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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*

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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.
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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.

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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 1×10^6 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

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Table 2
The summary of *Mycobacteria tuberculosis* strains used in this study.

ID	Strain type	MDR/XDR	Specimen type	Antibiogram
S-LMS	Clinical isolate	No	Bronchoalveolar lavage	RIF-S, INH-S, ETH-S, STR-S, PZA-S, CYC-S, AMI-S, OFX-S, ETA-S, KAN-S, CAM-S
GB2	Clinical isolate	MDR	Sputum	RIF-R, INH-R, ETH-S, STR-S, CYC-S, AMI-S, OFX-S, ETA-S, SPZ-S, KAN-S, CAM-S
WYC-I1	Clinical isolate	XDR	Sputum	RIF-R, INH-R, ETH-R, STR-R, PZA-R, AMI-R

RIF: rifampicin, INH: isoniazid, ETH: ethambutol, STR: streptomycin, OFX: ofloxacin, PZA: pyrazinamide, CYC: cycloserine, KAN: kanamycin, AMI: amikacin, ETA: ethionamide, CAM: chloramphenicol.

cells. Undifferentiated THP-1 cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Invitrogen). The cells were seeded at a density of 2×10^5 cells/well in a 24-well plate, and were differentiated to macrophage-like cells by exposure to 100 μM phorbol-myristate actate (PMA) for 24 h prior to experiment.

2.4. Ex vivo anti-TB assay of D-AMPs

Four peptides, D-LAK120-A, D-LAK120-AP13, D-LAK120-H and D-LAK120-HP13, were included in the *ex vivo* assay. The method was described in the previous study with modification [24]. In brief, differentiated THP-1 cells were inoculated with Mtb clinical strains at a multiplicity of infection (MOI) of 1 for 24 h. On the day of experiment (Day 0), the cells were thoroughly washed with phosphate buffered saline (PBS) three times to remove the extracellular mycobacteria. The infected macrophages were then incubated with either culture medium alone (control) or D-AMPs at a concentration of 6.25 μM for up to 10 days. At different time points (Day 0, 4, 7 and 10), the infected macrophages (both D-AMPs treated and untreated control) were lysed with 0.1% SDS. Cell lysates were plated on Middlebrook 7H10 agar plates supplemented with 10% OADC which were incubated at 37 °C in 5% CO_2 for four to six weeks before the CFUs were counted. The experiments were performed three times.

2.5. Effect of INH and D-AMP combination against MDR-TB

To investigate the anti-TB effect of the INH/D-AMPs combination against drug-resistant TB, INH (Sigma–Aldrich) and D-AMPs (D-LAK120-A and D-LAK120-HP13) were mixed at various concentrations and added to the MDR strain GB2 bacterial suspension in 96-well plate as described in Section 2.2. The growth of Mtb was visualized and imaged after three weeks of incubation to assess the inhibitory effect on bacterial growth of the combination. In addition, the CFUs of Mtb were calculated in a separate experiment to investigate the bactericidal effect of the combination. After the cells were incubated with the INH/D-AMPs combination in 96-well plate for three days, the content of each well was plated on Middlebrook 7H10 agar plates and incubated for further four to six weeks. The CFUs were counted and the percentage of Mtb growth of each treatment group comparing to the control groups was calculated. The experiments were performed three times.

2.6. Cytotoxicity assay

The cytotoxicity of the all six D-AMPs was evaluated by measuring the lactate dehydrogenase (LDH) activity of D-AMP treated THP-1 cells using the LDH cytotoxicity assay kit (Cayman Chemical), which measured the membrane integrity. THP-1 cells were seeded in 96-well plates at a cell density of 1×10^5 cells/well and differentiated. D-AMPs were diluted in RPMI-1640 medium supplemented with 10% FCS and incubated with the differentiated THP-1 cells for 4 h at 37 °C. 100 μl of supernatant from each well was transferred to a new 96-well plate, and the LDH activity of each sample was evaluated according to the manufacture protocol. Triton X-100 (0.15% v/v) treated cells and untreated cells were used as positive and negative controls respectively. The percentage of cell death was calculated according to the standard curve and presented as % relative to the controls. To determine the EC_{50} of the D-AMPs in the LDH assay, the percentage of cell death was plotted against log concentration of the peptide and fitted with a dose–response model (OriginPro 9 Software, Massachusetts, USA). The cytotoxicity of the INH/D-AMPs combination was assessed by the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay, which measured the cell viability. THP-1 cells were seeded and differentiated in 96-well plates at a cell density of 1×10^5 cell/well. INH and D-AMPs were diluted to the designed concentrations in RPMI-1640 medium supplemented with 10% FCS and incubated with differentiated THP-1 cells at 37 °C for 24 h. The cells were then incubated with 200 μl of pre-warmed MTT solution (0.8 mg/ml) per well for 4 h, followed by addition of 100 μl isopropanol per well to dissolve the formazan crystals formed by the MTT reagent. The percentage viability of THP-1 cells was calculated by comparing to the control group without any treatment. The experiments were performed three times.

2.7. Statistical analysis

All data presented in this study were statistically analyzed using program Prism (GraphPad Software). One way or two way ANOVA test was performed, followed by Bonferroni's post-hoc test. A value of $p \leq 0.05$ was used to establish statistical significance. All experiments were performed at least twice.

