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Biomaterials 35 (2014) 3102–3109

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Synthetic modifications of the immunomodulating peptide thymopentin to confer anti-mycobacterial activity

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ARTICLE INFO

Article history:
Received 27 November 2013
Accepted 19 December 2013
Available online 8 January 2014

Keywords:
Cationic peptides
Thymopentin
Drug resistance
Synergy
Immunotherapeutics
Mycobacterium smegmatis

ABSTRACT

Effective global control of tuberculosis (TB) is increasingly threatened by the convergence of multidrug-resistant TB and the human immunodeficiency virus (HIV) infection. TB/HIV coinfections exert a tremendous burden on the host's immune system, and this has prompted the clinical use of immunomodulators to enhance host defences as an alternative therapeutic strategy. In this study, we modified the clinically used synthetic immunomodulatory pentapeptide, thymopentin (TP-5, RKDVY), with six arginine residues (RR-6, RRRRRR) at the N- and C-termini to obtain the cationic peptides, RR-11 (RKDVYRRRRRR-NH_2) and RY-11 (RRRRRRRKDVY-NH_2), respectively. The arginine residues conferred anti-mycobacterial activity to TP-5 in the peptides as shown by effective minimum inhibitory concentrations of 125 mg/L and killing efficiencies of >99.99% against both rifampicin-susceptible and -resistant *Mycobacterium smegmatis*. The immunomodulatory action of the peptides remained unaffected as shown by their ability to stimulate TNF-α production in RAW 264.7 mouse macrophage cells. A distinct change in surface morphology after peptide treatment was observed in scanning electron micrographs, while confocal microscopy and dye leakage studies suggested bacterial membrane disruption by the modified peptides. The modified peptides were non-toxic and did not cause hemolysis of rat red blood cells up to a concentration of 2000 mg/L. Moreover, RY-11 showed synergism with rifampicin and reduced the effective concentration of rifampicin, while preventing the induction of rifampicin resistance. The synthetic peptides may have a potential application in both immunocompetent and immunocompromised TB patients.

1. Introduction

Tuberculosis (TB) is caused by the bacillus *Mycobacterium tuberculosis* and ranks as the second leading cause of death from a single infectious agent, after the human immunodeficiency virus (HIV) [1]. Although TB incidence and mortality rates have fallen globally, the rapid emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB continues to threaten decades of progress in global TB control. MDR-TB, with an estimated 450,000 incident cases in 2012, is defined as resistance to isoniazid and rifampicin, the two most powerful first-line anti-TB drugs. XDR-TB strains, which accounts for 9% of MDR-TB cases, are further generated when MDR-TB strains develop additional resistance to a fluoroquinolone and a second line injectable agent [2]. In addition, efforts to tackle MDR-TB are complicated by TB/HIV coinfections, which dramatically reduce host immunity and increase individuals' susceptibility to TB infection and reinfection, including with drug-resistant strains [3]. Malabsorption of anti-TB drugs, particularly rifampicin, in TB/HIV patients may also predispose them to acquisition of drug resistance [4]. Progress towards early diagnosis and the development of shorter, less toxic and more efficacious treatment regimens in MDR-TB is clearly a priority in the global management of TB.

Cationic host defense peptides (HDPs) are a diverse group of molecules produced by the innate immune system in response to infectious agents. They have recently been identified as a potential new class of anti-infectives for drug development, given their broad-spectrum activity through both direct bactericidal and adjunctive immunomodulatory actions [5]. The direct anti-

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http://dx.doi.org/10.1016/j.biomaterials.2013.12.049

microbial mechanism of HDPs is largely attributed to their ability to fold into amphiphilic structures with hydrophobic and cationic domains, facilitating physical interactions with the negatively charged bacterial cell membrane and in some cases, bacterial cell penetration to act on intracellular targets, leading to the disruption of membrane integrity and cellular processes, respectively [6]. Their aggregate actions on several components essential to bacterial cell survival thus make development of drug resistance much less likely [7,8]. Rational design of directly anti-microbial peptides based on structure-function relationships has thus been widely employed to produce candidates with greater bactericidal efficiency, although often at the expense of safety, which may explain why most peptides are used topically in clinical trials. The adjunctive immunomodulatory actions of HDPs, on the other hand, have been demonstrated in animal models to be important for pathogen clearance via the regulation of chemotactic activities of dendritic and T-cells, and induction of pro-inflammatory cytokines, leading to enhanced leukocyte recruitment to the site of infection [9]. Given their pleiotropic targets and effects and the lack of clear structural requirements for immunomodulation, rational design of immunomodulatory HDPs is relatively more challenging and lags behind the development of microbial HDPs. Nonetheless, immunomodulation is recognized as a highly effective strategy to combat MDR infections especially in immunocompromised patients, as the target of action is the immune system rather than the pathogen itself.

