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Methicillin Resistance *Staphylococcus aureus* Treatment by Targeting Ribosomal RNA using modified linezolid: A Structure Based CADD Analysis Approach

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ABSTRACT

Microbial drug resistance is increasing worldwide and currently it is considered as great threat to human health and wellbeing. Superbugs methicillin resistance *Staphylococcus aureus* (MRSA) and vancomycin- resistant *Enterococci* (VRE) are developing resistance to most of the drugs. Linezolid is often used as choice of the drug to control MRSA and VRE infections. Long term use of linezolid has led to peripheral neurotoxicity and kidney toxicity. An attempt was made to modify the antibiotic linezolid and to study its interaction with 23S rRNA. Three modified linezolid ligands (MLL) were chosen based on drug likeness score. AutoDock vina was used to perform the docking analysis between 23S rRNA and the MLLs. ADMET properties of the linezolid and the modified ligand molecules were analyzed using admetSAR online server. Among the three MLLs, MLL-1 interacted with 23S rRNA and showed the least binding energy of -8.8 Kcal/mol with 3 hydrogen bonds. MLL-1 demonstrated no mutagenicity, tumorigenicity, irritability and reproductive effects. The AutoDock vina analysis of MLL-1 with 23S rRNA revealed that, better drug-likeness, increased ADMET property and high affinity towards 23S rRNA suggesting MLL-1 is a better drug of choice for the treatment MRSA infections.

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Introduction

Bacterial drug resistance has become a huge threat to human healthcare. Among the drug resistant bacterial pathogens, Methicillin Resistance *Staphylococcus aureus* (MRSA) is termed as superbugs. These MRSA microbes show resistance to various antibiotics such as, aminoglycosides, tetracyclines, fluoroquinolones, macrolides and vancomycin (Green et al., 2012). *Staphylococcus aureus*, gram positive cocci can be present in pairs, chains, and tetrads forming grape like clusters. *S.aureus* commonly found in the upper respiratory tract, skin and nasal area and acts as an opportunistic pathogen, that can cause severe infections. *β*-lactam antibiotics are normally the first choice for the treatment of staphylococcal infections. But lately some strains of *S.aureus* developed resistance to *β*-lactam antibiotics due to the presence of *β*-lactamase, a plasmid mediated enzyme that inactivates the *β*-lactam ring. Methicillin was used to overcome *β*-lactam resistance which resulted in occurrence of new variety of methicillin resistant strains known as MRSA. It was already reported that MRSA strains are producing β-lactamases which is one of the main reasons for its resistant activity against β lactam antibiotics (Haq et al., 2011). MRSA is a major cause of community acquired infections and results in high mortality rate in hospital acquired infections (Rossolini et al., 2014). MRSA causes several severe

life threatening pneumonias and blood stream infections with resistance to various antibiotics which makes the treatment ineffective causing economical problem due to need for substitution of highly expensive antibiotics.

This methicillin resistance is identified to be due to over expression of an additional protein, Penicillin Binding Protein 2a (PBP2a) (Livermore, 2000). PBP2a performs the catalytic function of PBP1 and it has very low affinity to β-lactam antibiotics. Since the primary mechanism of action of β-lactam antibiotics is to inhibit PBP1 and thereby prevent cell wall synthesis, in case of MRSA the role of PBP1 is performed by PBP2a which does not bind with β-lactam antibiotics, hence conferring the resistance to those antibiotics that targets PBP1 (Pantosti et al., 2007). Infectious diseases caused by multi-drug resistant pathogens are rapidly increasing which makes the treatment very difficult (Mainous et al., 2006).

Vancomycin is the first choice of drug for the treatment of MRSA infections, which inhibits cellwall synthesis in bacteria by binding to D-ala-D-ala protein. But some MRSA strains display resistance against vancomycin leading to Vancomycin Resistant *Staphylococcus aureus* (VRSA). Daptomycin is the second line of treatment for MRSA infections, which non-specifically inhibits protein synthesis, DNA replication and also damages the cell wall. Linezolid is the third line of treatment for MRSA infections. It is a synthetic antibiotic found to be very effective against MRSA which specifically inhibits bacterial protein synthesis, by blocking the 23S rRNA and prevents the formation of a functional 70S complex. The main disadvantage of this antibiotic is high cost and it also has various side effects like thrombocytopenia, peripheral and optic neuropathy, and lactic acidosis in patients receiving prolonged therapy. Tigecycline is the 4th line of treatment and it targets 30S Ribosome. Quinupristin is the 5th line of treatment for MRSA infection and it also targets 23S Ribosome and prevents protein synthesis.