3. Results

3.1. In vitro anti-TB assay

The *in vitro* anti-TB activities of the D-AMPs were screened by visually comparing the growth and the morphology of the mycobacterial colonies with the control samples by light microscopy (Figure 1). All six D-AMPs tested in this study showed concentration-dependent antibacterial activity against the three clinical strains of Mtb. At low concentrations of D-AMPs, the mycobacteria appeared to be dispersed and scattered inside the culture well instead of remaining as colonies of aggregated bacteria as seen in the control wells. As the concentration of D-AMPs increased, the growth of mycobacteria was further inhibited. Effective concentrations of each peptide for different clinical strains were highlighted (framed) in the figures. For the drug susceptible strain S-LMS (Figure 1A), D-LAK120-A and D-LAK120-HP13 showed the highest potency, as the mycobacteria were not detected in the wells at the concentration of 50 μM or above. In contrast, D-LAK120-H and D-LAK120-AP13 appeared to be the least potent, as a considerable amount of mycobacteria could still be seen in the wells even at 100 μM. For the MDR strain GB2 (Figure 1B), although none of the D-AMPs managed to abolish the growth of bacteria even at the highest concentration used, D-LAK120 and D-LAK120-HP13 were the most effective D-AMPs against this strain,

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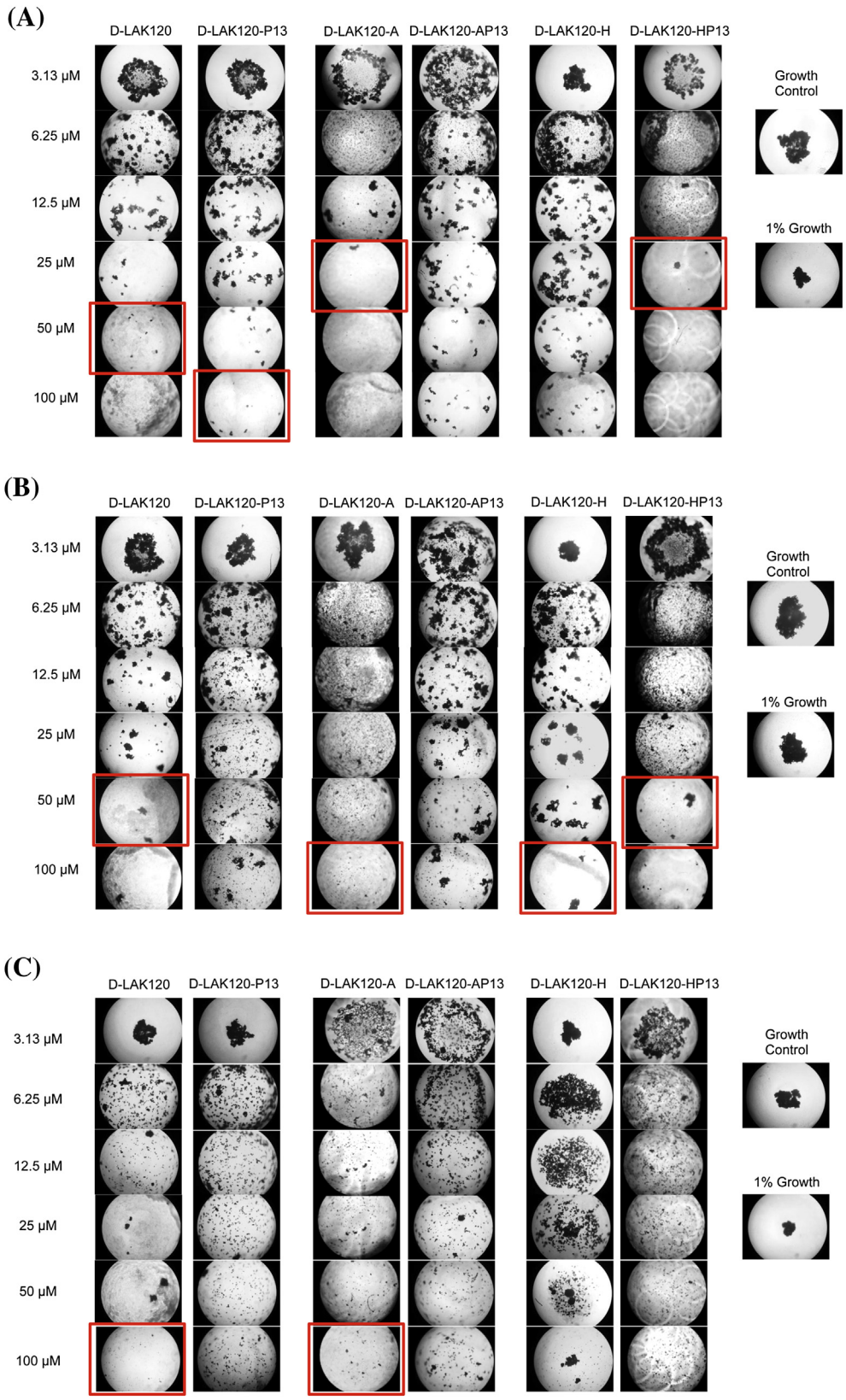

Figure 1. *In vitro* anti-TB screening of D-AMPs activity against Mtb clinical isolates: susceptible strain S-LMS (A); MDR strain GB2 (B); XDR strain WYC-I1 (C). Representative light microscope images show the growth condition of the bacteria at various concentrations of D-AMPs after four to six weeks of incubation. Dark areas indicated the bacterial colonies. 1% growth contained 1% bacteria (1×10^4 CFU/ml) comparing to growth condition (1×10^6 CFU/ml), The highlighted images (framed) indicate the effective concentrations of each peptide which are the lowest concentration of antimicrobials that inhibited 99% of bacterial growth (by eye) compared with the growth control. D-AMPs without highlighted images suggest that the peptides are ineffective in the concentration range tested.

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followed by D-LAK120-A and D-LAK120-H. For the XDR strain WYC-I1 (Figure 1C), D-LAK120 and D-LAK120-A were the most effective peptides. Again, none of the D-AMPs managed to eradicate the growth of WYC-I1 strain. In general, D-LAK120, D-LAK120-A and D-LAK120-HP13 were the three most potent D-AMPs against the clinical strains of Mtb *in vitro*.

3.2. Cytotoxicity of the D-AMPs

The LDH assay was used to assess the cytotoxicity of the D-AMPs by measuring membrane integrity. All peptides showed a concentration-dependent cytotoxic effect on macrophage-like THP-1 cells (Figure 2) with EC_{50} values in the range 14.4–32.3 μM (Table 3). Apart from D-LAK120 and D-LAK120-P13, no sign of cytotoxicity was observed for the peptides at concentration of 1.56 μM or below. Significant cytotoxic effects ($p < 0.05$) were observed at concentrations ≥ 3.13 μM for D-LAK120-HP13, and ≥12.5 μM for D-LAK120-H and D-LAK120-A. D-LAK120-AP13, being the least cytotoxic D-AMPs out of the six D-AMPs tested, showed noticeable cell death only at ≥ 25 μM. Comparison of the EC_{50} values reveals the alanine-rich peptides (D-LAK120-A and D-LAK120-AP13) and histidine-rich peptides (D-LAK120-H and D-LAK120-HP13) to have similar cytotoxic effects whereas substantially higher cytotoxicity was observed with D-LAK120 and D-LAK120-P13.

