Halide-selective, proton-coupled anion transport by phenylthiosemicarbazones†

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† Dedicated to Professor Lechosław Latos-Grażyński on the occasion of his 70th birthday.

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Highlights

• A new class of phenylthiosemicarbazone-based anion receptors have been synthesized and shown to function as pH-switchable transmembrane anion transporters.

• An intramolecular hydrogen bond locks the thiourea binding site and inhibits chloride/nitrate exchange at pH 7.2. Protonation of phenylthiosemicarbazones at pH 4.5 switches on chloride/nitrate exchange activity.

• Anion transport selectivity analysis reveals strong halide over oxyanion preference exhibited by protonated phenylthiosemicarbazones.

• The pH-switchable chloride/nitrate exchange is shown to originate from inhibition of rate-limiting nitrate transport at pH 7.2.

Abstract: Phenylthiosemicarbazones (PTSCs) are proton-coupled anion transporters with pH-switchable behaviour known to be regulated by an imine protonation equilibrium. Previously, chloride/nitrate exchange by PTSCs was found to be inactive at pH 7.2 due to locking of the thiourea anion binding site by an intramolecular hydrogen bond, and switched ON upon imine protonation at pH 4.5. The rate-determining process of the pH switch, however, was not examined. We here develop a new series of PTSCs and demonstrate their conformational behaviour by X-ray crystallographic analysis and pH-switchable anion transport properties by liposomal assays. We report the surprising finding that the protonated PTSCs are extremely selective for halides over oxyanions in membrane transport. Owing to the high chloride over nitrate selectivity, the pH-dependent chloride/nitrate exchange of PTSCs originates from the rate-limiting nitrate transport process being inhibited at neutral pH.

1. Introduction

Small-molecule transmembrane anion transporters are promising anti-cancer drug candidates due to their ability to disrupt pH/anion gradients and membrane potential across cell membranes and in organelles.[1, 2] The research of synthetic anion transporters was initially inspired by the natural product prodigiosin which facilitates efficient H+/-Cl− symport and as a result neutralizes acidic pH
inside vacuolar organelles including lysosomes, endosomes and Golgi apparatus.[3-5] While prodigiosin exhibits potent anion transport activity under both neutral and acidic conditions, synthetic anion transporters with pH-dependent transport activities have been designed with a potential to target the acidic intracellular environment of tumours and reduce the toxicity towards normal cells.[6-10]

Phenylthiosemicarbazones (PTSCs) are a class of pH-switchable anion transporters previously reported to exhibit up to 640-fold increase in chloride/nitrate exchange activity on going from pH 7.2 to 4.0.[11] This pH dependence was regulated by protonation of the imine nitrogen at acidic pH, which led to a conformational switch of the thiourea motif from an inactive anti-conformation to an active anion binding syn-conformation (Fig. 1a). For a better understanding of anion transport and pH switching mechanism of this class of transporters, we decided to study an expanded library of PTSCs and here report our serendipitous discovery of their extremely high halide over oxyanion transport selectivity leading to the pH-switchable chloride/nitrate exchange being regulated by the rate-limiting nitrate transport process.

![Fig. 1. (a) Mechanism for pH-switchable anion binding by phenylthiosemicarbazones (PTSCs). (b) Structures of new PTSC transporters 1–9.](image-url)
2. Experimental section

2.1 Materials

All chemicals and solvents were purchased from commercial sources (Alfa Aesar, Fisher Scientific and Sigma Aldrich). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was purchased from Corden Pharma GmbH. All deuterated solvents for NMR were purchased from Cambridge Isotope Laboratories. Commercially available reagent grade chemicals were used without further purification unless otherwise specified.

2.2 Instruments

$^1$H and $^{13}$C NMR spectra were recorded on Bruker Avance AVII400 and AVIIIHD400 FT-NMR spectrometers with 5 mm BBFO z-gradient probes, operating at a frequency of 400 MHz for $^1$H NMR and 101 MHz for $^{13}$C NMR respectively.

