Full Length Research Paper

Evaluation of the K9CATH peptide in the treatment of experimental pulmonary tuberculosis

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The antimicrobial activity of the peptide K9CATH against Mycobacterium tuberculosis H37Rv in vitro and its therapeutic potential against pulmonary tuberculosis in a murine model was evaluated. By the alamar blue colorimetric assay, a minimum inhibitory concentration (MIC) of 10.66 µg/ml was obtained. Also, K9CATH did not show to be cytotoxic at 32 µg/ml on lung cells (A549). Electron microscopy images obtained of M. tuberculosis treated with the peptide showed disruption of the membrane and condensation of the cytoplasmic content. Mice infected with M. tuberculosis H37Rv and treated with 32 µg/ml of K9CATH for 30 and 60 days showed a significant decrease (p < 0.5) in colony-forming units (CFU) and pneumonic area.

Key words: Antimicrobial peptides, pulmonary tuberculosis, K9CATH peptide.

INTRODUCTION

Tuberculosis is an infectious disease that represents one of the major public health problems in the world, and novel and more effective therapeutic approaches are critically needed. In 2009, the World Health Organization (WHO) reported about the existence of approximately 9.4 million new cases of tuberculosis in the world, and 1.3 million deaths for this cause (WHO, 2010). Also, it has been estimated that one third of the world population is infected and between 5 to 10% will develop clinical disease sometime in their lives, especially those affected by diabetes and HIV (Harries and Dye, 2006).

Current standard treatment for tuberculosis is based in the administration of a set of specific antibiotics. Although antibiotics can eliminate the disease with a good degree of success, they have to be taken for long periods of time (up to six months) and patients frequently abandon the treatment allowing bacteria susceptible to develop resistance. The emergence of multi-drug-resistant (MDR) Mycobacterium tuberculosis strains has created an imminent need for the development of more efficient, less toxic drugs that can reduce the intake period (Jia et al., 2001). A potential and innovative alternative could be the therapeutic use of antimicrobial peptides (AMP’s). These gene-encoded molecules are well recognized components of the innate immune system and can either act directly against bacteria, or perform their biological function in synergy with other anti-tuberculosis drugs, sometimes acting as immune modulatory components of the immune response. Recent studies have reported the antimicrobial potential of a variety of AMP’s against M. tuberculosis in vitro, however little is known about the use of AMP’s for the treatment of tuberculosis in an in vivo model (Kalita et al., 2004; Linde et al., 2001).

Cathelicidins are a group of cationic peptides found in leukocytes and epithelial cells that play a central role in the early innate immune defense against infection. The K9CATCH peptide is a canine cathelicidin of 38 amino acids (aa’s) present in neutrophil granule contents. Synthetic K9CATH displayed broad antimicrobial activity
in vitro against gram positive bacteria (Listeria monocyctogenes and Staphylococcus aureus), gram negative bacteria (Escherichia coli, Klebsiella pneumoniae, Salmonella serotype typhimurium, Pseudomonas aeruginosa, Proteus mirabilis, Salmonella serotype enteritidis and Neisseria gonorrhoeae), and yeast (Candida albicans) (Sang et al., 2007).

Due to the broad antimicrobial spectrum of K9CATH, the objective of the present study was to evaluate the antimicrobial activity of K9CATH in vitro against M. tuberculosis H37Rv as well as to evaluate its potential for the treatment of pulmonary tuberculosis in a murine model.

MATERIALS AND METHODS

Antimicrobial activity of K9CATH was evaluated in vitro by calculating the minimal inhibitory concentration (MIC) using the microplate-based Alamar Blue assay (MABA) method described by Franzblau (1998) with a minor modification that included the use of a solution of resazurin at 0.01% concentration to asses for color development, and the evaluation of antimicrobial peptides instead of conventional antibiotics. Briefly, after 15 days of incubation cultures of M. tuberculosis H37Rv were diluted to match the McFarland No.1 scale (300 × 10^6 bacteria/ml), and from here on a 1:20 dilution was prepared, to be used in the assay. A K9CATH working solution of 512 µg/ml was prepared and from here two fold dilutions were made in Middlebrook 7H9 broth (Becton Dickinson, France) to obtain the following concentrations: 128, 6, 32, 16, 8 and 4 µg/ml. Also, rifampicin at 2 µg/ml was included in the assay as the reference antibiotic.

