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Research Article

Identification and molecular characterization of drug targets of methicillin resistant *Staphylococcus aureus*

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Abstract
Antimicrobial resistance is a major world health concern and drug-resistant *Staphylococcus aureus* is a serious threat. Due to the emergence of multidrug-resistant bacterial strains, there is an urgent need to develop novel drug targets to meet the challenge of multidrug-resistant organisms. The main objective of the current study was to determine molecular targets against *S. aureus* using by computational approach. *S. aureus* was cultured in brain heart infusion broth medium and MRSA (Methicillin resistant *S. aureus*) protein was extracted acetone-sodium dodecyl sulfate method. The cell lysate was treated with various antibiotics and proteinase K stable proteins were analyzed. The molecular weight of Geninthiocin-targeted protein of interest in *S. aureus* ranged from 46 to 50 kDa. A prominent protein band in SDS-PAGE indicated that the protein corresponding 50 kDa was resistant against proteinase K. The SDS-PAGE separated sample was excised and trypsinated, and the peptides were characterized using Nano Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) analysis. Spectrum with clusters of molecular peptides and peptide fragments ranging from 110.0716 to 1002.7093 mass/charge ratio (m/z) were displayed against intensity or relative abundance in the excised gel band. The spectral data from nano LC-MS/MS was subjected to mascot search in the NCBIprot database (taxonomy-bacteria (eubacteria), resulting in seven bacterial proteins. Geninthiocin target proteins were determined against MRSA. To conclude, antibiotic target proteins were identified using a machine learning approach and these targets may have a lot of applications in developing a novel lead molecule against drug-resistant bacteria.

Keywords: Bacteria, Drug resistance, Drug target, Geninthiocin, Virulence

INTRODUCTION

Staphylococcus aureus causes both community-acquired and nosocomial infections. It is the primary cause of respiratory tract and surgical site infections and the second leading cause of infection worldwide (Richards *et al.,* 1999). The microbial resistance against various antimicrobials, including oxacillin, and methicillin, with *S. aureus* is challenging to treat with currently available antibiotics (Kifelew*et al*., 2020). Penicillin-resistant bacteria were determined among hospital pathogens to be very small; however, as the application of penicillin increased significantly, drug resistance spread to the community and into hospitals(Chambers, 2001). More than 80% of the hospital- and drug acquired *S. aureus* were highly resistant to penicillin in the 1960s. Then MRSA (Methicillin resistant *S. aureus*) emerged in recent times, and wave-like emergence pattern was reported(Strausbaugh*et al*., 1996). National Nosocomial Infections Surveillance system's data reported that the number of *S. aureus* strains that are resistant tomethicillin has dramatically increased to 59.5%-64.4% in intensive care units(Klevens*et al.,* 2006). The presently well-known drug Target of *Staphylococcus* sp. includes penicillin-binding protein of peptidoglycan biosynthesis pathway. Earlier, β-lactam antibiotics were highly effective against *Staphylococcus* sp.. Moreover, due to biosynthesis of a modified form of penicillin-binding protein and biosynthesis of β-lactamase, those drugs are not active in the present day (Kong*et al*.,2010).Worldwide attention is being made to examine the possibilities of a previously ex-

ploited important bacterial mechanism. The molecular basis for these targets has also been investigated (Takahashi and Nakashima, 2018).

