

**Unnatural amino acid analogues of membrane-active helical peptides with
anti-mycobacterial activity and improved stability**

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Running title: Enhanced selectivity of D-amino acid containing anti-mycobacterial peptides

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Abstract

Objectives: The emergence of MDR tuberculosis, coupled with shrinking antibiotic pipelines, has increased demands for new antimicrobials with novel mechanisms of action. Antimicrobial peptides have increasingly been explored as promising alternatives to antibiotics, but their inherent poor *in vivo* stability remains an impediment to their clinical utility. We therefore systematically evaluated unnatural amino acid modified peptides to design analogues with enhanced anti-mycobacterial activities.

Methods: Anti-mycobacterial activities were evaluated *in vitro* and intracellularly against drug-susceptible and MDR isolates of *Mycobacterium tuberculosis* using MIC, killing efficacy and intracellular growth inhibition studies. Toxicity profiles were assessed against mammalian cells to verify cell selectivity. Anti-mycobacterial mechanisms were investigated using microfluidic live-cell imaging with time-lapse fluorescence microscopy and confocal laser-scanning microscopy.

Results: Unnatural amino acid incorporation was well tolerated without an appreciable effect on toxicity profiles and secondary conformations of the synthetic peptides. The modified peptides also withstood proteolytic digestion by trypsin. The all D-amino acid peptide, i(lkk)₂i (II-D), displayed superior activity against all six mycobacterial strains tested with a four-fold increase in selectivity index as compared to the unmodified L-amino acid peptide in broth. II-D effectively reduced the intracellular bacterial burden of both drug-susceptible and MDR clinical isolates of *M. tuberculosis* after 4-days of treatment. Live-cell imaging studies demonstrated that II-D permeabilises the mycobacterial membrane, while confocal microscopy

revealed that II-D not only permeates the cell membrane, but also accumulates within the cytoplasm.

Conclusions: Unnatural amino acid modifications not only decreased the susceptibility of peptides to proteases, but also enhanced mycobacterial selectivity.

Introduction

According to the latest WHO figures, TB now ranks alongside HIV as the leading cause of death from an infectious disease worldwide, with 9.6 million new cases and 1.5 million deaths in 2014.¹ Multidrug-resistant TB (MDR-TB) accounted for 3.3% of the new cases, 20% were previously treated cases, and 9.7% were extensively drug-resistant TB (XDR-TB). The WHO recently developed a new framework (The End TB Strategy) to succeed the Millennium Development Goals after 2015, aimed at reducing TB death and incidence rates by 95% and 90% respectively.² However, given that second-line drugs only cure 60% of MDR-TB and 40% of XDR-TB, resistant forms of *Mycobacterium tuberculosis* pose a significant challenge to global efforts in controlling TB.³ While two new drugs, bedaquiline and delamanid, have recently been approved for MDR-TB treatment, rifampicin was the last first-line drug to be developed in 1967. Currently, there are only eight new or repurposed drugs undergoing late phase clinical trials, of which few, if any may eventually make it to the clinic.¹ Thus, it is crucial that a constant pipeline of novel anti-mycobacterial compounds is maintained to address these challenges and improve treatment outcomes.

Antimicrobial peptides (AMPs) have gained prominence over the past few decades as a promising class of therapeutics, due to their potent broad-spectrum activity encompassing

bacteria, fungi, viruses and parasites.⁴ Despite their structural diversity, AMPs possess key salient features including a net positive charge and a high proportion of hydrophobic residues, both of which are fundamental for AMP-mediated bacterial killing. Cationic AMPs adopt amphipathic structures upon contacting bacterial membranes, resulting in pore-formation via barrel-stave or toroidal-pore mechanisms, or widespread ‘micellization’ by carpet-like mechanisms.⁵ Alternatively, some peptides traverse the lipid bilayer, without inducing membrane permeabilisation, and inhibit enzymatic activity or impede cell wall, nucleic acid, or protein synthesis.⁵ Importantly, these bactericidal mechanisms are not limited to drug-susceptible bacteria, but also target their drug-resistant counterparts including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecalis*, MDR *Pseudomonas aeruginosa* and *M. tuberculosis*.⁶ In addition, the rapid membrane-lytic mechanisms of AMPs is likely to diminish their propensity for resistance development, while their ability to synergize with conventional antibiotics underlines their potential as adjuvants in combinatorial drug therapies for infection control.⁷

However, the transition of AMPs from bench to bedside has largely been hindered by their susceptibility to enzymes in biological fluids and degradation by bacterial proteases, resulting in short half-lives and loss of antimicrobial properties.⁸ Several strategies have been employed to enhance AMP stability including the incorporation of unnatural amino acids, N- and/or C-terminal modifications, cyclization, inclusion of non-peptidic backbones (peptidomimetics), and multimerization of AMP monomers to form dimers, oligomers and dendrimers.⁸ Previously, D-enantiomers were studied to determine the influence of peptide chirality on function, and the importance of stereospecific interactions with enzymes, receptors or lipids in membranes.^{9, 10} While both L- and D-enantiomers of peptides emerged equally active, suggesting a lack of specific peptide-receptors interactions, the latter were more resistant to

trypsin degradation. This in turn translated into longer half-lives *in vivo*, reinforcing their potential as therapeutic candidates.

Increasing evidence suggests that incorporation of D-amino acids not only confers protection against proteolysis by trypsin, but also against bacterial proteases such as staphylococcal aureolysin and V8 proteinase, thus rendering peptides more active.¹¹⁻¹³ *M. tuberculosis* is also known to secrete enzymes such as proteases, lipases, esterases, and dehydrogenases, which contribute towards its pathogenicity and persistence in the host.¹⁴ However there are limited reports systematically evaluating the impact of various stability-enhancing modifications on the anti-mycobacterial activity of synthetic AMPs.¹⁵

Recently, we demonstrated that the recurring sequence (XXYY)_n – (where X is a hydrophobic amino acid, Y is a cationic amino acid, and n is the number repeat units) – could be used to design short amphipathic α -helical anti-mycobacterial peptides.¹⁶ Subsequently, N- and C-terminal capping with hydrophobic residues revealed the importance of both hydrophobicity and helicity on the anti-mycobacterial activity, with I(LLKK)₂I emerging as the most selective peptide.¹⁷ These synthetic α -helical peptides primarily targeted the mycobacterial membrane, inducing rapid membrane permeabilisation and depolarization, resulting in leakage of intracellular contents. In this study, the effect of various unnatural amino acid substitutions on the anti-mycobacterial properties of the peptide, I(LLKK)₂I, were investigated. Peptide secondary structures were assessed by circular dichroism (CD) spectroscopy, while cytotoxicity was evaluated against human red blood cells and the murine macrophage RAW 264.7 cell line. Antimicrobial activities were studied against a panel of six mycobacterial strains using the broth microdilution method. The stability of the modified peptides was also evaluated against the serine protease trypsin. Since *M. tuberculosis* is a major intracellular

pathogen, the ability of peptides to activate macrophages and eliminate intracellular mycobacteria was also examined. Finally, the *in vitro* anti-mycobacterial mechanisms were studied using confocal and time-lapse fluorescence microscopy.