Furthermore the cytotoxicity of K9CATH was evaluated on human type II alveolar pneumocytes (A549; American Type Culture Collection (ATCC) reference number CCL185), as described by Rivas-Santiago et al. (2005). A minor modification consisted in exposing the pneumocytes to three different concentrations of K9CATH (12, 64 and 32 µg/ml) for 18 h. Cell viability was measured by adding Trypan blue (Sigma-Aldrich, St Louis, Mo, USA), and cells were observed under an inverted microscope at 40X. Viability was determined by calculating the mean between living (not stained) and dead (stained) cells.

Moreover, the effect of K9CATH peptide on M. tuberculosis H37Rv was examined by electron microscopy. Briefly, M. tuberculosis H37Rv was grown to mid log phase, and bacterial suspension was placed in a 1.5 ml vial and centrifuged at 10,000 rpm for 5 min. After removing the supernatant, the pellet was resuspended in peptone water, and 100 µl of K9CATH peptide solution at 128 µg/ml concentration was added and incubated at 37°C for 18 h. After centrifugation, the supernatant was removed and 100 µl of 2% glutaraldehyde dissolved in 0.1 M cacodilate were added an incubated at 4°C for 1 h. After removing the glutaraldehyde the pellet was washed twice with 0.1 M phosphate buffer and incubated 1 h with 1% osmium tetroxide. Finally, the pellet was dehydrated with increasing degrees of alcohol (ethanol at 30% for 10 min, ethanol at 60% for 10 min, ethanol at 90% for 10 min, ethanol at 100% for 10 min and ethanol 100% for 1 h), and embedded in Procure 812 resin. Thin sections of 100 nm were placed in nickel grids, stained with uranium salts and examined with an M-10 Zeiss electron microscope (Karl Zeiss, Jena Germany) (Hernandez-Pando et al., 1996).

Finally, the therapeutic effect of K9CATH peptide was evaluated in 6 to 8 week-old male BALB/c mice infected with M. tuberculosis H37Rv as previously described by Hernandez-Pando et al. (2010), with minor modifications. Briefly, once the infection was established (at day 60), mice were randomly distributed in two experimental groups of 10 mice each. Animals were maintained in cages fitted with microisolators connected to negative pressure. One group (treated group) received 32 µg of diluted K9CATH peptide in 100 µl of sterile water, and the other group (placebo group) received only 100 µl of sterile water. Treatments were infused intratracheally using a flushing cannula introduced by slowly moving the knob in the tip of the cannula through the tracheal rings. Treatments were applied three times a week. Mice were sacrificed at 30 and 60 days post-treatment and lungs collected to determine bacterial counts and percentage of pneumonia. The right lungs from 4 mice at each time point were snap-frozen in liquid nitrogen and then stored at -70°C for microbiological analysis. Lungs were homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland) in sterile 50-ml tubes containing 3 ml of isonic saline. Four dilutions of each homogenate were spread onto duplicate plates and incubated for 21 days prior to determination of CFU. The left lungs from 4 mice at each time point were fixed by intratracheal perfusion with absolute alcohol for 24 h and then sectioned through the hilus and embedded in paraffin. Sections 5 µm thick were stained with hematoxylin-eosin for the histological-morphometric analysis. The percentage of the pulmonary area affected by pneumonia was determined using an automated image analyzer Zidas Zeiss (Carl Zeiss Ltd, Herts, UK) y Q500W Leica. With the analyzer, the total lung area (corresponding to 100%) and the pneumonic areas were measured, and a rule of three was used to determine the percentage of pneumonic area (Hernandez et al., 2010). Data obtained from the treated and placebo group of animals were contrasted by the non-parametric procedure Kruskal-Wallis with the statistic program SAS version 9.2.