Actinomycetes, particularly *Streptomyces* species, have proven to be a novel, high-impact source of valuable compounds. They have resulted in many clinically critical antibacterial substances, including streptomycin, actinomycin, and streptothricin (Schneider *et al.,* 2018). *Streptomyces* species are Gram-positive bacteria that inhibit in various environments and exhibit physical characteristics comparable to fungal groupings. Layers of hyphae with spore chains are found in *Streptomyces* species which are Gram-positive forms that have a specialized, sophisticated, and interconnected metabolism and are particularly prone to this. Antifungal, antiviral, anti-hypertensive, and, most importantly, antibiotic and immunosuppressives are produced by *Streptomyces*species (Emerson *et al.,* 2012). *Streptomyces* species produce different types of thiopeptide and have antibiotic effects against numerous Gram-positive bacteria. The thiopeptide family has recently acquired popularity because of its effectiveness against various drug-resistant infections, including MRSA. Thiopeptides have attracted the interest of researchers and industry due to their distinctive activities and efficacy against Gram-positive bacteria, and they can be actively considered as an alternate for many drug-resistant infections(Just-Baringo *et al*., 2014). Geninthiocin is a thiopeptide having a macrocyclic core moiety of 35 members. It is effective against Gram-positive (G+) bacteria (Li *et al.,* 2019). The antibacterial agents have a small number of molecular targets, but they are almost all involved in macromolecular production pathways that cannot be supplied by feeding intermediate (Silver, 2016). A variety of approaches for identifying small-molecule targets have been developed, with affinity chromatography being the most often utilized (Pai *et al.,* 2015). Affinity for drugs DARTS is helpful for complex protein samples in which non-specific protein-ligand interactions occur despite the existence of a large number and variety of proteins in the mixture. In addition, any small molecule in its native form or chemical alterations to the ligand that is required for target identification can be employed in DARTS. Affinity chromatography and most other affinity-based approaches for target identification, on the other hand, necessitate chemical modification of each ligand, which poses a number of challenges (Lomenick *et al.,* 2011).

Nanoscale liquid chromatography linked to tandem mass spectrometry (nano LC-MS/MS) has become a critical instrument in proteomics study. It is more sensitive than traditional LC-MS/MS, allowing it to analyze peptide mixtures in sample-constrained scenarios e.g., proteolytically digested proteins isolated by two-dimensional gel electrophoresis, even though technical challenges associated with low flow rates of chromatographic separation are limited (Gaspari and Cuda, 2011).The main objective of the study was to analyze geninthiocin activity against multidrug-resistant *S. aureus* and to determine drug targets by computational approach.

MATERIALS AND METHODS

Microorganisms and culture condition

The American Type Culture Collection (ATCC) 33591 type MRSA strain was cultured on Brain Heart Infusion broth (BHI) Medium (Himedia, Mumbai, India), pH 7.4, which included (g/L) 20 g of calf brain infusion, 25 g of beef heart infusion, 10 g of protease peptone, 2 g of dextrose, 5 g of $NaCl_2$, and 2.5 g of disodium phosphate. Broth cultures were incubated at 37 °C on a rotatory shaking incubator at 160 rpm for 24 h (Vijayaraghavan *et al*., 2015)

Extraction of geninthiocin

A geninthiocin producing *Streptomyces* strain ICN19 was isolated in our earlier studies from marine sediment collected at a depth of two feet on the Chinnamuttom coast of Kanyakumari, India(Iniyan*et al.,* 2019). Geninthiocin was purified and characterized in our previous study; the purified compound and the purified antimicrobial compound were used for this study. The structure of Geninthiocin was determined by 1D and 2D Nuclear Magnetic Resonance spectra (PubChem 2021) and High-resolution electrospray ionization mass spectrometry analysis. The study sequenced the whole genome of strain ICN19^{T},whichwas9,010,366 bp in sizeand consisted of 98 RNA genes and 7420 protein-coding genes(Iniyan*et al.,* 2021).

Extraction of MRSA proteins by acetone/SDS method

The MRSA cells were separated from the BHI medium (M211, Hi-Media, Mumbai, India) after 24 h culture by centrifugation (7000 *g* × 10 min). It was then washed twice with phosphate-buffered saline (PBS, pH 7.4). After that, the cells were suspended in 5 mL of ice-cold acetone (analytical grade). It was allowed for 5 minutes on ice and centrifuged (7000 ×g). Then it was incubated for 2 minutes in 0.5 ml of 1% sodium dodecyl sulphate (SDS) (Saumya and Paul, 1983).Protein estimation was performed using bovine serum albumin as the standard (100 – 1000 µg).