In the present study, we explored synthetic modifications of a clinically used immunomodulator, thymopentin (TP-5), to confer cationicity to mimic the dual immunomodulatory and antimicrobial effects of HDP. TP-5 is a synthetic pentapeptide consisting of five amino acids, Arg-Lys-Asp-Val-Tyr, that correspond to the 32–36 amino acid sequence of the thymus hormone thymopoietin Refs. [10,11]. TP-5 reproduces the immunomodulatory activity of thymopoietin, which is responsible for thymocyte differentiation and maturation [10,11]. Besides inducing the phenotypic differentiation of T precursor cells *in vitro*, TP-5 can regulate the expression of CD4 and CD8 cell surface markers on human thymocytes [11,12]. With its ability to boost T-cell mediated immune response, TP-5 has been used in the clinical treatment of primary immunodeficiencies such as Acquired Immunodeficiency Syndrome (AIDS) [13–15], severe acute respiratory syndrome (SARS) [16], rheumatoid arthritis and atopic dermatitis [17–21]. By conjugating TP-5 with six arginine amino acid residues at either the N- or C-termini, we obtained cationic peptide candidates that were evaluated for antimycobacterial activity by minimum inhibitory concentration (MIC), killing efficiency and time-kill measurements in drug-susceptible and drug–resistant *Mycobacterium smegmatis*. Synergistic interactions were also determined by co-treatment of *M. smegmatis* with rifampicin and peptides via the chequerboard assay. A preliminary evaluation of the anti-microbial mechanism of the peptides was carried out using confocal and scanning electron microscopy and via their ability to induce leakage of an encapsulated fluorophore from large unilamellar vesicles composed of negatively charged phospholipids. Lastly, to confirm the retention of immunomodulatory activity in the modified peptides, we performed ELISA assays for the induction of tumor necrosis factor (TNF)-α in RAW 264.7 mouse macrophage cells.

2. Materials and methods

2.1. Materials

The peptide candidates TP-5 (RKDVY), RR-6 (RRRRRR), RR-11 (RKDVYRRRRRR-NH_2) and RY-11 (RRRRRRRKDVY-NH2) were custom synthesized by GL Biochem (Shanghai, China), and their molecular weights were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS, Model 4800, Applied Biosystems, USA), using α-cyano-4-hydroxycinnamic acid (CHCA) as matrix. *M. smegmatis* (ATCC No. 14468) was purchased from ATCC (USA). Nutrient broth (Acumedia No. 7146) and bacteriological agar (Acumedia No. 7176) were purchased from Neogen Corporation (Michigan, USA). Ethanol (analytical grade, 99%) and glutaraldehyde (synthetic grade, 50% in H_2O), FITC-labeled dextran (100 kDa), DMSO, and calcein were purchased from Sigma–Aldrich (Singapore). Dulbecco's modified Eagle medium (DMEM) and lipopolysaccharide (LPS) from the *Escherichia coli* 0111:B4 strain were purchased from Sigma–Aldrich (St Louis, MO, USA). Phosphate-buffered saline (PBS, pH 7.4) at 10× concentration was purchased from 1st Base (Singapore) and used after dilution to the desired concentration. The phospholipids 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (PG) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were obtained as dry powder from Avanti Polar Lipids, Inc (Alabaster, AL, USA). Rat red blood cells (rRBCs) used in this study were obtained from the Animal Handling Units of the Biomedical Research Centers (AHU, BRC, Singapore).

2.2. Peptide characterization

Synthesis of the peptides was carried out by Fmoc-solid phase protocol at GL Biochem (Shanghai, China). MALDI-TOF MS was carried out to further confirm the characteristics of the peptides. Molecular weights of the peptides were measured by spotting an equal volume of peptide solution (0.5 mg/mL in deionized water) and CHCA solution (saturated in acetonitrile/water mixture at 1:1 volume ratio) onto the MALDI ground-steel target plate. As shown in Table S1, there was close agreement between the measured and theoretical molecular weights of the peptides. Reverse phase (RP)-HPLC used by the manufacturer confirmed the purity of the peptides to be more than 98%.

2.3. Minimum inhibitory concentration (MIC) measurements

The MICs of the peptides were determined by the broth microdilution method as described previously [22–24]. Briefly, a 2-fold serial dilution of the peptides (1.95, 3.90, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, 1000 and 2000 mg/L) was prepared and added to an equal volume of bacterial solution (100 μL) containing approximately 10^5 CFU/mL in each well of a 96-well plate. The plates were incubated at 37 °C at a shaking speed of 200 rpm and read after 72 h. The MIC was defined as the lowest peptide concentration at which no microbial growth was observed visually or spectrophotometrically via readings of optical density (OD) at 600 nm using a microplate reader (TECAN, Switzerland). Growth media containing only microbial cells was used as the negative control. Each MIC test was carried out in 5 replicates and repeated 3 times.

2.4. Time kill and killing efficiency studies

The *in vitro* killing kinetics of the peptides was performed according to a previously reported method with slight modifications [25]. Flasks containing 10 mL of nutrient broth with RR-11 peptides at concentrations corresponding to ½×, 1× and 2× MIC were inoculated with *M. smegmatis* at a density of approximately 10^5 CFU/mL and incubated at 37 °C for 72 h with shaking at 200 rpm. To determine synergistic interactions of the peptides with rifampicin, similar studies were performed with RY-11 and rifampicin alone and in combination at ½× MIC. Aliquots were removed at time 0, 8, 24, 48 and 72 h post-inoculation and serially diluted in nutrient broth for the determination of viable counts. Diluted samples (100 μL) were plated in triplicates onto agar plates and total bacterial counts determined after incubation at 37 °C for 72 h. Killing efficiency studies were performed similarly with samples taken after 72 h. The results were expressed as mean log (CFU/mL) ± standard deviation.

