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DRUG DISCOVERY AND RESISTANCE

# Cationic amphipathic D-enantiomeric antimicrobial peptides with *in vitro* and *ex vivo* activity against drug-resistant *Mycobacterium tuberculosis*

Yun Lan [a], Jason T. Lam [b], Gilman K.H. Siu [c], Wing Cheong Yam [b], A. James Mason [d], Jenny K.W. Lam [a, *]

[a] *Department of Pharmacology & Pharmacy, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Laboratory Block, 21 Sassoon Road, Pokfulam, Hong Kong Special Administrative Region*
[b] *Department of Microbiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, University Pathology Building, Queen Mary Hospital, Pokfulam Road, Hong Kong Special Administrative Region*
[c] *Department of Health Technology and Informatics, Faculty of Health and Social Sciences, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong Special Administrative Region*
[d] *Institute of Pharmaceutical Science, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, United Kingdom*

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SUMMARY

Tuberculosis (TB) is the leading cause of bacterial death worldwide. Due to the emergence of multi-drug resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB), and the persistence of latent infections, a safe and effective TB therapy is highly sought after. Antimicrobial peptides (AMPs) have therapeutic potential against infectious diseases and have the ability to target microbial pathogens within eukaryotic cells. In the present study, we investigated the activity of a family of six AMPs containing all-D amino acids (D-LAK peptides) against MDR and XDR clinical strains of *Mycobacterium tuberculosis* (Mtb) both *in vitro* and, using THP-1 cells as a macrophage model, cultured *ex vivo*. All the D-LAK peptides successfully inhibited the growth of Mtb *in vitro* and were similarly effective against MDR and XDR strains. D-LAK peptides effectively broke down the heavy clumping of mycobacteria in broth culture, consistent with a 'detergent-like effect' that could reduce the hydrophobic interactions between the highly lipidic cell walls of the mycobacteria, preventing bacteria cell aggregation. Furthermore, though not able to eradicate the intracellular mycobacteria, D-LAK peptides substantially inhibited the intracellular growth of drug-resistant Mtb clinical isolates at concentrations that were well tolerated by THP-1 cells. Finally, combining D-LAK peptide with isoniazid could enhance the anti-TB efficacy. D-LAK peptide, particularly D-LAK120-A, was effective as an adjunct agent at non-toxic concentration to potentiate the efficacy of isoniazid against drug-resistant Mtb *in vitro*, possibly by facilitating the access of isoniazid into the mycobacteria by increasing the surface permeability of the pathogen.


## 1. Introduction

Tuberculosis (TB) is a severe infectious disease of which there were 8.6 million new cases and caused 1.3 million deaths globally in 2012 [1]. Although the absolute number and the incidence rate of TB has been falling in the last few years, cases of multi-drug resistant TB (MDR-TB) are rising, reaching almost 450,000 worldwide in 2012 [1]. The situation has been worsened by the *emergence* of extensively drug-resistant TB (XDR-TB). The average proportion of MDR-TB cases with XDR-TB is 9.6%. According to the Global Tuberculosis Report 2013 [1], the progress towards targets for diagnosis and treatment of MDR-TB is far off-track. The target treatment success rate of 75% or higher for patients with MDR-TB was reached by only 34 of 107 countries that reported treatment outcomes [1]. Within the UK, of the 8751 cases in 2012, 73% were non-UK born and the majority of cases were likely due to reactivation of latent TB infections, with 6.8% of cases in the UK resistant to isoniazid [2]. There is an urgent need to develop safe and effective TB therapies that can combat latent TB infections and/or MDR-TB.

* Corresponding author. Department of Pharmacology & Pharmacy, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, 21 Sassoon Road, Hong Kong Special Administrative Region. Tel.: +852 3917 9599; fax: +852 2817 0859.
*E-mail address:* jkwlam@hku.hk (J.K.W. Lam).

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TB is caused by *Mycobacterium tuberculosis* (Mtb), an intracellular pathogen which can survive inside human alveolar macrophages. The exact mechanisms underlying the virulence of Mtb have not been fully elucidated. Extensive studies have indicated that despite the antimicrobial activity of macrophages, Mtb has developed multiple strategies to interfere with the phagosomal maturation, manipulate the host machinery, neutralize toxic factors and eventually survive the harsh intracellular environment within the host macrophages [3–6]. Antimicrobial peptides (AMPs) are produced by a wide variety of living organisms, serving an essential role in the host innate immune system. Various studies have demonstrated that both naturally occurring AMPs and their synthetic analogues have a wide spectrum activity against pathogenic organisms, including Gram-positive and Gram-negative bacteria, as well as intracellular pathogens like *Plasmodium falciparum* and *M. tuberculosis* [7–11]. AMPs are usually positively charged amphipathic small peptides consisting around 12 to 50 amino acid residues [11–13]. Although their mechanisms of action have not been fully understood, it is generally believed that cationic AMPs can selectively bind to the negatively charged membrane components of the pathogens and destabilize and/or penetrate their plasma membrane. Subsequently, this membrane activity causes either leakage of the cellular fluid or facilitates additional intracellular nucleic acid or protein inhibition activity and eventually the death of the pathogenic microorganism [13–15].