Preparation of protein samples with geninthiocin, nalidixic acid, and amoxycillin

The cell lysate (99 µl) was mixed with 1 µl Dimethyl sulfoxide (DMSO). It was further mixed with Geninthiocin, Nalidixic acid, and Amoxycillin using a rotator for 15 –30 minutes at room temperature (28 ± 2 °C). Proteinase K was added with lysate at various concentrations

(2-3µl), and to the control enzyme was not incorporated. Phenylmethylsulfonyl fluoride was added to the non-digested sample. Samples were incubated at 28 ± 2 °C for 30 minutes with the proteinase K, and PMSF was used to stop the reaction and incubated on ice for 10 minutes(Pai *et al*., 2015).

Sodium dodecylsulfate-polyacrylamide(SDS-PAGE) gel electrophoresis

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Model : Dual Mini Gel System, Cat No. : 106724GB, GeNeI, Bangalore, India) analysis was performed to purify the cell lysate of MRSA. The separating gel (11%) was used for the separation of protein and stained with Coomassie Brilliant Blue-R250.

NanoLiquid chromatography with tandem mass Spectrometry(LC-MS/MS) analysis

A LC-MS machine, 1D nano-LC (Agilent),Nanomate Triversa (Advion), LTQ – Orbitrap Discovery (Thermo) Sonicator was used for analysis. Water (LC/MS grade-Fluka), Acetonitrile (LC/MS grade –Fluka), and Formic Acid (LC/MS grade Fluka) were LC/MS grade. The SDS-PAGE separated sample was excised, and trypsinated, and the peptides were reconstituted in a 0.1 percent formic acid. It was vortexed for 1 hour, then centrifuged at 10,000 rpm for 5 minutes.MS scan/survey scan was analyzed in FT mode at 30,000 FWHM and MS/MS in the linear ion trap (https://www.ccamp.res.in/mass_spectrometry_services/ sites/default/files/nano LC-MSMS%20prot ocol.pdf). MS1 mass range 300-2000 with lock mass: 445.1200 (corresponding to polysiloxane), DDA settings: 1 MS survey scan followed by 5 MS/MS scans, exclusion duration120.0sec conditions to trigger MS/MS: minimum signal intensity(Van Chi and Dung, 2012). The experimental data were searched using Mascot v1.8 software against the NCBIprot and protein database, in which the criteria were based on the manufacturer's definitions and the proteins were identified (http://www.matrixscience.com). The parameters for identification of the peptide spectrum were set as follows: enzymatic cleavage with trypsin; 2 potential missed cleavage; a peptide and fragment mass tolerance of ± 0.6Da, and fixed modification of carbamidomethyl (cysteine); variable modification Acetyl (Protein N-term) and oxidation (M). The proteins were identified using MOWSE scoring system with a confidence level of 95% was utilized, which revealed at least two matched peptides with a high score (http://www.matrixscience.com)

RESULTS AND DISCUSSION

The 2 D structure of Geninthiocinis shown in Fig.1 (Pub Chem 16129809) and the properties of predicted Geninthiocin are described in Table 1.

Analysis of geninthiocin activity against MRSA proteins using SDS-PAGE

Geninthiocin, which remained the antibiotic-thiopeptide of interest, was isolated from a marine *Streptomyces* ICN 19. It provided the highest protection to the potential protein therapeutic target by closely adhering to it against protease K. It revealed that Geninthiocin, a thiopeptide has effective antibacterial activity against MRSA and the result was described. The results of the pathogen's maximal output of cell proteins imply that the acetone-SDS chemical approach for protein extraction can be employed for protein target identification research (Bhaduri *et al.,* 1983). One-dimensional SDS PAGE was used to determine molecular weight and separation of protein subunits of interest (Fig. 2).

