In vitro activity of twelve antimicrobial peptides against *Mycobacterium tuberculosis* and *Mycobacterium avium* clinical isolates.

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Abstract

Tuberculosis (TB) remains a major threat to human health worldwide. The increasing incidence of non-tuberculous mycobacterial infections and particularly those produced by *Mycobacterium avium* has emphasized the need to develop new drugs. Additionally, high levels of natural drug resistance in non-tuberculous mycobacteria (NTM) and the emergence of multidrug-resistant (MDR) TB is of great concern. Antimicrobial peptides (AMPs) are antibiotics with broad-spectrum antimicrobial activity. The objective was to assess the activity of AMPs against *Mycobacterium tuberculosis* and *M. avium* clinical isolates. Minimum inhibitory concentrations (MIC) were determined using microtiter plates and the resazurin assay. Mastoparan and melittin showed the greatest activity against *M. tuberculosis*, while indolicidin had the lowest MIC against *M. avium*. In conclusion, AMPs could be alternatives for the treatment of mycobacterial infections. Further investigation of AMPs activity in combination and associated with conventional antibiotics and their loading into drug-delivery systems could lead to their use in clinical practice.

Keywords: antimicrobial peptides; resazurin assay; minimum inhibitory concentration; mycobacterial infections; *Mycobacterium tuberculosis; Mycobacterium avium*.

Tuberculosis (TB) remains a major health problem worldwide and is one of the leading causes of death by a single infectious agent, that is, *Mycobacterium tuberculosis*. According to the World Health Organization in 2016 there were an estimated 10.4 million new TB cases and 1.6 million TB deaths [1]. Furthermore, the emergence of multidrug-resistant (MDR) TB and extensively drug-resistant (XDR) TB has led to the need to develop new treatment options [2].

Infections produced by nontuberculous mycobacteria (NTM) have dramatically increased in the last years, mainly in immunocompromised patients and individuals with pre-existing pulmonary diseases. Among these NTM, *Mycobacterium avium* is of note and is gaining increasingly more relevant clinical significance. The high levels of natural drug resistance of NTM lead to poor treatment outcomes requiring novel drug regimens and compounds [3].

Antimicrobial peptides (AMPs) are powerful natural antibiotics produced by all life forms from microorganisms to humans [4-6]. AMPs tend to be relatively short (20-60 amino acid residues), amphipathic and positively charged. Four main structural AMPs classes have been defined: α-helix, β-hairpin, β-sheet, and linear, non α-helical [7]. In addition, several AMPs databases, such as the Antimicrobial Peptide Database (APD), Dragon Antimicrobial Peptide Database (DAMPD) and the database Linking Antimicrobial Peptide (LAMP) are available, providing comprehensive information about their antimicrobial activity and mechanisms of action [8, 7].

AMPs play an important role in the innate immune response and have the ability to modulate host defences. They promote pathogen clearance with their broad-spectrum antimicrobial activity against bacteria, viruses, parasites and fungi. Most AMPs target the cell wall by different modes of action, such as pore formation, thinning, altered curvature, localized perturbations and modified electrostatics [9]. Hence, the interaction between AMPs and the membrane may result in disruption of the bacterial membrane. Different studies have demonstrated that AMPs increase the permeability of the mycobacterial wall, which may facilitate the translocation of AMPs and other drugs across the membrane and into the cytoplasm. Additionally, acquisition of resistance to AMPs is very rare, and only a few bacteria show resistance. As a result, AMPs have promising clinical applications and the potential to combat mycobacterial infections [10-12].

The objective of the present study was to investigate the activity of 12 different AMPs (bactenecin, buforin I, mastoparan, indolicidin, histatin 5, histatin 8, magainin I, magainin II, cecropin PI, cecropin A, cecropin B and melittin) against clinical isolates of *M. tuberculosis* and *M. avium* using microtiter plates and the resazurin assay.

