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In Vitro Efficacy of Lipid Conjugated Peptidomimetics Against *Mycobacterium smegmatis*

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Abstract
Tuberculosis has become a cause of worldwide concern; emergence of resistance in various mycobacterial strains has led to an urgent demand for new therapeutic molecules. Antimicrobial peptides have emerged as potential candidates in antimicrobial drug development. We have tested a lead antibacterial peptide LP-23 and its cost-effective mimetic DP-23 for their antimicrobial effects in a non-pathogenic model of *Mycobacterium.* Their minimum inhibitory concentration (MIC) values against *M. smegmatis* were calculated by resazurin reduction assay. MIC of both peptidomimetics against *M. smegmatis* was found to be 6.25 µg/mL. In addition, hemolytic toxicity study of peptidomimetics suggested that the synthesized compounds were selective against bacteria. To better understand stability and mechanism of action of peptidomimetics, serum stability study and SEM analysis were carried out. Peptoid DP-23 was found to be more stable in serum then peptide LP-23, while SEM analysis indicated that these compounds target the cell membrane, impairing the membrane integrity of *M. smegmatis*. The activity and properties of LP-23 and DP-23 may make them new potential antibacterial agents against tuberculosis.

Keywords *Mycobacterium smegmatis* · Peptidomimetics · Peptoid · Peptide · Antibacterial

Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis which thrives in macrophages within host cells. Tuberculosis has become a cause of growing public health concern throughout the world (Deng et al. 2016). *M. tuberculosis* infects humans, and has the ability to escape host immune mechanisms (Chatrath et al. 2016). It has evolved various mechanisms that allow it to survive within the adverse environment of macrophages and establish infection in the host (Arya et al. 2013). Since *M. tuberculosis* is an airborne bacterium that is highly infectious, *Mycobacterium smegmatis* is generally employed as its surrogate for biological studies (Gough et al. 2017). *M. smegmatis* is a fast growing, non-pathogenic soil bacterium sharing numerous features with pathogenic mycobacteria, making it a suitable model organism (He and De Buck 2010).

Most anti-tubercular drugs have become ineffective against *M. tuberculosis* due to the emergence of multidrug-resistant mycobacterial strains. Thus, new molecules with improved therapeutic potential that is effective against drug susceptible as well as multidrug-resistant mycobacterial strains are required. Antimicrobial peptides (APs) have emerged as potential alternative antimicrobial agents against resistant microbes. There are diverse APs, but only a small number of them have been tested against mycobacteria (Jena et al. 2011). Synthetic short cationic antimicrobial peptides have shown promising results against *M. tuberculosis* even at low micromolar concentrations (Ramón-García et al. 2013). A study by Khara et al. (2014) also demonstrated that short amphipathic α-helical peptides have the ability to kill susceptible as well as drug-resistant *M. tuberculosis.* Despite credible advantages, APs have not been utilized to their full potential as these suffer from serious drawbacks of poor proteolytic stability (Bottger et al. 2017), toxicity and high cost of production (Lyu et al. 2016) and these factors are often considered as a major limiting factor in their therapeutic

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applications. To overcome limitations associated with peptides, their synthetic mimetics known as 'peptidomimetics' are generally designed. Peptidomimetics not only mimic the biological activity of APs, but also display extended proteolytic stability (Molchanova et al. 2017).

N-substituted glycine congeners (peptoids) are one of the mimetics of peptides in which side chains are connected to nitrogen atom of the peptoid backbone, making peptoids more stable to proteolysis (Zuckermann et al. 1992). In addition, peptoid synthesis allows for low-cost production of peptidomimetics; and a variety of structures can be attained (Hebert et al. 2013). In the present study, a lead lipopeptide (LP-23) from our previous study (Lohan et al. 2013), and its peptoid analogue (DP-23) were synthesized and evaluated against *M. smegmatis* for antimycobacterial activity.

Materials and Methods

Materials Used

Amino acids: Protected amino acids were purchased from Novabiochem and N-boc-1,3-diaminopropane was purchased from Alfa aeser.

Chemicals used: diisopropyl carbodiimide (Sigma), 4-methyl piperidine (Alfa aeser), dimethyl formamide (Alfa aeser), diethyl ether (Sigma), trifluoroacetic acid (Sigma), rink amide MBHA resin (Novabiochem), triisopropyl silane (Alfa aeser), acetonitrile HPLC Grade (Merck).

