Dissecting transmembrane bicarbonate transport by 1,8-di(thio)amidocarbazoles

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Abstract

Synthetic ionophores able to transport bicarbonate and chloride anions across lipid bilayers are appealing for their wide range of potential biological applications. We have studied the bicarbonate and chloride transport by carbazoles with two amido/thioamido groups using a bicarbonate-sensitive europium(III) probe in liposomes and found a highly remarkable concentration dependence. This can be explained by a combination of two distinct transport mechanisms: HCO_3^-/Cl^- exchange and a combination of unassisted CO_2 diffusion and HCl transport, of which the respective contributions were quantified. The compounds studied were found to be highly potent HCl transporters. Based on the mechanistic insights on anion transport, we have tested the antimicrobial activity of these compounds and found good correlation with their ion transport properties and a high activity against Gram-positive bacteria.

Introduction

Synthetic anion transporters, *i.e.* molecules which facilitate anion diffusion across phospholipid membranes, continue to attract considerable attention due to their wide range of biological activity. The selectivity of anion transporters and their preferred mechanism of action are important factors in guiding their medical applications. For instance, chloride and bicarbonate transporters are considered as particularly appealing for treating diseases arising from the malfunction of natural anion channels, such as cystic fibrosis.[1,2] Toxic side effects are expected to be minimised by using anionophores with high selectivity for targeted anions.[3,4] On the other hand, H^+Cl^- symporters show promise as candidates for anti-cancer agents.[5,6,7] Chloride transporters have been also shown to display promising antibacterial activity, notably against antibiotic resistant bacteria.[8,9]

Diamides and dithioamides based on the carbazole skeleton are known for their high oxyanion affinity,[10] outstanding optical properties[11,12] and ability to transport various biologically relevant anions, such as chloride,[10,13,14] organic phosphates,[15] aspirin,[15] and amino acids.[16] Owing to their modular synthesis, high anion transport activity, and good deliverability, these compounds are promising candidates for medicinal applications. This raised the question if these compounds could also act as bicarbonate transporters and if they would be selective for the transport of HCO_3^- and Cl^- over H^+ or OH^-. Preliminary HCO_3^- transport studies with 1,8-diamidocarbazoles and 1,8-dithioamidocarbazoles showed promising activity,[10,13] but the exact mechanism of HCO_3^- transport was not investigated.

Recently, some of us have developed a new assay to study HCO_3^- transport by fluorescence spectroscopy[17] using the europium(III) complex $[Eu.L^1]^+$ developed by Butler.[18] Studies using this EuL1 assay revealed that bicarbonate transport into liposomes can take place not only *via* the commonly assumed receptor-mediated HCO_3^- anion transport, but also *via* the unassisted diffusion of CO_2 accompanied by pH equilibration by an ionophore. The latter mechanism was found to be dominant for prodigiosin and (thio)ureas with a bis(CF_3)phenyl groups,[19,17] which made us wonder if this would also hold for other anionophores, such as di(thio)amidocarbazoles. These compounds show promise for different biological applications, depending on whether they can truly translocate bicarbonate anions across lipid bilayers or merely facilitate spontaneous CO_2 diffusion by dissipating the pH gradient.

Here we present mechanistic studies on HCO_3^- and Cl^- transport by four potent carbazole-based chloride transporters **1**-**4** (Fig. 1). Using the EuL1 assay, the contribution of various mechanisms to the overall HCO_3^- transport was quantified for the first time. Based on the outstanding activity of the four model carbazoles in H^+Cl^- symport (or OH^-/Cl^- antiport), with EC_{50} values in the nM range, their antibacterial activity was also investigated. The most active HCl transporters were found to be highly active against Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*.

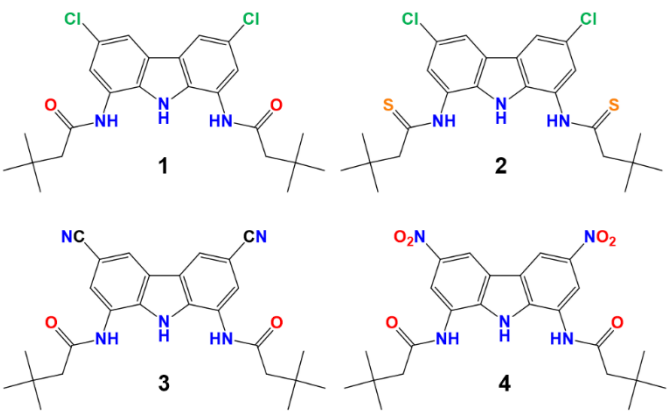

Figure 1. Structures of carbazole-based transporters **1**-**4**.

