

Appendix A

Synergistic Augmentation of Beta-Lactams: Exploring Quinoline-Derived Amphipathic Small Molecules as Antimicrobial Potentiators Against Methicillin-resistant *Staphylococcus aureus*

6.1 Abstract

The escalation of bacterial resistance against existing therapeutic antimicrobials has reached a critical peak, leading to the rapid emergence of multidrug-resistant strains. Stringent pathways in novel drug discovery hinder our progress in this survival race. A promising approach to combat emerging antibiotic resistance involves enhancing conventional ineffective antimicrobials using low-toxicity small molecule adjuvants. Recent research interest lies in weak membrane-perturbing agents with unique cyclic hydrophobic components, addressing a significant gap in antimicrobial drug exploration. Our study demonstrates that quinoline-based amphipathic small molecules, SG-B-52, and SG-B-22, significantly reduce MICs of selected beta-lactam antibiotics (ampicillin, amoxicillin) against lethal methicillin-resistant *Staphylococcus aureus* (MRSA). Mechanistically, membrane perturbation, depolarization, and ROS generation drive cellular lysis and death. These molecules display minimal *in vitro* and *in vivo* toxicity, showcased through hemolysis assays, cell cytotoxicity analysis, and studies on albino Wistar rats. SG-B-52 exhibits impressive biofilm-clearing abilities against MRSA biofilms, proposing a strategy to enhance beta-lactam antibiotics and encouraging the development of potent antimicrobial potentiators.

6.2 Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a predominant multi-drug-resistant bacteria, causing several human maladies ranging from mild skin conditions to severe bloodstream infections. Misuse, overuse, and improper knowledge about antibiotic consumption have led to the rapid upsurge of multi-drug resistant MRSA that have acquired high resistance against several antibiotics, including methicillin, oxacillin, and penicillin. MRSA has become a global issue, with high rates of infection in healthcare facilities, such as hospitals and long-term care facilities. Community-acquired MRSA infections are also on the rise and can occur in healthy individuals with no recent healthcare exposure. Risk factors for MRSA infection include recent hospitalization, surgery, dialysis, intravenous catheter use, and weakened immune system.^{1, 2} MRSA infections are projected to cause over a million deaths per year by 2050 with an associated global economic burden of one trillion US dollars.³

MRSA is resistant to several classes of antibiotics, including beta-lactams (penicillins, cephalosporins) chloramphenicol, lincomycin, aminoglycosides, tetracyclines, macrolides,

quinophthalones, sulfonamides and rifampicin, which is a grave problem in strategizing clinical treatment.⁴ Focusing on the beta-lactams, due to the presence of a unique penicillin-binding protein (PBP2a), MRSA is least affected by them. This modified PBP2a has a low affinity for beta-lactam antibiotics and can continue building the bacterial cell wall even in the presence of these antibiotics.⁵ The production of PBP2a is mediated by the *mecA* gene, which is carried on a mobile genetic element called the *staphylococcal chromosomal cassette* (SCCmec). The SCCmec element can transfer between different strains of *S. aureus*, allowing for the spread of *mecA*-mediated beta-lactam resistance.⁶ MRSA may also acquire additional resistance mechanisms, such as the production of beta-lactamase enzymes that can break down beta-lactam antibiotics. These enzymes are not present in all MRSA strains but may sometimes contribute to increased resistance.⁷ Because of its resistance to beta-lactam antibiotics, MRSA infections are typically treated with alternative antibiotics, such as vancomycin, daptomycin, or linezolid. However, resistance to these antibiotics is also a growing concern, highlighting the urgent need for new treatment options.⁴

Recent studies have demonstrated the potential of membrane-perturbing or disrupting synthetic small molecules (SMs) as novel therapies against MRSA. By targeting the bacterial cell membrane, these SMs offer a new approach to the management of such lethal infections facilitated by this pathogen.⁸ Alternatively, the development of antibiotic adjuvants/potentiators is also picking up pace, to synergize with existing antibiotics that have lost their efficacy against certain bacteria. The idea is to restrict or nullify the bacterial resistance towards the 'lost' antibiotics to restore their function. Recent literature suggests that the prime focus on membrane perturbing antibiotic adjuvant design and application has been placed on gram-negative priority pathogens like *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Escherichia coli* compared to gram-positive candidates like MRSA.⁹ Although an array of SMs like brominated carbazoles, 1,4-Napthoquinone, engineered antivirulence agents, and phytochemicals have been tested as antibiotic adjuvants for their synergistic antibacterial effect against MRSA,¹⁰⁻¹³ there is a significant research gap in the development of antibiotic membrane perturbers against the organism, and gram-positive pathogens as a whole. In addition, the application of weak membrane perturbers in the accentuation of antibiotic efficacy has been successfully reported in recent literature against gram-negative superbugs¹⁴ and the strategy has been underutilized in gram-positive bacteria.

This study involved the design and synthesis of a library consisting of ten novel analogs of quinoline and the 4-(trifluoromethyl/tert-butyl) benzene with varying hydrophobic and hydrophilic moieties. Growth inhibition studies, membrane perturbation assays, ROS assays, and microscopic investigations were performed to confirm the mechanism of inhibition. The antimicrobial potency of the lead molecules SG-B 22 and SG-B 52 alone and in combination with two beta-lactam antibiotics, ampicillin (AMP) and amoxicillin (AMOX), was meticulously probed against MRSA. Following the determination of the impact of the SM-antibiotic combinations on MRSA biofilms, the cytotoxic profiles of the compounds were examined on HRBCs and other mammalian cell lines (WI-38, HEK-293, and RAW 264.7). Albino Wistar rats were used to study the tolerance, survival, and *in vivo* organ-loading profiles of SG-B 52. The histopathological intervention was carried out to examine the efficacy of the SM-antibiotic combination in treating systemic MRSA infections.

6.3 Results and Discussion:

6.3.1 Small Molecule Library Design, Synthesis, and Characterization:

A series of novel analogs of quinoline scaffold (4, 6) and the 4-(trifluoromethyl/tert-butyl) benzene (9, 11) with varying hydrophobic and hydrophilic moieties were designed and synthesized as depicted in **Scheme 1 and 2**. The analogy behind choosing quinoline analogs as our adjuvants was inspired by prior literature where quinoline-based compounds exhibited potent antibacterial activity against gram-positive bacteria.¹⁵ Also, a combination of both hydrophilic and hydrophobic properties was incorporated to impart an amphipathic character to our designed molecules, providing an advantageous feature to combat gram-positive antibiotic-resistant bacterial strains. To investigate the influence of the amphipathic nature, encompassing both polar and non-polar functionalities on the central core, acyclic aliphatic hydrophobic groups with varying side chains (ranging from 2 – 11 carbons) were attached to the 2- and 3- positions. Additionally, polar hydrophilic groups, such as N- hydroxycarboxamide and positively charged primary amines were introduced in the 6- position. Furthermore, a distinct series of molecules featuring benzene as the core scaffold were designed. This series included hydrophobic electron-donating groups like tert-butyl and electron-withdrawing, as well as moderately hydrophobic groups like CF₃, introduced at one end. Simultaneously, hydrophilic groups like N-hydroxycarboxamide and positively charged primary amines were inserted at the other end of the benzene ring for the synthesis of molecules 9 and 11, as depicted in **Scheme 2**. It is important to note that although the molecule SG-B-11 has been previously reported as a PqsR antagonist against *P. aeruginosa*, it was tested in this study.¹⁶

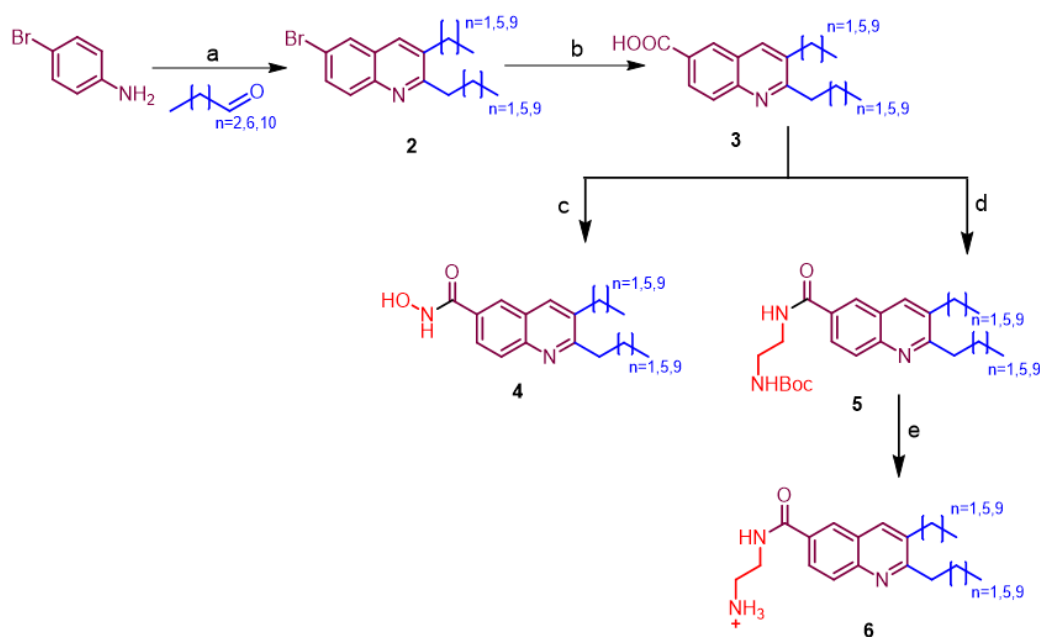


Figure 6.1: Scheme 1: Reaction conditions: a) 4(M) HCl, DMSO, 60°C, 4-5 h; (b) Pd (OAc)₂, X-phos, Et₃N, HCOOH, DIC, DMF, 120°C, 18 h; (c) HATU, DIPEA, NH₂OH.HCl, DMF, RT, overnight; (d) HATU, DIPEA, N-Boc-ethylenediamine, DMF, RT, overnight; (e) TFA, DCM, 0°C-RT, 4 h. (Red = hydrophilic end; Blue = hydrophobic end; Maroon: core quinoline scaffold).

Briefly, the synthesis of Scheme 1 commenced from commercially available 4-bromoaniline. The formation of the quinoline core and the introduction of the hydrophobic side chain in **2** took place simultaneously with different aliphatic aldehydes (butanal, octanal, dodecanal) in the presence of 4M HCl and DMSO. Next, a palladium-catalyzed carboxylation reaction using formic acid converted the 6-bromoquinolines (**2**) to their corresponding acids (**3**). Finally, the carboxylic acid group conjugated with either hydroxylamine or N-Boc-ethylenediamine via HATU coupling procured the desired compounds **4** and **5** respectively. The terminal Boc functionality in **5** was deprotected using TFA-DCM to obtain the desired product **6**. For **Scheme 2**, the commercially available aldehydes were converted to their corresponding acids via oxone oxidation to obtain the acids (**8**). Next, the final compounds **9**, and **11** were obtained following a similar procedure as described in **Scheme 1**.

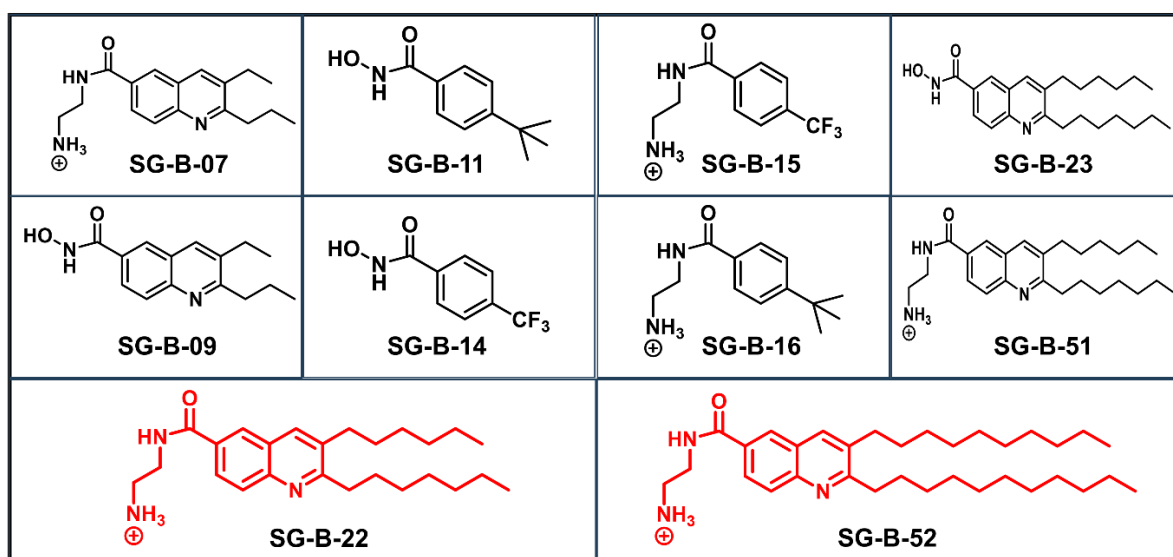


Figure 6.2: Antimicrobial adjuvant library: chemical structures of the antimicrobial adjuvant library with varying hydrophobic and hydrophilic moieties (the selected lead molecules have been colored red for reference).

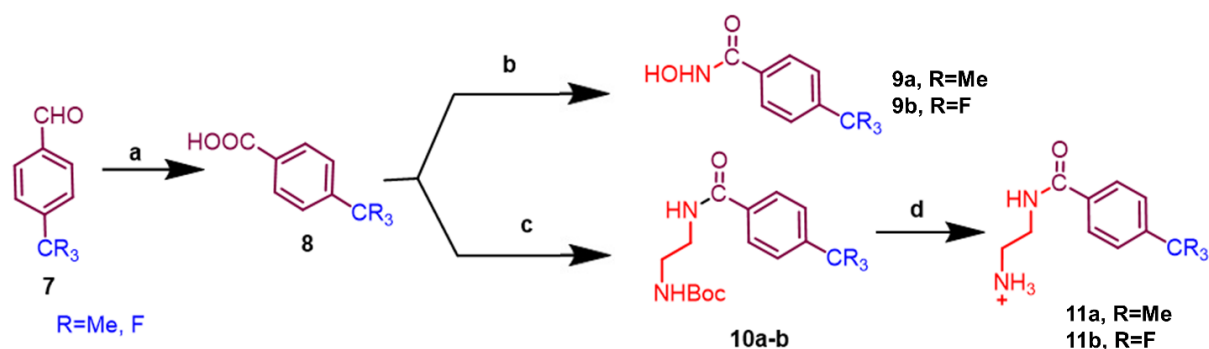
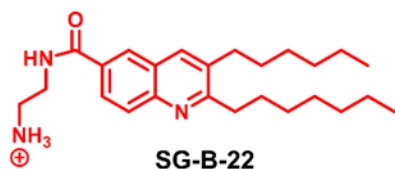
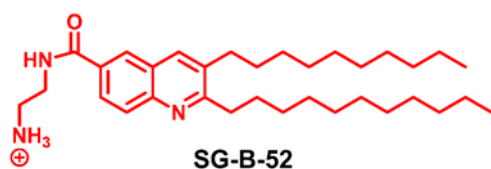


Figure 6.3: Scheme 2: Reaction condition: a) Oxone, DMF, RT, overnight; (b) HATU, DIPEA, NH₂OH.HCl, DMF, RT, overnight; (c) HATU, DIPEA, N-Boc-ethylenediamine, DMF, RT, overnight; (d) TFA, DCM, 0°C-RT, 4 h. (Red = hydrophilic end; Blue = hydrophobic end; Maroon: core scaffold)

All molecules in the library (Figure 6.2) were characterized using ^1H NMR, and ^{13}C NMR. The molecular masses of the synthesized molecules are ranged from 193-510 Da are obtained via ESI-MS, and they were purified using RP-HPLC with >95% purity. Characterization of two hit candidates SG-B-52 and SG-B-22 are given below. (Figure



2-Heptyl-3-hexyl-N-hydroxyquinoline-6-carboxamide, SG-B-22: ^1H NMR (DMSO- d_6 , 500 MHz) δ_{H} 8.92 (t, J = 5.5, 1H), 8.54 (d, J = 2.0 Hz, 1H), 8.48 (s, 1H), 8.21 (dd, J = 8.8, 1.8 Hz, 1H), 8.09 (d, J = 9.0, 1H), 7.89 (brs, 3H), 3.58 (q, J = 6.0 Hz, 2H), 3.06-3.03 (m, 4H), 2.85 (t, J = 7.8 Hz, 2H), 1.80-1.74 (m, 2H), 1.71-1.65 (m, 2H), 1.45-1.26 (m, 14H), 0.9-0.85 (m, 6H); ^{13}C NMR (DMSO- d_6 , 125 MHz) δ_{C} 162.9, 159.0, 158.7, 158.5, 136.2, 128.1, 127.7, 126.7, 117.4, 115.1, 39.0, 37.7, 31.6, 31.5, 31.1, 29.9, 29.4, 29.2, 28.9, 28.6, 28.9, 28.8, 22.5, 22.4, 14.4; LCMS calculated for $\text{C}_{25}\text{H}_{41}\text{N}_3\text{O}^+$ $[\text{M}+\text{H}]^+$, 399.3244; found, 399.3206.



2-(3-Decyl-2-undecylquinoline-6-carboxamido)ethan-1-aminium, SG-B-52: ^1H NMR (DMSO- d_6 , 500 MHz) δ_{H} 8.90 (t, J = 5.8, 1H), 8.48 (s, 1H), 8.30 (s, 1H), 8.15 (d, J = 9.0, 1H), 8.02 (d, J = 9.0 Hz, 1H), 7.96 (brs, 3H), 3.57 (q, J = 6.0 Hz, 2H), 3.05 (q, J = 6.0 Hz, 2H), 2.98 (t, J = 7.8 Hz, 2H), 2.80 (t, J = 8.0 Hz, 2H), 1.79-1.73 (m, 2H), 1.68-1.62 (m, 2H), 1.43-1.48 (m, 4H), 1.35-1.32 (m, 4H), 1.27-1.23 (m, 22 H), 0.84 (t, 6H); ^{13}C NMR (DMSO- d_6 , 125 MHz) δ_{C} 166.8, 163.4, 158.9, 158.6, 135.6, 132.1, 128.1, 127.8, 126.5, 39.1, 37.7, 34.6, 31.7, 31.6, 30.1, 29.5, 29.4, 29.3, 29.26, 29.25, 29.1, 22.5, 14.4; LCMS calculated for $\text{C}_{33}\text{H}_{56}\text{N}_3\text{O}^+$ $[\text{M}]^+$, 510.4418; found, 510.4436.

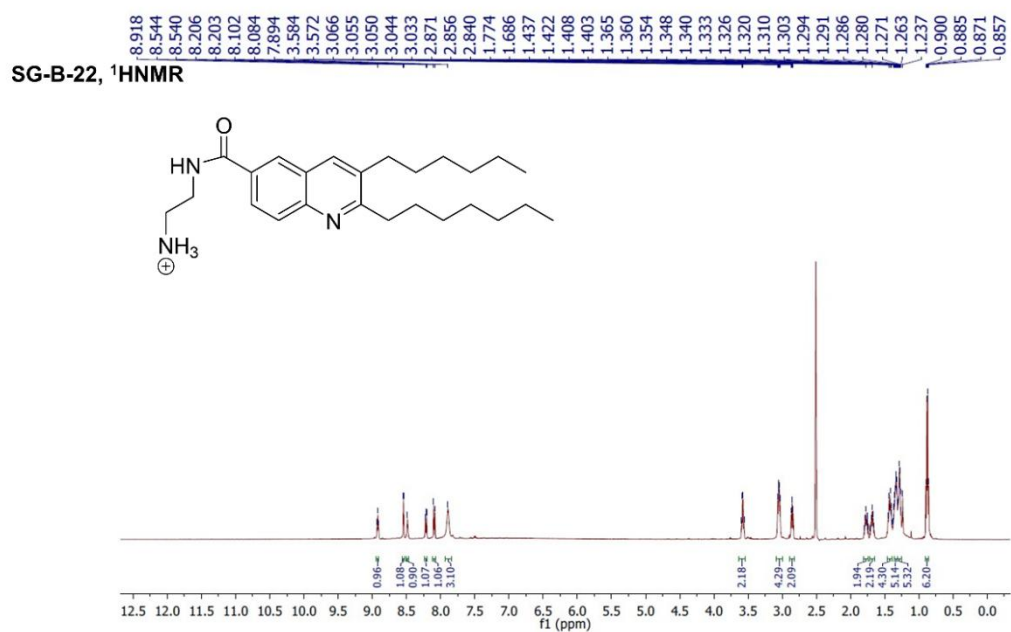


Figure 6.4: ^1H (500 MHz) NMR of SG-B-22.

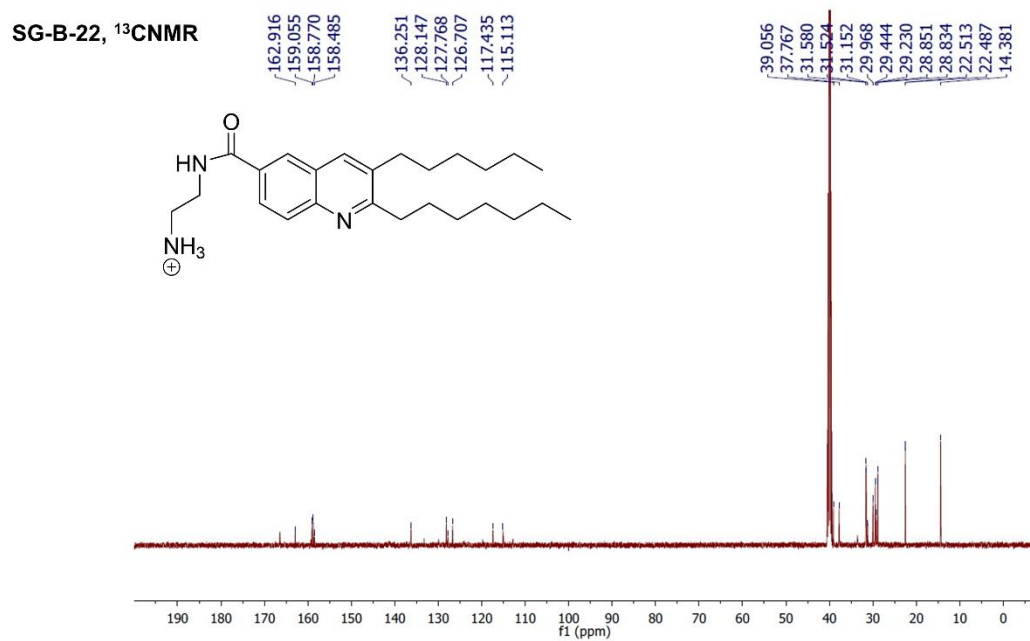


Figure 6.5: ^{13}C (125 MHz) NMR of SG-B-22.