Material and methods

Materials

Synthetic peptides, including the N-terminal FITC-labeled II-D, were synthesized by GL Biochem (Shanghai, China) and their purity was confirmed to be more than 95% by reverse-phase HPLC. DMEM, PBS solution, DMSO, rifampicin, moxifloxacin, Tween 80, glycerol, propidium iodide, HPLC grade water, Triton X-100 and lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 were from Sigma-Aldrich (St Louis, MO, USA). Hygromycin B was from Roche Diagnostics (Indianapolis, IN, USA). FBS was obtained from Labtech International (Sussex, UK). Difco Middlebrook 7H9 broth, Difco Mycobacteria 7H11 agar, BBL Middlebrook albumin-dextrose catalase (ADC) supplement, and OADC were from BD (Sparks, MD, USA). MTT was from Duchefa Biochemie (Haarlem, Netherlands), while the membrane dye FM4-64FX was from Molecular Probes, Inc (Eugene, OR, USA). N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-treated trypsin was from AB Sciex (Framingham, MA, USA). The mouse TNF- α ELISA Ready-SET-Go kit was from eBioscience (San Diego, CA, USA) and Griess Reagent System from Promega (Madison, WI, USA).

Mycobacterial strains and growth conditions

M. tuberculosis H37Rv (H37Rv) was from ATCC (USA) while *M. bovis* BCG *lux* (BCG), Montreal strain, was transformed with the reporter plasmid construct pSMT1 as previously described.¹⁸ mCherry-expressing BCG (BCG-mCherry) was produced by transforming *M. bovis* BCG Danish strain 1331 (Statens Serum Institut) with pCherry3 (Addgene plasmid #24659 encoding mCherry and conferring hygromycin resistance¹⁹) using established protocols for mycobacteria.²⁰ The MDR clinical isolate, *M. tuberculosis* CSU87 (CSU87), resistant to rifampicin, isoniazid, ethambutol, streptomycin and kanamycin, was also evaluated in this study. Three other clinical isolates were *M. tuberculosis* 173 (Mtb 173) from the Euro-American lineage and, *M. tuberculosis* 212 (Mtb 212) and *M. tuberculosis* 411 (Mtb 411), both from the East Asian/Beijing lineage. Liquid cultures of all mycobacterial strains were grown in Middlebrook 7H9 broth supplemented with 0.05% Tween 80, 0.2% glycerol and 10% ADC. Mycobacterial colonies were grown on solid media consisting of Middlebrook 7H11 agar supplemented with 0.5% glycerol and 10% OADC. BCG and BCG-mCherry were grown in 7H9 broth or 7H11 agar in the presence of 50 µg/mL hygromycin. Bacterial stocks, grown to log phase, were prepared in 15% glycerol and aliquots frozen at -80°C. For each experiment a fresh vial was defrosted, inoculated into 7H9 broth (as described above) and grown at 37°C in a shaking incubator (180rpm) until the culture reached mid-log phase.

Peptide characterization

Surface-enhanced laser desorption/ionization time-of-flight mass spectroscopy (SELDI-TOF MS) was performed to confirm the molecular weights of the synthetic peptides. ProteinChip® NP20 arrays (Bio-Rad Laboratories) were primed with 5 µL of HPLC grade water, and 5 µL of each peptide (0.5 mg/mL) was applied to the array surface and allowed to air-dry for 1 h at room temperature. The arrays were washed twice with 5 µL of HPLC grade water and allowed

to air-dry for another 15 min at room temperature. 20% α -cyano-4-hydroxycinnamic acid (CHCA) was applied twice ($2 \times 0.7 \mu\text{L}$) to each spot on the arrays, allowing the matrix to air-dry between each application. Time-of-flight spectra were generated using a PCS 4000 SELDI-TOF MS instrument (Bio-Rad Laboratories). Spectra from the peptide pools were obtained at a laser energy of 800 nJ, a focus mass based on the theoretical molecular weight of each individual peptide and the matrix attenuated to 500 Da. Ten shots were obtained per position and a total of 500 shots kept per array. Mass accuracy was calibrated externally using All-in-One Peptide (Bio-Rad Laboratories).

Circular Dichroism (CD) spectroscopy

CD spectra were recorded at room temperature with ChirascanTM CD Spectrometer (Applied Photophysics, Surrey, UK). Peptides were first prepared at a final concentration of 0.2 mg/mL by dissolving them in 25 mM sodium dodecyl sulfate surfactant. Peptide solutions were then added to a quartz cell with a path length of 1.0 mm and measurements were taken from a wavelength of 190 to 240 nm, at a scanning speed of 60 nm min⁻¹ and averaged from 3 runs per peptide. The acquired spectra were converted to mean residue ellipticity using the following equation:

$$\theta_M = \frac{\theta_{\text{obs}}}{10} \cdot \frac{M_{\text{RW}}}{c \cdot l}$$

where θ_M is the mean residue ellipticity (deg cm² dmol⁻¹), θ_{obs} is the observed ellipticity corrected for the blank at a given wavelength (mdeg), M_{RW} is residue molecular weight (M_w /number of amino acids), c is peptide concentration (mg/mL), and l is the path length (cm).

Minimum inhibitory concentration (MIC) measurements

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199 The anti-mycobacterial activities of the synthetic peptides, and rifampicin and moxifloxacin
200 were assessed using the standard broth microdilution method. Briefly, two-fold serial dilutions
201 of the drugs were prepared with 7H9 broth before adding 100 μ L to each well of the 96-well
202 plate. Mid-log phase bacterial cultures were diluted to a density of 10^6 cfu/mL and an equal
203 volume was seeded into each well. The plates were then incubated at 37°C in a shaking
204 incubator and read after 7 days. The MIC was defined as the lowest concentration with no
205 bacterial growth, determined both visually and by spectrophotometric measurements taken
206 with the iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Hertfordshire, UK)
207 at OD₅₉₅. Experiments were carried out in triplicate on 2 or 3 separate occasions.

208

209 *Haemolytic activity test*

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211 Synthetic peptides were assessed for toxicity against mammalian cells using freshly drawn red
212 blood cells (RBCs) from healthy adult donors. Prior to commencement, ethical approval was
213 granted from Imperial College London NHS Healthcare Tissue Biobank (sub-collection
214 reference number: Pae-SN-15-001) and written informed consent was obtained from all
215 participants. Haemolytic activity was tested on a 4% (v/v) suspension obtained by a 25x
216 dilution of RBCs with PBS. Peptides were serially diluted with PBS to give solutions with final
217 concentrations ranging from 0 to 1000 mg/L. Equal volumes of peptide solution and blood
218 suspension were then mixed to give a final volume of 600 μ L. Following incubation at 37°C
219 for 2 h, the mixtures were centrifuged at 1700 g for 5 min. 100 μ L of the supernatant was then
220 added to each well of the 96-well plate and haemoglobin release was measured
221 spectrophotometrically at 576 nm by the VersaMax Tunable microplate reader (Molecular
222 Devices, Sunnyvale, CA, USA). Untreated RBCs were included as the negative control while

RBCs treated with 1% Triton X-100 served as the positive control. Data are expressed as mean $\pm$ standard deviation for two independent runs performed in triplicate. The degree of haemolysis was calculated using the following equation:

$$\text{Haemolysis (\%)} = \frac{(OD_{576nm} \text{ of treated sample} - OD_{576nm} \text{ of negative control})}{(OD_{576nm} \text{ of positive control} - OD_{576nm} \text{ of negative control})} \times 100\%$$

In vitro killing efficiency test

The antimicrobial peptide II-D was evaluated for its ability to kill H37Rv and clinical isolates Mtb 411 and CSU87, as it possessed the highest selectivity for bacterial over mammalian cells. Peptides were serially diluted with 7H9 broth to give solutions with final concentrations ranging from 31.3 to 500 mg/L. 100 μ L of diluted bacterial culture, with an inoculum size of 10^6 cfu/mL, was added to an equal volume of peptide solution. Following 7 days of incubation at 37°C in a shaking incubator, samples were serially diluted in PBS and 10 μ L was plated out in duplicates onto 7H11 agar. Bacterial colonies were counted after incubation at 37°C for 14 days. Experiments were carried out on at least 2 separate occasions and results are expressed as mean log (cfu/mL) $\pm$ standard deviation of both runs.