Media used: Middle brook MB7H9 (Himedia), Mueller–Hinton Agar (Himedia), Luria–Bertani Agar (Himedia).

Bacterial strain: *Mycobacterium smegmatis* MC^2 155.

Standard drug used: Vancomycin HCl (Himedia).

Methods

Synthesis and Characterization of Peptidomimetics

Synthesis of LP-23: LP-23 was synthesized by solid phase synthesis method using rink amide MBHA resin (loading value 0.79 mmol/g) (Merrifield 1986). Rink amide MBHA resin was swollen in DMF (Dimethyl formamide) for 1 h. Deprotection of rink amide MBHA resin was carried out using 20% v/v 4-methyl piperidine in DMF (2 × 20 min). Resin was washed with DMF (5 × 2 mL) followed by coupling of 4 equivalent (equiv) Fmoc–Orn(Boc)–OH in the presence of 2 equiv DIC/HOBt in DMF for 4 h. Completion of coupling was confirmed by Kaiser test. This cycle was repeated desired number of times and finally (4 equiv) palmitic acid was conjugated to achieve the sequence of LP-23. After completion of sequence of LP-23, peptidomimetic was cleaved from the resin using 4 mL TFA cocktail (TFA: thioanisole: water, 95:2.5:2.5). Sample was freeze dried to obtain a powder. Synthesized peptidomimetic was characterized by RP-HPLC and mass spectroscopy. RP-HPLC was performed on a C18 column (Waters India) using 0–100% linear gradient of solvent B (0.1% TFA in acetonitrile) in solvent A (0.1% TFA in water) over 30 min and detection of peptidomimetic was done at 220 nm. Mass of LP-23 was confirmed by ESI–MS.

Synthesis of DP-23: Synthesis of DP-23 was carried out by submonomer method using rink amide MBHA resin (Bolt et al. 2017). Fmoc-protected Rink amide MBHA resin (100 mg) was swollen in DMF for 1 h. The resin was deprotected using 20% v/v 4-methyl piperidine in DMF (2 × 20 min) and washed with DMF (5 × 2 mL). Bromoacetylation was achieved using bromoacetic acid (1 mL, 0.6 M in DMF), DIC (0.2 mL, 50% v/v in DMF) for 2 × 30 min on a shaker platform at 200 rpm. The resin was washed with DMF (5 × 2 mL) and N-boc-1,3-diaminopropane (1 mL, 2 M in DMF) was added into reaction vessel and allowed to react for 4 h. Bromoacetylation and amine displacement steps were repeated until a desired sequence was complete and finally (4 equiv) palmitic acid was coupled to amine sequence using 0.2 mL DIC, 50% v/v in DMF. Methodology for cleavage of DP-23 from resin and its characterization was same as methodology mentioned above for LP-23.

Anti-mycobacterial Activity

- Determination of minimum inhibitory concentration (MIC)

  For determination of the antimycobacterial activity of LP-23 and DP-23 against *M. smegmatis,* inoculum of bacterial culture was prepared as per method given by Sirgel et al. (2009). *M. smegmatis* was inoculated in MB7H9 medium, 100 µL of inoculum and 100 µL of peptidomimetic dilution were added in round bottom microtitre plates so that the final density of inoculum in each well corresponds to 5×10^5 CFU/mL of *M. smegmatis* cells. Two fold dilutions of peptidomimetics were prepared and added to microtitre wells to obtain 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL and 3.125 µg/mL as final concentrations of DP-23 and LP-23. Mycobacterial cells along with media control was taken as positive control, Vancomycin HCl treated cells at 12.5 µg/mL was taken as drug control and sterile MB7H9 medium was taken as blank. The plates were covered to prevent media evaporation and incubated at 37 °C for 72 h.
- Minimal inhibitory concentration against: *M. smegmatis* by Resazurin assay

  Minimal inhibitory concentration (MIC) against *M. smegmatis* was determined using resazurin assay (Sieniawska et al. 2018). After incubation of microtitre

plates for 72 h, 20 µL of resazurin dye was added into each microtiter well. The plates were further incubated at 37 °C for 24 h, and observed for color development. A change from blue to pink indicated viability of bacterial cells as viable bacterial cells reduce resazurin (blue) to resorufin (pink). The experiments were performed in triplicates and MIC was determined as the lowest concentration of peptidomimetic that prevented development of pink color.

