pH Regulated Non-electrogenic Anion Transport by Phenylthiosemicarbazones

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ABSTRACT: Gated ion transport across biological membrane is an intrinsic process regulated by protein channels. Synthetic anion carriers (anionophores) have potential applications in biological research, however, previous reported examples are mostly nonspecific, capable of mediating both electrogenic and electroneutral (non-electrogenic) transport processes. Here, we show the transmembrane Cl‒ transport studies of synthetic phenylthiosemicarbazones mimicking the function of acid-sensing (proton-gated) ion channels. These anionophores have remarkable pH-switchable transport properties with up to 640-fold increase in transport efficacy on going from pH 7.2 to 4.0. This “gated” process is triggered by protonation of the imino nitrogen and concomitant conformational change of the anion binding thiourea moiety from *anti* to *syn*. By using a combination of two cationophore-coupled transport assays, with either monensin or valinomycin, we have elucidated the fundamental transport mechanism of phenylthiosemicarbazones which is shown to be non-electrogenic, inseparable H+/Cl‒ cotransport. This study demonstrates the first examples of pH-switchable non-electrogenic anion transporters.

INTRODUCTION

Transmembrane ion transport processes facilitated by protein channels and carriers across biological lipid bilayer membranes are essential for life.1 There has been much effort devoted to the development of small molecule anion carriers that have potential as future treatments for diseases such as cystic fibrosis or cancer.2 Prodigiosin is a natural product, best known as one of the most potent anion transporters that facilitate H+/Cl‒ cotransport, hence the transport of chloride can dissipate a transmembrane pH gradient (Figure 1).3 pH gradient dissipation has been observed within cancer cells and may be a trigger for apoptosis.4 The transport of protons and chloride are inseparable because protonated prodigiosin is unable to diffuse through the lipid bilayer in the absence of chloride5 (or another bound anion, e.g. Cl‒/NO3‒ exchange6); thus the effect of prodigiosin is non-electrogenic.5 There are other classes of structurally similar transporters that function in a similar fashion to prodigiosin, these include the tambjamines,7 perenosins8 and others.9 In seminal work by Pérez-Tomás, Quesada et al., this class of anionophores induced the combined effect of cytosolic acidification and hyperpolarization of cellular membranes on cancer stem cells, leading to selective elimination of affected cell population.7b

Prodigiosin and other similar classes of H+/Cl‒ cotransporters are active across the pH range of 4‒7, therefore it would be very beneficial for biological studies if synthetic carriers could facilitate H+/Cl‒ cotransport explicitly in acidic intracellular organelles such as lysosomes. Furthermore, transporters that can effectively switch ON in an acidic environment, but OFF at neutral pH would mimic the function of acid-sensing (proton-gated) ion channels.10 There are only a few reports of pH-dependent transporters,11 and carriers that possess a highly specific pH influenced ON/OFF function are still unavailable. Our group, in collaboration with Jolliffe and co-workers, has previously reported thiosquaramides and an oxothiosquaramide as pH-dependent anionophores.12 However, these carriers can mediate electrogenic transport, hence are capable of depolarizing membrane potential;13 unfortunately, this is undesirable for certain cellular studies.14 The challenge is to develop pH-switchable anionophores with truly prodigiosin-like transport properties.



**Figure 1.** Prodigiosin mediated H+/Cl‒ cotransport (symport) showing binding of chloride upon protonation at the inner membrane interface of liposomes, translocation as a neutral complex [Prod•H+⊃Cl‒], and dissociation of H+/Cl‒ to the external bulk, resulting in dissipation of pH gradient.