Cell culture and cytotoxicity testing

Peptides were evaluated for their cytotoxicity against the mouse macrophage cell line RAW 264.7 using the MTT assay. RAW 264.7 cells were maintained in DMEM supplemented with 10% FBS and cultured in humidified atmosphere at 37°C and 5% CO₂. The cells were first seeded at a density of 1×10^4 per well in 96-well plates and allowed to incubate for 24 h. Cells were then treated with peptides solutions ranging from 1.95 to 250 mg/L for an additional 24 h. Subsequently, the medium was replaced with 200 μ L of fresh DMEM and 40 μ L MTT

solution (5 mg/mL) and incubated for another 4 h at 37°C. The resultant formazan crystals were then dissolved using 150 µL DMSO and absorbance was measured spectrophotometrically at 595 nm in a VersaMax Tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was determined relative to untreated controls and expressed as $(A_{595nm} \text{ of treated sample}) / (A_{595nm} \text{ of control}) \times 100\%$. Data are expressed as mean $\pm$ standard deviation for two independent runs performed in triplicate.

Intracellular anti-mycobacterial activity

The intracellular activity of the antimicrobial peptide II-D was assessed against *M. tuberculosis* clinical isolates Mtb 411 and CSU87 as described previously.²¹ Briefly, RAW 264.7 cells were plated at a final concentration of 4×10^4 per well in 96-well plates and incubated for 24 h to allow for adherence. Prior to infection, mid-log phase bacterial cultures were washed twice with PBS, resuspended in DMEM and added at a final concentration of 4×10^5 cfu per well to achieve a multiplicity of infection (MOI) of 10:1. Plates were incubated at 37°C and 5% CO₂ for 4 h to allow for uptake of bacteria by macrophages. Subsequently, cells were washed thrice with prewarmed DMEM to remove extracellular bacteria before adding 200 µL of DMEM, with or without drugs, to each well. II-D was tested at concentrations corresponding to 0.5x, 1x, 2x and 4x MIC, while rifampicin and moxifloxacin were evaluated at 1x, 2x, 4x and 8x MIC. Each concentration was tested in triplicate. For determination of bacterial viability at various time points, macrophages were lysed with 200 µL of sterile water for 30 min, serially diluted with PBS and 10 µL was plated out in duplicate on 7H11 agar. Following incubation at 37°C for 14 days, bacterial colonies were enumerated and data are expressed as mean log (cfu/mL) $\pm$ standard deviation.

Nitric oxide (NO) production by peptide-treated macrophages

RAW 264.7 cells were seeded in 96-well plates at a density of 4×10^4 per well, followed by incubation at 37°C and 5% CO₂ for 24 h. The medium was removed and cells were treated with peptide solutions ranging from 7.81 to 250 mg/L for an additional 24 h. NO production was estimated by measuring nitrite concentrations in supernatants using the Griess reagent (0.1% N-1-naphthylethylenediamine dihydrochloride, 1% sulfanilamide and 5% phosphoric acid) according to the manufacturer's protocols. The absorbance was measured by at 540 nm and nitrite concentrations were determined using standard curves generated with NaNO₂ solutions. Macrophages stimulated with 100 ng/mL LPS served as positive controls while unstimulated macrophages served as negative controls. Data are expressed as mean $\pm$ standard deviation for two independent runs performed in triplicate.

TNF- α production by peptide-treated macrophages

RAW 264.7 cells were seeded in 96-well plates at a density of 4×10^4 per well, followed by incubation at 37°C and 5% CO₂ for 24 h. The medium was removed and cells were treated with peptides solutions ranging from 7.81 to 250 mg/L for an additional 24 h. TNF- α concentration in supernatants was determined using the mouse TNF- α ELISA kit as per the manufacturer's instructions. Absorbance readings were measured at 450 nm. Unstimulated macrophages served as negative controls, and positive controls were stimulated with 100 ng/mL LPS. Data are expressed as mean $\pm$ standard deviation for two independent runs performed in triplicate. The degree of TNF- α production was calculated as follows:

$$TNF - \alpha \text{ production (\%)} = \frac{(OD_{450nm} \text{ of treated sample} - OD_{450nm} \text{ of negative control})}{(OD_{450nm} \text{ of positive control} - OD_{450nm} \text{ of negative control})} \times 100\%$$

Protease stability assay

The proteolytic stability of the synthetic peptides was assessed by measuring their antimicrobial activity against BCG after pretreatment with the serum protease, trypsin, in accordance with methods described previously.¹² Briefly, peptide solutions at a fixed concentration of 4x MIC were incubated with trypsin at an enzyme to peptide ratio of 1:100. Following incubation at 37°C for 6 h, the solutions were heat-treated at 80°C for 10 min to inactivate the enzyme. 100 µL of the treated peptide solution was then added to an equal volume of bacterial culture (10^6 cfu/mL) and incubated at 37°C in a shaking incubator for 7 days. Inhibition of bacterial growth was determined by spectrophotometric measurements at OD₅₉₅. Data are expressed as mean ± standard deviation for two independent runs performed in triplicate.

Microfluidic live-cell imaging with time-lapse fluorescence microscopy

Live-cell imaging was performed using the automated CellASIC ONIX Microfluidic Platform with the CellASIC ONIX B04A-03 Microfluidic Bacteria Plates (EMD Millipore Corporation, Hayward, CA, USA). Mid-log phase cultures of BCG were first diluted with 7H9 broth to a final density of 10^7 cfu/mL, before adding 100 µL to each of the cell loading wells. To evaluate the antimicrobial mechanisms of action, the most selective peptide II-D, was serially diluted with 7H9 broth to give final concentrations corresponding to 4x and 8x MIC. Peptide solutions were then added to the inlet wells together with the membrane-impermeable dye, propidium iodide (10 µg/mL), in a total volume of 350 µL. Next, the microfluidic plate was vacuum-sealed to the F84 manifold and the CellASIC ONIX FG Software initiated. Loading and subsequent washing of un-trapped bacterial cells was carried out according to the

manufacturer's protocol.²² Peptide solutions were perfused into culture chambers at the recommended pressure of 2 psi for up to 4 h. The temperature was maintained at 37°C and bacterial cells treated with medium and propidium iodide alone served as negative controls. Phase contrast and fluorescent images of bacterial cells were captured with a 63x oil-immersion objective lens every 10 min using the Zeiss Axiovert 200M inverted microscope (Carl Zeiss Inc.). Microscopy was performed in the Facility for Imaging by Light Microscopy (FILM) at Imperial College London.

Confocal laser-scanning microscopy

BCG and BCG-mCherry cultures were grown up to mid-log phase, centrifuged at 2000 g for 10 min, washed, and resuspended in PBS at a cell density of 10^8 cfu/mL. Following treatment with 500 mg/L FITC-labelled II-D for 2 h, bacterial cells were centrifuged at 2000 g for 10 min and washed three times with PBS. To stain bacterial membranes, BCG cells were resuspended in PBS with 2 µg/mL FM4-64 for 10 min at 37°C with shaking. Cells were then pelleted, washed twice with PBS to remove any unbound dye, and fixed overnight with 4% formaldehyde at 4°C. Following fixation, cells were pelleted, resuspended in PBS and allowed to air dry on microscope slides for 20 min. Samples were mounted with Mowiol mounting medium and imaged with a 100x oil-immersion objective lens using a Zeiss LSM-510 inverted confocal microscope (Carl Zeiss Inc.). FITC was excited with a 488 nm laser and detected with a 505-530 nm band-pass filter while mCherry and FM4-64 were both excited with a 543 nm laser and detected with a 615 nm and 650 nm long-pass filters respectively.

