Tridecaptin M, a new variant discovered in mud bacterium shows activity against colistin-1 and extremely drug-resistant Enterobacteriaceae

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Running title: Tridecaptins: A hope against colistin-resistance
Abstract
WHO has categorized the Gram-negative superbugs, which are inherently impervious to many antibiotics, as critical priority pathogens due to lack of effective treatments. The breach in our last resort antibiotic (i.e. colistin) by extensively drug-resistant and pan drug-resistant Enterobacteriaceae demands the immediate development of new therapies. 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 the mode of action studies using various fluorescent probes and studied the haemolytic activity and mammalian cytotoxicity in two cell lines. Tridecaptin M displayed strong antibacterial activity (MIC 2-8 µg ml⁻¹) against clinical strains of K. pneumoniae (which were resistant to colistin, carbapenems, third-and fourth-generation cephalosporins, fluoroquinolones, fosfomycin and other antibiotics) and mcr-1 positive E. coli. Unlike polymyxins, tridecaptin M did not permeabilize the outer membrane or cytoplasmic membrane. It blocked the ATP synthesis in bacteria by dissipating the proton-motive force. The compound exhibited negligible acquired resistance, low in vitro cytotoxicity or haemolytic activity, and no significant acute toxicity in mice was observed. 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 XDR Enterobacteriaceae infections. The work also emphasizes the importance of natural products in our shrunken drug-discovery pipeline.
Introduction

The discovery of penicillin in 1928 transformed the world of medical science, saved millions of lives. This was followed by a ‘Golden-Era’ of antibiotics when many new classes were uncovered from the natural sources (1). But bacteria also developed a counter mechanism, during evolution, to neutralize the effect of antibiotics they were exposed to. Treating common infection with global capacity relies on the constant supply of effective antibiotics. If we compare the rate of spread of drug-resistance with the rate of new antimicrobials approved, the number has come down to only 9 antibiotics in the last decade from 29 in the 1980s and 23 in 1990s. Recently some innovative contributions added to our antibiotic arsenal, e.g. teixobactin, lugdunin, pseudouridimycin, malacidins (2-5). Notably, all these newly isolated classes of antibiotics exert their effect in Gram-positive bacteria only. As per the PEW Charitable Trust analysis on the antibiotic pipeline, only 11 antibiotics in clinical development could address the infections caused by critical pathogens mentioned in WHO’s priority list(6).

XDR and PDR strains of Enterobacteriaceae cause severe nosocomial and community infections which are now becoming untreatable due to the scarcity of effective antibiotics (7-9). Carbapenem-resistant Enterobacteriaceae forced clinicians to include in their drug regimens, a previously discarded antibiotic, polymyxin E (or colistin) in the early 2000s as the last resort antibiotic (10). Colistin is a cationic lipodecapeptide that exerts its bactericidal effect through initial electrostatic interactions with lipopolysaccharide (LPS), membrane permeabilization leading to cell contents leakage and eventually cell-death (11). But massive use of this important antibiotic in agriculture and poultry has elevated the resistance in clinical pathogens (12).

Colistin-resistance is attributed to alteration in lipid A biosynthetic pathway and modification to LPS (13-17). This breach in our last line of antibiotic defence by deadly pathogens is a serious public health issue. Colistin-resistance alarms the scientific community and pharmaceutical companies to develop new weapons against XDR and PDR strains (18-20). Smith et al. recently reported a new class of antibiotics, arylomycins which is effective against ESKAPE pathogens (21). Few more antibiotics are developed or in development stage such as POL7080, plazomycin (22) but they are all prone to the evolution of resistance. Hence, given the fewer chances of approval of a newly discovered antibiotic for final use, and the profound crisis in available therapies against Gram-negative pathogens, we must tackle this global challenge by continuously screening for new antibiotics.
Tridecaptins, initially discovered in 1978, is a group of non-ribosomally synthesized lipopeptides antibiotics which retain activity against Gram-negative bacteria (23-26). They possess a different mechanism of action than polymyxins, and kill the Gram-negative bacteria through binding to lipid II and causing membrane disruption (25). Even though these antibiotics show promising activity in Gram-negative pathogens, very few studies have been carried out to enlighten their potential in the shrunken drug-discovery pipeline. Further, no reports are available whether these antibiotics will work against colistin-resistant superbugs. Here, we show the discovery of a new variant of this family, tridecaptin M from a mud bacterium which intrigued us to study its antibacterial potential against colistin- and extremely drug-resistant K. pneumoniae isolates in vitro and in vivo.

Results

Isolation and structural characterization of tridecaptin M. The isolate M-152 inhibited both Gram-positive and Gram-negative bacteria and identified as Paenibacillus sp. based on 16S rRNA gene sequencing (99.5% similarity with P. jamilae) and MALDI-TOF-MS analysis. Our group was also involved in a parallel study of polymyxin A from P. dendritiformis (27). We hoped that polymyxin A would respond to colistin-resistant strains because no sufficient data was available on cross-resistance among polymyxin variants. But unfortunately, the activity of polymyxin A did not differ from that of colistin (and polymyxin B) in those strains. This phenomenon led us to conclude that a microbe which produces any polymyxin compound will, most likely, not inhibit the bacteria resistant to the same or any other polymyxin variant. We set up an assay with polymyxin-sensitive and –resistant strain of K. pneumoniae using crude fermentation extract of M152. The extract inhibited both the cultures at a similar concentration with the difference of just 2-4 folds whereas this difference appeared to be 32 folds for colistin. The clinical isolate (AH-16) used in the assay was resistant to almost every class of antibiotics (as described next). We hypothesized that this activity corresponds to either a new class of antibiotic which shares no cross-resistance with polymyxin (or any other antibiotic) or some kind of synergism. We proceeded further to identify the active ingredient(s) responsible for this phenomenon.

Through bioactivity-guided fractionation on cation-exchange chromatography and reversed-phase HPLC, we isolated one compound (initially named M152-P3) from the crude extract. The signal of compound appeared at an m/z of 1488.8246 [M+H] in HRMS (Supplementary Figure
suggesting the molecular ion as 1487.8167. LC-ESI-MS also revealed the monoisotopic mass of 1487.8344 Da (Supplementary figure S2). This data helped us to deduce the molecular formula, C_{68}H_{113}N_{17}O_{20} [calculated monoisotopic mass, 1487.8348]. We then pursued to solve the structure of this compound. The daughter ions generated in MS/MS spectroscopy were assigned using Peaks Studio (8.5) (28). Unfortunately, the software could not predict some of the ions due to the presence of unusual amino acids, so we manually curated the de novo sequence and assigned all the b and y ions (Supplementary figure S3) and further evaluated by amino acid analysis (Supplementary figure S4). The data were consistent except that tryptophan was not detected in the amino acid analysis. But the compound showed an absorbance at 280 nm which supported the presence of a tryptophan moiety in the compound. The amino acid composition and a tentative sequence derived from MS/MS suggested that this compound belongs to the tridecaptin family (23, 26, 29). The MS/MS sequence was consistent with tridecaptin B at all the positions except one. At position 11, M152-P3 displayed isoleucine while tridecaptin B has a valine at this place. Also, tridecaptin B possesses 6-methyloctanoic acid at N-terminus. But it was difficult to conclude whether the residual mass of 158 Da obtained in tandem MS of M152-P3 at N-terminus is 6-methyloctanoic acid or a linear nonanoic acid. To solve this puzzle and deduce the complete structural assignment of M152-P3, we performed 1D and 2D (HSQC, HMBC, COSY, TOCSY) NMR spectroscopy. Apparently, the NMR data removed all the ambiguities appeared at initial stages. We could clearly observe the tryptophan signals in the NMR spectra and also the presence of isoleucine at position 11 was confirmed. NMR spectroscopy also revealed that the fatty acid moiety at N-terminus was 6-methyloctanoic acid since a methyl group showing coupling to methine carbon (at position 6) of lipid tail was visible. The complete NMR assignment and the raw NMR spectra are provided in the supplementary information (Figure S5 to S12; Table S1). The stereochemistry of amino acids was assigned based on the Marfey’s analysis. The low biological yield (<1 mg L\(^{-1}\)) of M152-P3 in fermentation caused limitation in the analysis of stereochemistry of the lipid tail because we need a large amount of peptide to obtain enough fatty acid upon hydrolysis (less than 10% of the total compound) (26). The chiral methyl group present in the lipid chain of all the natural tridecaptin variants is S-configuration because, in the Bacillus genus, it is mostly derived from L-isoleucine (26, 30). Therefore, we considered the methyl group as S-configuration. Also, the stereochemistry of the methyl group does not contribute significantly to the activity (31).
1d shows the 2D structure of M152-P3. Considering mass spectrometry, amino acid analysis, and NMR data, we concluded that M152-P3 is a new variant of this family and differs from tridecaptin B at one amino acid position. We named this variant tridecaptin M (the letter ‘M’ derived from the village name from where the mud sample was collected).