Phenylthiosemicarbazones are structurally similar to phenylthioureas, however they contain an additional imine group directly adjacent to the thiourea anion binding site.15 Herein, we report three phenylthiosemicarbazones **1**‒**3** (Figure 2) as a new class of anionophores that have excellent pH-switchable anion transport properties. In order to evaluate the effect of electron donating or withdrawing substituents on the *para*-position of the phenyl ring, the unsubstituted **1** (σp 0.0), methoxy **2** (σp ‒0.27), and trifluoromethyl **3** (σp 0.54) were synthesized. The Hammett constant (σ)16 is a descriptor of the electron withdrawing or donating effect of a specific substituent; it has been used extensively to correlate the hydrogen bond donor acidity.17 Chloride anion binding and transmembrane transport activities of 1-hexylidene-4-phenylthiosemicarbazones **1**‒**3** were compared with the analogous 1-hexyl-3-phenylthioureas **4**‒**6**,17c and squaramides **7**‒**9**.12,18 Thiosemicarbazones **1**‒**3** were found to be more effective pH-switchable anion transporters (from neutral to acidic environment) than previously reported oxothiosquaramide **8** and thiosquaramide **9**. More importantly, we have shown that chloride and proton transport by thiosemicarbazones are inseparable electroneutral processes (elucidated by using the newly developed cationophore-coupled KCl efflux assays from our most recent work)19 in the same fashion as the H+/Cl‒ symport properties of prodigiosin. Therefore, we have developed the first examples of a new class of pH-switchable non-electrogenic anion transporters.

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**Figure 2.** Structures of anionophores used in this study: thiosemicarbazones **1**‒**3**, phenylthioureas **4**‒**6**, squaramides **7**‒**9**, and phenylamino-thiadiazole **10**.

RESULTS AND DISCUSSION

**Synthesis, solid-state structural analysis and anion binding studies.** Thiosemicarbazones **1**‒**3** were synthesized by the condensation of the corresponding phenylthiosemicarbazides with hexanal in absolute ethanol, with yields of 47‒52% after recrystallization from ethanol/pentane mixture. Phenylthiosemicarbazides (OCH3 and CF3 analogues) were prepared by the reaction of hydrazine monohydrate with the appropriate phenyl isothiocyanate in isopropanol. The rearrangement of thiosemicarbazones to 5-membered heterocyclic thiadiazoles is well documented;20 therefore, 2-phenylamino-5-pentyl-1,3,4-thiadiazole **10** was prepared from **1** via an oxidative cyclization and used as a control in the transmembrane transport studies.

The resolved X-ray structure of **10** confirmed the thiadiazole heterocyclic structure (see Supporting Information). Solid-state structures of all three thiosemicarbazones **1**‒**3** revealed that the thiourea moieties adopt a flat planar *anti*-conformation, presumably due to an intramolecular hydrogen bond (H-bond) between the thiourea-N(1)H and the π-electrons of imino-N(3), as shown in Figure 3a for compound **3** (see Supporting Information for structures of **1** and **2**). Additionally, crystal packing interactions of thiosemicarbazones are mainly established via H-bonds between thiourea N(2)H**···**S, and an imine N=CH**···**S interaction.



**Figure 3.** (a) Single-crystal X-ray structure of **3**: ORTEP diagram showing 50% probability anisotropic displacement ellipsoids at 100(2) K; inset: perspective packing diagram of two molecular structures, all H atoms are omitted for clarity, except thiourea-H and imine-NCH, showing intermolecular H-bonds (purple dash), selected H-bond (donor-acceptor) distances (Å): N(2)···S(1) 3.352(1), N(3)=C···S(1) 3.688(2). (b) Schematic equilibria of thiosemicarbazones binding conformation towards chloride anion.

