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Effect of antimicrobial peptides on ATPase activity and proton pumping in plasma membrane vesicles obtained from mycobacteria

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ABSTRACT

The potential usefulness of antimicrobial peptides (AMPs) as antimycobacterial compounds has not been extensively explored. Although a myriad of studies on AMPs from different sources have been done, some of its mechanisms of action are still unknown. Maganins are of particular interest since they do not lyse non-dividing mammalian cells. In this work, AMPs with well-recognized activity against bacteria were synthesized, characterized, purified and their antimycobacterial activity and influence on ATPase activity in mycobacterial plasma membrane vesicles were assessed. Using bioinformatics tools, a magainin-I analog peptide (MIAP) with improved antimicrobial activity was designed. The influence of MIAP on proton (H^+) pumping mediated by F_1F_0-ATPase in plasma membrane vesicles obtained from *Mycobacterium tuberculosis* was evaluated. We observed that the antimycobacterial activity of AMPs was low and variable. However, the activity of the designed peptide MIAP against *M. tuberculosis* was 2-fold higher in comparison to magainin-I. The basal ATPase activity of mycobacterial plasma membrane vesicles decreased approximately 24–30% in the presence of AMPs. On the other hand, the MIAP peptide completely abolished the F_1F_0-ATPase activity involved in H^+ pumping across *M. tuberculosis* plasma membranes vesicles at levels similar to the specific inhibitor N,N′ dicyclohexylcarbodiimide. These finding suggest that AMPs can inhibit the H^+ pumping F_1F_0-ATPase of mycobacterial plasma membrane that potentially interferes the internal pH and viability of mycobacteria.

1. Introduction

The discovery of new drugs against mycobacteria is one of the most important priorities in tuberculosis (TB) control. *Mycobacterium tuberculosis* is the leading cause of human TB and the cause of the most number of deaths by a bacterial pathogen worldwide. The World Health Organization (WHO) reported 8.9 million new cases and 1.7 million of deaths caused by TB in 2010 [59]. The treatment against TB is long and difficult due to the complex biology of the tubercle bacilli and latent infections, which sometimes reduce the activity of antituberculous drugs [37]. The increasing number of multidrug-resistant (MDR)-TB cases reflects the need to improve the current treatment. The identification of new drugs with diminished toxicity and improved activity against MDR strains are necessary. Thus, a deeper understanding about mycobacterial biology and the mechanism of drug actions is important.

Among others, resistance against antimycobacterial drugs is induced by the high content of lipids in the mycobacterial cell wall [13]. The low fluidity produced by the mycolic acid layer, and low content of porins in the mycobacterial cell wall make the uptake of antimycobacterial compounds difficult [45]. The particular structure of antimicrobial peptides (AMPs), their modulatory effects on the immune system, and their mode of action make them an interesting anti-TB tool [20]. AMPs function as a part of the innate immune response in a wide range of organisms, including humans [18,61]. Most AMPs are characterized by their low molecular weight, cationic charge, amphipathic α-helical structure, and affinity for the membranes of prokaryotic cells [24]. The positive charge, hydrophobicity and flexibility facilitate AMP interactions with anionic phospholipids of bacterial plasma membranes [58]. Once AMPs bind to plasma membranes or artificial bilayers, their hydrophobic and cationic aminoacids are able to be spatially distributed on surface by interacting with negatively charged lipids [31]. The α-helical structure of some AMPs can form channels or pores that interfere with the plasma membrane transport [49]. Lipids of the external membranes of vegetal and mammalian cells do not have charges that effect their interactions with AMPs, making these peptides selective for prokaryotic cells.

Little is known about the activity of AMPs against mycobacteria. Some studies have shown low *in vitro* activity of human

Abbreviations: AMPs, antimicrobial peptides; DCCD, N,N′ dicyclohexylcarbodiimide; MDR, multidrug resistant; MIAP, magainin-I analog peptide; MIC, minimal inhibitory concentration; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; TB, tuberculosis; TFA, trifluoroacetic acid.

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β-defensin-2 (HBD-2), human neutrophil peptide-1 (HNP1), protegrins, granulysins and cathelicidins against *M. tuberculosis* [16,35,46,47,50,57]. The *ex vivo* activity of HNP1 was estimated to be approximately 50–98% against *M. tuberculosis* inside macrophages [50]. In addition, combinations of protegrin-1 and human β-defensin-1 with isoniazid were observed to significantly increase anti-TB activity [16]. There are recent discoveries about the biological activity of α-helical AMPs against *M. tuberculosis*. The novo designed peptides consisting of all D-amino acid residues display bactericidal concentrations lower than 50 μg/mL against *M. tuberculosis* clinical isolates [25]. In some cases, the deleterious effect of AMPs on the mycobacterial cell membrane has been demonstrated. The synergistic killing of *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG by human placental lysosomal contents together with NK-2, the cationic core of the NK-lysin protein, or Ci-MAM-A2, a peptide derived from immune cells of *Ciona intestinalis*, has been observed by electron microscopy [23].

On the other hand, the use of AMPs as immunotherapy agents is becoming increasingly important in the development of new strategies to control mycobacterial infections. It highlights, for example, the potential use of induced β-defensin-1 as a novel immunotherapy/adjuvant in experimental murine TB has been proposed to be useful in TB control [40]. The production of hepcidin in epithelial cells induced by *M. tuberculosis* lipoglycans, or by stimulation of mouse macrophages with IFN-γ + *M. tuberculosis* has also been demonstrated [54]. In this context, the role of cathelicidins in the intracellular killing of *M. tuberculosis* has acquired relevance in the development of strategies for the *M. tuberculosis* control. Cathelicidins are a family of cationic peptides that are present in different human epithelia and the mechanism of action is by interacting with the cell membrane inducing pore formation, leaking of the cell cytoplasm and consequent bacteria cell death [33]. It has been recently demonstrated that vitamin D induces production of cathelicidins mediated by IFN-γ realizing in T-cells [16].

