Potent antimicrobial effect induced by disruption of chloride homeostasis

Luke E. Brennan,[a,b†] Lokesh K. Kumawat,[a†] Magdalena E. Piatek,[b,c] Airlie J. Kinross,[e] Daniel A. McNaughton,[e] Luke Marchetti,[a] Conor Geraghty,[a,b,d] Conor Wynne, [a,b] Hua Tong,[a] Oisín N. Kavanagh,[f] Finbarr O'Sullivan,[g,b] Chris S. Hawes,[h*] Philip A. Gale,[e,i*] Kevin Kavanagh [b,c,d*] and Robert B. P. Elmes.[a,b,d*]

[a] Department of Chemistry, Maynooth University, National University of Ireland, Maynooth, Co. Kildare, Ireland.
[b] Synthesis and Solid State Pharmaceutical Centre (SSPC), Ireland.
[c] Department of Biology, Maynooth University, National University of Ireland, Maynooth, Co. Kildare, Ireland.
[d] Kathleen Lonsdale Institute for Human Health Research, Maynooth University, National University of Ireland, Maynooth, Co. Kildare, Ireland.
[e] School of Chemistry, The University of Sydney, NSW 2006, Australia.
[f] School of Pharmacy, Newcastle University, Newcastle upon Tyne NE1 7RU, UK
[g] National Institute for Cellular Biotechnology, Dublin City University, D09 NR58 Dublin, Ireland
[h] School of Chemical and Physical Sciences, Keele University, Keele ST5 5BG, U.K.
[i] University of Technology Sydney, Faculty of Science, City Campus, PO Box 123, Broadway, NSW 2007, Australia

robert.elmes@mu.ie
kevin.kavanagh@mu.ie
philip.gale@uts.edu.au
c.s.hawes@keele.ac.uk

[†] Both authors contributed equally to this work

Summary

Artificial transmembrane ion transporters have proposed applicability to medicinal chemistry where perturbation of normal cellular homeostasis has already been shown to induce apoptosis in mammalian cells, however this effect has not been observed in bacteria. In this study, we report the synthesis and structural characterisation of a new class of fluorescent anionophores that effectively kill Gram+ bacteria by disrupting normal Na^+ and Cl^- concentration. So-called 'squindoles' take advantage of both NH and CH H-bonding interactions to bind chloride with high affinity and act as efficient anion transporters as measured by lipid vesicle transport assays. The most active transporter shows potent inhibitory activity against *Staphylococcus aureus* (SA) and *Methicillin-resistant Staphylococcus aureus* (MRSA). Cell-based assays and label-free quantitative proteomic profiling suggest that the mode of action is directly related to the anion transport ability where an influx of chloride into bacterial cells significantly affects their proteome and induces several known stress responses.

Introduction:

Each year anti-microbial resistant (AMR) bacteria kill over 1 million people and it is expected that this will increase to 10 million people by 2050 if new antibiotic drugs are not found.[1] Therefore, there is an urgent need for antimicrobial drugs that exhibit a distinct mode of action from traditional approaches. Anion transport has become an increasingly important area in this regard where it's proposed applicability to medicinal chemistry has stimulated significant research interest.[2-4] For example, Quesada *et al.,* recently reported a promising new approach for Cystic Fibrosis (CF) therapy with a series of tambjamine analogues capable of efficient transmembrane anion transport that also improved several aspects of CF pathophysiology in a lung epithelial cell model.[5] Several classes of synthetic anionophores have been utilised as vectors for the disruption of anion homeostasis *in cellulo*.[6-8] The dysregulation of chloride influx with perenosins can induce apoptosis and cell cycle arrest[9] while some squaramide transporters have been shown to disrupt autophagy through altering the lysosomal pH.[10] There has been less research focus on the applicability of anionophores that may function as antimicrobial agents.[11] This is somewhat surprising given the documented antimicrobial activity of many cationophore natural products such as monensin, salinomycin and lasalocid.[12] Quesada *et al.,* recently exploited a series of indol-7-yl-tambjamine conjugates where it was shown that these compounds exhibited anti-microbial activity against a panel of Gram-positive and Gram-negative strains, however, no link between anionophoric ability and anti-microbial activity was established.[13] Similarly, Share *et al.* reported a series of aminopyrrolic receptors that were shown to inhibit the growth of *S. aureus*.[14] Interestingly, while some correlation between anion transport activity and antimicrobial effect was observed for this series of compounds, the trend was imperfect and suggests that the observed activity might stem from more than one underlying mechanism. Thus, there remain several unanswered questions on the role of anionophores in promoting antimicrobial effects.

To date, the commonality amongst most known anion binding motifs is the use of NH bond donor systems that display high affinity for biologically relevant anions such as Cl^- or bicarbonate. For the most part, this has been achieved through exploiting functional groups such as ureas,[15-18] thioureas,[19-21] pyrroles,[22-24] thioamides[25] and squaramides[26-29] to name just a few examples. More recently, pnictogen-, chalcogen- and halogen-bonding receptors have been proposed as anion transport scaffolds and several examples using this approach have been reported,[30-33] while Flood *et al*., have demonstrated how a cryptand-like triazolo cage bearing only CH donors was capable of binding chloride with attomolar affinity (10^{17} M^{-1}).[34] Although not yet exploited for the purposes of anion transport, it is clear that CH interactions provide a new perspective in the pursuit of effective anion transporters.

Our groups have also exploited the squaramide functionality in the design of anion receptors,[35-37] sensors[38,39] and transporters.[40-43] Squaramides are particularly amenable to this purpose due to their strong H-bond donating ability, planar structure and the observed increase in aromaticity upon guest binding.[44,45] Moreover, their potential uses across a range of applications in the chemical sciences has seen them be exploited to a large extent in recent years.[46]

Herein, we describe a new class of squaramides that take advantage of both NH and CH bonding to recognise anions, and with the benefit of an incorporated indoline moiety, display bright green fluorescence. We expected that the combination of both anion binding ability and fluorescence would prove useful as either a potential new therapeutic or as a tool to 'track and trace' anion transport in biological environments. This new family of 'squindoles' (**1** – **4**, Scheme 1) differ in their aromatic substitution and modification of lipophilicity significantly affects anion transport ability. They are easily synthesised, display high chloride binding affinity, and exhibit efficient anion transport. Moreover, we demonstrate the potent anti-microbial activity of this class of compounds and, for the first time undertake a detailed study of the underlying mechanism of action. The results suggest a direct link between the anionophoric ability of compound **2** and its potent inhibitory activity in MRSA. We believe these findings lay the foundation for a new approach in the fight against bacterial resistance, where anionophores provide a viable alternative to known antibiotics.

Synthesis:

Compounds **1** - **4** were synthesised according to Scheme 1. Reaction of 1-ethyl-2,3,3-trimethyl-3H-indol-1-ium iodide with ethyl squarate under basic conditions gave the known semisquaraine intermediate **5** in 55% yield.[47] Subsequent reaction of the appropriate aniline with **5** in the presence of $Zn(OTf)_2$ afforded **1** - **4** in 48%, 45%, 47% and 50% yield, respectively after purification by column chromatography. The successful synthesis of all compounds was confirmed by ^1H and ^{13}C NMR, high-resolution mass spectrometry (HRMS), and IR spectroscopy (see Supporting Information for further details). The ^1H and ^{13}C NMR spectra showed signals for the indoline as well as the aniline components and importantly, well-resolved signals were clearly observed for the squaramide NH and indoline CH.

Scheme 1.

Crystals suitable for single crystal X-ray analysis were obtained for **1** - **4** by recrystallisation from concentrated DMSO solutions allowing the evaluation of their solid-state behaviour and also confirmation of the trans geometry of the indoline relative to the cyclobutene ring; an important consideration for successful anion binding (See Figure 1).

X-Ray Crystallography:

Each of the four hosts **1** – **4** crystallised as their DMSO solvates, and common features in molecular geometry, intermolecular interactions and crystal packing behaviour were observed in all four structures. The structural models for compound **1**, **2** and **3** (refined in the space groups $P2_1/c$, $P\text{-}1$ and $P2_1/n$, respectively) each contain one molecule of the host and a single DMSO molecule within their asymmetric unit, while the model for compound **4** in the space group $P\text{-}1$ contains two unique host molecules and two DMSO molecules. As shown in Figure 1, all four structures confirm the expected *trans* orientation of the indoline group, and the central NH and CH groups are oriented in a *cis* fashion to form a heterotopic central binding pocket reminiscent of that observed in symmetric squaramides. [48]

Figure 1.

In all four cases, the expected N-H···O hydrogen bond from the squaramide to the DMSO oxygen atom is observed, while weaker ancillary C-H···O contacts from the indoline C-H group are observed also involving the DMSO acceptor. Unsurprisingly, the C-H donor exhibits considerably longer donor···acceptor distances than the NH donor in all cases (Table 1). The N···O distances vary uniformly for the series, decreasing in the order **1**>**2**>**3**>**4** with the distance in **4** nearly 4% shorter than the equivalent distance in **1**. In contrast, the C···O distances show a more random distribution, and the difference between the two non-equivalent molecules in the asymmetric unit of **4** is considerably larger than any discrepancy observed between different hosts. These observations are consistent with the electronic influence of the phenyl substituent affecting the acidity of the directly attached NH group, while the contacts from the C-H group are likely more influenced by the local geometry and crystal packing forces.

Table 1: Key hydrogen bonding parameters for the bound DMSO species in compounds **1** – **4**.

