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Anionic Self-Assembling Supramolecular Enhancers of Antimicrobial Efficacy against Gram-Negative Bacteria

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As a result of the looming antimicrobial resistance crisis, there is an urgent need for novel antimicrobial treatments. This is particularly true for hard-to-treat Gram-negative bacteria, as many antimicrobial agents are unable to cross the cell membrane to gain access to the cell interior, and thus elicit a therapeutic response. Herein, evidence is provided of the use of anionic supramolecular self-associating amphiphiles (SSAs) as antimicrobial efficacy enhancers for commonly used antimicrobial agents, to which there is known resistance, against Gram-negative bacteria. The co-administration of the SSAs with antimicrobials is shown to sensitize traditionally hard to treat *Pseudomonas aeruginosa* to both rifampicin and novobiocin, from which structure activity relationships can be elucidated. Quantitative fluorescence microscopy is performed, indicating membrane permeabilization to be the likely mode of action of drug efficacy enhancement by the SSAs. These results offer an alternative strategy in antimicrobial adjuvant design, expanding focus beyond cationic peptides and into the realm of anionic small molecules. Finally, the self-assembly of the SSAs in the presence of these antimicrobials is investigated through a combination of quantitative NMR, tensiometry, dynamic light scattering, and zeta potential studies, demonstrating the impact of these agents on SSA self-association events.

1. Introduction

The development of antimicrobial resistance (AMR) is one of the greatest health threats facing humanity today, and as such has been termed the silent pandemic by some.[1] By 2050, it is predicted that AMR will have had a global cost of $100 trillion and have been directly responsible for 50 million deaths worldwide (cumulative values from 2014).[2]

Since the discovery of penicillin in 1928,[3] antibiotics have been a linchpin in humanities war on mortality. Unfortunately, as novel antimicrobial therapies have been developed, so too have bacteria evolved resistance mechanisms to evade their action.[4] However, the costs and poor market returns associated with novel antimicrobial development have rendered research into antibiotics an unattractive investment for the pharmaceutical industry.[5] Thus, academic institutions and small/medium-sized enterprises have become the primary source of novel antimicrobial development,[6] as highlighted by our own supramolecular self-associating amphiphile (SSAs **1**–**4**, **Figure 1**) technology.[7] Alongside small molecule approaches, alternative technologies such as cold atmospheric plasma treatments,[8] and bacteriophage-associated innovations have also been undergoing development.[9] In addition, there is an increasing body of evidence, which shows that the use of antimicrobial/antibiotic/antiseptic agents throughout the COVID-19 pandemic may have intersected with a further enhancement to the rise of AMR.[10]

A specific challenge for those working in the field of antimicrobial technology development, is the need to produce novel treatments against infections caused by Gram-negative bacteria.[11] This is due to the robust double layered outer membrane common to all Gram-negative bacteria.[12] Unlike the single layered outer membrane of Gram-positive bacteria, the double layered membrane is far more effective at limiting the permeability of antimicrobial agents, restricting cell entry via membrane permeation to all but the most hydrophobic of molecules.[13] However, it is the phospholipid composition of the double layered outer membrane which provides a target for the development of novel selective antimicrobial treatments Figure 1.

Typically, the phospholipid composition of the Gram-negative bacterial outer membrane is phosphatidylethanolamine (PE)

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DOI: 10.1002/adtp.202200024

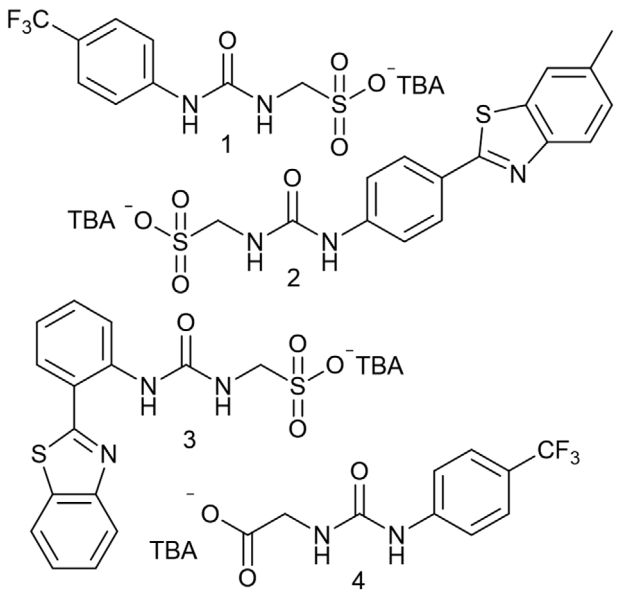

Figure 1. Chemical structures of SSA **1**–**4**. TBA = Tetrabutylammonium.

and phosphatidylglycerol (PG).[13b,14] This results in a negative net surface charge. However, the greater phosphatidylcholine (PC) content of mammalian cells yields a net neutral surface charge,[13b] thus enabling differentiation between the two types of cell membrane.