**Biosynthetic gene cluster.** To further confirm the production of tridecaptin M by the bacterium M-152, we analysed the whole-genome of this strain. The antiSMASH predicted a single tridecaptin biosynthetic gene cluster responsible for the synthesis of M152-P3 (Figure 1a) which was homologous to the previously reported gene clusters of tridecaptin A (32) and B (26). The cluster consisted of five genes encoding different proteins for synthesis and secretion of active peptide, eg trmA (a putative thioesterase), trmB and C (ABC transporters), trmD and E (NRPSs, non-ribosomal peptide synthetases). The two main biosynthetic enzyme complexes (trmD and E) together composed of 13 adenylation domains, each responsible for incorporation of one amino acid (Figure 1b). We could also observe the epimerase domains next to seven adenylation domains. This domain converts the stereochemistry of L-amino acid to D-form (33, 34). The positions of epimerase domains were consistent with the experimentally observed D-amino acids in the compound (Figure 1c) and with other variants of this family. To mention, we noticed some discrepancies in the specificities of some adenylation domains predicted by software programs (Supplementary Table S2). Similar results were reported for gene clusters of tridecaptin A (32) and B (26). The biosynthetic gene cluster of M152-P3 differed (10-15%) from other tridecaptin gene clusters.

**Tridecaptin M shows activity in colistin-resistant Gram-negative bacteria.** We next sought to determine the biological activity of this variant in different pathogens. M152-P3 showed narrow-spectrum activity against Gram-negative bacteria (Table-1) with better MIC (minimum inhibitory concentration) against the Enterobacteriaceae family. In *E. coli* and *K. pneumoniae* strains, the MIC varied from 0.5 to 8 µg ml$^{-1}$. The compound also displayed efficacy in MDR clinical isolates of *K. pneumoniae* which were all polymyxin-sensitive. Though the compound had two to four folds higher MIC than colistin and polymyxin B in polymyxin-sensitive strains, it exhibited a much better spectrum in polymyxin-resistant *K. pneumoniae* isolates (Table-2). Some of the clinical isolates displayed XDR profile, but they were all sensitive to M152-P3 (MIC 2 µg ml$^{-1}$), unlike polymyxins which had poor activity. M152-P3 was not active against Gram-positive bacteria or *Candida albicans* (No MIC was achieved up to 128 µg ml$^{-1}$). The MIC
was also determined in Ca-MHB supplemented with 5% (v/v) fetal bovine serum and MIC remained unchanged (data not shown) suggesting the stability of this compound.

**Time-kill kinetics and resistance studies.** We also studied the time-dependent killing of *K. pneumoniae* in the presence of M152-P3 and found that the compound killed the bacteria completely within four to eight hours even at a very low concentration (Figure 2a). There was no re-growth observed in time-kill assay after 24 h which suggested the ability of this compound to resist spontaneous mutation. These results prompted us to calculate the mutation prevention concentration (MPC) and to analyse the resistance mutation frequency. M152-P3 prevented spontaneous mutation in *K. pneumoniae* at a concentration of 16 µg ml⁻¹. The survivors appeared in four conditions below the MPC were selected for checking the susceptibility towards M152-P3, and none of the survivors showed resistance. Therefore, the frequency of resistance mutation was calculated as <2.5×10⁻⁹. Since mutation frequency was very low for M152-P3, we tried to develop acquired resistance through serial passaging of bacteria for 20 days in the presence of the sub-lethal concentrations of M152-P3 (Figure 2b). After 20 days, the mutant showed just four to eight folds increase in MIC whereas bacteria successfully developed resistance towards ciprofloxacin (128 folds change in MIC).

**Tridecaptin M kills the bacteria by blocking ATP synthesis through a cascade of reactions.**

The study further aimed at discerning the mode of action of M152-P3 in *K. pneumoniae*. Recently, Cochrane et al. showed that tridecaptin A₁ binds specifically to lipid II of Gram-negative bacteria (25). They also reported that LPS interaction is necessary to facilitate the crossing and entry of tridecaptin into the periplasmic space (where lipid II is situated). We performed a series of *in vitro* experiments to demonstrate the mode of action of M152-P3 and compared the results with those obtained for tridecaptins previously. In an attempt to study the effect of this compound on the outer membrane of Gram-negative bacteria, N-phenyl-1-naphthylamine (NPN) (35, 36) dye was used. Polymyxin B caused a rapid increase in fluorescence as compared to control cells (Figure 3a) which indicates the disruption of the outer membrane. This is a characteristic phenomenon of polymyxins in Gram-negative bacteria (27, 37, 38). On the contrary, M152-P3 showed poor or negligible permeabilization at 32 µg ml⁻¹ while this effect appeared to be more at higher concentration. This explains for the higher MIC of M152-P3 than polymyxins in polymyxin-sensitive strains. We then checked if M152-P3 acts similarly on the inner membrane. Two membrane-impermeable substrates, i.e. ortho-
nitrophenyl-β-galactoside (ONPG) and propidium iodide (PI) were used to see the large pore formation in the inner membrane. As visible in Figure 3b, M152-P3 did not facilitate the entry of ONPG inside the cells whereas polymyxin B treated cells had much greater uptake of ONPG. This data indicates that M152-P3 did not create large pores in the membrane. Similar results were obtained with propidium iodide (PI) (Figure 3c). This rise in intensity at prolonged incubation may be due to the cell population which is already dead (25). We also studied the alternation in membrane potential using localization of a voltage-sensitive fluorescent probe, i.e. 3, 3-dipropylthiacarbocyanine DiSC3(5) as a proxy. In Figure 3d, it is clearly visible that the addition of valinomycin caused fluorescence-leakage in a concentration-dependent manner. This validated our assay to study the membrane potential in the presence of M152-P3. After addition of M152-P3, fluorescent intensity enhanced to some extent in a concentration-dependent manner. This result was in controversy to what was previously reported for tridecaptin A1 (25). Cochrane et al. showed that tridecaptin A1 alters the proton motive force by creating proton-specific pores and concluded that disruption of proton-motive force would block the energy generation in bacteria, ultimately causing cell-death (25). We monitored the ATP synthesis in peptide-treated bacteria via luciferase-based ATP determination assay and compared the results with those obtained for non-treated cells. After two hours of treatment, OD600 of bacteria increased by almost two folds, indicating that peptide concentration used in the assay was not lethal to cells (Figure 3e). On the other hand, the untreated sample reached an OD600 of approximately 3.5. This data suggests that M152-P3 halted bacterial growth even at a sub-inhibitory concentration and caused prolonged lag-phase or adaptation-phase. This explanation was further supported by the luciferase assay. The bacteria accumulated low level of ATP in the presence of a sub-inhibitory concentration of M152-P3 (Figure 3f), showing the slowdown of this important pathway and initial retardation in growth. Other antimicrobial peptides have also been shown to create proton-specific pores in the membrane, e.g. nigericin and subtilisin (39, 40). Inhibition of ATP synthesis is, therefore, a consequence of this unregulated proton-motive force caused by the action of such peptides.

As mentioned in previous reports, tridecaptins behaved similarly with LPS as polymyxins (25). This was assumed to be the fundamental attack that helps them in disruption of the outer membrane and to reach their main target. But if this is the case then tridecaptins should not interact with LPS in its first place and be able to reach its target in polymyxin-resistant strains in
which LPS modification is the main cause of resistance. Since tridecaptins retain superior activity in spite of these modifications in LPS (leading to reduced binding with polymyxins), the results imply that their interactions with LPS at the molecular level are different from polymyxins’. However, this requires further investigations in details to confirm their molecular interactions with LPS and to compare the interaction site with that of polymyxins.

**Potentiation of tridecaptin M in the presence of colistin.** Considering the low outer membrane permeabilization potential of M152-P3, we postulated that this compound can exert its effect at a lower concentration, provided the outer membrane is compromised by other means. Teixobactin (a Lipid II inhibitor in Gram-positive bacteria) also showed activity in an *E. coli* strain which was genetically compromised for outer membrane, suggesting that outer membrane was a probable hindrance (2). Also, many other antibiotics, which are used for Gram-positive bacteria, have been reported to act in Gram-negative bacteria in synergy with colistin (20). This hypothesis exhorted us to check the potentiation of M152-P3 in the presence of colistin. Figure 3g explains the synergy between colistin and M152-P3 in colistin-resistant strains. Sub-inhibitory concentrations of colistin (also below clinical break-point for its resistance), reduced the MIC of M152-P3 up to 16 folds. In one strain, CF-23, the FICI for colistin was 0.31 which clearly demonstrates the synergy between these two classes. These results indicate that tridecaptins can be further optimized for its ability to penetrate the outer layer more efficiently.