Compound	*N···O distance (Å)*	*N-H···O angle (°)*	*C···O distance (Å)*	*C-H···O angle (°)*
1	2.925(2)	168	3.431(3)	167
2	2.900(5)	171	3.442(6)	160
3	2.873(3)	168	3.369(3)	165
4	2.820(3)	172	3.392(3)	163
	2.807(3)	161	3.657(4)	148

Beyond the key hydrogen bonding interactions, the remaining intermolecular forces observed in the structures of the DMSO solvates mainly involve π-π interactions, as shown in Figure 2. Given the out-of-plane steric bulk of the indoline group, these interactions primarily involve the squaramide core and phenyl ring where additional influence from the R_1 and R_2 substituents is observed. For all four solvates, the most notable intermolecular contact is a parallel head-to-tail π-π interaction between the phenyl rings. In compound **1**, the phenyl ring and squaramide group are essentially co-planar at a 3.3° angle, and adjacent molecules stack at an interplanar distance of 3.39 Å. A similar interplanar angle of 3.2° is observed in **2**, although the increased steric bulk from the trifluoromethyl groups results in a longer interplanar distance of 3.52 Å. Compound **3** shows the shortest interplanar stacking distance of 3.28 Å despite a larger interplanar angle of 14.8°. In compound **4**, the most prominent stacking interaction is between the two non-equivalent host molecules with overlap solely involving the phenyl rings, where the two rings are offset by 3° to one another and the shortest inter-atomic distance is 3.580(4) Å for C18-C42. The disfavoured stacking in **4** likely relates to the large phenyl-squaramide interplanar angles in both unique molecules, of 36.7 and 39.1°. A close contact between the squaramide carbonyl groups of adjacent molecules is also observed in the structure of **4**; the minimum inter-atomic distance of 3.171(4) Å is shorter than the overlap distances of the phenyl ring, although the interaction between squaramides is limited to the periphery of the ring only.

Figure 2.

Photophysical Properties:

The absorption and emission properties of **1** – **4** were evaluated in DMSO solution (10 μM) where all compounds exhibited two broad absorbance bands between 287 nm - 309 nm and 428 nm - 440 nm. In the case of the **2**, a third absorption band at *ca.* 540 nm was also observed, suggesting that some internal charge transfer (ICT) may be occurring between the electron rich indoline and the electron deficient 3,5-bis(trifluoromethyl)phenyl moieties. Similarly, the fluorescence emission of **1** – **4** was also measured in DMSO (10 μM) where excitation between 425 – 450 nm led to characteristic emission at *ca.* 490 nm in all cases.

Given the interesting absorption and emission characteristics of **1** – **4** we next wished to evaluate their response to anions. Addition of various anions (I^-, AcO^-, SO_4^-, Br^-, Cl^-, $H_2PO_4^-$ and NO_3^- as their tetrabutylammonium (TBA) salts) to a solution of the receptors in DMSO (10 μM) showed that the absorbance spectra of the compounds remained mostly unchanged. However, upon the addition of F^-, the absorbance showed a clear hypochromic shift for all compounds. In the case of **2**, there was also a clear hyperchromic shift at 298 nm and 536 nm (See Supporting Information). The observed results with F^- are likely due to deprotonation where there is now significant evidence that F^- is capable of deprotonating squaramides with aromatic substituents. Moreover, this theory is further supported by visible disappearance of the NH protons in the presence of F^- ions in ^{1}H NMR spectroscopic studies discussed below.

Anion Binding Properties:

To confirm the ability of **1** – **4** to bind anions, ^{1}H NMR spectroscopic studies were carried out in DMSO-d_6/H_2O (99.5:0.5) with the TBA salts of a selection halides and oxoanions. Treatment of the receptors with basic anions such as fluoride, sulfate, acetate and phosphate gave rise to deprotonation of the NH and alkene CH protons, indicated by the disappearance of the respective peaks and a broadening of the aromatic signals in the ^{1}H NMR spectra. No observable changes were seen upon treatment with iodide and nitrate, implying minimal hydrogen bond formation with these anions (See Supporting Info). Upon treatment of the receptors with chloride and bromide, a considerable shift downfield was observed for signals associated with the proposed binding site, indicating interaction and formation of host:guest complexes. The titration of compound **2** with TBACl is shown in Figure 3 as an example. The largest changes are observed for CH_b and NH_c (Δ_{ppm} = 0.7 and 1.7, respectively) with the methylene protons H_a and aromatic CH proton H_d also shown to undergo a downfield shift (Δ_{ppm} = 0.2 and 0.4, respectively). This suggests the main binding interaction is occurring at the squaramide NH with a significant contribution from the indoline CH. Similarly, it suggests that a weak interaction exists with the protons surrounding the binding site (H_a and H_d) and the guest anion. Weak C-H···anion binding has been reported previously.[49] From this titration data, association constants

were determined by fitting to a 1:1 binding model using BindFit,[50,51] the results of which are summarised in Table 2. (See Supporting Info for fitted data).

Table 2: Summary of the interaction between receptors **1** - **4** and the TBA salts of various anions and their association constants. All values represented are the association constant (K_a/M^{-1}) for the formation of a 1:1 host : guest complex. (d. = deprotonation occurred.)(n. = no interaction observed.)

	F^-	Cl^-	Br^-	I^-	SO_4^{2-}	NO_3^-	AcO^-	$H_2PO_4^{2-}$
1	d.	41	12	n.	d.	n.	d.	d.
2	d.	201	50	n.	d.	n.	d.	d.
3	d.	83	18	n.	d.	n.	d.	d.
4	d.	63	13	n.	d.	n.	d.	d.

Figure 3.

Overall, the data revealed that anion affinity for the receptors is in the order of **2** > **3** > **4** > **1**. This trend is congruent with previously reported receptors, where the substitution pattern of the aniline affects binding to a significant extent.[52] **1** displayed the lowest binding constant due to a lack of electron-withdrawing groups, while **2** has by far the highest binding affinity for both chloride and bromide due to the presence of two electron withdrawing CF_3 substituents.

Anion Transport Properties:

Once the ^{1}H NMR titration data had confirmed the affinity of this class of compounds towards chloride, their ability to transport this anion across a model lipid bilayer was investigated using the Cl^-/NO_3^- exchange assay. Vesicles consisting of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), prepared following literature methods,[53] were loaded with a pH-buffered sodium chloride internal solution (300 mM) and sodium nitrate external solution (300 mM). Each transporter was added in a portion of DMSO (10 μL) at a number of different molar concentrations, and subsequent chloride efflux monitored using a chloride ion-selective electrode (ISE) for 300 seconds. By plotting the efflux achieved after 270 seconds for each concentration and fitting to the Hill equation, an EC_{50} value can be generated - the gold standard measurement of transporter activity. The results are summarised in Table 3.

Table 3: A summary of the activity and rate measurements recorded for receptors **1** - **4** in the transport assay experiments. [a] EC_{50} at 270 s, shown as molar percentage with respect to lipid concentration. [b] The Hill coefficient. [c] The ratio of electrogenic transport character was performed via division of the valinomycin test initial rate ($k_{max(Val)}$) by the monensin test initial rate ($k_{max(Mon)}$). [d] Rate calculated by fitting to an exponential decay curve. [e] Rate calculated by fitting to a sigmoidal curve.

	Cl^-/NO_3^- Exchange Assay			*Cationophore Coupled Assay*		
	$EC_{50,270s}$[a] (mol%)	n[b]	k_{max} (% s^{-1})	$k_{max(Val)}$ (% s^{-1})	$k_{max(Mon)}$ (% s^{-1})	Ratio[c]
1	1.28 ± 0.05	2.8 ± 0.4	0.23[d]	2.51[e]	0.53[e]	4.7
2	0.21 ± 0.006	1.5 ± 0.10	0.82[e]	0.63[e]	0.49[e]	1.3
3	1.25 ± 0.05	1.7 ± 0.1	0.27[d]	0.74[e]	0.44[e]	1.7
4	0.74 ± 0.02	2.0 ± 0.1	0.50[e]	0.44[e]	0.38[e]	1.2

All four compounds were found to facilitate transport at low molar concentrations (with respect to the concentration of lipid), however compound **2** proved to be the most efficient transporter (Figure 4), with an EC_{50} value of 0.21 mol%. This can be partially attributed to the higher chloride binding affinity afforded by multiple electron-withdrawing groups as described above. The effect of the bis-CF_3 groups is amplified further when considering the enhanced lipophilicity afforded by this motif, which results in better partitioning of the transporters into the lipid bilayer.[54-56] The EC_{50} value for compound **2** is comparable to that of a number of the most active squaramide transporters tested, which include the symmetrical bis-CF_3 squaramide **6** (EC_{50} = 0.15 mol%) and structurally similar mono-CF_3 squaramide **7** (EC_{50} = 0.06 mol%).[10,52,57] These results indicate that the replacement of the potent hydrogen bonding NH group with an alkene CH hydrogen bond does not diminish transport activity. Notably, the trend reported in the titration data for compounds **3** and **4** is reversed for transport activity, with **3** exhibiting EC_{50} and k_{max} values similar to parent compound **1**. This again could be ascribed to the increased lipophilicity of the CF_3 leading to enhanced partitioning in to the lipid bilayer but is likely due to solubility issues associated with compound **3** which limits the amount of transporter that reaches the lipid bilayer.

Figure 4.

