergent (polyoxyethylene (8) lauryl ether) to release all chloride ions; the resulting value was considered to represent 100% release and used as such.

^{13}C NMR Assays

Materials. ^{13}C NMR spectra were recorded on a Bruker DRX500 instrument operating at 125.77 MHz, with chemical shifts reported in ppm. Egg yolk phosphatidylcholine (EYPC) lipids, nuclepore® polycarbonate membranes and membrane filters were purchased from Avanti Polar Lipids. Deuterated solvents were purchased from Cambridge Isotope Laboratories. Prodigiosin was a gift from the Development Theraputics Program at the National Cancer Institute, U. S. National Institutes of Health. All other chemicals were purchased from Sigma, Aldrich, Fisher, Fluka, or Acros and used without further purification.

Liposome Preparation for ^{13}C NMR Assays. Giant EYPC liposomes (5 μm) were prepared by evaporating a chloroform solution of EYPC (20 mg/mL) under reduced pressure, resulting in a thin film that was dried *in vacuo* overnight. The liposomes were then formed by rehydrating the lipid film with either 450 mM NaCl in 20 mM HEPES (pH 7.3) buffer (for the liposomes described in Figure 4 of main text, i.e., the Cl^--loaded liposomes), or 100 mM $NaH^{13}CO_3$ in 20 mM HEPES (pH 7.5) buffer (for

the liposomes described in Figure 5, i.e., the HCO_3^--loaded liposomes). Buffer solutions were prepared in a 9:1 H_2O/D_2O mixture. After 5 freeze/thaw cycles, the liposomes were extruded through a 5 μm polycarbonate membrane 41 times at room temperature using a high-pressure mini-extruder (Avanti). For the Cl^--loaded liposomes, the giant liposome suspension obtained after extrusion was used without further purification in the ^{13}C NMR transport assays. However, for the HCO_3^--loaded liposomes, the resulting giant liposome suspension was separated from extravesicular $NaH^{13}CO_3$ by size exclusion chromatography (SEC) (stationary phase: Sephadex G-25, mobile phase: 9:1 H_2O/D_2O, 20 mM HEPES, pH 7.3, 75 mM Na_2SO_4). The 30 mL suspension ($NaH^{13}CO_3$ inside, Na_2SO_4 outside) collected was centrifuged (Eppendorf Centrifuge 5804R) at 10,000 rpm for 30 minutes followed by the removal of the non-liposome containing buffer. The recovered giant liposome suspension was then diluted with the 75 mM Na_2SO_4 mobile phase buffer and used directly in the ^{13}C NMR transport assays. The stock concentrations obtained for the liposomes were 90.3 mM for the Cl^--loaded liposomes (assuming 100% lipid retention after extrusion) and 66.6 mM for the HCO_3^--loaded liposomes (assuming 80% lipid retention after gel filtration) respectively.

Bicarbonate Transport in Cl^--Loaded Liposomes Monitored by ^{13}C NMR. ^{13}C NMR spectra were recorded using a Bruker DRX500 spectrometer with a 5 mm broad band probe operating at 125.77 MHz, with chemical shifts reported in ppm. The instrument was locked on 9:1 H_2O/D_2O. Experimental conditions were: acquisition time, 0.93 s; spectrum width, 35211 Hz; 90º pulse width, 6.70 μs; relaxation delay, 0.2 s; number of scans, 160; temperature, 27 ºC. For each experiment, 230 μL of stock (90.3 mM) liposome solution was mixed with 340 μL of 150 mM Na_2SO_4 in 20 mM HEPES (pH 7.3) buffer in a 5 mm NMR tube to give a liposome suspension containing NaCl inside, and Na_2SO_4 outside. A $NaH^{13}CO_3$ pulse was then added to the mixture to give 35 mM and 50 mM final concentrations of liposome and $H^{13}CO_3^-$ respectively. The ^{13}C NMR of the preceding liposome mixture (NaCl inside, Na_2SO_4 and $NaH^{13}CO_3$ outside) was then taken. After data acquisition, a solution of $MnCl_2$ was added to give a final Mn^{2+} concentration of 0.5 mM (1:100 $Mn^{2+}/H^{13}CO_3^-$ ratio), and immediately followed by another set of data acquisition. Finally, a final set of ^{13}C NMR data was collected after the addition of a solution of the ligand (**1** or **4**) or DMSO to the mixture. Isophthalamide **4** was added in a 1 mol% ligand-to-lipid ratio, while prodigiosin **1** was added in a 0.1 mol% ligand-to-lipid ratio. For the DMSO control, 6 μL of the solvent was added corresponding to a 403 mol% DMSO-to-lipid ratio.