Results and discussion

Bicarbonate transport studies

The bicarbonate transport by **1**, **2** and related di(thio)amidocarbazoles has been previously studied indirectly, by monitoring chloride efflux from large unilamellar vesicles (LUVs) with a chloride selective electrode.[10,13] In order to examine the mechanism of bicarbonate transport by **1**-**4**, we used the recently reported EuL1 assay, which is based on a luminescent, HCO_3^--sensitive europium(III) complex (Fig. 2A).[17] Large unilamellar vesicles, made of POPC:cholesterol 7:3, were loaded with a buffered solution of $[Eu.L^1]^+$ probe (50 µM, 225 mM NaCl, 5 mM HEPES, pH 7.0) and suspended in an identical external solution without $[Eu.L^1]^+$. Anionophores **1**-**4** were added as a DMSO solution at different transporter to lipid molar ratios and anion transport was induced by a pulse of $NaHCO_3$ (see Fig. 2B,C for compounds **1** and **2**, and Fig. S2.2.3, S2.2.4 for compounds **3** and **4**). In control experiments, the H^+/M^+ antiporter monensin was used to demonstrate the possibility of apparent HCO_3^- transport in the absence of any anionophore (red curves in Fig. 2). As mentioned above, this is possible through unassisted CO_2 diffusion, followed by formation of H^+ and HCO_3^-, and subsequent transport of H^+ out of the vesicles by monensin (*via* H^+/Na^+ antiport). The addition of monensin to vesicles with carbazoles did not change the transport curves (Fig. S2.9.1, S2.9.2), indicating that compounds **1**-**4** can also act *via* a mechanism that involves CO_2 diffusion.

Transporter-independent rate-limiting process

When studying the rate of bicarbonate transport by **1**-**4** at different concentrations, we noticed a highly remarkable concentration dependence. For instance, the data for compound **2** in Fig. 2B show that the transport curves are nearly identical for transporter to lipid molar ratios ranging from 0.001 mol% to 0.1 mol%, thus over a 100-fold difference in concentration. The transport curves were fitted to a single exponential equation to determine the rate constants (see ESI, sections 2.4-2.8). In ion transport experiments, the rate of ion transport by mobile carriers should be directly proportional to the concentration of the transporter in the membrane. Figure 3 shows that in the case of transporters **1**-**4**, this dependence is highly non-

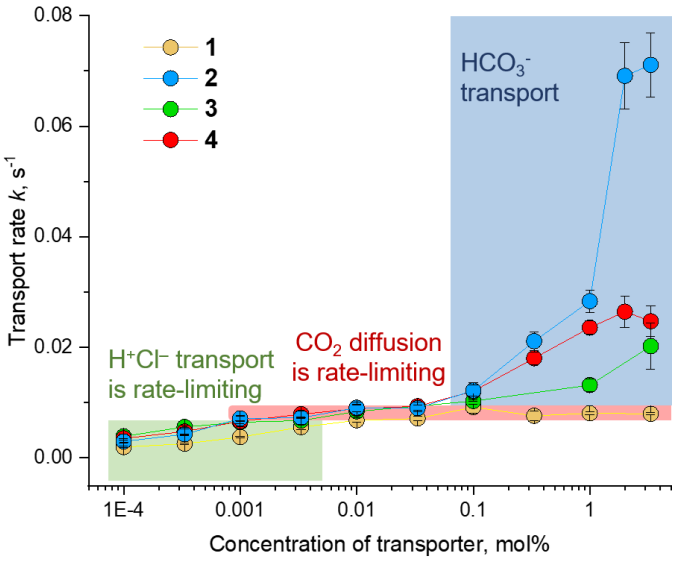

Figure 3. Plot of the overall bicarbonate transport rate *k* vs. concentration of transporters **1**-**4** in the lipid bilayer (logarithmic scale).