LR-MS samples were analysed using a Bruker amaZon SL mass spectrometer equipped with a quadrupole analyser. Samples were introduced to the mass spectrometer via direct injection. Low resolution mass spectra were recorded using positive/negative ion electrospray ionization.

Fluorescence measurements were performed using an Agilent Cary Eclipse Fluorescence Spectrophotometer equipped with a stirrer plate and a temperature controller.

X-ray diffraction data were collected at 100 K on a SuperNova Dual, Cu at home/near Atlas diffractometer.

2.3 Syntheses

The respective substituted $N$-phenyl thiosemicarbazide (~300 mg) was suspended in absolute ethanol (10 mL) and the mixture was treated with 4-butylbenzaldehyde (~1.1 equivalent) and stirred at room temperature under nitrogen atmosphere for > 8 h. The products were re-precipitated with a solvent mixture of $H_2O$/ethanol and were collected by filtration. Compound 9 was purified by column chromatography with silica gel and 1:1 ethyl acetate/hexane as the eluent. Characterization data for transporters 1–9 is provided in Supplemental Material.

2.4 Crystallographic analysis

The solutions of X-ray diffraction data for transporter 1 were obtained by direct methods using SHELXT[12] followed by successive refinements using full matrix least squares method against $F^2$ using SHELXL-2018/3.[13] The program OLEX2 [14] was used as a graphical SHELX interface. See Supplemental Material for crystal and data refinement parameters.

2.5 Membrane transport assays

Large unilamellar vesicles of POPC were prepared by hydrating a lipid film using an internal solution, followed by 9 freeze-thaw cycles and extrusion 25 times through a 200 nm polycarbonate membrane. Finally, the vesicles were subject to dialysis or Sephadex size-exclusion chromatography using the required external solution to remove unencapsulated chloride ions (ISE assay) or the fluorescence pH indicator 8-hydroxypyrene-1,3,6-trisulfonate (HPTS assay) to obtain vesicle stock suspensions for membrane transport studies.

The vesicles stock suspensions were diluted to POPC concentrations of 1 mM (ISE assay) and 0.1 mM using the required external solution. DMSO solutions of transporters were added to the vesicle suspensions and the membrane transport kinetics was studied by measuring the chloride...
concentrations in the external solutions using a chloride selective electrode (ISE assay) or by monitoring the ratiometric fluorescence response from intravesicular pH indicator HPTS (HPTS assay). See Supplemental Material for detailed assay conditions.

3. Results and Discussion

3.1 Synthesis of anion transporters 1–9 and crystallographic analysis of 1

Transporters 1–9 bearing aryl substituents on both sides of the PTSC core were synthesized by imine condensation of 4-pentylbenzaldehyde with the corresponding phenylthiosemicarbazide. Single crystals of transporter 1 (CCDC 2103182) of suitable quality for X-ray diffraction were obtained via slow evaporation of a DMSO solution. Transporter 1 crystallizes in the monoclinic space group P2₁/c with two formula units in the asymmetric unit. The crystal structure highlights the thione form to be predominant due to the CS bond lengths (1.6869(18) Å and 1.6788(19) Å) and the C=N–N angles (116.15(16)° and 114.90(16)°) being similar to other thione tautomeric structures. The presence of two NH and one carbazone CH hydrogen-bond donor group, permits the formation of multiple hydrogen bonds between pairs of 1.

Each pair is held together through phenyl ring edge-to-face C–H···π stacking interactions between aromatic rings (3.2106(10) Å and 3.295(10) Å). Close C(13)–H···N(6) (3.570(3) Å) and C(5)–H···S(2) (3.677(2) Å) interactions further stabilize the pairs of receptors. Pairs run parallel along the a-axis, 180° to one another, held through close hydrogen bonding interactions between the thiosemicarbazone groups. The \( R_2^2 \) (6) and \( R_2^2 \) (8) motifs present between the carbazone groups of adjacent pairs necessitates their parallel position. Overall, this arrangement forms 2D sheets running along the ab axis.