The development of molecular membrane interaction technologies has also led to the evolution of (typically peptide-based) molecular innovations,[15] which selectively increase the permeability of Gram-negative bacterial membranes toward antimicrobial agents and/or adjuvants.[16] For example, Brown and co-workers investigated the use of SPR741, a polymyxin B analog, as an antimicrobial efficacy enhancer which exhibits minimal inherent antimicrobial activity itself.[15c] This is favorable as minimizing the antimicrobial activity of a therapeutic efficacy enhancer is crucial to avoid the generation of AMR.[17] The authors demonstrated that SPR741 was capable of destabilizing the outer membrane of *Escherichia coli* (*E. coli*), a common Gram-negative pathogen. Nielsen and co-workers built upon these data, showing that the peptide mimic H-[NLys-^{t}BuAla]$_6$-NH_2 was capable of permeabilizing the membranes of a range of pathogenic bacteria including *E. coli, Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (*P. aeruginosa*).[18] Here, the authors showed that outer membrane permeabilization was concurrent with antimicrobial efficacy enhancement, observing an increase in the activity of three antibiotics, rifampicin, clarithromycin, and azithromycin against these bacteria. Beyond antimicrobial peptides, the small molecule antifungal drug pentamidine was investigated by Stokes et al. as an enhancer of antimicrobial activity against a panel of multidrug-resistant Gram-negative bacteria.[19] This work was built upon by Herrera-Espejo et al. who showed potential pentamidine to act as an adjuvant for *P. aeruginosa* treatments.[20] These activities are proposed to be due to the cationic nature of pentamidine interacting with the negatively charged bacterial membrane.

SSAs are a novel class of amphiphilic salt, the anionic component of which is able to undergo hydrogen bonding self-association events. These events result in the environmental-dependent formation of macromolecular superstructures that include dimers, spheroids, and hydrogels.[7d] However, as the presence of these spheroids are dependent on their solvent environment, visualizing these SSA self-associated species has only been successfully achieved using fluorescence microscopy, or when these spheroids have been coated in an inorganic salt.[7b,7d,21] The previously unpublished single-crystal X-ray structure of SSA **4**, **Figure 2**, exhibits urea-anion dimerization. Here, the anionic dimer is supported through the formation of four urea-anion hydrogen bonds. These dimers are then bridged by water molecules, through formation of further hydrogen bonds to the urea and carboxylate oxygen atoms.

Interestingly, the anionic component of these SSAs has been observed to interact with synthetic phospholipid membranes derived from Gram-negative bacteria,[22] a process we hypothesize to contribute to the antimicrobial mode of action against both Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) USA300 and Gram-negative *E. coli* DH10B, despite the obvious electrostatic repulsion effects.[7b,7d] The antimicrobial activity of these compounds has been linked to their inherent ability to self-assemble.[7b–7d] It has been shown previously that **2** arrives at the surface of the bacterial cell as self-assembled spherical aggregates, which subsequently coats the bacterial membrane before internalizing within the cells.[7b,7d] Inspired by the actions of antimicrobial peptides (AMPs),[23] a recent study by the co-authors of this manuscript has shown a member of this class of compounds capable of potentiating the activity of cisplatin, an anticancer DNA crosslinking agent, against *E. coli*,[7a] as well as cancer cell lines.[24] Here, we expand on this preliminary work and report the use of quantitative fluorescence imaging to study the effect of SSA **2** on the permeability of bacterial membranes, and demonstrate the ability of SSAs (**1** and **4**) to act as the first anionic antimicrobial efficacy enhancers for the treatment of *P. aeruginosa*. In a similar strategy to that employed by the antimicrobial potentiators reported by Nielsen et al., we seek to use the membrane destabilization effects of the SSAs to enhance the efficacy of antimicrobial treatment.[18]

Gram-negative *P. aeruginosa* represents the "P" in the ESKAPE pathogens, a group of potentially drug-resistant bacteria that have been listed as urgently requiring novel antimicrobials.[16a,25] Indeed, carbapenem-resistant *P. aeruginosa* has been placed in the highest priority list of bacteria requiring new treatments, as published by the World Health Organization (2017).[26] Therefore, there is a clear clinical need to: 1) develop new therapies active against *P. aeruginosa*, and/or 2) to re-sensitize this bacteria toward existing therapies to which resistance has been developed. The latter is particularly relevant to *P. aeruginosa* due to resistance now identified toward a variety of currently marketed antibiotics.[13a] Additionally, this approach has some advantages over the former, which include reduced developmental costs and reduced time to clinical use.

