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A novel molecular scaffold resensitizes multidrug-resistant *S. aureus* to fluoroquinolones

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Nosocomial infections arising from opportunistic pathogens, such as *Staphylococcus aureus*, are growing unabated, compounded by the rapid emergence of antimicrobial resistance. Herein, we demonstrate a new molecular design that exhibits excellent activity against multidrug-resistant *S. aureus* with no cytotoxicity and resensitizes fluoroquinolones (FQ) towards FQ-resistant methicillin-resistant *S. aureus* strains, with DNA gyrase B as the likely molecular target as determined by molecular dynamics (MD) simulations.

Staphylococcus aureus, a Gram-positive bacteria, is a versatile opportunistic pathogen possessing a remarkable tenacity to overcome antibiotic action, leading to emergence of drug-resistant phenotypes which are responsible for causing infections under communities as well as under hospital settings worldwide.[1-3] *S. aureus* biofilms are known to escape host immune response to cause endocarditis, osteomyelitis, skin and soft tissue infections to sepsis, all of which are becoming increasingly difficult to treat due to rapid spread of antimicrobial resistance (AMR).[4] Given the current trends in discovery and approval of new antibiotics, it is estimated that drug-resistant superbugs could cause up to 10 million deaths worldwide by 2050. Such an alarming situation necessitates that in addition to continued discovery of broad-spectrum antibiotics, emphasis should also laid on new molecules that could synergize with known antibiotics and could potentiate them through favourable action on a variety of mechanistic targets.[5] Thus, continuous discovery of innovative antibacterial molecules, acting via novel mechanisms is an urgent unmet need. In this context, antimicrobial peptides and peptide-based scaffolds represent an effective strategy for the discovery of new antimicrobials with broad-spectrum action and decelerated emergence of drug-resistant strains.[6] Such peptide-containing structures exhibit significant therapeutic potential through an interplay of hydrophobicity and cationic charges,[7] and as an added advantage, they are potentially less prone to resistance, can counter virulence factors and modulate host immune response.[8] However, limitations such as toxicity, degradation and scale-up issues have to be considered prior to full-scale clinical development. Needless to mention, there is considerable interest and urgent need to seek new scaffolds with selective antibacterial action to mirror success stories such as teixobactin,[9] and polymixin.[10-12]

The menace of multidrug resistant (MDR) bacterial strains poses serious health challenge necessitating prolonged chemotherapeutic intervention, which further adds to the pre-existing load of resistant strains. Given that the last line of antibiotics have also reached the verge of MDR regime, it is imperative that newer targets and active structures be unveiled to support viable chemotherapeutic alternatives.[13] In addition to discovery of new antibiotic pharmacophores and active structures, it is also recognized that adjuvants having potential to enhance the efficacy of existing antibiotics or the ability to resensitize antibiotic-resistant strains, also add to the molecular repertoire against MDR strains.[14,15] Thus, new entities augmenting bactericidal activity of antimicrobials for resensitization of resistant strains would be an added incentive in antimicrobial drug development.

In this communication, we present design of a new antibacterial based on tryptophan dipeptide platform, conjugated to rhodamine B to impart cationic character and membrane permeability,[16] with a contribution from lipoic acid fragment purported to provide protection toward oxidative stress.[17] It is notable that studies have documented favourable effect of lipoic acid on membrane-bound renal enzymes against gentamicin toxicity in bacteremic mice model, and recently, a lipoic acid derivative was shown to suppress multidrug resistance in clinical isolates of *P. aeruginosa* through anti-ROS action.[18,19] Based on this premise, new chemical entities **1** and **2** were designed to maintain a balance of charge, hydrophobicity and lipophilicity, that could mimic AMPs followed by their antibacterial assessment. Further, DL-lipoic acid in **1** was substituted with R-lipoic acid for conjugation with L-tryptophan and D-tryptophan containing dipeptides, to afford two derivatives **1a** and **1b**, respectively (Figure 1). These analogs were screened against the ESKAPE pathogen panel (***E**nterococcus faecium*, ***S**taphylococcus aureus*, ***K**lebsiella pneumoniae*, ***A**cinetobacter baumannii*, ***P**seudomonas aeruginosa*, ***E**nterobacter* species) and the results are presented in Table 1. Compounds **1**, **1a** and **1b** exhibited antibacterial activity against *S. aureus* (Table 1), while individual fragments were devoid of activity.