Image and statistical analysis

The Huygens Deconvolution software (Scientific Volume Image) was used to perform deconvolution of confocal images. Fluorescence intensity profiles along the bacterial cell length were obtained with the segmented line and multi-channel intensity line profile plot tools in the ImageJ software. Processing and compiling of phase and fluorescent images into movies at 2 frames per second, were carried out using the ImageJ software. All statistical analysis in this study was performed with GraphPad Prism 6 software (GraphPad Software Inc., CA, USA) using one-way analysis of variance (ANOVA), with Bonferroni's post hoc test for multiple comparisons. Significant differences between groups were indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Results and discussion

Peptide design and characterization

We previously reported that short 10 amino acid peptides with the backbone sequence X(LLKK)₂X (where X is a hydrophobic amino acid), assumed ordered α -helical conformations in a membrane-mimicking environment and were capable of eradicating mycobacteria *in vitro*.^{16, 17} In this study, systematic substitutions with unnatural or D-amino acids were performed in an attempt to design synthetic analogues with improved stability and selectivity indices. The isoleucine substituted analogue, I(LLKK)₂I or II, which emerged as one of the most selective peptides against mycobacteria, served as a framework for these structural modifications. To maintain sequence identity and structural integrity of the optimized lead peptide, the two main modifications involved (a) swapping all L-amino acid with D-amino acid residues and (b) substitution of lysine (Lys) residues with Lys analogues possessing shorter side-chains including ornithine (Orn), 2,4-diaminobutyric acid (Dab) or 2,3-diaminopropionic

acid (Dap). The C-terminal of all synthetic analogues was amidated to improve anti-mycobacterial activity by increasing net positive charge.²³ The sequences and abbreviated names of the five synthetic peptides evaluated in this study are listed in Table 1. In order to verify that the peptides were successfully synthesized according to the desired specifications, SELDI-TOF MS was performed for molecular weight determination. As shown in Table 1, the close agreement between the theoretical and measured molecular weights of the peptides served as confirmation of the fidelity of peptide synthesis.

Circular Dichroism spectroscopic studies

The influence of unnatural or D-amino acid substitutions on the secondary conformation of the synthetic peptides was investigated in a membrane-like environment consisting of 25 mM SDS micelle solution, and studied using CD spectroscopy. The CD spectra of the synthetic peptides are summarized in Figure 1. The presence of the characteristic absorption bands at ~208 and 222 nm suggests that all five synthetic peptides adopted α -helical structures in 25 mM SDS. The replacement of Lys residues with Orn, Dab or Dap, did not compromise the helical structure of peptides, evident from the comparable relative helicities and mean residue ellipticity values at 222 nm (θ_{222}) as shown in Table 1. As expected, II-D exhibited a CD spectrum of opposite sign to that of II, though not an exact mirror image. II-D recorded a lower absolute θ_{222} value than II (10033 versus 11290), indicative of a slight reduction in α -helical conformation. Since, D-allo-isoleucine was utilised during peptide synthesis, the observed differences in CD ellipticity at 222 nm could be attributed to II and II-D being diastereomers, rather than enantiomers of each other.²⁴

In vitro anti-mycobacterial activity

399 The antimicrobial activities of the synthetic helical peptides were evaluated by the broth
400 microdilution method against a panel of six mycobacterial strains and the results are
401 summarized in Table 2. Overall, structural modifications with unnatural or D-amino acids were
402 effective in producing analogues with improved anti-mycobacterial activities. A two to
403 fourfold reduction in the geometric mean (GM) of the MICs was observed for the modified
404 peptides as compared to II. Importantly, three of the synthetic peptides displayed a fourfold
405 reduction in MIC in comparison to II against the MDR clinical isolate CSU87. The replacement
406 of Lys with Orn proved least effective, with II-Orn demonstrating similar activity as II against
407 50% of the mycobacterial strains tested. This analogue was more active against only two of the
408 six strains, while substitution of Lys with Dab and Dap resulted in decrease in MICs against
409 five and four of the six strains respectively. The all D-amino acid analogue exhibited superior
410 efficacy as compared to II against all six mycobacterial strains, which translated into a fourfold
411 reduction in the GM of its MICs.

412 We had previously reported that both hydrophobicity and folding of peptides into amphipathic
413 helical structures was crucial for inducing mycobacterial membrane permeabilisation and
414 augmenting anti-mycobacterial activity.¹⁷ Given that II and II-D are diastereomers of each
415 other, their amino acid composition, charge, molecular weight, amphiphilicity and in turn
416 hydrophobicity is expected to be similar. However, the lower relative helicity of 0.89 for II-D
417 (Table 1) as compared to II would be expected to compromise its activity, but the converse was
418 true. The reduced hydrophobicities of II-Orn, II-Dab and II-Dap relative to that of II, also did
419 not translate into diminished activities. This suggests that factors unrelated to the
420 physiochemical properties of the peptides may also be responsible for the observed differences
421 in anti-mycobacterial efficacy of the synthetic peptides. A plausible explanation is that the
422 structural modifications employed in this study could potentially have enhanced peptide

stability to extracellular proteolytic enzymes of *M. tuberculosis*, in turn rendering them more active against the pathogen. *M. tuberculosis* culture filtrates containing proteases were found to diminish the anti-tubercular activity of the L- rather than D-enantiomer of peptides, with the latter demonstrating greater resistance to degradation, evident from the significantly higher recovery as compared to the L-enantiomer (89% versus 26%) after 7 days exposure.¹⁵ These findings suggest that stability-enhancing modifications such as the incorporation of unnatural amino acids may be crucial to developing AMPs with superior efficacies against slowly-replicating bacilli like *M. tuberculosis*.

Haemolytic activities, cytotoxicity and cell selectivities

The haemolytic activities of the synthetic peptides were evaluated using 4% (v/v) human blood from two healthy adult donors and the results are summarized in Figure 2(a). Even at the highest tested concentration of 1000 mg/L, all five peptides displayed minimal haemolysis of approximately 2%. The HC₅₀ values, defined as the lowest peptide concentration resulting in 50% haemolysis of RBCs, of all five peptides was found to be >1000 mg/L (Table 2). Similar observations were also made with respect to the cytotoxicity of the peptides against the murine macrophage cell line, RAW 264.7. As highlighted in Figure 2(b), cell viabilities remained in excess of 80% even at concentrations of 250 mg/L following 24 h exposure to synthetic peptides. Importantly, none of the modifications produced analogues of II with an inferior toxicity profile against mammalian cells. The selectivity indices (SIs) highlighted in Table 2, defined as the ratio of HC₅₀ to GM MIC values, are often utilised to identify analogues with superior antibacterial selectivities. The D-amino acid analogue of II emerged as the most selective for mycobacterial over mammalian cells, with a SI value of 20. This was fourfold greater than that of II and approximately 1.8 and 2.2 times higher than that of II-Dab and II-

Dap respectively. Since larger SI values are deemed as more desirable, the strategies presented in this study could therefore prove useful in designing more selective AMPs to treat *M. tuberculosis* infections.

Resistance to protease degradation

A major barrier limiting the clinical application of AMPs is their susceptibility to protease degradation in biological fluids.⁸ Trypsin specifically cleaves the C-terminal of both lysine and arginine, thus, peptides containing these cationic residues are likely to be rendered inactive. We therefore assessed the stability of the Lys-rich II and its unnatural amino acid substituted analogues by pre-treating the peptides with trypsin before evaluating their antimicrobial activities against BCG using the standard broth microdilution method. As shown in Figure 2(c), all five peptides inhibited bacterial growth after 7 days when no prior treatment with trypsin was performed. However, following pre-treatment with trypsin, the antimicrobial activity of only II was lost while that of the other four modified peptides was preserved. The modifications to II not only produced analogues with superior mycobacterial selectivity (Table 2), but also conferred protection against the serine protease trypsin, enhancing their clinical utility.