**Therapeutic efficacy in mice model and toxicity studies.** The in vitro efficacy of M152-P3 in colistin or polymyxin-resistant XDR strains urged us to study its therapeutic potential in more details. The haemolysis assay interpreted that this compound had no effects on erythrocytes up to a concentration of 100 µg ml\(^{-1}\) which is more than 25 folds higher than its MIC in bacteria (Figure 4a). In cytotoxicity experiments as well, the compound exhibited IC\(_{50}\) at >250 µg ml\(^{-1}\) in the human embryonic kidney cell line (HEK293) and mouse macrophage cell line (J774) (Figure 4b). These results are quite desirable for the antimicrobial compounds to be administered systemically in the humans. To mention, though the IC\(_{50}\) in cell-lines was significantly greater than its antibacterial concentration, this should be noted that approximately 25% of cell toxicity was observed at 100 µg ml\(^{-1}\). To further provide the insights into the toxicity of this compound, we examined the acute toxicity of M152-P3 in an animal model. M152-P3 or colistin was given to mice at 12 mg kg\(^{-1}\) every 2 h until an accumulated dose of 72 mg kg\(^{-1}\). No animal in the colistin group could survive the six doses (Figure 4c) and all the mice died within 24 h. The
results were reasonably similar to the previously observed effects of colistin where 8.5 mg kg\(^{-1}\) of intravenous injection of colistin showed lethality to mice (41). Also, the nephrotoxicity has been associated with polymyxins given subcutaneously at the same dose as used in the present study (41, 42). On the contrary, M152-P3 was well suited in mice and no death was observed for 24 h after the last dose at which mice were sacrificed for histopathology experiments. The mice behaved similar to the placebo group (PBS) and upon histopathology of major organs, no significant changes appeared (Figure 4d). We could not observe any nephrotoxicity for M152-P3 at the given dose. The toxicity results were more favourable over colistin in the \textit{in vivo} system since in our previous study colistin showed IC\(_{50}\) in HEK cell-line at >500 µg ml\(^{-1}\) (27) whereas it displayed severe toxicity in animals. This observation can be supported by the assumption that the tridecaptin M may bind (reversibly or irreversibly) to serum or plasma proteins to a greater extent than colistin, and thus its PK/PD profile may be different from that of colistin. Nevertheless, this needs to be confirmed by further studies. Finally, we wanted to know whether this compound would exert its effect \textit{in vivo} to eradicate the bacteria from the body. A thigh infection model was set up to assess the \textit{in vivo} efficacy of M152-P3 against polymyxin-sensitive \textit{K. pneumoniae} ATCC 700603 as described previously (20, 21). At 10 mg kg\(^{-1}\), colistin reduced the bacterial burden by almost 1.5 log CFU per thigh whereas M152-P3 reduced around 0.5 log CFU (Figure 4e). These results were expected since \textit{in vitro} MIC of colistin is nearly four folds less than M152-P3 against this strain. Nonetheless, this is noteworthy that M152-P3 successfully reached the tissue and showed efficacy against bacteria as compared to untreated control. Next, we infected the mice with colistin-resistant XDR strain (AH-16) and treated them with two doses (10 mg kg\(^{-1}\)) of colistin or M152-P3. Colistin failed to eradicate the bacteria in this model and bacteria were able to multiply in the muscle while M152-P3 displayed 90 per cent removal of the bacterial population (Figure 4e). These results indicate the excellent therapeutic efficacy of this class of antibiotics to develop a suitable candidate against colistin-resistant Enterobacteriaceae.

**Discussion**

The dwindled antibiotic pipeline and the growing non-responsiveness of antibiotic-resistant Gram-negative pathogens to colistin have propelled us to the verge of a ‘post-antibiotic era’. This scenario requires our immediate actions to tackle these bugs by developing new drugs and to find appropriate solutions to control the spread of resistance. The natural products built our antibiotic arsenal in ‘Golden era’ and further continued to provide new drugs with the
advancement of medicinal chemistry (1). Conventional approaches for isolating bacterial natural products soon faced the replication of already discovered compounds. No new class emerged from microbes after the 1980s until very recently when uncultured bacteria or microbes dwelling in unusual habitats proved to become a repertoire of novel antimicrobial entities (2, 3). The exemplary discovery of such antibiotics stimulated the resurgence in the exploitation of new natural products to fight hard-to-deal-with pathogens (43). The present study discusses the discovery of a new variant of the tridecaptin class which is effective against colistin-resistant Gram-negative superbugs. The producer strain M-152 was initially discarded because of the belief that it would secrete polymyxin because we analysed the whole genome sequence of a previously reported strain of *P. jamilae* (44) available at NCBI and observed the polymyxin biosynthetic gene cluster. This genus is very well known to produce polymyxins which are mainly active against Gram-negative bacteria (10, 27, 45). Later, its crude extract was found to inhibit both colistin-resistant and -sensitive *K. pneumoniae* at a similar concentration. This curiosity turned this study into a rational discovery of tridecaptin M, belonging to a class which was never assayed against colistin-resistant bacteria in past. The compound showed strong antimicrobial activity against polymyxin-resistant XDR clinical isolates of *K. pneumoniae* and *mcr-1* positive *E. coli*. The mode of action studies were consistent with previous reports (25) and thus further corroborate these assays. We observed some discrepancy in membrane potential assay. Previously, it was shown that tridecaptin A₁ did not cause depolarization in *E. coli* or *A. baumannii* when the anionic dye DiBAC₄ treated cells were incubated with the peptide (25, 46, 47). This dye enters depolarized cells and binds to proteins and membrane components. In contrast to DiBAC₄, DiSC₃(5) is a cationic dye that accumulates on polarized cells and embeds itself into the lipid membrane where its fluorescence is masked (47, 48). If depolarization of the membrane occurs, the accumulated dye moves back to the surrounding, causing an increase in fluorescence intensity. This leakage in fluorescence is a measure of change in membrane potential. Though the change in membrane potential in M152-P3 treated cells was not as high as in case of valinomycin, this can be argued with the fact that both K⁺ and H⁺ ions are responsible for the membrane potential (47, 49, 50) and valinomycin is specific to the transport of K⁺ ions down the electrochemical gradient (51). In normal respiring cells, H⁺ ions contribute most to the membrane potential gradient due to active electron transport chain (49, 50). Addition of valinomycin causes movement of K⁺ ions inside the cell and thus leading to a reduction in...
membrane potential or also called depolarization. We thought of another possibility, an inward movement of H$^+$ ions due to the formation of proton-specific pores will also lead to disturbance in membrane potential (39, 40). This could be a plausible explanation for the change in membrane potential after M152-P3 addition to the cells. Indeed this phenomenon was observed for tridecaptin A$_1$. One explanation for negative results with DiBAC$_4$ could be the interference of this dye with tridecaptins (47). Since this compound targets lipid II (which is not a protein) and ultimately makes the bacteria devoid of ATP generation, there are very rare chances of developing resistance towards this antibiotic. This was clearly observed in an in vitro experiment (Figure 2b). Vancomycin and a recently discovered antibiotic, teixobactin also target lipid II in Gram-positive bacteria and have been complemented with the ability to thwart the development of resistant mutants (2).

Tridecaptins were discovered in a time when the existence of colistin-resistance was rare and other safe antibiotics were available. Also, they shared a greater homology in structure with polymyxins. This led people to believe that they may possess similar nephrotoxicity and moreover, they showed decreased antibacterial activity when compared with polymyxins. This is one of the reasons this class remained underexplored over the years. Cochrane et. al (25) also found that tridecaptin A$_1$ bound to LPS in a similar fashion as polymyxins, and they might have assumed that LPS modification would restrict this class from its binding to LPS. On the contrary, the foundation of the present study was the activity of this class observed in colistin-resistant strains.