2.5. Dye leakage assays

Dye-filled large unilamellar vesicles (LUV) were prepared using the extrusion method as previously established [26,27]. Calcein dye was dissolved in a buffer containing 10 mM Na_2HPO_4 in H_2O at pH 7.0 to achieve a concentration of 40 mM. To prepare PE/PG 4:1 LUV, 476 μL PE and 127 μL PG (dissolved in 25 mg/mL $CHCl_3$) were combined to make up 2 mL of $CHCl_3$ in a clean round bottom flask. The $CHCl_3$ solvent was removed by rotary evaporator to obtain a thin lipid film, which was then hydrated by 1 mL of calcein solution. The mixture was left stirring on a rotary evaporator at atmospheric pressure for 1 h, after which it was subjected to ten freeze–thaw cycles (using dry ice/acetone to freeze and warm water to thaw). The suspension was then extruded twenty times through a polycarbonate membrane with 400 nm pore diameter. Sephadex G-50 column was used to remove the free dye using a buffer composed of 10 mM Na_2HPO_4 and 90 mM NaCl as eluent. Dye-filled LUV were diluted 2000 times with the eluent buffer to achieve a final lipid concentration of approximately 5.0 mM before use. To evaluate the ability of the peptides to rupture the dye-filled LUV, 90 μL of the diluted vesicles was mixed with 10 μL of peptide solution at various concentrations (50, 100 and 200 mg/L) in a 96-well plate and shaken for 1–2 h. The calcein fluorescence emission intensity I_t (Ex. = 490 nm, Em. = 515 nm) was measured by the microplate reader (TECAN, Switzerland). Calcein fluorescence without peptide treatment (I_0) was used as the baseline and fluorescence emission after addition of 10 μL Triton-X (20% in DMSO) (I_x) was taken as 100% leakage. The percentage of leakage was calculated by leakage (%) = $100[(I_t-I_0)/(I_x-I_0)]$. No leakage was shown with the treatment of pure DMSO.

Table 1
Minimum inhibitory concentration (MIC) of synthetic peptides, TP-5, RR-6, RR-11 and RY-11, against *M. smegmatis* and their 50% hemolysis concentration (HC_{50}). Modified peptides, RR-11 and RY-11, maintained the anti-mycobacterial ability of RR-6 against RIF-susceptible *M. smegmatis* and RIF-resistant *M. smegmatis* and no hemolysis was observed at concentration of 2000 mg/L for all peptides.

Peptides	Peptides sequence	MIC (mg/L)		HC_{50} (mg/L)
		RIF-susceptible *M. smegmatis*	RIF-resistant *M. smegmatis*	
TP-5	RKDVY-NH2	>1000	ND	>2000
RR-6	RRRRRR-NH2	125	125	>2000
RR-11	RKDVYRRRRRR-NH2	125	125	>2000
RY-11	RRRRRRRKDVY-NH2	125	125	>2000

2.6. Confocal microscopy

To 400 μL of *M. smegmatis* suspension seeded into an 8 well-cover slip chamber, an equal volume of PBS solution containing RR-11 (500 mg/L) and FITC-dextran (100 kDa, 250 mg/L) were added. The mixture was incubated for a predetermined period of time (0, 10 min, 30 min and 60 min) at 37 °C and with constant shaking at 200 rpm. After washing three times with PBS to remove the free FITC-dextran, images were acquired using the Zeiss LSM-510 META confocal microscope equipped with a 63 × 1.4 (oil) Plan apochromate lens.

2.7. Scanning electron microscopy (SEM)

Bacterial cells were prepared as in MIC measurements but treated with the peptide RR-11 (250 mg/L) for a shorter incubation time (2 h). After treatment, the bacteria were centrifuged at 4000 rpm for 5 min, and washed 3 times with PBS. Sample fixation was performed with 2.5% glutaraldehyde for 30 min, followed by washing with PBS and finally deionized water. Sample dehydration was carried out using a series of graded ethanol solutions (35%, 50%, 75%, 90% and 100%), followed by drying for two days. The dried samples were mounted on carbon tape, sputtered with platinum coating and analyzed under a field emission scanning electron microscope (JEOL JSM-7400F, Japan).

2.8. Hemolytic activity test

To assess the safety of the peptides against mammalian cells, their hemolytic activity was tested using freshly drawn rat red blood cells (rRBCs) obtained from Animal Holding Unit (AHU), Biomedical Research Center (BRC), Singapore. The rRBCs were diluted 25 times with PBS to give 4% blood content as reported previously [22–24]. Serial dilution of the peptides was prepared using PBS with concentrations ranging from 0 to 2000 mg/L. Equal volumes (250 μL) of the peptide solution and blood suspension were mixed. The mixtures were incubated at 37 °C for 2 h to facilitate the interactions between the rRBCs and peptides. Subsequently, the mixtures were centrifuged at 4000 g for 5 min and 100 μL of the supernatant was transferred into a 96-well microplate. Hemoglobin release was determined spectrophotometrically by measuring the absorbance of the samples at 576 nm via the microplate reader (TECAN, Switzerland). Untreated rRBCs served as a negative control while rRBCs treated with 1% Triton-X served as a positive control. Each test was carried out in 4 replicates and reproduced twice. The degree of hemolysis was calculated using the following formula: Hemolysis (%) = [(O.D.$_{576\ nm}$ of the treated sample-O.D.$_{576\ nm}$ of the negative control)/(O.D.$_{576\ nm}$ of positive control − O.D.$_{576\ nm}$ of negative control)] × 100%.

