ABSTRACT

Due to the extensive arsenal of mechanisms acquired by bacterial pathogens, during evolution, to become resistant to most or all of the current antibiotics, antimicrobial resistance (AMR) is now an alarming threat to our society, making common infections complicated and untreatable. At present, more than 700, 000 people per year across the globe succumb to drug-resistant infections and this number is expected to reach 10 million deaths annually (more than cancer and cardiac diseases) by the year 2050, if no action is taken. The World Health Organization (WHO) has announced a list of drug-resistant bacteria to pave the way for the development of new antibiotics. Among them, 'ESKAPE' (*Enterococcus faecium Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species*) pathogens are listed under the top priority bugs and are responsible for major nosocomial and community infections. The modern chemistry approaches and chemical genomics have been unsuccessful to provide enough antibiotics. In stark contrasts to this, the natural products have been gifted with remarkable chemical complexities and biological activities. Our modern antibiotic armamentarium was built from the natural products of microbes, especially *Streptomyces* spp, isolated in the golden era. After a long void of no new antibiotics, the striking discoveries, in the recent past, of the novel classes of antibiotics such as teixobactin and lugdunin from microbes re-surged a spark in the exploitation of natural products of bacteria.

In the present study we investigated the antimicrobial potential of microbial diversity isolated from two different ecological niches in India. Initially, two antimicrobial compounds named P1 and P2 were isolated from the soil isolate *Paenibacillus dendritiformis* strain PV3-16, obtained from Leh & Ladakh samples. The mass spectrometry, amino acid analysis and nucleic magnetic resonance spectroscopy together, with the help of whole genome analysis identified them as polymyxin A_2 and A_1, respectively. We tested their minimum inhibitory concentrations (MICs) against

multiple drug-resistant (MDR) clinical isolates, performed membrane permeabilization assays and determined their interaction with lipopolysaccharide (LPS). Finally, we studied their toxicity against human Leukemic monocyte cell line (THP-1) and embryonic kidney cell line (HEK293). Both compounds displayed equal efficacy when compared with standard polymyxins. P1 was 2–4 fold more active in most of the clinical strains tested. Moreover, P1 showed higher affinity toward LPS. In cytotoxicity studies, P1 had IC_{50} value (>1000 mg/ml) similar to colistin against HEK cells but immune cells, i.e., THP-1 cell lines were more sensitive to polymyxins. P1 showed less toxicity in THP-1 cell line than all other polymyxins checked. To sum up, P1 (polymyxin A_2) possessed better efficacy than polymyxin B and E and had least toxicity to immune cells. Since polymyxin A was not investigated thoroughly, we performed the comprehensive *in vitro* assessment of this molecule. Moreover, this is the first report of isolation and characterization of polymyxin A from *P. dendritiformis*. This compound should be further investigated for its *in vivo* efficacy and toxicity to develop it as a drug candidate.

Other than the Gram-negative bacteria, methicillin-resistant *Staphylococcus aureus* (called as MRSA) and vancomycin-resistant enterococci (VRE) cause serious skin infections, wound infections and are also associated with systemic infections. These bacteria have become resistant to the last line antibiotics and therefore, warrant the discovery and development of new and effective antimicrobial compounds. As an alternative to tradition small molecule based antibiotics, small cationic antimicrobial peptides (CAMPs) seem to be a promising therapeutic alternative to fight multi-drug resistance. In this study, we have examined the *in vitro* potential of a previously reported lantibiotic, paenibacillin, from the clinical perspective. An antimicrobial peptide, M152-P4, was isolated, purified and characterized from a mud isolate M-152, and its susceptibility was determined in clinical isolates of *Staphylococcus aureus* and *Enterococcus* spp. Time-kill kinetics, resistance, probable mode of action, haemolytic activity and mammalian cytotoxicity were investigated. The strain M-152 belonged to

Paenibacillus genus (99.5% similarity with *P. jamilae* based on 16S rRNA gene sequence). The peptide M152- P4 was identified as paenibacillin based on mass spectroscopy data, amino acid analysis and biosynthetic gene cluster analysis. It had potent antibacterial activity against the Gram-positive pathogens tested, with minimum inhibitory concentrations from 0.1 to 1.56 μM. It appeared very challenging for *S. aureus* to develop resistance to this compound. Also, paenibacillin penetrated the outer layer of bacteria, and depolarized the membrane completely by creating pores in the plasma membrane with better potential than nisin. Paenibacillin showed no haemolysis up to 60 μM, and the half maximal inhibitory concentration on mammalian cell lines was > 100 μM. These results highlight the excellent antibacterial properties of paenibacillin in clinically relevant pathogens. It is stable in the presence of serum, and non-haemolytic and non-cytotoxic even above the therapeutic concentration. Further research efforts regarding toxicity and in-vivo efficacy are necessary to develop paenibacillin as a next-generation therapeutic drug to overcome multi-drug resistance in Gram-positive pathogens.