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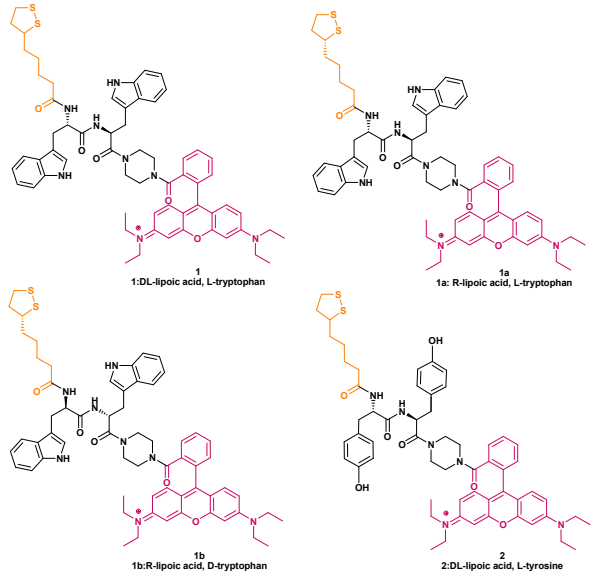

Figure 1: Molecular structures of **1**, **1a**, **1b** and **2**

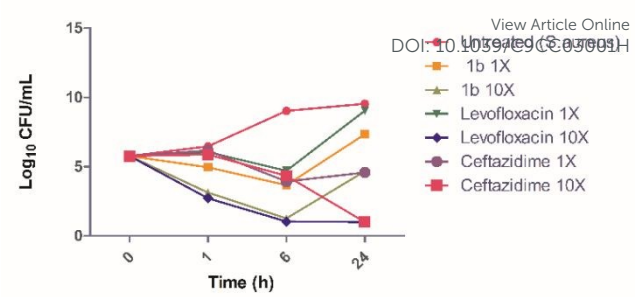

Figure 2: Time kill kinetics of **1b** against *S. aureus* (ATCC 29213). Each experiment was done in replicates and the values represent SD

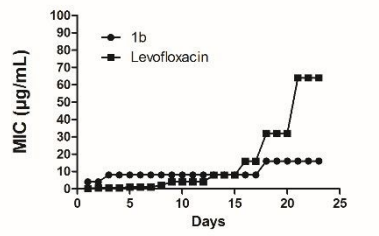

Figure 3: MIC values of levofloxacin and **1b** are shown against *S. aureus* ATCC 29213 along with the number of passages

Cytotoxicity of active compounds toward Vero cells was determined and the selected compounds were found to be non-toxic with high CC_{50} values, offering Selectivity Index >100 (Table 1). **3**, a control compound without dipeptide residue, with higher MIC value, was also toxic (Scheme S1, ESI; Table 1). Given the favourable antimicrobial activity and selectivity index of these three compounds, we continued with **1b** as presence of D-amino acid offers more stability. Consequently, antimicrobial action of **1b** was determined against several clinically drug-resistant strains of *S. aureus*. **1b** showed equipotent activity against methicillin-resistant *S. aureus* (MRSA) and vancomycin resistant *S. aureus* (VRSA), with MIC ranging from 2-4 µg/mL (Table 2). These results clearly suggested that observed antibacterial actions are possibly operating via other novel molecular target(s).

Time killing kinetics activity of **1b** with *S. aureus* ATCC 29213 was investigated, compared to ceftazidime and levofloxacin to determine whether observed antimicrobial activity was bacteriostatic or bactericidal in nature (Figure 2). Bactericidal antibiotics are desired over bacteriostatic since they eliminate infection faster as well as suppress development of drug resistance.[20,21] Upon examination, **1b** was found to exhibit conc. dependent bactericidal activity with ~6 log reduction in colony forming unit (CFU) in 24h, which was comparable to levofloxacin.

We further assessed the potential emergence of bacterial resistance against **1b**. The propensity of bacteria to generate resistance can be evaluated using serial exposure of organisms to antimicrobial agents.[22] We exposed *S. aureus* ATCC 29213 to serial passages of **1b** and levofloxacin and monitored the changes in MIC values over a period of 25 days. Despite repeated culturing in the presence of sub-inhibitory concentrations of **1b**, a stable resistant mutant was not isolated for **1b** and the MIC increased by only 2-fold (Figure 3). Comparatively, *S. aureus* expressed high level resistance to levofloxacin with a MIC of 32 and 64 µg/mL, within 17 and 20 days of culture, respectively. It must be noted that **1b** exhibits lower propensity to generate resistance as compared to levofloxacin.

DNA gyrase and topoisomerase IV are required for the processing of topological changes in bacterial DNA structure.