- Minimal bactericidal concentration against: *M. smegmatis*

  Minimal bactericidal concentration (MBC) against *M. smegmatis* was determined by incubating 10^5 CFU/mL cells of *M. smegmatis* with different concentrations of peptidomimetic ranging from 100 to 1.56 µg/mL for 24 h at 37 °C. Following incubation, 5 µL aliquot from each well was plated onto LB agar plates and incubated at 37 °C for 72 h. After 72 h, the plates were observed for the growth of *M. smegmatis*. MBC of compounds against *M. smegmatis* was determined as the lowest concentration of the compound at which no visible growth of *M. smegmatis* was observed. The MBC values were calculated as mean of triplicates.
- Growth kinetics

  Growth kinetics of *M. smegmatis* was determined by quantitative resazurin reduction assay (von Groll et al. 2010). *M. smegmatis* inoculum equivalent to 0.5 Mcfarland's standard was diluted 10 times. 100 µL of *M. smegmatis* culture was added to 100 µL of test compounds' solution to obtain a final concentration of 8x MIC in MB7H9 broth at 37 °C. Plates were incubated at 37 °C for 48 h. After 48 h of incubation resazurin dye was added and plates were re-incubated at 37 °C for 24 h. Optical density of resazurin dye was observed at two time points (24 h and 48 h) at 620 nm. Growth kinetics was established by plotting the difference in OD between inoculated and uninoculated wells vs. time of incubation. Results were expressed as mean ± SD of readings in triplicate.

Serum Stability Assay

Serum stability assay was carried out as per method described by Jenssen and Aspmo (2008). LP-23 and DP-23 were incubated with human serum at 37 °C. Aliquots were taken after 0 h, 3 h, 6 h and 24 h and processed further for analysis by RP-HPLC. Peptidomimetics were detected at 220 nm and percentage degradation was calculated using peak area at each time point relative to the initial peak area (at 0 min). Percentage degradation values for serum stability tests were calculated as mean of values in duplicates.

Hemolysis Assay

Hemolytic assay of synthesized compounds was carried out to determine their toxicity towards human RBCs by incubating a suspension of healthy human RBCs with serially diluted concentrations of the compounds. Briefly, red blood cells were washed three times by centrifugation at 2000 rpm for 10 min in normal saline and then a 4% v/v suspension of healthy human RBCs in normal saline was prepared. Dilutions of peptidomimetics in normal saline were added to micro centrifuge tubes, each tube contained 100 µL of peptidomimetic dilution and equal amount (100 µL) of RBCs' suspension to obtain concentrations of test compounds ranging from 500 to 50 µg/mL, followed by incubation at 37 °C for 60 min. Percent hemolysis was calculated by measuring optical density of supernatant at 570 nm using a microplate reader. Human erythrocytes incubated with 1% Triton X100 served as positive control, while suspension of human erythrocytes without treatment served as negative control. % hemolysis was calculated using following equation, where Abs stands for absorbance value obtained at 570 nm (de Menezes et al. 2014).

$$\%\text{Hemolysis} = \left(\frac{\text{Abs peptoid} - \text{Abs Blank}}{\text{Abs Triton} \times \text{Abs Blank}}\right) \times 100$$

Scanning Electron Microscopy (SEM) Analysis

SEM analysis was performed to determine the effect of LP-23 and DP-23 peptidomimetics on *M. smegmatis* cell morphology. Peptidomimetics were incubated with a high cell density bacterial culture, as a high cell density of about 10^8-10^{10} CFU/mL of bacterial cells is required to observe EM images (Hartmann et al. 2010). Briefly, *M. smegmatis* was cultured in MB7H9 media at 37 °C. 1 mL bacterial culture of *M. smegmatis* at late log phase (10^{10} CFU/mL) was inoculated with 0.25 mg/mL of LP-23 or DP-23 and incubated at 37 °C under shaking conditions at 200 rpm, with untreated culture serving as negative control. After 30 min of incubation, bacterial cultures were centrifuged at 3000 rpm for 15 min and washed thrice with phosphate buffered saline (PBS). The pellet formed was fixed with 2.5% glutaraldehyde for 1 h; followed by washing with PBS and dried in graded ethanol concentrations of 50, 70, 90, and 100% for 10 min each. Microscopy was performed with Hitachi (Japan) FESEM microscope.