linear (see also Section 3 in ESI for plots on a non-logarithmic scale). This peculiar concentration dependence can be explained by the co-existence of two mechanisms: *i*) unassisted transmembrane diffusion of CO_2 followed by dissipation of the pH gradient by **1**-**4** *via* H^+Cl^- symport (or Cl^-/OH^- antiport) and *ii*) HCO_3^-/Cl^- antiport. In the first mechanism, there are two possible rate-limiting processes to consider: CO_2 diffusion and pH gradient dissipation by **1**-**4**. This leads to the three different concentration regimes observed on Figure 3. At very low transporter concentration (*e.g.*, < 0.001 mol% for **2**) the pH equilibration by the transporter is slow and therefore turns out to be the rate-limiting process (*green regime*). At higher concentrations of **1**-**4**, the H^+Cl^- co-transport (or functionally equivalent Cl^-/OH^- antiport) becomes very fast, while the true HCO_3^- anion transport is still negligible. In this concentration regime, the overall HCO_3^- transport is limited by the rate of CO_2 diffusion and hence almost independent of the transporter concentration (*red regime*). This rate is nearly identical to that observed in the presence of monensin at 0.1 mol%, which was previously demonstrated to be limited by the rate of CO_2 diffusion.[17] At even higher concentrations of anionophores **2**-**4** (0.05-3.3 mol%, *blue regime*), the total HCO_3^- transport is clearly faster than the CO_2 diffusion-limited rate.

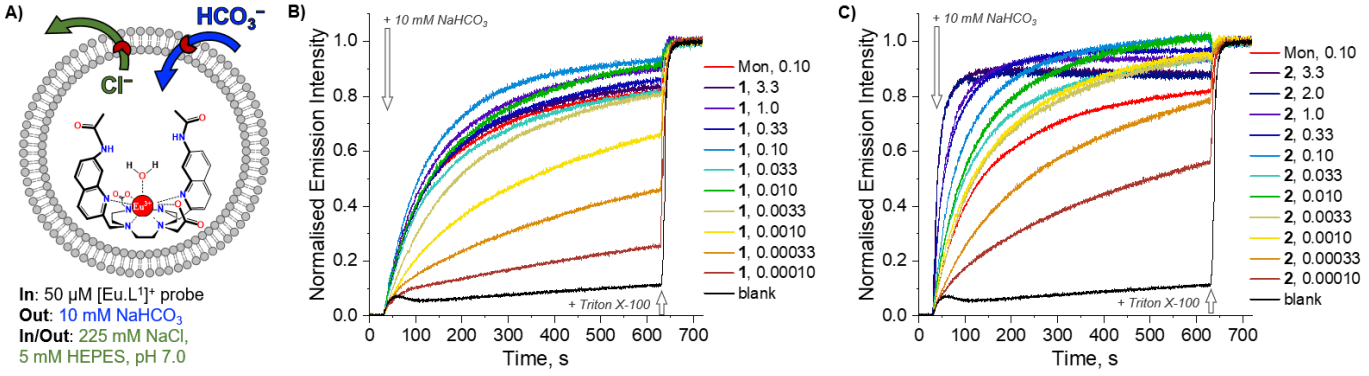

Figure 2. Transport of HCO_3^- by anionophores **1** and **2** in LUVs with encapsulated probe $[Eu.L^1]^+$ (50 µM), suspended in 225 mM NaCl with 5 mM HEPES at pH 7.0 (interior and exterior), upon addition of 10 mM $NaHCO_3$ (after 30 seconds) and lysis of the LUVs (after 10 minutes). A) Schematic representation of EuL1 assay to study the transport of HCO_3^-. Normalized transport curves for anionophore **1** (B) and **2** (C), post-inserted at various anionophore-to-lipid molar ratios (mol%) and for the cationophore monensin at 0.10 mol%.

Table 1. Contribution of the HCO_3^-/Cl^- mechanism to the total bicarbonate transport by compounds **2** and **4**.