![Fig. 2.](image)

**Fig. 2.** (a) Two crystallographically unique receptors 1 found in the asymmetric unit. (b) phenyl ring edge-to-face stacking observed between both crystallographically unique receptors. (c) Packing diagram of 1 shown along the a axis with carbon atoms from each receptor coloured either purple or orange for clarity. Black dashed bonds represent close H bonds holding together a pair of receptors while grey dashed bonds represent close H bonds between the pairs. Hydrogen atoms on butyl chains omitted for clarity. (d) Hydrogen bonding \( R_2^2 \) (6) and \( R_2^2 \) (8) motifs between receptor pairs.

3.2 pH-dependent Cl⁻/NO₃⁻ exchange studies

The transporters were initially evaluated for their pH switchability by comparing their Cl⁻/NO₃⁻ exchange activities under neutral and acidic conditions. POPC vesicles with internal NaCl (489 mM) buffered at pH 4.5 or 7.2 were suspended in a NaNO₃ (489 mM) external solution buffered at pH 4.5 or 7.2. Anion transporters were added to the vesicles as DMSO solutions and Cl⁻ efflux facilitated by an anion transporter was monitored using a chloride ion selective electrode (ISE). Similarly to
previously reported PTSCs, transporters 1–9 were inactive Cl\(^-\)/NO\(_3^-\) exchangers at pH 7.2 but became “switched ON” to facilitate rapid anion exchange at pH 4.5 (Figs. 3, S20–S37). To quantify the pH switchability, concentration-dependent Hill plot analysis were conducted to give an effective transporter concentration that facilitates 50% of Cl\(^-\) efflux at 270 s (EC\(_{50}\) values) at pH 4.5 and pH 7.2. As shown by Table 1, compound 1 was identified as the most active anion transporter, showing a low EC\(_{50}\) of 0.0175 mol% (with respect to lipids) at pH 4.5 and ~550-fold reduced activity at pH 7.0. For transporters 5 and 7–9, the Cl\(^-\)/NO\(_3^-\) exchange was almost completely suppressed at pH 7.2, with modest-to-high activities observed at pH 4.5. Compared with 1–3, the much weaker anion transport activities of 5, 8 and 9 under both pH conditions are attributed to the presence of electron-withdrawing substituents lowering the pK\(_a\) of the iminium group or increasing the strength of the intramolecular hydrogen bond, thereby inhibiting imine protonation as required for anion transport. The low Hill coefficients observed for those transporters are due to the poor delivery of transporters to the membrane when high concentrations were used. In contrast to those transporters containing strong electron-withdrawing substituents, transporter 7 demonstrated a potent activity similar to 1–3 at pH 4.5 consistent with the modest electron-withdrawing ability of a single fluorine substituent at the para- position. Transporter 6 precipitated upon addition to the vesicle suspensions as DMSO solutions which prohibited a detailed analysis of its pH switchability.

**Fig. 3.** (a) Schematic representation of the Cl\(^-\)/NO\(_3^-\) exchange assay. (b) Cl\(^-\)/NO\(_3^-\) exchange assay for transporter 1 and DMSO control using vesicles with internal and external solutions buffered at pH 4.5 (blue) and pH 7.2 (red). Error bars represent standard deviations from three runs.
Table 1 Hill analysis for Cl\(^{-}\)/NO\(_3\)^{-} exchange by PTSCs 1 – 9.