Nano LC-MS/MS analysis of expressed proteins

The separated proteins were analyzed using mass spectrometry to determine amino acid sequences and post-translational modifications (Gallagher, 2012). Although 1D gel analysis lacks the separation capacity of 2 D electrophoresis, it has certain advantages over 2 D analysis. The ability to distinguish hydrophobic proteins from those that can handle much higher protein loads and the capacity to determine proteins linearly over the pH scale are advantages (Lai *et al*., 2003). Denoising Algorithm based on Relevance network Topology (DARTS) involves treating aliquots of cell lysate with the drug of interest and either vehicle control or an inert analogue, then digesting the proteins in the cell lysate with proteases to a restricted extent. The proteins contained in each band were subsequently identified using Mass spectrometry (MS). Recent examples include identifying a novel protein target for disulfiram, an FDA-approved medicine used to treat chronic alcoholism,

Table 1. Computed properties of geninthiocin

Property Name	Property value
Molecular Weight	1132.092 g/mol
XLogP3-AA	1.2
Hydrogen Bond Donor Count	12
Hydrogen Bond Acceptor Count	21
Rotatable Bond Count	7
Exact Mass	1131.325 g/mol
Monoisotopic Mass	1131.325 g/mol
Topological Polar Surface Area	478 A^2
Heavy Atom Count	81
Formal Charge	0
Complexity	2700
Isotope Atom Count	0
Defined Atom Stereocenter Count	0
Undefined Atom Stereocenter Count	3
Defined Bond Stereocenter Count	1
Undefined Bond Stereocenter Count	0
Covalently-Bonded Unit Count	1

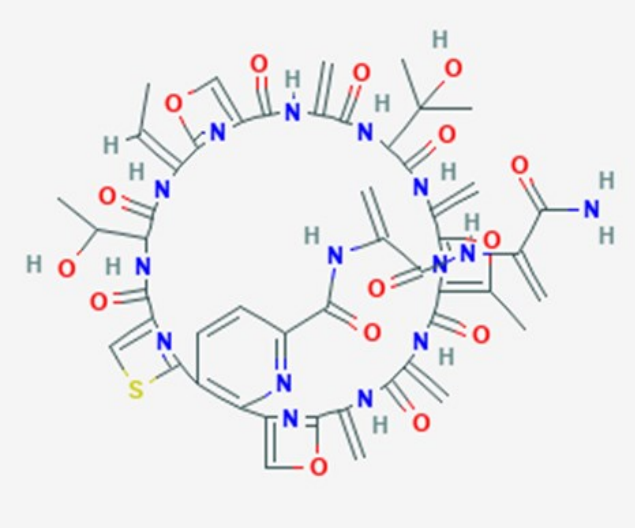

Fig. 1. *2-D structure of geninthicin isolated from a marine Streptomyces sp. ICN19 (PubChem 16129809)*

and a novel protein target for the metabolite - ketoglutarate, both using this unbiased DARTS technique. Potential small-molecule targets were identified via the mass spectrum. No thick protein bands were determined in lanes 4 and 5, which validated the experimental design. Amoxicillin and nalidixic acids did not show any protection against protease K. A prominent protein band in SDS-PAGE indicated that the protein corresponding 50 Da was resistant against proteinase K. This showed that the Geninthiocin-targeted protein has a molecular weight of 46 to 50 kDa. Spectrum with clusters of molecular peptides and peptide fragments ranging from 110.0716 to 1002.7093 mass/charge ratio (m/z) were displayed against intensity or relative abundance in the excised thick gel band in lane 3 was subjected to nano LC-MS/MS analysis (Fig. 3). The number of MS/MS spectra and peptide spectrum matches (PSMs) generated are critical indicators of metaproteomics data quality. The identification of protein groups is based on MS/MS spectra that match peptides in a database comparison, resulting in PSMs(Hinzke*et al.,* 2019).

