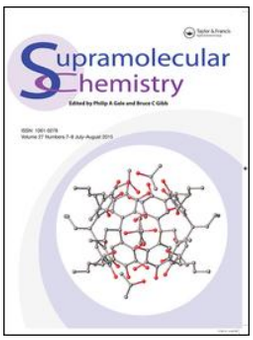

Supramolecular Chemistry

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/gsch20

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To cite this article: Patrick Wang, Xin Wu & Philip A. Gale (2021): Carbazole-based bis-ureas and thioureas as electroneutral anion transporters, Supramolecular Chemistry, DOI: 10.1080/10610278.2021.1946539

To link to this article: https://doi.org/10.1080/10610278.2021.1946539

SUPRAMOLECULAR CHEMISTRY
https://doi.org/10.1080/10610278.2021.1946539

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Carbazole-based bis-ureas and thioureas as electroneutral anion transporters

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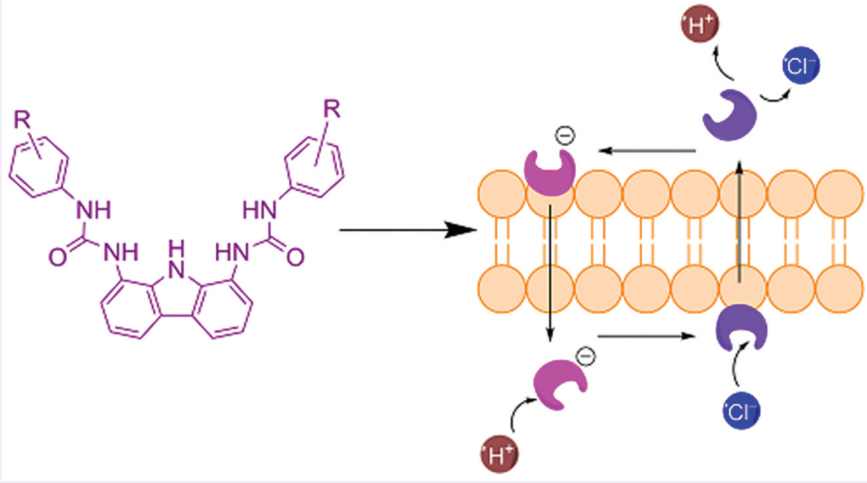
ABSTRACT

We report a series of easily accessible carbazole-based bis-ureas and thioureas as effective anion receptors and transporters. The compounds exhibit moderate Cl^- binding affinity in wet DMSO and Cl^- transport capabilities in phospholipid membranes predominantly through an electroneutral H^+/Cl^- and anion exchange mechanisms.



ARTICLE HISTORY
Received 15 May 2021
Accepted 17 June 2021

KEYWORDS
Supramolecular chemistry; anion transport; hydrogen bonding; carbazole

Introduction

Hydrogen-bond-based discrete molecular anion receptors have proven to be highly effective at facilitating the transport of a range of anions across both synthetic and biological lipid bilayer membranes [1] and are of interest due to their potential applications in the treatment of diseases such as cystic fibrosis [2,3] and cancer [4–7]. Some of the most effective transporters reported to date are bis-ureas and thioureas which may be constructed from simple or more complex diamines [8], including *ortho*-phenylenediamines [9–11], 1,8-diaminoanthracene [12], diaminodecalins [13], and steroid-based scaffolds [14]. The carbazole group has been shown to be an effective anion-binding motif in a variety of receptors to achieve high-affinity anion binding [15]. Chmielewski and co-workers have recently shown that carbazole bis-amides and bis-thioamides derived from 1,8-diaminocarbazole can function as anion transporters [16,17]. In contrast, although analogous carbazole bis-urea compounds were previously shown to be effective carboxylate receptors [18], membrane transport applications of these receptors have not been explored. We anticipate that compared with analogous anthracene or *ortho*-phenylene-based receptors, the carbazole bis(thio)urea would offer an additional hydrogen bond donor to the bound anion. This provides an additional opportunity to tune the anion-binding affinity, anion selectivity, and receptor-phospholipid headgroup interactions, which are important considerations for designing customised anion receptors to target a specific therapeutic application, such as cystic fibrosis or cancer [2,5]. We therefore decided to investigate the anion complexation and membrane transport properties of a series of