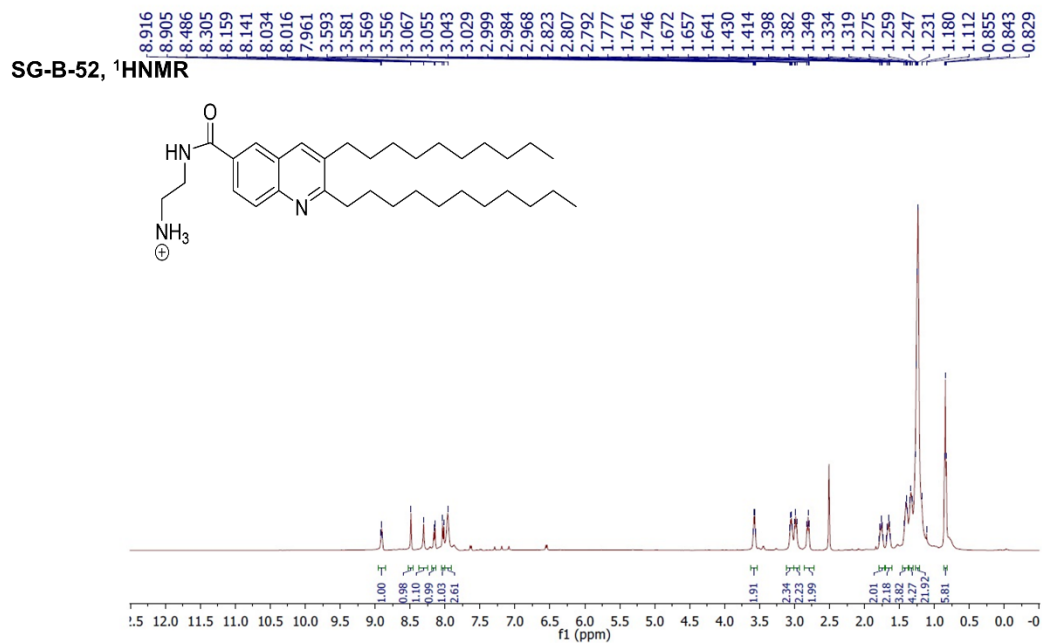


Figure 6.6: ^1H (500 MHz) NMR of SG-B-52.

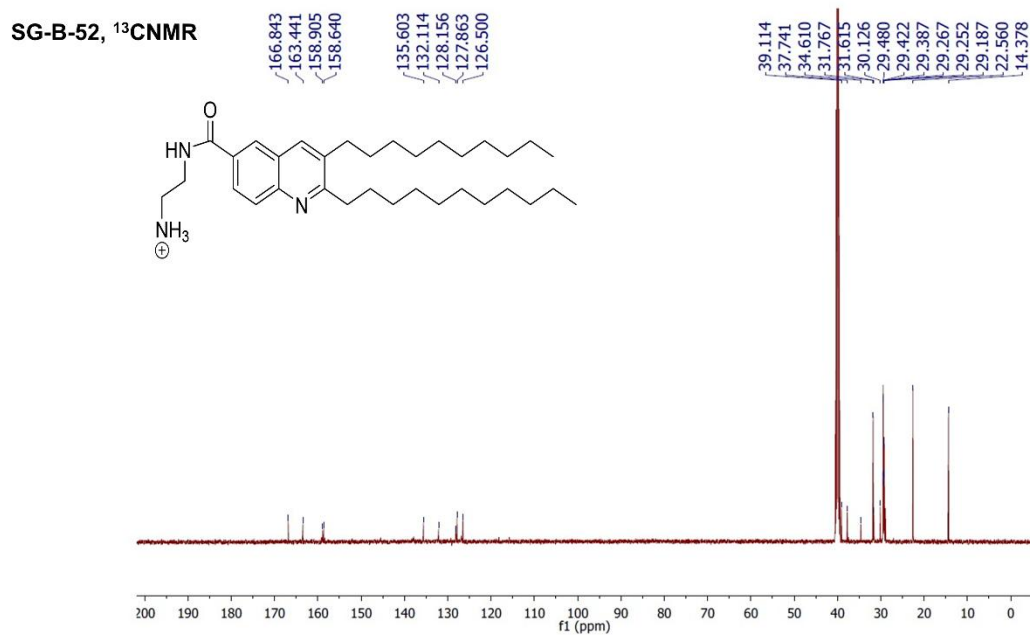


Figure 6.7: ^{13}C (125 MHz) NMR of SG-B-52.

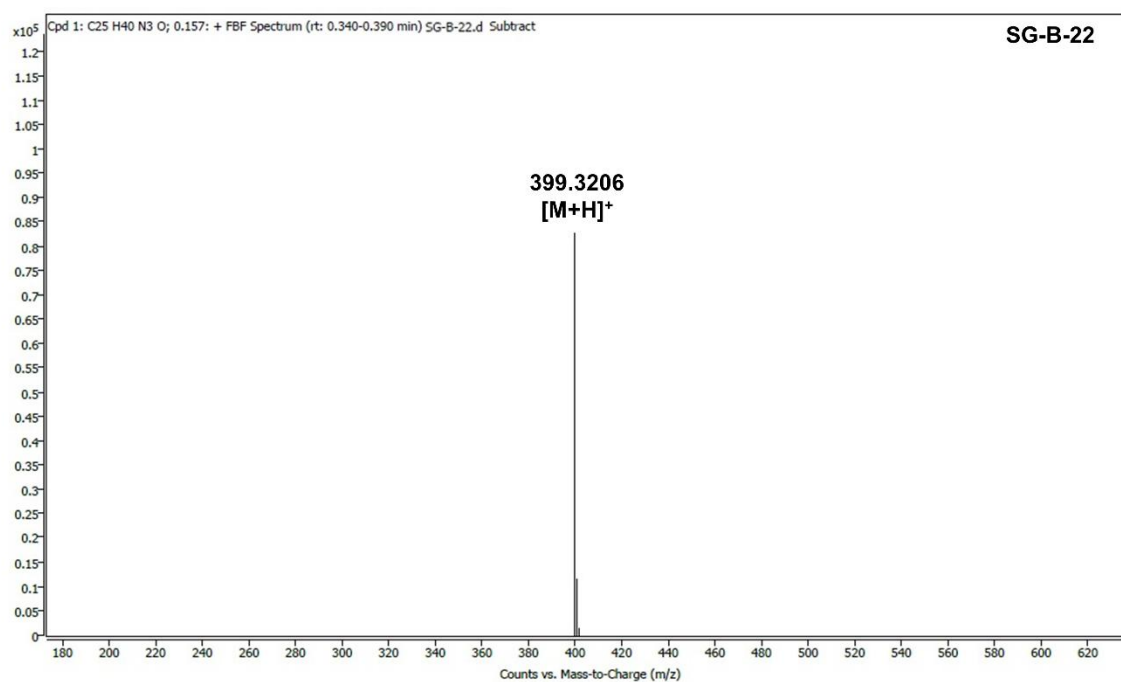


Figure 6.8: Mass of SG-B-22 as observed to be 399.3206 Da $[M+H]^+$ as determined by ESI-LC/MS.

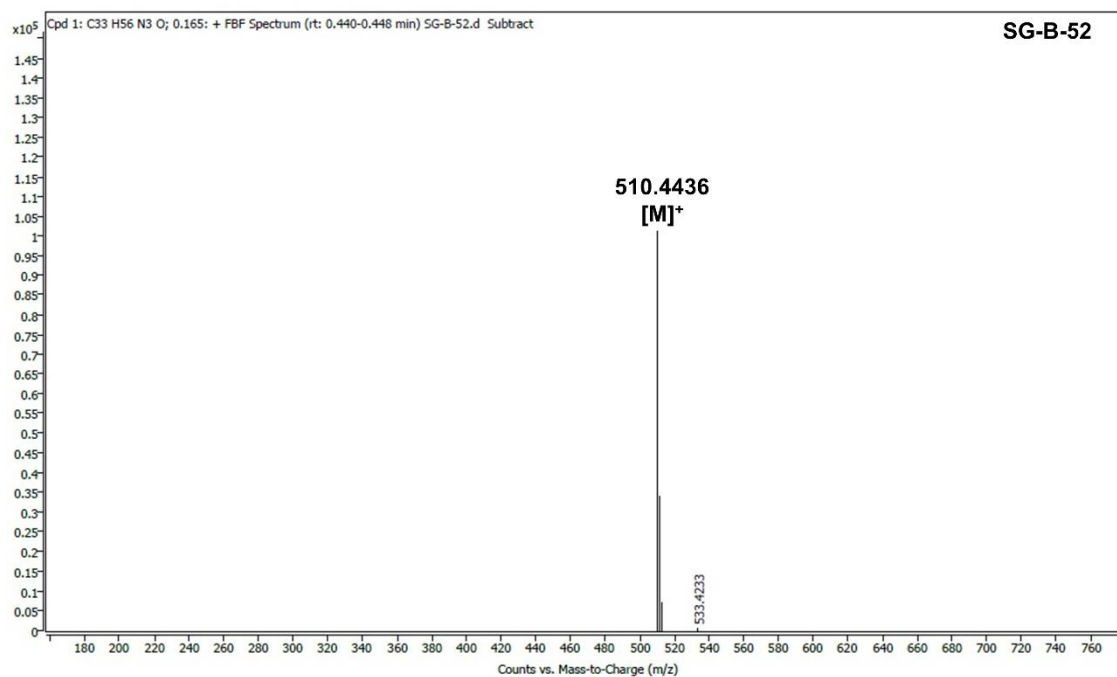
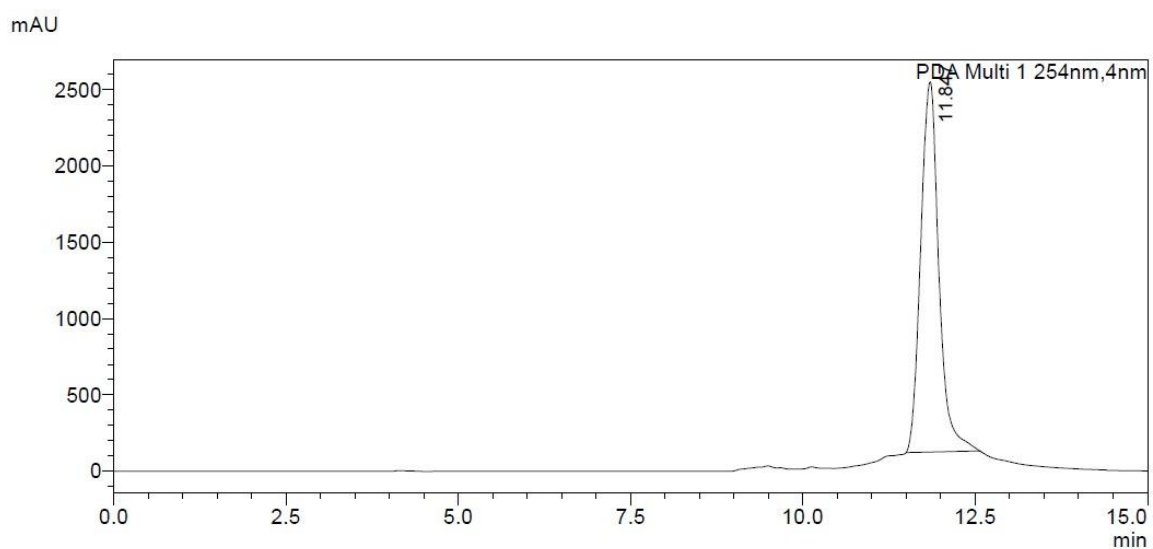
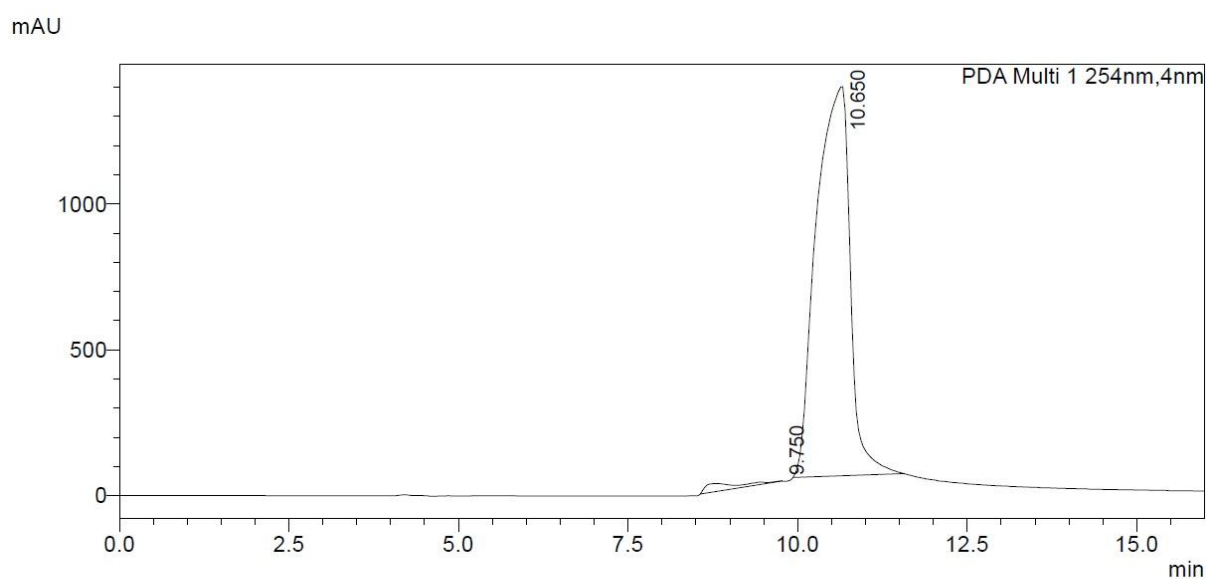


Figure 6.9: Mass of SG-B-52 as observed to be 510.4436 Da $[M]^+$ as determined by ESI-LC/MS.



Peak Table				
Peak#	Ret. Time	Area	Height	Area%
1	11.847	44454971	2424853	100.000
Total		44454971	2424853	100.000

Figure 6.10: HPLC profile of SG-B-22 as observed to be 100% pure.



Peak Table				
Peak#	Ret. Time	Area	Height	Area%
1	9.750	929773	414	1.969
2	10.650	46295944	1333513	98.031
Total		47225717	1333927	100.000

Figure 6.11: HPLC profile of SG-B-52 as observed to be 98% pure.

6.3.2 Antimicrobial Activity, and Synergy between SG-B 22/ SG-B 52 and AMP/AMOX

After screening the molecules of the synthesized library against *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and MRSA (Table 6.1), the MIC values of AMP, AMOX, and the two lead molecules SG-B 22 and SG-B 52 were obtained against the MDR strain of MRSA, the selected target organism.

The rationale behind the selection of the lead molecules was their ability to weakly inhibit the growth of MRSA (MIC of 220-240 μM), whereas the other library molecules had negligible anti-MRSA activity upto 300-350 μM . Out of the four MRSA strains tested, the target strain showed the highest MIC against AMP (180 μM) and AMOX (160 μM) and was thus selected for the study. The MICs displayed by the other three strains SGM1, SGM2, and SGM3 against AMP and AMOX were (80, 100, 100) and (60, 80, 60) respectively. Their MICs against SG-B 22 and SG-B 52 were also lower than the reported strain at (120, 120, 180) and (100, 120, 120) respectively. For AMP, the MIC was found to be 180 μM whereas for AMOX the MIC was 160 μM . The individual antibacterial activities of SG-B 22 and SG-B 52 were then investigated. Preliminary spotting assays indicated that SG-B 22 and SG-B 52 inhibited the growth of MRSA above 200 μM concentration when administered alone. The microdilution-based MIC assay revealed that while working alone, the MIC of SG-B 22 and SG-B 52 were 240 μM and 220 μM respectively (Table 6.2). The results indicated that the molecules were weak membrane perturbers, as seen in similar studies on gram-negative superbugs.¹⁴ This strategy to target the bacterial membrane presents some advantages. For instance, the uptake machinery of persister cells in resistant bacterial populations is tuned in a way, to repel most antimicrobials, making the cellular interior inaccessible.¹⁷ Additionally, targeting a specific gene/protein results in a higher probability of resistance acquisition by the bacteria.¹⁸ In theory, the weak membrane perturbation by the SMs should overwhelm the cells in such a way that the protective function of PBP2a should be neutralized, making way for the beta-lactams to target the susceptible PBPs of MRSA. Subsequently, the MBCs for MRSA were found to be 280 μM and 260 μM for SG-B 22 and SG-B 52 respectively (Table 6.3). For AMP, the MBC was observed to be 220 μM and for AMOX, the value was 200 μM .

S. No.	I.D.	MIC (EC) in μM	MIC (MRSA) in μM	MIC (PA) in μM	MIC (KP) in μM
1.	SG-B-07	>300	>300	>350	>350
2.	SG-B-09	>350	>350	>350	>350
3.	SG-B-11	>350	>350	>350	>350
4.	SG-B-14	>350	>350	>350	>350
5.	SG-B-15	>300	>300	>300	>350
6.	SG-B-16	>300	>350	>350	>350
7.	SG-B-22	>300	>200	>350	>350
8.	SG-B-23	>300	>300	>300	>300
9.	SG-B-51	>300	>300	>300	>350
10.	SG-B-52	>300	>200	>300	>300

Table 6.1: Preliminary spotting assay data for all the small molecules of the synthesized library against *Escherichia coli* (EC), *Methicillin-resistant Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* (PA), and *Klebsiella pneumoniae* (KP).

S. No.	I.D.	MIC of MRSA in μM
1.	SG-B-07	320
2.	SG-B-09	380
3.	SG-B-11	380
4.	SG-B-14	390
5.	SG-B-15	310
6.	SG-B-16	400
7.	SG-B-22	240
8.	SG-B-23	320
9.	SG-B-51	300
10.	SG-B-52	220

Table 6.2: MIC data for all the small molecules of the synthesized library against MRSA.

S. No.	I.D.	MBC of MRSA in μM
1.	SG-B-07	360
2.	SG-B-09	>400
3.	SG-B-11	>400
4.	SG-B-14	>400
5.	SG-B-15	350
6.	SG-B-16	>400
7.	SG-B-22	280
8.	SG-B-23	360
9.	SG-B-51	350
10.	SG-B-52	260

Table 6.3: MBC data for all the small molecules in the synthesized library against MRSA

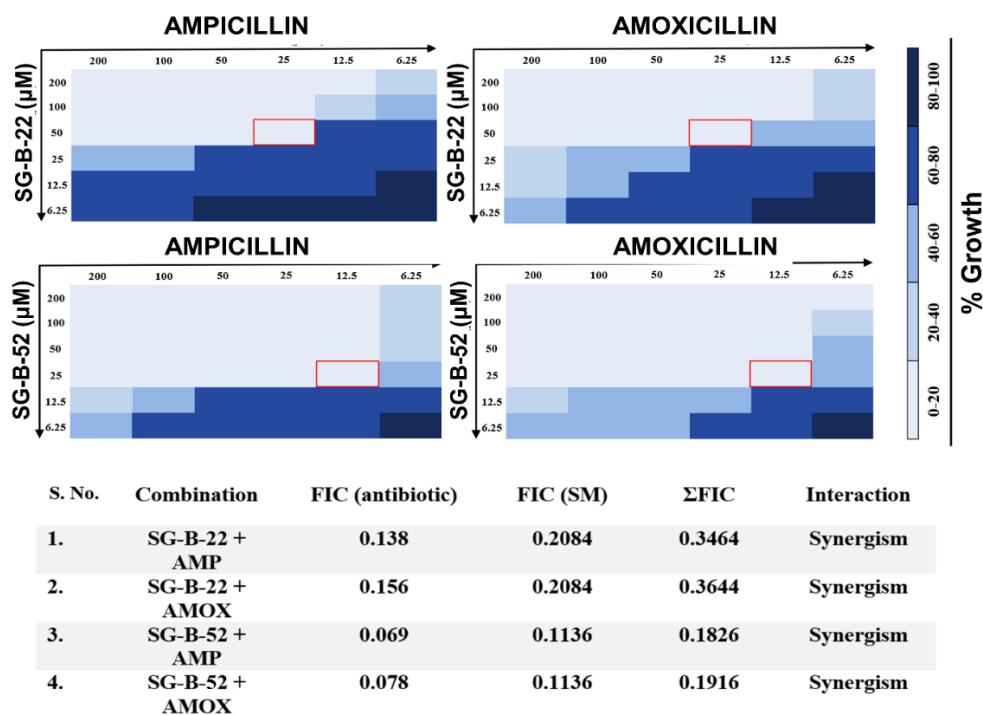


Figure 6.12: Potentiation of ampicillin and amoxicillin by SG-B-22 and SG-B-52 against Methicillin-resistant *Staphylococcus aureus* (MRSA): Checkerboard assays of SG-B-22 with ampicillin and amoxicillin (upper panel) and SG-B-52 with ampicillin and amoxicillin (lower panel) against MRSA along with the FIC results obtained with SM-antibiotic combinations by the checkerboard method as tabulated.

When applied in combination with AMP/AMOX, 4.8- and 8.8-fold reductions were observed in the MIC for SG-B 22 and SG-B 52 respectively (Figure 6.12). For the antibiotics, a 7.2-fold reduction was observed in AMP MIC, and a 6.4-fold reduction in AMOX MIC was observed in combination with SG-B 22. For SG-B 52 however, the fold reductions were more pronounced at 14.4 for AMP and 12.8 for AMOX. When expressing the results in terms of fractional inhibitory concentration (FIC), it became clear that all four SM+antibiotic combinations showed synergism.

Depending on the Σ FIC values obtained, the order of synergy was expressed as:

SG-B-52+AMP > SG-B-52+AMOX > SG-B-22+AMP > SG-B-22+AMOX.

These four combinations were found to be the most effective against MRSA and were considered EC or effective combinatorial concentrations and were used further in the study. A comprehensive analysis of the growth of MRSA was conducted, focusing on the dosage and time of growth of SG-B 22 (50 μ M), SG-B 52 (25 μ M), AMP (12.5 μ M, 25 μ M), AMOX (12.5 μ M, 25 μ M), and their combinations (EC). The study revealed that the growth of MRSA was inhibited by both the SMs and the antibiotics, whether used alone or in combination in a time-dependent manner (Figure 6.13).

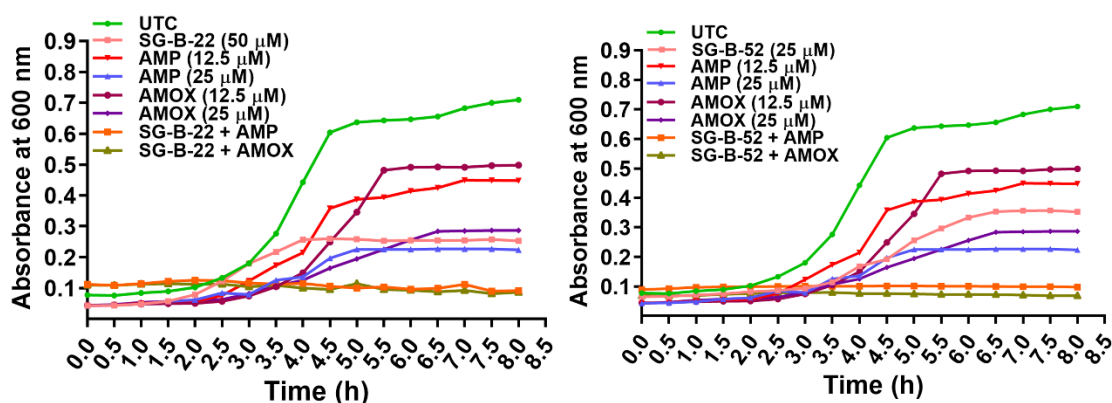


Figure 6.13: Growth inhibition studies: The decrease in optical density (OD_{600}) as a function of time (h) in MRSA samples treated with SG-G-22, SG-B-52, Ampicillin (AMP), Amoxicillin (AMOX), and SM+antibiotic combinations measured over 8h, indicating concentration and time-dependent inhibition of cell growth in Mueller-Hinton broth at 37°C.