3.3. Ex vivo anti-TB assay

The *ex vivo* assay investigated the survivability of the drug-resistant Mtb strains inside the macrophage-like THP-1 cells at different time points in the presence of D-AMPs. The two most cytotoxic peptides, D-LAK120 and D-LAK120-HP13, were excluded from the *ex-vivo* anti-TB assay according to the LDH results combined with the hemolysis test performed before. The remaining four peptides were assessed for their *ex vivo* effect. Based on the microscopic observation anti-TB assay, a concentration of 6.25 μM was established as having surface activity towards the Mtb for all four D-LAK peptides in the *in vitro* studies and was well tolerated by the macrophages (a concentration approximately five times lower than the EC_{50} in the LDH cytotoxicity assay). Starting from day 7, all four D-AMPs showed significant inhibition of intracellular growth of drug-resistant Mtb as compared with the untreated control (Figure 3). However, none of the D-AMPs were able to eradicate the mycobacteria, leading to a small increase of CFU over time for all the D-AMPs. For the MDR strain GB2 (Figure 3A), the D-AMPs caused a 1.6- to 3.5-fold of reduction in CFU on day 10 compared with the control, although there was no significant difference in CFU observed between the four D-AMPs. For the XDR strain WYC-I1 strain (Figure 3B), there was a 3.3- to 5.3-fold of reduction in CFU on day 10 with the D-AMPs treated groups compared with the control. It is notable that the WYC-I1 strain grew at a slower rate than the GB2 strain; a probable reason for the large difference in CFU values between the two sets of data.

Table 3
Summary of EC_{50} (50% of effective concentration) of D-AMPs of the LDH assay performed in THP-1 cells. Results are an average of three independent repeated experiments.

D-AMPs	EC_{50} (μM)
D-LAK120	14.4 ± 3.3
D-LAK120-P13	17.9 ± 1.7
D-LAK120-A	31.9 ± 9.5
D-LAK120-AP13	32.1 ± 4.8
D-LAK120-H	32.2 ± 5.5
D-LAK120-HP13	32.3 ± 4.7

3.4. Anti-TB assay of INH/D-AMP combination

To investigate the effect of combining INH and D-AMPs against drug-resistant Mtb, D-LAK120-A or D-LAK120-HP13 and INH were added together to the MDR strain GB2 at various concentrations. The bacterial inhibitory effect of the combination was evaluated by visual comparison in a 96-well plate culture (Figure 4), and the bactericidal effect of the combination was assessed by comparing the CFU values on agar plate (Figure 5). The microscopic images showed that the inhibitory effect on bacterial growth of the combination was better than INH alone or the D-AMPs alone at respective concentration. At 6.25 μM of D-LAK120-A but in the absence of INH, the growth of Mtb could still be detected in the well. Similarly at 6.25 μM of D-LAK120-HP13 alone, the D-AMP could clearly de-aggregate the Mtb but the presence of Mtb in the well was evident. On the other hand, at 10 μg/ml of INH in the

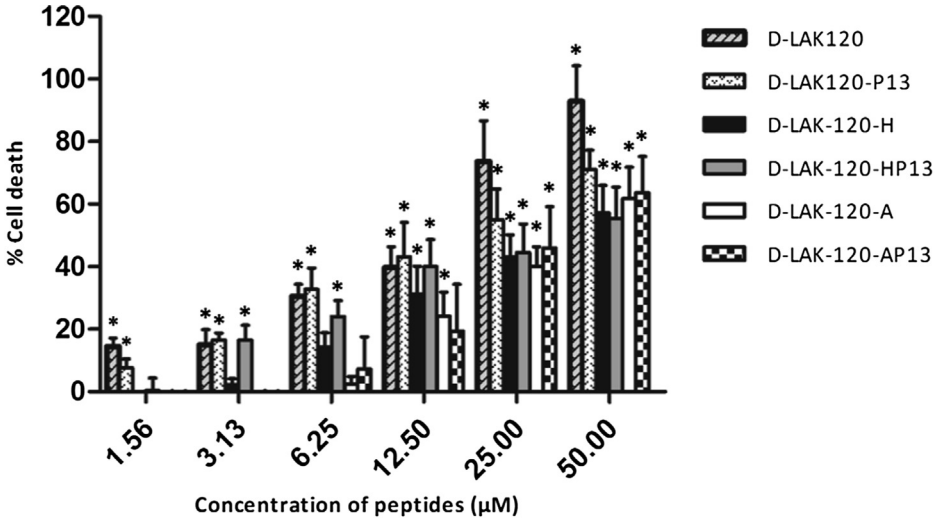

Figure 2. Cytotoxicity of D-AMPs on THP-1 cells. LDH assay was performed after 4 h of incubation with D-AMPs. Results were presented as % of cell death relative to peptide-free controls (0% cell death) and 0.15 %Triton X 100 treated groups (100% cell death). Bar shown as mean ± standard error of four independent experiments performed in duplicates. Significance difference was determined by comparing the peptide and the control (peptide-free), using two way ANOVA analysis followed by Bonferroni's post-hoc test, *<0.05.