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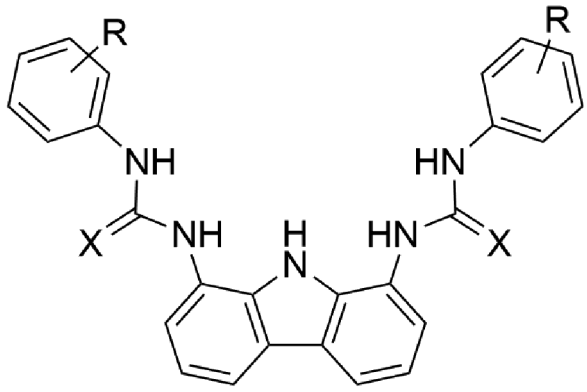
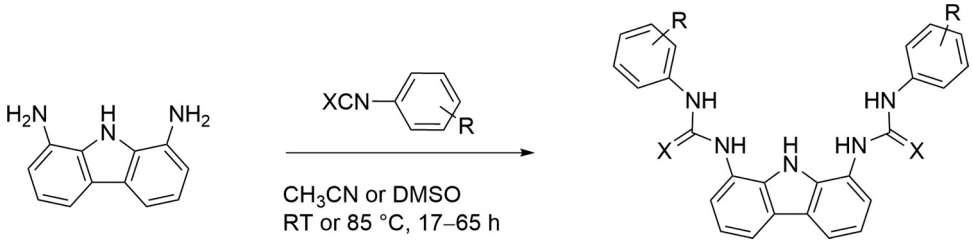
Supplemental data for this article can be accessed here.

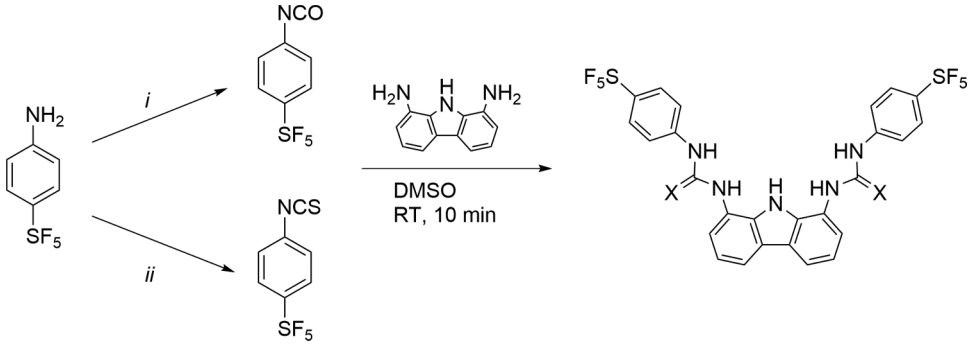


1 X = O, R = H
2 X = O, R = *p*-NO_2
3 X = O, R = *p*-CF_3
4 X = O, R = 3,5-bisCF_3
5 X = O, R = *p*-SF_5
6 X = S, R = H
7 X = S, R = *p*-NO_2
8 X = S, R = *p*-CF_3
9 X = S, R = 3,5-bisCF_3
10 X = S, R = *p*-SF_5

Figure 1. Structures of the carbazole compounds investigated in this work.



Scheme 1. Synthesis of carbazole compounds **1–4** and **6–9** from the starting carbazole diamine.