2.9. Chequerboard assay

Chequerboard assays were performed to determine the effect of combining the peptide RY-11 and rifampicin treatments against *M. smegmatis* [28]. A set of mixtures of 100 μL each were prepared by varying RY-11 and rifampicin concentrations according to Table S2 and added to 100 μL of *M. smegmatis* suspension (containing approximately 10^5 CFU/mL) in each well of a 96-well plate. The plates were then incubated at 37 °C and read after 72 h. Assessment of microbial growth was done visually or spectrophotometrically via OD readings at 600 nm (TECAN, Switzerland). The fractional inhibitory concentration index (FICI) was calculated for each combination using this equation: FICI = FIC_A + FIC_B, where FIC_A = MIC of drug A in combination/MIC of drug A alone, and FIC_B = MIC of drug B in combination/MIC of drug B alone. FICI of ≤0.5 was interpreted as synergy, 0.5 < FICI ≤ 1.0 as additive, 1.0 < FICI ≤ 4.0 as indifferent, and FICI > 4.0 as antagonism [29,30].

2.10. Simulation of drug resistance

Drug resistance was induced in *M. smegmatis* by repeated treatments with RR-11 or RY-11 for 10 passages and established via MIC measurement as abovementioned. For each treatment, bacterial cells exposed to a sub-MIC concentration ($^1/_8$ of MIC at that particular passage) were re-grown to the log phase and reused for the following passage's MIC measurement. Changes in the MIC were depicted by normalizing the MIC at passage *n* to that of the first passage.

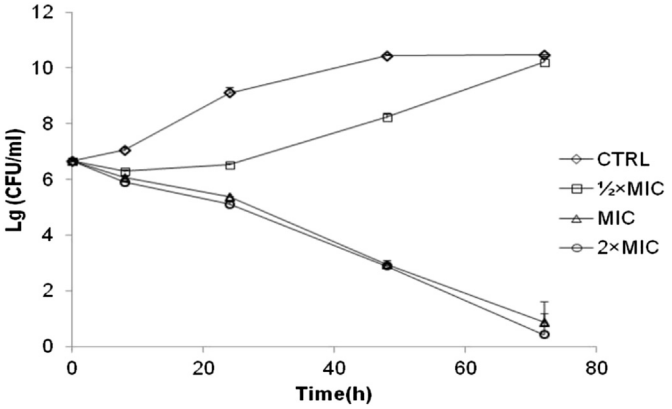

Fig. 1. Colony formation units (CFUs) of *M. smegmatis* after incubation with RR-11 for various periods of time. Experiments were performed in triplicate and the bactericidal activities are expressed as mean lg (CFU/mL) ± standard deviations shown by the error bars. The bactericidal effect RR-11 at MIC and 2 × MIC was validated by the 3 $\log_{10}$ of CFU/ml comparing with initial inoculums.

2.11. TNF-α stimulation assay

The ability of the peptides RR-11 and RY-11 as well as TP-5 to modulate immune response was evaluated by measuring the production of the proinflammatory cytokine tumor necrosis factor (TNF-α) in RAW 264.7 mouse macrophage cells. The cells were kindly provided by Dr Ho Han Kiat (Department of Pharmacy, National University of Singapore, Singapore) and cultured in DMEM supplemented with 10–15% FBS, 10 U/mL penicillin G and 100 μg/mL streptomycin in a humidified atmosphere at 37 °C containing 5% CO_2. The cells were seeded at density of 4 × 10^5 cells/mL in 24-well plates for 24 h, after which they were treated with TP-5, RR-11 and RY-11 at a final concentration of 5 μM in serum-free medium for 1 h. The media was then collected and centrifuged at 5000 g for 5 min to spin down any cells. This cell-free supernatant was used to conduct TNF-α ELISA assay as per manufacturer's instructions. Lipopolysaccharide (LPS), a bacterial cell wall component, which is known to stimulate TNF-α production in RAW 264.7 cells was used at a concentration of 500 ng/mL as the positive control. Absorbance readings were taken at 450 nm with wavelength correction at 570 nm using the Infinite® 200 Pro spectrophotometer (TECAN, Switzerland). The amount of TNF-α released upon peptide treatment was quantified using the regression equation from the generated standard curve. All the experiments were performed in triplicates and reported as mean ± S.E.M.

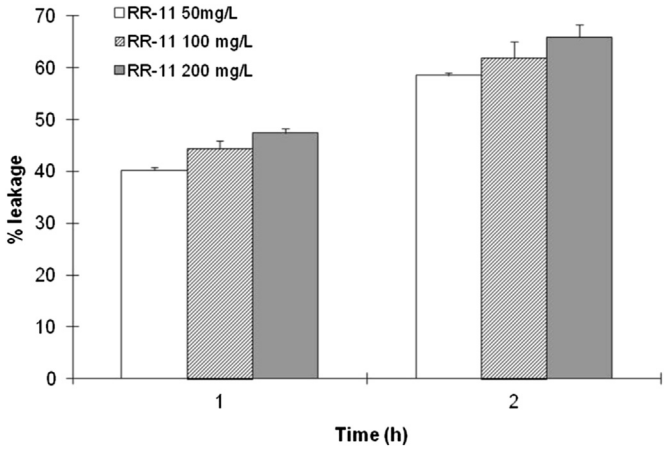

Fig. 2. Comparison of dye leakage between 1 h and 2 h treatment of RR-11 on PE/PG (membrane mimic of bacteria) vesicles. Dye leakage was caused by membrane damage. An increased leakage indicated more efficient membrane disruption caused by antimicrobial polymer treatment. The concentration- and time-dependent increase in dye leakage indicates the ability of the peptide to disrupt the microbial cell membrane.