Six clinical isolates of *M. tuberculosis* susceptible to first-line anti-TB drugs and 4 clinical isolates of *M. avium* were studied. Two of the *M. avium* isolates were susceptible to clarithromycin, rifampicin and amikacin but resistant to ethambutol. The other 2 *M. avium* isolates were susceptible to clarithromycin, rifampicin amikacin and ethambutol. All of the isolates were obtained from the Laboratory of Microbiology of the Hospital Clínic of Barcelona (Barcelona, Spain).

Of the 12 AMPs studied, histatin 5, magainin I, magainin II, cecropin PI, cecropin A and cecropin B were provided by Sigma-Aldrich (St. Louis, MO, USA). On the other hand, bactenecin, buforin I, mastoparan, indolicidin, histatin 8 and melittin were purchased from BionovaCientifica (S.L., Madrid, Spain) (Table 1) [12]. The lyophilized AMPs were dissolved in sterile distilled water and sterilized by filtration. Then, they were stored at -20°C in aliquots until use. All the AMPs were tested at concentrations ranging from 128 µg ml^{-1} to 0.125 µg ml^{-1}. The AMPs were selected based on the activity shown against mycobacteria and other bacterial species described in previous studies as well as their availability and affordability [8].

M. tuberculosis isolates were grown in Lowenstein-Jensen medium slants (Becton Dickinson, Sparks, MD) and *M. avium* isolates were grown in BD™ Columbia Agar with 5 % Sheep Blood plates (Becton Dickinson). Afterwards, all of the isolates were subcultured in Middlebrook 7H9 liquid medium (Becton Dickinson) supplemented with 10 % oleic acid-albumin-dextrose-catalase (OADC) (Comercial Bellés, Tarragona, Spain) and 0.25 % Tween 80 (Merck, Darmstadt, Germany) to avoid bacilli clump formation. After 7 days of incubation, *M. tuberculosis* cultures were subjected to disaggregation techniques by being vigorously vortexed in a tub containing glass beads (Sigma-Aldrich) and the use of insulin syringes (Becton Dickinson). *M. avium* cultures were homogenised by agitation. Finally, the inoculum was adjusted to 1.5 x 10^8 cells ml^{-1} using a nephelometer (CrystalSpec™; Becton Dickinson).

The minimum inhibitory concentration (MIC) of each peptide was determined using the resazurin assay in 96-well microtiter plates (Smartech Biosciences, Barcelona, Spain) as described by Palomino *et al*. [13]. Schena *et al.* [14] demonstrated that the resazurin assay is useful for the rapid and accurate MIC determination of delamanid in *M. tuberculosis* isolates, showing great concordance with the agar reference method. Briefly, 100 µl of Middlebrook 7H9 liquid media were added to each well. Then, serial dilutions of the AMPs ranging from 128 µg ml^{-1} to 0.125 µg ml^{-1} were made. Finally, 100 µl of inoculum at a final concentration of 5 x 10^5 cells ml^{-1} were added. Positive control wells consisted of 100 µl of Middlebrook 7H9 and 100 µl of inoculum (5 x 10^5 cells ml^{-1}). Negative control wells were prepared by adding 200 µl of Middlebrook 7H9. Plates were incubated at 37 °C in a 5 % CO_2 atmosphere for 7 days, after which 20 µl (10 % of the final volume) of fresh resazurin were added (alamarBlue® Invitrogen, Life Technologies, Belgium). Plates were covered with aluminium foil for

protection from light. After overnight incubation, visual reading was performed. Resazurin is a colorimetric reagent used to assess cell viability. This active agent, is blue in color and is reduced to resorufin (pink in color) by viable cells. A colorimetric change from blue to pink was interpreted as mycobacterial growth. The MIC was interpreted as the first concentration at which there was no colorimetric change. All the experiments were performed in duplicate for each isolate.

Twelve different AMPs were tested against clinical isolates of *M. tuberculosis* and *M. avium*. The AMPs tested showed moderate activity against *M. tuberculosis* and *M. avium* clinical isolates.