In vitro and intracellular killing efficiencies

The D-amino acid analogue of II was not only more resistant to enzymatic degradation, but also exhibited the highest SI of all peptides evaluated in this study. Hence, II-D was evaluated for its *in vitro* killing efficiency against laboratory strain H37Rv, the drug-susceptible clinical isolate Mtb 411, and the MDR clinical isolate CSU87. II-D displayed killing efficiencies of >90% at the respective MICs for all three strains (Figure S1). At 2x the respective MIC values,

II-D killing efficiency was >99% against both H37Rv and CSU87 and >97% against Mtb 411. The intracellular activity of II-D was subsequently evaluated against both drug-susceptible and MDR clinical isolates of *M. tuberculosis* during macrophage infection, at concentrations corresponding to 0.5x, 1x, 2x and 4x MIC. The first-line anti-tubercular drug, rifampicin, and second-line fluoroquinolone, moxifloxacin, served as controls for Mtb 411 and CSU87, respectively. As shown in Figures 3(a) and 3(b), II-D and rifampicin resulted in significant reductions in intracellular Mtb 411 counts following 4-day treatment as compared to untreated controls ($p < 0.0001$). Both II-D and rifampicin demonstrated concentration-dependent killing of Mtb 411, although peptide treatment was more effective in rapidly eliminating the intracellular pathogen. At their respective MICs, rifampicin and II-D reduced bacillary loads within 3 days by 36% and 84%, respectively. Furthermore, exposure to II-D at 250 mg/L (4x MIC) resulted in approximately 1 log reduction of intracellular bacterial burden by days 3 and 4 (Figure 3a). This effect was less pronounced with rifampicin as bacterial counts decreased by 66% and 70% on days 3 and 4 respectively, even up to concentrations corresponding to 8x MIC (0.016 mg/L). Similarly, II-D and moxifloxacin also induced significant reductions in intracellular CSU87 counts after 4 days in comparison to untreated controls ($p < 0.0001$). II-D brought about a 62% decrease in bacterial load within 4 days, even at sub-MIC of 31.3 mg/L (Figure 3c). II-D was also more effective than moxifloxacin in clearing intracellular CSU87 at equivalent concentrations of 1x, 2x and 4x MIC. Given that II-D demonstrated minimal toxicity towards RAW 264.7 macrophage cell line, with high cell viability of 90% even up to concentrations of 250 mg/L (Figure 2b), the observed reductions in intracellular mycobacterial load were a direct consequence of peptide treatment rather than cytotoxic effects on macrophages. Overall, these findings reinforce the applicability of AMPs for the treatment of intracellular pathogens such as *M. tuberculosis*.

In vitro and intracellular antimicrobial mechanisms

The bactericidal activity of AMPs is largely mediated by non-specific membrane-lytic mechanisms, which induces pore formation and/or membrane destruction, leading to membrane depolarization and outflow of cytoplasmic contents.⁴ In this study, the antimicrobial mechanisms of II-D were investigated against BCG using microfluidic live-cell imaging by employing the CellASIC™ ONIX Microfluidic Platform. Bacterial cells were treated with peptides at 4x and 8x MIC, in the presence of the DNA intercalating dye propidium iodide (PI) for up to 4 h. As illustrated in Movies S1 and S2, exposure of BCG to II-D at 4x and 8x MIC resulted in fluorescence staining of bacterial cells within 10 min. The uptake of PI was concentration-dependent, with ~50% of bacterial cells exhibiting fluorescence after 210 min at 8x MIC of II-D (Figure 4) while a much lower proportion of cells were stained over the same time period at 4x MIC of II-D (Figure S2). Given the membrane-impermeant nature of PI, the dye is generally excluded from viable cells with intact bacterial membranes. Hence, the observed fluorescence provides evidence of the membrane-permeabilising activity of II-D, resulting in the loss of membrane integrity which in turn facilitates intracellular diffusion and binding of PI to DNA. Furthermore, II-D demonstrated bactericidal activity at both these concentrations against BCG with a >2 log reduction in RLU readings after 7 day treatment as compared to the initial inoculums (Figure S3a). Taken together, these findings suggest that the bactericidal activity of II-D may partly be mediated by disrupting the structural integrity of the mycobacterial membrane.

Increasingly, the ability of AMPs to modulate the host innate immunity has been explored as an alternative strategy for effective infection control.²⁵ To evaluate the capacity of II-D to modulate host immunity by activating macrophages, unstimulated RAW 264.7 cells were treated with up to 250 mg/L of the peptide for 24 h and proinflammatory responses were

quantified by analysing NO and TNF- α production relative to that of LPS-stimulated macrophages. As shown in Figure 5, II-D neither induced NO nor TNF- α production, suggestive of its inability to effect macrophage activation. Thus, the observed intracellular anti-mycobacterial activity of II-D is likely due to direct bactericidal mechanisms rather than the induction of secondary proinflammatory responses.

Cellular localization of II-D

To ascertain the site of action of II-D, BCG was exposed to FITC-labeled II-D for 2 h using the CellASIC™ ONIX Microfluidic Platform and its localization was visualized by fluorescence microscopy. FITC-II-D was tested at 500 mg/L as the killing efficacy against BCG was similar to that of II-D at 2x MIC (Figures S3b and S3c). As shown in Figure 6(a), treatment of BCG with 500 mg/L of FITC-II-D resulted in peptide accumulation within the cytoplasm as evidenced by the bright green fluorescence signal. This was confirmed by confocal laser-scanning microscopy studies with mCherry-expressing BCG where the co-localization of FITC-II-D with the cytosolic fluorescence protein mCherry was observed (Figure 6b). Bacterial cells unaffected by FITC-II-D demonstrated strong red fluorescence signal across the entire cell length without any detectable green fluorescence peptide signal (Figures 6b, S4b and S4c), while peptide penetration into bacterial cells resulted in stronger green than red intracellular fluorescence intensity profile along the cell length (Figure S4a). This observed reduction in red fluorescence mCherry signal in cells which internalized FITC-II-D was likely due to peptide-mediated membrane permeabilisation resulting in leakage of cytosolic contents.

Next, co-labeling studies with the membrane-binding dye FM4-64 in BCG revealed that the red FM4-64 signal was predominantly located on the bacterial cell surface, and showed the

same pattern of staining as the green fluorescence of FITC-II-D (Figure 6c). Moreover, the fluorescence intensity profile of FM4-64 across the cell width matched that of FITC-II-D (Figures S5a and S5b). Previous studies have reported that the cell-penetrating peptide, buforin II, accumulates in cytoplasm without inducing the influx of PI into bacterial cells, suggestive of a non-membrane permeabilising mode of action.²⁶ Following internalization, buforin II promotes cell death by inhibiting cellular functions through binding strongly to DNA and RNA.²⁷ In contrast, the membrane-permeabilising α -helical peptide magainin II, binds to the bacterial membrane, inducing pore formation and promoting the influx of PI.²⁶ In this study, fluorescence and confocal microscopy analysis revealed that II-D permeates the mycobacterial cell membrane and accumulated in the cytoplasm (Figure 6). Additionally, membrane integrity studies with PI provided evidence of the membrane-lytic mechanisms of action (Figure 4). Taken together, the killing mechanisms of II-D could potentially be attributed to both pore-formation and targeting of intracellular components. Recently, non-membrane lytic, cell-penetrating synthetic AMPs (SAMPs) were reported to selectively kill *M. smegmatis* by binding bacterial DNA and consequently inhibiting DNA-dependent processes including replication and transcription.²⁸ Hence, the ability of the membrane-active peptide II-D to interact with intracellular targets such as bacterial DNA and RNA warrants further investigation.