Drug target identification by computational approach

The isolated spectral data from nano LC-MS/MS was subjected to mascot search in NCBI-prot database (taxonomy-bacteria (eubacteria) resulted in seven bacterial proteins. Thechromatographic separation of proteins from *S. aureus*with mass Spectrophotometer studywas used for the characterization of various target proteins, including *S. aureus* surface protein G (Gatlin *et al*., 2006). HPLC-MS/MS-based method was used for the determination of expressed proteins from *S. aureus* (Schelli *et al*., 2017).

There are several methods for identifying the proteins in the sample, and the most commonly used method is the search for uninterpreted MS/MS data. Raw data of peptide spikes obtained from nano LC-MS/MS analyzed using MASCOT in NCBIprot with a search type of MS/MS Ion search, obtained seven possible proteins, and accession numbers were listed based on overall protein score. The obtained spectrum was analyzed with the Mascot search engine, which predicted seven possible proteins of the charged fragment ions from the protein database. The predicted proteins are described in Table 2. Spectral data is converted into protein entities by Mascot, which is a software package from Matrix Science. The software compares the observed spectra to a database of known proteins and determines the most likely matches (Asmi *et al*., 2021). The mascot program calculates an overall Protein Score, which is the sum of all detected mass spectra scores. The amino acid sequences within that protein can be compared to these scores. As described in Table 2, the protein scores for the seven potential proteins were very high. In this study, the excised gel band showed about 50kDa, which remained to be a match for the mass of the proteins obtained after the spectral analysis; except for 50 S ribosomal protein L2 and Ton B- dependant receptor, which may be evidence of a post-translational modification or cleavage event. The seven proteins obtained after the search with NCBIprot (taxonomy-bacteria) indicated the number of MS/MS spectra that matched this protein.

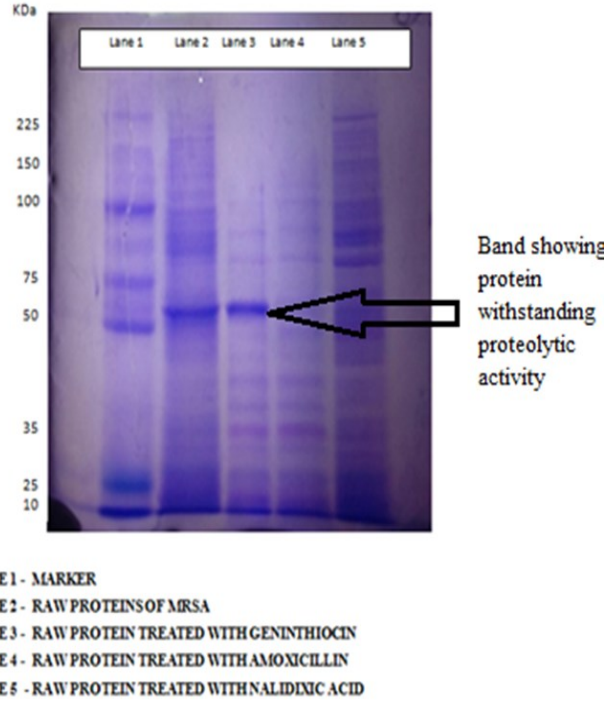

Fig. 2. *SDS-PAGE with treated proteins subjected to isolation resolved by 1D standard SDS– 11% PAGE, stained with Coomassie brilliant blue R-250 and showing the Target Protein withstanding the proteolytic activity*

Table 2. Nano LC-MS/MS spectrum analysis of proteins and predicted proteins

Protein Description	Sequence coverage	Mascot Score	Mass	Accession no.
Dihydrolipoyl dehydrogenase	0.26	472	50080	RCL62640.1
TonB-dependent receptor	0.2	358	121979	EKY30101.1
Acetyl-CoA hydrolase	0.4	331	53676	ABP80444.1
Amino acid ABC transporter substrate-binding protein	0.33	283	33400	OHC17518.1
homogentisate 1,2-dioxygenase	0.32	263	43398	WP_011912060.1
50S ribosomal protein L2	0.37	195	30291	WP_000511580.1
Polyamine ABC transporter substrate-binding protein	0.31	183	40098	WP_011911317.1