1H NMR titration studies of **1**‒**3** in DMSO-*d*6/0.5% H2O with tetrabutylammonium (TBA) chloride salt did not result in an observable change in the chemical shifts (Δδ), indicative of no binding. When using acetone-*d*6 as a less competitive solvent,21 titration of **1**‒**3** with TBA-Cl induced a downfield Δδ on the resonance signals of thiourea-NHβ and imine-NCH, indicating Cl‒ binding to the thiosemicarbazones via a cleft formed by NH/CH hydrogen donors. This suggests that the conventional anion binding thiourea moiety is ‘locked’ by the intramolecular H-bond interaction between NHα and the π-electrons of imino nitrogen (Figure 3b). The resulting *K*a (Table 1) derived from global fitting analysis22 revealed weak binding towards both Cl‒ and NO3‒ with similar trends (**3** > **1** ≈ **2**). Expectedly, the binding of phenylthioureas **4**‒**6** to Cl‒ in acetone-*d*6 was significantly stronger than previously reported studies in DMSO-*d*6/0.5% H2O mixture, and the derived *K*a for binding to both Cl‒ and NO3‒ were two to three orders of magnitude higher than the analogous thiosemicarbazones. The binding of Cl‒ and NO3‒ to squaramide **7** in acetone-*d*6 was very strong, whilst *K*a for Cl‒ was too strong (> 105 M‒1) to be accurately determined by 1H NMR titration techniques.22a

Table 1. Overview of association constants (*K*a) for the complexation of 1‒9 toward Cl‒ and NO3‒ (as TBA salts), obtained from 1H NMR titrations (400 MHz) at 298 K in DMSO-*d*6/0.5% H2O and acetone-*d*6; Cl‒/NO3‒ transport activity at pH 7.2 and 4.0 obtained from Hill analysis and calculated log *P* values.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Compound | *K*a Cl‒ [M‒1]  DMSO-*d*6 | *K*a Cl‒ [M‒1]  acetone-*d*6 | *K*a NO3‒ [M‒1]  acetone-*d*6 | Cl‒/NO3‒ at pH 7.2 | |  | Cl‒/NO3‒ at pH 4.0 | |  | *EC*50(pH7.2)/ *EC*50(pH4.0)*b* | c log *Pc* |
| *EC*50 [mol%]*a* | *na* |  | *EC*50 [mol%]a | *n*a |  |
| **1** | -*d* | 16*e* | 3.3*e,h* | 4.7 | 2.3 |  | 0.0074 | 1.3 |  | 640 | 3.81 (0.52) |
| **2** | -*d* | 18*e* | 3.2*e,h* | 1.8 | 1.3 |  | 0.0073 | 1.2 |  | 250 | 3.74 (0.58) |
| **3** | -*d* | 31*e* | 4.3*e,h* | >> 10*j* | -*j* |  | 0.13 | 0.7 |  | >> 77 | 4.70 (0.52) |
| **4** | 13*k* | 9.2 × 103*f* | 250*f* | 2.7*k* | 0.9*k* |  | 2.2 | 0.9 |  | 1.2 | 3.63 (0.35) |
| **5** | 11*k* | 3.8 × 103*f* | 120*f* | 5.5*k* | 1.3*k* |  | 4.3 | 1.0 |  | 1.3 | 3.64 (0.42) |
| **6** | 26*k* | 2.5 × 104*f* | 780*f* | 0.44*k* | 1.7*k* |  | 0.51 | 1.3 |  | 0.86 | 4.62 (0.31) |
| **7** | 460*k* | > 105 *g,i* | 1.7 × 104 *g* | 0.065*k* | 1.2*k* |  | 0.077*k* | 1.3*k* |  | 0.84 | 4.43 (0.90) |
| **8** | 60*k* | -*m* | -*m* | 0.68*k* | 0.8*k* |  | 0.013*k* | 6.3*k* |  | 52 | 5.39 (0.69) |
| **9** | 470*l* | -*m* | -*m* | 0.22*l* | 1.9*l* |  | 0.027*l* | 2.6*l* |  | 8.1 | 4.94 (0.66) |