In summary, synthetic helical AMPs modified with unnatural amino acids demonstrated improved anti-mycobacterial activities and stability to trypsin degradation as compared to the all L-amino acid peptide, II. The incorporation of D-amino acids proved to be the most effective modification, with the diastereomer II-D exhibiting a fourfold increase in SI as compared to II, likely due to the enhanced stability to *M. tuberculosis* proteases. In addition to its *in vitro* inhibitory activity against both drug-susceptible and MDR *M. tuberculosis*, II-D was equally, if not more effective, at clearing intracellular mycobacteria than both rifampicin

and moxifloxacin at MIC equivalent concentrations. Mechanistic studies revealed that the site of action of II-D may not be limited to the mycobacterial membrane, but could potentially involve intracellular targets following internalization of the peptide. Overall, these findings reiterate the importance of unnatural amino acid substitutions towards enhancing protease stability while highlighting their applicability in designing AMPs with superior mycobacterial selectivity.

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Transparency declarations

None to declare.

Supplementary data

Movies S1 and S2, and Figures S1 to S5 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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Tables and Figures

Table 1. Design of α -helical peptides modified with unnatural amino acids and their molecular weights.

Antimicrobial peptide	Peptide sequence ^a	Theoretical M _w	Measured M _w ^b	[θ] ₂₂₂ ^c	Relative helicity ^d
II	I(LLKK) ₂ I-NH ₂	1208.67	1209	11290	1.00
II-D	i(lkk) ₂ i-NH ₂	1208.67	1209	10033	0.89
II-Orn	I(LL-Orn-Orn) ₂ I-NH ₂	1152.56	1153	11432	1.01
II-Dab	I(LL-Dab-Dab) ₂ I-NH ₂	1096.70	1097	11081	0.98
II-Dap	I(LL-Dap-Dap) ₂ I-NH ₂	1040.35	1041	11765	1.04

^aD-amino acids are represented by lower case letters. Ornithine, 2,4-diaminobutyric acid and 2,3-diaminopropionic acid are abbreviated as Orn, Dab and Dap respectively.

^bMolecular weight (M_w) determined by surface-enhanced laser desorption/ionization time-of-flight mass spectroscopy (SELDI-TOF MS).

^cThe mean residue ellipticity values at 222 nm (θ_{222}) were measured in 25 mM SDS solution by circular dichroism spectroscopy.

^dThe helical content of modified peptides relative to the mean residue ellipticity values at 222 nm of II.

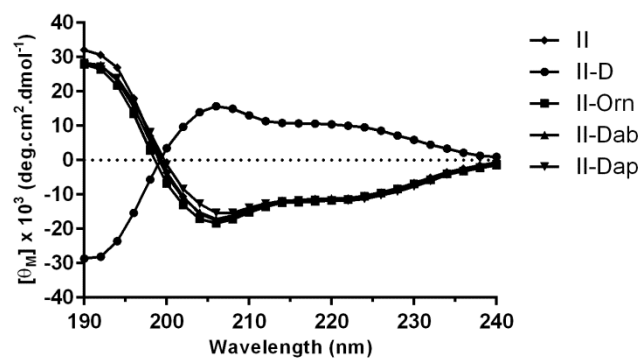


Figure 1. CD spectra demonstrating α -helical secondary conformations of II and its synthetic analogues with unnatural amino acid substitutions in 25 mM SDS micelle solution. Data are expressed as mean of three runs per peptide.

Table 2. Minimum inhibitory concentrations (MICs) and selectivity indices (SIs) of helical peptides against various mycobacterial strains including drug-susceptible and MDR clinical isolates of *Mycobacterium tuberculosis*

Antimicrobial peptide	MIC ^a (mg/L)						GM ^b (mg/L)	HC ₅₀ (mg/L)	SI ^c
	BCG	H37Rv	Mtb 173	Mtb 212	Mtb 411	CSU87			
II	125	500	>500	>500	250	250	397	>1000	5
II-D	31.3	250	125	250	62.5	62.5	99	>1000	20
II-Orn	62.5	>500	>500	500	250	250	354	>1000	6
II-Dab	31.3	>500	500	500	62.5	62.5	177	>1000	11
II-Dap	62.5	>500	> 500	500	62.5	62.5	223	>1000	9
Rifampicin	0.001	0.015	0.008	0.004	0.002	>32	0.004	-	-

^aMIC results are representative of 2 to 3 independent experiments.

^bThe geometric mean (GM) of the MICs against the 6 mycobacterial strains. A value of 1000 mg/L was used for SI calculations when no anti-mycobacterial activity was observed at the highest tested concentration of 500 mg/L.

^cSelectivity index (SI) is determined as follows: (HC₅₀/GM). When no detectable haemolysis was observed at the highest tested concentration of 1000 mg/L, a value of 2000 mg/L was used for SI calculations.

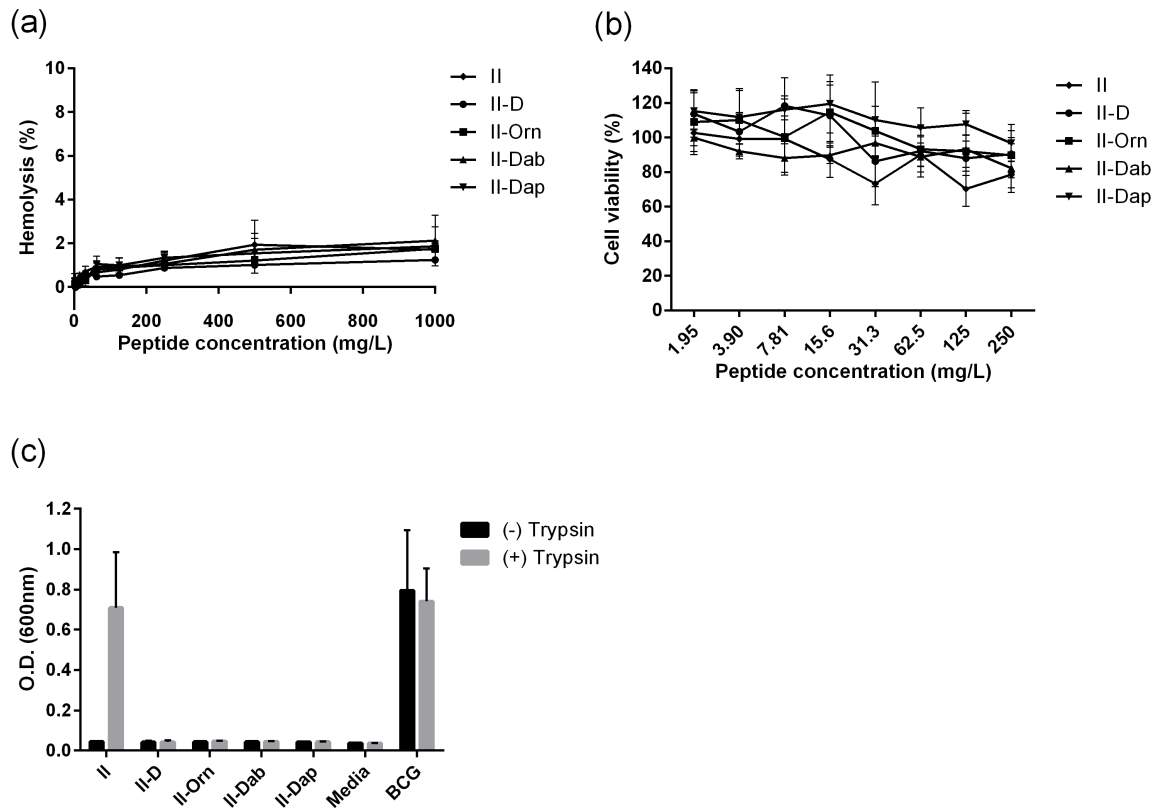


Figure 2. Toxicity profiles of synthetic peptides modified with unnatural amino acids against (a) human red blood cells and (b) the mouse macrophage cell line RAW 264.7. Peptides displayed minimal haemolytic activity and cytotoxicity against mammalian cells at various concentrations tested. (c) Inhibitory activity of synthetic peptides against BCG at concentrations of 4x MIC following 6 h treatment with trypsin at a ratio of 1:100. Only the unmodified peptide, II, did not inhibit bacterial growth after 7 days. Data are expressed as mean and standard deviation for two independent experiments.