C, mol%	$k_{experimantal}$ by **2**, s^{-1}	$k_{HCO3-\ transport}$ by **2**, s^{-1}	$k_{HCO3-\ transport}$ by **2**, % [(a)]	$k_{experimantal}$ by **4**, s^{-1}	$k_{HCO3-\ transport}$ by **4**, s^{-1}	$k_{HCO3-\ transport}$ by **4**, % [(a)]
0.00010	0.0030	-	0	0.0035	-	0
0.00033	0.0043	-	0	0.0048	-	0
0.0010	0.0072	-	0	0.0066	-	0
0.0033	0.0073	-	0	0.0079	-	0
0.010	0.0091	0.0011	12.0	0.0089	0.00090	10.2
0.033	0.0090	0.0010	11.3	0.0094	0.0014	14.5
0.10	0.012	0.0042	34.4	0.012	0.0041	33.7
0.33	0.021	0.013	**62.1**	0.018	0.010	**55.7**
1.0	0.028	0.020	**71.8**	0.024	0.016	**66.1**
2.0	0.069	0.061	**88.4**	0.027	0.019	**69.8**
3.3	0.071	0.063	**88.7**	0.025	0.017	**67.6**

[(a)] Relative share (in %) of the transporter-dependent bicarbonate transport by **2** and **4**, calculated as $k_{HCO3-\ transport} \cdot 100\% / k_{experimantal}$.

It suggests that under these conditions an additional mechanism of bicarbonate transport by **2**-**4** is operational, most likely the HCO_3^-/Cl^- antiport. Diamide **1** has the lowest transport activity among all four tested carbazoles and does not transport bicarbonate anions to any noticeable degree. The presence of strong electron-withdrawing groups in receptors **3** and **4** results in a higher transport activity,[14] what manifests in Fig. 3 as an increase in the rate constant at high concentrations of transporters, beyond the CO_2 diffusion-limited rate. This behaviour is even more pronounced in the case of dithioamide **2**, which is known as potent oxyanion transporter[15] and here also shows the highest activity in HCO_3^-/Cl^- transport.

Determining the contributions of different transport mechanisms

Assuming that both bicarbonate transport mechanisms are independent from each other and that both follow simple exponential kinetics, the overall experimental transport rate constant $k_{experimental}$ should be the sum of rate constants of the two mechanisms:

$$k_{experimental} = k_{CO_2\ diffusion} + k_{HCO_3^-\ transport}$$

The maximum rate constant for the increase of the interior HCO_3^- concentration due to CO_2 diffusion can be estimated from an independent experiment with monensin and was found to be 0.0080 s^{-1}. Thus, if the concentration of carrier is high enough (in the *red* and *blue* regimes), the rate of transporter-mediated HCO_3^-/Cl^- transport can be calculated as:

$$k_{HCO_3^-\ transport} = k_{experimental} - k_{CO_2\ diffusion} = k_{experimental} - 0.0080$$

The values thus obtained for the most active compounds **2** and **4** are reported in Table 1. It is apparent that already at a concentration of 0.01 mol% the true anion transport mechanism contributes significantly to the overall bicarbonate transport and that at 0.33 mol% it starts to dominate (Table 1).

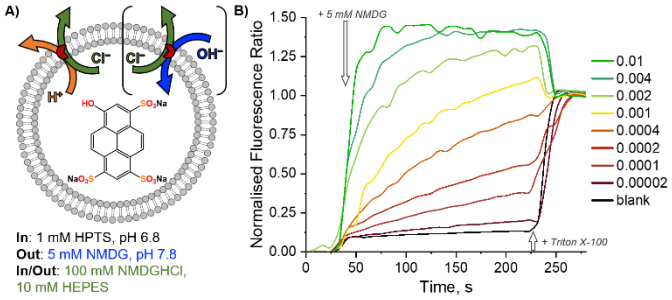

Figure 4. H^+Cl^- symport (or OH^-/Cl^- antiport) by anionophore **2** in LUVs with encapsulated HPTS (1 mM), suspended in 100 mM NMDGHCl with 10 mM HEPES, upon addition of 5 mM NMDG after 30 seconds and lysis of the LUVs after 200 seconds. A) Schematic representation of HPTS assay. B) Normalized transport curves for anionophore **2** post-inserted at various anionophore-to-lipids ratios.