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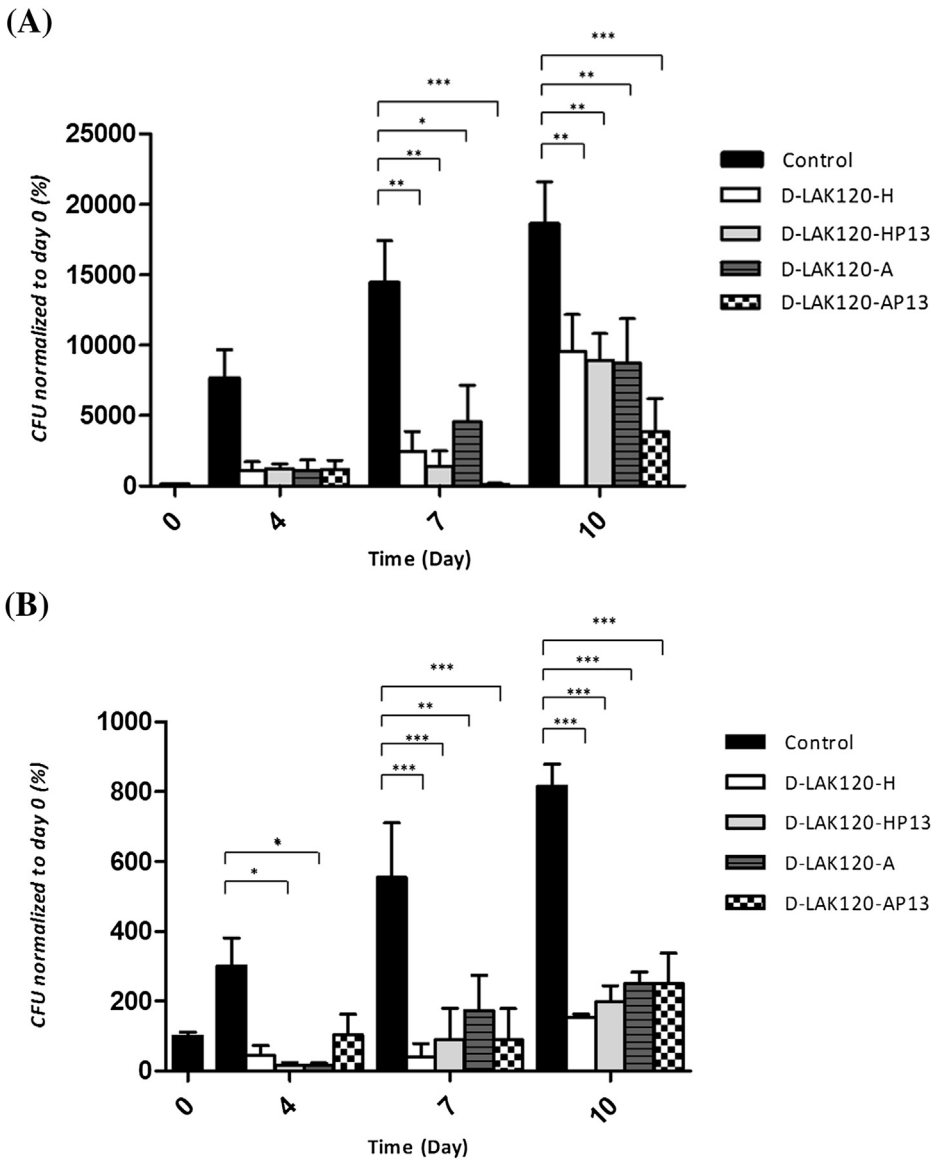

Figure 3. *Ex vivo* anti-TB assay of D-AMPs against intracellular Mtb clinical isolates MDR strain GB2 (A); XDR strain WYC-I1 (B). The CFUs of Mtb after challenged with 6.25 μM D-AMPs at different time points were compared to the CFU of untreated controls of the same day. Bar shown as mean ± standard error of repeated experiments performed in duplicates. Significant difference was determined using one way ANOVA analysis followed by Bonferroni's post-hoc test, *<0.05, **<0.01, ***<0.001.

absence of D-AMPs, the effect of INH on the growth of Mtb was indiscernible. With the combination of INH and D-AMPs, the growth of Mtb was markedly inhibited. The CFU values also showed a similar trend. In the absence of D-AMPs, the CFU decreased as the INH concentration increased, but INH failed to eradicate Mtb at a concentration of 1 μg/ml. In the absence of INH, at 0.01 μg/ml and 0.1 μg/ml of INH, the CFU gradually decreased as the concentration of D-AMPs increased. At 1 μg/ml of INH, there was no significant difference in CFU value between the different concentrations of D-AMPs. The results suggested that both D-LAK120-A and D-LAK120-HP13 could indeed improve the potency of INH against MDR strain GB2 when INH was used at low concentrations.

3.5. Cytotoxicity of INH/D-AMPs combination

The MTT assay was used to assess the cytotoxicity of the INH/D-AMPs combinations (Figure 6). When D-LAK120-A was added alone, there was a significant reduction of cell viability at concentration 3.13 μM and 6.25 μM but the cell viability remained over 80%. For INH/D-LAK120-A combination, no significant reduction of cell viability was observed at all tested concentrations (1.56, 3.13 and 6.25 μM). For INH/D-LAK120-HP13 combination, there was a significant reduction of cell viability, and the percentage of viable cells decreased as the concentration of D-LAK120-HP13 increased.

4. Discussion

The extensive use and, particularly, mismanagement of TB antibiotics in the clinic has favored the emergence of drug-resistant strains of mycobacteria. There is an urgent need for the development of new classes of antimicrobial agents particularly for the treatment of drug-resistant TB which is very challenging. Our group is currently investigating a series of linear cationic amphipathic α-helical AMPs, LAK peptides, which have the potential to be developed into therapeutic agents for clinical use [7]. Here we aim to determine and optimize the properties of LAK based AMPs