AMPs that assume an α-helical structure are widespread and abundant in nature. The selectivity, potency and safety of α-helical AMPs heavily depend on their physicochemical properties including their amphipathic nature, net charge, charge angle, overall hydrophobicity and conformational flexibility [8]. In addition, the use of D-conformation peptides is preferred because it has been shown that the L to D-amino acid substitution of AMPs confers resistance to the activity of proteolytic enzymes and therefore improves AMP biostability, without impairing antimicrobial activity [16,17]. In our pervious study, we have already identified that a charge angle of 120° conferred the highest potency against Mtb using the attenuated laboratory strain H37Ra as a model [7]. When proline is incorporated to the hydrophilic face of the amphipathic α-helical AMPs, an improvement of antibacterial activity and reduction of hemolytic effect were observed, as well as increasing the conformational flexibility [18,19]. In addition, increasing the conformational flexibility within the peptide backbone, through incorporation of a proline residue at position 13 has been shown to enable effective killing of *P. falciparum* within an erythrocyte host while minimizing collateral toxicity as determined by hemolysis [7]. We therefore tested the hypothesis that the same modifications to the peptides would promote activity against Mtb within a macrophage host while mitigating toxicity to macrophages.

In this study, the anti-TB activity of six structurally similar AMPs of the D-LAK family, with different physicochemical properties, was evaluated both *in vitro* and *ex vivo* (Table 1). These six D-AMPs consist of 25 D-enantiomer amino acid residues in a primary sequence designed to adopt a left-handed α-helix conformation with charge angle of 120°. Each of them contains eight lysine residues with a nominal charge of +9 at neutral pH. The major difference between these six D-AMPs is the complement of histidine, alanine and presence/absence of proline residues, which in turn affects their overall hydrophobicity and conformational flexibility. We tested the D-AMPs against clinical strains of Mtb, including MDR-TB and XDR-TB. In addition, since AMPs possess membrane active properties, they may facilitate the access of anti-TB drugs such as isoniazid (INH) into the Mtb, potentiating the efficacy of the anti-TB drugs [20,21]. Therefore D-LAK peptides were also used in combination with INH to investigate whether these peptides can enhance the efficacy of INH against drug-resistant TB *in vitro*.

**Table 1**
Comparison of D-AMPs used in this study. The average hydrophobicity of D-AMPs is shown according to the combined consensus scale. All peptides contain eight lysine residues and are amidated at the C terminus. Significant changes to the sequences are highlighted: histidine, alanine are highlighted in bold while proline residues are in bold and underlined.

| D-AMPs | Sequence | Average hydrophobicity |
|---|---|---|
| D-LAK120 | KKLALLALKKWLLALKKLALLALKK | 1.26 |
| D-LAK120-H | KKLAL**H**ALKKWL**H**ALKKLA**H**LALKK | −0.35 |
| D-LAK120-A | KKLAL**A**LAKKWL**A**LAKKLAL**A**LAKK | −0.02 |
| D-LAK120-P13 | KKLALLALKKWL**P**ALKKLALLALKK | 0.87 |
| D-LAK120-HP13 | KKALA**H**ALKKWL**P**ALKKLA**H**ALAKK | −1.07 |
| D-LAK120-AP13 | KKLAL**A**LAKKWL**P**LAKKLAL**A**LAKK | 0 |

Although other mycobacteria such as *Mycobacterium smegmatis* or *Mycobacterium marinum* are commonly used as a surrogate model as they grow faster than tuberculosis and require a lower biosafety level for experiment, they are also less relevant [22,23]. Therefore clinical isolates of drug-resistant Mtb strains were employed in our study, allowing us to give a more representative examination of the potential of the D-AMPs for clinical development.

## 2. Materials and methods

### 2.1. Antimicrobial peptides

Six structurally similar D-AMPs (Table 1) were synthesized using standard manual Fmoc (*N*-(9-fluorenyl)methyoxycarbonyl) solid state chemistry as previously described [7]. High performance liquid chromatography (HPLC) purification was performed using acetonitrile/water gradients on an Agilent 1100 system using a SymmetryPrep™ C8 7 μm, 19 × 300 mm column (Waters, Milford, MA) and the identity of the product was confirmed by matrix assisted laser desorption ionization mass spectrometry. Peptides were lyophilized from 10% acetic acid to remove the trifluoroacetic acid counter ion.

### 2.2. In vitro anti-TB assay of D-AMPs

The antibacterial activities of six D-AMPs against Mtb were screened using a broth micro-dilution assay in 96-well plates. Three different clinical isolates were used: (i) drug susceptible strain S-LMS; (ii) MDR strain GB2; and (iii) XDR strain WYC-I1. Their antibiograms were determined using the agar proportion method according to Clinical Laboratory Standard Institute (CLSI) recommendations (Table 2). D-AMPs were first diluted in Middlebrook 7H9 Broth supplemented with 10% Middlebrook oleic albumin dextrose catalase (OADC) growth supplement (BD Difco). 180 μl of D-AMPs (in serial dilution from 3.13 μM to 100 μM) and 20 μl of bacterial suspensions were added to the well to obtain a final bacterial concentration of $1 \times 10^6$ colony forming units (CFUs)/ml. Untreated bacterial suspensions were used as negative controls. The plates were then incubated at 37 °C for four to six weeks. The growth of Mtb in the presence of D-AMPs was visualized and imaged using a digital camera (Canon DIGITAL IXUS 70) mounted on a light microscope (Nikon Eclipse TS100 40 × 20 × 10x ELWD 0.3/OD75 C-W10x/22 inverted microscope). The effective concentration for Mtb, which is the lowest concentration of antimicrobials that inhibited 99% of bacterial growth compared with the growth control, was determined for each peptide by visual inspection.

### 2.3. Cell culture

Human monocytic cells THP-1 (ATCC) were used as a macrophage model after they were differentiated into macrophage-like