The Hill coefficient (n) indicates the stoichiometry of the complex formed between the receptor and the anion during transmembrane movement.[58] A value of 2, approximately, was reported for each compound, suggesting that the movement of chloride across the bilayer is facilitated via encapsulation by two transporter molecules. The maximum rate of efflux achieved by each transporter at 1 mol% loading (k_{max}) was found to be in agreement with the trend reported for activity. The transport capabilities of receptors **1 - 4** were also probed using the NMDG-Cl assay, which again returned the same pattern of activity (Results can be found in the supplementary information).

The transport mechanism for this class of transporters was subsequently investigated using cationophore coupled assays. In this assay, vesicles are loaded with potassium chloride solution (200nm) and suspended in a solution of potassium gluconate (200 mM), both buffered to pH 7.2 using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The highly hydrophobic gluconate anion is unable to cross the lipid bilayer, meaning the specific chloride transport mechanism can be probed. Efflux achieved by the free transporter is again recorded, followed by separate runs accompanied by two cationophores. Valinomycin is a potassium uniport transporter and, when coupled to a chloride transporter, enables the molecule to solely transport chloride out of the vesicle, also known as electrogenic transport (Figure 5(a)). By comparison, monensin acts as a K^+/H^+ exchanger. This means that an electroneutral H^+/Cl^- symport mechanism is facilitated because monensin balances pH by transporting H^+ ions in the reverse direction (Figure 5(b)). Each experiment was run at a concentration of transporter which corresponded to 80% efflux in the previous ISE experiments.

Figure 5.

This assay revealed that coupling of compounds **1** - **4** to either cationophore resulted in an increased rate of transport. This suggests that these compounds are capable of both Cl^- uniport and H^+/Cl^- symport, however the predominant mode of transport is consistent across the series. The ratio of the maximum rate of Cl^- efflux achieved when coupled to either cationophore ($k_{max(Val)}$ / $k_{max(Mon)}$) was calculated,[59] and all receptors returned a value greater than **1**. These results indicate a preference for chloride transport over H^+/Cl^- transport, with the parent compound **1** displaying the most electrogenic character of the compounds tested.

Antimicrobial Susceptibility Assays:

With the observation of strong chloride affinity and potent transmembrane chloride transport for compounds **1 – 4** we next wished to ascertain whether this anionophoric ability might result in the compounds possessing some antimicrobial properties. Each compound was screened for activity across a range of pathogens (*Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae),* through the use of a modified antibacterial susceptibility assay.[60] From these experiments, a degree of growth inhibition by **2** against both *S. aureus*, and MRSA was observed. No activity was observed for any of the Gram-negative pathogens tested suggesting that the compounds under study could not effectively permeate the secondary membrane present in these organisms. Informed by this – an antimicrobial susceptibility assay, in line with CLSI defined standards, was performed to ascertain minimal inhibitory concentrations (IC_{50}/IC_{80}) of **2** against both *S. aureus* and MRSA. The IC_{50} and IC_{80} values are defined as the minimal concentrations of **2** required to inhibit growth of *S. aureus* by 50% or 80%, respectively. IC_{50}/IC_{80} values were determined experimentally through varying treatment concentrations in a 96-well plate where cells were grown at 37°C and measured at 600 nm after 24 h.

A concentration of 2.5 μM resulted in 51% growth inhibition of *S. aureus*, relative to the control – indicating an approximate IC_{50} value of 2.5 μM for **2** against *S. aureus*. Furthermore, the highest concentration – 80 μM inhibited growth of *S. aureus* by approximately 75%. Concentrations of 3.1 μM and 1.6 μM gave 60% and 40% growth inhibition of MRSA relative to control, respectively, thus the IC_{50} value for compound **2** against MRSA is approximately 2.35 μM. This value correlates to the definitive IC_{50} value of 2.5 μM observed against *S. aureus*. This compares well to a recently reported IC_{50} value of 6 μM against *S. aureus* and MRSA for Monensin[12], indicating that **2** has a clinically relevant activity when compared against known antimicrobials. Furthermore, a concentration of 100 μM resulted in 80% growth inhibition relative to the control, and as such 100 μM can be considered the IC_{80} value for compound **2** against MRSA (Figure 6).

Figure 6.

Antimicrobial susceptibility assays were also performed with compounds **6** and **7** which are known anion transporters.[52] **7** has also been shown to facilitate chloride transport in to mammalians cells resulting in a disruption

of autophagy and induction of apoptosis by perturbing cellular chloride homeostasis.[61] It was expected that if the antimicrobial effect was due solely to anionophoric ability, **6** and **7** should also exhibit significant activity. This was not the case. When susceptibility assays were carried out for **6** and **7** against *S. aureus* a significantly lower level of activity was observed compared to **2**. At a concentration of 200 μM, 50% growth relative to control was exhibited in both cases. This indicates that the antimicrobial characteristics of **2** may be as a result of multiple factors and not just anion transport efficiency. Nonetheless, it is likely that the effect is linked to its anionophoric ability across the bacterial membrane and we wished to investigate this further.

Another concern regarding the marked activity of **2** was whether *S. aureus* or MRSA would develop resistance to **2**. To ascertain this, *S. aureus* was exposed to compound **2** at a concentration of 2.5 μM for a period of 10 days, with repeated daily subculturing to allow for sufficient adaptation. After this period, we found an appreciable but low level of acquired resistance to compound **2**, where the IC_{50} increased from 2.5 μM to 4.69 μM (see supporting info). When compared to other clinical antimicrobials, this level of acquired resistance is extremely low. For example, when compared to the degree of change in IC_{50} for Ciprofloxacin, when resistance is acquired through point mutation of *grlA* and *gyrA*. This mutation yields an IC_{50} increase from 0.125 – 0.25 μg/mL to 16 – 32 μg/mL, which corresponds to a 128 fold increase in IC_{50}. This increase in inhibitory concentration is of much higher severity than the acquired resistance to **2** (approx. 2-fold increase). This result also suggests that the efficacy of **2** may be due to a non-canonical mechanism of action and highlights its ability to evade resistance.

To ascertain the clinical applicability of **1** - **4**, we carried out *in-vitro* cytotoxicity assays against HaCaT cell lines using an Almar blue assay. Compounds **1** – **4** showed no marked toxicity to the cell line until a concentration of approx. 10 μM, 4x the concentration of the IC_{50} for *S. aureus* growth (figure S43). The order of cytotoxicity for compounds **1** - **4** was determined to be **2**>**4**>**1**>**3**. Encouraged by this result, *in-vivo* toxicity was also investigated using the model organism *Galleria mellonella*.[62-66] *G. mellonella* were treated with a concentration gradient of **2** from 3 – 300 μM, where even at the highest administered dose there was no observed toxicity. With this encouraging information, an infection model was also established in *G. mellonella*. Larvae were initially infected with a previously determined LD_{50} dose of *S. aureus*, and after a 30 min incubation time, to allow for establishment of infection were treated with a dose of **2** at either 50 or 150 μM. After a period of 96 h it was observed an increased 40% survival rate of *G. mellonella* when compared to a 27% rate of survival for those infected and untreated with **2** (Figure S44). This is an important result as it highlights the low cytotoxicity of compound **2** while also showing its ability to prolong the survival of larvae when exposed to an infection of *S. aureus*.

Bacterial uptake of 2:

Given the exciting results discussed above, we wished to further investigate the underlying mechanism of action. The inherent emission characteristics of compound **2** allowed the use of fluorescence microscopy confirm bacterial membrane permeability and uptake. An overnight culture of *S. aureus* treated with **2** at concentrations of 3, 10, 50, and 100 μM for 30 mins resulted in clearly observable fluorescent signals (λ_{exc} = 420 nm) localised to bacterial cells (Fig. 7). Interestingly, bacteria were observed to be self-adhering, forming clumped aggregates in response to **2**. This behaviour was not observed in control samples. It has been well documented that bacterial auto-aggregation is a protective mechanism brought about by chemical stressors being introduced to the environment.[67,68] These results confirm both the uptake of **2** in to *S. aureus* but also further confirm the potent effect it has on bacterial behaviour. Moreover, Z-stack experiments confirmed that the observed fluorescence was shown to be uniformly in three dimensions and corresponded to **2** being located inside the bacterial aggregates and not concentrated on the periphery.

Figure 7.

In an effort to ascertain whether the anionophoric ability of **2** results in an increase in cytosolic Cl^- concentration, an assay previously outlined by Ko *et. al.* was performed.[70] An overnight culture of *S. aureus* was pre-treated with *N*-ethoxycarbonylmethyl-6-methoxyquinolinium bromide (MQAE), for 1 h at 37 ˚C before treatment with compound **2** at various concentrations for 5 min, whereafter fluorescence emission was measured. Treatment with **2** (3, 10, and 30 μM) resulted in a rapid, concentration dependent decrease in fluorescence output from MQAE, centred around 460 nm, indicating a rapid increase in cytosolic Cl^- concentration in response to **2** (Figure S45).

The observed decrease in MQAE fluorescence, taken together with the results of the lipid vesicle experiments suggests **2** acts to rapidly import Cl^- into bacterial cells. Whilst known in bacteria, CLC chloride channels and transporter proteins are poorly understood and are observed in relatively low abundance endogenously.[71,72] Thus we suspect that the fluorescence quenching response observed from MQAE is primarily a direct result of facilitated transport by **2**. Moreover, in the absence of compound **2** basal cytosolic chloride concentration remains unchanged – resolved using time dependent fluorescent measurement of MQAE fluorescence. This further indicates that the reduction in fluorescence observed is likely correlated to **2**'s propensity for anion transport.