2. Results and Discussion

2.1. Antimicrobial Activity of SSAs

The antimicrobial efficacy of four structurally related, stepwise altered SSAs (**1**–**4**) was investigated against *E. coli* DH10B and *P.*

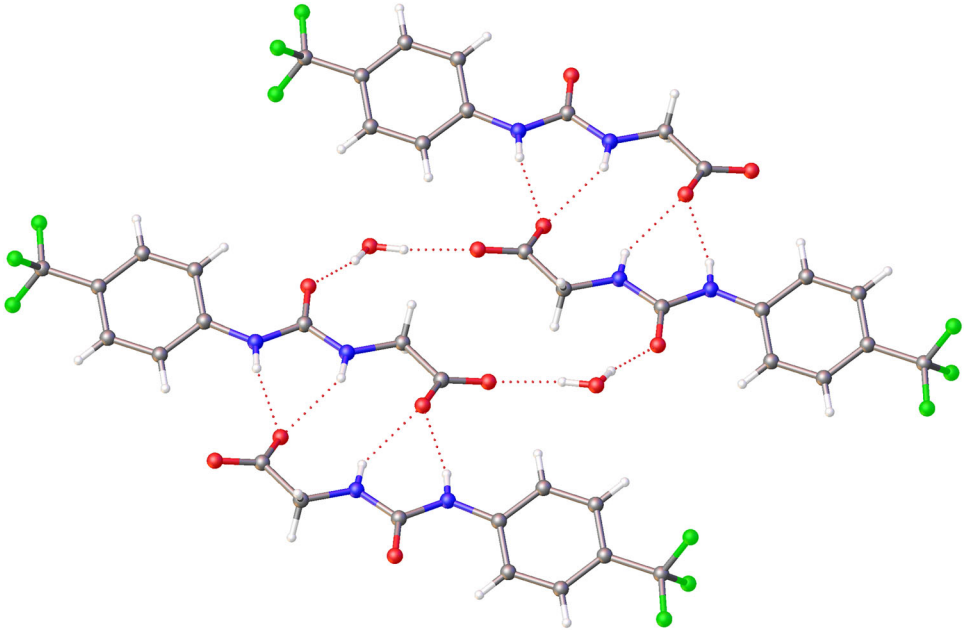

Figure 2. Single crystal X-ray structure of **4**: red = oxygen; blue = nitrogen; white = hydrogen; gray = carbon; red dashed lines = hydrogen bonds. CCDC 2122929. Here, the TBA counter cations have been omitted for clarity.[‡]

Table 1. The MIC of compounds **1**–**4** and **5**–**7** against Gram-negative bacteria *E. coli* DH10B and *P. aeruginosa* PAO1 at an initial calibrated cell concentration equal to $\approx 1 \times 10^6$ bacteria mL^{-1}, after 900 min.

Compound	MIC[$\times 10^{-3}$ M]	
	*E. coli*DH10B	*P. aeruginosa*PAO1
1	> 10[a)]	> 10[a)]
2	> 10[a)]	> 10[a)]
3	> 10[a)]	> 10[a)]
4	10	> 10[a)]
5	0.00078	0.0031
6	1.6	3.2
7	0.019	0.019

[a)] Compounds exhibited limited solubility above 10×10^{-3} M.

aeruginosa PA01, through calculation of the respective minimum inhibition concentration (MIC) value. This is the concentration of antimicrobial agent required to inhibit bacterial growth. The results of these studies are summarized in **Table 1**. The lack of antimicrobial activity demonstrated for **1**–**4**, supports potential use of these compounds as antimicrobial efficacy enhancers. Of these four SSAs, only **4** displayed an MIC value against *E. coli* DH10B, low enough to be measured at 10×10^{-3} M. SSAs **1**–**3** showed no effect against *E. coli* DH10B at their maximum solubility (> 10×10^{-3} M) (Figures S118–S121, Supporting Information). When tested against *P. aeruginosa* PAO1, no SSA tested exhibited antimicrobial activity within the available molecular solubility range (Figures S111–S114, Supporting Information). It is crucial that adjuvants do not display inherent antimicrobial activity, as this will drive the formation of bacterial resistance;[27] in this respect, the SSAs show great promise as potential antimicrobial adjuvants. This is further supported by previous investigation into **1**–**4** which showed these agents to be nontoxic against normal human dermal fibroblasts.[24]

2.2. SSA Antimicrobial Co-Formulation

Three structurally diverse but commonly used antimicrobials (**5**–**7**, **Figure 3**) were chosen to investigate the activity of **1**–**4** as antimicrobial efficacy enhancers. These antimicrobials were chosen due to their varied modes of action and, in the case of novobiocin, their inability to penetrate Gram-negative membranes.[28] Octenidine (**5**), a membrane active antiseptic, has previously shown antagonism in combination with **1** against *E. coli* DH10B.[7a] Novobiocin (**6**) and rifampicin (**7**) have intracellular modes of action. Novobiocin (**6**) is a topoisomerase IV inhibitor,[28] while **7** is known to inhibit RNA polymerase,[29] both of which target the molecular machinery inside bacteria that enables replication. As *P. aeruginosa* is known to be resistant to **6**,[30] this will serve as a good indicator of the ability of **1**–**4** to act as antimicrobial efficacy enhancers.[31] MIC values for **5**–**7** against *E. coli* DH10B and *P. aeruginosa* PAO1 are summarized in Table 1.

Surface targeting, broad spectrum, **5** displayed the greatest activity against *E. coli* and *P. aeruginosa* of those antimicrobial agents **5**–**7** tested (MIC = 0.78×10^{-6} and 3.1×10^{-6} M, respectively). Novobiocin (**6**), was shown to be the least effective antimicrobial agent against both bacteria tested, exhibiting MIC values in the millimolar range, while **7** presented identical MIC values (0.019×10^{-3} M) against both bacterial species.