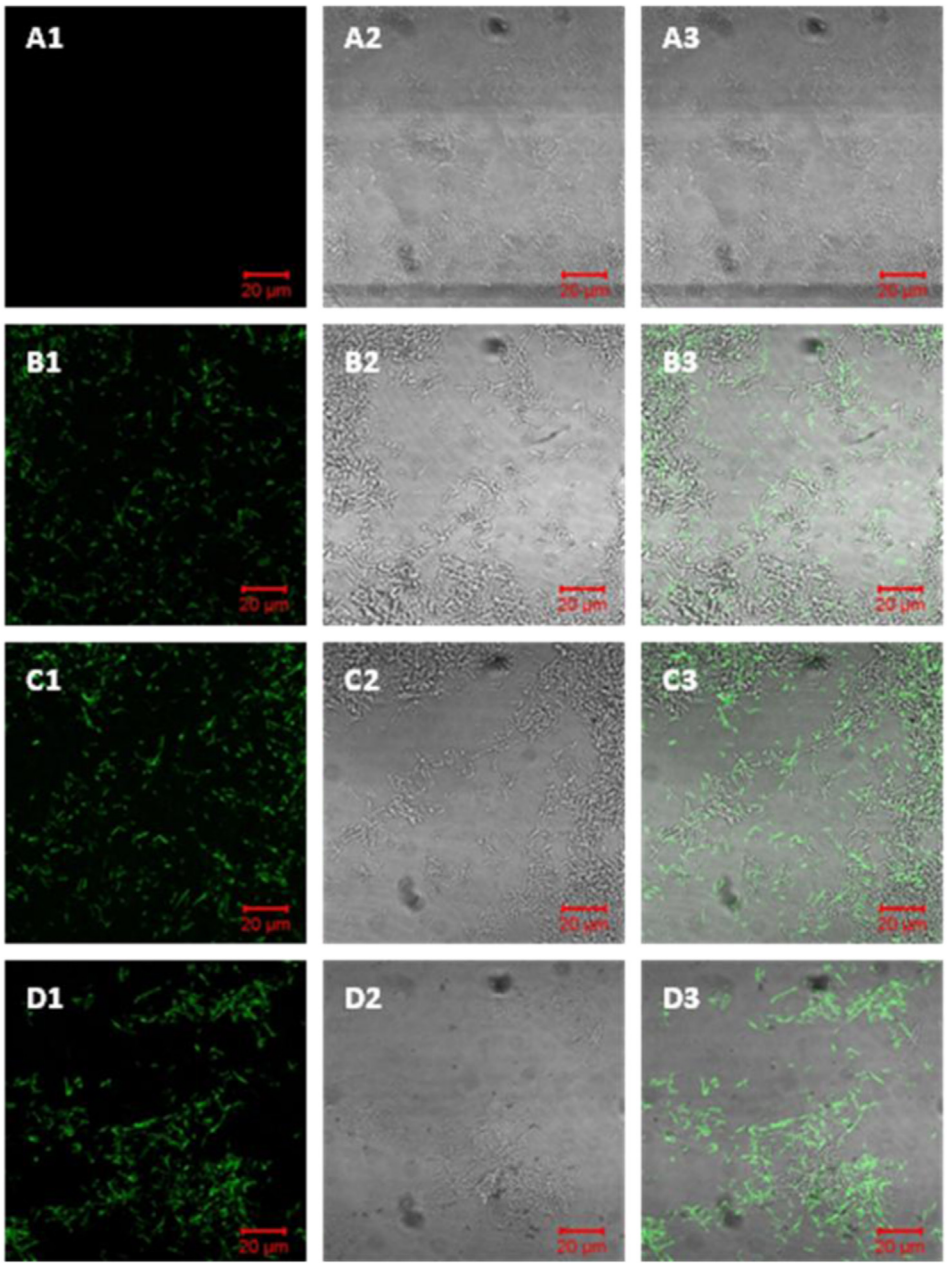

Fig. 3. Confocal microscopic images of *M. smegmatis* incubated with FITC-conjugated dextran (250 mg/L) in the presence of PBS (A) or RR-11 (500 mg/L) for 10 min (B), 30 min (C) and 1 h (D). Notes: A1-D1: Green represents fluorescence of FITC-conjugated dextran. A2-D2: Bright field. A3-D3: Overlapping images. Scale bar: 20 μm. The entry of FITC-conjugated dextran indicates the microbial cell membrane damage after treatment with the peptide. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.12. Statistical analysis

Data were analyzed using one-way ANOVA followed by Dunnett's post-hoc analysis (SPSS, Chicago, IL). The difference between values for the treatments was considered to be statistically significant at $p < 0.001$.