Bicarbonate Transport in HCO_3^--Loaded Liposomes Monitored by ^{13}C NMR. Instrument details are the same as described above for the Cl^--loaded liposomes. The instrument was locked on 9:1 H_2O/D_2O. Experimental conditions were: acquisition time, 0.93 s; spectrum width, 35211 Hz; 90º pulse width, 6.70 μs; relaxation delay, 0.2 s; number of scans, 196; temperature, 27 ºC. For each experiment, an initial ^{13}C NMR spectrum of 520 μL of the giant liposome solution was acquired. Then, a NaCl

pulse resulting in final extravesicular concentrations of 58 mM and 50 mM for the giant liposomes and Cl^- respectively was added to the NMR tube. The ^{13}C NMR of the preceding liposome mixture ($NaH^{13}CO_3$ inside, Na_2SO_4 and NaCl outside) was taken followed by the addition of a solution of the ligand (**1** or **4**) or DMSO to the cocktail. Again, isophthalamide **4** was added in a 1 mol% ligand-to-lipid ratio, while prodigiosin **1** was added in a 0.1 mol% ligand-to-lipid ratio. For the DMSO control, 10 μL of the solvent was added corresponding to an 870 mol% DMSO-to-lipid ratio. A ^{13}C NMR spectrum of the ligand-containing cocktail was then acquired before and after the addition of a solution of $MnCl_2$ (0.5 mM final Mn^{2+} concentration corresponding to 1:100 Mn^{2+}/Cl^- ratio).

ACKNOWLEDGMENTS

J.T.D. and P.A.G. thank the NSF/EPSRC for funding this research through the "International Collaboration in Chemistry" program under CHE-0822244 and EP/G002576/1. R.Q. thanks the Spanish Ministerio de Educación y Ciencia for support "Jose Castillejo" mobility grant and "Ramon y Cajal" contract. T.T. thanks DGI of Spain (CTQ2006-15456-C04-04BQU) for funding. O.A.O thanks the University of Maryland Graduate School for the Mabel Spencer Fellowship award. P.A.G thanks Christine C. Tong for the mass spectrometry study with prodigiosin and Matthew G. Fisher for fitting the NMR titration data with prodigiosin.

AUTHOR CONTRIBUTIONS

J.T.D., P.A.G. and R.Q conceived this project, experiments, analyzed data and prepared the manuscript; O.A.O. and R.Q. conducted experiments and analyzed data; P.P. and T.T. contributed reagents, materials, analysis tools and supervised the synthesis; J.C.I.S. and R.Q. synthesized new compounds.

References

1. Cleland, W. W., Andrews, T. J., Gutteridge, S., Hartman, F. C. & Lorimer, G.H. Mechanism of Rubisco: The Carbamate as General Base. *Chem. Rev.* **98**, 549-561 (1998).
2. Hempling, H.G. Intracellular Water and the Regulation of Cell Volume and pH. in *Principles of Medical Biology* Eds: Bittar, E.E. & Bittar, N. Elsevier 1996.
3. Lipscomb, W. N. & Strater, N. Recent Advances in Zinc Enzymology. *Chem. Rev.* **96**, 2375-2433 (1996).
4. Florman, H. M., Jungnickel M. K. & Sutton, K.A. What can we learn about fertilization from cystic fibrosis? *Proc. Nat. Acad. Sci.-USA* **104**, 11123-11124 (2007).
5. Norris, F.A. & Powell, G.L., Characterization of CO_2/carbonic acid mediated proton flux through phosphatidylcholine vesicles as model membranes. *Biochim & Biophys. Acta.* **1111**, 17-26 (1992).