Table 1: Minimum Inhibitory Concentration (MIC) in µg/mL and Selectivity Index (SI) values against ESKAPE pathogen panel of compounds **1**, **2**, **3**, **1a**, and **1b** and their components

Compound	*E. coli* ATCC 25922	*S. aureus* ATCC 29213	*K. pneumoniae* BAA-1705	*A. baumannii* BAA 1605	*P. aeruginosa* ATCC 27853	Cell cytotoxicity (CC_{50}) against Vero cells (µg/mL)	Selectivity Index (CC_{50}/MIC)
1	>64	2	>64	>64	>64	> 1000	>500
2	>64	16	>64	>64	>64	<100	<6.25
1a	>64	4	>64	>64	>64	>400	>100
1b	>64	4	>64	>64	>64	>1000	>250
3	>64	8	>64	>64	>64	<80	<10
Rhodamine B	>64	>64	>64	>64	>64	ND	ND
DL-lipoic acid	>64	>64	>64	>64	>64	ND	ND
Levofloxacin	0.015	0.125	64	8	0.5	ND	ND

ND-Not determined

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Table 2: MIC (μg/mL) of **1b** against clinical multidrug resistant *S. aureus* strains including MRSA and VRSA along with control antibiotics

Bacteria	Strain	**1b**	Levofloxacin	Meropenem	Methicillin	Vancomycin
S. aureus	ATCC 29213	4	<0.5	<0.5	2	1
Vancomycin	VRS 1	4	32	>64	>64	>64
resistant	VRS 4	4	>64	>64	>64	>64
S. aureus (VRSA)	VRS 12	4	32	32	>64	>64
Methicillin resistant	NRS100	4	<0.5	>64	>64	1
S. aureus (MRSA)	NRS119	4	4	>64	>64	1
	NRS 129	4	<0.5	32	>64	1
	NRS 186	4	4	32	>64	1
	NRS 191	4	16	>64	>64	1
	NRS 192	4	8	64	>64	1
	NRS 193	4	32	>64	>64	1
	NRS 194	2	<0.5	8	32	1
	NRS 198	4	32	>64	>64	1

DNA gyrase are heterotetrameric proteins composed of two subunits, referred as A and B, and they serve as a validated antibacterial target for existing antibiotics as fluoroquinolones (FQ) that target DNA gyrase A.[23,24] In comparison, there is no clinically utilized antibiotic targeting gyrase B. In order to understand the possible mechanism of action of **1b**, computational studies were carried out to explore the binding of **1b** with DNA gyrase A (PDBID **5CDQ**), DNA gyrase B (PDBID **4P8O**), and ParE (Topoisomerase IV; PDBID **4URN**). Molecular docking studies were first conducted with **1b** against DNA gyrase A and gyrase B, which revealed that it favourably binds to gyrase B ATP binding site with a binding energy of -11 kcal/mol (Table S4, ESI). Interestingly, the affinity of **1b** was higher compared to known gyrase B inhibitors such as novobiocin and aminobenzimidazole by ~2-3 kcal/mol (Table S4, ESI).

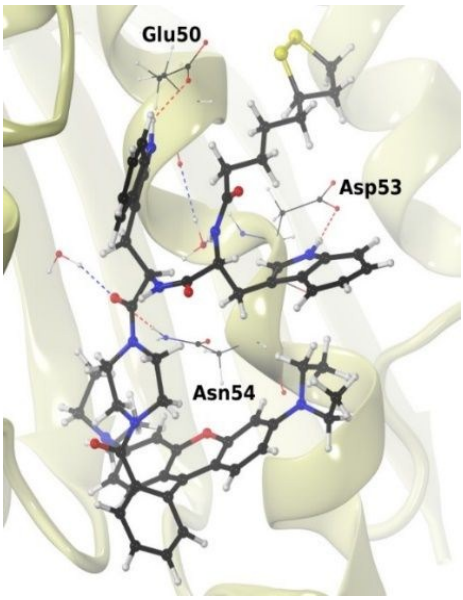

Figure 4: Snapshot from MD simulation of solvated **1b**: gyrase B complex. Crucial interactions of the ligand with the protein are identified. Hydrogen bond with protein are indicated in red dotted lines, while water mediated hydrogen bonds are drawn in blue dotted lines

On the other hand, intercalation of **1b** in DNA gyrase A (in complex with DNA) was found to be unfavourable (Figure S1, ESI). Further, the binding affinities of **1b** and novobiocin against ParE were also found to be similar (-9 kcal/mol, Table S5 and Figure S3, ESI). To further validate the binding mechanism, an all-atom molecular dynamics (MD) simulation was performed at 300 K for the docked conformation of **1b** in gyrase B, which was fully solvated with explicit water molecules. During the 50 ns trajectory, **1b** was found to reside at the active site of gyrase B without intermittent detachments (Figure 4; Figure S4, Movie S1, ESI), which indicates a strong affinity between **1b** and gyrase B, Asp53:OD1--N6:1b, Glu50:OE1/OE2--N4:1b, and Asn54:ND2--O1:1b (Figure 4; Figure S2, Table S6, ESI). Cumulatively, the aforementioned hydrophobic and hydrophilic interactions of **1b** with DNA gyrase B active site residues**,** as deciphered from molecular dynamics simulation, explain strong binding affinity observed in our docking computations.