Ribosomal ribonucleic acid (rRNA) is the RNA component of the ribosome that is essential for the synthesis of proteins in all living organisms. Ribosome consists of approximately 60% rRNA and 40% proteins. In prokaryotes a small 30S ribosomal subunit contains 16S ribosomal RNA and the large 50S ribosomal subunit contains two rRNA species 5S and 23S rRNAs. Among these, 23S rRNA has ribosomal peptidyl transferase activity (Mueller et al., 2000). They have also hypothesized that, antibiotics targeting bacterial cell wall synthesis seems to be less effective compared to the rRNA targeting antibiotics with regards to MRSA, due to rapid development of resistance against these antibiotics.

Computer-aided drug discovery/design now commonly known as CADD is the use of computational methods to design new drug molecules or to improve the already existing drug molecules. CADD has two types, first one is structure-based and second one is ligand-based. Structure-based CADD is to develop drug molecules specific for a particular target protein, while ligand-based CADD is to design new drug molecules in reference to the already existing drug molecules and to study its activity (Sliwoski et al., 2014). Poor pharmacokinetics and toxicity analysis are major reason for late-rejection in drug development. Good absorption, distribution, metabolism, excretion and toxicity (ADMET) properties are mandatory for any small molecule to be approved as drug (Waterbeemd and Gifford, 2003). Linezolid is a highly effective antibiotic against treatment of MRSA infections, but it has a huge drawback, i.e., high cost of production, and toxicity over long term use.In the present study, structure-based CADD was applied along with the ADMET analysis against bacterial 23S ribosomal RNA to develop an effective anti-MRSA drug.

Materials and methods

MRSA Culture

The Methicillin Resistant *Staphylococcus aureus* strain - ATCC 43300 was purchased from American Type Culture Collection (ATCC) and revived as per the guidelines given by the ATCC using the specific medium. Once pure colonies were obtained on the agar medium, a single colony was picked and inoculated in nutrient broth medium and incubated at 37˚C for overnight. This broth culture was used as inoculum for further experiment.

Antibiotic susceptibility testing (AST)

Disc diffusion assay was performed to study the antibiotic susceptibility of the ATCC 43300 strain. The inoculum was spread evenly across the Mueller Hinton Agar medium plate (Lawn culture) using a sterile cotton swab. The plate was left to stand for 10min, after which the antibiotic discs were placed on the medium. Cefoxitin, vancomycin, methicillin, linezolid, tetracycline and ciprofloxacin discs were used for this assay. The AST was performed based on the CLSI guidelines.

Table 1 Antibiotic susceptibility testing of MRSA strain- ATCC 43300

Antibiotic disc	Zone of Inhibition (mm)	Inference	Protein Target
Cefoxitin	12	Resistant	PBP1
Vancomycin	12	Resistant	D-ala-D-ala
Methicillin	17	Resistant	D-ala-D-ala
Linezolid	24	Sensitive	30S ribosome
Tetracycline	24	Sensitive	23S ribosome
Ciprofloxacin	25	Sensitive	DNA Gyrase