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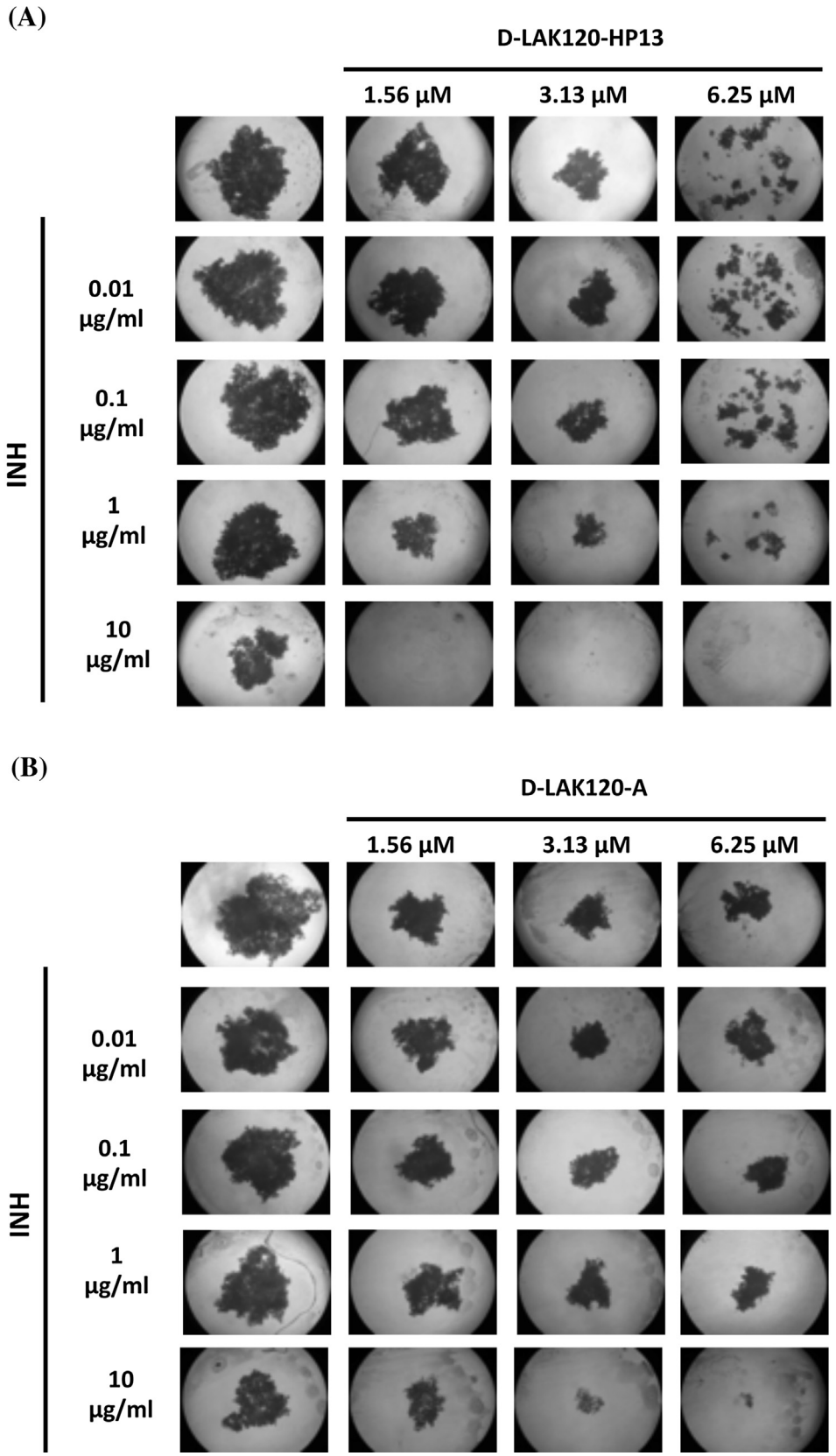

Figure 4. *In vitro* inhibitory effect on bacterial growth of INH/D-LAK120-HP13 combination (A) and INH/D-LAK120-A combination (B) against Mtb clinical isolates MDR (GB2). Representative light microscope images show the growth condition of the bacteria at various concentrations of D-AMPs and INH after three weeks of culture on 96-well plates in broth condition. Dark areas indicated the bacterial colonies.

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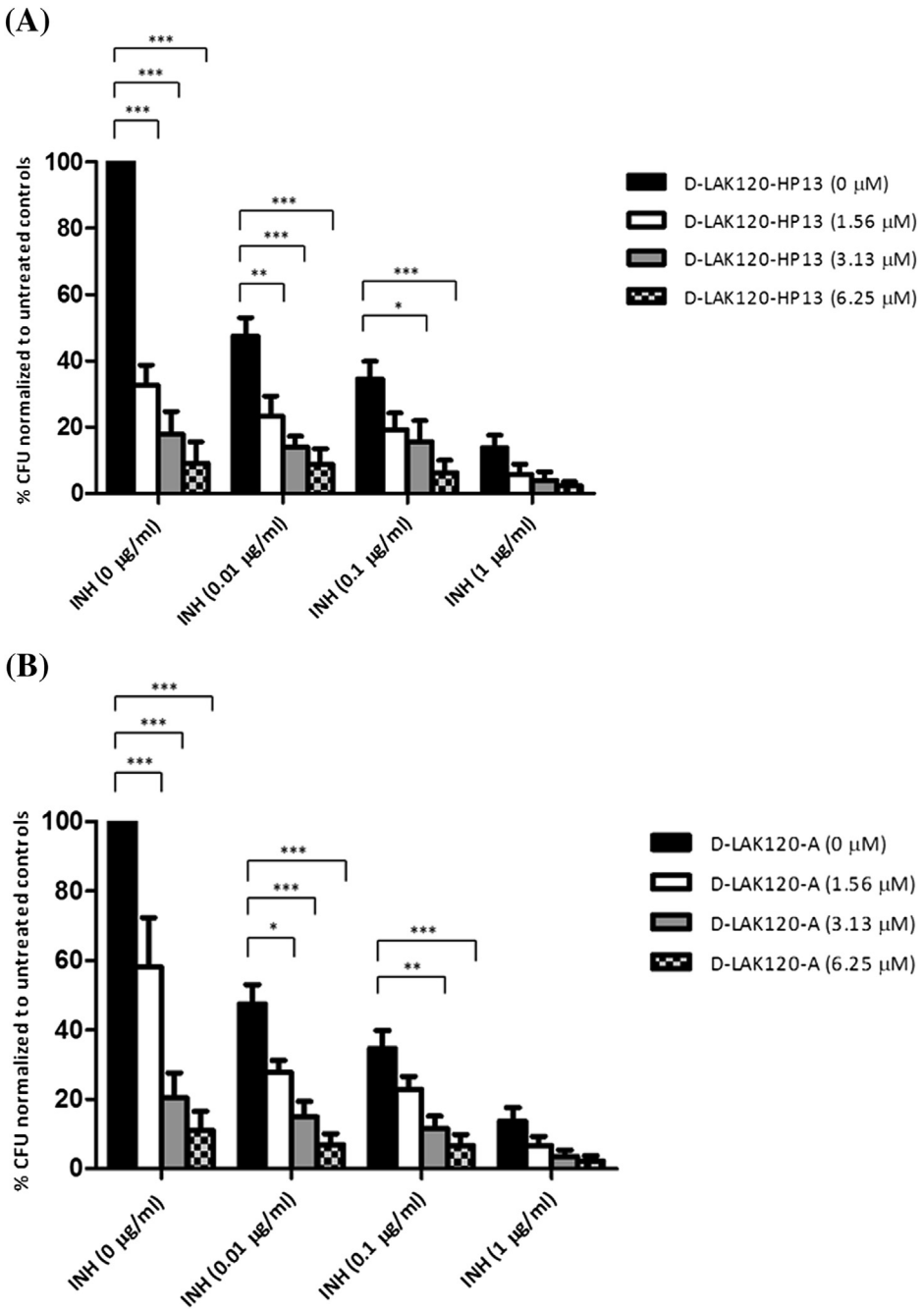