The most effective inhibition was observed when the SMs were combined with the beta-lactam antibiotics tested, while the SMs and antibiotics used alone resulted in less noticeable inhibition. In the case of both SG-B 22 and SG-B 52, the SM and antibiotics combinations yielded flat lines, announcing the fact that at EC, both the SMs were extremely competent in inhibiting the growth of MRSA. The growth response in MHA at the 8h mark showed that except for the SM+antibiotic combinations, all the other treated/untreated cells were capable of growth post-incubation at 37°C for 12h, indicating bacteriostasis (Figure 6.14). But for the combinations, no growth was observed, indicating bacterial death.

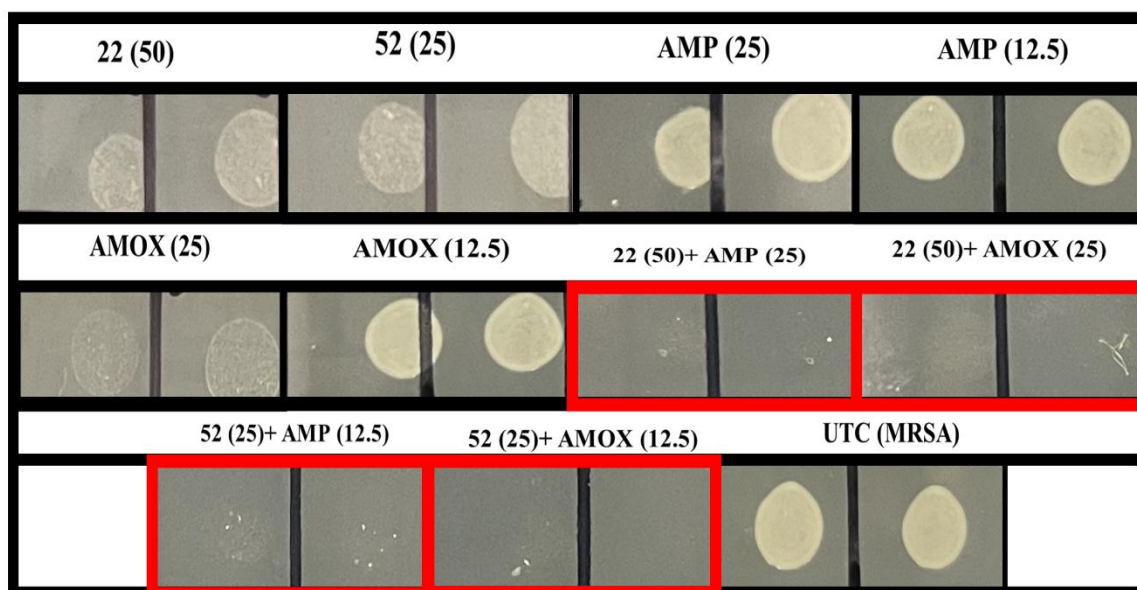


Figure 6.14: Bacterial growth assay to check the viability of Methicillin-resistant *Staphylococcus aureus* (MRSA) post-treatment with SG-B-22, SG-B-52, Ampicillin (AMP), Amoxicillin (AMOX), and the SM + antibiotic combinations in Mueller-Hinton agar plates incubated for 12h at 37°C; UTC = untreated control.

6.3.3 Molecules SG-B 22/ SG-B 52 in combination with AMP/AMOX perturb the MRSA cell membrane and cause cellular disruption

The leakage of bioendogenous DNA/RNA/Proteins on treatment with a membrane perturbing or degrading agent can present an excellent early indication of bacterial membrane damage.¹⁹ These bioendogenous materials can be detected in the cell-free media by monitoring the UV absorbance at 260 nm. Likewise, the cellular leakage was observed for 4h treated MRSA cells with AMP, AMOX, SG-B 22, SG-B 52, and their combinations with AMP/AMOX. Compared with the untreated control, there was a significant elevation in cellular leakage in all the treated cells, whether alone or in combination, prominently seen from the 2h time point ($p < 0.05$) (Figure 6.15 A). When the comparison was made between AMP/AMOX alone or in combination with SG-B 22 and SG-B 52, the combinations yielded significantly higher OD_{260} , indicating superior cellular damage at all time points from 2h onwards ($p < 0.01$). These results were encouraging and presented a persuasive case to probe the membrane further.

All the compounds tested using the fluorescence potentiometric probe DiSC₃(5) displayed varying degrees of membrane depolarization against MRSA. One common facet among all the tested SMs, as well as antibiotics, was the rapidity of the depolarization effect. For SG-B 22, the depolarising caused by the individual molecule as well as the antibiotic combinations

was lower than the positive control gramicidin (Figure 6.15 B) but reached the maximum intensity as early as 60-150 sec.

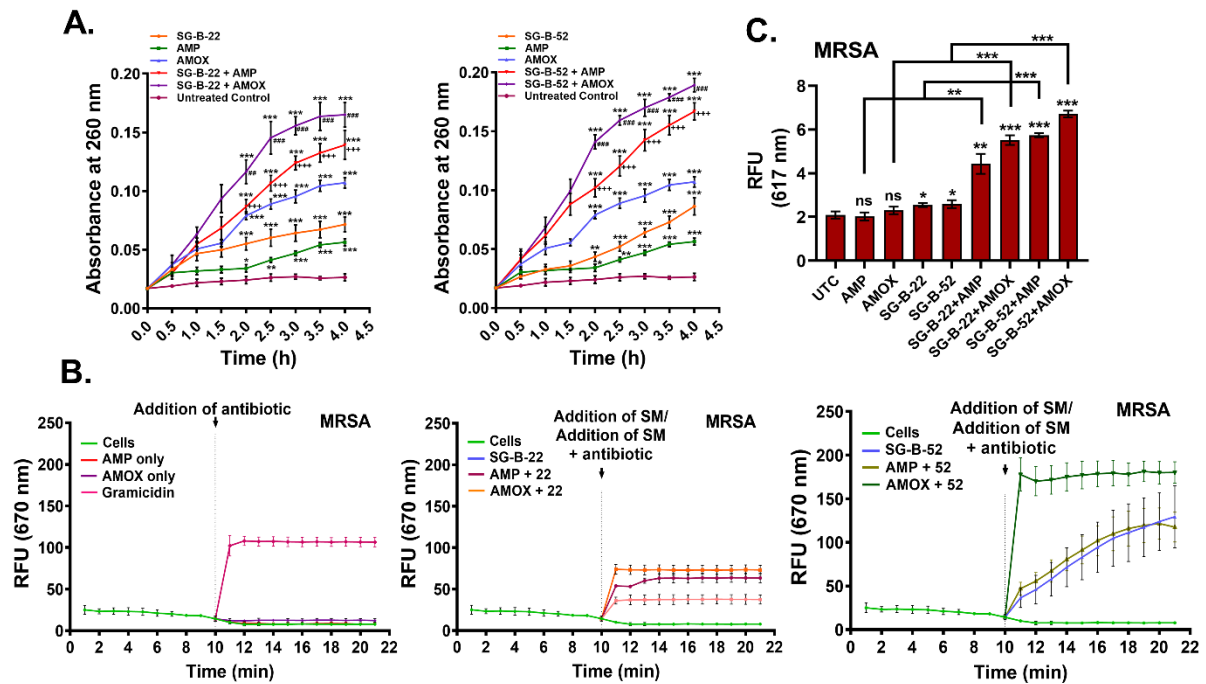


Figure 6.15: Membrane perturbation: A) Cellular leakage induced by SG-B-22, SG-B-52, Ampicillin (AMP), Amoxicillin (AMOX), and their combinations as a function of absorbance with time (h) at OD₂₆₀ in methicillin-resistant *Staphylococcus aureus* (MRSA); B) Effect of AMP, AMOX, SG-B-22, SG-B-52, and their combinations on the cytoplasmic membrane depolarization of MRSA, indicated by the enhanced fluorometric emission at 670 nm of the voltage-sensitive cyanine dye DiSC₃(5) at stipulated concentrations with time (h), keeping gramicidin as a positive control; C) Results of the PI assay demonstrating the ability of AMP, AMOX, SG-B-22, SG-B-52, and their combinations to permeabilize the cell membranes of MRSA compared to the untreated controls; the mean RFU values of AMP/AMOX were also compared with their SM combinations; p -value < 0.05 = *|#|+, p -value < 0.01 = **|##|+++, p -value < 0.001 = ***|###|++++, ns = not significant; + denotes statistical significance between SM + AMP and AMP alone; # denotes statistical significance between SM + AMOX and AMOX alone.

For SG-B 52 however, a gradual and continuous increase in membrane depolarisation was observed for the SM alone and in combination with AMP for the tested period. The SG-B 52+AMOX combination showed the most intense and immediate depolarization among all others. This urgency in membrane depolarisation was by all probability due to the rapid K^+/Na^+ movement across the membrane, similar to the effect caused by the positive control gramicidin (gramicidin ABCD), a potent K^+/Na^+ membrane channel former.²⁰ The gradual increase in depolarization for SG-B 52 and SG-B 52+AMP can be explained by the progressive dissipation of the H^+ gradient across the membrane, replaced by an increase in the K^+ gradient, a typical indicator of bacterial membrane depolarization.²¹ The results indicated that SG-B 52 in combination with AMOX caused a rapid dissipation of membrane potential in MRSA, even outperforming gramicidin, whereas the depolarization caused by SG-B 52 alone and in

combination with AMP was greater than gramicidin at the 21 min time point, reaching there gradually.

The idea of exploiting the amphipathicity of possible drug molecules for the penetration of bacterial membranes has stemmed from antimicrobial peptides (AMPs). AMPs are generally cationic and amphipathic, meaning they can interact with the negatively charged bacterial outer membranes (and inner membranes for gram-negative organisms), binding at the membrane-water interface.²¹ The insertion of the hydrophobic moieties to the apolar core of the bacterial membrane, while interacting with the head-group region of the membrane through the positively charged amino acids, disrupts the surface tension of the outer leaflet, facilitating the formation of pores, over a threshold peptide concentration.²² To address the toxicity, stability, and bioavailability concerns of AMPs, a wide range of amphipathic peptide mimics have been synthesized. Termed membrane-active small molecules (MASMs), many have shown independent antibacterial activity²³ while some have been reported to act as adjuvants of conventional antimicrobials, similar to SG-B 22 and SG-B 52.⁹ In this present scenario, we believe that the weak membrane perturbation of the two SMs facilitates the access of AMP/AMOX to the beta-lactam susceptible PBPs (PBP1-PBP4) of MRSA (neutralizing the protection conferred by PBP2a),²⁴ resulting in the prevention of selection of high beta-lactam resistant strains from heterogeneous MRSA populations (populations with high and low beta-lactam resistant MRSA cells) to form extensively resistant homogenous sub-populations.²⁵ It must be mentioned that it is a possible hypothesis and the exact mode of synergistic action is subject to further research. The results of the PI assay justified the intended cause. Expectedly, AMP and AMOX had a negligible membrane-damaging effect on MRSA while working alone ($p > 0.05$) (Figure 6.15 C). Interestingly, both SG-B 22 and SG-B 52 were able to damage the MRSA membranes weakly, but independently ($p < 0.05$). On combining the SMs with AMP/AMOX, there was a significant accentuation of the damaging effect concerning the independent activity of the antibiotics and SMs. The PI fluorescence intensity went up considerably for all four combinatorial treatments in comparison to the individual SMs and antibiotics, signifying increased membrane damage ($p < 0.001$).

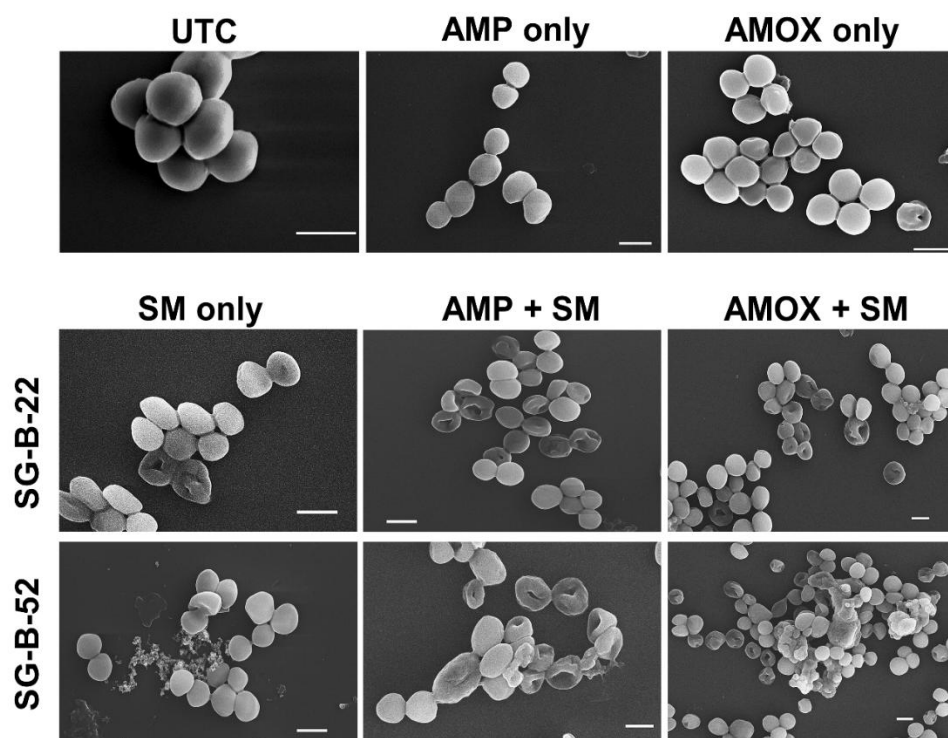


Figure 6.16: Morphological disruption of MRSA: (top panel) FESEM micrographs of untreated (UTC), AMP, and AMOX-treated Methicillin-resistant *Staphylococcus aureus* (MRSA) showing that the cells are round and intact with a few damaged cells (in the AMOX treated samples); **(bottom panel)** In the SG-B-22 and SG-B-52 treated MRSA cells, some perforations in the cells can be observed, but mostly, the cells are unaffected; cells treated with SM+AMP and SM+AMOX show deep craters and predominantly lysed cells; most cells are deformed or devoid of cellular materials signifying complete lysis and loss of cellular integrity; Scale Bar = 1 μm .

The Field Emission Scanning Electron Microscopy (FESEM) data provided comprehensive validation that the SMs in combination with AMP/AMOX had a lethal membranolytic effect on MRSA. Round and intact bacterial cells were observed for the untreated control sets (Figure 6.16). Like the untreated controls, the AMP-treated cells showed undisturbed cells with proper cellular morphology. In the AMOX-treated samples, some cells appeared with distorted membranes but were negligible in number. Similar observations were made for the SG-B 22 treated cells where a small proportion of the cells showed signs of membrane damage. A few heavily damaged cells appeared in the SG-B 52 treated samples with complete loss of cellular materials but overall, the populations consisted of cells with normal shape and integrity. The combinatorial treatment sets of both SG-B 22 and SG-B 52 with AMP and AMOX however, were composed of cells that showed an increased frequency and severity in wrinkling, blebbing, and deformation, all of them arising due to the damage inflicted to their membranes. Most of the cells showed an RBC-like appearance with a central depression indicating a complete loss of cellular integrity. Although the damage to the cell membranes was evident from these micrographs, their viability status needed to be verified.

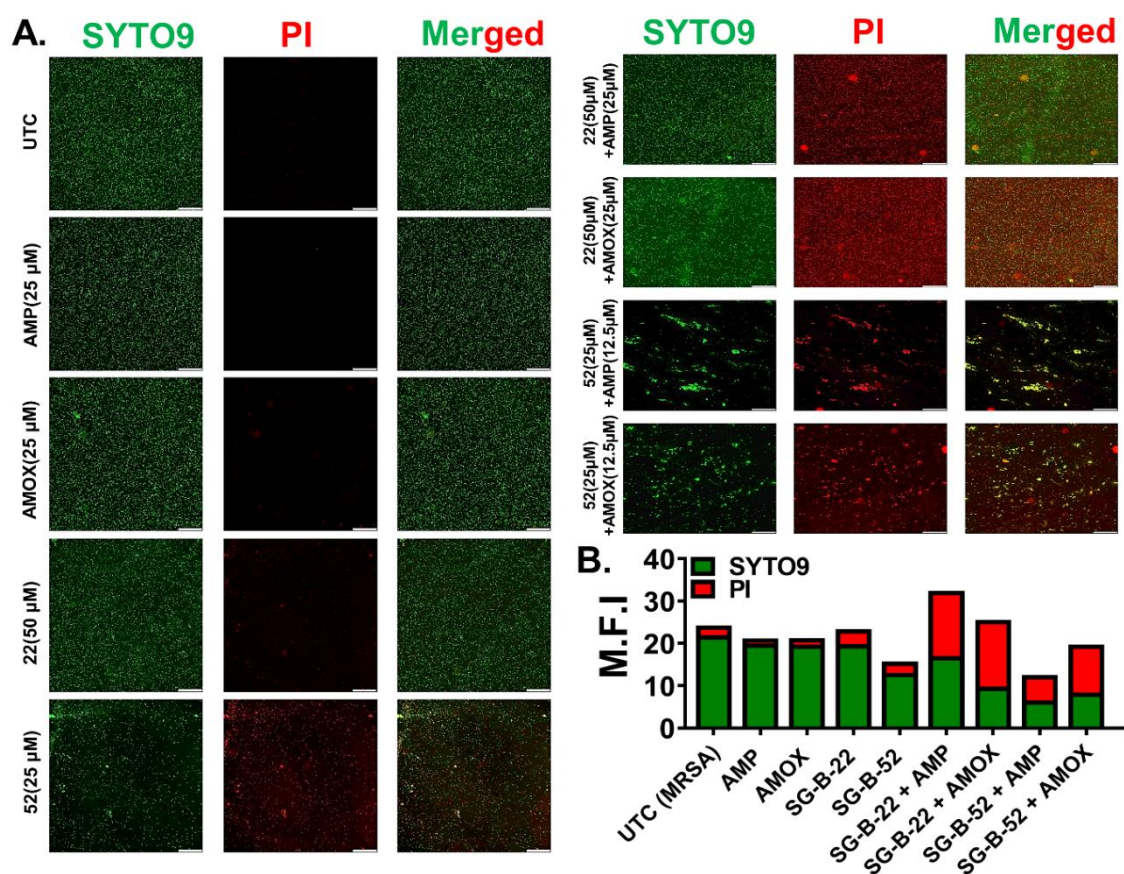


Figure 6.17: Bacterial Live/Dead Assay: A. The SYTO9/PI mediated live/dead assay of methicillin-resistant *Staphylococcus aureus* (MRSA) cells treated with AMP (25 μ M), AMOX (25 μ M), SG-B-22 (50 μ M), SG-B-52 (25 μ M), or their AMP/AMOX combinations (EC) treated for 30 min indicating extensive cellular damage to the SM+antibiotic treated cells compared with untreated, AMP, AMOX, SG-B-22, and SG-B-52 controls as evident from the fluorescence micrographs; B. Quantification of fluorescence intensities of AMP, AMOX, SG-B-22, SG-B-52, or their AMP/AMOX combination treated MRSA cells, expressed as MFI; M.F.I = Mean Fluorescence Intensity; Scale Bar = 200 μ m.

The viability status of the MRSA cells was verified using the Live/Dead assay²⁶ after treatment with AMP (25 μ M), AMOX (25 μ M), SG-B 22 (50 μ M), SG-B 52 (25 μ M), SM+antibiotic combinations (EC). In the untreated controls, the amount of SYTO9 stained cells was abundant, where PI penetration was determined to be minimal (Figure 6.17 A). The results emphasized that the cellular damage in the untreated control cells was minor, preventing PI from effectively penetrating the cells and producing insignificant red fluorescence, as opposed to SYTO9, which is cell permeable and easily stained the live cells and produced green fluorescence. Similar observations were made for the AMP, AMOX, SG-B 22, and SG-B 52 treated cells.

Nonetheless, there was visible damage in the cells treated with the SM+antibiotic combinations. As per the quantification data, for all four combinations, both the membrane-permeant dye SYTO9 and the impermeant dye PI stained the cells, indicating disruption of the bacterial membrane and likely death (Figure 6.17 B). It must be noted that the SG-B 52+AMP/AMOX samples yielded comparatively weaker/inconsistent fluorescence signals as

extensive cellular clumping was observed, compared with the uniformly distributed cells in the other samples.

6.3.4 Molecules SG-B 22/ SG-B 52 in combination with AMP/AMOX show minimal cytotoxicity towards HRBCs, HEK-293, RAW 264.7, and WI38 cell lines:

The extent of hemolysis of RBCs is now considered an important metric in determining the cytotoxicity of a prospective antimicrobial adjuvant.¹⁴ Both SG-B 22 and SG-B 52 (along with their AMP/AMOX combinations) were assayed up to 100 μ M with an 8% v/v⁻¹ HRBC suspension to test the ability of the molecules to lyse the erythrocytes. At a relatively high molar concentration of 100 μ M, SG-B 22 caused 34.1 % hemolysis whereas SG-B 52 lysed 16 % of the cells, compared to the positive control Triton-X 100 (Figure 6.18 A). This indicates that the HC₅₀ values for both SG-B 22 and SG-B 52 were greater than 100 μ M. Even in combination with AMP/AMOX, these values did not go up, indicating hemolytic safety. At EC, the maximum hemolysis was obtained for SG-B 52+AMP at 17.1 % whereas the other three combinations yielded values less than 10%.

The lowest mammalian cell cytotoxicity was noted against the lung fibroblast cell line WI38 with >80% cellular viability at all tested concentrations (1.5-50 μ M) of SG-B 22 and SG-B 52 (Figure 6.18 B). For the mouse macrophage cell line RAW 264.7, the highest toxicity was noted for SG-B 22 at a concentration of 50 μ M with a viability percentage of 74.4%. For all other concentrations of SG-B 22 and SG-B 52, the percent viability of the cells ranged from 78.5 – 96.4 %, indicating robust biocompatibility. When tested on the human embryonic kidney cell line HEK 293, a certain degree of toxicity was noted as 51.2 % of cells remained viable after treatment with the highest concentration of SG-B 52, i.e., 50 μ M. However, at 25 μ M (the concentration used in combination with AMP/AMOX), 67.5 % of cells survived. Percent viabilities of ~80 % or >80 % were obtained at all other tested concentrations of SG-B 22 and SG-B 52.