<table>
<thead>
<tr>
<th>PTSC</th>
<th>Cl(^{-})/NO(_3)^{-} at pH 7.2</th>
<th>Cl(^{-})/NO(_3)^{-} at pH 4.5</th>
<th>EC(<em>{50}) (pH 7.2) / EC(</em>{50}) (pH 4.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC(_{50}) / mol% n</td>
<td>EC(_{50}) / mol% n</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9.6 1.2</td>
<td>0.018 1.7</td>
<td>550</td>
</tr>
<tr>
<td>2</td>
<td>3.9 0.92</td>
<td>0.020 1.8</td>
<td>190</td>
</tr>
<tr>
<td>3</td>
<td>2.8 1.6</td>
<td>0.044 0.82</td>
<td>64</td>
</tr>
<tr>
<td>4</td>
<td>– a</td>
<td>8.5 0.35</td>
<td>– a</td>
</tr>
<tr>
<td>5</td>
<td>– a</td>
<td>0.17 0.44</td>
<td>– a</td>
</tr>
<tr>
<td>6</td>
<td>– a</td>
<td>– a</td>
<td>– a</td>
</tr>
<tr>
<td>7</td>
<td>– a</td>
<td>0.035 1.0</td>
<td>– a</td>
</tr>
<tr>
<td>8</td>
<td>– a</td>
<td>3.7 0.36</td>
<td>– a</td>
</tr>
<tr>
<td>9</td>
<td>– a</td>
<td>1.1 0.66</td>
<td>– a</td>
</tr>
</tbody>
</table>

*Too inactive for Hill analysis.

3.3 pH-dependent studies of Cl\(^{-}\) uniport and H\(^{+}\)/Cl\(^{-}\) symport

With the pH-dependent Cl\(^{-}\)/NO\(_3\)^{-} exchange confirmed for the new PTSCs, we next investigated the Cl\(^{-}\) uniport and H\(^{+}\)/Cl\(^{-}\) symport activities of transporters 1 – 9 at pH 4.5 and pH 7.2 using our previously developed cationophore-coupled ISE assays.[15] These assays complement the Cl\(^{-}\)/NO\(_3\)^{-} exchange assay by providing additional insights into electrogenic/electroneutral transport phenomena and revealing the potentially slow NO\(_3\)^{-} transport that could rate-limit the Cl\(^{-}\)/NO\(_3\)^{-} exchange process.[16] POPC vesicles were loaded with KCl (300 mM) at pH 4.5 or 7.2, and suspended in an external solution containing inert K\(_2\)SO\(_4\) (150 mM) at pH 4.5 or 7.2. Anion transporters were added along with a cationophore (valinomycin or monensin) to facilitate KCl efflux. In the presence of K\(^{+}\) uniporter valinomycin, the anion transporter needs to facilitate Cl\(^{-}\) uniport to give KCl efflux (Fig. 4a bottom). By contrast, in the presence of K\(^{+}\)/H\(^{+}\) exchanger monensin,[17] H\(^{+}\)/Cl\(^{-}\) symport facilitated by the anion transporter is required to generate net KCl efflux (Fig. 4a top). Similar to prodigiosin, the previously reported PTSCs were shown to facilitate H\(^{+}\)/Cl\(^{-}\) symport (coupling to monensin) but no Cl\(^{-}\) uniport (coupling to valinomycin) at pH 4.5.[11] This was attributed to the inability of the cationic protonated PTSC\(^{+}\) (decomplexed transporter) to diffuse through the membrane without a bound anion. Back diffusion of the decomplexed transporter is a necessary step for Cl\(^{-}\) uniport but not for anion exchange or H\(^{+}\)/Cl\(^{-}\) symport.[18] Previously, the cationophore-coupled assays were not conducted at pH 7.2.