Figure 5. *In vitro* bactericidal effect of INH/D-LAK120-HP13 combination (A) and INH/D-LAK120-A combination (B) against Mtb clinical isolates MDR (GB2). Mtb were cultured on 96-well plates in broth condition at various concentrations of the combinations for three days. Samples were then transferred to agar plate for growth for a further four to six weeks before CFUs were counted. Bar shown as mean ± standard deviation of three independent experiments. Significance difference was using two way ANOVA analysis followed by Bonferroni's post-hoc test, $^{*}<0.05$, $^{**}<0.01$, $^{***}<0.001$.

specifically against drug-resistant TB, targeting the bacteria within their macrophage host.

Typically, in broth culture condition, the morphology of Mtb often appears as aggregated clumps of colonies, as seen in our control samples of the *in vitro* anti-TB assay. This is mainly due to the presence of the hydrophobic waxy cell wall, which is rich in mycolic acids, a distinctive characteristic of mycobacteria. The mycolic acids have a critical role in the survival and virulence of mycobacteria. They also contribute to the development of resistance to anti-TB drugs by reducing permeability to drugs [9,25]. One of the interesting phenomenon observed in the *in vitro* study is that all D-AMPs have the ability to break down the heavily clumped bacteria so that they appeared to be more dispersed and scattered inside the culture well. This was not observed in the INH treated group, in which the mycobacteria remained aggregated. This unique phenomenon of D-AMPs may be explained by their amphipathic properties. Although the overall hydrophobicity of the different D-AMPs varies, all of them are able to produce a 'detergent-like effect' by reducing the hydrophobic interaction between the cell walls of the bacteria and preventing cell clumping. At high concentrations of D-AMPs, some of the peptides may even affect the composition of the cell envelope and reduce the virulence of

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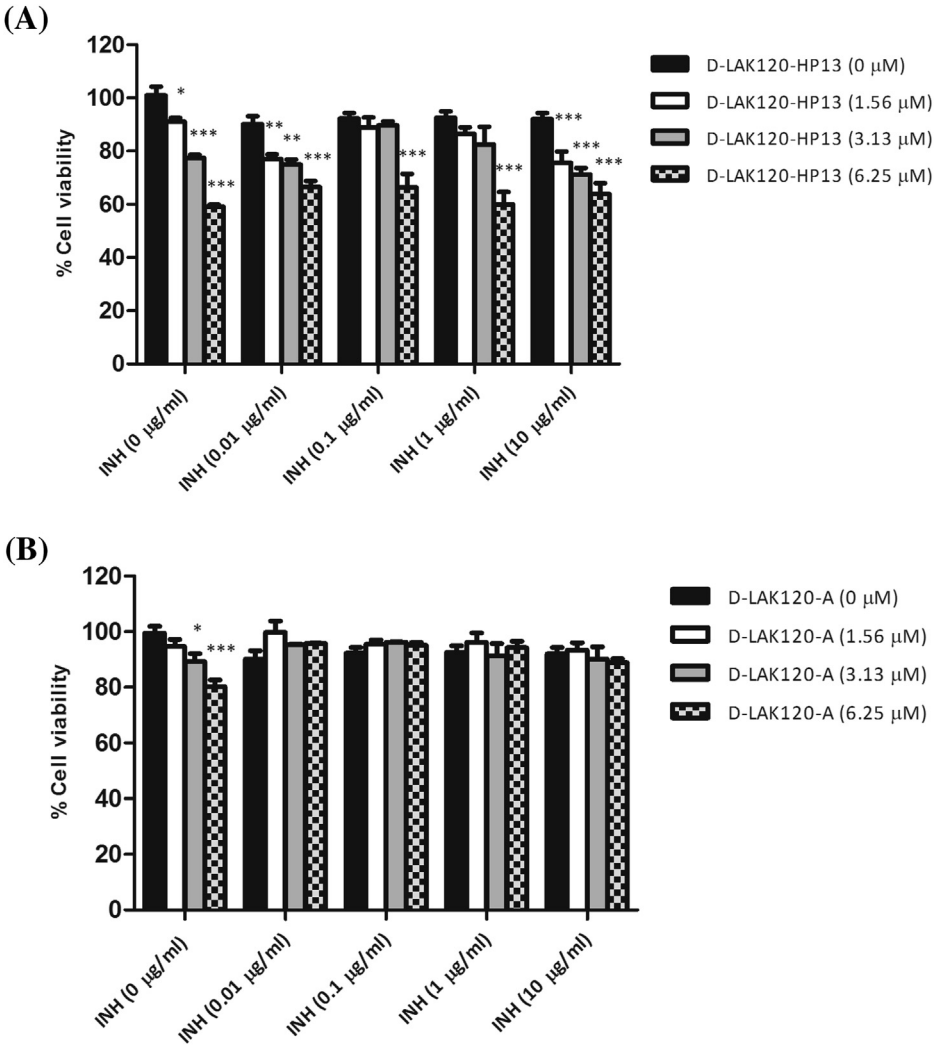