H^+Cl^- or Cl^-/OH^- transport studies

As the HCO_3^- transport studies in the EuL1 assay indicated that **1**-**4** can dissipate the pH gradient caused by CO_2 diffusion, the HPTS assay was used to verify if **1**-**4** can indeed facilitate H^+Cl^- symport (or OH^-/Cl^- antiport) and to estimate its rates. POPC:cholesterol 7:3 liposomes were loaded with a buffered solution of the pH-sensitive dye HPTS (1 mM in 100 mM NMDGHCl,[20] 10 mM HEPES, pH 6.8) and suspended in an identical external solution without HPTS. Anionophores **1**-**4** were added to this suspension as a solution in DMSO and anion transport was induced by a pulse of NMDG base that increased the external pH to 7.8 (Fig. 4 and Section 4.3 in ESI). All of the studied compounds **1**-**4** turned out to be very efficient H^+Cl^- co-transporters, even at low concentrations. EC_{50} values indicate concentrations at which half of the maximum effect after 200 s is observed and were found to be in the nM range (Table 2). The obtained EC_{50} values correspond well to the concentration ranges where the $H^+(OH^-)/Cl^-$ transport by **1**-**4** is rate-limiting in the EuL1 assay. This corroborates our assumption that the bicarbonate transport rate in the '*green*' concentration regime is limited by the rate of pH equilibration.

While it is difficult to compare the transport activities of anionophores studied under different conditions, it is apparent that **1**-**4** belong to the most active H^+Cl^- transporters reported to date. For example, the previously reported carbazole-based 1,8-diureas and 1,8-dithioureas were studied in pure POPC vesicles,[21] where somewhat higher rates can be expected due to higher membrane flexibility. Despite that, bis(thio)amides **1**-**4** were found to exhibit 2 to 4 orders of magnitude higher transport activities than the carbazole-based bis(thio)ureas. The lowest EC_{50} values for H^+Cl^- transport were reported for a fluorinated tetraurea macrocycle developed by Gale and co-workers ($0.67 \cdot 10^{-4}$ mol%) and the anionophore prodigiosin ($0.61 \cdot 10^{-4}$ mol%).[22] These values, however, were also obtained in pure POPC vesicles. This comparison shows that **1**-**4** are among the most potent H^+Cl^- transporters reported to date.

Table 2. Overview of transport activities and antibacterial properties of compounds **1**-**4**.

Transporter	POPC/cholesterol 7:3 LUVs		Gram-positive bacteria		Gram-negative bacteria	
	$EC_{50,\ 200\ s}$		*B. subtilis*	*S. aureus*	*E. coli*	*A. baumannii*
	mol%	nM	MIC, μM	MIC, μM	MIC, μM	MIC, μM
1	$7.1 \cdot 10^{-4}$	0.71	75	>150	>150	>150
2	$4.4 \cdot 10^{-4}$	0.44	0.3	0.07	>150	>150
3	$7.0 \cdot 10^{-4}$	0.70	2.3	>150	>150	>150
4	$3.4 \cdot 10^{-4}$	0.34	0.3	>150	>150	>150

Biological studies

The outstanding activity of carbazoles **1**-**4** in H^+Cl^- co-transport prompted us to investigate their antibacterial properties. Bacterial cell membranes are much more complex than the lipid bilayer of a synthetic liposome and contain various proteins, lipopolysaccharides and other biomolecules that can interfere with the activity of synthetic transporters. The ability of synthetic anionophores to disturb ion homeostasis may have a profound effect on cell functions. In fact, the antibacterial properties of synthetic anionophores often correlate with their anion transport activity.[8,9,23,24,25,26,29] However, some deviations from this rule[27] indicate, that the rate of anion transport is not the only factor responsible for antimicrobial activity of synthetic ionophores. Further biological studies with transporters that act *via* different mechanisms may be helpful in order to learn more about the origins of the antibacterial properties of ionophores.