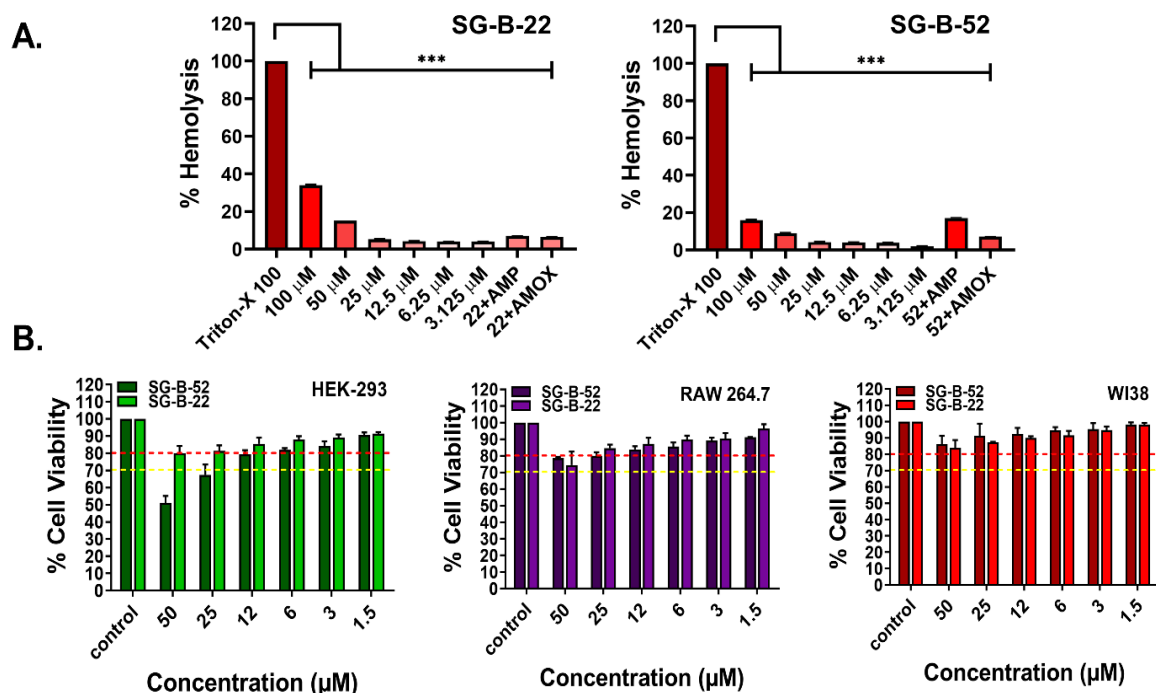


Figure 6.18: Hemolysis and cytotoxicity: A. Hemolytic activity of SG-B-22 and SG-B-52 (100 – 3.125 μM) and SM+antibiotic combinations (EC) as assessed towards human red blood cells (HRBCs); B. Cytotoxicity profiles of SG-B-22 and SG-B-52 against mammalian cell lines HEK 293, RAW 264.7, and WI-38 (at 1.5 - 50 μM concentrations); The red (top) and yellow (bottom) dotted lines indicate 80 % and 70 % cell viabilities respectively; three replicates of each sample type were tested, and the experiment was performed thrice (n = 3); p -value < 0.001= ***.

6.3.5 Molecules SG-B 22/ SG-B 52 in combination with AMP/AMOX causes the generation of endogenous ROS in MRSA:

Antimicrobials can cause the inhibition of essential metabolic processes in bacteria, resulting in the generation of endogenous ROS, including hydroxyl, peroxide, and superoxide radicals.²⁷ These highly reactive molecules have the potential to damage cellular DNA, RNA, lipids, and proteins.²⁸ Recent literature has shown how membrane-active amphipathic antimicrobials like daptomycin can interact with the *S. aureus* membranes and stress-related proteins, causing an overproduction of ROS, and eventually killing the cells.²⁹ Owing to the amphipathic structures of SG-B 22 and SG-B 52, the elicitation of ROS by these molecules and their AMP/AMOX combinations were investigated. Independently, both the SMs and the beta-lactam antibiotics (AMP/AMOX) were found to elicit moderate ROS generation, as anticipated (Figure 6.22A). In combination, however, there was a significant increase in the levels of ROS in the MRSA cells ($p < 0.01$). There was an anticipated lowering of cellular ROS in the ascorbic acid (AA, 40 μM) treated cells (negative control) for both the AA-treated and the SM + AMP/AMOX treated cells (Figure 6.19). When compared with AMP/AMOX alone, The SG-B 52 + AMP/AMOX + AA treated cell showed an insignificant increase in ROS and for SG-B 22 + AMP/AMOX + AA treated cells, the decrease was significantly lower ($p < 0.001$ = ***).

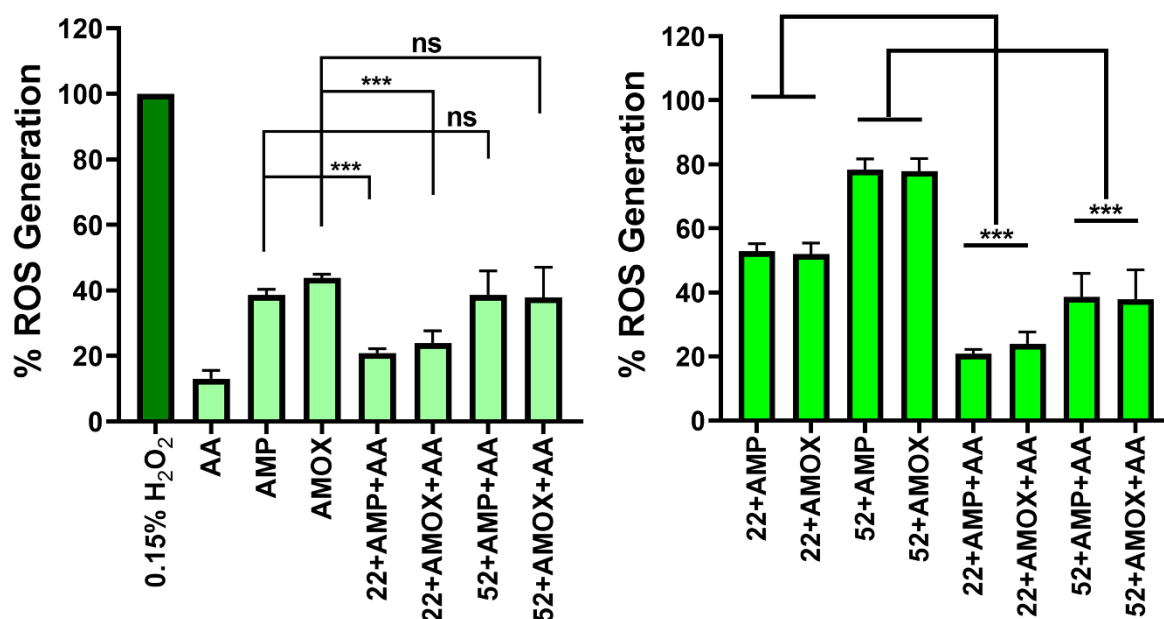


Figure 6.19: (left) The decrease in cellular ROS in the AMP/AMOX + ascorbic acid (AA, 40 μ M) treated cells compared to only AMP/AMOX treated MRSA; (right) Reduction in cellular ROS in AMP/AMOX + SM + AA combinations compared to AMP/AMOX + SM; p -value < 0.001= ***, ns = not significant.

When the comparison was made between SM + AMP/ AMOX and SM + AMP/ AMOX + AA treated cells, the drop in cellular ROS was clearly observable due to the antioxidant action of AA. For both SG-B **22** and SG-B **52**, their AMP/ AMOX combinations yielded significantly higher cellular ROS ($p < 0.001$ = ***) compared to the SM + AMP/ AMOX + AA treated cells.

ROS has been shown to possess protective functions in bacteria at low cellular concentrations but can become lethal at high levels.³⁰ Thus, we believe that this increase in the levels of ROS on treatment with the SM+antibiotic combinations must have a role in the accentuation of bacterial killing, compared to independent SM or beta-lactam treatment. When comparing the ROS elicitation by SG-B **22** and SG-B **52**, the AMP/ AMOX combinations of the latter instigated significantly more ROS generation ($p < 0.001$) than the SG-B **22**+AMP/ AMOX combinations, hinting at a higher cellular lethality. It has been shown previously that beta-lactam antibiotics like ampicillin can elicit ROS in *E. coli* by a downstream process involving the binding of the ampicillin molecule with its targets. i.e. the PBPs.³¹ We believe that, SG-B **22** and SG-B **52** destabilises the MRSA membranes and cell walls, facilitating the binding of AMP/ AMOX to their target PBPs by a yet unknown mechanism. This leads to the generation of generous amounts of endogenous ROS, contributing significantly to the biocidal outcome. The exact mode of action of AMP/ AMOX post-potentialiation by the adjuvant molecules SG-B **22** and SG-B **52** is an interesting area of research and needs further exploration.

6.3.6 Molecules SG-B 22/ SG-B 52 coupled with AMP/AMOX disrupt new and established MRSA biofilms, prevent biofilm formation, and kills biofilm embedded cells

The use of tuned amphipathic or amphiphilic cationic small molecules is not new in anti-biofilm applications.³² The primary aim is to inhibit the colonization of mammalian cell surfaces by the biofilm-forming pathogens for the prevention of potentially fatal diseases like cystic fibrosis, endocarditis, dermal infections, and periodontitis.^{33,34} This attribute can be particularly useful in disinfecting nosocomial abiotic surfaces like medical implants and catheters.³⁵

A broad variety of surface translocation modalities enable bacteria to locomote on solid surfaces via the flagellum-independent sliding process.³⁶ This type of motility is presumably crucial to the propensity of gram-positive and non-flagellated organisms like MRSA to colonize surfaces in both the host and the environment, resulting in the formation of biofilms.³⁷ Here, the treated and untreated cultures of MRSA with AMP, AMOX, SG-B 22, SG-B 52, and the SM+antibiotic combinations were spotted on MHA plates, followed by incubation for 48h at 37°C. The sliding motility of MRSA treated with SG-B 22+AMP, SG-B 22+AMOX, SG-B 52+AMP, and SG-B 52+AMOX was dramatically decreased, as evident from the colony diameters, in comparison to the untreated control (Figure 6.20). The untreated control's colony size and shapes were larger and nearly spherical whereas the combinatorial treatment sets of the SMs and the antibiotics yielded smaller and deformed colonies (Table 6.4). The colonies of the AMP, AMOX, SG-B 22, and SG-B 52 showed near-similar colony appearances, indicating unaltered sliding motility, as compared with the untreated controls.

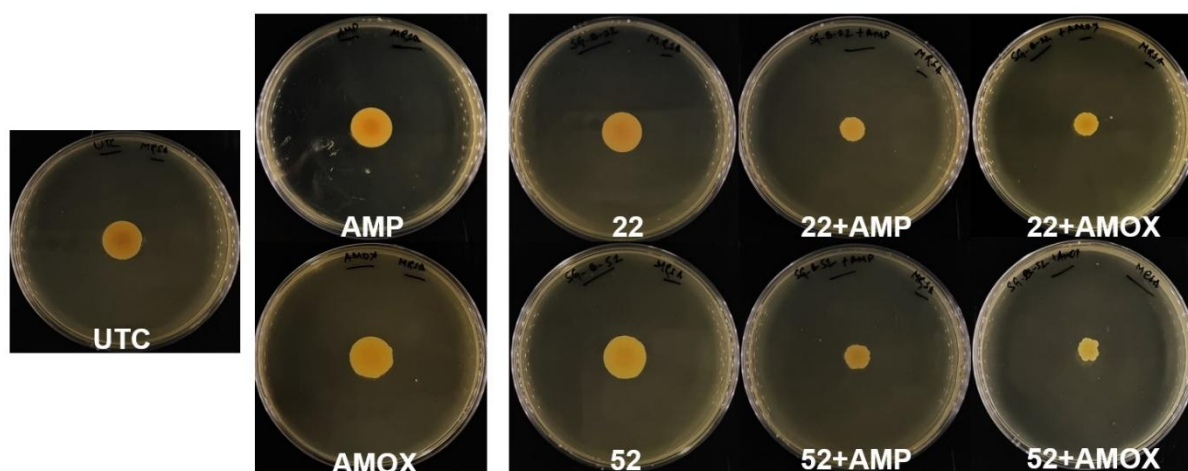


Figure 6.20: Pictorial representation showing the effect of AMP, AMOX, SG-B-22, SG-B-52, and the SM+antibiotic combinations on the sliding motility of MRSA along with the untreated control. An alteration in the sizes of the colonies was noted with the application of the SM+antibiotic combinations, compared to the SM (only) or antibiotic (only) treated samples; UTC = untreated control.

The results of the CV assay showed that compared to only AMP, AMOX, SG-B 22, and SG-B 52 treated cells, there were insignificant differences in percent biofilm inhibition for 1/4EC and 1/2EC of the AMP/AMOX + SG-B 22 and SG-B 52 combinations ($p > 0.05$) (Figure 6.22 B). However, when the treatment concentration was increased to EC of the respective SMs and antibiotics, a dramatic rise in percent biofilm inhibition was observed. For the SG-B 22 + AMP and SG-B 22 + AMOX combinations, the percentages were observed to be 47.3% and 72.8% respectively whereas for the SG-B 52 + AMP and SG-B 52 + AMOX combinations, the inhibition percentages were 34.8% and 91.5% ($p < 0.001$).

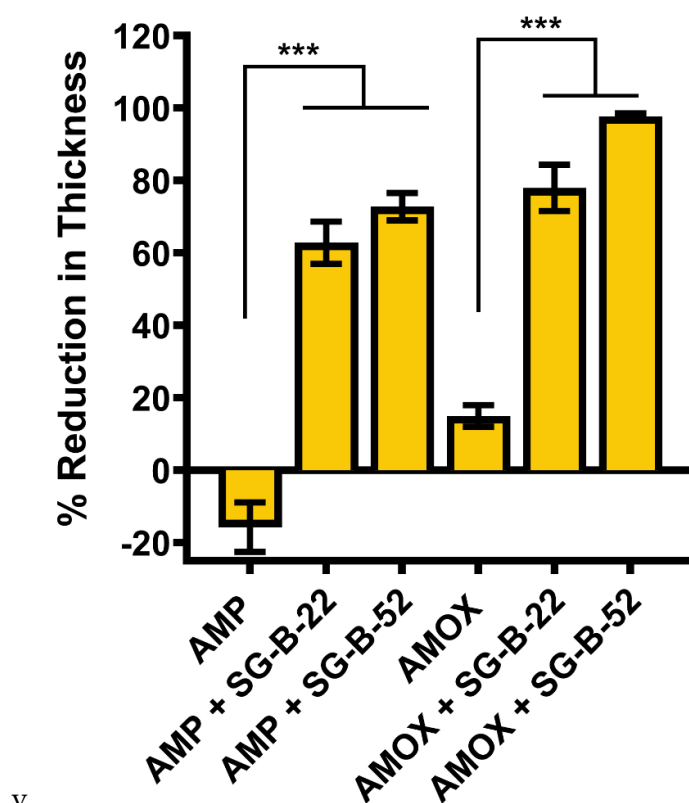


Figure 6.21: Percent (%) reduction in thickness of biofilm matrix when treated with AMP, AMOX, and the SM+AMP/AMOX combinations as compared with untreated biofilms; The graph has been plotted based on the AFM data; p -value < 0.001 = ***.

Experiment	Mean Colony Diameter (mm)								
	UTC	AMP	AMOX	SG-B-22	SG-B-52	SG-B 22+AMP	SG-B 22+AMOX	SG-B 52+AMP	SG-B 52+AMOX
Sliding Motility Assay	18.2	18.1	18.5	18.2	18.6	7.7	7.4	7.9	7.1

Table 6.4: Bacterial colony diameters obtained from the sliding motility assay of MRSA in the presence of AMP, AMOX, SG-B-22, SG-B-52, and the SM + antibiotic combinations. UTC: Untreated Control.

For 24h grown MRSA biofilms, the effect of SG-B 22 and its AMP/ AMOX combinations was negligible as they possessed no biofilm clearance attribute whatsoever (Figure 6.22 C) with insignificant differences in mean percent biofilm eradication ($p > 0.05$) between SG-B 22 alone, AMP (only), AMOX (only), and the SM+antibiotic combinations. The data suggests that SG-B 52 and its AMP/AMOX combinations were capable of eradicating pre-formed mature biofilms of MRSA.

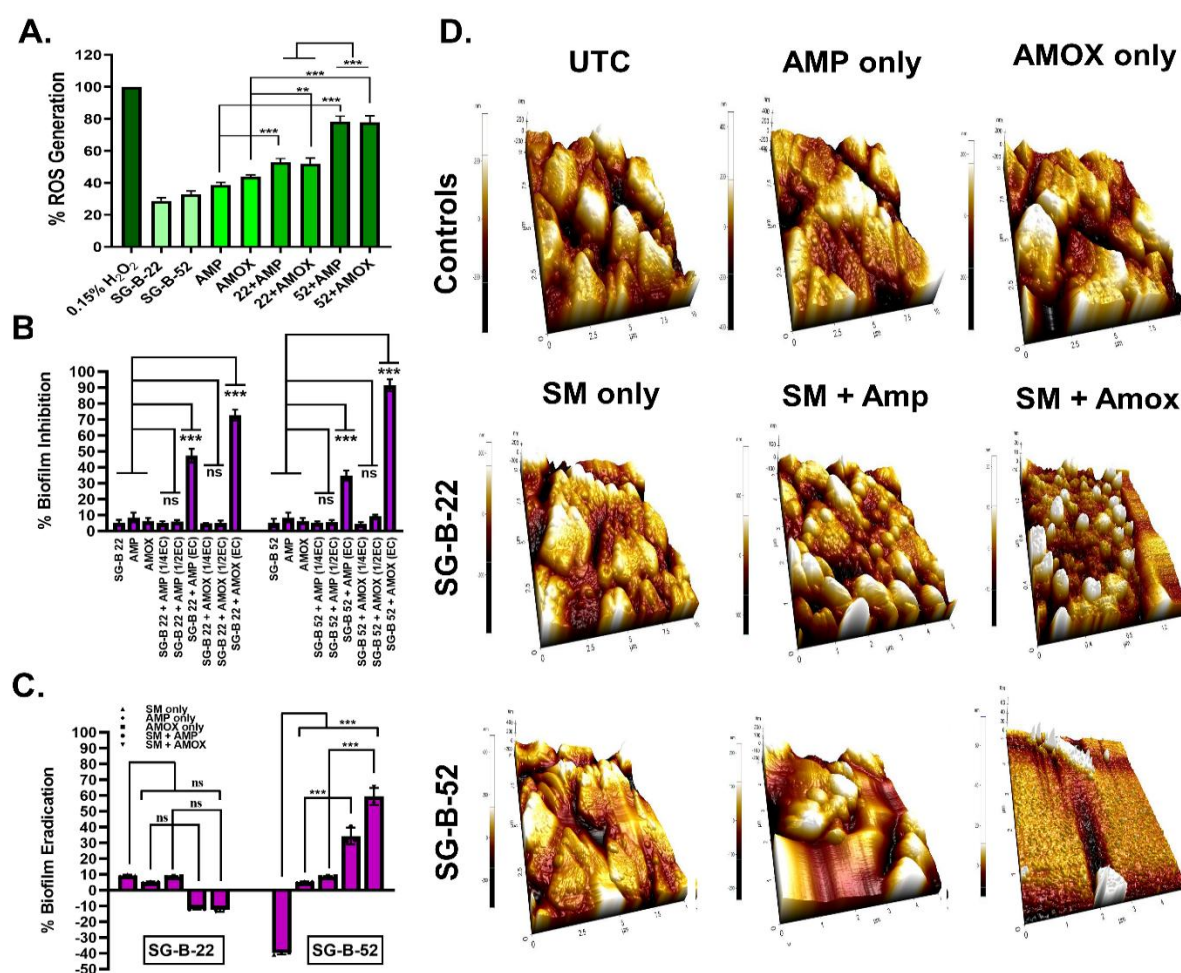


Figure 6.22: ROS generation and anti-biofilm activity: A. Percent (%) ROS generated by AMP (25 μ M), AMOX (25 μ M), SG-B-22 (50 μ M), SG-B-52 (25 μ M), and SM+antibiotic combinations (EC in μ M: for SG-B 22 + AMP: 50 + 25; SG-B 22 + AMOX: 50 + 25; SG-B 52 + AMP: 25 + 12.5; SG-B 52 + AMOX: 25 + 12.5) treated MRSA cells (4h) by measurement of the fluorescence intensity of DCFH-DA emission; 0.15% H₂O₂ was used as positive control; B. CV assay to quantify the biofilm inhibition potential of AMP, AMOX, SG-B-22, SG-B-52, and SM+antibiotic combinations at EC, 1/2EC, and 1/4EC against neoformative MRSA biofilms, expressed as percent (%) biofilm inhibition with respect to the untreated controls; C. CV assay to quantify the biofilm eradication potential of AMP, AMOX, SG-B-22, SG-B-52, and SM+antibiotic combinations against 24h mature MRSA biofilms, expressed as percent (%) biofilm eradication with respect to the untreated controls; C. Atomic Force Micrographs of neoformative biofilms (inhibition of new biofilm formation) of MRSA treated with AMP, AMOX, SG-B-22, SG-B-52, and SM+antibiotic combinations at concentrations mentioned in the ROS generation assay; UTC = untreated control; p -value < 0.01= **, p -value < 0.001= ***, ns = not significant.

Compared to the individual administrations of SG-B **52**, AMP, and AMOX, the combinations showed statistically notable biofilm eradication trends with 34.4% (SG-B-52+AMP) and 59.5% (SG-B-52+AMOX) ($p < 0.001$). For biofilm neoformation, the atomic force micrographs revealed thick, compact EPS matrices with embedded MRSA cells in the untreated control, AMP, AMOX, SG-B **22**, and SG-B **52** treated samples (Figure 6.22 D), indicating minimum interference of these molecules towards the establishment of new MRSA biofilms. When treated in combination, however, SG-B **22**+AMP/AMOX caused visible clearance of the biofilm matrices, exposing the MRSA cells. The AFM micrographs provided intangible evidence that significant matrix thickness was reduced for the SM + AMP/AMOX combinations (Figure 6.21) compared to AMP/AMOX alone ($p < 0.001$). For AMP, on synergistic application with SG-B **22**, a 62.8% reduction in biofilm thickness was noted (compared to only AMP-treated biofilms at -15.7%), whereas for AMP + SG-B **52** treated biofilms the reduction was 72.8%. When AMOX was co-treated with SG-B **22** and SG-B **52**, the percent reduction in thickness was found to be 78% and 97.7% respectively as opposed to 15% for only AMOX. The best anti-neoformative effect was observed for the SG-B **52**+AMP/AMOX combinations. When co-administered with AMP, the biofilm matrices appeared dispersed with scanty zones of EPS. Along with AMOX, SG-B **52** completely inhibited the formation of MRSA biofilms showing excellent efficiency.

The ability of pathogenic strains of MRSA to colonize a range of substrata depends on a variety of virulence attributes, one of which is the ability to adhere to hydrophobic surfaces via hydrophobic surface proteins.³⁸ Compared to the SMs and AMP/AMOX alone, the combinatorial treatment of the SMs with AMP/AMOX yielded significantly greater inhibition of cell surface hydrophobicity (Figure 6.23) with p values < 0.001 . A simple visual evaluation of the data suggested that SG-B **52** acted as a better inhibitor of cell surface hydrophobicity of MRSA compared to SG-B-**22** in combination with AMP and AMOX.