Figure 6. Cytotoxicity of INH/D-AMPs on THP-1 cells. MTT assay was performed after 24 h of incubation with the combinations (A) INH/D-LAK120-HP13 combination; (B) INH/D-LAK120-A combination. Results were presented as % cell viability compared to untreated control. Bar shown as mean ± standard error of four independent experiments performed in duplicates. Significance difference was determined by comparing the combinations and the control, using two way ANOVA analysis followed by Bonferroni's post-hoc test, $^{*}<0.05$, $^{**}<0.01$, $^{***}<0.001$.

Mtb, as previously observed in other detergents [26]. Since the interaction between the outermost constituents of the mycobacterial cell wall and the phagocyte partly determines the immunological response of the host, any disruption of surface lipid composition may affect the pathogenesis of the mycobacteria. This could be further investigated by an *ex vivo* anti-TB study which explores the efficacy of the D-AMPs on the intracellular Mtb.

It is widely accepted that the quantification of mycobacterial biomass is difficult when bacteria are cultured in a liquid medium and form clumps of various sizes [27]. Therefore the level of mycobacterial growth cannot be accurately evaluated by turbidity measurements using simple spectrophotometric methods. Instead the *in vitro* growth of Mtb was assessed by visual comparison in this study without further analysis. In addition, since Mtb is an intracellular pathogen that typically resides in alveolar macrophages, it is more relevant to investigate the effect of D-AMPs on the intracellular growth of Mtb. The present *ex vivo* study using THP-1 cells as macrophage model was performed to provide a more accurate reflection of natural conditions.

To test the activity of the D-AMPs against the intracellular Mtb, D-AMPs were used at a non-toxic concentration ensuring that they would not kill the host cells. Otherwise the survivability of the intracellular Mtb will be affected by the viability of its host. Due to the membrane activity of D-AMPs, erythrocyte hemolysis is a commonly used method to assess the toxicity of AMPs to eukaryotic cells. Our previous study showed that D-LAK120 and D-LAK120-P13 were more hemolytic than the other four peptides [7]. For this reason, although D-LAK120 was effective against all the three clinical strains of Mtb *in vitro*, they were excluded in the *ex vivo* assay. Only D-LAK120-A, D-LAK120-AP13, D-LAK120-H and D-LAK120-HP13 were tested for their *ex vivo* anti-TB activity. In addition, an LDH assay was carried out on THP-1 cells to evaluate the cytotoxicity of the peptides as well as to determine the suitable concentration for *ex vivo* study. The LDH assay measures the activity of LDH released into the extracellular medium following loss of membrane integrity, which is used as an indication of the level of cell death. A concentration of 6.25 μM was chosen in the *ex vivo* study because at this concentration, no significant cytotoxicity effect was observed in D-LAK-120A, D-LAK-120-AP13 and D-LAK120-H, and no more than 25% of cell death was seen in D-LAK120-HP13; and these four peptides showed some level of surface activity against the three clinical strains of Mtb *in vitro*. Furthermore, this

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concentration was at least three times lower than the 50% hemolytic concentration (HC_{50}) value of each peptide measured in the previous hemolytic study [7], and was about five times lower than the EC_{50} value of each peptide measured in the LDH cytotoxicity study here.

Although the D-AMPs could barely de-aggregate the Mtb clumps without obvious inhibition of the *in vitro* growth of Mtb at 6.25 μM, it was surprising that all the four peptides tested were effective in inhibiting the intracellular growth of both MDR strain GB2 and XDR strain WYC-I1. To exert the intracellular anti-TB activity, the D-AMPs need to gain access inside the infected macrophages, either by direct penetration through the membrane of the host cells or by endocytosis/phagocytosis. Since the LDH assay indicated that the membrane of THP-1 cells remained intact when treated with D-LAK120-A, D-LAK120-AP13 or D-LAK-120-H at 6.25 μM, these amphipathic peptides may self-aggregate and enter the cells through endocytosis/phagocytosis. Once inside the macrophages, the peptides exerted their anti-TB activity by disrupting the constituents of the highly lipidic cell wall of the mycobacteria which in turn affected the interaction between the pathogens and the host macrophages. However, the exact antimicrobial mechanism of the D-AMPs remains to be elucidated.

In order to optimize the efficacy of the D-AMPs, it is necessary to rationalize their activities in terms of their structural properties. Amongst the six D-AMPs tested, D-LAK120, D-LAK120-A and D-LAK120-HP13 have the highest potency against Mtb *in vitro*. According to the overall hydrophobicity index of each peptide, D-LAK120 is more hydrophobic, D-LAK120-A is neutral, whereas D-LAK120-HP13 is more hydrophilic. It appears that the hydrophobicity does not have a direct determinant effect on the anti-TB activity. Similar to our previous study against the attenuated H37Ra strain [7], the incorporation of a proline residue did not appear to improve the activity against the drug-resistant Mtb either *in vitro* or *ex vivo*. However, the presence of the proline residue could dramatically reduce the hemolytic potential of the peptides [7]. In the present study, the incorporation of proline residue did not affect the cytotoxicity to THP-1 cells as shown in the LDH assay. Instead, we noticed that the histidine-rich peptides (D-LAK120-H and D-LAK120-HP13) were more cytotoxic than the alanine-rich peptides (D-LAK120-A and D-LAK120-AP13) to THP-1 cells. D-LAK120 and D-LAK120-P13 were the two most cytotoxic peptides being tested. Since both the anti-TB activity and the cytotoxicity of peptides are important properties to consider in developing AMPs for clinical use, we conclude that D-LAK120-A and D-LAK120-HP13 are our optimal candidates against drug-resistant Mtb for further development.