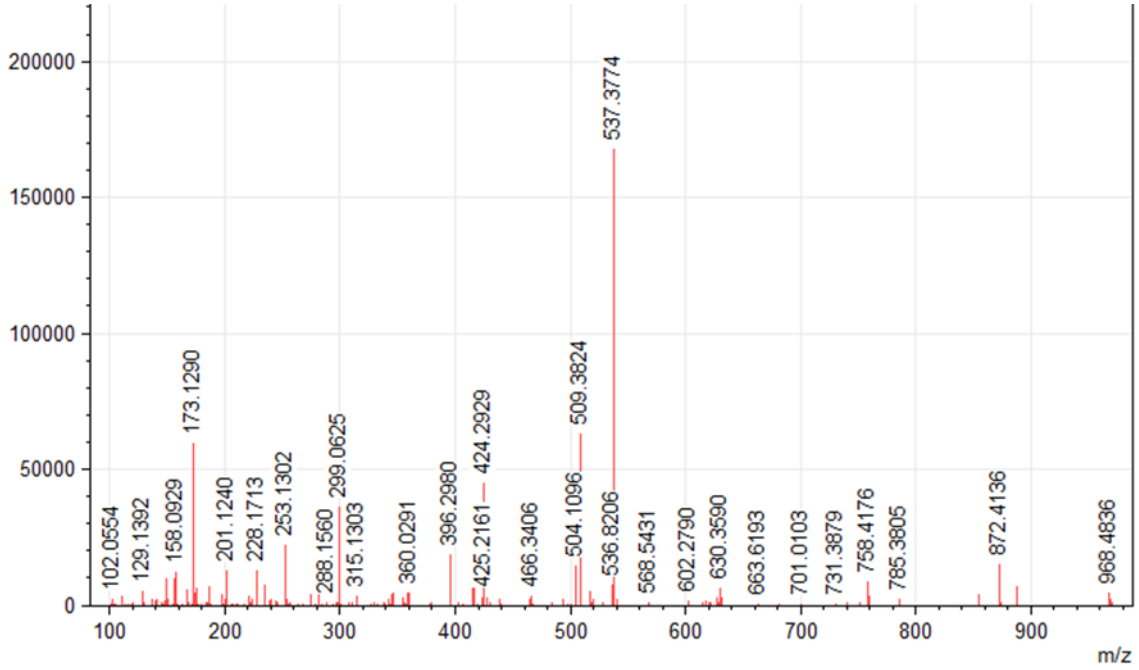

Fig. 3. *Showing Nano LC-MS/MS spectrum of peptides from the excised band from the SDS - PAGE*

Conclusion

Treatment of MRSA infection is highly complicated and causes various ailments (including septicemia), necessitating the development of a new medication that can be utilized efficiently. Geninthiocin emerged as a novel therapeutic alternative in the search for an efficient antibiotic since it exhibited anti-MRSA efficacy, despite its disadvantages, such as a large molecular weight and poor water solubility. As most bacterial drug targets are proteins, this study aimed to attempt MRSA targets for Geninthiocin. Drug Affinity Responsive Target Stability was used in this study because it can be utilized more successfully for small molecule target identifications. The Mascot-NCBIprot database analysis showed seven potential protein targets. Although the potential MRSA protein targets for geninthiocin were determined in this study, furthermore study is needed to determine the affinity of geninthiocin and clinical studies that could pave the way for geninthiocin to be used to treat MRSA infections.

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Conflict of interest

The authors declare that they have no conflict of interest.

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