As cytosolic Cl^- concentration is evidently increased by the presence of **2**, we wished to further investigate its biological impact on bacterial cells. A triphenyl-tetrazolium chloride (TTC) assay to monitor cellular respiration was carried out to ascertain whether the activity of **2** is reliant on the abundance of free Cl^- ions in solution, where the "into-cell" transport of chloride gives rise to therapeutic effect.

Assessment of cellular respiration using the TTC assay:

It has been established that the unanticipated or facilitated influx of Cl^- ions into mammalian cells can exert a toxic effect leading to cell death.[73,74] Thus with the observed potent anionophoric activity displayed by **2**, we sought to elucidate whether the absence of free Cl^- ions in solution would attenuate its efficacy. Following standard procedures, a TTC assay for monitoring cellular respiration was carried out in three buffer systems: Hank's buffered salt solution (HBSS), Cl^- free HBSS and Na^+ free HBSS. *S. aureus* was treated with **2** for 5 h before growing in the presence of 0.5 mg/mL TTC. Samples were incubated at 37 °C for 18 h, whereafter formazan production was quantified – indicating cellular respiration levels after treatment. In HBSS buffer *S. aureus* respiration was decreased in a dose-dependent fashion, with 100 μM **2** resulting in a 75% reduction in cellular respiration. When the experiment was repeated in HBSS buffer where the Cl^- was replaced with equimolar gluconate, the effect on cell viability was completely attenuated. Similarly, the replacement of Na^+ with choline also gave rise to an equal attenuation of activity. Taken together, these experiments suggest that the effect of compound **2** is dependent on the presence of sufficient extracellular Cl^- and Na^+ ions, and acts as an "into-cell" transporter. This is believed to be a result of charge dependency with regards to transport, where sodium influx is also integral to the observed activity of **2**. It has previously been reported the influx of Na^+ cations in response to synthetic transporter induced Cl^- influx is dictated largely by the presence of native sodium ion channels on cell membranes.[70] We hypothesise this dependency on both Cl^- and Na^+ ionic abundance for activity is due to the transport coupling between synthetic transporter and native ion channels to maintain a net-neutral charge and seek ionic balance within cells.

Figure 8.

The above results suggest that both Na^+ and Cl^- ions are required for **2** to exert its inhibitory effect and that this effect is closely correlated to its ionophoric activity (Figure 8). In an effort to further elucidate the effect of **2** and the associated ionic dysregulation, label free quantitative (LFQ) proteomics was carried out.

Proteomic analysis of *S. aureus* cellular response to treatment with compound 2:

LFQ proteomics is used to compare proteomes of a cell or organism in response to a stress, or stimulus and it is an invaluable technique in the characterisation of potential modes of action of compounds. In order to investigate the *S. aureus* proteomic response to compound **2** and further evaluate its mode of action, *in-vitro* LFQ proteomics was performed on whole cell lysates, as per literature procedures with minor modifications.[63] In an effort to elicit a change in the proteome in response to treatment, *S. aureus* was grown in the presence of compound **2** (3 μM) for a total of 6 h, to early stationary phase. This concentration was chosen based on the IC_{50} value determined through treatment with varying concentrations of **2**.

The experiment identified a total of 1351 proteins via Perseus, and following filtration steps to remove potential contaminants and misidentified peptides, a total of 548 proteins remained. Filtered proteins were subjected to a principal component analysis which demonstrated a large degree of difference between treatment and control (Figure S47). A total variance of 90.6% was found between treatment and control, illustrating a large proteomic shift in response to treatment with **2**. A total of 534 proteins were identified as present in both samples, with 10 proteins being exclusive to control, and a further four exclusive to treatment. Post-imputation, it was determined a total of 228 proteins were statistically significant and differentially abundant (SSDA), pairwise test with a cut-off of $p < 0.05$ and a minimum fold change of 1.5. Samples were subjected to hierarchical clustering of z-score normalised intensity values. All SSDA proteins (n = 228) were resolved as such, and subsequently visualised by heatmapping. Perseus allows for the generation of protein intensity heatmaps based on their median expression trends. Whereupon, a set of two distinct major protein clusters, 1 & 2, with three replicates from relevant conditions in each were identified. SSDA proteins observed in lower abundance in treatment compared to control are clustered in protein group 1, where SSDA proteins observed in higher abundance in treatment compared to control samples are clustered in protein group 2 (Figure S48).

The volcano plot (Figure 9) illustrates the distribution of all 548 filtered proteins and altered protein abundance levels in treatment versus control samples. When treated with **2**, the most statistically significant, differentially abundant proteins which were observed in higher abundance include: Glycyl-glycine endopeptidase LytM (16.66 – fold) associated with cell-wall organisation, ArsA family transcriptional repressor (13.73 – fold), Chaperonin (11.89 – fold) which is linked to stress-response protein folding, Starvation-inducible DNA-binding protein (10.39 – fold), Superoxide dismutase 1 (10.25 – fold), Cold shock protein CspA (8.09 – fold), Fibronectin-binding protein A (8.09 – fold) which relays a self-adhesion response, Thioredoxin reductase (7.01 – fold) which regulates cell redox homeostasis & detoxifies, Cold-shock protein CspC (5.61 – fold), 50s ribosomal protein L29 (4 – fold), Organic hydroperoxide resistance protein (3.31 – fold) which induces a response to oxidative stress, and Alkaline shock protein 23 (2.85 – fold).

Conversely, the most statistically significant, differentially abundant proteins which were observed in lower abundance in response to treatment with compound **2** were: Lipase 1 (62.82 – fold) which is responsible for lipid breakdown at

the cellular frontier, Carbamoyl-phosphate synthase large chain (23.94 – fold) & small chain (20.16 – fold) which are both responsible for UMP, pyrimidine & arginine biosynthesis via the metabolism of glutamine, alpha-hemolysin (19.9 – fold) which is an extracellular virulence factor, Leukocidin LukS (16.44 – fold) which causes hemolysis of other organisms, N-acetylmuramoyl-L-alanine-amidase (11.67 – fold) which is responsible for the breakdown of cell-wall peptidoglycans, amino acid ABC transporter AA-binding protein (7.96 – fold) which is responsible for ligand-gated transmembrane ion transport, Succinyl-CoA ligase subunit alpha (6.56- fold) & beta (6.95 – fold) associated with the TCA cycle, and Leukotoxin LukD (4.54 – fold) & LukE (4.37 – fold). A table summarising protein group 1 and protein group 2 is shown in the supporting info and graphically represented in Figure 9.

Figure 9.

In an effort to identify biological pathways and networks of proteins which are linked between SSDA proteins increased and decreased in abundance, the STRING database was used to compile and visualise protein networks. Resultant pathways decreased in abundance were found to be involved in; *'de-novo'* amino acid biosynthesis, 50s ribosomal protein synthesis, virulence factors and aerobic respiration (TCA cycle) (Figure 10a). Protein networks which were observed in higher abundance upon treatment include: 30s ribosomal protein synthesis and translation, transmembrane glucose transport, self-adhesion and clumping factors, compensatory protein folding and cellular responses to oxidative stress (Figure 10b).

Figure 10.

Taking the above results together it is apparent, through the use of LFQ proteomics, that *S. aureus* cells experience a dramatic alteration to their proteome in response to treatment with **2**. This technique has provided further evidence toward the mechanism of action of **2** and the cellular response brought about by its anionophoric ability. The proteomic response to treatment appears to predominantly be membrane localised activity which we suspect is a result of homeostatic imbalance. With regard to membrane localised activity, SSDA proteins which indicate this activity include: Lipase 1 (66.82 – fold decrease), *N*-acetylmuramoyl-L-alanine-amidase (11.67 – fold decrease), and Glycyl-glycine endopeptidase LytM (16.66 – fold increase). This decrease in Lipase 1 abundance is believed to diminish lipid membrane metabolism. Lipase 1 plays an integral role in the breakdown of lipids which are essential for membrane stability,[75] and thus lower lipase 1 abundance may infer an attenuation of membrane lipid metabolism. Additionally, Glycyl-glycine Endopeptidase LytM is a cell wall localised endopeptidase which acts to stimulate cell wall organisation in response to cell division or dramatic environmental changes.[76] Moreover, LytM plays an integral role as a bifunctional autolysin where its autolytic pathogenic function is elicited in response to an attenuation of other virulence proteins in *S. aureus*. It is also clear that **2** elicits a dramatic decrease in virulence factor abundance. Supporting this is the decreased abundance of *N*-acetylmuramoyl-L-alanine-amidase which is a bifunctional autolysin which plays an integral role in the hydrolysis of cell wall peptidoglycans, and exerts a lytic effect on host cells.[77] This attenuation of virulence factor production can be rationalised by as an induced stress response to **2**. *S. aureus* has been noted to be "disarmed" by various therapeutic agents, where this attenuation of virulence factor activity allows for host cells to deal with the infection more expeditiously.[78,79] It has also been noted that molecules which possess the capacity to attenuate virulence in *S. aureus* and other species are of particular interest for the management and treatment of infections.[78-80]

It has previously been demonstrated that therapeutic agents which exert activity by increasing cytosolic chloride content can induce ROS formation.[73,74] Whilst not intrinsically linked, it has also been shown that rationally designed anionophores cause increase of ROS in cells.[70] In the case of **2**, we see evidence for this. From LFQ proteomic data we see an increased abundance of proteins that sequester and remediate ROS under oxidative stress. Exemplary SSDA proteins observed, linked to ROS remediation include: Cold shock protein CspA (8.09 – fold), Cold-shock protein CspC (5.61 – fold), Superoxide dismutase 1 (10.25 – fold), Thioredoxin (7.01 – fold), Organic hydroperoxide resistance protein (3.31 – fold), and Alkaline shock protein 23 (2.85 – fold). Whilst not cognately linked to ROS remediation in *S. aureus*, it has been widely documented that Cold Shock and Alkaline Shock proteins relay an oxidative stress response towards ROS in cells.[81,82] However, Superoxide dismutase 1, Thioredoxin reductase, and Organic hydroperoxide resistance protein are all critical in the cellular response to oxidative stress and in dealing with intracellular ROS.[83-85] In addition to these two mechanistic insights, LFQ proteomics indicates that **2** induces a decreased level of cell division, protein biosynthesis, and as previously noted, an attenuation of virulence.