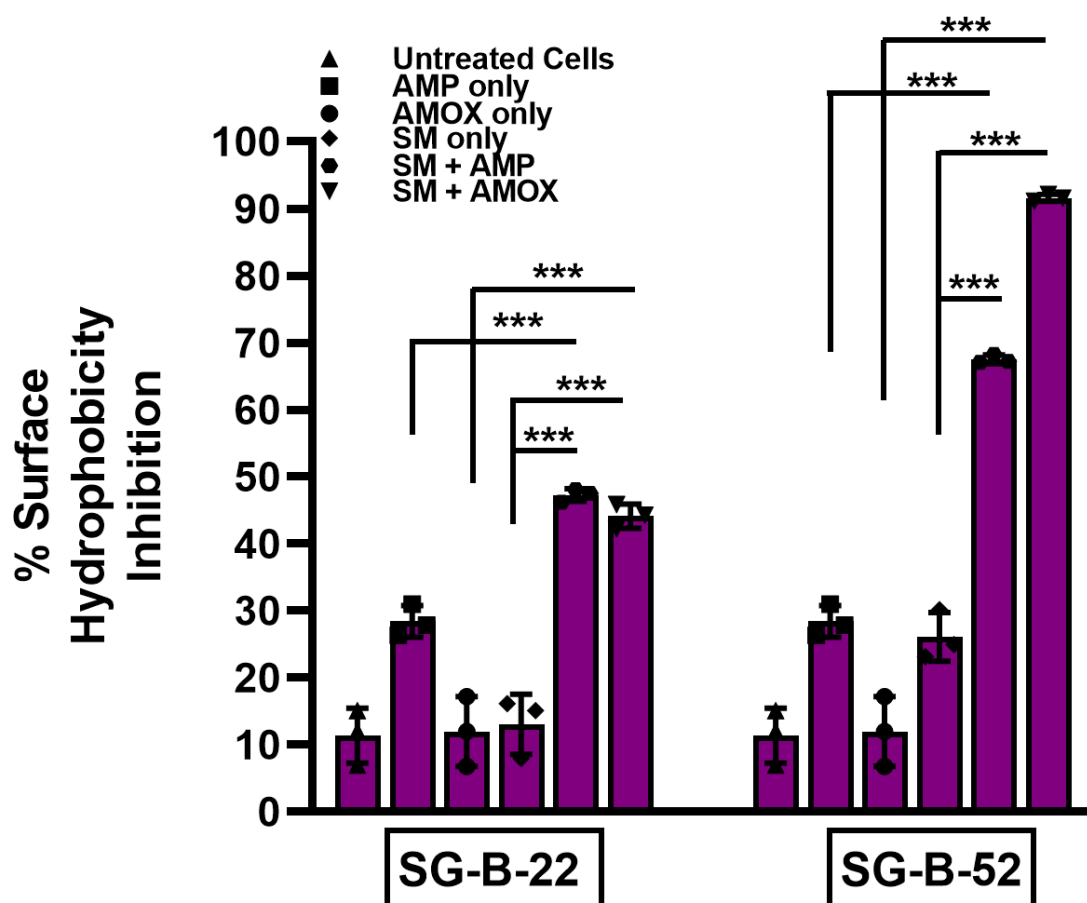


Figure 6.23: Effect of SG-B-22, SG-B-52, AMP, AMOX, and the SM + antibiotic combinations on cell surface hydrophobicity of MRSA as determined by MATH assay; p -value < 0.001 = ***.

Auto-aggregation refers to bacterial cells settling on solid surfaces that stimulate biofilm development. Proteins like Bap and Bap-related proteins have been reported to play a major role in biofilm development, even in the absence of biofilm-instigating cellular substances like polysaccharide intercellular antigen or PIA.³⁹ The bacterial biofilm is reinforced on solid surfaces through the adhesion of neighboring cells. The potential of inhibiting such auto-aggregation by individual and synergistic efforts of SG-B-22 and SG-B 52 was observed along with AMP/AMOX. As we know, scattered and non-aggregated cells would register a higher OD₆₀₀ compared to the aggregated cells, which would settle at the bottom, leaving clear media at the top. It was observed that both the SMs alone had no anti-aggregating tendencies whatsoever, whereas the antibiotics AMP and AMOX prevented nominal aggregation of the cells (Figure 6.24). When the SMs and the antibiotics were combined (EC), there was a significant increase in the OD₆₀₀, indicating a high number of dispersed cells ($p < 0.001$). The anti-aggregation effects of the SG-B 52+AMP/AMOX combinations were much superior compared to SG-B 22+AMP/AMOX as evident from the higher OD₆₀₀ values.

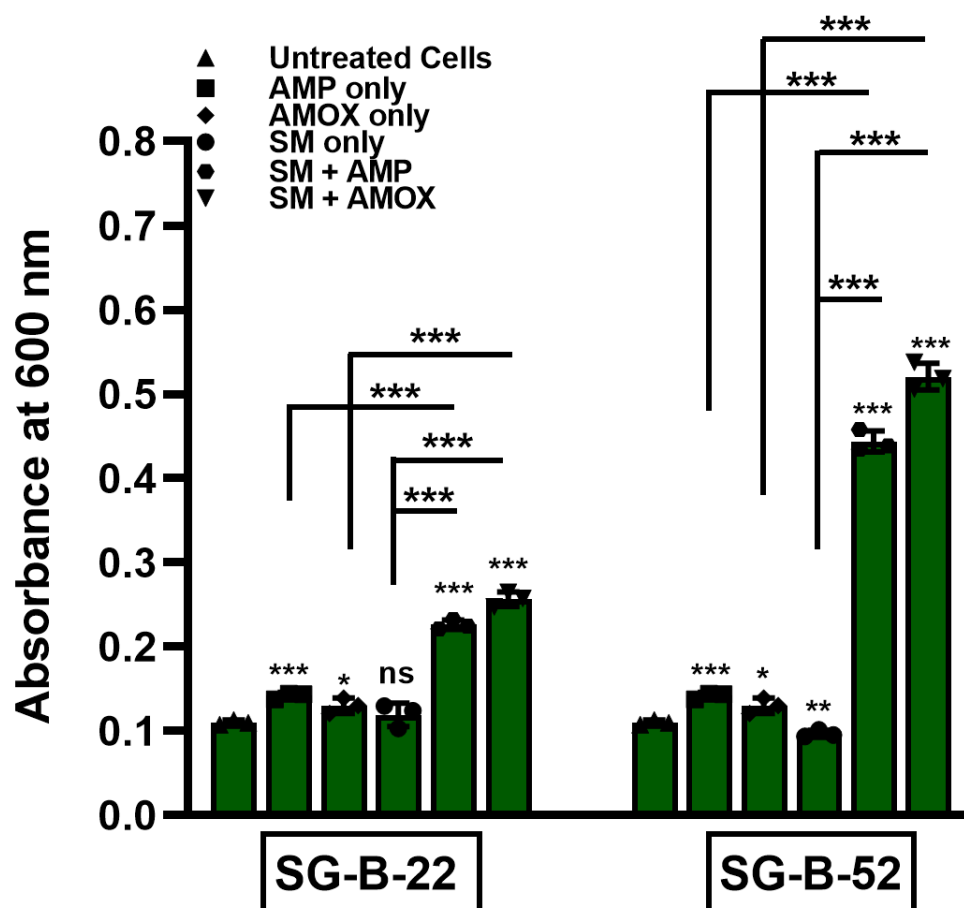


Figure 6.24: Determination of efficacy of SG-B-22, SG-B-52, AMP, AMOX, and the SM + antibiotic combinations in prevention of auto-aggregation of MRSA cells; the auto-aggregation potential of MRSA cells was determined as a measure of the absorbance value at 600 nm concerning the untreated control; data comparisons were also made between the antibiotics/SMs and their combinations; p -value < 0.05 = *, p -value < 0.01 = **, p -value < 0.001 = ***, ns = not significant.

Slime Formation is yet another trait that confers MRSA the ability to adhere to an array of biotic and abiotic surfaces. The deposition of the Congo red dye on the slime of prolific biofilm producers renders the colonies a characteristic blackish-red colour whereas the inhibition of slime formation is indicated by a Bordeaux red plate, comprising transparent MRSA colonies. In figure 6.25A, UTC, AMP, AMOX, SG-B 22, and SG-B 52 plates displayed the typical blackish-red appearance of the MRSA colonies, indicating slime generation and minimal antimicrobial action. Even in the SG-B 22+AMP, SG-B 22+AMOX, and SG-B 52+AMP plates, some Congo red uptake was evident. However, a perfect Bordeaux red plate was obtained for the SG-B 52+AMOX combination, clearly demonstrating that SG-B 52 in combination with AMOX was the most efficient concoction in preventing slime formation by MRSA.

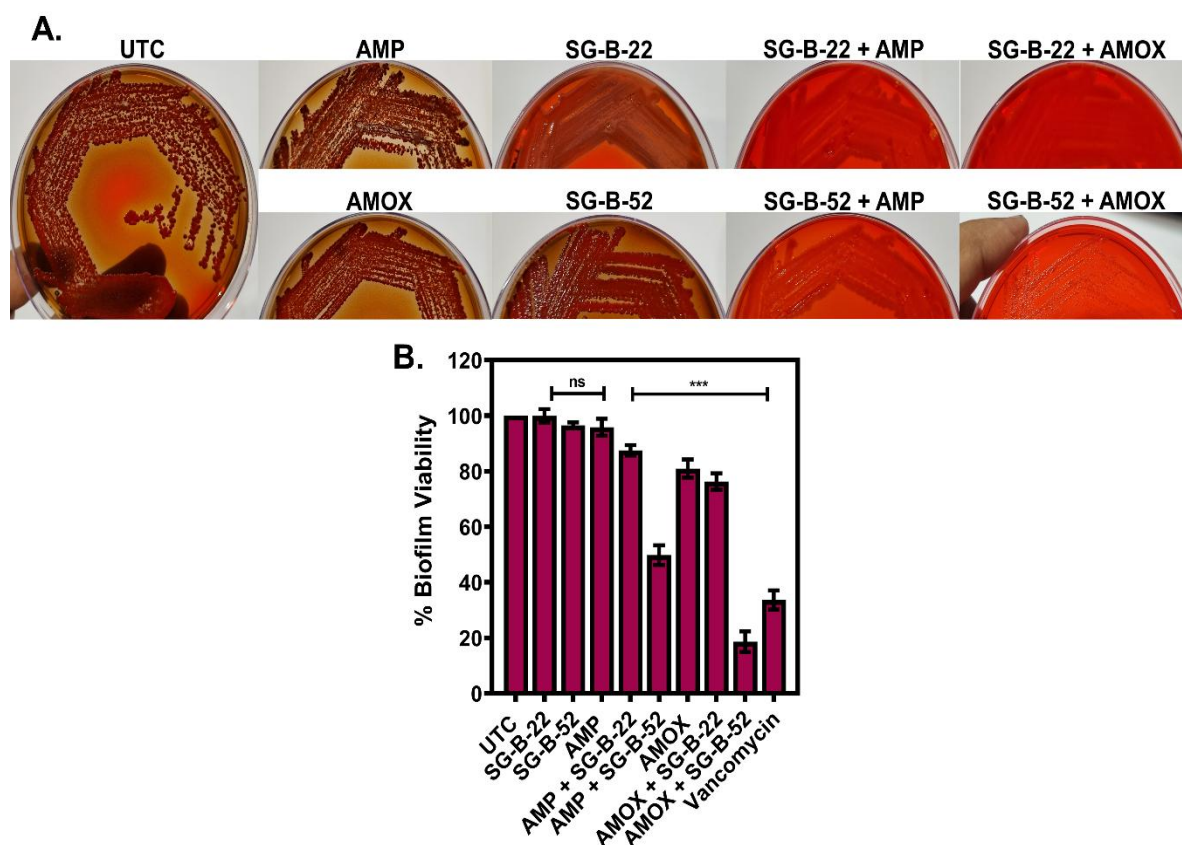


Figure 6.25: Assessment of slime production and bacterial viability of biofilm embedded cells: A. Qualitative determination of slime production of treated and untreated MRSA cells by 0.08% Congo red (w/v) agar plating assay; the gradual change in colour of the colonies from blackish red to Bordeaux red specifies the reduction in slime production in SG-B-22, SG-B-52, AMP, AMOX, and the SM + antibiotic combinations treated MRSA cells; B. MTT assay to determine the percent bacterial viability of biofilm embedded MRSA cells on treatment with SG-B-22, SG-B-52, AMP, AMOX, and the SM + antibiotic combinations; p -value < 0.001= ***, ns = not significant.

The biofilm viability assay indicated that the combinatorial treatment of the SMs with AMP/AMOX caused a considerable reduction in the viability of the matrix-embedded MRSA cells (Figure 6.25B). The SMs and AMP alone failed to show any significant killing of the biofilm cells whereas AMOX was found to kill ~20% of the cells alone. When used in combination, however, all four antibiotics + SM combinations significantly killed bacteria embedded in the biofilms (p -value < 0.001= ***). Among the combinations, the lowest biofilm viability was noted for AMOX + SG-B 52 at 18.6% (which was lower than the positive control vancomycin (33.7%)) and the highest was observed for AMP + SG-B 22 at 87.5%. The percent biofilm viabilities for AMP + SG-B 52 and AMOX + SG-B 22 were observed to be 49.8% and 76.3% respectively.

Despite having impressive antimicrobial traits, the failure to clear mature MRSA biofilms made the further study of SG-B 22 inconsequential. Even when the percent biofilm viabilities were compared, SG-B 52 easily outperformed SG-B 22. It has been reported in the literature that amphipathic peptidomimetic molecules with lipid tails can form micelles above a certain concentration and can then bind to polyanionic exopolysaccharide matrices of bacterial

biofilms, resulting in their penetration and eradication ⁴⁰. As the overall structures of SG-B 22 and SG-B 52 are identical except for the size of their hydrophobic tails (7C tail for SG-B 22 and 11C tail for SG-B 52), we believe that the potential for micellar structure formation is superior in SG-B 52 compared to SG-B 22, resulting in better biofilm penetration and eradication. It is the most probable explanation of the obtained results in the biofilm eradication studies. Nevertheless, further studies are warranted to substantiate the claims. These results were crucial as biofilms constitute a vital virulence trait of MRSA, *in vivo*.⁴¹ The outcome of the anti-biofilm assays helped us narrow our focus toward SG-B 52 for the animal studies to follow.

6.3.7 Molecule SG-B 52 is found to be stable in human plasma:

Before selecting an appropriate animal model to assess the *in vivo* antibacterial properties of SG-B 52, a thorough evaluation was conducted to determine its serum stability. The presence of several hydrolytic enzymes, including lipases, cholinesterases, and phosphatases within the human blood plasma makes the molecules susceptible to hydrolysis. This results in a swift reduction of their plasma concentration, thus limiting their therapeutic effectiveness.⁴² The serum stability of SG-B 52 was assessed by conducting an assay over a 3h period. The results indicated that the molecule was entirely unaltered at the start of the test. After 3h, however, a decent 40% of the compound remained unchanged in human serum. The assessment also revealed that 72.5%, 60.6%, and 52.8% of the initial concentration of the molecule was still present and unaltered at the 0.5h, 1h, and 1.5h time points, respectively (Figure S36), indicating that degradation occurred progressively over time. The RP-HPLC purified fractions collected at 0h and 3h underwent further analysis using ESI-LC/MS, and their masses were found to be consistent with SG-B 52 (Figure 6.26).

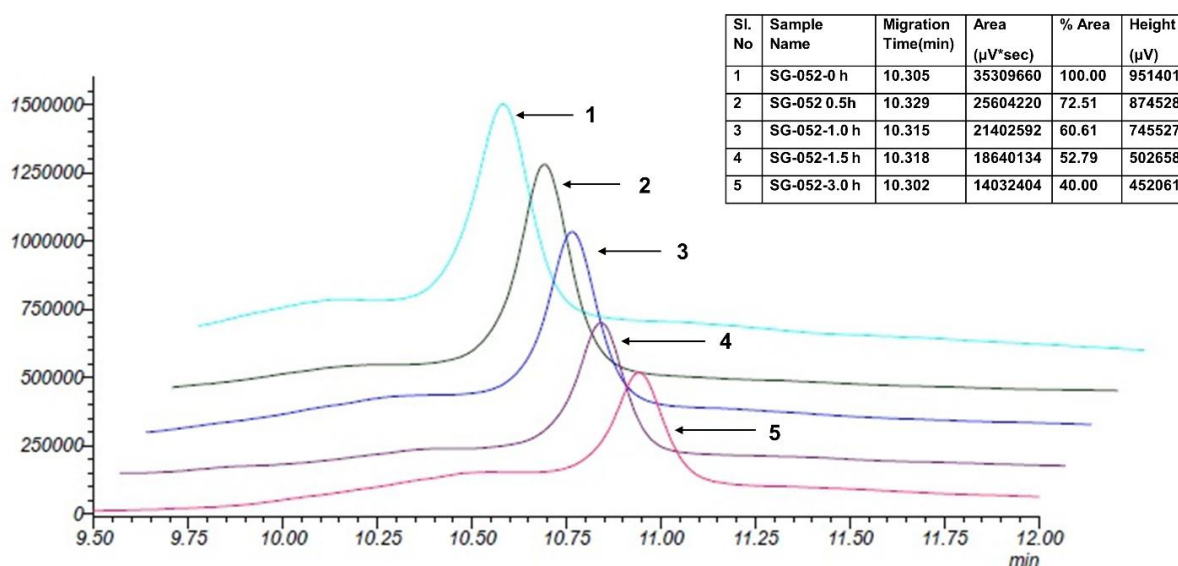


Figure 6.26: The serum stability profile of SG-B-52 in 25 % human serum (in PBS); the amount of molecule remaining after incubation at different time intervals (0h, 0.5h, 1h, 1.5h, and 3h) was quantified by HPLC; the percent molecule remaining at each time point was determined by integrating the peak area related to the molecule's chromatographic profile at t = 0h.

In Vivo Tolerance, Organ Loading, and Histopathological Analysis:

Based on the results obtained in the study *in vitro*, the *in vivo* investigations followed (Figure 10A). The status and survival rates of MRSA-inoculated albino Wistar rats were monitored for 7 consecutive days to determine the preliminary toxicity of SG-B 52 and SG-B 52+AMP/AMOX, *in vivo*. SG-B 52 (20 and 10 mg kg⁻¹) and SG-B 52+AMP/AMOX combinations (10 mg kg⁻¹+10 mg kg⁻¹) were injected intraperitoneally (IP) into adult albino

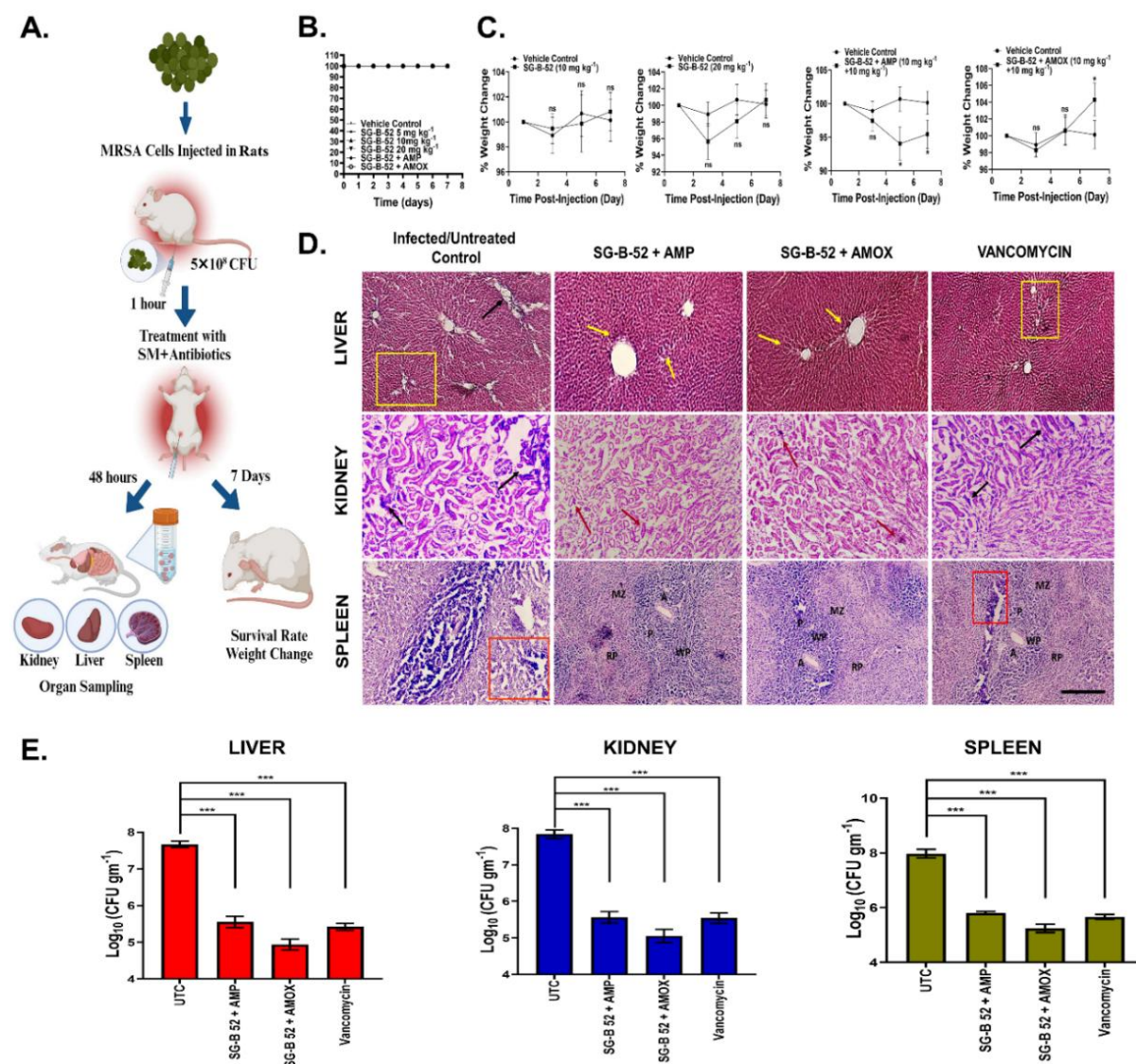


Figure 6.27: Effect of SG-B-52+AMP/AMOX on albino Wistar rats: A. Schematic illustration depicting the *In vivo* studies; B. Percent (%) survival of albino Wistar rats after administration of 10/20 mg kg⁻¹ SG-B-52, SG-B-52+AMP/AMOX (10+10 mg kg⁻¹) observed over a 7-day time frame compared with the vehicle control; C. Percent (%) weight change observed in albino Wistar rats after administration of 10/20 mg kg⁻¹ SG-B-52, SG-B-52+AMP/AMOX (10+10 mg kg⁻¹) as well as the positive control vancomycin (5 mg kg⁻¹) observed over a 7-day time frame compared with the vehicle control; D. *In vivo* antibacterial activity of SG-B-52 in combination with AMP/AMOX based on hematoxylin and eosin (H&E) staining (visualized at 100x) of livers, kidneys and

spleens of MRSA inoculated rats treated with SG-B-52+AMP and SG-B-52+AMOX (compared with untreated control animals); albino Wistar rats were inoculated with the clinical MRSA isolate ($\sim 5 \times 10^8$ CFU mL⁻¹) by intraperitoneal injection (only saline injected for the blank group) and then treated with SG-B-52+AMP/AMOX (10 mg kg⁻¹+10 mg kg⁻¹) and vancomycin (5 mg kg⁻¹) after 30 min; yellow boxes indicates region of localised necrosis; black arrows indicate heavy polymorphonuclear and mononuclear inflammatory cell infiltration; yellow and maroon arrows indicate moderate polymorphonuclear and mononuclear inflammatory cell infiltration and Kupffer cell activation; red boxes indicate hyaline deposits in the tissue; A = central artery; MZ = marginal zone; P = peri-arteriolar lymphocyte sheath; RP = red pulp; WP = white pulp; E. Bacterial load in the liver, spleen, and kidneys of MRSA-infected albino Wistar rats treated with SG-B 52 + AMP (10+10 mg kg⁻¹), SG-B 52 + AMOX (10+10 mg kg⁻¹) and vancomycin (5 mg kg⁻¹) compared with untreated controls (UTC); Scale bar = 50 μ m. *p*-value < 0.05 = *, *p*-value < 0.001=***, ns = not significant.

Wistar rats. Over the 7-day observation period, all the rats (including the vehicle controls) were in good health and no anomalies were seen. The survival rate of the rats at the tested concentrations was 100% after IP injection of SG-B 52 and SG-B 52+AMP/ AMOX at the specified dosages (Figure 10B). It must be mentioned here that the percent weight change profiles showed that rats treated with SG-B 52+AMP lost significant weight post-injection, evident from the 5-day time point onwards, whereas, rats treated with SG-B 52+AMOX gained weight at the 7-day time point (Figure 10C). The animals did not show any sign of stomach distress or altered food/grooming habits during the study. None of the animals showed elevated body temperature/fever. The data and observations demonstrate that SG-B 52 and SG-B 52+AMP/ AMOX had minimal *in vivo* toxicity.