Potential targets and physiological effects of AMPs on the mycobacterial envelope have not been fully identified. However, it is known that the interaction of AMPs on the cell surface interferes with the normal ion transport, inhibiting mycobacterial cell growth [50]. Some proteins of the mycobacterial plasma membrane are responsible for maintaining an adequate level of metal ions and protons (H^+) inside mycobacteria that are essential for enzyme function and mycobacterial survival. In bacteria, ionic transport is carried out by efflux pumps that belong to either the P-type ATPase superfamily, ATP binding cassettes (ABC transporters) and by metallic ion/H^+-antiporter systems in which ion transport depends on the energy supplied by the H^+ gradient [3,40]. ATPases help maintain the ionic gradients responsible for cellular volume control and the function of vital intracellular proteins in eukaryotic cells [14]. However, their distribution and function in prokaryotic cells are not fully understood. ATPases hydrolyze ATP, releasing energy that is used in the transport of ions against electrochemical gradients in plasma membranes. Particularly, P-type ATPases, which can be inhibited by vanadate, undergo a phosphorylation/dephosphorylation of an aspartate residue within the DKTGTLT consensus sequence during the catalytic cycle. This modification induces conformational changes in the enzymes, resulting in different affinities and cation transport [5]. Other types of ATPases, known as type F, have dual functions of ATP hydrolysis coupled to reversible H^+ pumping by electrochemical gradients [9]. This mechanism is coupled to pH homeostasis to prevent intracellular acidification [48]. F-type ATPases, which are inhibited by N,N′ dicyclohexylcarbodiimide (DCCD), consist of F_1 and F_0 subunits that function as ATPases and synthases, respectively [8]. When the *M. smegmatis* and *M. bovis* BCG F_1F_0-ATPase is inhibited by DCCD, the transmembrane H^+ gradient (ΔpH) diminishes, decreasing cellular viability [43].

Table 1
Sequences of antimicrobial peptides.

Peptide	Aminoacid sequence	Reference
Magainin I	GIGKFLHSAGKFGKAFVGEIMKS	60
Magainin II	GIGKFLHSAKKFGKAFVGEIMNS	12
Melittin	GIGAVLKVLTTGPALISWIKRKRQQ	56
Mastoparan	INLKALAALAKKIL	21
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK	4
Cecropin B	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL	29
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPRC	28
Bombin	GIGALSAKGALKGLAKGLAQHFAN	26
Protamine	PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR	38
Protamine-2	PRRRRSSSRPIRRRRPRRASRRRRRRGGRRRR	44
MIAP	GIGKFLKSKGKFGKA	This study

The identification of H^+ ATPase as a rational target against mycobacterial survival can be considered in the development of new anti-TB drugs. In the present study, the effects of AMPs on ATPase activity correlated to intracellular pH are assessed.

2. Materials and methods

2.1. Mycobacterial strains and growth conditions

M. tuberculosis H37Ra (ATCC 25177), *M. smegmatis* mc^2155 [53] and *M. tuberculosis* 74, 85, 114 and 121 clinical isolates (Departamento de Microbiología, Facultad de Medicina, Universidad Nacional de Colombia, Bogotá) were used in this study. Mycobacteria were grown at 37 °C with shaking at 70 rpm in Middlebrook 7H9 (Difco) supplemented with ADC (0.5% bovine serum albumin, 0.2% dextrose, 0.0003% beef catalase, Difco) and 0.05% Tween 80. Cultures were grown until an OD_{600} of approximately 0.5 was reached for both radial diffusion experiments and minimal inhibitory concentration (MIC) determination, and 0.4 for the extraction of mycobacterial plasma membrane. For radial diffusion assays, precultured mycobacteria were plated on Middlebrook 7H10 (Difco) plates supplemented with OADC (ADC plus 0.005% oleic acid, Difco).

2.2. Peptides chemical synthesis and characterization

Ten natural peptides [4,12,21,26,28,29,38,44,56,60] (names and sequences are shown in Table 1) and the designed peptide MIAP were synthesized by the standard solid-phase peptide synthesis technique proposed by Merrifield [34] with modifications according to Houghten [22], using good manufacturing practices. p-Methylbenzhydrylamine-resin (0.7 meq/g), t-Boc amino acids (Bachem, USA) and low-high cleavages were used in the synthesis of peptides. Once synthesized, the peptides were extracted with 10% acetic acid in water. The peptides were subsequently purified by reverse-phase HPLC, lyophilized and dissolved in bidistilled water. Crude peptides were purified by RP-HPLC on a semipreparative Vydac 218TP1022 column (Alltech, Templemars, France), and their purity was determined by RP-HPLC on an analytical Lichrosorb C18 column using 0.05% trifluoroacetic acid (TFA) in water (solvent A) and 0.05% TFA in acetonitrile (solvent B) to generate a mobile phase of 0–70% solvent B over 30 min. The molecular masses of the peptides were determined on a Bruker Protein MALDI-TOF mass spectrometer (Autoflex Bruker Daltonics, Germany).

2.3. Radial diffusion assay and antimicrobial peptide susceptibility testing

The antimycobacterial activities of the peptides were evaluated using a modified diffusion assay previously developed for the analysis of defensins activity on mycobacteria [30]. Briefly,