Table 1 Results of characterization, antibacterial studies and hemolysis assay

Compound name	Retention time (min)	Percent purity	Calculated mass (M+1)	Observed mass (M+1)	MIC (μg/mL)	MBC (μg/mL)	HC_{50} (μg/mL)
LP-23	5.103	> 95	712.5	712.5	6.25	12.5	490
DP-23	5.073	> 95	712.5	712.5	6.25	12.5	390

MIC minimum inhibitory concentration, *MBC* minimum bactericidal concentration, HC_{50} concentration of test compound causing 50% hemolysis of human RBCs

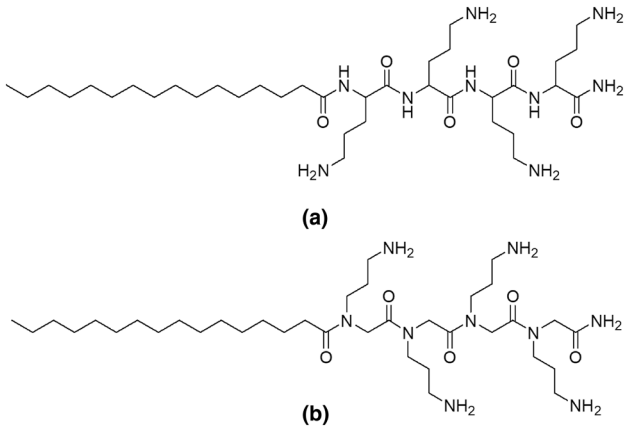

Fig. 1 Chemical structures of LP-23 (**a**) and DP-23 (**b**)

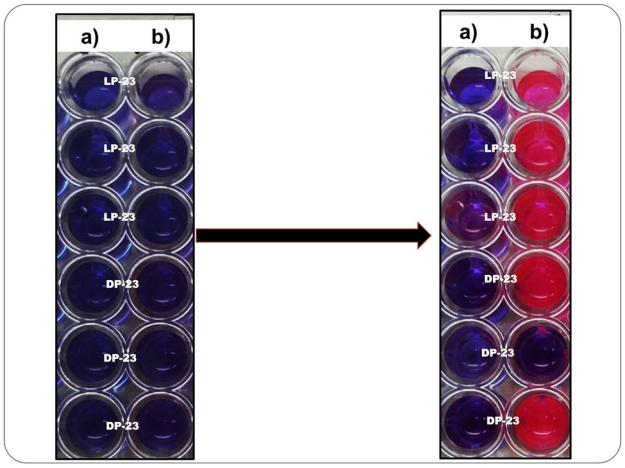

Fig. 2 MIC of peptidomimetics against *M. smegmatis* (conversion of resazurin (blue) to resorufin (pink) by viable cells after 24 h of incubation; **a** 6.25 μg/mL and **b** 3.125 μg/mL concentration of tested compound (Color figure online)

Results and Discussion

Synthesis and Characterization

The lead from previous study (LP-23) is an ornithine rich peptide with palmitic acid as hydrophobic residue (Lohan et al. 2013). DP-23, a short amphiphilic peptoid was based on LP-23 lipopeptide. In DP-23, peptoid backbone was achieved by submonomer synthesis using bromoacetic acid; side chains were introduced by displacement of bromide by Boc-1, 3-diaminopropane. Overall cationic charge in both peptidomimetics was +4 (Table 1, Fig. 1). LP-23 and DP-23, both were synthesized manually on rink amide MBHA resin by solid phase synthesis (Scheme 1, supplementary file). They were characterized by RP-HPLC and mass spectroscopy. Molecular mass of both compounds was found to be 712.5 $(M+1)^+$ and their percentage purity as per analytical RP-HPLC was found to be > 95%. Results of ESI–MS and RP-HPLC (Table 1) suggested that both peptidomimetics were synthesized successfully (see Supplementary Files for HPLC chromatograms and mass spectrums).