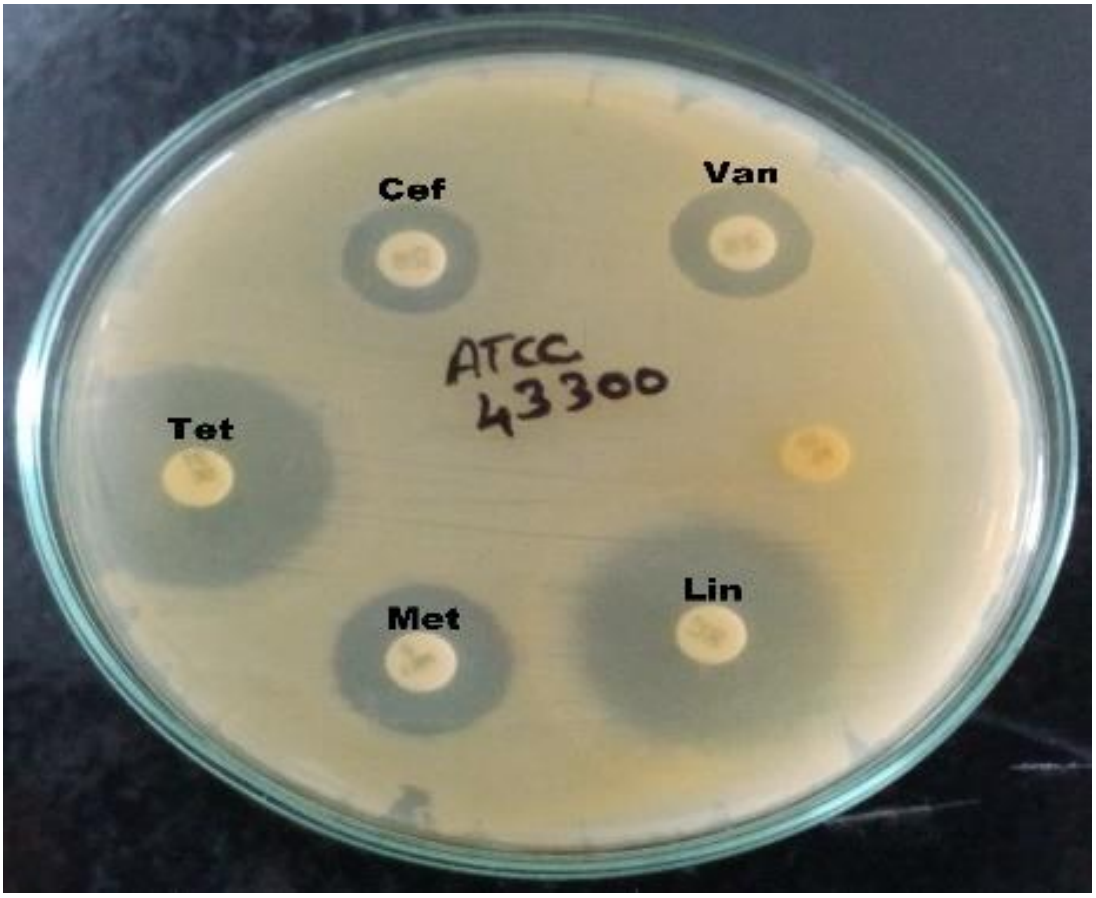

Fig. 1. Antibiotic susceptibility (zone of inhibition) testing of MRSA strain-ATCC 43300

[Cef:Cefoxitin, Van:Vancomyin, Met:Methicillin, Lin:Linezolid, Tet:Tetracycline]

Table 2. Osiris Property Prediction results of linezolid and the modified ligand molecules

Parameter	Linezolid	MLL-1	MLL- 2	MLL-3
Mutagenic	GREEN	GREEN	GREEN	GREEN
Tumorigenic	GREEN	GREEN	GREEN	GREEN
Irritant	GREEN	GREEN	GREEN	GREEN
Reproductive Effects	GREEN	GREEN	GREEN	GREEN
Druglikeness	-4.08	-2.61	0.46	1.91
Drug Score	0.45	0.48	0.73	0.86

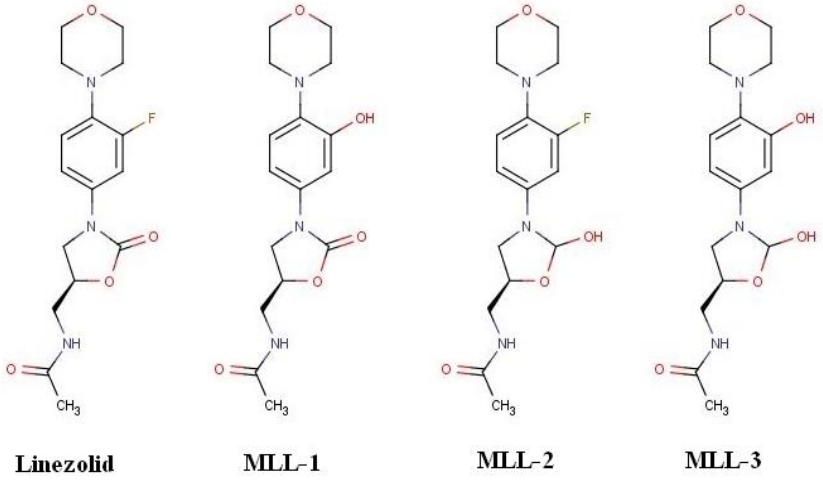

Figure 2. Chemical structure of linezolid and modified ligand molecules

Drug-likeness analysis

Linezolid structure was taken as the lead molecule and modifications to this lead molecule was carried out within the Osiris Molecular Property Prediction tool to obtain increased drug-likeness [www.organic-chemistry.org]. The modified structure that displayed better drug-likeness and good drug score was then drawn in Marvin sketch tool and saved as .pdb files for docking studies.