Taken together, this evidence suggests a sodium chloride-dependent activity exerted by **2** that has been shown to induce oxidative and osmotic stress through the use of LFQ proteomics These results are strong evidence to support the use of **2** as a non-canonical therapeutic motif for the management and treatment of *S. aureus* infections.

As a final experiment to check the validity of our results, LFQ proteomics was repeated, this time in the absence of chloride. Given our conclusions from the previous section we expected that experiments in the absence of chloride should give a significantly different proteomic response of the bacteria.

LFQ proteomics was again performed on whole cell lysates. *S. aureus* was grown in the presence of compound **2** (3 μM) for a total of 6 h, to early stationary phase, but this time in Hanks Buffered salt solution containing no chloride (HBSS-Cl free). In addition, untreated cells were grown in HBSS-Cl free, and Nutrient broth solutions. This was done in an effort to replicate the previous set of conditions, and to allow for visualisation of proteomic shifts in response to the

varying media, and treatment conditions. On this occasion, 1677 proteins in total were identified via Perseus, where following filtration steps to remove likely contaminants and misidentified peptides, a total of 1616 proteins remained. From this, all proteins were identified as present in all three samples, with 0 proteins being exclusive to their relevant dataset. Following imputation steps, it was determined a total of 1261 proteins were SSDA, (multiple sample T test) (ANOVA, p ,0.05) with a minimum fold change of 1.5. As before, filtered proteins were subjected to PCA which demonstrated a moderate degree of variance amongst all three conditions (Figure S49). A total variance of 65.8% was found between all three sample sets. Interestingly, the variance between nutrient broth and both HBSS sample sets is more pronounced than the disparity between HBSS Cl^- free and HBSS Cl^- free w/ treatment which we ascribe to the differing nutrient composition in NB, and the lack thereof in HBSS media.

Based on the clustering of z-score normalised intensity values, samples were again subjected to hierarchical clustering. All SSDA proteins (n = 1261) were resolved as such, and subsequently visualised by heatmapping as before. A set of three distinct major protein clusters, 1, 2, and 3, with four replicates from relevant conditions in each were identified. This clustering visualised by heatmapping illustrates a somewhat disordered intensity trend within each distinct group (Figure S50). Indeed, group 1, 2, and 3 protein intensities all show a lack of trend with respect to alterations in intensity. Indeed, this lack of trend with regard to median expression suggests that treatment with **2** in Cl^- free media is not the main cause of proteome alteration of *S. aureus* in these samples. When datasets from HBSS Cl^- free conditions were subjected to a STRING analysis to compare protein pathways observed in higher or lower abundance (See supplemental info), it was noted that the primary cellular pathways decreased in abundance upon treatment with **2** were; primarily those related to ribosomal biogenesis, and amino acid biosynthesis. Proteins observed in lower abundance were similar to original data sets, despite the removal of Cl^- from the media and thus can be deemed as less important than proteins which were observed in higher abundance. These were; the TCA cycle, peptidoglycan biosynthetic processes, cell cycle, and cell division. Indeed, having observed active cell division upon treatment with **2** but in the absence of Cl^-, further supports our assertion that **2** requires the presence of Cl^- for activity. Had this not been the case, inhibition of the cell cycle and active cell division would still have been possible in the presence of **2** under Cl^- free conditions. Further protein networks observed in increased abundance, are located primarily at the periphery of the cell, at the envelope and cytoplasmic cell membrane, further supporting the replication process observed. Whilst there is an increase in proteins related to oxidoreductase activity, as a result of treatment with **2**, this heightened presence is not entirely unexpected. The heightened presence of these proteins can be ascribed to the nutrient deficient nature of the media, HBSS. However, the results also indicate (although poorly defined) some secondary effects that may be brought about by **2** and further experiments and further experiments will be required to fully elucidate any alternatives modes of action.

Overall, however, the results of LFQ proteomics carried out in the absence of chloride does not show the same proteomic response seen in the presence of chloride and suggests that an abundance of chloride is integral the main antimicrobial activity of **2**.

Conclusions:

In conclusion, we report the synthesis and characterisation of a new anion binding motif – so-called 'squindoles' that take advantage of both NH and CH interactions to bind chloride with high affinity. X-ray crystallography of all compounds confirmed the desired trans geometry of the indoline relative to the cyclobutene ring that forms a heterotopic central binding pocket reminiscent of that observed in symmetric squaramides. All compounds **1** – **4** display bright green fluorescence that is not modulated in the presence of less-basic anions such as chloride. ^{1}H NMR anion binding titrations confirmed high affinity chloride binding in all cases and lipid vesicle transport assays also confirmed their ability to transport chloride across a model lipid bilayer. Compound **2**, that contains a 3,5-bis(trifluoromethyl)aniline in its structure is shown to be amongst one of the most efficient anionophores reported to date (EC_{50} = 0.21 mol%) acting through a combination of Cl^- uniport and H^+/Cl^- symport. Importantly, these results confirm that replacing the potent hydrogen bonding NH group with an alkene CH hydrogen bond does diminish binding or transport activity to any great extent. A series of antimicrobial susceptibility assays demonstrated that **2** is also a potent anti-microbial agent against both *S. aureus* and MRSA with a clinically relevant IC_{50} value of 2.5 μM. Subsequent experiments utilising confocal microscopy, scanning electron microscopy and fluorescence spectroscopy confirmed that **2** is rapidly internalised by bacterial cells, promotes an influx of Cl^- into the cell and triggers a significant change in cell morphology. Furthermore, assessment of cellular respiration in the presence of **2** confirms a dose dependent toxicity towards *S. aureus* that is completely attenuated in the absence of either Cl^- or Na^+ ions. This important result confirms the pivotal role NaCl concentration has in the activity of **2** as an antimicrobial agent. Finally, LFQ proteomics revealed that *S. aureus* cells experience a dramatic alteration to their proteome in response to treatment with **2** with a decreased level of cell division, reduced protein biosynthesis and an attenuation of virulence factors. Taken together, the results suggest a sodium chloride-dependent activity that induces membrane perturbation and oxidative stress and ultimately cell death. LFQ proteomics carried out in the absence of Cl^- did not show the same proteomic response and confirms the necessity of Cl^- to the antimicrobial activity of **2**.

Overall, this study represents the first detailed mechanistic insight into the anti-microbial activity of synthetic transmembrane anion transporters. It confirms their potential as a new approach to fighting AMR and suggests a mechanism of action directly linked to a perturbation of sodium chloride homeostasis that circumvents resistance mechanisms in MRSA. Given the promising results seen from this small family of compounds, we are currently undertaking an expansion of the structural diversity of squidoles with a view to identifying further lead compounds.

We are hopeful that with some synthetic modification, we will identify more efficacious compounds that can be progressed into more complex in vivo models. The results of this work will be communicated in due course.

EXPERIMENTAL PROCEDURES

Resource availability

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Robert B. P. Elmes (robert.elmes@mu.ie).

Materials availability

All reagents used in this study and full experimental details can be found in the supplemental information.

Data and code availability

Full experimental procedures are provided in the supplemental information.Crystallographic data of **1** – **4** have been deposited in the Cambridge Crystallographic Data Centre under accession CCDC numbers 2167535 - 2167538. These data can be obtained free of charge via https://www.ccdc.cam.ac.uk/structures/

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at

ACKNOWLEDGMENTS

L.E.B. thanks the Irish Research Council for a Government of Ireland Postgraduate Scholarship (GOIPG/2020/78). L.K.K. thanks the Irish Research Council for a Government of Ireland Postdoctoral Research Fellowship (GOIPD/2017/1091). CG acknowledges Maynooth University and the Kathleen Lonsdale Institute for Human Health Research for PhD funding. L.M acknowledges Maynooth University for a Hume Scholarship. R.E., K.K, F.O.S, C.W., O.K. and M.P acknowledge funding from Science Foundation Ireland (SFI), grant number 12/RC/2275/P2, which is co-funded under the European Regional Development Fund. SFI are also acknowledged for the funding of the NMR facility (12/RI/2346/SOF) and Q-Exactive mass spectrometer (12/RI/2346(3)) through the Research Infrastructure Programme and the Advion Compact Mass Spec through the Opportunistic Infrastructure Fund (16/RI/3399). D.A.M., A.J.K 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 and the University of Technology Sydney. P.A.G. thanks the Australian Research Council, Australia (DP200100453), the University of Sydney and the University of Technology Sydney for funding.

