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.