3. Results and discussion

3.1. In vitro anti-mycobacterial activity

It has been reported that anti-microbial activity of peptides could be enhanced by replacing the acidic residues by basic ones to increase the net positive charge [31,32] Hence, in order to amplify the anti-microbial ability, it is reasonable to increase the cationicity by including positive charged amino acids in the peptide sequence. Furthermore, peptide consisting of six or more arginine entered cells far more effectively than peptides of equal length containing lysine, ornithine or histidine. However, peptides of fewer than six amino acids were ineffective [33,34]. Therefore, we engineered a short peptide composed of six arginine residues alone (RR-6) and this short peptide was capable of inducing pronounced growth inhibition of both rifampicin (RIF)-susceptible and RIF-resistant *M. smegmatis* with an MIC value of 125 mg/L (Table 1, Fig. S1). RIF-resistant *M. smegmatis* was developed by multiple treatments of drug-susceptible *M. smegmatis* with rifampicin at a sub-MIC concentration (($^1/_8$ of MIC) and the MIC concentration of rifampicin increased from 7.81 mg/L to 500 mg/L (Fig. S2). As expected, TP-5 itself could not inhibit bacterial growthup to the highest concentration used (1000 mg/L). However, upon addition of six

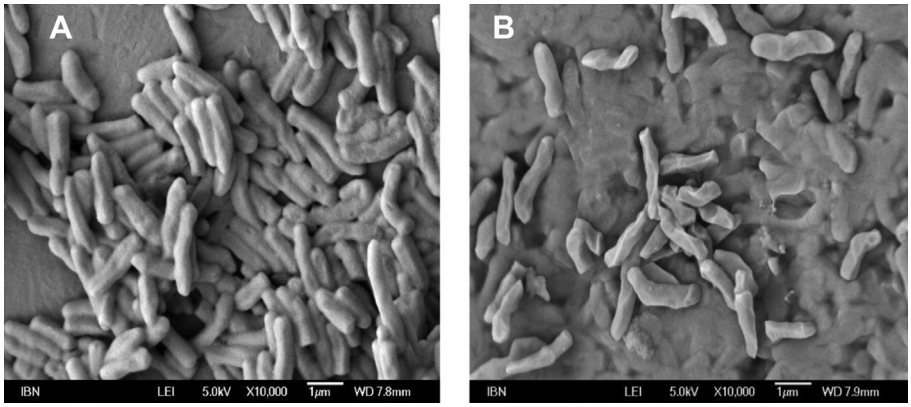

Fig. 4. FE-SEM images of *M. smegmatis* showing the difference on the morphology between untreated (A) and treated (B) bacteria. A flaccid and shrunken structure with distorted surfaces was seen upon treatment with RR-11 (250 mg/L) for 2 h, in comparison to the untreated control.

arginine residues at either C- or N-terminus of TP-5, the modified peptides, RR-11 and RY-11, became active against *M. smegmatis* at MIC similar to RR-6. In addition, killing efficiencies greater than 99.99% were achieved at respective MICs, suggesting a bactericidal effect of the peptides against RIF-susceptible and RIF-resistant *M. smegmatis* (Fig. S3). This effect was further validated using *in vitro* time-kill assays to analyze the fractional cell survival upon RR-11 peptide treatment at none, ½× MIC, MIC and 2× MIC over 72 h Fig. 1 shows that RR-11 was bactericidal (defined as $\geq$3 $\log_{10}$ decrease in the initial inoculum [35]) at 48 h at both MIC and 2× MIC. Our results support the successful conferment of anti-microbial activity on the immunomodulator TP-5 via increasing the cationic charge density of the peptide.

3.2. Anti-mycobacterial mechanism of action

LUVs were employed to contain the fluorescent calcein dye and to mimic the loss of cellular content upon membrane rupture in bacteria. A concentration- and time-dependent increase in dye leakage was observed with RR-11 peptide treatment, indicating that the peptide was highly efficient in disrupting the integrity of the simulated bacterial membrane (Fig. 2). To verify the potential of RR-11 in damaging bacterial cell membrane, *M. smegmatis* was exposed to RR-11 (500 mg/L) for 10 min, 30 min and 60 min in the presence of fluorescent FITC-labeled 100 kDa dextran molecules (250 mg/L) and observed under the confocal microscope for evidence of dextran uptake (Fig. 3). In contrast to the control group incubated with PBS alone, FITC-dextran gained entry into *M. smegmatis* readily after 10 min incubation with RR-11 to give a fluorescent signal, which becomes more intense with a longer incubation. The entry of the FITC dye indicated that the microbial cell membrane was damaged after the treatment with RR-11. These observations were supported by SEM visualization of cell lysis, which demonstrated the change of surface morphology in *M. smegmatis* from a rod-shaped structure with smooth surface to a flaccid and shrunken structure with distorted surfaces after 2 h incubation with RR-11 (250 mg/L) (Fig. 4).

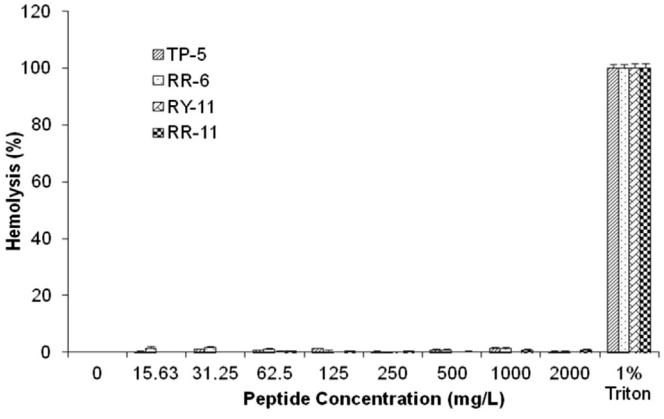

Fig. 5. Hemolytic activity of the anti-mycobacterial peptides. All peptides displayed minimal hemolytic effect up to the concentration of 2000 mg/L.