ADMET analysis

ADMET properties of the lead molecule and the modified ligand molecules were analyzed using admetSAR online server [http://lmmd.ecust.edu.cn:8000]. This is a comprehensive source and free tool for evaluating the chemical ADMET properties.

AutoDock vina

AutoDock vina was used to perform the docking analysis between 23S rRNA from MRSA strain and linezolid. 23S rRNA from PDB database (PDB ID: 5ADY) was taken as target macromolecule. Linezolid structure was downloaded from ZINC database (http://zinc.docking.org) with ZINC ID: 2008866. The modified ligands were sketched in Marvin sketch tool and saved as .pdb files for docking analysis. The results of the dockings were analyzed using PyMol software.

Results

Antibiotic susceptibility testing

Among the six tested antibiotics, the MRSA strain ATCC 43300 was resistant to cefoxitin, vancomycin and methicillin. The strain was sensitive/susceptible to linezolid, tetracycline and ciprofloxacin. Antibiotics susceptibility of MRSA strain is given in Table 1. The zone of inhibition exhibited by antibiotics against MRSA strain 43300 is shown in Figure 1. AST was performed and the results were interpreted according

Table 3. ADMET SAR analysis of Linezolid and modified ligand molecules

Parameters	Linezolid	MLL-1	MLL-2	MLL-3
Absorption	BBB+	BBB-	BBB+	BBB+
Human Intestinal Absorption	HIA+	HIA+	HIA+	HIA+
Caco-2 Permeability	Caco2+	Caco2-	Caco2+	Caco2-
P-glycoprotein Substrate	Substrate	Substrate	Substrate	Substrate
P-glycoprotein Inhibitor	Inhibitor	Non-inhibitor	Inhibitor	Inhibitor
	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
Renal Organic Cation Transporter	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
Metabolism				
CYP450 2C9 Substrate	Non-substrate	Non-substrate	Non-substrate	Non-substrate
CYP450 2D6 Substrate	Non-substrate	Non-substrate	Non-substrate	Non-substrate
CYP450 3A4 Substrate	Substrate	Substrate	Substrate	Substrate
CYP450 1A2 Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
CYP450 2C9 Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
CYP450 2D6 Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
CYP450 2C19 Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
CYP450 3A4 Inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
CYP Inhibitory Promiscuity	High CYP Inhibitory Promiscuity	Low CYP Inhibitory Promiscuity	High CYP Inhibitory Promiscuity	Low CYP Inhibitory Promiscuity
Toxicity				
Human Ether-a-go-go-Related Gene Inhibition	Weak inhibitor	Weak inhibitor	Weak inhibitor	Strong inhibitor
	Inhibitor	Non-inhibitor	Inhibitor	Non-inhibitor
AMES Toxicity	Non AMES toxic	Non AMES toxic	Non AMES toxic	Non AMES toxic
Carcinogens	Non-carcinogens	Non-carcinogens	Non-carcinogens	Non-carcinogens
Fish Toxicity	High FHMT	High FHMT	High FHMT	High FHMT
Tetrahymena Pyriformis Toxicity	High TPT	High TPT	High TPT	High TPT
Honey Bee Toxicity	Low HBT	Low HBT	Low HBT	Low HBT
Biodegradation	Not ready biodegradable	Not ready biodegradable	Not ready biodegradable	Not ready biodegradable
Acute Oral Toxicity	III	III	III	III

Table 4. AutoDock vina analysis of linezolid and modified ligand molecules

Lignad	BindingEnergy (Kcal/Mol)	Number of Hydrogen bonds
Linezolid	-8.5	6
MLL-1	-8.8	3
MLL-2	-8.7	1
MLL-3	-8.7	2