AUTHOR CONTRIBUTIONS

RE, KK, PAG, DAM, CSH, LKK and LEB designed the study and wrote the manuscript. RE, KK and PAG supervised the study. LKK and LEB synthesized and characterized the compounds and carried out spectroscopic titrations. CSH performed crystal structure analysis and also associated data refinement. D.A.M., A.J.K performed anion transport assays. LEB and MP carried out the biological studies and proteomics analysis. FOS contributed to the fluorescent microscopy results and mammalian cell cytotoxicity data. CW, CG, HT, LM and OK contributed to compound characterisation and data gathering. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Scheme 1: The synthesis of compounds **1** – **4**. (i) Iodoethane, toluene, reflux, 24 hr, 85%; (ii) 3,4-diethoxy-cyclobut-3-ene-1,2-dione, triethylamine, EtOH, reflux, 8 hr, 55%; (iii) substituted aniline, $Zn(OTF)_2$, EtOH, reflux, 24 hr, 45 - 50%.

Figure 1: Structures of the DMSO solvates of compounds **1** – **4** with partial atom labelling scheme and ADPs rendered at 50% probability level. Crystallographic disorder on the DMSO sulfur atoms in **3** and **4** and the indoline and DMSO methyl groups in **2**, and selected hydrogen atoms are omitted for clarity.

Figure 2: Comparison of the key parallel π-π interactions between the phenyl groups observed in the DMSO solvates of compounds **1** – **4**. Selected hydrogen atoms and crystallographic disorder are omitted for clarity.

Figure 3: ^{1}H NMR stack of receptor **2** with 0.0 – 10.0 eq. TBACl in DMSO-d_6/0.5% H_2O.

Figure 4: Hill analysis of Cl^-/NO_3^- exchange facilitated by **2**. Each point is the average of three repeats with error bars to show standard deviation. A run of pure DMSO was used as a control.

Figure 5: Determination of the mode of transport by **1** – **4**. a) Experimental set up to determine electrogenic transport capability. This type of transport occurs when coupled to valinomycin (red). b) Experimental set up to determine electroneutral transport capability. This type of transport occurs when coupled to monensin (turquoise).

Figure 6. Percentage growth of *S. aureus* (a) and MRSA (b) in the presence of compound **2** relative to the control. Cultures grown overnight were treated with compound **2**, incubated at 37 °C and growth was quantified after 24 h. All values are the mean ± S.E of eight samples.

Figure 7. Fluorescent microscopy images of *S. aureus* samples upon treatment with Compound **2** at varying concentrations. A) 3 μM, B) 10 μM, C) 50 μM, D) 100 μM. Images were acquired using a Photometrics CoolSnap HQ2 camera, mounted on a Nikon TiE fluorescent microscope. Images were acquired through a 60x objective oil immersion lens, where the scale bar represents a distance of 20 μm.

Figure 8. Percentage cellular respiration relative to the control, upon treatment with Compound **2** in various buffer systems. Blue = Hank's buffered salt solution (HBSS), Orange = Cl^- free HBSS buffer, Green = Na^+ free HBSS buffer. Cells were treated with varying concentrations of Compound **2** for 5 h before incubation with TTC at 37 ^{0}C for 18 h. All values represented are the mean ± S.E of 24 samples.

Figure 9. Volcano plot for visualisation of protein abundance from *S. aureus* in response to treatment with compound **2** for 6 h. Protein distributions are based on their relative significance ($Log_{10}P$ value)(y) graphed against the fold change (Log_2 LFQ intensity difference)(x). Proteins of statistical significance (p value < 0.05) are found above the horizontal orange line. Proteins of interest are annotated with those increased in expression shown as orange or to the right of the blue vertical line and those decreased in expression shown as blue or to the left of the vertical blue line. Proteins of interest have a fold change of ≥1.5.

Figure 10. STRING analysis of protein networks decreased (a), & increased (b) in abundance in *S. aureus* treated with 3 μM SQS-IND A3 for 6 h Versus untreated controls. Data accrued from the STRING database using UniProt IDs from SSDA proteins from multiple sample *t* tests (p < 0.05) details the interactions between individual proteins, or corresponding pathways (highlighted in distinct colours & labelled). (A) Protein pathways observed to be downregulated in response to treatment. (B) Protein pathways observed in increased abundance upon treatment with compound **2**.

Supplemental Data Items

Supplemental dataset 1: All identified proteins.

Supplemental dataset 2: Heirarchical clustering of proteins.

Supplemental dataset 3: Proteomics data in varied buffers.

References

1. Murray, C. J. L.; Ikuta, K. S.; Sharara, F.; Swetschinski, L.; Aguilar, G. R.; Gray, A.; Han, C.; Bisignano, C.; Rao, P.; Wool, E.; et al. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet, 399, 629-655.
2. Wu, X.; Gilchrist, A. M.; Gale, P. A. (2020). Prospects and Challenges in Anion Recognition and Transport. Chem, 6, 1296-1309.
3. Davis, J. T.; Gale, P. A.; Quesada, R. (2020). Advances in anion transport and supramolecular medicinal chemistry. Chem. Soc. Rev., 49, 6056-6086.
4. Akhtar, N.; Biswas, O.; Manna, D. (2020). Biological applications of synthetic anion transporters. Chem. Commun., 56, 14137-14153.
5. Gianotti, A.; Capurro, V.; Delpiano, L.; Mielczarek, M.; García-Valverde, M.; Carreira-Barral, I.; Ludovico, A.; Fiore, M.; Baroni, D.; Moran, O.; Quesada, R.; Caci, E. (2020). Small Molecule Anion Carriers Correct Abnormal Airway Surface Liquid Properties in Cystic Fibrosis Airway Epithelia. International Journal of Molecular Sciences, 21, 1488.
6. Akhtar, N.; Saha, A.; Kumar, V.; Pradhan, N.; Panda, S.; Morla, S.; Kumar, S.; Manna, D. (2018). Diphenylethylenediamine-Based Potent Anionophores: Transmembrane Chloride Ion Transport and Apoptosis Inducing Activities. ACS Applied Materials & Interfaces, 10, 33803-33813.
7. Rodilla, A. M.; Korrodi-Gregório, L.; Hernando, E.; Manuel-Manresa, P.; Quesada, R.; Pérez-Tomás, R.; Soto-Cerrato, V. (2017). Synthetic tambjamine analogues induce mitochondrial swelling and lysosomal dysfunction leading to autophagy blockade and necrotic cell death in lung cancer. Biochem Pharmacol, 126, 23-33.
8. Hernando, E.; Soto-Cerrato, V.; Cortés-Arroyo, S.; Pérez-Tomás, R.; Quesada, R. (2014). Transmembrane anion transport and cytotoxicity of synthetic tambjamine analogs. Org. Biomol. Chem, 12, 1771-1778.
9. Jowett, L. A.; Howe, E. N. W.; Soto-Cerrato, V.; Van Rossom, W.; Pérez-Tomás, R.; Gale, P. A. (2017). Indole-based perenosins as highly potent HCl transporters and potential anti-cancer agents. Scientific Reports, 7, 9397.