Consistent with previous findings, the new PTSCs 1 – 9 induced efficient Cl\(^{-}\) efflux with monensin and low-to-negligible Cl\(^{-}\) efflux with valinomycin at pH 4.5 (Fig. 4 blue, Table 2), indicating the strong preference for H\(^{+}\)/Cl\(^{-}\) symport over Cl\(^{-}\) uniport for these transporters. Significant Cl\(^{-}\) uniport activities (relative to H\(^{+}\)/Cl\(^{-}\) symport activities) however, could be observed for polyfluorinated transporters 6, 8 and 9 at pH 7.2. This can be attributed to the neutral form of PTSCs facilitating Cl\(^{-}\) uniport via Cl\(^{-}\) binding in the anti-conformation (Fig. 1a left) followed by transmembrane diffusion of the anionic complex, release of Cl\(^{-}\) and the back diffusion of the neutral uncomplexed PTSC. This alternative mechanism required a significant anion binding affinity of the neutral form (in which only one thiourea
NH and a weak imine CH donor are available for anion binding) and therefore was only observed for the more acidic transporters in the series.

Fig. 4. (a) Schematic representation of $\text{H}^+/\text{Cl}^-$ symport (top, anion transporter T coupling to monensin Mon, 0.1 mol%, and $\text{Cl}^-$ uniport (bottom, anion transporter T coupling to valinomycin, Vln, 0.1 mol%) assays. (b) $\text{H}^+/\text{Cl}^-$ symport (red and blue) and $\text{Cl}^-$ uniport (green and pink) assays for transporter 1 at pH 4.5 (blue and green) and pH 7.2 (red and pink). The $\text{Cl}^-$ efflux induced by the anion transporter or cationophore alone is negligible (Fig. S38). Note that activity of monensin ($pK_a$ reported to be 7.6 in 66% DMF/H$_2$O, ref 17) is compromised at pH 4.5 due to the low concentration of its deprotonated form required for $\text{K}^+$ transport. Therefore, the monensin concentration (0.1 mol%) used is likely insufficient to fully reveal the $\text{H}^+/\text{Cl}^-$ symport potency of 1 at pH 4.5. Error bars represent standard deviations from three runs.
# Table 2 Cationophore-coupled assays for PTSCs 1–9.

<table>
<thead>
<tr>
<th>PTSC</th>
<th>Concentration</th>
<th>pH 7.2</th>
<th>pH 4.5</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Cl(^-) efflux at 300 s / %</td>
<td>Cl(^-) efflux at 300 s / %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mon(^a)</td>
<td>Vln(^b)</td>
</tr>
<tr>
<td>1</td>
<td>1 mol%</td>
<td>92</td>
<td>9.4</td>
</tr>
<tr>
<td>2</td>
<td>1 mol%</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>1 mol%</td>
<td>99</td>
<td>6.2</td>
</tr>
<tr>
<td>4</td>
<td>10 mol%</td>
<td>49</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>10 mol%</td>
<td>50</td>
<td>8.3</td>
</tr>
<tr>
<td>6</td>
<td>10 mol%</td>
<td>9.4</td>
<td>8.4</td>
</tr>
<tr>
<td>7</td>
<td>1 mol%</td>
<td>95</td>
<td>19</td>
</tr>
<tr>
<td>8</td>
<td>10 mol%</td>
<td>35</td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>10 mol%</td>
<td>49</td>
<td>28</td>
</tr>
</tbody>
</table>

\(^a\)Cl\(^-\) efflux by PTSC in the presence of monensin (Mon, 0.1 mol%), which indicates the H\(^+\)/Cl\(^-\) symport activity of PTSC.

\(^b\)Cl\(^-\) efflux by PTSC in the presence of valinomycin (Vln, 0.1 mol%), which indicates the Cl\(^-\) uniport activity of PTSC.

Surprisingly, while these PTSCs are inactive Cl\(^-\)/NO\(_3\)\(^-\) exchangers at pH 7.2, we observed significant H\(^+\)/Cl\(^-\) symport (coupling to monensin) for all compounds at pH 7.2 (Fig. 4b red, Table 2). No enhancement of H\(^+\)/Cl\(^-\) symport activity was observed on acidifying the vesicle suspensions from 7.2 to 4.5. These results appear to indicate the lack of pH switchability for the H\(^+\)/Cl\(^-\) symport facilitated by PTSCs in the tested pH range, contrasting the strong pH switchability in the Cl\(^-\)/NO\(_3\)\(^-\) exchange assay (Fig. 3, Table 1).