Scheme 2. Synthesis of carbazole transporters **5** and **10**. *Reagents and conditions*: i) triphosgene, toluene, pentane, TEA, 70 °C, 2 h; ii) thiophosgene, DCM, $NaHCO_3$, RT, 12 h.

carbazole-based bis(thio)urea containing anion transporters (Figure 1).

Results and discussion

1,8-Diaminocarbazole was prepared according to the literature methods [19]. Compounds **1**–**4** and **6–9** were prepared from their respective iso(thio)cyanates and the starting diamine in either acetonitrile or DMSO (Scheme 1). Compounds **5** and **10** were prepared with a variant of pre-existing literature methods [20], and the iso(thio)cyanate intermediates were used without further purification to obtain the *para*-SF_5 compounds (Scheme 2). Full synthetic details and characterisation data are available in the ESI.

The chloride-binding properties of **1–10** were investigated using ^{1}H NMR titration techniques in DMSO-d_6 /0.5% H_2O. Tetra-*n*-butylammonium chloride (TBA-Cl) was titrated against the compounds, resulting in chemical shift changes of both (thio)urea NH protons and the carbazole NH proton, indicative of chloride binding. The change in the chemical shift of the NH peaks was followed over the addition of 300 mM of TBA-Cl, and fitted to a 1:1 and 1:2 model using BindFit v0.5 [21,22]. For all receptors, both 1:1 and 1:2 models could be fitted to the data; however, a 1:2 model was determined to be the correct model (see Table S1 in the ESI). The receptors show moderate binding to Cl^-, with K_{11} binding constants in the range of 100–300 M^{-1} for the urea receptors (Table 1). In distinction to these results, the thioureas showed much weaker Cl^- binding affinities than the analogous ureas, with K_{11} binding constants in the range of 60–120 M^{-1}. Despite the presence of an additional NH hydrogen bond donor, the Cl^- affinities of the carbazole bis-ureas are similar to the analogous *ortho*-phenylenediamine-based bis-ureas while being substantially lower than those of the anthracene and decalin-based bis-ureas. This may be due to the relatively large central binding cavity of the 1,8-diaminocarbazole scaffold not being optimal for chloride binding. The lower binding constants for the thioureas were attributed to the larger sulfur atom distorting the planarity of the compound, thus incurring an energy cost in order to bind chloride in a favourable geometry. For all the compounds, it was observed that the nitro- and bis-CF_3 derivatives possessed the highest binding constants, consistent with the electron-withdrawing nature of these groups leading to the strongest enhancement of the (thio)urea NH proton acidity.

Table 1. K_{11} and K_{12} binding constants (M^{-1}) at 298 K of receptors **1–10** with Cl^- (added as TBA-Cl) in DMSO-d_6/0.5% H_2O. BindFit errors are <5%. The data were fitted to a 1:2 model as determined by the covariance of fit analysis (Table S1) and also indicated by a reversal of shift direction for the carbazole NH peak.

	Cl^- association constants				
Compound	K_{11}	K_{12}	Compound	K_{11}	K_{12}
1	122	6	**6**	67	5
2	233	11	**7**	102	6
3	133	11	**8**	83	5
4	222	12	**9**	119	8
5	180	12	**10**	66	4

Interestingly, while all (thio)urea NH protons shifted downfield with increasing chloride concentrations initially, the carbazole NH proton stopped its downfield shift at approximately 60 mM of TBA-Cl, before shifting slightly upfield with further additions. Since this reversal in the direction of shift was not observed with the (thio)urea protons, we propose that at higher host/guest ratios, the 1:1 receptor/chloride complex transformed to a 1:2 complex which involves a conformational change with the (thio)urea NH groups directed exo to the 1:1 binding site (Figure 2).