3.3. Hemolytic activity

With its influence on the structural integrity of the bacterial membrane, we were interested to find out the relative specificity of the peptide on mammalian cells. This is evaluated by hemolysis tests, which measure the ability of the peptides to lyse rat red blood cells *in vitro*. Fig. 5 shows that the peptides displayed minimal hemolytic effects even up to a concentration as high as 2000 mg/L, a concentration which is 16 times that of the MIC of peptides (125 mg/L) (Table 1). This confirms that the peptides have high selectivity towards bacterial cells, which is desirable to expand the therapeutic window for clinical application.

3.4. Synergism with first-line anti-TB drug rifampicin

Cationic peptides are considered as promising antibiotic candidates based on the fact that these peptides are less likely to develop drug resistance and have broad spectrum of activity. However, in spite of the advantages, the clinical usage is limited due to drawbacks such as poor potency, specificity and *in vivo* stability [36]. To overcome the shortcomings and translate laboratory discovery to the clinic, synergistic effect between peptides and traditional antibiotics can be applied as synergy can reduce the dose of each drug in combination, prevent drug resistance and result in greater antibacterial effect than the sum of the effects due to single agent [37,38]. Rifampicin has been reported to act synergistically when administered in conjunction with anti-microbial peptides, possibly as a consequence of the peptide-mediated membrane disruption, which in turn enhances uptake of the drug [36,39,40]. To determine the interactions between RY-11 and rifampicin, synergy studies were performed using the chequerboard assays. Fig. 6 shows that the minimum effective concentrations for each agent to be used in combination to achieve synergy in inhibiting the growth of *M. smegmatis* was ¼× MIC of RY-11 and ¼× MIC of rifampicin. The calculated FICI value was 0.5. Time-kill studies were performed to

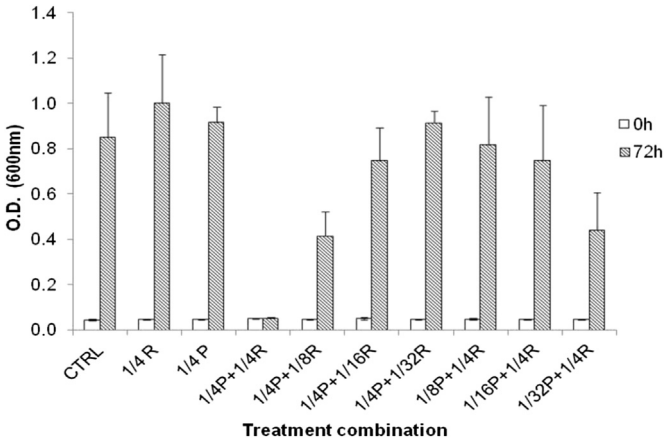

Fig. 6. MICs for various combinations of RIF (R) and RY-11 (P) against *M. smegmatis*. For example, "1/4P + 1/8R" indicates that the bacteria were treated with the combination of peptide and RIF at concentration of ¼× MIC and $^{1}/_{8}$ × MIC, respectively. Combination of ¼× MIC of RY-11 and ¼× MIC of rifampicin achieves synergy in inhibiting the growth of *M. smegmatis*.

validate the synergistic effect. Fig. 7 shows that rifampicin and RY-11 used alone as a single agent at ¼× MIC could not inhibit the growth of *M. smegmatis*, while their combination successfully inhibited the bacterial growth. Synergy was defined as $\geq$2 $\log_{10}$ decrease in CFU/ml by the drug combination when compared to its most active constituent [35]. Synergistic activity was observed for combination group at 48 h and 72 h, confirmed by the reduction in viable colony counts of >2 $\log_{10}$ as compared to the single most active constituent.

3.5. Effect in drug-resistant M. smegmatis

The induction of drug resistance was used to evaluate the possibility of peptides to induce drug resistance in mycobacteria after multiple treatments with low sub-MIC concentrations. Fig. 8 shows that after repeated treatments of rifampicin at $^{1}/_{8}$ × MIC concentration, *M. smegmatis* became resistant to rifampicin as early as passage 2 and the MIC against *M. smegmatis* rose dramatically by approximately 30 fold by passage 10. In contrast, RR-11 and RY-11 remained equally active from passage 1 to 10, as demonstrated by

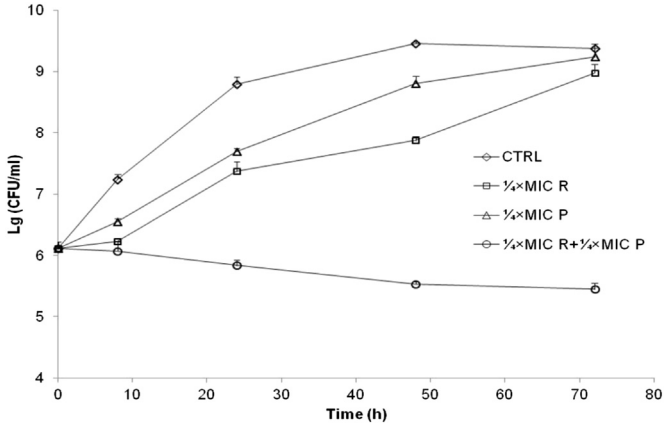

Fig. 7. Colony formation units (CFUs) of *M. smegmatis* after incubation with ¼× MIC of RIF (R), ¼× MIC of RY-11 (P) and combination of ¼× MIC of RIF (R) with ¼× MIC of RY-11 (P) for various periods of time. Experiments were performed in triplicate and the bactericidal activities are expressed as mean lg (CFU/mL) ± standard deviations shown by the error bars. Synergistic activity was observed for combination group at 48 h and 72 h, indicated by the reduction in viable colony counts of >2 $\log_{10}$ as compared to the single most active constituent.