Previous work has demonstrated that SSAs are able to actuate the activity of cisplatin against *E. coli*, which features an

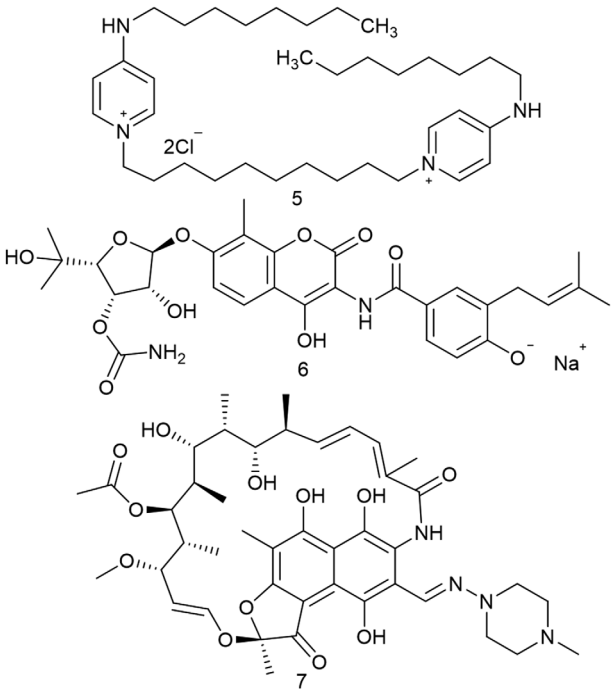

Figure 3. Antimicrobials octenidine dihydrochloride (**5**), novobiocin (**6**), and rifampicin (**7**).

intracellular target,[32] yet decreased the antimicrobial activity of membrane acting agents (octenidine),[33] an observation attributed to competitive membrane interaction events. A detailed physiochemical analysis of SSA:antimicrobial co-formulation has therefore been conducted, in order to ascertain the effect that the antimicrobial agents **5**–**7** have on the self-assembly properties of SSAs **1**–**4**, building on our previous work in this area.[34]

2.3. Physiochemical Investigation

Physiochemical examination of SSAs **1**–**4** with antimicrobials **5**–**7** in a 1:1 ratio was performed in both polar organic solvent DMSO-d_6/0.5% H_2O and a H_2O/5.0% EtOH solution in line with previous studies.[7a,34] These co-formulations were assigned alphabetical values, as recorded in **Table 2** and compared to control solutions, containing the appropriate SSA only. Full experimental detail and supporting results can be found in the Supporting Information. In a H_2O/5.0% EtOH solution, the results of dynamic light scattering (DLS) studies showed the presence of octenidine (**5**) to increase the hydrodynamic diameter (D_h) of those self-associated aggregates formed, when compared to the results of analogous studies containing the SSA only at a comparative concentration. In contrast, novobiocin (**6**) and rifampicin (**7**) caused a decrease in aggregate size, with the exception of co-formulations **e** and **f**, where an increase in D_h was noted. These changes in D_h are further evidence toward the formation of heterogenous self-associated SSA aggregates. Unlike the SSA self-associative events observed in a DMSO-d_6/0.5% H_2O solution,

Table 2. Overview of average DLS intensity particle size distribution peak maxima (D_h at 0.56×10^{-3} M), zeta potential (0.56×10^{-3} M), and CMC, measurements obtained for a H_2O/5.0% EtOH solution of SSAs (**1–4**) and co-formulations (**a–l**) at 298 K. Self-association dimerization constants (K_{dim}) and SSA anion D_h (112×10^{-3} M) calculated in an analogous DMSO-d_6/0.5% H_2O solution at 298 K were also obtained for comparison. These values were obtained from the fitting of ^{1}H NMR dilution study data refined to the EK/dimerization model using BindFit v0.5[35] and the substitution of the SSA diffusion constant obtained from ^{1}H NMR DOSY data into the Stokes–Einstine equation, respectively.

Entry	Compound combination	DMSO-d_6/0.5% H_2O		H_2O/5.0% EtOH		
		D_h[nm]	K_{dim}[M^{-1}]	Zeta potential [mV]	CMC[$\times 10^{-3}$ M]	D_h[nm]
1	-	1.15[36]	2.7[36]	−66[36]	10.4[36]	142[36]
2	-	1.64[37]	2.7[37]	−101[37]	0.5[37]	122[37]
3	-	1.38	0.6[37]	−79[37]	9.5[37]	295[37]
4	-	1.28[37]	41.4[36]	−37[36]	11.2[36]	220[36]
a	**1**+**5**	1.69[7a]	2.9[7a]	+76[7a]	5.6[7a]	240[7a]
b	**1**+**6**	2.30	2.2[c)]	+1		98
c	**1**+**7**	1.91	2.9	+6		136
d	**2**+**5**	[a)]	[a)]	+33	[b)]	391
e	**2**+**6**	3.20	2.1[c)]	−46	3.1	240
f	**2**+**7**	2.53	2.0[c)]	−44	1.0	636
g	**3**+**5**	1.14	3.9[c)]	−23	[b)]	586
h	**3**+**6**	1.99	1.3[c)]	−1	2.1	160
i	**3**+**7**	2.09	4.1[c)]	−26	[b)]	116
j	**4**+**5**	1.57	39.1	+44	[b)]	360
k	**4**+**6**	2.56	1.6[c)]	−1	3.0	174
l	**4**+**7**	1.72	6.6[c)]	+1	[b)]	160