The MIC values obtained are shown in Table 2. When tested against *M. tuberculosis*, mastoparan and melittin showed the best activity, with MIC values ranging from 32-64 μg ml^{-1}. Indolicidin showed MIC values of 32 μg ml^{-1} against 2 of the isolates and of 64 μg ml^{-1} against 1 isolate. The other AMPs showed higher MICs (>128 μg ml^{-1}).

Regarding *M. avium*, indolicidin had the greatest activity with MIC values of 128 μg ml^{-1}. All the remaining AMPs showed MICs >128 μg ml^{-1}.

To our knowledge, there are few studies on the *in vitro* effect of the AMPs investigated in the present study against *M. tuberculosis*, and especially against *M. avium*. Human neutrophil peptides (HNP) are the most frequently studied against mycobacteria [15]. Sharma *et al*. [16] reported that HNP-1 has effective bactericidal activity against *M. tuberculosis* H37Rv *in vitro* as well as mycobacteria replicating within macrophages. Furthermore, Ogata *et al*. [17] demonstrated that HNP-1 and HNP-2 killed all of the *M. avium-M. intracellulare* isolates. Therefore, the present study aimed to assess the antimicrobial activity of 12 AMPs with antibacterial activity against other bacteria in *M. tuberculosis* and *M. avium* clinical isolates.

The activity of AMPs against mycobacteria is probably lower than in Gram-negative and other Gram-positive bacteria [15]. In the present study, we tested a set of 12 AMPs previously tested against other bacteria but rarely studied or with unknown activity against mycobacteria. As shown in Table 2, mastoparan and melittin showed better results against *M. tuberculosis* than the other AMPs. In a previous study these two AMPs also showed a good activity against *Acinetobacter baumanii* [12]. However, these AMPs were less effective against the *M. avium* isolates. Differences in membrane composition may affect the activity of the AMPs. Therefore, we hypothesized that they may present a different efficacy in other mycobacterial species, suggesting that each AMP should be specifically tested in each species. Moreover, the mycobacterial cell membrane has a distinct architecture with a lipid-rich envelope, and taking into account the cationic and amphipathic nature of these peptides AMPs targeting mycobacterial membrane lipids could be further explored.

Several AMPs have shown to cause membrane permeabilization, which would facilitate the entry of drugs into the mycobacterial cell and allow interaction with intracellular targets [18]. From this point of view, AMPs could have greater activity or even synergism when combined with antibiotics. Sharma *et al.* [19] observed that the bacterial peptide deformylase (PDF) showed synergism with isoniazid and rifampicin against *M. tuberculosis* H37Rv. Therefore, further studies are needed to stablish the potential synergism of any AMP in combination with antimycobacterial antibiotics [20]. Combination therapy including AMPs and common antibiotics would be an excellent therapeutic option to facilitate antibiotic uptake and decrease the MICs. Moreover, this novel treatment could

reduce the therapeutic dose, prevent the appearance of resistance and reduce side effects. In a recent study, Li *et al*. [21] demonstrated that the combination of PA-824 (pretomanid) and *Cordyceps sinensis* had greater bacteriostatic activity against *M. tuberculosis* than pretomanid alone.

It is mandatory to find novel drugs with minimum or no toxicity to human cells. In general, many AMPs are considered to have low toxicity to eukaryotic cells. In addition, AMPs are cell selective, having the ability to kill bacterial cells without being toxic to human host cells. Furthermore, AMP selectivity could be increased by modifying the molecule, thus altering the physicochemical properties of the AMPs which would thereby enhance the safety profile [9, 7]. Nonetheless, some studies have described the *in vitro* cytotoxicity of some AMPs. For instance, Fu *et al.* [22] reported that HNP-1 exhibits important cytotoxicity to different types of cells when found at high concentrations. On the other hand, in order to improve their activity and to target AMPs to specific sites of infection, they could be loaded in drug-delivery systems such as liposomes and nanoparticles. These methods of drug administration reduce side effects by allowing the delivery of high drug concentrations directly to the site of infection. However, this system of administration should be further optimized [7].