To conclude, M152-P3 appeared safe to RBCs and mammalian cell-lines at concentrations much higher than its effective concentration in bacteria. Also, no acute toxicity was observed in the animal model at the administered dose. Above all, the compound exhibited remarkable efficacy in a thigh infection model with the colistin-resistant strain of \textit{K. pneumoniae}. Nevertheless, this compound requires detailed investigations on its pharmacokinetics and pharmacodynamics (PK/PD). Though some structural-activity relationship (SAR) studies have been carried out on this class (26, 31, 52, 53), more efforts are needed to design a suitable clinical drug-candidate. It is also worthwhile to study how this class can be optimized to improve its activity in other Gram-negative bacteria. Overall, we demonstrate the desirable therapeutic indices of this class of antibiotics against toughest superbugs which immediately demand new and effective drug...
The study also emphasizes the significance of natural products as pioneering drugs to fight pathogens expressing resistance to even last-line antibiotics.

Materials and Methods

Bacterial strain and isolation of antimicrobial compound M152-P3

Bacterial isolates obtained from the mud sample (described in another manuscript under communication) were screened for antimicrobial activity. One mud bacterium, named M-152 inhibited the growth of both Gram-negative and Gram-positive bacteria. The inoculum was prepared in 100 ml MHB and log-phase grown culture was transferred in 2L-flasks containing 700 ml sterile MHB (2% inoculum). The fermentation was carried out in 5.6 L batches and lasted for 24 h. The culture was harvested by centrifugation. Then we prepared the crude extract using Diaion-HP 20 resin (27). The antimicrobial compound was partially purified by bioactivity-guided fractionation on cation-exchange chromatography with the conditions: SP-sepharose column (bed height, 5 cm x 15 cm, column volume 300 ml), flow rate 10 ml/min, 10 mM ammonium acetate buffer with pH 5.0±0.1. 1 M NaCl in the same buffer was used as an eluent solution. A two-stage linear gradient of 0 to 0.5 M NaCl in 900 ml and 0.5 to 1 M in 300 ml was used to elute the bound components. 100 ml fractions were collected and assayed for antimicrobial activity against K. pneumoniae. The active fractions were further purified by reversed-phase HPLC on C18 column (21.2 mm x 250 mm, Phenomenex, 5 µm) using 20% acetonitrile in water and 100% acetonitrile (both containing 0.075% TFA) as solvent A and B respectively. The gradient was ramped from 0% B to 18% B in 25 min, and 18% B to 80% B in 11 min. All peaks were collected and assayed for bioactivity. The active peak was further purified by the second round of HPLC on C4 column (Phenomenex, Jupiter, 10 mm x 250 mm, 5 µm, 300 Å) with solvent A as 20% acetonitrile and 80% of 5 mM ammonium acetate buffer (pH ~5.2) and solvent B as 100% acetonitrile. The gradient of solvent B was ramped from 5% to 30% in 25 min. The pure compound, named M152-P3 was lyophilized after desalting to obtain the off-white powder.

Structural characterization by mass spectrometry, amino acid analysis and NMR spectroscopy

M152-P3 was subjected to LC-ESI-MS, ESI-HRMS and LC-ESI-MS/MS analysis to know the monoisotopic mass and fragmentation pattern. The daughter ions series generated in the tandem mass spectrum were assigned manually and de novo sequencing was performed to deduce the
probable amino acid sequence. For amino acid analysis, 100 µg of the compound was hydrolysed and derivatized as per the manufacturer’s protocol (PICO-TAG amino analysis system, Waters). The hydrolysate was analyzed on HPLC and the retention times of amino acids were compared with standard amino acids as per the protocol. For NMR spectroscopy, 10-12 milligrams of pure compound was dissolved in deuterated dimethylsulfoxide (DMSO-d6) and 1H, 13C, COSY, TOCSY, DEPT, HMBC and HSQC spectra were acquired on Bruker 600 MHz spectrometer.

**Marfey’s analysis**

Marfey’s analysis (Marfey’s reagent, L-1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA), Thermo scientific, Catalogue number: 48895) was performed to determine the stereochemistry of the amino acids. Three milligrams of the compound was hydrolysed to its amino acid constituents derivatised as per manufacturer’s instructions. For detection of FDAA derivatives of amino acids, reversed-phase HPLC was employed (XBridge, waters, C18, 5µm, 130 Å, 4.6x250 mm) with the mobile phase system: solvent A, water containing 0.075% TFA and solvent B, acetonitrile containing 0.075% TFA. A linear gradient from 18% B to 46% B in 60 min was used. The retention time of standard amino acids was compared with those obtained in M152-P3 chromatogram.

**Biosynthetic gene cluster identification**

The whole genome sequence was obtained using a hybrid assembly of Illumina reads and Nanopore data. The biosynthetic gene cluster was located with the help of antiSMASH version 3.0 (54). The preference of the adenylation domain towards specific amino acids was checked using NRPSpredictor2 and other programs provided in antiSMASH and curated manually with the help of experimentally determined structure.

**Minimum inhibitory concentration (MIC)**

MIC was determined using micro broth-dilution assay in Cation-adjusted MHB according to CLSI guidelines. Briefly, bacterial cell concentration was adjusted to approximately 4x10^5 colony forming unit (CFU) per ml. The experiment was performed in a 96-well polystyrene tissue-culture grade plate (Falcon, 353072) in 200 µl. The bacteria were incubated with two-fold varying concentrations of M152-P3 for 18 h at 37°C. The lowest concentration with no visible growth was considered as MIC. The standard ATCC cultures were procured from HiMedia laboratory, India. The antibiotic susceptibility of all the clinical isolates was examined using either the disk-diffusion method or micro broth-dilution method as per CLSI guidelines. The data
is given in supplementary information or referenced to previous studies from our group for some 
isolates.

**Time-dependent killing**

*K. pneumoniae* ATCC 700603 was grown till log-phase and OD<sub>600</sub> was adjusted to 0.35 (4x10<sup>8</sup> CFU per ml). This culture was diluted 1000 times in fresh MHB containing with 2xMIC and 4xMIC concentration of the M152-P3 or colistin and incubated at 37°C. 50-100 µl of the culture was spread plated on Mueller-Hinton agar plates at different time intervals. The culture without antimicrobial compound was taken as positive control. CFU per ml was calculated and plotted against time. The experiment was performed in triplicate with two biological repeats.

**Mutation prevention concentration (MPC) and resistance frequency analysis**

*K. pneumoniae* ATCC 700603 was grown till log phase and adjusted to an OD<sub>600</sub> of ~1.0 (10<sup>9</sup> CFU ml<sup>-1</sup>). 100 µl of this culture was plated on cation-adjusted MHA plates supplemented with different concentration of M152-P3 (0.5 MIC, 1.0 MIC, 1.5 MIC, 2.0 MIC and 4 MIC). The plates were incubated at 37°C for 24 h. The plates were observed for the survivors and the concentration at which no colony appeared was considered as the mutation prevention concentration. To calculate the resistance mutation frequency, the survivors appeared after 24 h (at lower concentrations) were again plated (two colonies from each concentration) on a fresh agar plate with a similar concentration of M152-P3 from which survivor emerged. After reconfirmation of the survivors, 4 to 6 representatives from each condition were assayed for their susceptibility towards M152-P3. A survivor was considered resistant if the MIC increased a minimum of 4 folds since a two-fold change in MIC can occur due to intrinsic variation in MIC determination (2). The mutation frequency was determined (55) as per the following equation:

\[
\text{Frequency of resistance (FOR)} = \frac{\text{number of resistant mutants}}{\text{number of conditions} \times \text{CFU plated}}
\]

**Resistance studies**

To study the development of acquired resistance towards M152-P3, we conducted the sequential passaging (2, 25) of *K. pneumoniae* ATCC 700603 in the presence of sub-lethal concentrations of M152-P3 or ciprofloxacin. Four different concentrations i.e. 0.25xMIC, 0.5xMIC, 1xMIC and 2xMIC in Ca-MHB were used to grow the culture for 24 h. Afterwards, the highest concentration showing a minimum growth of OD600 ≥0.3 was transferred (3 to 4 µl in 200 µl broth in a 96-
well plate) into the fresh broth containing higher concentrations of antibacterial compounds the following day. This sequential culturing was performed for 20 days with antibiotic and next three days without antibiotic to check the stability of acquired resistance. The MIC was determined every day for M152-P3 or ciprofloxacin and fold-change in MIC was measured.