RESULTS

The peptide K9CATH inhibited M. tuberculosis H37Rv with a MIC of 10.66 µg/ml, and for the antibiotic of reference, rifampicin was 0.1043 µg/ml. In addition, K9CATH was not cytotoxic since a 95% A549 cell viability was obtained at a concentration of 32 µg/ml. Electron microscopy images showed disruption on the membrane of M. tuberculosis H37Rv. Also, strong cytoplasmatic changes were observed inside the bacteria (Figure 1A and B).

In the murine model, a significant decrease (p < 0.5) in the CFU was shown between the treated and placebo groups of mice at 30 and 60 days post-treatment (Table 1). Furthermore, a significant decrease (p < 0.5) in the pneumonic area was observed between the treated and the placebo groups at 30 and 60 days post-treatment (Figure 2A and B).

DISCUSSION

The MIC for rifampicin (0.1043 µg/ml) agrees with the MIC reported by Acosta et al. (2004) of 0.11 µg/ml, indicating that dilution of the antimicrobial peptide and the antibiotic for the assay was adequate. Similar to K9CATH, the peptides PR-39 (Linde et al., 2001), sringopeptin (Grgrurina et al., 2005), and the synthetic peptide Nisin S (Carroll et al., 2010) have shown inhibition of M. tuberculosis by this colorimetric assay.
Regarding CFU and percentage of pneumonic area peptides are no cytotoxic to eukaryotic cells.

Any membrane damage that could be caused by the hypersensitivity reactions or an increase in antibody titles studies with the peptides cepronin, PR-17.28 11.41 H37Rv treated with K9CATH (A) and

Electron microscopy images of Percentage of pneumonia Data obtained from the murine pulmonary tuberculosis.

Table 1. Data obtained from the murine pulmonary tuberculosis.

<table>
<thead>
<tr>
<th>Days post-infection</th>
<th>CFU Placebo (CFU/ml) Treated (CFU/ml)</th>
<th>Percentage of pneumonia Placebo (%) Treated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>$8.91 \times 10^6$ $1.05 \times 10^6$</td>
<td>17.28 11.41</td>
</tr>
<tr>
<td>120</td>
<td>$11.74 \times 10^6$ $0.58 \times 10^6$</td>
<td>28.1 8.31</td>
</tr>
</tbody>
</table>

(Zerbini et al., 2006).

The lack of cytotoxicity could be due to the presence of cholesterol on the membrane of normal eukaryotic cells that has been suggested to be cytoprotectant, preventing any membrane damage that could be caused by the highly cationic antimicrobial peptides (Boman, 1995). Previous in-vitro studies with the peptides cepronin, PR-39 and NK-lisin, did not show cytotoxicity to the cell lines 3B6, K562, U932 and EL-4 (Catrina et al., 2009). Also, BALB/C mice treated with the P34 peptide did not show hypersensitivity reactions or an increase in antibody titles (Vaucher et al., 2011), demonstrating that antimicrobial peptides are no cytotoxic to eukaryotic cells.

Disruption of the membrane is a common mechanism of action of the AMP’s. Structural changes in the bacteria are similar to the mechanism of action observed with the peptides B1 y SMAP29 (Chen et al., 2011). This could be due to the positive charges of the peptides that are electrostatically attracted to teichoic acid, lipoteichoic acid or lipopolysaccharides (Téllez y Castaño, 2010). Changes in the cytoplasm suggested that K9CATH penetrated the membrane and accumulated inside the cell, where as suggested by Kragol et al. (2001), may interfere with intracellular metabolic pathways such as bacterial protein synthesis (Kragol et al., 2001).

Regarding CFU and percentage of pneumonic area

Figure 1. Electron microscopy images of *M. tuberculosis* H37Rv exposed to K9CATH.

Figure 2. Pneumonic area at day 60 post-infection with *M. tuberculosis* H37Rv treated with K9CATH (A) and placebo (B).
which was significantly lower in mice treated with the K9CATH compared to the control mice, this is the first time that an intratracheal treatment with an antimicrobial peptide is applied for the treatment of pulmonary tuberculosis in a mice model.

Conclusion

K9 CATH showed to be effective in vitro and in vivo against M. tuberculosis H37Rv. However, further studies are needed to evaluate the effect of K9CATH against MDR and hyper-virulent strains as well as the synergy of K9CATH with other antituberculosis drugs.

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REFERENCES