The liver sections of the MRSA untreated/infected control animals showed regions of heavy polymorphonuclear and mononuclear inflammatory cell infiltration. Areas of hepatocellular necrosis were evident in the livers of the untreated and vancomycin-treated animals. Moderate inflammatory cell infiltration and Kupffer cell activation were observed in the liver sections of the SG-B 52+AMP and SG-B 52+AMOX-treated animals. The SG-B 52+AMP and SG-B 52+AMOX animals showed normal lobule architecture of the liver along with a prominent, non-constricted central vein surrounded by healthy hepatocytes. The increased sinusoidal space in MRSA-infected rat sections indicates severe liver damage whereas the morphology of the SG-B 52+AMP, SG-B 52+AMOX, and vancomycin-treated rat liver sections signifies good organ health.

While observing the H&E stained kidney sections of the untreated/infected control and vancomycin-treated rats, a very high inflammatory cell infiltration was noted, whereas, moderate infiltration was observed for the SG-B 52+AMP and SG-B 52+AMOX treated sections. The untreated/infected control and vancomycin-treated sections showed abnormal glomerular and tubular histology, clearly signifying MRSA-induced tissue, and organ damage. In contrast, the renal parenchyma in the SG-B 52+AMP and SG-B 52+AMOX treated rat kidney sections looked comparatively unaffected by the MRSA invasion. The moderate infiltration of interstitial peritubular mononuclear inflammatory cells signifies good pathogen clearance as well as controlled inflammation in the kidneys, considerably reducing the risks of organ damage.

The spleen sections of the SG-B 52+AMP, SG-B 52+AMOX, and vancomycin-treated rats showed a decently organized white pulp with an easily discernible peri-arteriolar lymphocyte sheath, germinal center, mantle zone, and marginal zone. On the contrary, the untreated/infected sections showed a white pulp consisting of hyperplastic, reactive

lymphoid follicles with a very high number of tissue macrophages, that can be considered as definitive indications of organ sepsis.⁴² The central artery in the infected/untreated sections looked heavily distorted and was nearly indiscernible from its surroundings. The hyaline deposits were also prominent in the untreated and vancomycin-treated sections.

Next, we proceeded to assess its effectiveness in a live animal model of sepsis using rats infected with the clinical MRSA strain.⁴³ An intraperitoneal injection of a sublethal dose (5×10^8 CFU mL⁻¹) of MRSA was administered to albino Wistar rats. 30 min after infection, a combination of molecule SG-B 52 with AMP/AMOX ($10 \text{ mg kg}^{-1} + 10 \text{ mg kg}^{-1}$) was administered to the rats. The reference drug utilized in this study was vancomycin at a dosage of 5 mg kg^{-1} . After a duration of 48h, it was observed that the septic rats treated with SG-B 52+AMP, SG-B 52+AMOX, and vancomycin demonstrated a noteworthy decrease in the bacterial load of each organ when compared to the untreated control group ($p < 0.001$) (Figure 10E). The effectivity of the SG-B 52 + AMP combination was found to be like that of the drug control vancomycin whereas the clearance shown by the SG-B 52 + AMOX combination was observed to be better than the control drug.

In summarizing the *in vivo* efficacy of SG-B 52, it must be acknowledged that at the specified combinatorial dosage of the molecule with AMP and AMOX ($10 + 10 \text{ mg kg}^{-1}$), the therapeutic outcome was better than the commercially available antibiotic vancomycin (5 mg kg^{-1}). Thus, molecule SG-B 52 demands further research inquest for its development as an anti-MRSA therapeutic, and investigations of its combinatorial effects with other antibiotics are warranted.

6.4 Materials and Methods:

6.4.1 Synthesis of Quinoline-Derived Small Molecules:

General Procedure for the synthesis of 2,3-dialkyl-6-bromoquinolines 2, Scheme1, Step a:

4-bromo aniline (1g) was dissolved in DMSO, followed by the addition of Aldehyde (2.5 eq) and 4M HCl in dioxane (0.05 eq) and allowed to stir under air at 60°C for 4-5h. After consumption of the starting material as monitored by TLC, the reaction mixture was diluted with saturated NaHCO₃ and H₂O and was later extracted by ethyl acetate (3×20 mL), and combined organic extracts were dried over anhydrous sodium sulphate and evaporated under reduced pressure to give the crude product. Purification of the crude product was performed by silica-gel column chromatography to get the desired compound 2 in (50-60) % yield at (0-4) % ethyl acetate/hexane.⁴⁴

General Procedure for 2,3-dialkylquinoline-6-carboxylic acids 3, Scheme 1, Step b:

The purified product 2 (1 eq) obtained in step 1 was taken in an oven-dried sealed tube, dissolved in dry DMF (3 mL), and allowed to stir for 5 min. Next, the reaction vial was charged with Palladium acetate (0.03 eq), X-phos (0.03 eq), and triethylamine (2 eq), and the reaction mixture was allowed to degas under a nitrogen atmosphere for 15 min. Next Formic acid (7 eq) was added followed by the addition of DIC (0.2 eq) and allowed to heat at 120°C for the next 18h. After consumption of the starting material as monitored by TLC, the reaction mass

was filtered over a celite bed, diluted with cold water, and extracted with ethyl acetate (2×30 mL). The combined organic extracts were dried over sodium sulphate and evaporated under reduced pressure to give the crude compound. Purification of the crude was performed by silica gel column chromatography to get the desired compound **3** in (50-60) % yield at 50-60% ethyl acetate/hexane.⁴⁵

General Procedure for 2,3-Dialkyl-N-hydroxyquinoline-6-carboxamide 4, Scheme1, step c:

Substituted acid derivatives **3** (1 eq) as obtained from the previous step were dissolved in dry DMF (3 mL) followed by the addition of DIPEA (2 eq) and HATU (1.5 eq) and allowed to stir at room temperature for 30 min. After activation of acid, NH₂OH.HCl (2 eq) was added and the reaction mixture was allowed to stir at RT overnight. After consumption of the starting material as monitored by TLC, the reaction mass was diluted with ice-cold water, and extracted with (3×30) mL of ethyl acetate. The combined organic extracts were dried over anhydrous sodium sulphate and evaporated under reduced pressure to get the crude compound. Later, the column purification was performed by silica gel column chromatography to get the desired compound **4** in (60-65) % yield at 60-70% ethyl acetate/hexane.⁴⁶

General Procedure for tert-butyl (2,3-dialkylquinoline-6-carboxamido) ethyl) carbamate 5, Scheme1, step d:

Substituted acid derivatives **4** (1 eq) as obtained from the previous step were dissolved in dry DMF (3 mL) followed by the addition of DIPEA (2 eq) and HATU (1.5 eq) and allowed to stir for 30 min at RT. After activation of acid, N-Boc-ethylenediamine (2 eq) was added and the reaction mass was allowed to stir at RT overnight. After consumption of the starting material as monitored by TLC, the reaction mass was diluted with ice-cold water, and the organic layer was extracted with (3×30) mL of ethyl acetate. The organic layer was dried over anhydrous sodium sulphate and dried under reduced pressure to get the crude compound. Later, the column purification was performed by silica gel column chromatography to get the desired compound **5** in (60-70) % yield at 60-70% ethyl acetate/hexane.⁴⁶

General Procedure for N-(2-aminoethyl)-2,3-dialkylquinoline-6-carboxamides 6, Scheme1, step e:

The N-Boc derivatives **5** (1 eq) were dissolved in DCM (2 mL) cooled at 0°C. Then TFA (200 µL) was added slowly to the reaction mass under stirring conditions at the same temperature (0°C). The ice bath was removed and the reaction mixture was allowed to stir at RT for 4h. After consumption of the starting material as confirmed by TLC, the reaction mass was triturated several times using DCM and evaporated under reduced pressure to completely remove TFA. The residue was then washed with diethyl ether and dried under a high vacuum to obtain a pale-yellow solid as the desired final product **6** (65-70) % yield which was used without further purification.⁴⁷

General procedure for 4- (tert-butyl/ trifluoromethyl) benzoic acid 8, Step a Scheme 2:

4-trifluoro/trimethyl substituted benzaldehydes **7** (1 eq) was dissolved in DMF, followed by the addition of oxone (3 eq), and allowed to stir at RT overnight. After completion of starting material as monitored by TLC, the reaction mixture was diluted with 1N HCl to dissolve the salts and extracted with ethyl acetate (2x30 mL). The organic layer was washed again with 1N HCl to remove any residual salt. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure to afford the title compound **8** in (60-70) % yield.⁴⁸

General procedure for the synthesis of 9a-b, 10a-b, 11a-b Scheme 2, Step b-d:

9a-b was synthesized following the same procedure as step c of Scheme 1, and the products were obtained in 70% (**9a**) and 73% (**9b**) yield. **11a-b** was obtained following the same procedure from **10a-b** as step e of scheme 1. The products were obtained in 62% (**11a**) and 64% (**11b**) yield. The synthesized molecules were purified using RP-HPLC (Shimadzu LC- 20AP, Japan). The molecular masses of the molecules were determined using ESI-LC/MS (Agilent 6530, USA). The structural elucidation of the library was performed using ¹H and ¹³C NMR (Bruker-Avance, USA).

6.4.2 Bacterial Strains, Growth Conditions, and Preliminary Screening, MIC, and MBC of MRSA:

This study primarily employed a clinical pathogenic MDR MRSA strain obtained from AIIMS, Jodhpur along with 3 other MRSA strains (patient-derived). The *in vitro* antibacterial screening of the SM candidates was done against *Escherichia coli* (ATCC CRM- 11229) (EC), methicillin-resistant *Staphylococcus aureus* (patient-derived strains) (MRSA), *Pseudomonas aeruginosa* (patient-derived) (PA), and *Klebsiella pneumoniae* (ATCC BAA-1705) (KP). The bacterial growth media were purchased from Himedia, India. EC and PA were maintained in NB, supplemented with 1% each of glucose and NaCl whereas KP and MRSA were cultivated in TSB and BHI respectively (37°C was maintained for all). The antimicrobial assays were carried out in MHA/MHB (37°C, under moderate shaking where applicable).

Preliminarily, the molecules synthesized in the library were screened against EC, MRSA, PA, and KP employing a simple spotting assay. The organisms (5×10⁵ CFU) were treated with 50-400 µM of each molecule in the library, overnight, in flat-bottomed 96-well plates at 37°C under moderate shaking. Post-incubation, samples from each well were spotted onto fresh MHA plates and incubated further for 24h at 37°C.

The MIC of AMP/AMOX, as well as the screened molecules SG-B **22** and SG-B **52**, was determined against MRSA using a CLSI-approved microdilution protocol with a few modifications.⁴⁹ 5×10⁵ CFU mL⁻¹ MRSA was seeded in 96 well microtiter plates (Tarsons, India) and treated with a range of concentrations (400 – 10 µM) of AMP, AMOX, SG-B **22** and SG-B **52** along with 150 µL sterile MHB followed by incubation at 37°C for 24h (shaking at 80-120 rpm). After 24h the growth was measured (OD₆₀₀) in a microtiter plate reader. Adequate untreated controls (without antibiotic/SM) and negative controls (without antibiotic/SM and bacterial cells) were maintained for each experimental replication. Fresh MHA plates were inoculated using incubated broth from the wells of the plates from the MIC experiment, which

had no observable growth, and these plates were then incubated at 37°C for 24h to assess the MBC of the bacteria against AMP, AMOX, SG-B **22** and SG-B **52**. The concentration deemed to be the MBC was that which produced no discernible MRSA growth in the MHA plates post-incubation.

6.4.3 Synergy Checkerboard Assays:

Using the well-known checkerboard assays, the potentiation effectiveness of adjuvants with antibiotics was evaluated in MHB. In each well of a 96-well plate, a solution 25 μ L solution of AMP/AMOX (200 – 6.25 μ M) was placed along the abscissa and SG-B **22** and SG-B **52** (200 – 6.25 μ M) were added along the ordinate. Next, 150 μ L of MRSA suspension ($\sim 10^5$ CFU mL⁻¹) was added, and the mixture was incubated at 37°C for 18h–24h. Sterile MHB and bacterial suspension alone were used as the respective negative and positive growth controls. The assays were repeated twice. The effective concentrations of the SMs, as well as the antibiotics, were noted.

The calculation of the summation of the fractional inhibitory concentrations (Σ FICs) of each SM+antibiotic combination was performed by adding FIC 1 and FIC 2, where FIC 1 refers to the ratio of the minimum inhibitory concentration (MIC) of antibiotic in the combination to the MIC of antibiotic alone, and FIC 2 is the ratio of the MIC of SM in the combination to the MIC of SM alone. The combination is classified as synergistic when Σ FIC is less than or equal to 0.5, indifferent when Σ FIC is greater than 0.5 but less than 2, and antagonistic when Σ FIC is equal to or greater than 2.²¹

6.4.4 Antibacterial Activity

Growth Curves:

Log phase cultures of MRSA (in MHB) were treated with SG-B **22** (50 μ M) and SG-B **52** alone (25 μ M) or in combination with AMP/AMOX (EC) with untreated MRSA cells as controls. The turbidimetric readings (OD₆₀₀) of the cultures were measured at 60 min intervals in a Varioskan LUX 96-well plate reader (Thermo Scientific, USA) using the kinetic loop function, with shaking at 120 rpm (37°C). After acquiring the readings for 8h, samples from each well were spotted onto fresh MHA plates without any SM or antibiotic to check the viability of the bacteria. The plates were incubated at 37°C for 12h.

6.4.5 Membrane Perturbation:

6.4.5.1 Cellular Leakage Assay:

Suspensions of MRSA ($\sim 10^9$ CFU mL⁻¹) were prepared in PBS and incubated with SG-B **22** (50 μ M), SG-B **52** (25 μ M), or their AMP/AMOX combinations (EC), along with positive control of 10 μ M gramicidin, for 4h at 37 °C. At 30 min intervals, equal volumes of the incubated culture were collected, and the supernatant was centrifuged at 5000 rpm for 5 min. The absorbance of the supernatant was measured at 260 nm, which corresponds to the λ_{max} of DNA

and RNA, indicating their presence in the supernatant along with some cellular proteins/peptides that can absorb at 260 nm.

6.4.5.2 Membrane Depolarization Assay:

The membrane potential-sensitive dye DiSC₃(5) was used in independent cytoplasmic membrane depolarization assays on SG-B **22**, SG-B **52**, and their combination sets with AMP/AMOX (TCI India). To study the membrane depolarization, the following protocol was followed.⁵⁰ MRSA was grown up to an OD₆₀₀ of 0.3 at 37°C. After centrifugation, the bacterial cells were twice washed in washing buffer (20 mM glucose, 5 mM HEPES, pH 7.2), then resuspended in the same solution to achieve an OD₆₀₀ of 0.05 with the addition of 0.1 mol L⁻¹ KCl. To stabilize the fluorescence, 100 µL of resuspended cells were gradually given 1 µM DiSC₃(5) before being incubated for 10 min. The ability of SG-B **22**, SG-B **52** or their AMP/AMOX combinations to depolarize the bacterial membrane was assessed by observing the change in the fluorescence emission intensity of DiSC₃(5) (λ_{ex} = 622 nm, λ_{em} = 670 nm) for 11 min after adding the SM alone (50 µM for SG-B **22** and 25 µM for SG-B **52**) or SM+antibiotic (EC) combination solutions while using 5µM gramicidin as the positive control.

6.4.5.3 Membrane Permeabilization Assay:

MRSA was cultured until it reached an OD₆₀₀ of 0.1. Afterward, they were treated with SG-B **22** (50 µM) and SG-B **52** (25 µM) alone or in combination with AMP/AMOX (EC) for 60 min at 37°C. Following treatment, the cells were washed twice with a 5mM HEPES-Glucose buffer (pH 7.2) using centrifugation at 5000 rpm for 10 min and then resuspended in the same. Next, 1 mL of the resuspended cells was mixed with 5 µL of a 1 mM solution of PI, and the mixture was incubated in the dark for 30 min. The cells were then transferred to a 96-well polypropylene plate and the fluorescence intensity was measured using a 96-well plate reader with excitation and emission wavelengths of 535 nm and 617 nm, respectively.

6.4.5.4 Bacterial Live/Dead Assay:

Mid-log phase cultures of MRSA were centrifuged (4000 rpm, 5 min) and the medium was discarded. The cell pellets were resuspended in 0.9% saline solution after being twice washed in sterile PBS. Following this, SG-B **22** and SG-B **52** (50 µM and 25 µM respectively) alone or in combination with AMP/AMOX (EC) were given to the cells and incubated for 30 min at 37°C. Following incubation, the cell suspensions were treated with the fluorescent probes SYTO9 (Invitrogen, USA) and PI (Sigma Aldrich, USA) at final concentrations of 3 µM and 15 µM, respectively, and incubated for 20 min in the dark. The cells were then resuspended in the saline solution after being spun down to remove the unattached dye molecules. Using a fluorescence microscope (IX83, Olympus, Tokyo, Japan) with a 10× objective and an excitation and emission wavelength range of 450–490 nm and 515–560 nm, cell suspensions (5 µL) from the treated and untreated samples were then examined. The fluorescence intensities of the images were quantified using the utility tool imageJ.

6.4.5.5 Field Emission Scanning Electron Microscopy:

For the FESEM analysis, SG-B **22** (50 μ M), SG-B **52** (25 μ M), or their AMP/AMOX combinations (EC) were used to treat 5×10^5 CFU of MRSA for 60 min. To get rid of any remaining media, the cells were spun down (5000 rpm, 6 min) and twice rinsed in sterile PBS. Finally, 200 μ L of PBS was used to resuspend the cells before adding them to wells (12-well plates) lined with sterile 12 mm glass coverslips. After 40 min, the extra liquid was drained from the wells, and each well received a gentle addition of 1 mL of a 2.5% GA solution (Sigma Aldrich, Germany) in PBS. The samples were fixed in GA at 4°C overnight before being rinsed twice with PBS and distilled water. After washing, The samples were exposed to an ethanol gradient (25%, 50%, 75%, 95%: 1 $\times$ 5min, 100%: 3 $\times$ 10 min) for controlled dehydration. The coverslips were then carefully removed from the wells, dried in a vacuum for 6h, sputtered with gold, and imaged with a FESEM (Apreo 2S, Thermo Fischer Scientific, USA).

6.4.6 Cytotoxicity Profile:

6.4.6.1 Haemolytic Activity:

Erythrocyte pellets were prepared from fresh male blood by centrifuging the whole blood at 5000 $\times$ g for 10 min at 4°C. The pellet was then washed three times with sterile PBS (pH 7.4) and resuspended in the same solution to create an 8% (vv⁻¹) suspension. Equal volumes of SG-B **22** (100-3.125 μ M), SG-B **52** (100-3.125 μ M), and SG-B **22**/ SG-B **52**+AMP/ AMOX (EC) were incubated with this for 30 min at 37°C (with shaking at 150 rpm). To assess HRBC lysis and heme release, samples were again centrifuged at 5000 rpm for 2 min at 4°C. The absorbance of the supernatant was then measured in a multiplate reader at 540 nm. Triton X-100 (0.1% vv⁻¹) was taken as a positive control. The HC₅₀ values were properly recorded.

6.4.6.2 Mammalian Cell Cytotoxicity of SG-B 22 and SG-B 52:

WI-38, RAW 264.7, and HEK-293 cells were seeded at a density of 5000 cells per well in 96-well plates with 100 μ L of DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution. The cells were then incubated for 24h at 37°C with 5% CO₂. Following this, the medium was replaced with fresh medium containing different concentrations of SG-B **22** and SG-B **52** (ranging from 1.5 to 50 μ M), and the cells were incubated for an additional 24-48h at 37°C. To assess cell viability, the old medium was replaced with 100 μ L of fresh medium, and 10 μ L of MTT solution (5 mg mL⁻¹ in DMSO) was added to each well. The plates were then incubated for an additional 4h. Next, the MTT solution was removed, and 100 μ L of DMSO was added to each well and incubated for 40 min in the dark. Finally, the absorbance of the solutions was recorded at 570 nm.

6.4.7 ROS generation assay:

MRSA suspensions ($\sim 5 \times 10^5$ CFU mL⁻¹) in MHB were treated with SG-B **22** (50 μ M), SG-B **52** (25 μ M), SG-B **22**+AMP/AMOX (EC), SG-B **52**+AMP/AMOX (EC), only AMP (25 μ M), only AMOX (25 μ M), and 0.15% H₂O₂ (positive control) and incubated at 37°C for 4h (shaking at

150 rpm). Another set of MRSA cells was treated with a negative (antioxidant) control, i.e., ascorbic acid (only cells + AA, AMOX/AMOX + SMs +AA) to demonstrate the lowering of endogenous ROS elicited by AMOX/AMOX + SMs. The treated cultures (2mL) were centrifuged at 10000×g for 5 min before being washed with PBS. Pellets were incubated for 1.5h at 37°C in the dark with shaking after being resuspended in 1 mL of 100 μM DCFH-DA in PBS (from 20 mM stock in DMSO). The cells were once more pelleted followed by a PBS wash and resuspended in fresh PBS. Five triplicate measurements of each of the distinct treatments were made at excitation and emission wavelengths of 495 and 527 nm respectively. The background fluorescence of PBS devoid of bacteria was also measured. Using 100 μL of DCFH-DA in PBS, spontaneous fluorescence was tracked. Percent ROS generation was then calculated accordingly:

$$\% \text{ ROS generation} = 1 - (\text{RFU}_{\text{TREATED}} / \text{RFU}_{\text{CONTROL}}) \times 100$$

6.4.8 Anti-Biofilm Activity:

6.4.8.1 Inhibition of Sliding Motility of MRSA:

To study the inhibition of sliding motility,³⁷ MRSA cells (10^7 CFU mL⁻¹) were cultivated in sterile test tubes with 2 mL of BHI medium and treated with SG-B **22** (50 μM), SG-B **52** (25 μM), SG-B **22**+AMP/AMOX (EC), SG-B **52**+AMP/AMOX (EC), only AMP (25 μM), and only AMOX (25 μM) in addition to the untreated control set. Following a 4h incubation period (an incubation period of 24h is preferred but we have incubated the samples for 4h as the cells treated with the SM+antibiotic combinations completely lost their viability at 24h), 5 μL of suspension from the treated and untreated samples were spot-inoculated in the center of the BHI plates (2.4%) followed by aseptic air drying for 5 min. The sliding motility of each Petri plate was evaluated by measuring the colony diameter post-48h incubation (37°C).

6.4.8.2 Crystal Violet Assay:

To study biofilm inhibition, MRSA cultures that had grown overnight (10^5 CFU mL⁻¹) were transferred into the wells of a sterile microtiter plate. The plate was then incubated at a constant temperature of 37°C for 24h after the addition of varying concentrations of SG-B **22**/SG-B **52** (50μM and 25μM respectively) or their AMP/AMOX combinations (1/4EC, 1/2EC, and EC). After Incubation, the media was disposed away, and the wells were rinsed with PBS to eliminate any unadhered bacterial cells. Afterward, 200μL of methanol was introduced into each well for a duration of 15 min to fix the biofilms. 200μL of CV (2% (wv⁻¹)) was then added to each well and left for 5 min. Following the staining process, the surplus stain was extracted and the wells were rinsed with deionized water. The wells were subsequently dried at ambient temperature. The solubilization of CV attached to the biofilm matrix was conducted using 100μL of 33% (v⁻¹) glacial acetic acid. The absorbance of each well was measured at a wavelength of 522 nm.