**Outer membrane permeabilization assay**

For outer membrane permeabilization, N-phenyl-1-naphthylamine (NPN) assay was used (27). *K. pneumoniae* ATCC 700603 cells were grown in MHB and washed with 5 mM HEPES buffer (containing 5mM glucose, pH ~7.4) twice. Final cell concentration was adjusted to an OD$_{600}$ of 0.063. NPN was dissolved in acetone at a concentration of 20 mM and then diluted in HEPES buffer to make a working stock of 40 µM. 130 µl of the cells were incubated with 50 µl of NPN (final concentration of 10 µM) and 20 µl of M152-P3 or polymyxin B at different concentrations (0.5 to 64 µg ml$^{-1}$). The wells without antibiotic were taken as a negative control. Polymyxin B was used as a standard because of its strong membrane permeabilizing properties. The fluorescence was measured in a time-dependent manner on a fluorescence microplate reader (Biotek, Synergy H1) with excitation at 350 nm and emission at 420 nm. NPN plus antibiotic without cells was also taken to check the interference in fluorescence. The experiment was performed in triplicates with two biological repeats.

**Inner membrane permeabilization assay**

For inner membrane permeabilization, ortho-Nitrophenyl-β-galactoside (ONPG) assay was performed (56). *K. pneumoniae* was grown in lactose medium and the cells were washed twice with PBS and adjusted to an OD$_{600}$ of 0.05 (5×10$^7$ CFU/ml). 180 µl of the cells were incubated with 1.5 mM ONPG and 20µl of M152-P3 or polymyxin B at different concentrations in a 96-well plate at 37°C. The absorbance was measured on a microplate reader (BioTek) at 420 nm from 0-45 min with 3 min interval. The experiment was conducted in triplicate. Cells without peptide were considered as a negative control. Three biological repeats were performed. Cytoplasmic membrane permeabilization was also studied with a membrane impermeable fluorescence dye, propidium iodide [(PI), Thermo Fisher Scientific, India]. The protocol mentioned in LIVE/DEAD® BacLight Bacterial Viability Kit (L7012, Molecular Probes, purchased from Thermo Scientific, India) was optimized initially with some modifications. Bacterial cells were grown in MHB and washed three times with normal saline (0.9% NaCl). Cell concentration was normalized to 10$^8$ CFU/ml or OD$_{600}$ 0.1. The cells were treated with
different concentrations of M152-P3 or polymyxin B in a 1.5 ml microcentrifuge tube. After 30 min, PI was added at a final concentration of 2.5 µg ml\(^{-1}\) and kept in the dark at 4°C for 15-20 min. The samples were centrifuged at 10,000 xg for 10 min to remove the unbound dye and excess antibiotics. The cells were re-suspended in 350 µl of normal saline and mixed well. The cells (100 µl per well) were dispensed in a 96-well plate (suitable for fluorescence) and the fluorescence was measured on a fluorescence microplate reader with excitation at 535 nm and emission at 617 nm. The experiment was conducted in triplicate and at least three biological repeats were performed. The cells without any treatment were considered as PI-negative control.

3, 3-dipropylthiacarbocyanine DiSC\(_3\)(5) assay

Membrane depolarization was studied using membrane-potential sensitive probe 3, 3-dipropylthiacarbocyanine (57-59) [DiSC\(_3\)(5); Sigma-Aldrich]. K. pneumoniae cells were prepared as described for NPN assay. The cells were incubated with DiSC\(_3\) dye (0.4 µM) and 100 mM KCl in a 96-well black plate (total volume 180 µl) and checked for a stable reduction in fluorescence as measured by a microplate reader. Cells were incubated with KCl to equilibrate the cytoplasmic and external K\(^+\) concentrations. This was followed by the addition of different concentrations of M152-P3 or polymyxin B (20 µl) and an incubation of 30 min. The fluorescence leakage was measured with excitation at 622 nm and emission at 670 nm. The experiment was performed in triplicate.

ATP determination

K. pneumoniae ATCC 700603 was grown till mid-log phase and inoculated in 5 ml LB broth in 100 ml Erlenmeyer flask to achieve 5×10\(^7\) CFU per ml. The medium contained 10 mM glucose and M152-P3 at 16 µg ml\(^{-1}\). The flasks were incubated at 37°C, 200 rpm. Control flasks contained no antibiotic. At two hour, samples (2 ml) from each flask were taken and OD\(_{600}\) was adjusted to obtain similar CFU per ml (variation in the number of bacteria may interfere with the results) for each set. The culture was pelleted and washed with PBS. The pellet was dissolved in lysis buffer (500 µl) and cells were lysed using either ultrasonic water bath for 15 min at 4°C or freeze-thaw method. In freeze-thaw method, the pellet was frozen at -80°C for 15 min and immediately kept in boiling water for 15 min. The cell lysate was centrifuged at 4°C and the supernatant was assayed for total intracellular ATP synthesis in treated and non-treated bacteria using ATP determination kit (Invitrogen™, Molecular Probes®, Part no. A22066).

M152-P3 potentiation assay
E. coli strains (mcr-1 positive, CF-23, CF-45 and CF-47) and K. pneumoniae AH-16 were used to study synergy between M152-P3 and colistin. Colistin was used at concentrations below its clinical breakpoints (≤2 µg ml⁻¹) and M152-P3 was diluted in a 2-fold manner. The fold modulation was calculated as the reduction in M152-P3 MIC with or without colistin.

Haemolysis and mammalian cytotoxicity

Haemolysis experiment was performed as explained previously (60) Briefly, fresh rabbit red blood cells (RBCs) were washed thrice with PBS and re-suspended in PBS at a concentration of 4% (v/v). 180 µl of RBCs was added to the wells of a 96-well U-bottom plate. 20 µl of M152-P3 was added resulting in a final concentration ranging from 0 to 200 µg ml⁻¹. After one hour incubation at 37°C, cells were centrifuged at 1,000g and 100 µl of the supernatant was transferred to 96-well flat-bottom plate. The release of haemoglobin was measured by the absorbance at 570 nm in the microplate reader. The experiment was performed in triplicate with two biological repeats. Mammalian cytotoxicity was performed on two different cell lines; human embryonic kidney cell line (HEK293) and mouse macrophage cell line (J774) as described elsewhere (manuscript under communication). The cells were treated with M152-P3 for 24 h at a concentration ranging from 0 to 250 µg ml⁻¹.

Animal studies

All the animal experiments were conducted at the animal facility, CSIR- Institute of Microbial Technology and were in compliance with the ethical standards of the Institutional Animal Ethics Committee (IAEC) of the Institute of Microbial Technology (Approval no. IAEC/17/11). Female Balb/c mice (6-8 weeks old) were used in all studies. Randomization or blinding was not deemed necessary in efficacy experiments. In histopathology analysis, the observer was unaware of the treatment groups.

In vivo toxicity

In vivo toxicity was studied at explained previously for polymyxins (42). Mice were injected subcutaneously with M152-P3 or colistin in normal saline at a dose of 12 mg per kg. Six doses were given at 2 h interval until a final accumulation of 72 mg per kg. 20 h after the last dose, mice were sacrificed and all major organs i.e. kidney, spleen, liver, brain were isolated and fixed in 10% buffered formalin (pH 7.2). The organs were submitted for histological staining. Four mice were taken for each group. One group received only normal saline (vehicle control). All the mice were also observed for survival until euthanization since colistin-receiving mice died before...
the last dose. The data was presented as any changes in the kidney or other organs, and the Kaplan–Meier survival (61) plot in terms of acute toxicity.

**Mouse thigh infection model**

In vivo efficacy of M152-P3 was assessed in a mouse thigh infection model (21) against *K. pneumoniae* ATCC 700603 (polymyxin-sensitive) and clinical isolate *K. pneumoniae* AH-16 (polymyxin-resistant and XDR strain). Female Balb/c Mice (n=6) were rendered neutropenic by giving two doses of cyclophosphamide i.e. 150 mg per kg and 100 mg per kg four and one day prior to infection respectively. Afterwards, mice were injected intramuscularly with 5x10⁵ CFU per mouse into the right thigh muscle. At 4 h post infection, all the mice were administered subcutaneously with colistin or M152-P3 (5 or 10 mg per kg). The control group received vehicle (normal saline). At 20 h after treatment, mice were sacrificed and CFU in muscle was determined on MHA plates through serial dilution in PBS after homogenization of muscle tissue.

In infection model against AH-16 strain, two doses (10 mg per kg) were given for both colistin and M152-P3 at 2 and 11 h post infection. At 20 h after the 1st dose, the mice were sacrificed and CFU per thigh was calculated as mentioned above.