*a*Chloride efflux (Cl‒/NO3‒ antiport) measured by chloride-ISE from POPC LUVs (mean diameter 200 nm) loaded with NaCl (~500 mM) and suspended in NaNO3 (~500 mM), buffered to pH 7.2 and 4.0 with phosphate (5.0 mM) and citrate (5.0 mM) sodium salts respectively. Hill analysis was performed to obtain the effective concentration to achieve 50% Cl‒ efflux (*EC*50) at 270 s for each carrier, shown as carrier:lipid molar percent; and Hill coefficient (*n*) reveals the stoichiometry of active carrier*n*:anion supramolecular complex mediating the transmembrane transport.23 *bEC*50(pH7.2)/*EC*50(pH4.0); an indicator for pH-switchable transport efficacy. *c*Averaged log *P* values (with error in parentheses) calculated via VCCLab.24 *d*No change in Δδ observed. *eK*a derived from global fitting analysis of Δδ for NHβ and NCH signals; *f*or NHα and NHβ signals; *g*or NH and ArH signals to the 1:1 binding model.22 *h*Very weak binding (*K*a < 5 M‒1), association constant values may be inaccurate. *i*Very strong binding (*K*a > 105 M‒1), the calculated association constant is beyond the upper limit that can be reliably obtained from 1H NMR titration studies.22a *j*Too inactive for Hill analysis, 10 mol% loading of **3** resulted in 15% Cl‒ efflux at 270 s. *k*Previously reported by Busschaert et al.12a,17c,18 *l*Previously reported by Elmes et al.12b *m*Not determined.

**pH regulated anion transport studies.** The pH-dependent chloride transport activities of compounds **1**‒**3** and **10** were investigated using the same liposome-based anion exchange assay (Cl‒/NO3‒) reported for anionophores **4**‒**9**.12,17c Briefly, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) large unilamellar vesicles (LUVs, mean diameter 200 nm) were loaded with NaCl (~500 mM) and suspended in NaNO3 (~500 mM), buffered to pH 4.0, 5.0, 6.0, 7.2 or 10.0 with appropriate buffer reagents (5.0 mM). The Cl‒/NO3‒ exchange process was initiated by addition of carriers in a DMSO solution, and the rate of Cl‒ efflux to the external bulk was monitored using a chloride ion selective electrode (ISE).

The results of the pH-dependent anion transport studies at pH 4.0 and 7.2 are shown in Figure 4 (see Supporting Information Section S7.2 for transport rates at other pH values). The anion transport activities of thiosemicarbazones **1**‒**3** (1.0 mol%) are clearly regulated by pH, significantly enhanced (switches ON) when going from neutral (pH 7.2) to acidic (pH 4.0) media. Compound **10** (control) did not mediate any significant transport activity at the pHs studied (See Supporting Information Figure S45). A control Cl‒/SO42‒ exchange assay in POPC liposomes, and Cl‒/NO3‒ exchange assay in POPC:cholesterol (7:3) liposomes were studied with **1**‒**3** (1.0 mol%) at pH 4.0 (see Supporting Information Section S7.4‒7.5 for details and brief discussion).

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**Figure 4.** Plot of Cl‒ efflux from POPC LUVs (mean diameter 200 nm) at pH 7.2 and 4.0, facilitated by thiosemicarbazones **1**‒**3** (1.0 mol%, carrier:lipid) monitored over a period of 5 min using chloride-ISE. Detergent was added to lyse the vesicles at 300 s, to release remaining encapsulated Cl‒ for the 100% chloride concentration at 420 s. Anionophores were added as DMSO solutions (10 μL) to the POPC LUVs suspension (5.0 mL), and lipid concentration used was 1.0 mM. Solid and dash lines drawn through the data points are fitted curves to calculate the initial rate of Cl‒ efflux; error bars corresponding to the SD from three repeats. DMSO was used as a control. Vertical dotted lines indicating the enhanced transport activities from pH 7.2 to 4.0.

Dose-response studies of anionophores at different concentrations were carried out to perform Hill analysis.23a This was to obtain the Hill coefficient (*n*), which indicates the stoichiometry of active carrier*n*:anion supramolecular complex mediating transport and the effective concentration of transporter required to achieve 50% anion transport at 270 s (*EC*50) for the quantitative transport activity.23b,c Hill analyses for **1**‒**3** were performed at both pH 7.2 and 4.0, and **4**‒**6** at pH 4.0 only. In order to evaluate the neutral to acidic pH-switchable transport efficacy of the anionphore, we also reported the ratio of *EC*50 at pH 7.2 to *EC*50 at pH 4.0. The Hill analysis derived results for **1**‒**9** are summarized in Table 1.