Percent (%) Biofilm Inhibition was calculated accordingly:

$$\text{Percent (\%)} \text{ Biofilm Inhibition} = [(ABS_{\text{control}} - ABS_{\text{treated}}) / ABS_{\text{control}}] \times 100$$

To study the biofilm disruption using the CV assay,⁵¹ MRSA cultures were grown in BHI to obtain cell suspensions of 10^5 CFU mL⁻¹. The suspensions were then added to the wells of a sterile 96-well plate and incubated at 37°C for 24h at stasis. Post-incubation, the spent media was discarded and replaced with fresh BHI containing varying concentrations of SG-B **22**/SG-B **52** (50µM and 25µM respectively) or their AMP/AMOX combinations (EC) and incubated further for 24h at 37°C, maintaining stasis (suitable untreated controls were maintained). Post-incubation, wells were washed with PBS to remove the unattached cells followed by fixing with 200 µL methanol for 15 min. 200 µL Hucker crystal violet (CV) (2% (wv⁻¹)) was then added to each well and kept for 5 min. Post-staining, the excess stain was aspirated from the wells and washed with deionized water followed by drying at room temperature. The CV bound to the biofilm matrix was then solubilized using 100 µL 33 % (vv⁻¹) glacial acetic acid and the absorbance was measured at 522 nm.

Percent (%) Biofilm Eradication was calculated accordingly:

$$\text{Percent (\%)} \text{ Biofilm Eradication} = [(ABS_{\text{control}} - ABS_{\text{treated}})/ABS_{\text{control}}] \times 100$$

6.4.9 Atomic Force Microscopy:

AMP/AMOX (25 µM), SG-B **22** (50 µM), and SG-B **52** (25 µM) along with SM+antibiotic combinations (EC) were added to cleaved mica disks along with 10^5 CFU mL⁻¹ MRSA and allowed to grow for 24h for biofilm formation in 6 well cell culture plates at 37°C. After 24h, the biofilms were fixed overnight with 2.5 % phosphate-buffered GA at 4°C. Post-fixation, the disks were washed twice with PBS and air-dried. An XE70 atomic force microscope (Park Systems, South Korea) was then used in the non-contact mode for the acquisition of the AFM images followed by analysis using the XEI software.

Further, based on the AFM data, percent (%) reduction in biofilm thickness was calculated using the following formula:

$$\text{Percent (\%)} \text{ Reduction in Thickness} = [(Thickness_{\text{Control}} - Thickness_{\text{Treated}})/Thickness_{\text{Control}}] \times 100$$

6.4.10 Microbial Adhesion to Hydrocarbons Assay:

For the MATH assay, all the treated (AMP, AMOX, SG-B **22**, SG-B **52**, and their combinations) and untreated MRSA (mid-log-phase) samples were grown overnight at 37°C (shaking at 160 rpm). To the overnight grown samples, the addition of toluene was done in a 1:1 ratio. This was followed by vortexing the samples for 2 min. The samples were allowed to rest for 10 minutes and the aqueous phase from the top was collected carefully. The absorbance of each of the samples before and after the addition of toluene was recorded at 600 nm in a microplate reader. The % hydrophobicity inhibition of MRSA was calculated accordingly:

$$\text{Percent (\%)} \text{ of hydrophobicity inhibition} = [(OD_{600} \text{ before vortexing} - OD_{600} \text{ after vortexing})/OD_{600} \text{ before vortexing}] \times 100$$

6.4.11 Auto-Aggregation Assay:

Overnight grown MRSA cells were added to test tubes containing 5 mL BHI medium with and without treatments (SG-B 22 (50 μ M), SG-B 52 (25 μ M)), SG-B 22+AMP/ AMOX (EC), SG-B 52+AMP/ AMOX (EC), only AMP/ AMOX (25 μ M)). This was followed by the incubation of the samples for 24h at 37°C with moderate shaking. Post-incubation, the samples were allowed to settle for 30 min. The upper clear layer of the sample suspensions was collected carefully and assessed for absorbance at 600 nm, without disturbing the pellet.

6.4.12 Slime Production Assay:

For the slime production assay, the CRA plates contained 3.7% (w v⁻¹) BHI broth, 3.6% (w v⁻¹) sucrose, 1.5% (w v⁻¹) agar powder, 0.08% (w v⁻¹) Congo red, 1.5% (w v⁻¹) NaCl, and 2% (w v⁻¹) glucose. In test tubes containing 2 mL BHI broth medium, the MRSA cultures (10⁷ CFU mL⁻¹) were grown for 24h at 37°C in the presence and absence of the SMs, antibiotics, or their combinations. The untreated as well as treated samples were streaked on the CRA plates after 4h incubation and were then incubated for a further 24h at 37°C, post-streaking. Following that, the change in color of the colonies was noted and documented pictorially.

6.4.13 Bacterial Viability in Biofilms:

24 h established biofilms of MRSA were subjected to overnight treatment with SG-B 22 (50 μ M), SG-B 52 (25 μ M), SG-B 22+AMP/ AMOX (EC), SG-B 52+AMP/ AMOX (EC), only AMP (25 μ M), only AMOX (25 μ M), and 10 μ M Vancomycin (positive control). All samples were then treated with a concentration of 0.5mg per mL of MTT and kept at a temperature of 37°C for 10 min. The media was discarded and the formazan crystals were dissolved in a mixture of DMSO and methanol in a 1:1 ratio. The absorbance of all samples was measured at a wavelength of 570 nm.

Percent (%) Biofilm Viability was calculated using the formula:

$$\% \text{ biofilm viability} = (\text{OD}_{\text{UTC}} - \text{OD}_{\text{Treated}}) \times 100$$

6.4.14 Serum Stability Assay:

Fresh male blood serum was collected and diluted with PBS to 25%. To extract the lipids, the diluted serum was centrifuged at 12000 rpm for 10 min at 4°C. The resulting supernatant was incubated at 37°C for 15 min. SG-B 52 was added at a final concentration of 1 mM to the supernatant and further incubated at 37°C. 200 μ L aliquots of this incubated mixture were withdrawn and placed on ice at intervals of 0h, 0.5h, 1h, 1.5h, and 3h. The serum proteins were precipitated from the aliquots using 40 μ L of 15% TCA and the tubes were centrifuged at 13,000 rpm for 10 min. The supernatant was recovered and kept at -20°C in fresh microcentrifuge tubes. For HPLC analysis, 5 μ L of the supernatant from each time point was

used and the amount of intact SG-B 52 in serum was determined by integrating the peak area associated with the observation of the small molecule's chromatographic profile at 0h.

6.4.15 Murine Tolerance Study, *In Vivo* Organ Loading, and Histopathology:

6.4.15.1 *In Vivo* Safety Studies:

All animal studies reported in this publication were conducted on approval from the Institutional Animal Ethics Committee (IAEC) of JNVU, Jodhpur, Rajasthan, India (Approval No: UDZ/IAEC/2023/5). 4-5 weeks old, healthy, female albino Wistar rats (120-130 gm) were used for the study. The doses of SG-B 52 were tested for safety *in vivo* using the drug safety evaluation assay. SG-B 52 was solubilized in DMSO (within permissible limits), saline, and 0.1% Tween 20 (vehicle). In this study, the rats were separated into 5 groups of six animals each comprising two SG-B 52 treatment groups (10 mg kg⁻¹ and 20 mg kg⁻¹), two groups of SG-B 52+AMP/AMOX combinations (10 mg kg⁻¹+10 mg kg⁻¹), and one vehicle control. Anaesthesia in the form of xylazine (20 mg kg⁻¹ of body weight) and ketamine (80 mg kg⁻¹ of body weight) was maintained throughout the procedure. After receiving the intraperitoneal injections, the rats were monitored for 7 days to determine their survival rates. The weight changes, feeding habits, excretion, agility, and sleep patterns of the animals were closely monitored daily.

6.4.15.2 *In Vivo* Organ Loading:

To assess the *in vivo* bactericidal activity SG-B 52 in combination with AMP and AMOX, a murine model of sepsis was used.⁴¹ Female albino Wistar rats (n = 6) were randomly assigned to 4 groups for the organ loading experiment, and each group received an intraperitoneal injection of 100 µL of MRSA suspension (~5×10⁸ CFU mL⁻¹). The rats were given intraperitoneal injections of SG-B 52+AMP/AMOX (10+10 mg kg⁻¹) as well as the positive control vancomycin (5 mg kg⁻¹) after 60 min of pathogen injection. Only saline was administered to the blank control group. The anaesthesia was maintained as mentioned in the previous section. Following a two-day observation period, the animals were euthanized by cervical dislocation (with anaesthesia). The livers, kidneys, and spleens of the animals (1-5 gm of tissue) were extracted for studying the bacterial load of each organ (n = 3) and for cryosectioning and H&E staining.

6.4.16 Histopathological Intervention:

For the histopathological studies,⁵² the animals were euthanized, and their livers, kidneys, and spleens were collected 2 days after the pathogen injection. The collected tissues were fixed in 4% paraformaldehyde in phosphate buffer and stored at 4°C for 48h. To further prepare the tissues for histological evaluation, they were dehydrated through a sucrose gradient, with concentrations of 10%, 20%, and 30% used over a period of 24h each until the tissues sank. The dehydrated tissues were then embedded in Fischer Healthcare's Tissue plus® optimal cutting temperature (OCT) compound for sectioning on a microtome-cryostat. Subsequently,

10 μm sections were obtained using an FSE cryotome (Thermo Scientific, USA) and subjected to hematoxylin and eosin staining for histopathological examination.

6.5 Conclusion:

In conclusion, the findings presented in this paper highlight the potential of SG-B **22** and SG-B **52** in the field of antimicrobial adjuvant research. These molecules were found to possess both weak membrane perturbation capability and good AMP/AMOX potentiation ability against MRSA, as demonstrated by various assays. They were also found to be biocompatible, as evidenced by mammalian cell cytotoxicity and hemolysis assays while being considerably stable in human plasma. SG-B **22** and SG-B **52** alone exhibited weak membrane perturbation capability and moderately caused the elicitation of endogenous ROS. This suggests that they may have limited direct antimicrobial activity, making them relatively unimportant for the bacteria to target. Furthermore, it was revealed that both SG-B **22** and SG-B **52** exhibited good AMP/AMOX potentiation capability. This implies that these molecules can enhance the effectiveness of ineffective conventional antimicrobial agents, such as AMP and AMOX, in combating MRSA infections. SG-B **52** in conjunction with AMP/AMOX demonstrated excellent anti-biofilm activity, which is a significant finding considering the impact of biofilms on chronic infections and their increased resistance to antimicrobial agents. The final notable finding of this study was the successful clearance of MRSA infections in albino Wistar rats when SG-B **52** was combined with AMOX. Further research and development are warranted to explore the therapeutic applications of SG-B **22** and SG-B **52**, including their potential for combating other drug-resistant bacterial pathogens and treating biofilm-associated infections.

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Appendix B



38th ACS National Medicinal Chemistry Symposium
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June 26, 2024

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This is to confirm that

Mr Surojit GHOSH
IIT Jodhpur
India

Registration number: **NMCS24/056**

has attended the **38th National Medicinal Chemistry Symposium NMCS 2024** in Seattle, WA, United States, from June 23-26, 2024.

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Discovery of Quinazoline and Quinoline-Based Small Molecules as Utrophin Upregulators via AhR Antagonism for the Treatment of Duchenne Muscular Dystrophy

Surojit Ghosh,[#] Mohammad Umar Arshi,[#] Satyajit Ghosh, Moumita Jash, Samya Sen, Kamel Mamchaoui, Sudipta Bhattacharyya, Nirmal Kumar Rana, and Surajit Ghosh*

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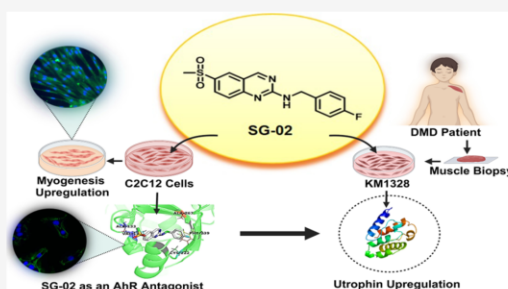
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ABSTRACT: Duchenne muscular dystrophy (DMD) is a fatal muscle-wasting disease caused by the absence of a dystrophin protein. Elevating utrophin, a dystrophin paralogue, offers an alternative therapeutic strategy for treating DMD, irrespective of the mutation type. Herein, we report the design and synthesis of novel quinazoline and quinoline-based small molecules as potent utrophin modulators screened via high throughput In-Cell ELISA in C2C12 cells. Remarkably, lead molecule SG-02, identified from a library of 70 molecules, upregulates utrophin 2.7-fold at 800 nM in a dose-dependent manner, marking the highest upregulation within the nanomolar range. SG-02's efficacy was further validated through DMD patient-derived cells, demonstrating a significant 2.3-fold utrophin expression. Mechanistically, SG-02 functions as an AhR antagonist, with excellent binding affinity ($K_d = 41.68$ nM). SG-02 also enhances myogenesis, as indicated by an increased MyHC expression. ADME evaluation supports SG-02's oral bioavailability. Overall, SG-02 holds promise for addressing the global DMD population.



1. INTRODUCTION

Duchenne muscular dystrophy (DMD) is a rare genetic disorder characterized by progressive muscle degeneration and weakness affecting approximately 1 in 3500 males. DMD arises from a mutation in the dystrophin gene located on the X chromosome and results in the absence or deficiency of the dystrophin protein.^{1,2} Dystrophin plays a significant role in facilitating the link between the intracellular cytoskeleton of muscle cells and the extracellular matrix, forming a dystrophin-associated protein complex (DAPC). DAPC assists in the maintenance of muscle fiber structure and plays a vital role in muscle contraction and relaxation.³ Symptoms of DMD usually emerge in early childhood, showing delayed motor skills, muscle weakness, and ambulation difficulties. Progression of the disease leads to a loss of ambulation during adolescence. DMD not only affects skeletal muscles but also involves cardiac and respiratory muscles, resulting in complications, such as cardiomyopathy and respiratory insufficiency. Ultimately, DMD causes significant disability, reduced life expectancy, and premature death for those affected.^{4,5}

After the identification of the gene associated with DMD,⁶ various pharmacological strategies have been explored for treating this disorder.⁷ Therapies such as premature stop-codon read-through using ataluren and exon skipping with drugs such as Eteplirsen (for exon 51) and Golodirsen (for

exon 53) have received conditional approval. This drug targets only certain subpopulations of DMD patients based on their specific genetic mutations.⁸ Currently, gene therapy approaches are under development, where they aim to deliver a functional copy of the DMD gene to muscle cells. Viral vectors and gene editing techniques like CRISPR/Cas9 are being investigated in this context.⁹ However, in the majority of cases, the current therapeutic paradigm for managing DMD primarily emphasizes symptomatic treatment. These aim to mitigate issues such as inflammation and fibrosis through clinical management strategies by employing corticosteroids. Although these symptomatic and supportive treatments have shown limited success in improving the life expectancy of individuals with DMD, there is no currently available treatment that can modify the course of the disease and treat the universal DMD population.^{8,10,11} Consequently, there is a dire need for a

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Synergistic Augmentation of Beta-Lactams: Exploring Quinoline-Derived Amphipathic Small Molecules as Antimicrobial Potentiators against Methicillin-Resistant *Staphylococcus aureus*Surojit Ghosh,[±] Samya Sen,[±] Moumita Jash, Satyajit Ghosh, Aniket Jana, Rajsekhar Roy, Nabanita Mukherjee, Dipro Mukherjee, Jayita Sarkar,* and Surajit Ghosh*Cite This: *ACS Infect. Dis.* 2024, 10, 1267–1285

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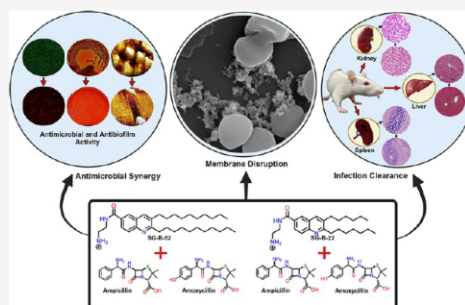
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ABSTRACT: The escalation of bacterial resistance against existing therapeutic antimicrobials has reached a critical peak, leading to the rapid emergence of multidrug-resistant strains. Stringent pathways in novel drug discovery hinder our progress in this survival race. A promising approach to combat emerging antibiotic resistance involves enhancing conventional ineffective antimicrobials using low-toxicity small molecule adjuvants. Recent research interest lies in weak membrane-perturbing agents with unique cyclic hydrophobic components, addressing a significant gap in antimicrobial drug exploration. Our study demonstrates that quinoline-based amphipathic small molecules, SG-B-52 and SG-B-22, significantly reduce MICs of selected beta-lactam antibiotics (ampicillin and amoxicillin) against lethal methicillin-resistant *Staphylococcus aureus* (MRSA). Mechanistically, membrane perturbation, depolarization, and ROS generation drive cellular lysis and death. These molecules display minimal *in vitro* and *in vivo* toxicity, showcased through hemolysis assays, cell cytotoxicity analysis, and studies on albino Wistar rats. SG-B-52 exhibits impressive biofilm-clearing abilities against MRSA biofilms, proposing a strategy to enhance beta-lactam antibiosis and encouraging the development of potent antimicrobial potentiators.

KEYWORDS: antimicrobial resistance, small molecules, quinolines, membrane disruption, antibiotic adjuvants, superbugs



Methicillin-resistant *Staphylococcus aureus* (MRSA) is a predominant multidrug-resistant bacteria, causing several human maladies ranging from mild skin conditions to severe bloodstream infections. Misuse, overuse, and improper knowledge about antibiotic consumption have led to the rapid upsurge of multidrug-resistant MRSA that have acquired high resistance against several antibiotics, including methicillin, oxacillin, and penicillin. MRSA has become a global issue, with high rates of infection in healthcare facilities, such as hospitals and long-term care facilities. Community-acquired MRSA infections are also on the rise and can occur in healthy individuals with no recent healthcare exposure. Risk factors for MRSA infection include recent hospitalization, surgery, dialysis, intravenous catheter use, and weakened immune system.^{1,2} MRSA infections are projected to cause over a million deaths per year by 2050 with an associated global economic burden of one trillion US dollars.³

MRSA is resistant to several classes of antibiotics, including beta-lactams (penicillins and cephalosporins), chloramphenicol, lincomycin, aminoglycosides, tetracyclines, macrolides, quinophthalones, sulfonamides, and rifampicin, which is a grave problem in strategizing clinical treatment.⁴ Focusing on the beta-lactams, due to the presence of a unique penicillin-

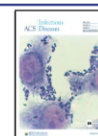
binding protein (PBP2a), MRSA is least affected by them. This modified PBP2a has a low affinity for beta-lactam antibiotics and can continue building the bacterial cell wall even in the presence of these antibiotics.⁵ The production of PBP2a is mediated by the *mecA* gene, which is carried on a mobile genetic element called the staphylococcal chromosomal cassette (SCCmec). The SCCmec element can transfer between different strains of *S. aureus*, allowing for the spread of *mecA*-mediated beta-lactam resistance.⁶ MRSA may also acquire additional resistance mechanisms, such as the production of beta-lactamase enzymes that can break down beta-lactam antibiotics. These enzymes are not present in all MRSA strains but may sometimes contribute to increased resistance.⁷ Because of its resistance to beta-lactam antibiotics, MRSA infections are typically treated with alternative anti-

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Multi-Faceted Antimicrobial Efficacy of a Quinoline-Derived Bidentate Copper(II) Ligand Complex and Its Hydrogel Encapsulated Formulation in Methicillin-Resistant *Staphylococcus aureus* Inhibition and Wound Management

Samya Sen,[#] Surojit Ghosh,[#] Aniket Jana,[#] Moumita Jash, Satyajit Ghosh, Nabanita Mukherjee, Dipro Mukherjee, Jayita Sarkar,^{*} and Surajit Ghosh^{*}



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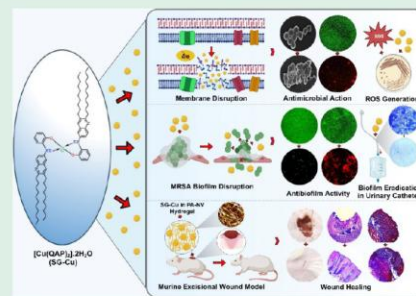
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ABSTRACT: The emergence of antimicrobial resistance, exemplified by methicillin-resistant *Staphylococcus aureus* (MRSA), poses a grave threat to public health globally. Over time, MRSA has evolved resistance to multiple antibiotics, challenging conventional treatment strategies. The relentless adaptability of MRSA underscores the urgent need for innovative and targeted antimicrobial approaches to combat this resilient pathogen. Ancient knowledge and practices, along with scientific evidence, have established that metallic copper, and its organic coordination complexes can act as potential antibacterial substances. In search of a smart and effective antimicrobial against MRSA, we designed, synthesized, and characterized a bidentate copper(II) ligand complex (SG-Cu) utilizing a comprehensive array of analytical techniques, including ESI-MS, elemental analysis, X-ray photoelectron spectroscopy, electron paramagnetic resonance spectroscopy, and others. Antibacterial efficacy and mechanism of action of the complex were assessed through bacterial growth analyses, bacterial membrane perturbation assays, ROS elicitation assays, and field emission scanning electron microscopy. SG-Cu was found to maintain robust biocompatibility against the mammalian cell lines HEK-293, WI-38, and NIH/3T3. Remarkably, SG-Cu demonstrated significant biofilm disruptive tendency evidenced by the retardation of sliding motility, reduction in slime production, reduction in biofilm viability, and enhanced biofilm eradication, both *in vitro* and in urinary catheters. *In vivo* studies on murine excisional wounds, with SG-Cu impregnated in a palmitic acid conjugated NAVSIQ hexapeptide (PA-NV) hydrogel, revealed the sustained release of SG-Cu from the gel matrix, facilitating accelerated wound healing and effective wound disinfection. This multifaceted investigation highlights the potential of SG-Cu as a versatile option for combating MRSA infections and promoting wound healing, solidifying its claim to be developed into a viable therapeutic.