Anion transport properties of the compounds were assessed using two assays: the Cl^-/NO_3^- exchange assay and the 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) assay. The Cl^-/NO_3^- assay was performed using the chloride ion-selective electrode (ISE) method [23]. Synthetic vesicles were prepared using POPC lipids and loaded with a 487 mM NaCl solution, suspended in a 487 mM solution of $NaNO_3$, both buffered to pH 7.20 in 5 mM sodium phosphate salts. Chloride efflux induced by the addition of a transporter, in DMSO, was recorded over 300 s. EC_{50} values were calculated using the Hill

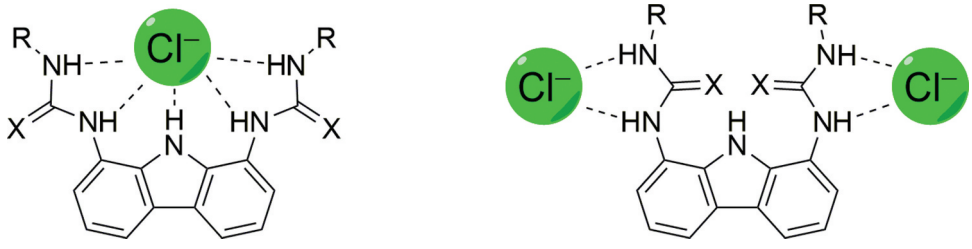


Figure 2. Proposed binding structures of the carbazole receptor to Cl^- in a 1:1 fashion (left, low Cl^- concentrations) or 1:2 fashion (right, high Cl^- concentrations). Hydrogen bonds are shown as dashed lines.

Table 2. EC_{50} values and Hill coefficients (n) for compounds **1–10** in the Cl^-/NO_3^- assay. EC_{50} values were calculated using a modified Hill equation to analyse incomplete Cl^- efflux.

	ISE NaCl/$NaNO_3$ EC_{50} (mol%) and Hill coefficient (n)				
Compound	EC_{50}	n	Compound	EC_{50}	n
1	14.7 ± 4.9	1.02	**6**	2.56 ± 0.30	0.81
2	0.108 ± 0.012	0.91	**7**	0.0352 ± 0.0026	2.11
3	0.208 ± 0.036	1.15	**8**	0.03800 ± 0.00073	2.20
4	0.94 ± 0.19	0.78	**9**	0.0984 ± 0.0088	1.92
5	0.357 ± 0.059	0.74	**10**	0.0864 ± 0.0041	1.81

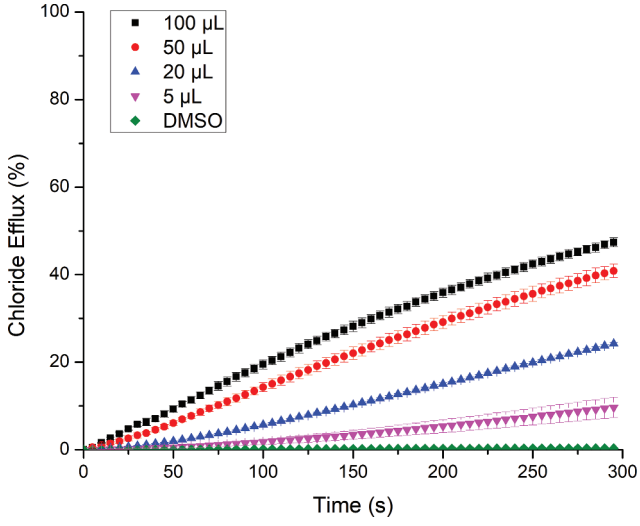


Figure 3. Volume-dependent transport activity of compound **1** in the Cl^-/NO_3^- assay at a fixed concentration of 2 mol%.

equation and a modified Hill equation (Equation S1 in ESI), representing the concentration (as a mol % of the total lipid concentration) of compound required to obtain 50% efflux.