In acidic aqueous media, the transport activities of thiosemicarbazones switches ON; as a result, **1** and **2** became the most potent anionophores at pH 4.0 from this study, with extremely low *EC*50 values of 0.0074 and 0.0073 mol%. The Hill coefficients obtained for **1**‒**3** are mostly ~1, except for **1** at pH 7.2 which has a Hill coefficient of ca. 2, suggesting a different binding mode for anion transport by **1**; i.e. 1:1 complex at pH 4.0 (**1**:Cl‒) and 2:1 complex at pH 7.2 (**1**2:Cl‒). Interestingly, the pH-switchable properties of thiosemicarbazones are significantly better than **8** and **9**, as **1** emerged as the best pH-switchable transporter with a remarkable *EC*50(pH7.2)/*EC*50(pH4.0) ratio of 640, followed by **2** and **3** with 250 and >>77 respectively, all larger than those of **8** (51-fold) and **9** (8-fold). As anticipated, there were negligible differences in the transport activities of phenylthioureas **4**‒**6** between pH 7.2 and 4.0 (*EC*50(pH7.2)/ *EC*50(pH4.0) ≈ 1), similar to squaramide **7**.

**Conformational control by protonation on imino nitrogen.** Previously reported compounds **8** and **9** have low p*K*a values of 5.3 and 6.6 respectively, hence the squaramide NH groups are deprotonated at neutral pH.12 However, the thiourea NH groups of **1**‒**3** are much less acidic (p*K*a > 11, see Supporting Information), therefore the pH-switchable transport mechanism of the thiosemicarbazones is likely due to protonation of the imine group, triggering a conformational change. It is well documented that thiosemicarbazones can complex with transition metals *via* the thiourea sulfur and imino nitrogen atoms,25 and this can facilitate the rearrangement of the thiourea moiety to the favorable *syn*-conformation.15b p*K*b (protonation of imine) measurements attempted using potentiometric methods were unsuccessful, possibly due to the values being too low. Results from 2-D 1H‒1H NOESY NMR studies of **1**‒**3** in DMSO-*d*6 are consistent with the *anti*-conformation observed in the solid-state X-ray structures. In the presence of one equivalent of tetrafluoroboric acid (HBF4) as a proton source, chemical exchange between the NH resonances of thiourea leads us to suggest that protonation of imine ‘unlocks’ the intramolecular (NHα**···**N=C) H-bond interaction (see Supporting Information Section S8 and Figures S65‒70 for details and brief discussion).



**Figure 5.** Selected partial 1H NMR (400 MHz, 298 K) spectra of compound **1** (11.2 mM) in (CD3)2CO, titrated with aliquots of HBF4•(CH3CH2)2O solution, showing the slow exchange speciation of the neutral and protonated states of **1**. “\*” and “Et2O” denote peaks of NMR solvent and diethyl ether, respectively.

In acetone-*d*6, 1H NMR titration studies with HBF4 resulted in equimolar protonation of **1**‒**3**, observable from the slow exchange between the neutral and protonated states (Figure 5, see Supporting Information Figures S71‒73 for more details). Proton chemical exchange between the protonated imino-NH and the proximal thiourea-NHβ with H2O in acidic environment was too fast to be observed on the NMR time scale.26 Nonetheless, the apparent changes in chemical shifts of the aromatic-CHs, thiourea-NHα, imine-CH and methylene-CH2 signals are evidence that lead us to suggest that the protonation of imino nitrogen perturbed the *anti-*conformation of the neutral state. The reversibility between the neutral and protonated states of **1**‒**3** was firmly established by 1H NMR titration studies with HBF4 (towards saturation of protonated state at one equivalent), followed by titration with tetrabutylammonium hydroxide up to one equivalent to achieve full recovery of the neutral state (see Supporting Information Figures S75‒77 for details). It should be noted that free aldehyde (hexanal) was not observed during the NMR studies, providing evidence that the protonated thiosemicarbazones do not undergo imine hydrolysis during these experiments (see Supporting Information Figure S74). Furthermore, UV-vis studies of **1**‒**3** at pH 4.0 demonstrated that these thiosemicarbazones are stable in the presence of liposomes, monitored over 30 min (see Supporting Information Section S10 and Figures S78‒85 for details and brief discussion), and likewise corroborate the stability of **1**‒**3** over the duration of the anion transport experiments.