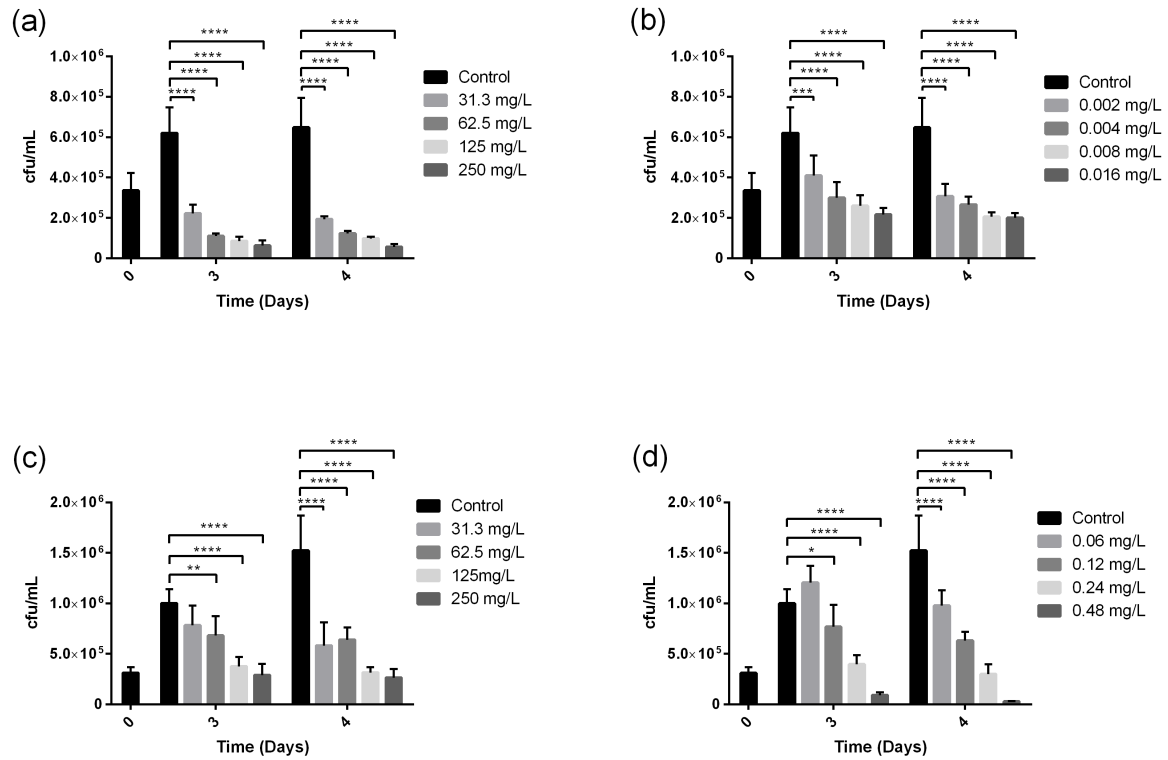


Figure 3. Intracellular killing of the drug-susceptible clinical isolate *Mtb* 411 by (a) antimicrobial peptide II-D and (b) rifampicin, and the MDR clinical isolate CSU87 by (c) antimicrobial peptide II-D and (d) moxifloxacin. One-way ANOVA followed by Bonferroni's post-hoc test was applied for the determination of significant differences, where * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Data are expressed as mean and standard deviation from one representative experiment.

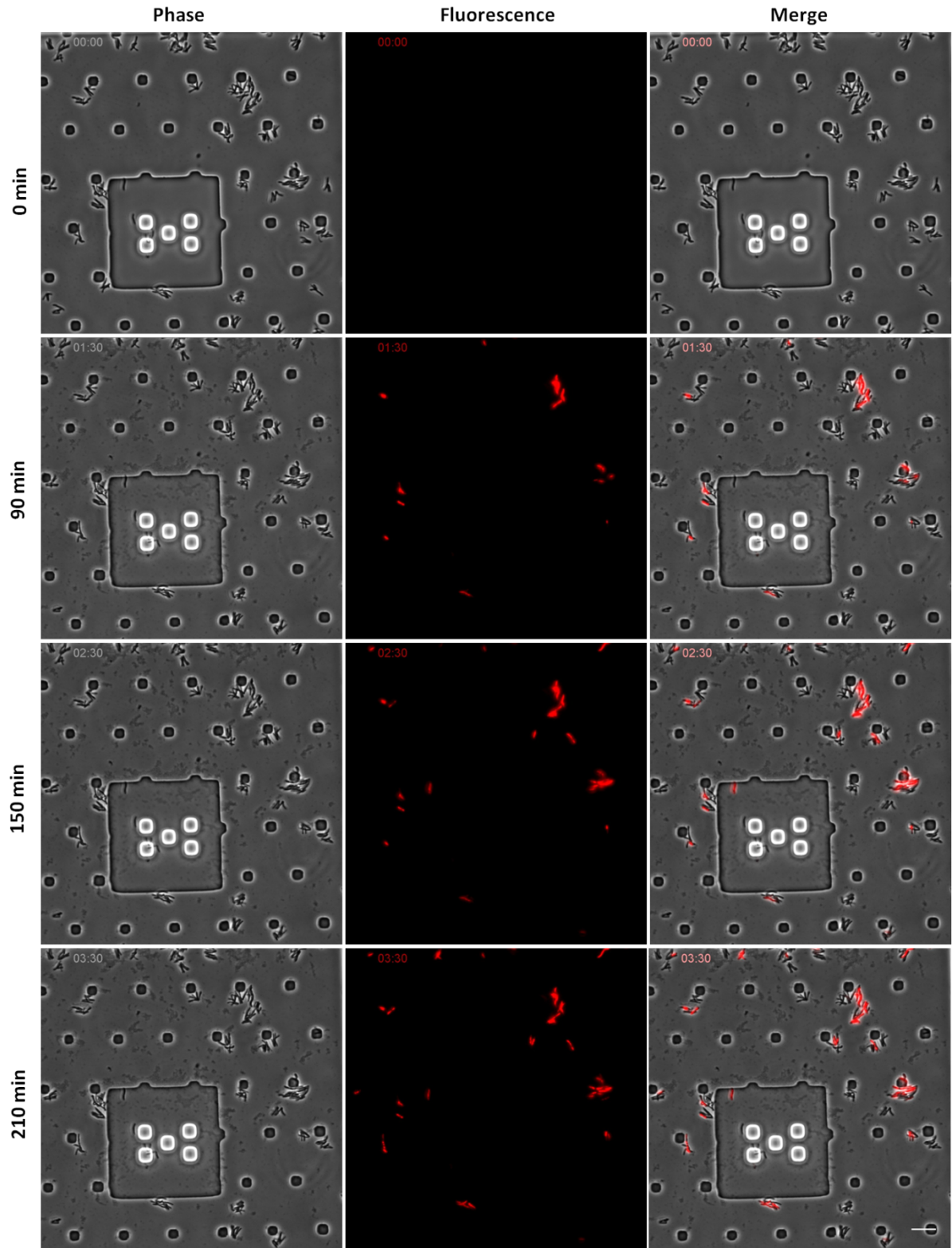


Figure 4. Time-lapse fluorescence microscopy images of BCG following treatment with antimicrobial peptide II-D at 8x MIC in the presence of the membrane-impermeable dye, propidium iodide (PI). Peptide-mediated membrane disruption promoted uptake of PI into

724 bacterial cells. Scale bar = 10 μm . Images are presented from one representative experiment.
725 This figure appears in colour in the online version of *JAC* and in black and white in the printed
726 version of *JAC*.