10. Busschaert, N.; Park, S.-H.; Baek, K.-H.; Choi, Y. P.; Park, J.; Howe, E. N. W.; Hiscock, J. R.; Karagiannidis, L. E.; Marques, I.; Félix, V.; Namkung, W.; Sessler, J. L.; Gale, P. A.; Shin, I. (2017). A synthetic ion transporter that disrupts autophagy and induces apoptosis by perturbing cellular chloride concentrations. Nat Chem, 9, 667–675.
11. Elie, C. R.; David, G.; Schmitzer, A. R. Strong (2015). Antibacterial Properties of Anion Transporters: A Result of Depolarization and Weakening of the Bacterial Membrane. J. Med. Chem., 58, 2358-2366.
12. Hickey, E. E.; Page, S. W.; Trott, D. J. (2020). In vitro efficacy and pharmacodynamic profiles of four polyether ionophores against methicillin-resistant Staphylococcus spp. J Vet Pharmacol Ther, 43, 499-507.
13. Carreira-Barral, I.; Rumbo, C.; Mielczarek, M.; Alonso-Carrillo, D.; Herran, E.; Pastor, M.; Del Pozo, A.; García-Valverde, M.; Quesada, R. (2019). Small molecule anion transporters display in vitro antimicrobial activity against clinically relevant bacterial strains. Chem. Commun., 55, 10080-10083.
14. Share, A. I.; Patel, K.; Nativi, C.; Cho, E. J.; Francesconi, O.; Busschaert, N.; Gale, P. A.; Roelens, S.; Sessler, J. L. (2016). Chloride anion transporters inhibit growth of methicillin-resistant Staphylococcus aureus (MRSA) in vitro. Chem. Commun., 52, 7560-7563.
15. Moore, S. J.; Haynes, C. J. E.; González, J.; Sutton, J. L.; Brooks, S. J.; Light, M. E.; Herniman, J.; Langley, G. J.; Soto-Cerrato, V.; Pérez-Tomás, R.; Marques, I.; Costa, P. J.; Félix, V.; Gale, P. A. (2013). Chloride, carboxylate and carbonate transport by ortho-phenylenediamine-based bisureas. Chem. Sci., 4, 103-117.
16. Jurček, O.; Valkenier, H.; Puttreddy, R.; Novák, M.; Sparkes, H. A.; Marek, R.; Rissanen, K.; Davis, A. P. (2018). Anion Recognition by a Bioactive Diureidodecalin Anionophore: Solid-State, Solution, and Computational Studies. Chem. Eur. J., 24, 8178-8185.
17. Dias, C. M.; Valkenier, H.; Davis, A. P. (2018). Anthracene Bisureas as Powerful and Accessible Anion Carriers. Chem. Eur. J., 24, 6262-6268.
18. Wu, X.; Wang, P.; Turner, P.; Lewis, W.; Catal, O.; Thomas, D. S.; Gale, P. A. (2019). Tetraurea Macrocycles: Aggregation-Driven Binding of Chloride in Aqueous Solutions. Chem, 5, 1210-1222.
19. Wu, X.; Judd, Luke W.; Howe, Ethan N. W.; Withecombe, Anne M.; Soto-Cerrato, V.; Li, H.; Busschaert, N.; Valkenier, H.; Pérez-Tomás, R.; Sheppard, David N.; Jiang, Y.-B.; Davis, Anthony P.; Gale, Philip A. (2016). Nonprotonophoric Electrogenic Cl− Transport Mediated by Valinomycin-like Carriers. Chem, 1, 127-146.
20. Jowett, L. A.; Ricci, A.; Wu, X.; Howe, E. N. W.; Gale, P. A. (2019). Investigating the Influence of Steric Hindrance on Selective Anion Transport. Molecules, 24, 1278.
21. Grauwels, G.; Valkenier, H.; Davis, A. P.; Jabin, I.; Bartik, K. (2019). Repositioning Chloride Transmembrane Transporters: Transport of Organic Ion Pairs. Angew. Chem. Int. Ed., 58, 6921-6925.
22. Martínez-Crespo, L.; Sun-Wang, J. L.; Ferreira, P.; Mirabella, C. F. M.; Aragay, G.; Ballester, P. (2019). Influence of the Insertion Method of Aryl-Extended Calix[4]pyrroles into Liposomal Membranes on Their Properties as Anion Carriers. Chem. Eur. J., 25, 4775-4781.
23. Clarke, H. J.; Howe, E. N. W.; Wu, X.; Sommer, F.; Yano, M.; Light, M. E.; Kubik, S.; Gale, P. A. (2016). Transmembrane Fluoride Transport: Direct Measurement and Selectivity Studies. J. Am. Chem. Soc., 138, 16515-16522.
24. Hernández, P. I.; Moreno, D.; Javier, A. A.; Torroba, T.; Pérez-Tomás, R.; Quesada, R. (2012). Tambjamine alkaloids and related synthetic analogs: efficient transmembrane anion transporters. Chem. Commun., 48, 1556-1558.
25. Pomorski, R.; García-Valverde, M.; Quesada, R.; Chmielewski, M. J. (2021). Transmembrane anion transport promoted by thioamides. RSC Adv., 11, 12249-12253.
26. Picci, G.; Kubicki, M.; Garau, A.; Lippolis, V.; Mocci, R.; Porcheddu, A.; Quesada, R.; Ricci, P. C.; Scorciapino, M. A.; Caltagirone, C. (2020). Simple squaramide receptors for highly efficient anion binding in aqueous media and transmembrane transport. Chem. Commun., 56, 11066-11069.
27. Qin, L.; Vervuurt, S. J. N.; Elmes, R. B. P.; Berry, S. N.; Proschogo, N.; Jolliffe, K. A. (2020). Extraction and transport of sulfate using macrocyclic squaramide receptors. Chem. Sci., 11, 201-207.
28. Li, Z.; Deng, L.-Q.; Chen, J.-X.; Zhou, C.-Q.; Chen, W.-H. (2015). Does lipophilicity affect the effectiveness of a transmembrane anion transporter? Insight from squaramido-functionalized bis(choloyl) conjugates. Org. Biomol. Chem, 13, 11761-11769.
29. Edwards, S. J.; Valkenier, H.; Busschaert, N.; Gale, P. A.; Davis, A. P. (2015). High-Affinity Anion Binding by Steroidal Squaramide Receptors. Angew. Chem. Int. Ed., 54, 4592-4596.
30. Sánchez-Sanz, G.; Trujillo, C. (2018). Improvement of Anion Transport Systems by Modulation of Chalcogen Interactions: The influence of solvent. The Journal of Physical Chemistry A, 122, 1369-1377.
31. Lee, L. M.; Tsemperouli, M.; Poblador-Bahamonde, A. I.; Benz, S.; Sakai, N.; Sugihara, K.; Matile, S. (2019). Anion Transport with Pnictogen Bonds in Direct Comparison with Chalcogen and Halogen Bonds. J. Am. Chem. Soc., 141, 810-814.
32. Bickerton, L. E.; Sterling, A. J.; Beer, P. D.; Duarte, F.; Langton, M. J. (2020). Transmembrane anion transport mediated by halogen bonding and hydrogen bonding triazole anionophores. Chem. Sci., 11, 4722-4729.
33. Lim, J. Y. C.; Beer, P. D. (2018). Sigma-Hole Interactions in Anion Recognition. Chem, 4, 731-783.
34. Liu, Y.; Zhao, W.; Chen, C.-H.; Flood, A. H. (2019). Chloride capture using a C–H hydrogen-bonding cage. Science, 365, 159-161.
35. Elmes, R. B. P.; Jolliffe, K. A. (2014). Amino acid-based squaramides for anion recognition. Supramol. Chem., 27, 321-328.
36. Elmes, R. B. P.; K. Y. Yuen, K.; Jolliffe, K. A. (2014). Sulfate-Selective Recognition by Using Neutral Dipeptide Anion Receptors in Aqueous Solution. Chem. Eur. J., 20, 7373-7380.
37. Kumawat, L. K.; Abogunrin, A. A.; Kickham, M.; Pardeshi, J.; Fenelon, O.; Schroeder, M.; Elmes, R. B. P. (2019). Squaramide–Naphthalimide Conjugates as "Turn-On" Fluorescent Sensors for Bromide Through an Aggregation-Disaggregation Approach. Frontiers in Chemistry, 7.