Compounds **1** and **6** had significantly lower transport activities compared to the other compounds, with EC_{50} values more than 10 times higher than their respective analogues (Table 2). This was partly due to solubility issues with the receptor at concentrations >1 mol%. The assay was modified by increasing the volume ratio of DMSO (as the transport delivery medium) to 2% to address the observed low solubility (Figure 3). Furthermore, the electron-withdrawing groups (EWGs) in compounds **2–5** and **7–10** improve their solubility in the aqueous phase, improving the compounds' ability to solubilise between the hydrophobic and hydrophilic environments. The improved aqueous solubility was reflected in the EC_{50} values of compounds with EWGs compared to the parent phenyl group. This relationship was also observed with the thiourea compounds **7–10**, which exhibit lower EC_{50} values than the urea analogues. This result was rationalised through the lipophilic nature of the sulfur atom in the thiourea group, which is known to increase overall transport activity when compared to equivalent urea transporters [12]. In addition, the EWGs also facilitated delocalisation of the charge of the bound chloride anion, thus improving the overall lipophilicity of the bound complex [24].

The mechanism of anion transport was investigated using a modified ISE assay involving valinomycin (VLN) and monensin. POPC vesicles were loaded with 300 mM KCl and suspended in a 300 mM solution of either potassium gluconate or KNO_3, buffered to pH 7.20 in potassium dihydrogen phosphate. Gluconate cannot be transported across the bilayer because it is large and polar; thus, compound-mediated Cl^- transport is dependent on the coupling to either VLN or monensin. Both cationophores transport K^+ across the bilayer via different mechanisms and indicate different modes of Cl^- transport.

VLN strictly uniports K^+, thus coupling to VLN indicates electrogenic transport of Cl^- facilitated by the tested transporter. Only compounds **1** and **6** showed substantial activity in the presence of VLN. The chloride uniport transport process required for coupling to VLN (Figure 4) involves a step where the free transporter diffuses across the lipid bilayer. This diffusion process is hindered by the transporter binding to the phospholipid head groups [25]. This uniport inhibition mechanism is less likely for compounds **1** and **6** than for the remaining compounds in the library that contain EWGs. To investigate the transporter-phospholipid head group interactions, a titration was performed on compounds **1** and **3** with POPC. This was performed in a 75% $CDCl_3$/24.5% DMSO-d_6/0.5% H_2O solvent system, following Busschaert's method [26]. Unfortunately, the data could not be fitted to a simple binding model (Figure 5). A qualitative analysis indicates that both compounds show interactions with POPC; however, it was not possible to determine which compound exhibited stronger binding. Nevertheless, this evidence provides support for phospholipid head group interactions with the transporters. Based on the chloride-binding constants, we hypothesise that compounds **2–5** and **7–10** will form stronger complexes with the phospholipid head groups due to their higher acidity, leading to inhibition of chloride uniport activity and hence the failure to couple to VLN.

In contrast to VLN, monensin exchanges K^+ and H^+ across the phospholipid bilayer; thus, coupling to monensin is indicative of the electroneutral symport of Cl^-/H^+ out of the vesicle facilitated by the anion transporter. Proton efflux may be facilitated by the deprotonation of the transporter, with the deprotonated form then diffusing back across the bilayer before repeating the transport cycle (Figure 4). All the compounds showed coupling to monensin, indicating electroneutral H^+/Cl^-