To gain further structural insights, density functional theory (DFT) calculations (SMD-M06-2X/6-31+G(d))27 were performed for **1**‒**3** (neutral and protonated) and their Cl‒ complexes. The optimized structures for all three thiosemicarbazones gave the same outcomes; therefore, only structures of **1** are illustrated in Figure 6. While the neutral state of **1** showed the same *anti*-conformation, the host-guest complex of **1⊃**Cl‒ correlates with the 1H NMR binding studies. The structure of **1**•H+ revealed that protonation of the imino nitrogen perturbs the intramolecular (NHα**···**N=C) H-bond. Furthermore, the proximal planar arrangement of the imino hydrogen towards the thiourea sulfur suggests an intramolecular N+H**···**S H-bond, which contributes to the stabilization of the *syn*-conformation. As a result, the protonated state of **1** binds Cl‒ via three H-bonds to form an overall neutral complex **1**•H+**⊃**Cl‒. Calculated binding energies in gas phase revealed stronger Cl‒ binding towards the protonated states of **1**‒**3** compared to the neutral species (see Supporting Information Section S11 for details and discussion).This conformational control mechanism via protonation emulates the intrinsic allosteric properties of acid-sensing (proton-gated) ion channel proteins.10



**Figure 6.** DFT (M06-2X/6-31+G(d)) optimized structures of **1** in neutral and protonated states, and the respective Cl‒ complexes, with intramolecular and intermolecular H-bonds shown as purple dash.

We also carried out high-level *ab initio* calculations28 to compute the p*K*a (in parentheses) of the protonated conjugate acids for **1** (2.01), **2** (1.91) and **3** (1.53) in water (with uncertainty of ± 2, see Supporting Information). Although the p*K*a can differ significantly in lipid bilayers,29 the trend of **1** ≈ **2** > **3** will remain similar; the trend suggests that the optimal pH for anion transport by **3** is the lowest, which provides a rationale for **3** being less active with a higher *EC*50. The acidity of thiourea NHα (governed by the electron donating or withdrawing substituents) will directly affect the strength of intramolecular (NHα**···**N=C) H-bond; consequently, resulting in the trend of predicted p*K*a values from competing intramolecular H-bond and solvation effects. Since NHα of **1** (-H) is more acidic than **2** (-OCH3), the NHα**···**N=C H-bond present in compound **1** is thermodynamically more stable, which inhibits perturbation of intramolecular H-bond and the concomitant *anti* to *syn* reorganization (see Supporting Information Section S11 for details and discussion). The overall result is the imino nitrogen of **2** can be expected to be more basic in both aqueous and lipid bilayer membrane environments, therefore, compound **1** is a better pH-switch than **2**, as shown by the higher *EC*50(pH7.2)/*EC*50(pH4.0) ratio. Compound **3** (-CF3) is more acidic than **1**, and this is demonstrated by the fully switched-OFF transport activity at pH 7.2 (Figure 4). The transport activities for **1**‒**3** are all fully switched OFF at pH 10.0 (see Supporting Information Figure S38), indicating that the thiosemicarbazones must be protonated to mediate transport of anions.