Selectivity of Peptidomimetics

Antibacterial activity and hemolysis assay were carried out to determine the therapeutic potential of synthesized compounds. Results of antibacterial activity against *M. smegmatis* (Table 1) suggested that both DP-23 and LP-23 had similar anti-mycobacterial activity towards *M. smegmatis* with MIC at 6.25 μg/mL (Fig. 2) and MBC at 12.5 μg/mL. Growth kinetics study was carried out to determine the effect of peptidomimetics on the growth of *M. smegmatis* at two time points (24 h and 48 h). DP-23 displayed comparable effect like LP-23 on the growth kinetics of *mycobacterium.* Results of growth kinetics study of peptidomimetics on *M. smegmatis* (Fig. 3) suggested that DP-23 has the potential to mimic the inhibitory effects of LP-23 on growth of *mycobacterium.* Overall results of antibacterial studies (MIC, MBC and growth kinetics study) of synthesized compounds suggested that peptoid DP-23 retained the antibacterial activity of lipopeptide LP-23.

On the other hand, hemolysis assay was carried out to determine the toxicity of peptidomimetics towards human RBCs. The HC_{50} values of DP-23 (390 μg/mL) and LP-23 (490 μg/mL) suggested that both compounds were selective towards bacteria, as these values (Table 1) were much greater than their MIC values against *M. smegmatis*. Both compounds (LP-23 and DP-23) are rich in cationic charge

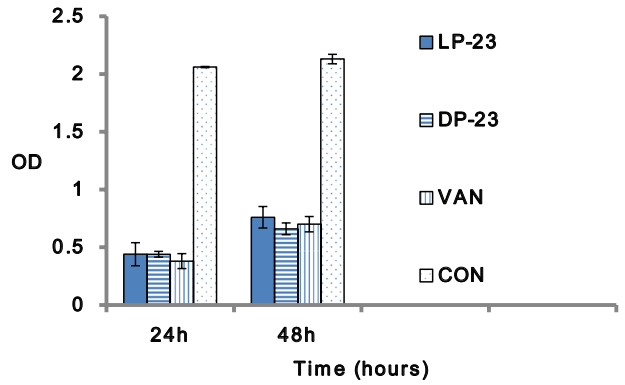

Fig. 3 Effect of LP-23, DP-23, vancomycin HCl and without treatment (control) on growth kinetics of *M. smegmatis*

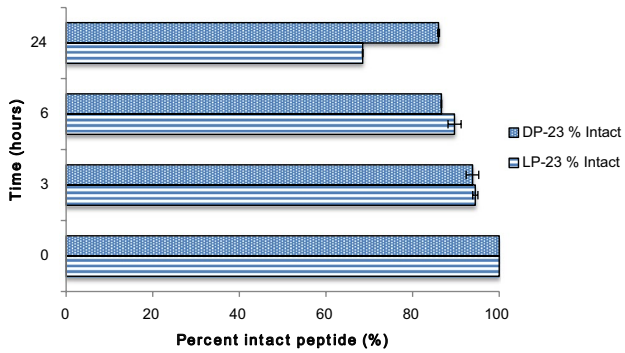

Fig. 4 Serum stability profile of DP-23 and LP-23

and have amphipathic structure; studies performed confirm that the synthesized compounds were selective towards *M. smegmatis*.

Serum Stability Study

Serum stability assay was used to gather information about the peptidomimetic's ability to resist proteolytic degradation by enzymes present in serum. Percent degradation after 24 h of incubation with human serum for DP-23 was 14% in comparison to 31.53% for LP-23. Results of serum stability assay suggested that DP-23 was more stable than LP-23 (Fig. 4). The reason behind this stability lies in the difference in structures of peptoid and peptide. In peptoid the side chains are connected to the nitrogen atom of the amide backbone while in case of peptides side chains are connected to α -carbon atom of amide backbone. Thus, making peptoids more stable to proteolysis in comparison to peptides (Culf and Ouellette 2010; Webster and Cobb 2018).