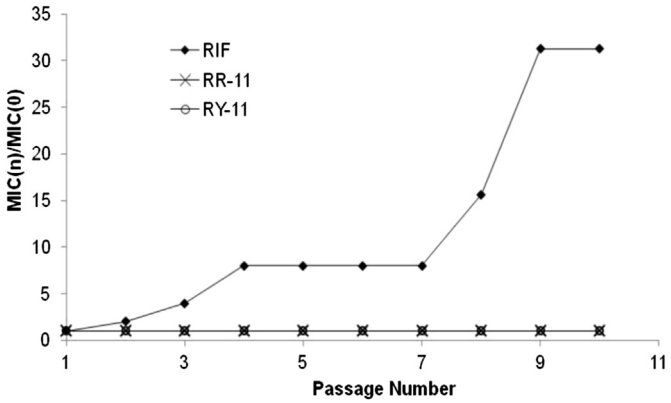

Fig. 8. Changes in MICs of anti-mycobacterial substances upon multiple sub-MIC dose exposures, signifying rifampicin (RIF) resistant development as a result of evolutionary survival *M. smegmatis*. Multiple treatments with low sub-MIC, RR-11 and RY-11 did not induce drug resistance by passage 10.

the maintenance of MIC value throughout. This result supports the hypothesis that the different mechanisms of anti-mycobacterial action may be exploited in combination therapy to evade drug resistance to a single agent.

3.6. TNF-α stimulation assay

TP-5 has been shown to induce the cytokine TNF-α production in unstimulated macrophages as part of its immunomodulatory functions [41]. Hence, to address the retention of biological activity of TP-5 in the modified peptides RR-11 and RY-11, ELISA assays were performed to measure and compare the level of TNF-α released after peptide treatment in RAW 264.7 macrophage-like cells. The amount of TNF-α released did not differ between the peptides although the levels were comparable to that released in response to LPS stimulation of the macrophages ($p > 0.05$) (Fig. 9). These levels were significantly higher than when the peptides were absent in the cell media ($p < 0.001$). Our results show that despite the structural modifications, the biological activity of TP-5 remained unperturbed in RR-11 and RY-11. Notably, it has been reported that TNF-α is essential in controlling TB infection [42]. In inflammatory bowel disease (IBD) patients who were also latently infected with *M. tuberculosis*, the approach to counteract the increased serum levels of TNF-α with TNF-α-neutralizing agents such as infliximab has led to TB reactivation [43,44]. The ability to induce TNF-α

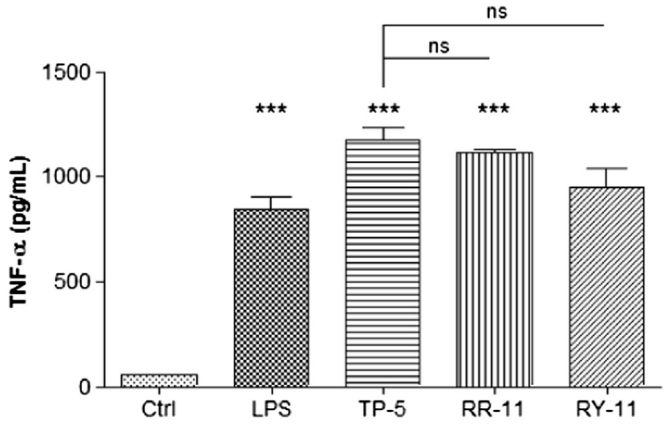

Fig. 9. TNF-α production of RAW 264.7 macrophage cells after treated with peptides at 5 μM "***" represents $p < 0.001$; "ns" represents not significant. The ability to induce TNF-α production of TP-5 was maintained in our modified peptides.

production specifically in our modified peptides may thus complement their anti-mycobacterial actions for a more effective therapeutic management of TB.

4. Conclusion

The present study demonstrates that cationic charges bestowed by repeated arginine residues have conferred anti-mycobacterial activity against both drug-susceptible and drug-resistant strains on the clinically used immunomodulator TP-5. Mechanistic investigations using dye leakage assays and microscopy reveal that the modified peptides function via membranolytic actions, which may account for their synergistic actions with the first-line anti-TB drug rifampicin. Despite the structural modifications, hemolysis hence toxicity is kept low and the biological activity of TP-5 is well retained as indicated by its ability to stimulate TNF-α production in macrophages. These results demonstrate that synthetic peptides, which possess dual anti-mycobacterial and immunodulatory effects may have the potential to evade drug resistance in both immunocompetent and immunocompromised TB patients.

Acknowledgments

The authors would like to acknowledge research funding and facilities provided by the National University of Singapore (NUS) and the Institute of Bioengineering and Nanotechnology (Biomedical Research Council, Agency for Science, Technology and Research, Singapore). This research is supported by the Singapore Ministry of Health's National Medical Research Council under its Individual Research Grant Scheme (NMRC/1298/2011) awarded to P.R. Ee and Y.Y. Yang and NUS research scholarships to Y. Wang, X. Ke, J.S. Khara and P. Bahety.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.12.049.

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