Another interesting observation is that the D-AMPs appear to be more effective against the XDR strain than the MDR strain, both *in vitro* and *ex vivo*. The acquisition of resistance of Mtb often requires mutation of essential genes targeted by antibiotics, and the mutation may affect the original function of the gene, leading to the reduction of virulence and growth of the bacteria. Comparing to MDR-TB, which is resistant to both first-line antibiotics INH and rifampicin (RIF), XDR-TB is also resistant to fluoroquinolones plus at least one of the injectable antibiotics. The considerable resistance of XDR is often associated with a loss of fitness in the host macrophages, and hence is less virulent [28–31]. The *ex vivo* anti-TB assay demonstrated the different virulence of two drug resistant clinical strains in THP-1 cells, where a hundred-fold difference was observed in CFU between the control of MDR strain GB2 and XDR strain WYC-I1 on the equivalent day of experiment despite the MOI ratio being the same to begin with. The different degree of fitness of the drug-resistant strains in the host cells may partially explain why the WYC-I1 strain was more sensitive to D-AMPs in general, compared with the GB2 strain. It is generally believed that AMPs exert their action by interfering with the microbial plasma membrane and other cellular targets. The Mtb envelope consists of three major parts including the plasma membrane, the cell wall core and the outermost layer [32]. One distinct feature of the Mtb envelope is the high and diverse lipid contents, which contribute to the virulence of the bacteria. D-AMPs have to pass through the cell wall before reaching the targeted membrane. Several studies have used electron microscopy to demonstrate thinning and disruption of the Mtb cell wall after exposure to AMPs [33–35]. In addition to the observation that D-AMPs have the ability to break down the heavily aggregated Mtb, the present study also suggests there may be an association between the potency of D-AMPs and the variation of bacterial cell wall properties known to exist between different Mtb strains. Considerable differences in cell wall thickness between drug-susceptible strains and drug-resistant strains were reported in another study [36], where XDR strains have the thickest cell wall, followed by MDR strain, and drug-susceptible strains have the thinnest cell wall. How these compositional changes might affect the action of AMPs on mycobacterial capsule is not clearly understood due to the complexity of the capsule structure and diverse mechanism of AMPs. Nevertheless, a better understanding of this apparent relationship would inform the next stage of development of anti-TB AMPs.

Although none of the D-AMPs tested were effective in completely eradicating the intracellular drug-resistant Mtb, they may be useful as an adjunct agent to potentiate the effect of other existing anti-TB drugs. This concept has been demonstrated by other groups using natural AMPs including human beta-defensin-1 (HBD-1), protegrin-1 (PG-1) and human neutrophil peptide-1 [20,21]. It is known that the highly lipid-rich cell wall of mycobacteria contributes to the resistance to anti-TB drugs by reducing their permeability [9]. INH is a pro-drug that targets mycolic biosynthesis and is activated inside the mycobacteria. A previous study found that resistance of *Mycobacteria avium* to INH was caused by the bacterial cell envelope, with the cell wall and the outer layer acting as an exclusion barrier [37]. This barrier was efficient in excluding the hydrophilic INH which could not penetrate a wall matrix formed with various highly lipidic substances. Mtb that are resistant to 1.0 μg/ml INH in 7H10 agar are regarded as high-level resistant, whereas Mtb that are resistant to 0.2 μg/ml INH but sensitive to 1.0 μg/ml INH are regarded as low-level resistant [38]. Here we combined the INH (up to 10 μg/ml for inhibitory study and 1 μg/ml for bactericidal study) with D-LAK120-A or D-LAK120-HP13 (up to 6.25 μM) and evaluated their effect against MDR clinical strain GB2. We demonstrate that both D-LAK120-A and D-LAK120-HP13 have the ability to potentiate the effect of INH against drug-resistant TB *in vitro*, possibly by interfering the hydrophobic cell wall interaction. This may in turn lead to the increase in the permeability of the mycobacterial cell walls of the drug-resistant Mtb. In particular, D-LAK120-A was able to enhance the antibacterial effect of INH at non-toxic concentration. Although it is known that cell wall barrier alone does not produce significant level of INH resistant and a synergistic contribution from a second factor such as *katG* or *inhA* mutation is often required [38,39], it is plausible that by increasing permeability of INH, its efficacy against drug resistant Mtb can be enhanced. INH is not generally recommended in confirmed MDR-TB case. However, there is increasing evidence suggested that continued use of high dose INH may be of value, that the high-dose INH (10–20 mg/kg) may enhance the effectiveness of second-line treatment in cases of MDR-TB [40–42]. Besides, INH remains the most effective and cheapest anti-TB drug available.

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With the current study that demonstrates the beneficial effect of the INH/D-AMPs combination, INH could remain useful in MDR-TB even when used at low-dose to reduce the risk of hepatotoxic and neurotoxic effects which are associated with high dose INH. Furthermore, it will be interesting to investigate whether D-AMPs can improve the efficacy of other anti-TB drugs such as RIF to overcome the problem caused by the multi-drug resistant TB. This approach of combining D-AMPs with anti-TB drugs may also reduce the effective dose of anti-TB drugs against drug susceptible bacteria, minimize adverse effects and improve treatment success rate.

In conclusion, we have demonstrated that D-LAK based AMPs can successfully inhibit the growth of drug-resistant clinical strains of Mtb *in vitro* as well as *ex vivo* to a certain extent. The amphipathic properties of the peptides could also effectively break down the heavily aggregated Mtb by interacting with the highly lipidic cell surface of the mycobacteria. While the relationship between structural properties of D-AMPs and their anti-TB activity could not be established in this study, and the mechanism of their intracellular antimicrobial activity remain to be elucidated, we have identified D-LAK120-A as the optimal peptide within the family based on the balance between anti-TB activity and the low cytotoxicity towards THP-1 cells. Although the D-AMPs alone may not be sufficiently potent at their non-toxic concentrations as anti-TB agent, the combination of INH/D-AMPs showed a beneficial effect by potentiating the anti-TB efficacy. The results suggested that the D-AMPs can be used as adjunct agent to improve the efficacy of the existing antibiotics against drug-resistant TB.

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Ethical approval: Not required.

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