Effect on Cell Morphology

APs as anti-mycobacterial compounds have not been explored extensively, therefore its mechanisms of action against mycobacteria are still not clearly known (Santos et al. 2012). However, few synthetic cationic antimicrobial peptides modified with different hydrophobic amino acids have been found to kill mycobacteria via a lytic mechanism on bacterial membrane (Khara et al. 2014). To investigate whether LP-23 and DP-23 synthesized in this study, act on the mycobacterial cell membrane or not, bacterial cells were treated with supra-MIC concentrations of peptidomimetics, and later the effect of peptidomimetics on cell morphology was visualized using scanning electron microscopy. SEM microphotographs (Fig. 5) of DP-23 and LP-23 treated *M. smegmatis* show alterations in cell membrane morphology in comparison to control treated cells. SEM microscopy results revealed that both peptidomimetics DP-23 and LP-23 disrupt the cell membrane of *Mycobacterial* cells. DP-23 formed pores on the bacterial cell membrane, whereas LP-23 caused damage over the entire cell membrane of *M. smegmatis.* However, more studies are required at molecular level to understand the exact difference in mode of action of LP-23 and DP-23.

Conclusion

Mycobacterium tuberculosis is a highly infectious pathogen; therefore fast growing *M. smegmatis* was used as a model mycobacterial organism as it shares numerous features with pathogenic mycobacteria (He and De Buck 2010). As multicellular organisms are constantly exposed to numerous pathogenic microbes, the innate and adaptive immune system plays an important role in the control of microbial infections (Gupta et al. 2015). Natural antimicrobial peptides are proposed to act as the first line of mucosal host defense as they exert microbicidal activity against various pathogenic microbes (Cole et al. 2000). This is the motivation behind the design of short peptoids as potential candidates for the efficacious eradication of bacterial pathogens that show increasing resistance towards traditionally used antibiotics (Mojsoska et al. 2015). Here, a lipopeptoid DP-23 was designed based on previously developed lead antimicrobial lipopeptide LP-23, and both were evaluated for their in vitro activity against *M. smegmatis.* Unlike other traditional antimicrobials, most of which act on cellular targets, these compounds act via unique mechanism by damaging cell membrane integrity. Hence, DP-23 and LP-23 may be optimized as potential lead compounds against *M. tuberculosis.*

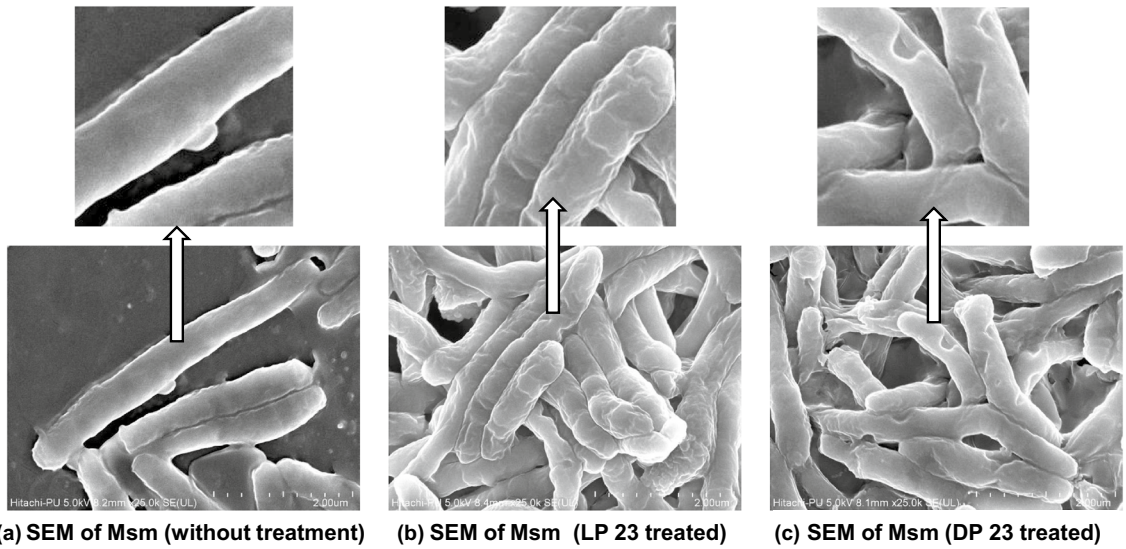

Fig. 5 SEM microphotograph of control (without treatment), DP-23 and LP-23 treated *M. smegmatis* (Msm)

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Compliance with Ethical Standards

Conflict of interest There is no conflict of interest.

Ethical Approval All procedures and protocols mentioned in this article have been approved by Institutional Biosafety Committee of Jaypee University of Information Technology, Waknaghat. The study does not involve any human participant or animal.

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