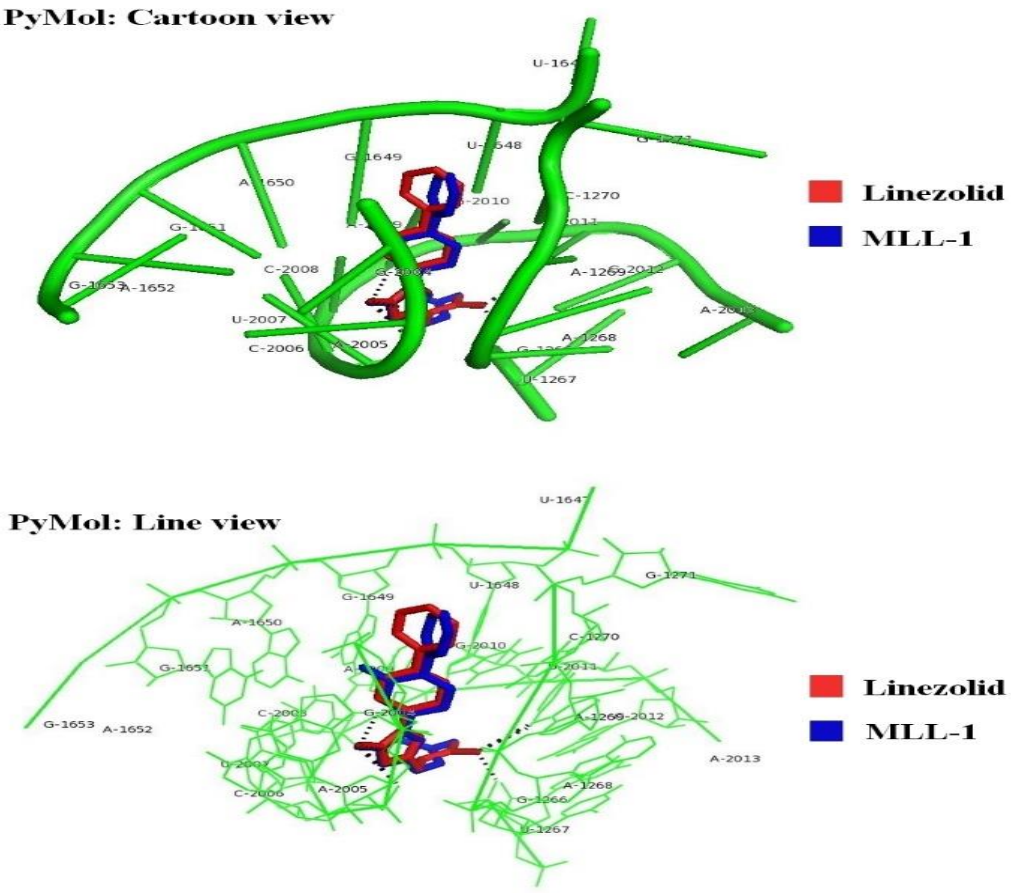

Figure 3. Binding sites of linezolid and MLL-1with 23S rRNA

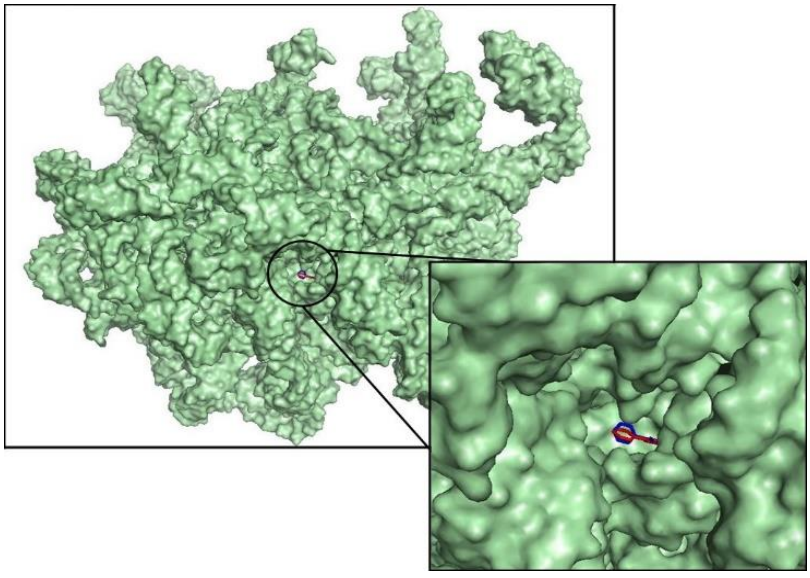

Figure 4. Positioning of Linezolid and MLL-1 on the 23S rRNA

to the guidelines of National Committee for Clinical Laboratory Standards (NCCLS).