[a)] Could not be calculated due to compound solubility [b)] Solubility prevented CMC calculation [c)] These values should be treated with caution due to the possible complex nature of events.

we hypothesize that within a H_2O/5.0% EtOH the co-formulant does interact with the SSA, resulting in the formation of SSA:co-formulant aggregates that differ significantly in their physical properties to that of the SSA alone. For example, for aggregated structures containing SSA **2**, a fourfold increase in D_h from 122 to 636 nm was observed in the absence and presence of rifampicin (**7**), respectively. Zeta potential measurements performed under identical experimental conditions to the analogous DLS studies, showed that self-associated aggregates formed by SSAs **1**–**4** decreased in stability in the presence of **6**–**7**, with the exception of co-formulations **a** and **j**, where an increase in stability was observed.

A change in solubility was also observed when an antimicrobial agent (**5**–**7**) and SSA were combined, preventing the calculation of critical micelle concentration (CMC) values for the majority of co-formulations **a**–**l**. Of those CMCs calculated, for co-formulations containing SSAs **1**, **3**, and **4**, the presence of antimicrobials **6**–**7** decreased the CMC when compared to the SSA alone. However, the reverse was true for co-formulations involving SSA **2**, which showed the majority of the self-association events to be disrupted, rather than enhanced by the presence of these molecular co-formulants/cargo.

To verify the presence of any hydrogen-bonded self-association events, these co-formulations were also studied in DMSO-d_6/0.5% H_2O solutions. This solvent condition was chosen to reduce the presence of higher-order self-associated species and thus allow analysis of the individual molecular components. A series of ^{1}H NMR DOSY (diffusion-ordered spectroscopy) experiments performed for SSAs **1**–**4** alone support the formation of lower-order anionic hydrogen-bonded species (e.g., SSA anionic dimers such as those exemplified in Figure 2), with hydrodynamic diameters of ≤ 1.64 nm obtained for these species under these solvent conditions.[36,37] When SSAs **1**–**4** are co-formulated with antimicrobial agents (**5**, **6**, and **7**), small increases in the hydrodynamic diameter of the anionic components are noted, these sizes however are still suggestive of lower-order self-associated structures (Table 2). Therefore, the data obtained from ^{1}H NMR dilution studies, containing 1:1 co-formulations **a**–**l**, were also fitted to the dimerization (EK) binding isotherms using BindFit v0.5.[35] The K_{dim} values obtained for co-formulations **a**, **c**, and **j** were found to reproduce those values obtained for the SSA alone, within experimental limitations. This indicates that the antimicrobial co-formulant is not likely to be involved in any molecular association with the SSA anionic component under these experimental conditions. Fitting data obtained from the remaining ^{1}H NMR dilution studies to the same dimerization (EK) isotherm resulted in a change in the K_{dim} values observed, beyond that which could be attributed to experimental limitations. This leads us to believe that in these instances, the antimicrobial co-formulants are involved in molecular association events with the SSA anion.

2.4. Antimicrobial Potentiation

For the purpose of our SSA antimicrobial efficacy enhancer studies, the SSA (**1**–**4**) and antimicrobial (**5**–**7**) were added separately to: i) prevent destabilization of the SSA self-associated structures, ii) increase co-formulation component solubility, and iii) maintain experimental uniformity. To elucidate the