Of note, colistin is considered as the last resort antibiotic to treat the carbapenem- and multiple drug-resistant (MDR) or extensively drug-resistant (XDR) Gram-negative bacteria. But the resistance to colistin is emerging very fast. Several reports have been published worldwide to highlight the detrimental effects of colistin-resistant bacteria. Without an iota of doubt, we are thrilled by the dissemination of colistin-resistance in *Enterobacteriaceae* and other Gram-negative bacteria. In the present study, we report the discovery of tridecaptin M, a new addition to the family, and its potential against colistin-resistant *Enterobacteriaceae in vitro* and *in vivo*. Also, we performed mode-of-action studies using various fluorescent probes and studied the hemolytic activity and mammalian cytotoxicity in two cell lines. Tridecaptin M displayed strong antibacterial activity (MICs of 2 to 8 μg/ml) against clinical strains of *Klebsiella pneumoniae* (which were resistant to colistin, carbapenems, third- and fourth-generation cephalosporins, fluoroquinolones, fosfomycin, and other antibiotics) and

mcr-1-positive *Escherichia coli* strains. Unlike polymyxins, tridecaptin M did not permeabilize the outer membrane or cytoplasmic membrane. It blocked ATP synthesis in bacteria by dissipating the proton motive force. The compound exhibited negligible acquired resistance, low *in vitro* cytotoxicity and hemolytic activity, and no significant acute toxicity in mice. It also showed promising efficacy in a thigh infection model of colistin-resistant *K. pneumoniae*. Altogether, these results demonstrate the future prospects of this class of antibiotics to address the unmet medical need to circumvent colistin resistance in extensively drug-resistant *Enterobacteriaceae* infections.

Tridecaptins belong to non-ribosomally synthesized peptide class, akin to polymyxins and bacitracins. The flexibility of the adenylation domains of non-ribosomal peptide synthetases (NRPSs) complex to different amino acid substrates creates a diversity of structurally similar peptides. In the present study, we also investigated the antimicrobial activity of different natural variants synthesized by tridecaptin M gene cluster and performed the *in vitro* drug kinetics on this class. The natural variants were isolated and characterized using MALDI-MS and tandem mass spectrometry. All the peptides were studied for their antimicrobial activity in different pathogens, including colistin-resistant bacteria, and for haemolytic activity. Furthermore, *in vitro* drug kinetics was performed with tridecaptin M (or M_1, the major product of the gene cluster). The natural variants displayed a varying degree of bioactivity, with M_{11} showing the most potent antibacterial activity (MIC, 1–8 μg/ml), even against *A. baumannii* and *P. aeruginosa* strains. The *in vitro* kinetic studies revealed that tridecaptin M at a concentration of 16 μg/ml eradicated the bacteria completely in high-density culture. The compound demonstrated desirable post-antibiotic effect after two-hour exposure at MIC concentration. We also observed the reversal of resistance to this class of antibiotics in the presence of efflux pump inhibitor, carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP). The study demonstrated that tridecaptins are an excellent drug candidate against drug-resistant Gram-negative bacteria. The variant M_{11} showed remarkable activity in all the pathogens. Future studies are required to

design a superior tridecaptin by investigating the interactions of different natural variants with the target. The work also emphasizes the importance of natural products in our shrunken drug discovery pipeline.

At last, we tried to enhance the production of tridecaptin M in fermentation broth using media optimization strategies. Using response surface methodology, we found that addition of complex nitrogen sources such as soya peptone and meat extract had positive effect on productivity. Also, NaCl and ammonium sulphate were found to increase the production of antibiotic. The optimized medium yielded 13 mg/L of tridecaptin, in comparison to 1 mg/L in Mueller-Hinton Broth (MHB). However, strain improvement using genetic engineering and further optimization of media and fermentations conditions in fermenter will be necessary in order to develop a sustainable technology for tridecaptin production. This study will help to generate large scale production of this compound for its preclinical development.

Keywords: Antimicrobial compounds, antibiotic resistance, Gram-negative bacteria, colistin-resistance, polymyxin A, tridecaptin M, *Paenibacillus* sp., reversed-phase HPLC, lipopeptides, antimicrobial peptides, natural products, paenibacillin, media optimization, minimum inhibitory concentration, drug kinetics.