**Non-electrogenic transport mechanistic studies.** By using the combination of two complementary cationophore-coupled KCl efflux assays (Figure 7a and 7b), we can effectively identify two different types of anion transport mechanisms that cannot be revealed using Cl‒/NO3‒ exchange experiments ‒ electrogenic chloride transport and electroneutral H+/Cl‒ cotransport. In an electrogenic process there is a net flow of charge across a membrane (Figure 7b). In an electroneutral process (Figure 7a), the charge is balanced either by back transport of a species with the same charge (i.e. Cl‒/OH‒ exchange) or cotransport of a species with opposite charge (H+/Cl‒). Monensin and valinomycin are naturally occurring cationophores, but their underlying transport mechanisms are fundamentally different. Monensin has a single carboxylic acid group that deprotonates upon metal complexation to form a pseudo macrocyclic complex. It therefore functions as a M+/H+ antiporter (exchanger) in lipid bilayer membrane transport, with negligible activity in facilitating M+ transport.30 This is an electroneutral cation exchange process and will result in perturbation of luminal pH. In contrast, valinomycin is a K+-selective carrier, it facilitates electrogenic K+ transport without directly affecting the transmembrane pH gradient.31



**Figure 7.** Overview of cationophore-coupled KCl efflux assays, measured by chloride-ISE from POPC LUVs (mean diameter 200 nm) loaded with KCl (300 mM) and suspended in K2SO4 (100 mM), adjusted to pH 4.5 with KOH in citrate (5.0 mM); (a) structure of monensin, and schematic diagrams showing overall KCl net flux induced by the combination of electroneutral transport processes, including monensin’s K+/H+ exchange and the anionophore’s H+/Cl‒ cotransport or Cl‒/OH‒ exchange; (b) structure of valinomycin, and schematic diagram showing overall KCl net flux from the combination of electrogenic Cl‒ efflux facilitated by anionophore, coupled to the electrogenic K+ transport mediated by valinomycin; (c)‒(f) Plots of Cl‒ efflux facilitated by anionophores (prodigiosin, thiosemicarbazones **1** and **2**, and squaramide **7**) in the absence or presence of cationophores (monensin or valinomycin) monitored over a period of 5 min, and detergent was added to lyse the vesicles at 300 s, to release remaining encapsulated Cl‒ for the 100% chloride concentration at 420 s. All ionophores were added as DMSO solutions (10 μL) to the POPC LUVs suspension (5.0 mL), lipid concentration used was 1.0 mM and loading concentrations of ionophores are shown as carrier:lipid molar percent. Solid lines are fitted curves using exponential functions to calculate the initial rate (*k*initial) of Cl‒ efflux, shown as %.s‒1; error bars corresponding to the SD from two repeats. Same DMSO, monensin and valinomycin controls are used in all four plots.

Liposomes were loaded with KCl and suspended in K2SO4 external solution, with both solutions buffered to pH 4.5 with citrate. Either monensin (0.1 mol%) or valinomycin (0.1 mol%) was added alone or in combination with an anionophore (0.2 mol%) or prodigiosin (0.005 mol%) and the rate of Cl‒ efflux was monitored by a chloride ion selective electrode. In these cationophore-coupled assays, chloride efflux is driven by the large Cl‒ concentration gradient. In the presence of an H+/Cl‒ cotransporter alone, no measureable Cl‒ efflux could occur due to the build-up of transmembrane pH gradient. For this reason, prodigiosin must couple with monensin to combine H+/Cl‒ symport and K+/H+ antiport, resulting in formal KCl efflux (Figure 7c). No measureable Cl‒ efflux was observed when prodigiosin was added with valinomycin (Figure 7c), consistent with the non-electrogenic nature of prodigiosin.5

Figure 7f shows that compound **7** can couple with both monensin and valinomycin to facilitate KCl efflux. We have previously shown that this is because simple neutral hydrogen bonding anionophores such as **7** are nonspecific, it can function both as an electrogenic chloride transporter and so can couple with valinomycin, and also as a H+ transporter or functionally equivalent OH‒ transporter; and hence these processes together with Cl‒ transport can couple with K+/H+ transport by monensin.19