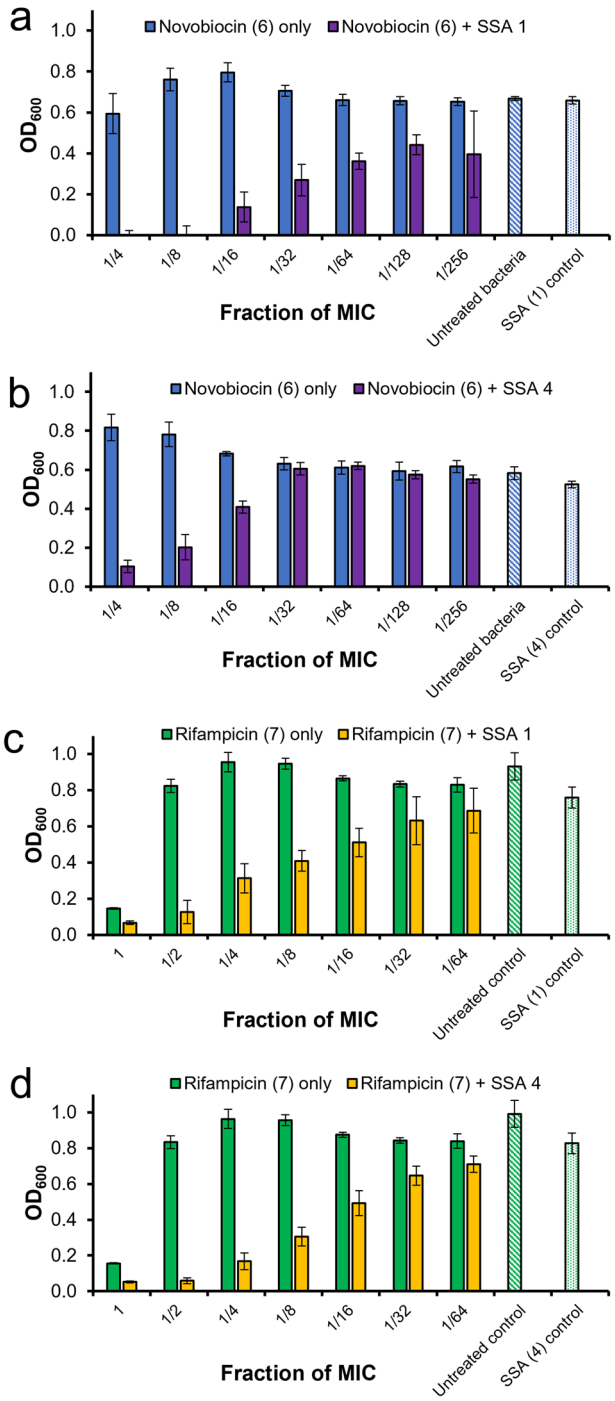

Figure 4. The change in bacterial growth (as a function of the OD_{600}) of *P. aeruginosa* PAO1 in response to incubation with the appropriate SSA (8×10^{-3} M) for 10 min at 37 °C, before the addition of the appropriate antimicrobial agent (**5**–**7**), at decreasing concentrations (as fractions of its MIC) for combination treatments of: a) **1**+**6**; b) **1**+**7**; c) **4**+**6**; and d) **4**+**7**, respectively, compared to a negative control of bacterial growth alone and in the presence of the appropriate SSA. Data recorded at 25 °C.

Table 3. Summary of inhibition assay outcomes. Antagonism is defined as an increase in OD_{600} observed when an antimicrobial is combined with SSA compared to the antimicrobial alone. Potentiation is defined as a decrease in OD_{600} observed when drug is combined with SSA compared to drug alone. Here, the SSA was supplied at a concentration where no antimicrobial effect could be demonstrated.

Combination treatment	*E. coli* DH10B	*P. aeruginosa* PAO1
a	No effect	Antagonism
b	No effect	Potentiation
c	No effect	Potentiation
d	Antagonism	Antagonism
e	Antagonism	No effect
f	Antagonism	Antagonism
g	Antagonism	Antagonism
h	Antagonism	No effect
i	Antagonism	Antagonism
j	No effect	Antagonism
k	No effect	Potentiation
l	No effect	Potentiation

antimicrobial efficacy of **a–j** compared to **1–4** and **5–7** alone, a modified inhibition assay was performed. In brief, a solution of **5–7** (100 μL, starting at 1 × MIC in lysogeny broth—LB media) was first serially diluted across the 96 microwell plate. To each well, 100 μL of bacteria subculture was then added, which had been preincubated with **1–4** at 37 °C for 10 min. In the case of *E. coli*, it was observed that while below the MIC, concentrations of **1** and **4** above 0.6×10^{-3} M negatively impacted bacterial growth, however **2** and **3** showed no such inhibition of *E. coli* growth up to 10×10^{-3} M (see the Supporting Information). As an ideal efficacy enhancer of antimicrobials should have no inherent antimicrobial effect, the modified inhibition assay was conducted at an SSA concentration of 0.6×10^{-3} M for *E. coli*. The analogous MIC experiments conducted with **1–4** against *P. aeruginosa* showed no inhibition of growth up to 10×10^{-3} M, therefore an arbitrary SSA concentration of 8×10^{-3} M was selected for ease of solubility to perform the analogous modified inhibition assay against this bacterium.

The 96 microwell plates were then incubated, with shaking at 37 °C for 18 h, then optical density (OD) measurements were recorded at 600 nm to determine the degree of microbial growth at the endpoint of this experiment. The key findings from these studies have been summarized in **Figure 4** and **Table 3**, full data sets can be found within the Supporting Information (Figures S125–S155, Supporting Information).

SSAs **1** and **4** showed antimicrobial potentiation of both **6** and **7** against *P. aeruginosa* PAO1, as shown in Figure 4. Combination treatment **a** offered the greatest increase in the activity of the antimicrobial novobiocin (**6**) (Figure 4a), demonstrating a dramatic eightfold decrease in the MIC when compared to that of the antimicrobial agent alone. Additionally, we also observe an antimicrobial dose-dependent decrease in bacterial growth at concentrations of **6** more than 100 times lower than that of its MIC. Figure 4b–d shows that a similar, although less dramatic, antimicrobial dose-dependent decrease in bacterial growth is also achievable with **c**, **f**, and **l**. Unexpectedly, combination treatments **d–i**