In conclusion, AMPs are promising alternatives in the treatment of mycobacterial infections. Nonetheless, since the AMPs studied may exhibit even greater activity, further evaluation of these AMPs in combination and particularly associated with conventional antibiotics would be of great interest. Moreover, new methods of drug administration may improve the therapeutical potential of AMPs.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Ethical statement

Ethical approval was received from the Ethical Committee of the Hospital Clínic de Barcelona (Barcelona, Spain) [ref.no. 2013/8147].

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Table 1. Characteristics of the antimicrobial peptides used in the present study

Antimicrobial peptide	Sequence	Length	Source	Activity
Bactenecin	RLCRIVVIRVCR	12	*Bos taurus*	Antibacterial
Buforin I	AGRGKQGGKVRAKAKTRSSRAGLQFPVGRVHRLLRKGNY	39	*Bufo bufo gargarizans*	Antibacterial, Antifungal
Mastoparan	INLKALAALAKKIL	14	*Vespula lewisii*	Antibacterial
Indolicidin	ILPWKWPWWPWRR	13	*Bos taurus*	Antibacterial, Antifungal, Antiviral
Histatin 5	DSHAKRHHGYKRKFHEKHHSHRGY	24	*Homo sapiens*	Antibacterial, Antifungal, Antiviral
Histatin 8	KFHEKHHSHRGY	12	*Homo sapiens*	Antibacterial, Antifungal
Magainin I	GIGKFLHSAGKFGKAFVGEIMKS	23	*Xenopus laevis*	Antibacterial, Antiviral
Magainin II	GIGKFLHSAKKFGKAFVGEIMNS	23	*Xenopus laevis*	Antibacterial, Antifungal, Antiviral, Antiparasitic
Cecropin PI	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	31	*Ascaris suum*	Antibacterial
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK	37	*Hyalophora cecropia*	Antibacterial, Antiviral, Antiparasitic
Cecropin B	KWKIFKKIEKVGRNIRNGIIKAGPAVAVLGEAKAL	35	*Antheraea pernyi*	Antibacterial, Antifungal
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	26	*Apis mellifera*	Antibacterial, Antifungal, Antiviral, Antiparasitic

The AMPs features were obtained from the Antimicrobial Peptide Database (APD, http://aps.unmc.edu/AP/main.php).

Table 2. Minimum inhibitory concentrations of the 12 antimicrobial peptides tested against *M. tuberculosis* clinical isolates

Isolate	Antimicrobial peptide MICs (μg ml^{-1})											
	Bac	Buf I	Mas	Ind	His 5	His 8	Mag I	Mag II	Cec PI	Cec A	Cec B	Mel
M. tuberculosis 1	>128	>128	64	>128	>128	>128	>128	>128	>128	>128	>128	64
M. tuberculosis 2	>128	>128	64	>128	>128	>128	>128	>128	>128	>128	>128	64
M. tuberculosis 3	>128	>128	64	>128	>128	>128	>128	>128	>128	>128	>128	64
M. tuberculosis 4	>128	>128	32	32	>128	>128	>128	>128	>128	>128	>128	32
M. tuberculosis 5	>128	>128	64	64	>128	>128	>128	>128	>128	>128	>128	64
M. tuberculosis 6	>128	>128	32	32	>128	>128	>128	>128	>128	>128	>128	64

MICs, minimum inhibitory concentrations; Bac, bactenecin; Buf I, buforin I; Mas, mastoparan; Ind, indolicidin; His 5, histatin 5; His 8, histatin 8; Mag I, magainin I; Mag II, magainin II; Cec PI, cecropin PI; Cec A, cecropin A; Cec B, cecropin B; Mel, melittin.