When thiosemicarbazones were examined in these assays, it was found that Cl‒ efflux is only observed in the presence of monensin but not valinomycin. This experiment demonstrates that K+/H+ antiport facilitated by monensin couples to H+/Cl‒ cotransport facilitated by the thiosemicarbazone, resulting in overall KCl efflux from the liposomes. Importantly, as no chloride efflux is observed in the presence of valinomycin, this experiment demonstrates that the H+ and Cl‒ transport facilitated by the thiosemicarbazone are inseparable processes (Figure 7d and 7e). The initial rates (*k*initial) of Cl‒ efflux from these cationophore-coupled assays gave the same trend (e.g. **1** faster than **2**) to the Cl‒/NO3‒ exchange assay discussed above, indicating the electrogenic Cl‒ or electroneutral H+/Cl‒ (or Cl‒/OH‒) transport facilitated by anionophores is the rate limiting flux process with 0.1 mol% of cationophore loading. Therefore, thiosemicarbazones are prodigiosin-like, in which chloride cannot be transported without a proton also being transported, and the protonated thiosemicarbazones cannot diffuse through the lipid bilayer without a counter anion.



**Figure 8.** Schematic overview for the acid-sensing (proton-gated) ON/OFF chloride transport mechanism of thiosemicarbazones.

These results taken together are evidence that thiosemicarbazones must be protonated at the imino nitrogen to switch ON transmembrane transport of chloride (Figure 8). This results in cotransport of H+/Cl‒, mimicking the non-electrogenic H+/Cl‒ symport mechanism of prodigiosin (Figure 1), which is fundamentally different from previously reported pH-dependent nonspecific transporters, which are able to mediate both electroneutral and electrogenic transport processes. This is important as electrogenic transporters are known to depolarize mitochondria membrane potential13 which can complicate biological studies of cellular processes.14 Furthermore, the *EC*50 of thiosemicarbazones **1** and **2** (Table 1) are significantly lower than the analogous phenylthioureas **4** and **5**, and are also comparable to some of the most potent synthetic anionophores reported to date,32 hence suggesting that an overall neutral complex (e.g. **1**•H+**⊃**Cl‒) is favorable in the transport process.

CONCLUSIONS

In conclusion, we have shown that phenylthiosemicarbazones are the first examples of non-electrogenic anion transporters that show pH-switching behavior between neutral and acidic pH conditions. The pH-switchable transport properties are very significant, in particular compound **1** which has an *EC*50 value 640-fold times lower at pH 4.0 than at pH 7.2 (i.e. it is a significantly better transporter under acidic conditions). The non-electrogenic H+/Cl‒ cotransport mechanism was shown to mimic that of prodigiosin by using cationophore-coupled KCl efflux assays. In the search for more effective anionophores tailored for different biological studies, this work in conjunction with our most recent work (on the study of electrogenic anionophores)19 have shown the importance of elucidating the fundamental transport mechanism as most reported synthetic anionophores are nonspecific, capable of mediating both electrogenic and non-electrogenic processes. The explicit pH-switchable transport at pH 4.0 demonstrated by this class of transporters could potentially be employed for targeted anion efflux from acidic lysosomal vesicles in cells, gaining new insights in cellular process induced by anionophores.

ASSOCIATED CONTENT

**Supporting Information**. Crystallographic data (CIF); details of synthesis, characterization, NMR and MS spectra, and single-crystal X-ray analysis of **1**‒**3** and **10**; methodology of anion binding studies, and 1H NMR titration fitted binding isotherms; 2-D NOESY 1H‒1H NMR spectra of conformational studies; 1H NMR spectra of HBF4 titration studies; UV-Vis spectra of stability studies; details on computational and p*K*a studies; details on anion transport studies in various liposome-based assays, along with fitted plots of Hill analysis and initial rate studies. This material is available free of charge via the Internet at <http://pubs.acs.org>. The data underlying this paper has been made available online at http://dx.doi.org/10.5258/SOTON/396680 to comply with the EPSRC open data policy.

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