38. Elmes, R. B. P.; Turner, P.; Jolliffe, K. A. (2013). Colorimetric and Luminescent Sensors for Chloride: Hydrogen Bonding vs Deprotonation. Org. Lett., 15, 5638-5641.
39. Marchetti, L. A.; Mao, N.; Krämer, T.; Kitchen, J. A.; Elmes, R. B. P. (2018). A long wavelength colourimetric chemosensor for fluoride. Supramol. Chem., 30, 795 - 805.
40. Busschaert, N.; Elmes, R. B. P.; Czech, D. D.; Wu, X.; Kirby, I. L.; Peck, E. M.; Hendzel, K. D.; Shaw, S. K.; Chan, B.; Smith, B. D.; Jolliffe, K. A.; Gale, P. A. (2014). Thiosquaramides: pH switchable anion transporters. Chem. Sci., 5, 3617-3626.
41. Elmes, R. B. P.; Busschaert, N.; Czech, D. D.; Gale, P. A.; Jolliffe, K. A. (2015). pH switchable anion transport by an oxothiosquaramide. Chem. Commun., 51, 10107-10110.
42. Qin, L.; Hartley, A.; Turner, P.; Elmes, R. B. P.; Jolliffe, K. A. (2016). Macrocyclic squaramides: anion receptors with high sulfate binding affinity and selectivity in aqueous media. Chem. Sci., 7, 4563-4572.
43. Busschaert, N.; Kirby, I. L.; Young, S.; Coles, S. J.; Horton, P. N.; Light, M. E.; Gale, P. A. (2012). Squaramides as Potent Transmembrane Anion Transporters. Angew Chem Int Ed., 51, 4426-4430.
44. Saez Talens, V.; Englebienne, P.; Trinh, T. T.; Noteborn, W. E. M.; Voets, I. K.; Kieltyka, R. E. (2015). Aromatic Gain in a Supramolecular Polymer. Angew. Chem. Int. Ed., 54, 10502-10506.
45. Saez Talens, V.; Davis, J.; Wu, C.-H.; Wen, Z.; Lauria, F.; Gupta, K. B. S. S.; Rudge, R.; Boraghi, M.; Hagemeijer, A.; Trinh, T. T.; Englebienne, P.; Voets, I. K.; Wu, J. I.; Kieltyka, R. E. (2020). Thiosquaramide-Based Supramolecular Polymers: Aromaticity Gain in a Switched Mode of Self-Assembly. J. Am. Chem. Soc., 142, 19907-19916.
46. Marchetti, L. A.; Kumawat, L. K.; Mao, N.; Stephens, J. C.; Elmes, R. B. P. (2019). The Versatility of Squaramides: From Supramolecular Chemistry to Chemical Biology. Chem, 5, 1398-1485.
47. Collot, M.; Kreder, R.; Tatarets, A. L.; Patsenker, L. D.; Mely, Y.; Klymchenko, A. S. (2015). Bright fluorogenic squaraines with tuned cell entry for selective imaging of plasma membrane vs. endoplasmic reticulum. Chem. Commun., 51, 17136-17139.
48. Rotger, M. C.; Piña, M. N.; Frontera, A.; Martorell, G.; Ballester, P.; Deyà, P. M.; Costa, A. (2004). Conformational preferences and self-template macrocyclization of squaramide-based foldable modules. J. Org. Chem, 69, 2302-2308.
49. Li, Y.; Yang, G.-H.; Shen, Y.-Y.; Xue, X.-S.; Li, X.; Cheng, J.-P. (2017). N-tert-Butyl Sulfinyl Squaramide Receptors for Anion Recognition through Assisted tert-Butyl C–H Hydrogen Bonding. J. Org. Chem, 82, 8662-8667.
50. Thordarson, P. (2011). Determining association constants from titration experiments in supramolecular chemistry. Chem. Soc. Rev., 40, 1305-1323.
51. Brynn Hibbert, D.; Thordarson, P. (2016). The death of the Job plot, transparency, open science and online tools, uncertainty estimation methods and other developments in supramolecular chemistry data analysis. Chem. Commun.,52, 12792 - 12805.
52. Busschaert, N.; Kirby, I. L.; Young, S.; Coles, S. J.; Horton, P. N.; Light, M. E.; Gale, P. A. (2012). Squaramides as Potent Transmembrane Anion Transporters. Angew. Chem. Int. Ed., 51, 4426-4430.
53. Jowett, L. A.; Gale, P. A. (2019). Supramolecular methods: the chloride/nitrate transmembrane exchange assay. Supramol. Chem., 31, 297-312.
54. Purser, S.; Moore, P. R.; Swallow, S.; Gouverneur, V. (2008). Fluorine in medicinal chemistry. Chem. Soc. Rev., 37, 320-330.
55. Busschaert, N.; Wenzel, M.; Light, M. E.; Iglesias-Hernández, P.; Pérez-Tomás, R.; Gale, P. A. (2011). Structure–Activity Relationships in Tripodal Transmembrane Anion Transporters: The Effect of Fluorination. J. Am. Chem. Soc., 133, 14136-14148.
56. Jowett, L. A.; Howe, E. N. W.; Wu, X.; Busschaert, N.; Gale, P. A. (2018). New Insights into the Anion Transport Selectivity and Mechanism of Tren-based Tris-(thio)ureas. Chem. Eur. J., 24, 10475-10487.
57. Bao, X.; Wu, X.; Berry, S. N.; Howe, E. N. W.; Chang, Y.-T.; Gale, P. A. (2018). Fluorescent squaramides as anion receptors and transmembrane anion transporters. Chem. Commun., 54, 1363-1366.
58. Bhosale, S.; Matile, S. A. (2006). simple method to identify supramolecules in action: Hill coefficients for exergonic self-assembly. Chirality, 18, 849-856.
59. Gilchrist, A. M.; Chen, L.; Wu, X.; Lewis, W.; Howe, E. N. W.; Macreadie, L. K.; Gale, P. A. (2020). Tetrapodal Anion Transporters. Molecules, 25, 5179.
60. Bauer, A. W.; Kirby, W. M.; Sherris, J. C.; Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol, 45, 493-496.
61. Ko, S.-K.; Kim, S. K.; Share, A.; Lynch, V. M.; Park, J.; Namkung, W.; Van Rossom, W.; Busschaert, N.; Gale, P. A.; Sessler, J. L.; Shin, I. (2014). Synthetic ion transporters can induce apoptosis by facilitating chloride anion transport into cells. Nat Chem, 6, 885-892.
62. Piatek, M.; Sheehan, G.; Kavanagh, K. (2021). Galleria mellonella: The Versatile Host for Drug Discovery, In Vivo Toxicity Testing and Characterising Host-Pathogen Interactions. Antibiotics (Basel), 10, 1545.
63. Piatek, M.; Griffith, D. M.; Kavanagh, K. (2020). Quantitative proteomic reveals gallium maltolate induces an iron-limited stress response and reduced quorum-sensing in Pseudomonas aeruginosa. J Biol Inorg Chem, 25, 1153-1165.
64. Dolan, N.; Gavin, D. P.; Eshwika, A.; Kavanagh, K.; McGinley, J.; Stephens, J. C. (2016).Synthesis, antibacterial and anti-MRSA activity, in vivo toxicity and a structure-activity relationship study of a quinoline thiourea. Bioorg Med Chem Lett., 26, 630-635.
65. Kavanagh, K.; Sheehan, G. (2018).The Use of Galleria mellonella Larvae to Identify Novel Antimicrobial Agents against Fungal Species of Medical Interest. J Fungi (Basel), 4, 113.
66. Piatek, M.; Sheehan, G.; Kavanagh, K. (2020). Utilising Galleria mellonella larvae for studying in vivo activity of conventional and novel antimicrobial agents. Pathog Dis., 78, ftaa059.
67. Trunk, T.; Khalil, H. S.; Leo, J. C. (2018). Bacterial autoaggregation. AIMS Microbiol., 4, 140-164.

68. Klebensberger, J.; Rui, O.; Fritz, E.; Schink, B.; Philipp, B. (2006). Cell aggregation of Pseudomonas aeruginosa strain PAO1 as an energy-dependent stress response during growth with sodium dodecyl sulfate. Arch Microbiol., 185, 417-427.
69. Martin, H.; Goyard, D.; Margalit, A.; Doherty, K.; Renaudet, O.; Kavanagh, K.; Velasco-Torrijos, T. (2021). Multivalent Presentations of Glycomimetic Inhibitor of the Adhesion of Fungal Pathogen Candida albicans to Human Buccal Epithelial Cells. Bioconjugate Chem., 32, 971-982.
70. Ko, S. K.; Kim, S. K.; Share, A.; Lynch, V. M.; Park, J.; Namkung, W.; Van Rossom, W.; Busschaert, N.; Gale, P. A.; Sessler, J. L.; Shin, I. (2014). Synthetic ion transporters can induce apoptosis by facilitating chloride anion transport into cells. Nat. Chem., 6, 885-892.
71. Jentsch, T. J.; Pusch, M. (2018). CLC Chloride Channels and Transporters: Structure, Function, Physiology, and Disease. Physiol Rev., 98, 1493-1590.
72. Iyer, R.; Iverson, T. M.; Accardi, A.; Miller, C. (2002). A biological role for prokaryotic ClC chloride channels. Nature, 419, 715-718.
73. Yu, L.; Jiang, X. H.; Zhou, Z.; Tsang, L. L.; Yu, M. K.; Chung, Y. W.; Zhang, X. H.; Wang, A. M.; Tang, H.; Chan, H. C. (2011). A protective mechanism against antibiotic-induced ototoxicity: role of prestin. PLoS One, 6, e17322.
74. Tsukimoto, M.; Harada, H.; Ikari, A.; Takagi, K. (2005). Involvement of chloride in apoptotic cell death induced by activation of ATP-sensitive P2X7 purinoceptor. J Biol Chem, 280, 2653-2658.
75. Zechner, R.; Zimmermann, R.; Eichmann, T. O.; Kohlwein, S. D.; Haemmerle, G.; Lass, A.; Madeo, F. (2012). FAT SIGNALS--lipases and lipolysis in lipid metabolism and signaling. Cell Metab., 15, 279-291.
76. Ramadurai, L.; Lockwood, K. J.; Nadakavukaren, M. J.; Jayaswal, R. K. (1999). Characterization of a chromosomally encoded glycylglycine endopeptidase of Staphylococcus aureus. Microbiol-Uk, 145, 801-808.
77. Oshida, T.; Sugai, M.; Komatsuzawa, H.; Hong, Y. M.; Suginaka, H.; Tomasz, A. (1995). A Staphylococcus aureus autolysin that has an N-acetylmuramoyl-L-alanine amidase domain and an endo-beta-N-acetylglucosaminidase domain: cloning, sequence analysis, and characterization. Proc Natl Acad Sci., 92, 285-289.
78. Rasmussen, T. B.; Givskov, M. (2006). Quorum-sensing inhibitors as anti-pathogenic drugs. Int J Med Microbiol., 296, 149-161.
79. Williams, P. (2002). Quorum sensing: an emerging target for antibacterial chemotherapy? Expert Opin Ther Targets, 6, 257-274.
80. Gordon, C. P.; Williams, P.; Chan, W. C. (2013). Attenuating Staphylococcus aureus virulence gene regulation: a medicinal chemistry perspective. J Med Chem., 56, 1389-1404.
81. Muller, M.; Reiss, S.; Schluter, R.; Mader, U.; Beyer, A.; Reiss, W.; Marles-Wright, J.; Lewis, R. J.; Pfortner, H.; Volker, U.; Riedel, K.; Hecker, M.; Engelmann, S.; Pane-Farre, J. (2014). Deletion of membrane-associated Asp23 leads to upregulation of cell wall stress genes in Staphylococcus aureus. Mol Microbiol., 93, 1259-1268.
82. Sun, W.; Wang, Z.; Cao, J.; Cui, H.; Ma, Z. (2016). Cold stress increases reactive oxygen species formation via TRPA1 activation in A549 cells. Cell Stress Chaperones, 21, 367-372.
83. Arner, E. S. J.; Holmgren, A. (2000). Physiological functions of thioredoxin and thioredoxin reductase. Eur J Biochem., 267, 6102-6109.
84. Tsang, C. K.; Liu, Y.; Thomas, J.; Zhang, Y. J.; Zheng, X. F. S. (2014). Superoxide dismutase 1 acts as a nuclear transcription factor to regulate oxidative stress resistance. Nat Commun., 5, 3446.
85. Lesniak, J.; Barton, W. A.; Nikolov, D. B. (2003). Structural and functional features of the Escherichia coli hydroperoxide resistance protein OsmC. Protein Sci., 12, 2838-2843.