**Author contribution**

HN, MJ, and MK and designed the study. MJ isolated and purified the compound, performed mass spectroscopy and amino acid analysis and analysed the results. MJ and MK performed the *in vitro* bioactivity assays. MJ, MK, NK and RT did the *in vivo* efficacy studies and analysed the results. MJ, MK and NK performed the *in vivo* toxicity. SKM and RR carried out NMR experiments and structural assignment with assistance from MJ. AG isolated and initially characterized colistin-resistant clinical isolates of *K. pneumoniae* and *mcr-1* positive *E. coli* strains from food samples. MJ and MK wrote and edited the manuscript with input from all the authors. All authors approved the manuscript before submission.

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**Transparency declarations**

The authors declare no conflict of interest.

**Ethics**

The animal experiments were conducted in compliance with the ethical standards of the Institutional Animal Ethics Committee (IAEC) of the Institute of Microbial Technology (Approval no. IAEC/17/11).

**Data availability**

The whole genome sequence of the producer strain M152 has been deposited at GenBank under accession no. CP034141 (https://www.ncbi.nlm.nih.gov/nuccore/CP034141). Additional data that support the findings of this study are available from the corresponding author upon request.

**References**

1. Brown ED, Wright GD. 2016. Antibacterial drug discovery in the resistance era. Nature 529:336.
2. Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP, Mueller A, Schäberle TF, Hughes DE, Epstein S. 2015. A new antibiotic kills pathogens without detectable resistance. Nature 517:455.
3. Zipperer A, Konnerth MC, Laux C, Berscheid A, Janek D, Weidenmaier C, Burian M, Schilling NA, Slavetinsky C, Marschal M. 2016. Human commensals producing a novel antibiotic impair pathogen colonization. Nature 535:511.
4. Chellat MF, Riedl R. 2017. Pseudouridimycin: the first nucleoside analogue that selectively inhibits bacterial RNA polymerase. Angewandte Chemie International Edition 56:13184-13186.
5. Hover BM, Kim S-H, Katz M, Charlop-Powers Z, Owen JG, Ternei MA, Maniko J, Estrela AB, Molina H, Park S. 2018. Culture-independent discovery of the malacidins as calcium-dependent antibiotics with activity against multidrug-resistant Gram-positive pathogens. Nature microbiology 3:415.
6. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, Pulcini C, Kahlmeter G, Kluymans J, Carmeli Y. 2018. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. The Lancet Infectious Diseases 18:318-327.
7. Krapp F, Ozer EA, Qi C, Hauser AR. Case Report of an Extensively Drug-Resistant Klebsiella pneumonias Infection With Genomic Characterization of the Strain and Review of Similar Cases in the United States, p ofy074. In (ed), Oxford University Press US,
8. Bathoorn E, Tsioutis C, da Silva Voorham J, Scoulia E, Ioannidou E, Zhou K, Rossen J, Gikas A, Friedrich A, Grundmann H. 2016. Emergence of pan-resistance in KPC-2 carbapenemase-
producing Klebsiella pneumoniae in Crete, Greece: a close call. Journal of Antimicrobial Chemotherapy 71:1207-1212.

9. Bi W, Liu H, Dunstan RA, Li B, Torres VVL, Cao J, Chen L, Wilksch JJ, Strugnell RA, Lithgow T. 2017. Extensively drug-resistant Klebsiella pneumoniae causing nosocomial bloodstream infections in China: molecular investigation of antibiotic resistance determinants, informing therapy, and clinical outcomes. Frontiers in microbiology 8:1230.

10. Landman D, Georgescu C, Martin DA, Quale J. 2008. Polymyxins revisited. Clinical microbiology reviews 21:449-465.

11. Newton B. 1956. The properties and mode of action of the polymyxins. Bacteriological reviews 20:14.

12. Davies M, Walsh TR. 2018. A colistin crisis in India. The Lancet Infectious Diseases 18:2.

13. Olaitan AO, Morand S, Rolain J-M. 2014. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Frontiers in microbiology 5:643.

14. Wright MS, Suzuki Y, Jones MB, Marshall SH, Rudin SD, Van Duin D, Kaye K, Jacobs MR, Bonomo RA, Adams MD. 2015. Genomic and transcriptomic analyses of colistin-resistant clinical isolates of Klebsiella pneumoniae reveal multiple pathways of resistance. Antimicrobial agents and chemotherapy 59:536-543.

15. Jaidane N, Bonnin RA, Mansour W, Girlich D, Creton E, Cotellon G, Chaouch C, Boujaafar N, Bouallegue O, Naas T. 2018. Genomic insights into colistin-resistant Klebsiella pneumoniae from a Tunisian teaching hospital. Antimicrobial agents and chemotherapy 62:e01601-17.

16. Baron S, Hadjadj L, Rolain J-M, Olaitan AO. 2016. Molecular mechanisms of polymyxin resistance: knowns and unknowns. International journal of antimicrobial agents 48:583-591.

17. Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. The Lancet infectious diseases 16:161-168.

18. Sonnevend Á, Ghazawi A, Hashmey R, Haidermota A, Girgis S, Alfaresi M, Omar M, Paterson DL, Zowawi HM, Pál T. 2017. Multi-hospital occurrence of pan-resistant Klebsiella pneumoniae ST147 with an ISEcp1-directed blaOXA-181 insertion into the mgrB gene in the United Arab Emirates. Antimicrobial agents and chemotherapy: AAC. 00418-17.

19. Chen L, Todd R, Kiehlbauch J, Walters M, Kallen A. 2017. Notes from the Field: Pan-Resistant New Delhi Metallo-Beta-Lactamase-Producing Klebsiella pneumoniae-Washoe County, Nevada, 2016. MMWR Morbidity and mortality weekly report 66:33-33.

20. MacNair CR, Stokes JM, Carfrae LA, Fiebig-Comyn AA, Coombes BK, Mulvey MR, Brown ED. 2018. Overcoming mcr-1 mediated colistin resistance with colistin in combination with other antibiotics. Nature communications 9:458.

21. Smith PA, Koehler MF, Girgis HS, Yan D, Chen Y, Chen Y, Crawford JJ, Durk MR, Higuchi RI, Kang J. 2018. Optimized arylomycins are a new class of Gram-negative antibiotics. Nature 561:189.

22. Project AR. 2018. Antibiotics Currently in Global Clinical Development. The PEW Charitable Trusts, Philadelphia, USA.

23. SHOJI JI, HINO H, SAKAZAKI R, KATO T, WAKISAKA Y, MAYAMA M, MATSUURA S, MIWA H. 1978. Isolation of tridecaptins A, B and C. The Journal of antibiotics 31:646-651.