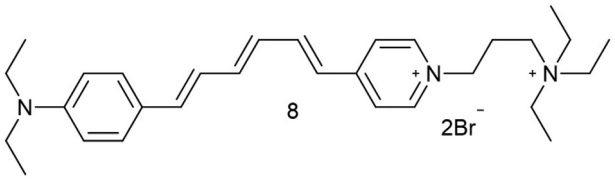

Figure 5. Chemical structure of the lipophilic fluorescent dye, FM 4–64 (**8**).

containing SSAs **2** and **3** showed no significant enhancement of antimicrobial activity, nor was any antimicrobial enhancement observed across all experimental conditions with *E. coli* (Table 3). In agreement with previous observations, there was no potentiation of **5** observed with either bacterial species.[7a] Indeed, antagonistic effects were observed for **5** in combination with each SSA **1–4** and *P. aeruginosa*, with **1** increasing the MIC of **5** to 40-fold higher than that of the antiseptic agent alone. It is hypothesized that this antagonistic effect may be due to the combination of microbial surface coordination effects between the SSA and octenidine (**5**).

The stepwise structural alterations present in the anionic component of SSAs **1–4** enables elucidation of basic molecular structure-antimicrobial efficacy enhancer relationships. Alteration of the hydrophobic unit from a 4-trifluorophenyl moiety (**1** and **4**) to a benzothiazole derivative (**2** and **3**) confers a clear reduction in antimicrobial potentiation, regardless of the position of the benzothiazole derivative. Interestingly, despite previous evidence indicating that the incorporation of a carboxylate, rather than a sulfonate anionic group, infers greater antimicrobial activity toward Gram-negative bacteria,[7b] the opposite effect is observed in respect to antimicrobial efficacy enhancement activity. Differences between the membrane phospholipid composition of *E. coli* and *P. aeruginosa* may provide an explanation as to their differences in efficacy enhancer susceptibility. The outer-membrane of *P. aeruginosa* PAO1 contains 59.7% (± 2.9%) PE, 27.1% (± 1.7%) PG, and 13.2% (± 2.8%) PC,[38] whereas the membranes of *E. coli* are reported to contain 70–80% PE, 20–25% PG, and <5% cardiolipin.[39]

2.5. Microscopy Studies

To elucidate SSA efficacy enhancer mode of action, quantitative fluorescence microscopy was employed, with a focus on intrinsically fluorescent SSA **2**. As antimicrobials **5–7** do not exhibit any appropriate fluorescence properties, these agents were substituted for the nonspecific membrane staining styryl dye FM 4–64 (**8**) (**Figure 5**). This dye was chosen due to an inability to cross the lipophilic bacterial membrane unaided and to exhibit complementary florescence properties to those exhibited by SSA **2**. Additionally, it is important to note that only when bound within a lipophilic membrane is the fluorescence of **8** able to be visualized.[41]

In short, *P. aeruginosa* PA01 and *E. coli* DH10B cells were incubated for 4 h with either FM 4–64 or SSA **2** alone, or FM 4–64 followed by **2** added ≈1 min apart. Due to the differences in fluorescence emission and excitation properties of FM 4–64 and **2**, these separate molecular entities can be visualized independently of one another using either 640/50 nm (red channel) or

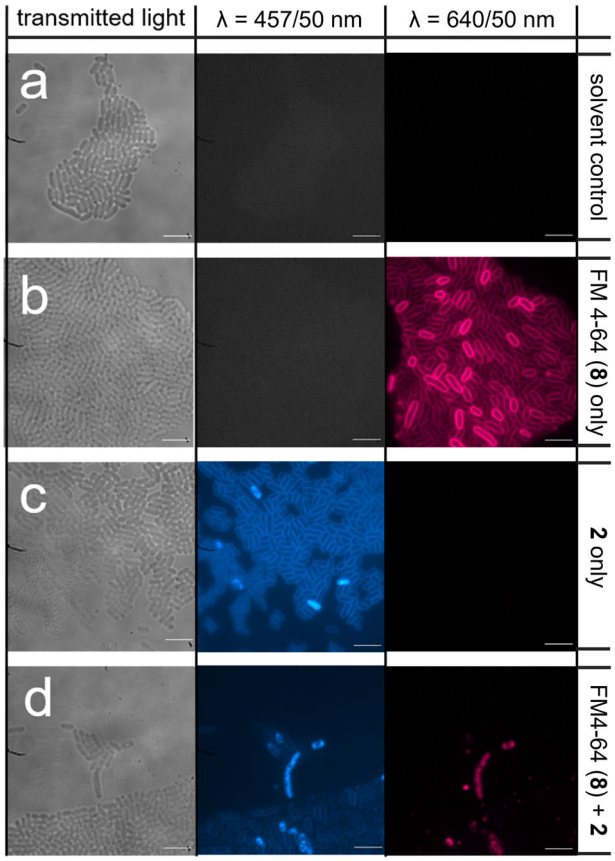

Figure 6. Fluorescence images of *E. coli*: a) in the absence of any compound; b) incubated with the lipophilic dye FM 4–64 (**8**) (red) for 4 h; c) incubated with **2** (blue) for 4 h; d) incubated initially with FM 4–64 (**8**) (1 min) followed by the addition of **2** (for 4 h). Scale bars: 10 μm.