KEYWORDS: antibacterial, multidrug resistance, copper complexes, antibiofilm, wound healing, hydrogels



1. INTRODUCTION

The rising threat of multidrug-resistant superbugs has driven mankind to an alarming crossroads for the development of novel, more efficient, and effective therapeutics to deal with these organisms. Multidrug-resistant superbugs are bacteria that are resistant to three or more classes of antibiotics. According to the O'Neill Report, a review published in 2014, and commissioned by the United Kingdom government, it was predicted that by 2050, infections from bacteria will cause around 10 million more deaths than cancer and diabetes combined.¹ Methicillin-resistant *Staphylococcus aureus* (MRSA) is a Gram-positive superbug from the ESKAPE group of pathogens responsible for the deadliest hospital-acquired infections (HAIs). The Gram-positive superbug gains resistance against conventional antibiotics through two major avenues; either it can produce β -lactamases which cause

enzymatic degradation of antibiotics or it can reduce the susceptibility of its target site PBP (penicillin-binding protein) by changing the genetic makeup of PBP genes or by the acquisition of extracellular DNA.² MRSA is an established opportunistic pathogen as well as a well-known human commensal. In the past few decades, the organism has gained resistance against most classes of antibiotics including glycopeptides.³ Along with vancomycin-resistance capability,

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An overview of key potential therapeutic strategies for combat in the COVID-19 battle

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Aniket Jana,[†] Ramkamal Samat,^{§,a} Nabanita Mukherjee,[†] Rajsekhar Roy^a
and Surajit Ghosh^{†,abc}

The sudden ravaging outbreak of a novel coronavirus, or SARS-CoV-2, in terms of virulence, severity, and casualties has already overtaken previous versions of coronaviruses, like SARS CoV and MERS CoV. Originating from its epicenter in Wuhan, China, this mutated version of the influenza virus with its associated pandemic effects has engulfed the whole world with awful speed. In the midst of this bewildering situation, medical and scientific communities are on their toes to produce the potential vaccine-mediated eradication of this virus. Though the chances are really high, to date no such panacea has been reported. The time requirements for the onerous procedures of human trials for the successful clinical translation of any vaccine or potential therapeutics are also a major concern. In order to build some resistance against this massive pandemic, the repurposing of some earlier antiviral drugs has been done, along with the refurbishment of some immune-responsive alternative avenues, like monoclonal antibody mediated neutralization, interferon treatment, and plasma therapy. New drugs developed from the RBD domain of the virus spike protein and drugs targeting viral proteases are also undergoing further research and have shown potential from preliminary results. The sole purpose of this review article is to provide a brief collective overview of the recent status of therapeutics advances and approaches, and their current state of implementation for the management of COVID-19.

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Introduction

As COVID-19 continues to spread around the globe, researchers and drug manufacturers are moving towards the development of potential therapeutics into clinical trials at a dizzying pace. The situation has been declared an emergency by the World Health Organization (WHO) and governments across the world have given urgent consideration to controlling the transmission of this disease. Worldwide the generally accepted plan to combat this pandemic has already been taken by imposing a “lockdown” aiming to prevent the spread of virus, as SARS-CoV-2 is hugely a human-to-human contagious disease, which has also been catastrophic to the medical infrastructure of all countries. Along with governments, the people who are working as the frontline warriors in the campaign against the novel coronavirus are

scientists and doctors. Researchers and medical experts are working very hard to find a promising outcome to treat this deadly disease. As we all know, viruses are non-living infectious species, which require a host cell to divide and sustain themselves. Here SARS-CoV-2, the virus causing COVID-19, is a single-stranded RNA virus, which utilizes its spike-like receptor binding domain to interact with the host cell.¹ In the lungs, the virus targets cells expressing angiotensin-converting enzyme 2 (ACE2), which are situated in the lining of the lungs (called pneumocytes), and cause respiratory disorder.² This results in a reduction in oxygen levels in the blood, which can finally lead to a fatal condition. A recent report reveals that the virus can interfere with the iron-containing compound of blood.³ Another study also proposes that patients with blood group A are more susceptible to SARS-CoV-2 in comparison to others.⁴ In addition, there is prevailing evidence that patients living with heart disease and diabetes are more vulnerable to this disease due to over-expression of a protein called ACE2, which SARS-CoV-2 can bind to and later use the host cell machinery for producing its duplicate copies. Hence, this further increases the rate of infection for these patients.⁵ Currently, the most generally adopted approach to treat COVID-19 infected people is to ease the patient's symptoms (which feature pneumonia), while the campaign to develop a complete cure for the disease is still a major challenge. SARS-CoV-2, being an RNA virus, can be

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[§] Aniket Jana and Ramkamal Samat are co-third authors.



Potential Broad-Spectrum Antimicrobial, Wound Healing, and Disinfectant Cationic Peptide Crafted from Snake Venom

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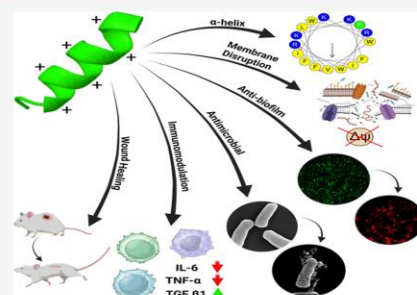
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ABSTRACT: Antimicrobial cationic peptides are intriguing and propitious antibiotics for the future, even against multidrug-resistant superbugs. Venoms serve as a source of cutting-edge therapeutics and innovative, unexplored medicines. In this study, a novel cationic peptide library consisting of seven sequences was designed and synthesized from the snake venom cathelicidin, batroxicidin (BtxC), with the inclusion of the FLPII motif at the N-terminus. SPIV3_1 demonstrated exceptional antibacterial effectiveness against *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* and destroyed the bacteria by depolarizing, rupturing, and permeabilizing their membranes, as evident from fluorescence assays, atomic force microscopy, and scanning electron microscopy. SPIV3_1 was observed to modulate the immune response in LPS-elicited U937 cells and exhibited good antibiofilm activity against MRSA and *K. pneumoniae*. The peptide promoted wound healing and disinfection in the murine model. The study demonstrated that SPIV3_1 is an exciting peptide lead and may be explored further for the development of better therapeutic peptides.



1. INTRODUCTION

The rapid advent of multidrug-resistant superbugs (bacteria capable of resisting at least three classes of antibiotics) has taken an alarming lead in the race against novel antimicrobial developmental pipelines.¹ Each year, more than 2.8 million antibiotic-resistant illnesses occur in the U.S., leading to more than 35,000 fatalities.² The O'Neill report, a review published in 2014 and commissioned by the United Kingdom government, estimated that by 2050, bacterial infections would cause 10 million more deaths than diabetes and cancer combined.³ Currently, bacterial resistance poses a serious threat to human health and is the leading cause of death in American hospitals, killing millions of people worldwide. Contemporary medicines and techniques are needed to treat recalcitrant infections since bacteria are becoming increasingly resistant to most antibiotics. The ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* sp.) in particular are a major concern because they are resistant to most of the medications that are currently available including β -lactams, oxazolidinones, fluoroquinolones, lipopeptides, macrolides, tetracyclines, β -lactam- β -lactamase inhibitor combinations, carbapenems, glycopeptides, and polymyxins.^{4,5} The present therapeutic antibiotics are constrained by their broad and deleterious off-target effects due to the lack of distribution methods that efficaciously release payloads at the site of infection. The rapidly increasing resistance to conventional

antibiotics suggests that we are on the verge of a “post-antibiotic era,” in which previously effective therapeutic strategies will be rendered obsolete.⁶

Antimicrobial peptides (AMPs), used as the first line of defense, are naturally occurring molecules produced by all types of multicellular organisms and have a wide range of activity including the ability to kill bacteria, yeasts, fungi, viruses, and even cancer cells, directly.⁷ AMPs are small peptide molecules with fewer than 50 amino acid residues that can have one or more secondary structures, such as α -helix, β -sheet, or coiled conformations,⁸ and can be cationic, anionic, or amphipathic.⁹ Venoms are being investigated as an untapped potential source of new antibiotics, as they can interact with a variety of targets, such as cellular receptors, membranes, and ion channels, to produce their effects.¹⁰ Their capacity to switch from an unstructured to a helical conformation is correlated with their antibacterial activity,¹¹ and even minor changes in the sequence affect their structural tendency and biological activity. Since antiquity, venoms have been known to have curative properties, making the snake a

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Short Peptoid Evolved from the Key Hydrophobic Stretch of Amyloid- β 42 Peptide Serves as a Potent Therapeutic Lead of Alzheimer's Disease

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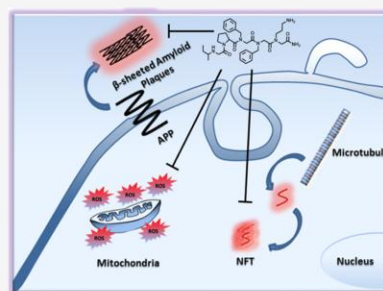
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ABSTRACT: Amyloid- β 42 (A β 42), an enzymatically cleaved (1–42 amino acid long) toxic peptide remnant, has long been reported to play the key role in Alzheimer's disease (AD). A β 42 also plays the key role in the onset of other AD-related factors including hyperphosphorylation of tau protein that forms intracellular neurofibrillary tangles, imbalances in the function of the neurotransmitter acetylcholine, and even generation of reactive oxygen species (ROS), disrupting the cytoskeleton and homeostasis of the cell. To address these issues, researchers have tried to construct several strategies to target multiple aspects of the disease but failed to produce any clinically successful therapeutic molecules. In this article, we report a new peptoid called RA-1 that was designed and constructed from the hydrophobic stretch of the A β 42 peptide, ¹⁶KLVF²¹. This hydrophobic stretch is primarily responsible for the A β 42 peptide aggregation. Experimental study showed that the RA-1 peptoid is stable under proteolytic conditions, can stabilize the microtubule, and can inhibit the formation of toxic A β 42 aggregates by attenuating hydrophobic interactions between A β 42 monomers. Furthermore, results from various intracellular assays showed that RA-1 inhibits A β 42 fibril formation caused by the imbalance in AChE activity, reduces the production of cytotoxic reactive oxygen species (ROS), and promotes neurite outgrowth even in the toxic environment. Remarkably, we have also demonstrated that our peptoid has significant ability to improve the cognitive ability and memory impairment in *in vivo* rats exposed to AlCl₃ and D-galactose (D-gal) dementia model. These findings are also validated with histological studies. Overall, our newly developed peptoid emerges as a multimodal potent therapeutic lead molecule against AD.

KEYWORDS: peptoid, A β 42 aggregation, Alzheimer's disease (AD), AChE inhibition, neuroprotection, primary cortical neurons, ROS, microtubule stabilization



1. INTRODUCTION

Toxic aggregation of amyloid- β 42 (A β 42) peptide in the extracellular region is considered the most important hallmark of Alzheimer's disease (AD).¹ Senile plaques formed of A β 42 peptide deposited in the synaptic region cause impairment in the synaptic plasticity and finally cause neuronal death.² Previously, the nucleation of this A β 42 peptide monomer only to the final fibrillary aggregates was thought to be the main reason, but recently, oligomers were found to be much more toxic. The propagation of aggregation of the monomer was thought to be initiated through the hydrophobic core, i.e., ¹⁶KLVF²¹.³ Hence, several strategies were developed that revolved around blocking the interaction of hydrophobic cores of A β 42 monomers. In this regard, short sequences of peptides called β -sheet breaker (BSB) peptid that can form direct interactions with the hydrophobic core and disrupt the aggregation at an early stage were synthesized. Soto and colleagues in their work took a pioneering approach to

modulate a β -sheet breaker peptide by modifying the sequence ¹⁷LVFFA²¹ and generating LPFFD, a short 5-amino acid peptide that can even disassociate preformed A β aggregates.⁴ Furthermore, Chalifour et al. replaced the L-amino acids with their D-enantiomers and showed enhancement in the stability and also increased effectiveness against the aggregation.⁵ Extrapolating this work, Chacón et al. modified LPFFD by adding acetyl protection to the N-terminal and amine protection to the C-terminal end.⁶ By doing this, they increased the serum stability and blood–brain barrier permeability that enhanced the effect in *in vivo* mice models.

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EphA4 Targeting Peptide-Conjugated Extracellular Vesicles Rejuvenates Adult Neural Stem Cells and Exerts Therapeutic Benefits in Aging Rats

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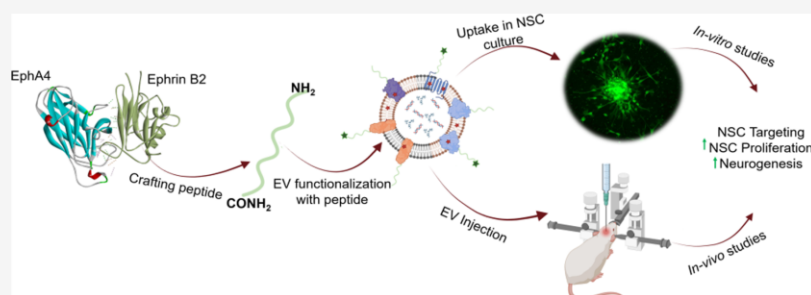
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ABSTRACT: Aging and various neurodegenerative diseases cause significant reduction in adult neurogenesis and simultaneous increase in quiescent neural stem cells (NSCs), which impact the brain's regenerative capabilities. To deal with this challenging issue, current treatments involve stem cell transplants or prevention of neurodegeneration; however, the efficacy or success of this process remains limited. Therefore, extensive and focused investigation is highly demanding to overcome this challenging task. Here, we have designed an efficient peptide-based EphA4 receptor-targeted ligand through an in silico approach. Further, this strategy involves chemical conjugation of the peptide with adipose tissue stem cell-derived EV (Exo-pep-11). Interestingly, our newly designed engineered EV, Exo-pep-11, targets NSC through EphA4 receptors, which offers promising therapeutic advantages by stimulating NSC proliferation and subsequent differentiation. Our result demonstrates that NSC successfully internalized Exo-pep-11 in both in vitro culture conditions as well as in the in vivo aging rats. We found that the uptake of Exo-pep-11 decreased by ~2.3-fold when NSC was treated with EphA4 antibody before Exo-pep-11 incubation, which confirms the receptor-specific uptake of Exo-pep-11. Exo-pep-11 treatment also increases NSC proliferation by ~1.9-fold and also shows ~1.6- and ~2.4-fold increase in expressions of Nestin and ID1, respectively. Exo-pep-11 also has the potential to increase neurogenesis in aging rats, which is confirmed by ~1.6- and ~1.5-fold increases in expressions of TH and Tuj1, respectively, in rat olfactory bulb. Overall, our findings highlight the potential role of Exo-pep-11 for prospective applications in combating age-related declines in NSC activity and neurogenesis.

KEYWORDS: adult neurogenesis, engineered extracellular vesicle, neural stem cell, neural stem cell targeting and rejuvenation

INTRODUCTION

Adult neurogenesis refers to the intricate process wherein neural stem cells (NSC) located in distinct brain regions, namely, the subventricular zone (SVZ) and the subgranular zone (SGZ), give rise to new neurons. Specifically, the SVZ contributes interneurons to the olfactory bulb, while the SGZ contributes neurons to the hippocampal dentate gyrus.^{1,2} The proliferation of the NSC within the SVZ occurs along the medial wall of the lateral ventricle (LV). Stem cells undergo division, producing transit-amplifying cells. These amplifying cells, in turn, give rise to neuroblasts that embark on a migratory journey through the rostral migratory stream (RMS), reaching their ultimate destination in the olfactory

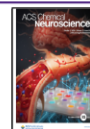
bulb (O.B.).³ Every day, more than 30,000 neuroblasts emerge from the mouse SVZ, journeying anteriorly. They cover distances of up to 5 mm in rodents and 2 cm in primates, propelled at speeds of approximately 122 mm/h toward the olfactory bulb.^{4–8} Upon reaching the O.B., neuroblasts unite

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Curriculum Vitae

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Ph.D. July 2019-Present (Tentative Date for Thesis Submission November 2024)

Inter Disciplinary Research Division, Smart Health Care, Indian Institute of Technology, Jodhpur

Supervisor: Prof. Surajit Ghosh, Department of Bioscience and Bioengineering, IIT Jodhpur

Thesis Title: *Smart-Engineered Small Molecule-Based Potential Therapeutic Development for Duchenne Muscular Dystrophy*

Research Interest:

Drug Design and Discovery, Small molecule and Peptide based Therapeutic development, Synthetic organic chemistry, Bio conjugation Techniques for oligonucleotides delivery, Metal Complexes posing therapeutic benefits, Development of Biomaterials for antibacterial applications, Development of nano micellar framework for smart delivery of therapeutic leads.

Academic Qualifications:

- Ph.D. in Science: Thesis entitled 'Smart-Engineered Small Molecule-Based Potential Therapeutic Development for Duchenne Muscular Dystrophy' under the supervision of Prof. Surajit Ghosh, Inter Disciplinary Research Division, Smart Health Care, Indian Institute of Technology, Jodhpur. **(July 2019-Present)** (Tentative date for Thesis Submission November-2024)
 - M.Sc. in Chemistry (Specialization in Organic Chemistry) from Ramakrishna Mission Vivekananda Centenary College, Rahara under the West Bengal State University with 74.2% marks. Year: 2016
 - B.Sc. (Honors) in Chemistry under University of Calcutta with 60.0 % marks. Year: 2014
-

Research Expertise:

- During doctoral studies, my research initiatives focused on design and synthesis of heterocyclic quinoline and quinazoline based molecular libraries targeting AhR protein for Utrophin upregulation, in the treatment of rare genetic disorder Duchenne Muscular Dystrophy (DMD).
- My research focusses on bio conjugation techniques for advancing antisense oligonucleotides (AON) delivery. Herein, we functionalized AON with newly designed short non-cationic cell penetrating peptides employing click chemistry.
- I have also gained expertise in the design and synthesis of peptide-based therapeutics aimed at enhancing myogenesis and mitigating fibrosis to address DMD pathophysiology. Additionally, my research includes the delivery of lead peptides identified for treating muscle injury.
- My research area further broadens by the development of micellar framework as nano carrier for the delivery of oligonucleotides and drug candidate together in a controlled fashion.

- Apart from my thesis work, during my tenure of doctoral work I also have developed some antibacterial metal complexes, small molecules as antibiotic adjuvants and wound healing biomaterials.
 - Apart from this I have also gained knowledge of routine cell culture techniques and animal handling.
-

Skills and Techniques:

- Design of novel drug candidates through *in-silico* docking analysis.
 - Scheme designing and process development of multistep organic molecule synthesis. Solvent Drying and Solvent Distillation. Expertise in synthesis of molecular library from mg to gram scale.
 - Handling of useful catalysts and pyrophoric reagents used in organic synthesis, includes conducting very moisture sensitive reactions.
 - Analysis Techniques: ^1H , ^{13}C nuclei 1D and 2D NMR spectroscopy, mass spectrometry, IR, UV-Vis spectroscopy, HPLC (analytical, preparative), XPS, EPR, TGA and more routine chromatographic techniques.
 - Solid phase peptide synthesis using both manually and microwave assisted peptide synthesizer. And its further modification for the development of peptide based hydrogels.
 - Expertise in Bio conjugation Techniques for efficient delivery of Antisense Oligonucleotides. Synthesis of metal complexes and its crystallization techniques.
 - Design and development of micellar core structures for the co-delivery of oligonucleotides and drug candidates in a controlled manner.
 - Capable of handling various software like Mestrenova, Topspin, Chem Draw, Origin, Graphpad Prism for routinely solving research data.
 - Proficient in routine cell culture techniques and animal handling.
 - Proficient in writing and design of manuscript and patents.
-

Industrial Experience in R&D Sector:

- As Research Associate at Jubilant Chemsys, Noida from March 2018 to November 2018.
 - As Research Chemist at TCG Life sciences Private Limited, Kolkata from January 2017 to February 2018.
-

Instrument Handled:

- NMR Spectrometer (Bruker 500 MHz for ^1H and ^{13}C Nuclei).
- Mass -ESI-LCMS (Agilent Technologies for routine identification of small molecules, peptides, Oligonucleotides and proteins)
- HPLC Analytical and Preparative (Shimadzu)
- Microplate Reader
- UV-Vis Spectrophotometer
- Solid Phase Peptide Synthesizer (CEM)

- Combiflash chromatography
 - Isothermal Calorimetry (TA instruments)
 - IR Spectrometer (Bruker)
-

Key Publications:

1. **Ghosh, S[#]**, Arshi, M.U[#], Ghosh, S., Jash, M., Sen, S., Mamchaoui, K., Bhattacharyya, S., Rana, N.K. and Ghosh, S., Discovery of Quinazoline and Quinoline-Based Small Molecules as Utrrophin Upregulators via AhR Antagonism for the Treatment of Duchenne Muscular Dystrophy. *J. Med. Chem.*, **2024**, 67, 11, 9260-9276.
2. **Ghosh, S[#]**, Sen, S[#], Jash, M., Ghosh, S., Jana, A., Roy, R., Mukherjee, N., Mukherjee, D., Sarkar, J. and Ghosh, S., Synergistic Augmentation of Beta-Lactams: Exploring Quinoline-Derived Amphipathic Small Molecules as Antimicrobial Potentiators against Methicillin-Resistant Staphylococcus aureus. *ACS Infect. Dis.*, **2024**, 10(4), pp.1267-1285.
3. Sen, S[#], **Ghosh, S[#]**, Jana, A[#], Jash, M., Ghosh, S., Mukherjee, N., Mukherjee, D., Sarkar, J. and Ghosh, S., 2024. Multi-Faceted Antimicrobial Efficacy of a Quinoline-Derived Bidentate Copper (II) Ligand Complex and Its Hydrogel Encapsulated Formulation in Methicillin-Resistant Staphylococcus aureus Inhibition and Wound Management. *ACS Appl. Bio Mater.* **2024**, 7, 4142-4161.

Manuscript Communicated and under Preparation

1. **Ghosh, S.**, Arshi, M.U, Ghosh, S., Jash, M., Mukherjee, N., Jana, A., Shastry, A., Karanth, D., Nishad.K., Rana, N.K., Bhattacharyya, S., and Ghosh, S., Advancing Antisense Oligonucleotide Delivery through Chemical Conjugation with Designed Short Non-Cationic Cell Penetrating Peptide using Click Chemistry for Duchenne Muscular Dystrophy. (*Manuscript under Revision*)
2. **Ghosh, S.**, Arshi, M.U., Ghosh, S., Jana, A., Roy, R and Surajit Ghosh. Development of mitochondrial targeted peptide based therapeutics for the treatment of Duchenne Muscular Dystrophy pathophysiology via enhancing myogenesis and mitigating fibrosis. (*Manuscript under Preparation*)
3. **Ghosh, S.**, Ghosh, S., Arshi, M.U., Shastry, A., Jana, A., Roy, R and Surajit Ghosh. Development of Micelles as Nano Delivery Vehicles for smart delivery of therapeutic leads for Treating Duchenne Muscular Dystrophy. (*Manuscript under Preparation*)

[#] Equal Contribution

Key Patents:

1. Ghosh, Surajit; **Ghosh, Surojit**; Arshi, Mohammad Umar; Ghosh, Satyajit; Rana, Nirmal; Bhattacharya, Sudipta. Development of Novel Utrrophin Upregulators for the treatment of Duchenne Muscular Dystrophy. Indian Pat. Appl. **IN 202311028857**.
2. Ghosh, Surajit; **Ghosh, Surojit**; Shastry, Arun; Arshi, Mohammad Umar; Ghosh, Satyajit; Jash, Moumita; Mukherjee, Nabanita; Jana, Aniket. Short Cell Penetrating Peptide Conjugated Antisense Oligonucleotides: A Potential therapeutic tool for the smart delivery of oligonucleotides for the treatment of Duchenne Muscular Dystrophy. Indian Pat. Appl. **IN202411032223**.
3. Ghosh, Surajit, **Ghosh Surojit**, Sen Samya, Jash Moumita, Roy Rajsekhar, Jana Aniket, Ghosh Satyajit , Mukherjee Nabanita , Sarkar Jayita. Quinoline derived small molecules SG-B-22 and SG-B-52 as beta lactam adjuvants. Indian Pat. Appl. **IN 202311054071**.
4. Ghosh, Surajit, Rana Nirmal Kumar, Bhattacharyya, Shastry Arun, Jain Anshul, Yadav Vinay Kumar, **Ghosh Surojit**. Composition of various small molecules for the treatment of Duchene Muscular Dystrophy. Indian Pat. Appl. **IN 202411000547**.

Conferences and Seminars Attended:

- Presented a poster at the American Chemical Society National Medicinal Chemistry Symposium (ACS-NMCS) at Seattle, Washington, USA, in June 2024.
-

Declaration:

I hereby declare that the information provided in this CV is true and accurate to the best of my knowledge and belief.

Signature: *Surajit Ghosh*

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