24. Kato T, HINoo H, SHOJI JI. 1978. The Structure of Tridecaptin A. The Journal of antibiotics 31:652-661.

25. Cochrane SA, Findlay B, Bakhtiyari A, Acedo JZ, Rodriguez-Lopez EM, Mercier P, Vederas JC. 2016. Antimicrobial lipopeptide tridecaptain A1 selectively binds to Gram-negative lipid II. Proceedings of the National Academy of Sciences 113:11561-11566.
26. Cochrane SA, Lohans CT, van Belkum MJ, Bels MA, Vederas JC. 2015. Studies on tridecaptin B 1, a lipopeptide with activity against multidrug resistant Gram-negative bacteria. Organic & biomo
cr. 2015:6073-6081.
27. Janga M, Randhawa HK, Kaur M, Srivastava A, Maurya N, Patil P, Jaswal P, Arora A, Patil 
PB, Raje M. 2018. Purification, characterization and in vitro evaluation of polymyxin A from 
Paenibacillus dendritiformis: An underexplored member of the polymyxin family. Frontiers in 
microbiology 9:2864.
28. Zhang J, Xin L, Shan B, Chen W, Xie M, Yuen D, Zhang W, Zhang Z, Lajoie GA, Ma B. 2012. PEAKS 
DB: de novo sequencing assisted database search for sensitive and accurate peptide 
identification. Molecular & Cellular Proteomics 11:M1111.010587.
29. Kato T, SAKAZAKI R, HINO H, SHOJI JI. 1979. The Structures of Tridecaptins B and C. The Journal 
of antibiotics 32:305-312.
30. Kaneda T. 1967. Fatty acids in the genus Bacillus I. Iso-and anteiso-fatty acids as characteristic 
constituents of lipids in 10 species. Journal of bacteriology 93:894-903.
31. Cochrane SA, Lohans CT, Brandelli JR, Mulvey G, Armstrong GD, Vederas JC. 2014. Synthesis and 
structure–activity relationship studies of N-terminal analogues of the antimicrobial peptide 
tridecaptin A1. Journal of medicinal chemistry 57:1127-1131.
32. Lohans CT, van Belkum MJ, Cochrane SA, Huang Z, Sit CS, McMullen LM, Vederas JC. 2014. 
Biochemical, structural, and genetic characterization of tridecaptin A1, an antagonist of Campylobacter jejuni. ChemBioChem 15:243-249.
33. Keating TA, Marshall CG, Walsh CT, Keating AE. 2002. The structure of VibH represents 
nonribosomal peptide synthetase condensation, cyclization and epimerization domains. Nature 
Structural and Molecular Biology 9:522.
34. Li J, Jensen SE. 2008. Nonribosomal biosynthesis of fusaricidins by Paenibacillus polymyxa PKB1 
involves direct activation of a D-amino acid. Chemistry & biology 15:118-127.
35. Loh B, Grant C, Hancock R. 1984. Use of the fluorescent probe 1-N-phenyl-2-naphthylamine to 
study the interactions of aminoglycoside antibiotics with the outer membrane of Pseudomonas 
aeruginosa. Antimicrobial Agents and Chemotherapy 26:546-551.
36. Muheim C, Götzke H, Eriksson AU, Lindberg S, Lauritsen I, Nørholm MH, Daley DO. 2017. 
Increasing the permeability of Escherichia coli using MAC13243. Scientific reports 7:17629.
37. Tsubery H, Ofek I, Cohen S, Eisenstein M, Fridklin M. 2002. Modulation of the hydrophobic 
domain of polymyxin B nonapeptide: effect on outer-membrane permeabilization and 
lipopolysaccharide neutralization. Molecular pharmacology 62:1036-1042.
38. Yu Z, Qin W, Lin J, Fang S, Qiu J. 2015. Antibacterial mechanisms of polymyxin and bacterial 
resistance. BioMed research international 2015.
39. Noll KS, Sinko PJ, Chikindas ML. 2011. Elucidation of the molecular mechanisms of action of the 
natural antimicrobial peptide subtilosin against the bacterial vaginosis-associated pathogen 
Gardnerella vaginalis. Probiotics and antimicrobial proteins 3:41-47.
40. Ahmed S, Booth IR. 1983. The use of valinomycin, nigericin and trichlorocarbanilide in control of 
the proton motive force in Escherichia coli cells. Biochemical Journal 212:105-112.
41. Cui A-L, Hu X-X, Gao Y, Jing J, Yi H, Wang X-K, Nie T-Y, Chen Y, He Q-Y, Guo H-F. 2018. Synthesis 
and Bioactivity Investigation of the Individual Components of Cyclic Lipopeptide Antibiotics. 
Journal of medicinal chemistry 61:1845-1857.
42. Roberts KD, Azad MA, Wang J, Horne AS, Thompson PE, Nation RL, Velkov T, Li J. 2015. 
Antimicrobial activity and toxicity of the major lipopeptide components of polymyxin B and 
colistin: last-line antibiotics against multidrug-resistant Gram-negative bacteria. ACS infectious 
diseases 1:568-575.
43. Clardy J, Fischbach MA, Walsh CT. 2006. New antibiotics from bacterial natural products. Nature biotechnology 24:1541.

44. Midha S, Bansal K, Sharma S, Kumar N, Patil PP, Chaudhry V, Patil PB. 2016. Genomic resource of rice seed associated bacteria. Frontiers in microbiology 6:1551.

45. Shaheen M, Li J, Ross AC, Vederas JC, Jensen SE. 2011. Paenibacillus polymyxa PKB1 produces variants of polymyxin B-type antibiotics. Chemistry & biology 18:1640-1648.

46. Vila-Farres X, Chu J, Inoyama D, Ternei MA, Lemetre C, Cohen LJ, Cho W, Reddy BVB, Zebroski HA, Freundlich JS. 2017. Antimicrobials inspired by nonribosomal peptide synthetase gene clusters. Journal of the American Chemical Society 139:1404-1407.

47. te Winkel JD, Gray DA, Seistrup KH, Hamoen LW, Strahl H. 2016. Analysis of antimicrobial triggered membrane depolarization using voltage sensitive dyes. Frontiers in cell and developmental biology 4:29.

48. Ehrenberg B, Montana V, Wei M, Wuskell J, Loew L. 1988. Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. Biophysical journal 53:785-794.

49. Mitchell P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature 191:144-148.

50. Saraste M. 1999. Oxidative phosphorylation at the fin de siecle. Science 283:1488-1493.

51. Shapiro HM. 1994. Cell membrane potential analysis, p 121-133, Methods in cell biology, vol 41. Elsevier.

52. Cochrane SA, Findlay B, Vederas JC, Ratemi ES. 2014. Key Residues in Octyl-Tridecaptin A1 Analogues Linked to Stable Secondary Structures in the Membrane. ChemBioChem 15:1295-1299.

53. Ballantine RD, Li Y-X, Qian P-Y, Cochrane SA. 2018. Rational design of new cyclic analogues of the antimicrobial lipopeptide tridecaptin A1. Chemical Communications 54:10634-10637.

54. Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Müller R, Wohlleben W. 2015. antiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic acids research 43:W237-W243.

55. Stokes JM, MacNair CR, Ilyas B, French S, Côté J-P, Bouwman C, Farha MA, Sieron AO, Whitfield C, Coombe BK, Brown ED. 2017. Pentamidine sensitizes Gram-negative pathogens to antibiotics and overcomes acquired colistin resistance. Nature microbiology 2:17028.

56. Sun C, Li Y, Cao S, Wang H, Jiang C, Pang S, Hussain M, Hou J. 2018. Antibacterial Activity and Mechanism of Action of Bovine Lactoferricin Derivatives with Symmetrical Amino Acid Sequences. International journal of molecular sciences 19:2951.

57. Wu M, Maier E, Benz R, Hancock RE. 1999. Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of Escherichia coli. Biochemistry 38:7235-7242.

58. Morin N, Laneluc I, Connin N, Cottencemain M, Pons AM, Sablé S. 2011. Mechanism of bactericidal activity of microcin L in Escherichia coli and Salmonella enterica. Antimicrobial agents and chemotherapy 55:997-1007.

59. Cheng M, Huang JX, Ramu S, Butler MS, Cooper MA. 2014. Ramoplanin at bactericidal concentrations induces bacterial membrane depolarization in Staphylococcus aureus. Antimicrobial agents and chemotherapy 58:6819-6827.

60. Stark M, Liu L-P, Deber CM. 2002. Cationic hydrophobic peptides with antimicrobial activity. Antimicrobial agents and chemotherapy 46:3585-3590.

61. Goel MK, Khanna P, Kishore J. 2010. Understanding survival analysis: Kaplan-Meier estimate. International journal of Ayurveda research 1:274.
Ghafur A, Shankar C, GnanaSoundari P, Venkatesan M, Mani D, Thirunarayanan M, Veeraraghavan B. 2019. Detection of chromosomal and plasmid-mediated mechanisms of colistin resistance in Escherichia coli and Klebsiella pneumoniae from Indian food samples. Journal of global antimicrobial resistance 16:48-52.

**Figure Legends**

**Figure 1** Predicted biosynthetic gene cluster of M152-P3 and the 2D structure determined by Mass and NMR spectroscopy. **a**, Organisation of biosynthetic genes for M152-P3. *trmB* and *C* are ABC transporters encoding genes; *trmD* and *E* encode NRPS. *trmA* is a putative thioesterase gene. **b**, Different modules of NRPS. Domains: C, condensation; T, thiolation or peptide carrier domain; E, epimerase and TE, thioesterase. Adenylation domains are represented by the amino acid codes. **c**, the linear peptide sequence of tridecaptin M encoded by NRPS, also showing the position of D-amino acids. **d**, Two-dimensional structure of tridecaptin M (or M152-P3).

**Figure 2** Time-kill assay of M152 and resistance acquisition study. **a**, *K. pneumoniae* ATCC 700603 was grown in the presence of M152-P3 or colistin and CFU per ml was determined by the plating method. M152-P3 is a slow killer as compared to colistin but definitely bactericidal in nature. No re-growth observed after 24 h even at a low concentration of the peptide. The experiment was conducted in triplicate and data plotted as Mean±SD. Two biological repeats were performed and similar results were obtained. **b**, Serial passaging of *K. pneumoniae* in the sub-lethal concentration of M152-P3 or ciprofloxacin for 20 days. Afterwards, three days subculturing was performed without any antibiotic to confirm the stability of resistance. M152-P3 showed an increase of just four to eight folds in MIC whereas ciprofloxacin caused a 128-fold increase.