Osiris Drug-likeness analysis

Among all the modifications analyzed, three modified ligands with improved or better drug-likeness and drug score was considered for further studies. These ligands were designated as modified linezolid ligand 1 (MLL-1), modified linezolid ligand 2 (MLL-2) and modified linezolid ligand 3 (MLL-3). These selected four modified linezolid ligand molecules demonstrated no mutagenicity, tumorigenicity, irritability and reproductive effects. Osiris drug-likeness properties for the selected molecules are tabulated in Table 2. The chemical structure of the analyzed molecules are represented in Figure 2.

ADMET analysis

Based on the admetSAR analysis, all the three modified ligand molecules were safe to be considered as drug molecules. Notably, MLL-1 molecule alone demonstrated negativity to cross the blood brain barrier. Signifying reduction in the side effects related to nervous system was observed. Results of admetSAR analysis are given in Table 3.

AutoDock vina Results

The molecular docking analysis demonstrated that, all three modified molecules has increased affinity / inhibition potential towards the 23S rRNA. The anti-MRSA activity of linezolid and modified molecules is given in Table 4. Notably, MLL-1 interacted with 23S rRNA and demonstrated the least binding energy of -8.8 Kcal/mol with 3 hydrogen bonds. The binding site of linezolid and MLL-1 are the same, but MLL-1 has increased affinity (Figure 3). Both linezolid and MLL-1 are binding to the centre pocket in the 23S rRNA (Figure 4). MLL-2 interacted with 23S rRNA and showed the binding energy of -8.7 Kcal/mol with 1 hydrogen bond. MLL-3 interacted with 23S rRNA and showed the binding energy of -8.7 Kcal/mol with 2 hydrogen bonds.

Discussion

Interpretations from the AST assay suggests that, the studied MRSA strain ATCC 43300 is resistant to all the tested antibiotics that target the cell wall synthesis, while it was susceptible for antibiotics that target protein synthesis and DNA replication. Several reports are available that, MRSA are capable of expressing β-lactamases that makes it resistant to β- lactam antibiotics. Hence, targeting bacterial cell wall synthesis might not be effective in controlling multi-drug resistant pathogens such as MRSA. As it is evident from the AST analysis, MRSA strain ATCC 43300 is highly susceptible to linezolid and tetracycline, both targeting the bacterial ribosome and inhibits protein synthesis in bacteria. The importance of ribosome targeting antibiotics and the mutations that are causing resistance in bacteria against these antibiotics was already been reported (Lambert, 2012). A review report was also available on the importance of ribosome targeting antibiotics and their effectiveness in control of multi-drug resistant pathogens (Wilson, 2014). The review also summarizes the recent reports on ribosomal targeting antibiotics, resistance mechanisms and ribosomal target specific drug development methods. Ribosomal targeting antibiotics are becoming quite popular in the recent drug development studies due to its effectiveness against multi-drug resistant pathogens. Considering these facts, it could be suggested that, ribosomal targeting antibiotics could be a promising approach for treatment of MRSA infections.

Linezolid is a well- known synthetic antibiotic that effectively controls the MRSA infections. The major drawback of linezolid is its high production cost and its mild neural toxicity over long term use. Based on the observed results, the MLL-1 shows better affinity and lower toxicity than that of linezolid. Substitution of fluorine atom instead of OH has increased the affinity towards 23S rRNA and has increased the drug likeness and ADMET properties, suggesting that the MLL-1 would be a better choice of drug molecule against MRSA. MLL-1 lost its ability to cross the blood brain barrier and this ADMET property could be responsible for its toxicity reduction. Initial screening in Osiris property predictor, also suggested that the MLL-1 has increased drug-likeness and drug score compared to linezolid. Comparison of computational analysis results, MLL-1 is considered to be highly effective than the parent molecule and is observed to have better drugability and reduced toxicity.

Conclusion

This study concludes that, targeting bacterial rRNA is an effective approach in treatment of MRSA infections. The results of AutoDock vina analysis of MLL-1 with 23S rRNA revealed that, better drug-likeness, increased ADMET property and high affinity towards 23S rRNA suggesting MLL-1 is a better drug of choice for the treatment MRSA infections. Also, the target specific drug design for 23S rRNA would greatly increase the chances of obtaining novel antibiotics for

better treatment of MRSA.

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Conflict of Interest
Authors declare that there is no conflict of interest.

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