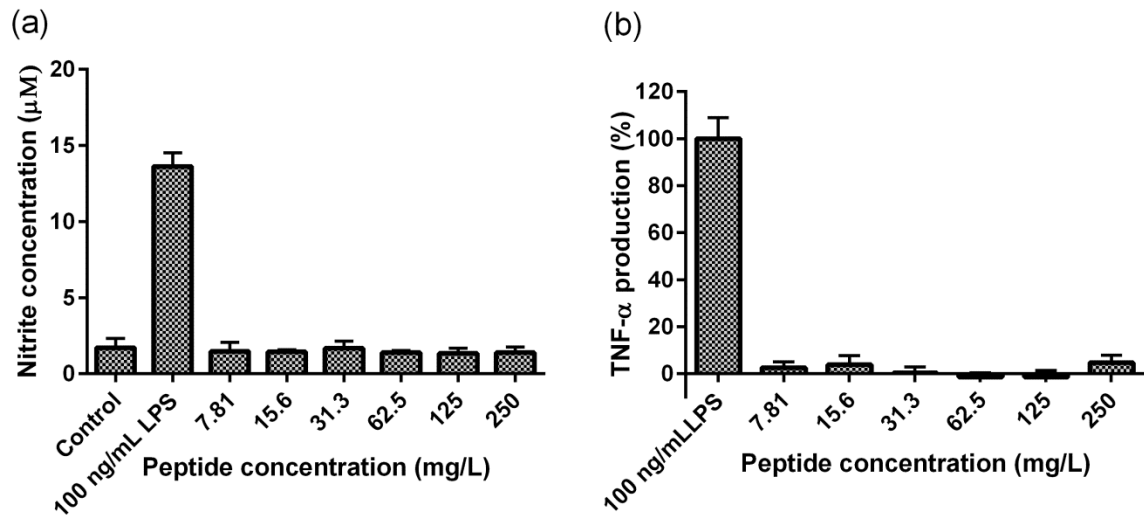


Figure 5. The ability of antimicrobial peptide II-D to promote (a) nitric oxide (NO) and (b) tumour necrosis factor- α (TNF- α) production in unstimulated RAW 264.7 mouse macrophage cells following 24 h treatment. II-D did not induce NO or TNF- α as compared to cells stimulated with 100 ng/mL LPS, which served as positive controls. Data are expressed as mean and standard deviation for two independent experiments.

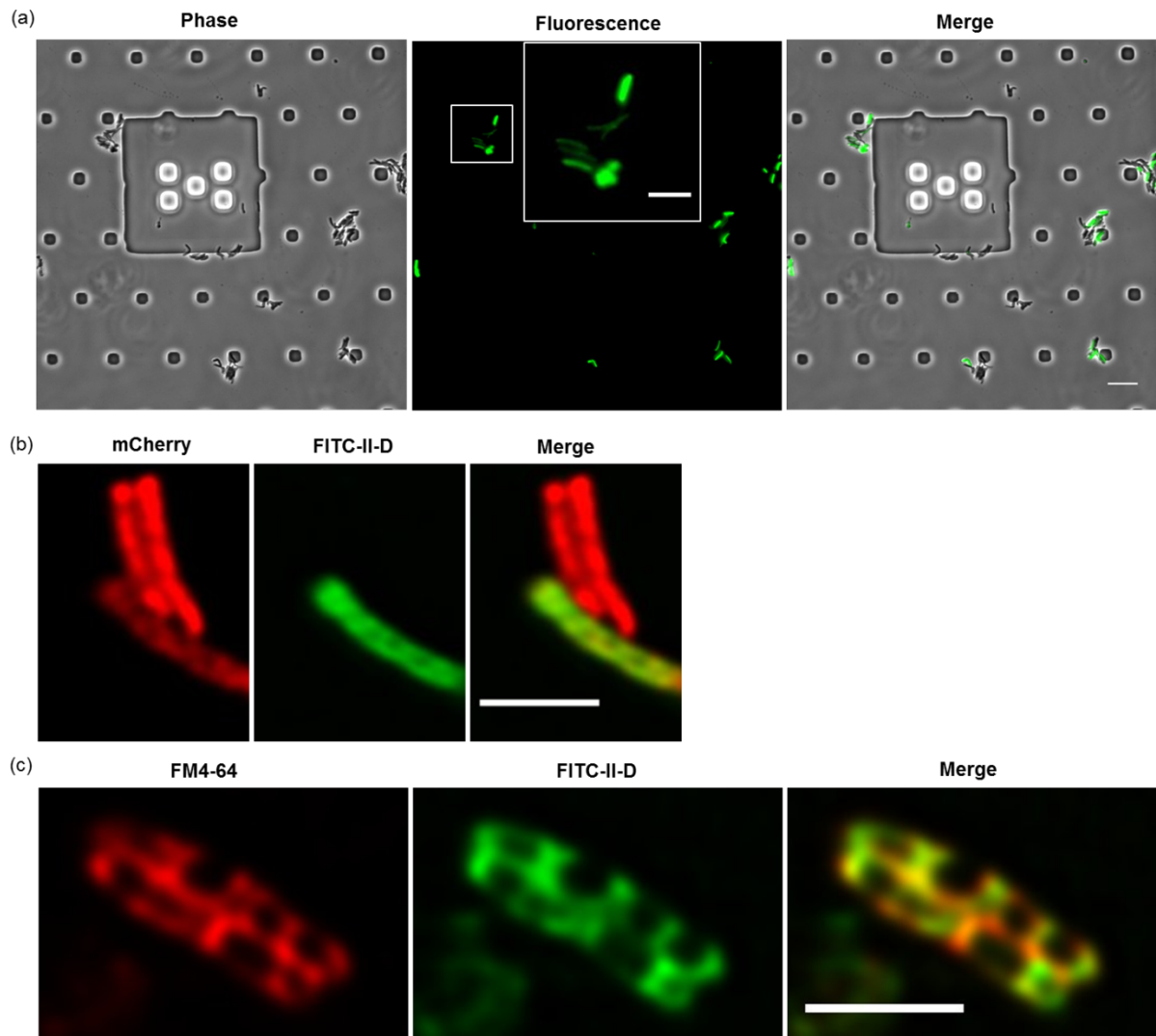


Figure 6. (a) Fluorescence microscopy images of BCG treated with 500 mg/L FITC-labelled II-D for 2 h using the CellASIC ONIX Microfluidic Platform. Scale bar = 10 μm (inset scale bar = 5 μm). Confocal laser scanning microscopy images following incubation of 500 mg/L FITC-labelled II-D for 2 h with (b) BCG-mCherry and (c) BCG in the presence of 2 $\mu\text{g/mL}$ membrane dye FM4-64. Scale bar = 2 μm . Images are presented from one representative experiment. This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC*.