Finally, we performed synergy experiments with FQ and **1b** against FQ-susceptible and FQ-resistant (FQ-R) MRSA NR10193 and NR10198 strains (Table S1-S3, ESI; Table 3), to give insight whether FQ and **1b** could "resensitize" FQ-resistant strain to FQ in active screens. **1b** and FQ synergized to "resensitize" NR10193 and NR10198 to various FQ drugs, when used in combination, to suggest putative medicinal applications of **1b** and FQ combination in treating FQ-resistant MRSA infections.

To further validate the observed synergy, time kill analysis was performed using 1X MIC of ciprofloxacin and **1b** against NRS10193 and NRS10198. The results of drug combination time kill analysis are depicted in Figure 5. As can be seen in Figure 5(a), the combination of 1X MIC **1b** (4 μg/mL) and ciprofloxacin (128 μg/mL) reduces ~1.5 $\log_{10}$ more CFU than either drug alone against NRS10193 at 24 h. A similar trend is also visible in NRS10198 where the combination reduces ~0.85 $\log_{10}$ more CFU than either compound alone at 24 h (Figure 5b). Taken together, it is clear that the combination of **1b** and FQ is synergistic and "resensitizes" FQ against FQ-resistant strains of *S. aureus.* Thus, **1b** and derivatives could serve as adjunct therapeutics for potentiating established antibiotics, since former targets a novel antibacterial target (gyrase B), thereby offering a double-pronged strategy for resistant strains.

Table 3: Synergism of **1b** against fluoroquinolones against FQ-resistant NRS10193 and NRS10198 (FIC: Fractional Inhibitory Concentration)

Drug in combination of **1b**	FQ-resistant NRS10193				FQ-resistant NRS10198			
	FIC A	FIC B	FICI----(FIC-A +FIC-B)	Inference	FIC A	FIC B	FICI----(FIC-A +FIC-B)	Inference
Levofloxacin	0.5	0.003	0.50	Synergic	0.25	0.007813	0.25	Synergic
Ciprofloxacin	0.5	0.001	0.50	Synergic	0.25	0.5	0.75	No interaction
Moxifloxacin	0.25	0.001	0.25	Synergic	0.25	0.25	0.5	Synergic

FIC A = MIC of **1b** in the combination with drug/MIC of **1b** alone, FIC B = MIC of drug in the combination with **1b**/MIC of drug alone, FICI-FIC Index

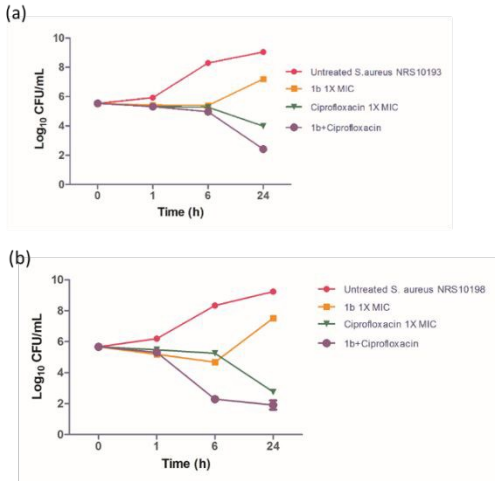

Figure 5: Combination time kill kinetics of **1b** against FQ-resistant strains (a) NRS 10193 and (b) NRS 10198. Each experiment was done in replicates and the values represent SD.

In conclusion, **1b** demonstrated potent *in vitro* MIC against multiple multidrug-resistant *S. aureus* strains, concentration-dependent rapid *S. aureus* bactericidal effect and found nontoxic toward mammalian cells up to 1000 µg/mL, as well as exhibited low propensity of generating resistance. In addition, we also demonstrated that **1b** potentiates FQ against FQ-resistant *S. aureus* with a possibility of developing combination therapy regime.[25] Taken together with molecular dynamics data, possible binding of **1b** to ATP-binding site of DNA gyrase B and its synergism with FQ antibiotics, and action against FQ-R *S. aureus* strains, **1b** could serve as an adjunct therapeutic molecule to potentiate different classes of antibiotics.

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Conflicts of interest

There are no conflicts of interest.

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