We hypothesised that the seemingly contradictory pH-dependent results in the two assays could be related to different rate-limiting steps for H\(^+\)/Cl\(^-\) symport and Cl\(^-\)/NO\(_3\)\(^-\) exchange. As shown by our previous studies, most hydrogen bond-based anion transporters facilitate the transport of the more lipophilic anion NO\(_3\)\(^-\) faster than Cl\(^-\) as governed by the ease of anion dehydration. However, exceptions have been found for a few transporters, most notably the tripodal tris-(thio)ureas [16, 19]. These transporters are Cl\(^-\) > NO\(_3\)\(^-\) selective due to their structural mismatch for binding planar NO\(_3\)\(^-\) ions, leading to underestimated Cl\(^-\) transport activity when tested under the Cl\(^-\)/NO\(_3\)\(^-\) exchange assay.

## 3.3 Anion transport selectivity studies

To understand the pH-dependent Cl\(^-\)/NO\(_3\)\(^-\) exchange property of the PTSCs, we determined the anion transport selectivity of 1 as a representative example under an HPTS assay utilising a transmembrane anion gradient to induce a pH gradient (Fig. 5a).[20] In this assay, vesicles containing NaCl (100 mM) and the pH indicator HPTS (1 mM) buffered at pH 7.0 were suspended in NaX (100 mM, X = Cl\(^-\), Br\(^-\), NO\(_3\)\(^-\), I\(^-\), or ClO\(_4\)\(^-\)) at pH 7.0. Transporter 1 was added to induce a pH gradient where an acidification
of the vesicular interiors indicates faster HX influx than HCl efflux (i.e., X⁻ > Cl⁻ selectivity) and vice versa. The assay revealed a selectivity pattern of I⁻ > Br⁻ > Cl⁻ > ClO₄⁻ > NO₃⁻ (Fig. 5b). Compared with a neutral mono-thiourea transporter showing a Hofmeister-type selectivity pattern (i.e., ClO₄⁻ > I⁻ > NO₃⁻ > Br⁻ > Cl⁻),[20] the selectivity profile of 1 features a strong halide-over-oxyanion preference with the selectivity sequence within each category (halides or oxyanions) governed by the ease of anion dehydration. This halide selectivity presumably originated from the covalent character of the strongly doubly ionic hydrogen bonds[21] in anion binding by protonated PTSCs. As the lone pair orbitals (HOMOs) of the halide ions are of higher energies than those of the tested oxyanions (ClO₄⁻ and NO₃⁻), charge transfer between the anion HOMO and the antibonding orbital (LUMO) of the hydrogen bond donor is more favourable for halides than for the oxyanions, which accounts for the halide over oxyanion selectivity here observed for doubly ionic hydrogen bonds. Corroborating the abovementioned hypothesis, a similar halide over oxyanion selectivity has been observed with a Pd(II)-based anion transporter in which anion binding (via metal coordination) features a strong covalent character.[22] Unfortunately, we were unable to determine the anion binding constants for the protonated PTSCs to evaluate the anion binding selectivity because of their instability.[11] To quantify the Cl⁻ vs NO₃⁻ transport selectivity of 1, a pH gradient dissipation assay[23] was conducted by applying a transmembrane pH gradient (pH 7.0 inside and pH 8.0) and measuring the rate of pH gradient dissipation under NaCl⁰⁰/NaCl⁰⁰¹ out and NaNO₃⁰⁰/NaNO₃⁰⁰¹ out conditions. These assays have been designed to evaluate H⁺/X⁻ symport activities of anion transporters (Fig. 5b). The results demonstrate rapid H⁺/Cl⁻ symport and no discernible H⁺/NO₃⁻ symport from DMSO control baseline for transporter 1 used at the same concentration (Fig. 5d), confirming its extremely high Cl⁻ > NO₃⁻ selectivity at neutral to slightly basic pH. By using an osmotic response assay, we were able to determine H⁺/X⁻ symport rates at pH 4.5 without using an intra-vesicular pH indicator and found that transporter 1 remains Cl⁻ > NO₃⁻ selective under acidic conditions (Fig. S65).