6. Fujinaga, J., Loiselle, F. B. & Casey, J. R. Transport activity of chimaeric AE2-AE3 chloride/bicarbonate anion exchange proteins. *Biochem. J.* **371**, 687-696 (2003).
7. Choi, J. Y. *et al.* Aberrant CFTR-dependent HCO_3^- transport in mutations associated with cystic fibrosis. *Nature* **410**, 94-97 (2001).
8. Casey, J. R. Why Bicarbonate? *Biochem. Cell Biol.* **84**, 930-939 (2006)
9. Koropatkin, N.M., Koppenaal, D.W., Pakrasi, H.B. & Smith, T.J. The Structure of a Cyanobacterial Bicarbonate Transport Protein, CmpA. *J. Biol. Chem.* **282**, 2606-2614 (2007).
10. Koulov, A.V. *et al.* Chloride transport across vesicle and cell membranes by steroid-based receptors. *Angew. Chem. Int. Ed.* **42**, 4931-4933 (2003).
11. Davis, A.P., Sheppard, D.N. & Smith, B.D. Development of synthetic membrane transporters for anions. *Chem. Soc. Rev.* **36**, 348-357 (2007)
12. Okunola, O.A., Seganish, J.L., Salimian, K.J., Zavalij, P.Y. & Davis, J.T. Membrane-active calixarenes: toward 'gating' transmembrane anion transport. *Tetrahedron* **63**, 10743-10750 (2007).
13. You, L. & Gokel, G.W. Fluorescent, synthetic amphiphilic heptapeptide anion transporters: Evidence for self-assembly and membrane localization in liposomes. *Chem. Eur. J.* **14**, 5861-5870 (2008).
14. Izzo, I. *et al.* Cationic calix[4]arenes as anion-selective ionophores. *Chem. Commun*. 2986-2988 (2008).
15. Whitmarsh, S.D., Redmond, A.P., Sgarlata, V. & Davis A.P. Cationic cyclocholamides; toroidal facial amphiphiles with potential for anion transport. *Chem. Commun*. 3669-3671 (2008).
16. Gale, P.A. *et al.* Co-transport of H^+/Cl^- by a synthetic prodigiosin mimic, *Chem. Commun.* 3773-3775 (2005).
17. Gale, P. A., Garric, J., Light, M.E., McNally, B.A. & Smith, B.D. Conformational control of HCl co-transport: imidazole functionalised isophthalamide *vs.* 2,6-dicarboxamidopyridine. *Chem. Commun.* 1736-1738 (2007).
18. Fürstner, A. Chemistry and biology of roseophilin and the prodigiosin alkaloids: A survey of the last 2500 years. *Angew. Chem., Int. Ed.,* **42**, 3582-3603 (2003).
19. Gerber, N.N. Prodigiosin-like pigments. *Crit. Rev. Microbiol.* **3**, 469-485 (1975).
20. Bennett, J.W. & Bentley R. Seeing red: The story of prodigiosin. *Adv. Appl. Microbiol.* **47**, 1-32 (2000).
21. Williamson, N.R. *et al.* Anticancer and immunosuppressive properties of bacterial prodiginines. *Future Microbiol*., **2**, 605–618 (2007).
22. Nguyen, M. *et al.* Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. *Proc. Natl. Acad. Sci. U. S. A.*, **104**, 19512–19517 (2007).
23. Yamamoto, C. *et al.* Cycloprodigiosin hydrochloride, a new H^+/Cl^- symporter, induces apoptosis in human and rat hepatocellular cancer cell

lines *in vitro* and inhibits the growth of hepatocellular carcinoma xenografts in nude mice. *Hepatology* **30**, 894-902 (1999).