The antibacterial activity of the carbazole-based transporters **1**-**4** was thus evaluated on two Gram-positive bacterial species: *Bacillus subtilis* and *Staphylococcus aureus* and two Gram-negative: *Escherichia coli* and *Acinetobacter baumannii*, each pair consists of model and pathogenic bacteria. The bacteria were grown overnight in LB medium and diluted in fresh medium to an optical density of 0.05 and mixed with DMSO solutions of **1**-**4** at various concentrations. The DMSO content was kept constant at 2% (v/v) in all experiments. The minimum inhibitory concentration (MIC) was determined as the concentration of a receptor in which the bacterial growth is inhibited by ≥80% as measured spectroscopically.[28]

Compounds **1**-**4** show strong antibacterial activity towards both Gram-positive strains. The growth of *B. subtilis* is significantly inhibited by all tested transporters, with MICs of **2**-**4** even lower than that of antibiotic tetracycline (MIC = 11.3 μM).[29] Noteworthy, the MIC values for **1**-**4** correlate with their H^+Cl^- transport activity in liposomal assays. Remarkably, thioamide **2** exhibits particularly strong antibacterial properties against *S. aureus,* the second leading pathogen responsible for antimicrobial resistance-associated deaths in 2019.[30] Transporter **2** was found to be almost 10-times more effective than the tren-based tris-thiourea, the best inhibitor of *S. aureus* growth among previously reported synthetic anionophores (MIC = 0.85 μM),[8] and almost 5-times more effective than natural antibiotic vancomycin (MIC = 0.345 μM for ATCC 25923 reference strain used in this study).[31]

On the other hand, the two Gram-negative strains, *E. coli* and *A. baumannii*, were found to be almost completely resistant (MICs > 150 μM) to the compounds **1**-**4**. The selectivity towards Gram-positive bacteria is often observed both for synthetic anionophores[9] and for antibiotics,[32] and can be attributed to the presence of double cell membrane in the Gram-negative bacteria.

Conclusions

Synthetic bicarbonate transporters are highly desirable due to their potential use in treating channelopathies associated with the disruption of natural transport proteins. However, until very recently, the kinetics of bicarbonate transport by synthetic receptors was studied only indirectly, by measuring chloride transport. A novel, direct method revealed an alternative transport mechanism, where non-polar and uncharged CO_2 crosses the membrane, while the transporters merely dissipate the resulting pH gradient. This calls into question whether various anion transporters are actually able to transport bicarbonate anions at all.

Using the newly developed direct assay we showed that carbazole-based amides and a thioamide facilitate the transport of bicarbonate by two independent mechanisms: 1) unassisted transmembrane diffusion of CO_2 coupled with pH equilibration *via* H^+Cl^- symport (or OH^-/Cl^- antiport), and 2) true bicarbonate anion transport *via* HCO_3^-/Cl^- antiport. Furthermore, we were able to estimate the relative contributions of the two transport mechanisms in a wide concentration range. It turned out that CO_2 diffusion dominates at low concentrations of transporters, whereas high concentrations give rise to the true HCO_3^-/Cl^- antiport. The ability of **1**-**4** to equilibrate the pH gradient across the lipid membrane was confirmed by the HPTS assay and especially **2** and **4** are among the most potent H^+Cl^- or OH^-/Cl^- transporters published.

Based on these results, the antibacterial properties of **1**-**4** were investigated on four model strains. Compounds **2**-**4** were very effective against *B. subtilis* and **2** also showed a very low MIC against *S. aureus*. These results are in line with the trends in transport activity, where **2** is the most efficient, followed by **3** and **4**. In contrast, no significant effect of **1**-**4** was observed against Gram-negative bacteria. This high selectivity against gram-positive bacteria indicates the crucial importance of cell membrane structure.

This study has furthermore demonstrated that investigating the transport mechanisms of highly potent anion transporters can guide their biological applications. In the case of di(thio)amidocarbazoles **2**-**4**, this has revealed their highly promising antibacterial properties.

Author Contributions

K. M.-J.: synthesis of receptors, anion transport experiments, data analysis, visualization, writing and editing of the manuscript. A. C.: anion transport experiments, data analysis. A. M.: MICs determination. I. I.: MICs determination. S. J. B.: providing the $[Eu.L^1]^+$ probe. R. S.: supervision of biological studies, data analysis, writing and editing of the biological part of the manuscript. M. J. C.: funding acquisition, data analysis, writing and editing of the manuscript. H. V. conceptualisation, funding acquisition, supervision of anion transport studies, methodology, data analysis, writing and editing of the manuscript. All authors have contributed to, seen and approved the manuscript.

Conflicts of interest

There are no conflicts to declare.

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