*Fig. 5.* Determination of anion transport selectivity of 1 using an anion gradient (pH gradient induction) assay (a, c) and a pH gradient dissipation assay (b, d). In (c) purple line, efficient Cl⁻ /I⁻ exchange led to...
rapid dissipation of the initially induced pH gradient. Note that in (d) 1 was tested at a higher concentration because of the lowered transporter activity under the assay conditions (pH 8.0 outside). Error bars represent standard deviations from two runs.

The abovementioned selectivity results indicate the rate-limiting NO$_3^-$ transport process as the determinant for the pH switchable Cl$^-$/NO$_3^-$ exchange by PTSCs. We here propose three mechanisms that likely contribute to the high Cl$^-$/NO$_3^-$ exchange activities of PTSCs at pH 4.5 and inhibition of Cl$^-$/NO$_3^-$ exchange at pH 7.2. (1) In lipid-water biphasic systems, NO$_3^-$ binding to the protonated PTSCs is expected to be drastically weaker than Cl$^-$ binding. As a result, a lower solution pH is required to shift the equilibrium from the free neutral PTSC to the PTSCH$^+$--X$^-$ ion pair complex for NO$_3^-$ binding than for Cl$^-$ binding and therefore significant NO$_3^-$ transport only occurs at pH 4.5. (2) Alternatively, the PTSCH$^+$ might not need to facilitate NO$_3^-$ transport to give Cl$^-$/NO$_3^-$ exchange at pH 4.5. By using an osmotic response assay, we found that HNO$_3$ (pK$_a$ = -1.4) can diffuse through the membrane without any anion transporter at pH 4.5 (Fig. S65). This process is negligible at pH 7.2 due to the low concentration of HNO$_3$ in equilibrium with NO$_3^-$ (Fig. S66). Given the extremely low NO$_3^-$ transport activities of PTSCH$^+$ (Fig. 5d blue), it is possible that simple diffusion of HNO$_3$ becomes the dominant NO$_3^-$ transport pathway at pH 4.5. (3) At neutral (external) pH under the NaCl$^+$/NaNO$_3$ out ISE assay conditions, the vesicle interiors are basified due to selective HCl efflux induced by PTSCs (Fig. 3c blue), which further reduces the ability of the PTSCs to form protonated anion complexes and contributes to transport inhibition at pH 7.2.

4. Conclusions

In summary, we have developed an expanded class of phenylthiosemicarbazones as pH-switchable Cl$^-$/NO$_3^-$ exchangers with suppressed activity at pH 7.2 and enhanced activity at pH 4.5. We showed that these transporters are active H$^+$/Cl$^-$ symporters and inactive Cl$^-$ uniporters at pH 4.5 similar to the behaviour of prodigiosin. Most importantly, we demonstrate the strong halide over oxyanion transport selectivity of the phenylthiosemicarbazones presumably due to the covalency of the doubly ionic hydrogen bond interactions leading to the preference for halide ions over oxyanions with low HOMO energies. As a result, while Cl$^-$ transport was efficient at both pH 4.5 and pH 7.2, the slow NO$_3^-$ transport turned out to be the rate-limiting factor for the suppressed Cl$^-$/NO$_3^-$ exchange at pH 7.2 and the activation at pH 4.5. Our results provide important insights for understanding the behaviour and improving the robustness of pH-dependent anion transporters aimed at therapeutic applications. In particular, the study demonstrates the limitation of the anion exchange assay and the benefit of measuring pH-dependent H$^+$/Cl$^-$ symport and Cl$^-$ uniport activities when studying pH-switchable transporters.

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