457/50 nm (blue channel) emission filters, respectively. The results from those studies involving *P. aeruginosa* showed that SSA **2** did not adhere to the microbial surface (not shown), consistent with the results of MIC and antimicrobial potentiation studies, in which this SSA showed no antimicrobial or efficacy enhancer activity. However, previous competition assays have shown that if **2** is added to *E. coli* cells that have been preincubated with FM 4–64 ($T = 1$ min) then, after 30 min this SSA is able to out compete the lipophilic dye from the surface of this bacteria, causing it to be displaced from the cell in favor of **2**.[8]

We now report the results of an analogous set of experiments (**Figure 6**), performed against *E. coli*, which show that both the nonspecific membrane labeling dye **8** (Figure 6b) and SSA **2** (Figure 6c) adhere to the external bacterial surface and, in the case of SSA **2**, to become internalized within the bacteria. After the extended 4 h experimental time period, **8** (Figure 6b) cannot be seen within the bacterial cells. Intriguingly, when added together as a combination therapy, SSA **2** was shown to disrupt outer membrane organization, resulting in FM 4–64 fluorescence associated with aberrant cytosolic membrane structures (Figure 6d). Further to this, both compounds co-localized within the *E. coli* cytosol (**Figure 7**). Although **2** did not show efficacy enhancer activity with *E. coli* and **5**–**7**, we believe the results of these studies

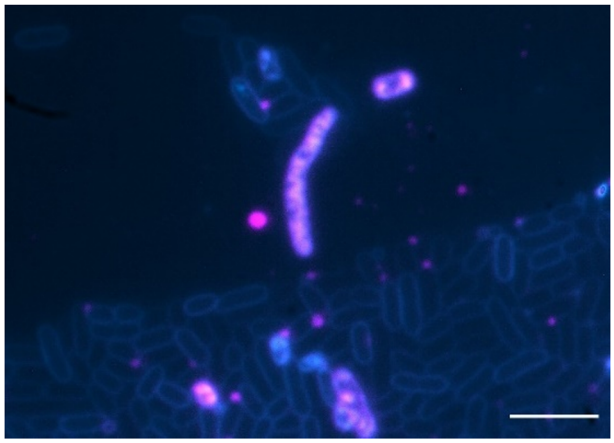

Figure 7. Overlay of fluorescence images shown in Figure 6d. This shows that SSA **2** and FM 4–64 (**8**) to have co-localized within *E. coli*. Scale bar 10 μm.

do offer a glimpse as to the potential mode of efficacy enhancer activity associated with SSAs **1** and **4**.

3. Conclusions

We have shown that SSAs are able to act as antimicrobial efficacy enhancers against *P. aeruginosa*, for the commonly used antibiotics novobiocin and rifampicin. Further to this we also demonstrate that this efficacy enhancer activity is dependent on the molecular structure of the SSA itself, with 4-trifluoromethyl-substituted SSAs **1** and **4** demonstrating a clear enhancement of antimicrobial efficacy of **6** and **7**, when compared to the activity of the individual agents alone. Here, the SSA was added to the microbial culture 10 min before the antimicrobial agent (**5**–**7**) due to the results of solution state physicochemical analysis. This group of experiments showed in general that directly co-formulating the SSA with an antimicrobial agent destabilized SSA self-associated structures thought to contribute to the observed biological activity and decrease co-formulation component solubility.

To ratify this observation, we performed a number of fluorescence microscopy studies with SSA **2** and lipophilic dye, FM 4–64 (**8**). Here, we were able to show **2** to effectively enable the internalization of FM 4–64, demonstrating a potential mode of action for the successful SSA:antimicrobial efficacy enhancer studies.

In summary, this work has shown that SSAs are capable of acting as antimicrobial efficacy enhancers, despite their anionic nature. To the best of the author's knowledge, this is the first evidence of an anionic construct being used to potentiate antimicrobial treatment against *P. aeruginosa*. This demonstrates the potential for small molecular constructs to compete alongside traditional antimicrobial peptide technologies and cationic small molecules in the field of adjuvant/efficacy enhancer development, offering another potential solution to the current AMR crisis.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

J.H., D.M., and L.W. thank the UKRI for JH's Future Leaders Fellowship (MR/T020415/1). J.B. thanks UK HSA and the University of Kent for funding her Ph.D. N.A. and K.H. thank the University of Kent for funding.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

J.E.B.: Investigation; Validation; Writing—original draft. G.T.W.: Conceptualization; Investigation; Supervision; Validation; Writing—original draft; Writing—review & editing. N.A.: Investigation; Validation. L.J.W.: Investigation; Validation. K.L.F.H.: Investigation; Writing—original draft. P.I.A.P.: Investigation. D.P.M.: Funding acquisition; Project administration; Supervision; Writing—review & editing. J.R.H.: Conceptualization; Funding acquisition; Project administration; Supervision; Writing—review & editing.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Keywords

antimicrobial chemotherapy, antimicrobial resistance, fluorescence microscopy, membrane binding, self-assembly, supramolecular chemistry

Received: January 25, 2022
Revised: March 3, 2022
Published online: March 31, 2022

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