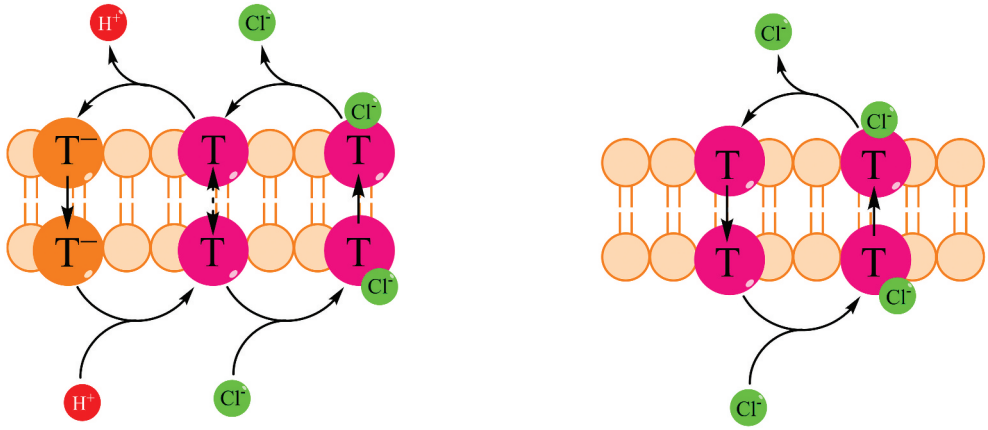


Figure 4. Modes of Cl^- transport facilitated by coupling to monensin (left) and valinomycin (right). The transporter must be able to freely diffuse back across the bilayer in electrogenic transport, which can be hindered by phospholipid head group interactions, but not in electroneutral transport.

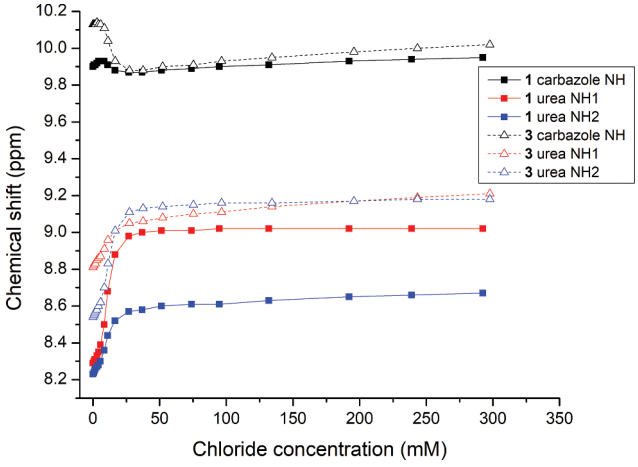


Figure 5. Chemical shifts (ppm) of the carbazole NH and both urea peaks in compounds **1** and **3** when titrated against up to 300 equivalents of POPC in 75% $CDCl_3$/24.5% DMSO-d_6/0.5% H_2O at 298 K. Peaks were referenced against $CDCl_3$ (7.24 ppm).

Table 3. EC_{50} values for compounds **1–10** in the HPTS assay. EC_{50} values were calculated using the Hill equation.

	HPTS EC_{50} (mol%)		
Compound	EC_{50}	Compound	EC_{50}
1	0.112 ± 0.019	**6**	0.118 ± 0.010
2	0.02160 ± 0.00066	**7**	0.000840 ± 0.000043
3	0.057 ± 0.013	**8**	0.00378 ± 0.00051
4	0.22 ± 0.12	**9**	0.008 ± 0.014
5	0.068 ± 0.019	**10**	0.0105 ± 0.0014

symport as a viable pathway. It is unlikely that the transporter directly co-transports H^+/Cl^- as HCl as the carbazole NH is not a likely protonation site, in addition to the EWGs which would further enhance the acidity of the overall compound.

As all the compounds exhibited electroneutral H^+/Cl^- co-transport, this mode of transport was further investigated using the HPTS assay. In this assay, POPC vesicles were loaded with a solution of 1 mM HPTS in 100 mM KCl, buffered to pH 7.00 with 5 mM HEPES, and suspended in an identical external solution without HPTS. Anion transport was induced by the addition of an aliquot of NaOH, followed by the compound. Like the ISE assay, the thiourea series exhibited lower EC_{50} values than the urea analogues (Table 3), which could be attributed again to increased acidity of the thiourea NH protons. Compounds **2** and **7** showed the lowest EC_{50} values of 0.02 and 0.0008 mol%, respectively, highlighting the propensity of the nitro-derivatives to be potent anion transporters.