24. Tanigaki, K. *et al.* BE-18591 as a new H^+/Cl^- symport ionophore that inhibits immunoproliferation and gastritis. *FEBS Lett.* **524**, 37-42 (2002).
25. T. Sato, *et al.*, Prodigiosins as a New Group of H^+/Cl^- symporters That uncouple proton translocators. *J. Biol. Chem.*, **273**, 21455-21462 (1998).
26. Gottlieb, R.A., Nordberg, J., Skowronski, E. & Babior, B.M. Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 654-658 (1996).
27. S. Ohkuma, *et al.* Prodigiosins uncouple vacuolar-type ATPase through H^+/Cl^- symport. *Biochem. J.* **334**, 731-741 (1998).
28. Sessler, J.L. *et al.* Synthesis, anion-binding properties, and *in vitro* anticancer activity of prodigiosin analogues. *Angew. Chem. Int. Ed.* **44**, 5989-5992 (2005).
29. Seganish, J.L. & Davis, J.T. Prodigiosin is a chloride carrier that can function as an anion exchanger. *Chem. Commun.,* 5781-5783 (2005).
30. Diaz, R.I.S. *et al.* Chloride anion transport and copper-mediated DNA cleavage by C-ring functionalized prodigiosenes. *Chem. Commun.* 2701-2703 (2007).
31. Santacroce, P.V. *et al.* Conformational Control of Transmembrane Cl^- Transport. *J. Am. Chem. Soc.* **129**, 1886-1887 (2007).
32. Kavallieratos, K.; Bertao, C.M. & Crabtree R.H. Hydrogen bonding in anion recognition: A family of versatile, non-preorganized neutral and acyclic receptors. *J. Org. Chem.* **64**, 1675-1683 (1999).
33. Li, X., Shen, B., Yao, X.Q. and Yang, D. A Small Synthetic Molecule Forms Chloride Channels to Mediate Chloride Transport across Cell Membranes. *J. Am. Chem. Soc.* **129**, 7264-7265 (2007).
34. Melvin, M.S. *et al.* Influence of the A-Ring on the Proton Affinity and Anticancer Properties of the Prodigiosins, *Chem. Res. Toxicol.* **15**, 734-741 (2002).
35. Stefayne, D. Halochromism studies on prodigiosin. *J. Org. Chem.* **25**, 1261-1262 (1960)
36. Rizzo, V., Morelli, A., Pinciroli, V., Sciangula, D. & D'Alessio, R. Equilibrium and Kinetics of Rotamer Interconversion in Immunosuppressant Prodigiosin Derivatives in Solution, *J. Pharm. Sci.* **88**, 73-78 (1999).
37. Hofmeister, F. Zur Lehre von der Wirkung der Salze, Zweite Mittheilung. *Arch. Exp. Pathol. Pharmakol.* **24**, 247-260 (1888).
38. Steinle, E.D., Schaller, U. & Meyerhoff, M.E. Response Characteristics of Anion-Selective Polymer membrane Electrodes Based on Gallium(III), Indium(III) and Thallium(III) Porphyrins, *Anal. Sciences*, **14**, 79-84 (1998).

39. Riddell, F.G., Arumugam, S. & Patel, A. Chloride transport through model biological membranes studied by ^{35}Cl NMR. *Chem. Commun.* 74-76 (1990).
40. Riddell, F.G. & Zhou, Z. Mn^{2+} as a contrast reagent for NMR studies of $^{35}Cl^-$ and $^{81}Br^-$ transport through model biological membranes, *J. Inorg. Biochem.* **55**, 279-293 (1994).
41. Gout, E. Bligny, R., Pascal, N. & Douce, R. ^{13}C nuclear magnetic resonance studies of malate and citrate synthesis and compartmentation in higher plant cells, $^{81}Br^-$ transport through model biological membranes, *J. Biol. Chem.* **268**, 3986-3992 (1993).
42. Chang, K. & Roberts, J.K.M. Quantitation of rates of transport, metabolic fluxes, and cytoplasmic levels of inorganic carbon in maize root tips during K^+ ion uptake, *Plant Physiol.* **99**, 291-297 (1992).