**Figure 3** Mode of action studies in *K. pneumoniae* ATCC 700603 and synergistic activity of M152-P3 with colistin. **a**, Outer membrane permeabilization assay with NPN. 32 and 64 in parentheses show the concentration in µg ml⁻¹. An increase in fluorescence indicates the localization of NPN in periplasm and phospholipid membrane suggesting disruption of the outer membrane. The data is representative of three independent experiments. Data plotted as Mean±SD of three replicates. **b**, ONPG assay for large pore formation in the inner membrane. ONPG is metabolized by β-galactosidase intracellularly to produce ortho-nitrophenol which absorbs at 420 nm. Polymyxin B shows inner membrane lysis which is characteristic of this class of antibiotics. No lysis observed in M152-P3 treated cells. Data shown here are mean±SD of three replicates. The experiment is representative of four independent experiments. **c**, PI uptake assay. Both compounds were used at a concentration of 32 µg ml⁻¹. M152-P3 shows negligible membrane lysis in 30 min whereas polymyxin B completely lysed the cells. The data plotted is Mean±SD of three replicates. **d**, Membrane depolarization using fluorescence leakage of DiSC₃(5). M152-P3 shows fluorescence leakage to some extent at higher concentrations. The experiment was performed in at least two biological repeats and plotted as Mean±SD of three replicates of one representative. **e, f**, ATP determination assay where (e) shows the growth of the untreated culture or treated with a sub-inhibitory concentration of M152-P3 after two hours.
After treatment, OD$_{600}$ for both samples was adjusted to achieve an equal number of bacteria and then intracellular ATP synthesis was compared by measuring bioluminescence (f) based on the luciferase assay. The experiment was performed in duplicates and the average value of three independent experiments is plotted here with standard deviation. Significance was calculated using a paired student’s t-test with two-tailed distribution, $P<0.05$. g. Potentiation assay of M152-P3 in the presence of colistin (at clinical breakpoint concentration). CF-23, CF-45 and CF-47 are mcr-1 positive E. coli strains; AH-16 is clinical K. pneumoniae. The numbers in the label, i.e. 0, 1 and 2 are colistin concentration in µg ml$^{-1}$. In E. coli strains, the MIC of M152-P3 was reduced by 16 folds at 2 µg ml$^{-1}$ of colistin whereas no synergy was observed in AH-16. FICI for colistin in strain CF-23, CF-45 and CF-47 was 0.31, 0.56 and 0.56 respectively when used at 2 µg ml$^{-1}$.

Figure 4 M152-P3 shows low in vitro and in vivo toxicity and is efficacious in thigh infection models of colistin-resistant K. pneumoniae. a, Haemolysis assay with rabbit RBCs. No lysis of RBCs up to 100 µg ml$^{-1}$. The data plotted as Mean±SD of three replicates. b, In vitro mammalian toxicity in HEK293 and J774 cell lines. The data shown here is Mean±SD of three replicates. c, Acute toxicity study in mice (n=4). Six doses (12 mg per kg) of M152-P3 or colistin were given subcutaneously at a two-hour interval. No mouse in colistin group could tolerate the six doses whereas M152-P3 showed no death at the administered dose and mice behaved similarly to the placebo group. d, Histopathology of major organs of mice after six-dose administration. M152-P3 showed no significant changes in any of the organs and results were comparable to PBS. e, In vivo efficacy of M152-P3 in thigh infection model (six mice in each group). K. pneumoniae ATCC 700603 was injected intramuscularly in the right thigh and single-dose treatment was given subcutaneously after 4 h. CFU per thigh was determined after 24 h post infection. Vehicle group received PBS. In the case of colistin-resistant K. pneumoniae AH-16, two-dose treatment was given at 2 h and 11 h post-infection and mice were sacrificed at 24 h post-infection. CFU for each mouse was calculated in duplicates and the average value was plotted. Each point in the graph represents one mouse. The data is plotted as Mean±SD.
Table 1 Antibacterial spectrum of M152-P3 in various pathogens

| Strain Name                                         | MIC (µg ml⁻¹) | M152-P3 | Polymyxin B | Colistin |
|-----------------------------------------------------|---------------|---------|-------------|----------|
| Klebsiella pneumoniae ATCC 700603                   | 4             | 1       | 1           |          |
| K. pneumoniae ATCC BAA-1705                         | 2             | 1       | 1           |          |
| K. pneumoniae ATCC BAA-1706                         | 4             | 1       | 1           |          |
| K. pneumoniae ATCC BAA-2146                         | 2             | 1       | 1           |          |
| K. pneumoniae ATCC 15380                            | 1             | 1       | 1           |          |
| K. pneumoniae ATCC 29665                            | 0.5           | 1       | 1           |          |
| K. pneumoniae subsp. rhinoscleromatis ATCC 13384    | 4             | 1       | 0.5         |          |
| K. oxytoca MTCC 8295                               | 2             | ND      | 1           |          |
| Enterobacter aerogenes MTCC 10208                   | 4             | ND      | ND          |          |
| E. cloacae MTCC 509                                | 4             | ND      | ND          |          |
| Escherichia coli ATCC 25922                         | 4             | 1       | 1           |          |
| Escherichia coli ATCC 35218                         | 4             | ND      | ND          |          |
| Escherichia coli 9062 (clinical isolate)            | 4             | 1       | 1           |          |
| Escherichia coli 7932 (clinical isolate)            | 4             | 1       | 1           |          |
| Pseudomonas aeruginosa ATCC 27853                   | 16            | 2       | 1           |          |
| Acinetobacter baumanii ATCC 19606                   | >32           | 2       | 1           |          |
| Salmonella enterica ATCC 10708                      | 4             | ND      | 1           |          |
| K. pneumoniae MDR clinical isolates (polymyxin-sensitive) | 2-8         | 1-2     | 0.5-2       |          |

Number of clinical strains = 19
Table-2 Activity of M152-P3 in colistin- and multidrug-resistant Enterobacteriaceae

| Strain | Resistance phenotype | MIC (µg/ml) |
|--------|----------------------|-------------|
|        | K. pneumoniae (clinical isolates) | M152-P3 | Pol B | Col |
| AH-1   | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 32 | 32 |
| AH-2   | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 8 | 8 |
| AH-3   | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 4 | 8 |
| AH-4   | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 4 | 8 |
| AH-5   | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 4 | 4 |
| AH-6   | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 4 | 8 |
| AH-7   | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 4 | 8 |
| AH-8   | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 4 | 4 |
| AH-9   | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 4 | 8 |
| AH-10  | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 4 | 8 |
| AH-11  | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 16 | 16 |
| AH-12  | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 16 | 32 |
| AH-13  | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 4 | 8 |
| AH-14  | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 4 | 8 |
| AH-15  | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 4 | 4 |
| AH-16  | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 4 | 4 |
| AH-17  | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 32 | 32 |
| AH-18  | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 32 | 32 |
| AH-19  | G, CIP, CTR, MRP, AMC, TOB, FO, CAZ, NIT, AK, P/T, NX, A/S, AT, PI, CTX, IMP | 2 | 32 | 32 |

E. coli (food isolates)

| Strain | mcr positive | MIC (µg/ml) |
|--------|--------------|-------------|
| CF-23  | mcr positive | 4 | ND | 8 |
| CF-45  | mcr positive | 4 | ND | 4 |
| CF-47  | mcr positive | 4 | ND | 4 |

*G, Gentamicin; CIP, Ciprofloxacin; MRP, Meropenem; AMC, Ampicillin; TOB, Tobramycin; FO, Fosfomycin; CAZ, Ceftazidine; NIT, Nitrofurantoin; AK, Amikacin; TE, Tetracycline; P/T, Piperacillin-Tazobactam; NX, Norfloxacin; A/S, Ampicillin-sulbactam; AT, Aztreonam; PI, Piperacillin; CTX, Cefotaxime; IMP, Imipenem. ‘I’ in parentheses is intermediate resistance.

*These strains were isolated from food samples and are sensitive to most of the other antibiotics (62). They were included in the study because they are colistin-resistant due to the presence of mcr-1 gene. The Klebsiella isolates have mutations in the mgrpB gene. ND, not determined; Pol B, polymyxin B; Col, colistin.
a. TrmD

```
A B C D E
Gly TEC Dab TEC Gly TEC Ser TEC Trp TEC Ser TC Dab TC Dab TEC Ile TC Glu TC
```

b. TrmE

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Ile TC alle TEC Ser TTE
```

c. 6-methyloctanoic acid - Gly - D-Dab - Gly - D-Ser - D-Trp - L-Ser - L-Dab - D-Dab - L-Ile - L-Glu - L-Ile - D-alle - L-Ser

d. [Chemical structure image]