To minimise the impact of fatty acid flip-flop contributing to the symport of H^+/Cl^-, the HPTS assay was repeated at EC_{50} concentrations in the presence of bovine serum albumin (BSA) [27]. Contrary to previous studies [28], none of the compounds showed a decrease in transport activity, while compounds **2–5** and **8–10** showed increased activity. This was unexpected as the removal of fatty acids would typically decrease transport activity as the fatty acid flip-flop mechanism of proton transport is removed. Furthermore, increased activity after fatty acid sequestration suggests that the compounds bind to fatty acid carboxylate groups, which compete with the chloride transport process. The rate-limiting step in the H^+/Cl^- symport process can also be probed by adding an ionophore, either the K^+ ionophore VLN or the H^+ ionophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), where enhanced activity in the presence of VLN and CCCP indicates $H^+ > Cl^-$ and $Cl^- > H^+$ selectivity, respectively.

It should also be noted that for an anion transporter to couple to either VLN or CCCP, the transporter needs to be capable of facilitating H^+ or Cl^- uniport. A dramatic acceleration of pH gradient dissipation by VLN was found for compound **6**, the unsubstituted thiourea, indicating its high $H^+ > Cl^-$ selectivity. For the analogous urea, compound **1**, the VLN-induced acceleration was less pronounced, suggesting lower H^+/Cl^- selectivity, which is rationalised based on its weaker H^+ transport activity due to the lower acidity of the urea group compared to the thiourea group. For the remaining anion transporters, VLN and CCCP did not significantly accelerate the pH dissipation and in some cases, decreased activities were observed. As discussed previously, these anion transporters are ineffective uniporters due to transporter-phospholipid head group interactions, which accounts for the failure of VLN and CCCP to accelerate the pH dissipation.

To investigate the anion selectivity of the compounds, a modified HPTS assay was carried out based on our recently reported method [29]. POPC vesicles were loaded with a solution of 1 mM HPTS in 100 mM NaCl, buffered to pH 7.00 with 10 mM HEPES, and suspended in different external solutions with an isotonic buffer, but with Cl^-, Br^-, NO_3^-, I^- and ClO_4^- anions. The majority of anion transporters facilitate the transport of hydrophobic anions such as I^- and ClO_4^- faster than Cl^- following the Hofmeister series. For the carbazole bis(thio)urea transporters, none showed preferential chloride selectivity (ESI Figures S4.51–S4.60). However, this was not unexpected, as a high degree of pre-organisation is typically required to achieve anion selectivity against the Hofmeister series [30]. An interesting trend is that the more acidic transporters **7–10** demonstrate I^- selectivity (Figures S4.57–S4.60), whereas the less acidic transporters **1–6** are selective for the more hydrophobic anion ClO_4^- (Figures S4.51–S4.56).

Conclusions

We have synthesised a series of carbazole-based bis-ureas and thioureas with aromatic sidearms bearing various EWGs. Compounds with more potent EWGs show stronger binding affinities to Cl^- when compared to the parent phenyl, with the *p*-nitro and 3,5-bisCF_3 groups displaying the strongest binding. Electrogenic Cl^- uniport capability was found only in compounds **1** and **6**; however, all the compounds displayed H^+/Cl^- symport and Cl^-/NO_3^- exchange activity. While not suitable for applications that rely on electrogenic transport of chloride, the ease of synthesis of the compounds may prove useful in future applications where electroneutral transport is required, such as in pH gradient disruption in putative anti-cancer treatments.

Acknowledgments

P.W., X.W., and P.A.G. acknowledge and pay respect to the Gadigal people of the Eora Nation, the traditional owners of the land on which we research, teach and collaborate at the University of Sydney. P.A.G. thanks the University of Sydney and the Australian Research Council (DP200100453) for funding.

Disclosure statement

The authors declare no competing interests.

Funding

This work was supported by the Australian Research Council [DP200100453].

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