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Reclaiming hijacked phagosomes: Hybrid nano-in-micro encapsulated MIAP peptide ensures host directed therapy by specifically augmenting phagosome-maturation and apoptosis in TB infected macrophage cells

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ABSTRACT

TB-Superbugs have emerged as one of the most challenging global health threat due to the decrease in effectiveness of conventional antibiotics. Meanwhile, Host defense peptides (HDP) have evolved as an alternative to classical therapeutics with lesser susceptibility of resistance. We describe the potential of nano-encapsulated synthetic Magainin-I analog peptide (MIAP) as Host Directed Therapy against TB. Micron-sized inhalable platform "Porous Nanoparticle Aggregates Particles (PNAP)" with nano-scale physiognomies were developed to improve the delivery of MIAP-peptide to the lungs and enhance its stability. This particle engineering enabled more control over aerodynamic characteristics and bioactive release. Antimicrobial and mechanistic studies were carried out against virulent H37Rv TB bacteria. These MIAP-PNAP nano-assemblies demonstrated dose and time dependent antibacterial action against virulent *M.tb* for at least 96 h, with up to ~3.03-log CFU reduction in numbers of viable bacteria compared to untreated group. These MIAP-PNAP at concentration of 50 μM and above showed significant antibacterial effects on *M.tb* after 48–96 h of incubation. Mechanistically, MIAP nano-formulation enhanced host defense mechanism by averting bacteria-induced inhibition of phagosomal-lysosome fusion (Lysostracker) and apoptosis (Annexin-FITC) as shown by confocal microscopy and flow-cytometry. Encapsulated MIAP may serve for adjunctive host-directed TB therapy which may also synergizes the efficacy of standard anti-TB drugs.

1. Introduction

Despite of recent advances in treatment modalities, tuberculosis (TB) remains a major source of mortality throughout the world and the emergence of "TB-Superbugs" worsen the scenario. The rise of multi drug-resistant (MDR), extensively drug-resistant (XDR) and completely resistant strains, is creating a concern regarding, how to effectively treat the TB infections caused by these recalcitrant strains (Cohn et al., 1997; Eldholm et al., 2015; Espinal et al., 2001). These hard-to-treat resistant strains progressively gaining foothold in the society. Conventional antimicrobials do not work on resistant TB-strains and there is slow progress in development of new anti-TB agents. More innovations and pragmatic approaches with novel drugs or combinations in TB therapeutics need to be pursued to combat this fatal threat.

Host-directed therapies have emerged as a potential alternative to combat TB by reducing treatment time and preventing development of resistance (Sahl, 2006; Wallis and Hafner, 2015; Zumla et al., 2015). Endogenous host defense peptides (HDP) are well documented elements of the innate immunity and have been suggested to have an important role in TB infections (Hancock and Sahl, 2006). There are several reports of the immuno-modulatory effects of these peptides in TB and other models (Hancock et al., 2016). Such peptides possibly inhibit microbial growth either directly through membrane/nucleic acid disruption or indirectly through immune-modulation to efficiently clear the infection. Magainins are 21–27 amino acid length non-hemolytic HDP found on the skin of frog (*Xenopus laevis*), possess broad spectrum antimicrobial properties and cytolytic activity at micro-molar concentrations (Bessalle et al., 1990; Humblot et al., 2009; Zasloff, 1987). Magainin-I analog peptide (MIAP) (sequence: GIGKFLKSKGKFGKA), an analogue of the Magainin HDP, has significant activity against TB

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strains with poorly-understood mechanism of action (Santos et al., 2012). Due to moderately simple peptide-sequence of this HDP, it is convenient to synthesize using solid-phase synthesis method.

However, due to lack of specificity toward target cells, short half-life, salt sensitivity, presence of proteases, poor pharmacokinetics and first pass metabolism, it is challenging to introduce MIAP in biological system in a controlled manner by using conventional delivery systems. To kill the bacilli residing in alveolar macrophages, it is necessary to deliver an adequate amount of MIAP into the lungs and alveolar macrophages where bug actually resides. The applicability of HDPs as antimicrobial therapeutic agents against TB is impeded due to lack of appropriate delivery system which can carry and deliver required payload of HDPs to the targeted site. Specifically targeting the lungs, and thus restricting non-selective delivery, provides an alternative way to overcome their limitations of arbitrary bioavailability and consequential toxic effects.

Porous Nano-Particles Aggregates (PNAP) (Micron-sized peptide-containing nano-assemblies) were generated and evaluated against virulant TB bacteria. Micron size (1–5 μm) is supposed to imparts optimum aerodynamic properties for maximal deposition, whereas nanoparticles (~300 nm) efficiently entrap peptides and facilitates dispersion in lung lumen (Labiris and Dolovich, 2003). The PNAP physiognomies are suitable for efficient accumulation into lungs, macrophage targeting and extended drug release. Hence, it was hypothesized, lung delivery of MIAP-nanoparticles (MIAP-NP) using inhalable Porous Nanoparticle-Aggregate Particles (PNAPs) could provide benefits of better aerodynamic properties in chemotherapy of TB.

The survival and pathogenesis of *Mycobacterium tuberculosis (M.tb)* inside macrophages and monocytes depends on its ability to control the host cell machinery and evade host's defense mechanisms (Flannagan et al., 2009). TB bacteria (virulent H37Rv) successfully reside in macrophages due to their ability to "Hijack" phagolysosome biogenesis, inhibition of pro-inflammatory effector molecules and suppression of host-cell apoptosis, as their survival strategy within the host macrophage (Clemens and Horwitz, 1995; Keane et al., 2000). Inside the macrophages, mycobacterium escapes the lethal effects of lysosomal enzymes and free radicals by halting phagosomal maturation (Flannagan et al., 2009). From this perspective, Desjardins et al., proposed an interesting "Kiss and Run" model for phagosome maturation in which microbe bearing phagosomes can momentarily/partially fuse (Kiss) with endosomes to permit exchange of some selective solutes, which is followed by quick fission (Run) that does not result in complete and irreversible fusion of two membranes (Desjardins, 1995). In principle, macrophages kill microbes by mechanisms which involve participation of lysosomal contents, hence we focused on lysosomes present in macrophages. Further, several reports illustrate that the virulent strain (H37Rv) inhibits apoptosis of host macrophages to facilitates its survival (Keane et al., 2000). Recognition of the innate immunity components (effector molecules) that contribute to various defense phenomenon like phagosome-lysosome fusion (PLF) and apoptosis phenomenon will allow rational design of novel approaches to the treatment and prevention of TB.

Working on above propositions, we highlighted the effect of PNAP-encapsulated MIAP on macrophage activation during TB infection *via* phago-lysosome fusion and specific apoptosis induction, thereby making an attempt to "reclaim hijacked phagosome" and kill the pathogen, which has not been underscored to date. These results may have important implications for the adjunct host directed therapy for the treatment of TB with inhaled HDP.

2. Material and methods

2.1. Chemicals and reagents

Biodegradable 50:50 poly (D, L-lactic acid) (PLGA), Mw = 7000–17,000, acid terminated with an intrinsic viscosity of 0.24 dl/g, Polyvinyl alcohol (PVA), Isoniazid (INH), Cyclohexamide (CHx), Dulbecco's Modified Eagle medium (DMEM), Fetal bovine serum(FBS), 2, 7-Dichlorofluorescein Di-acetate (DCFDA), Fluorescein Isothiocyanate (FITC), 4′, 6-Diamidino-2-Phenylindole (DAPI), AnnexinV-Propidium iodide apoptosis detection kit, MTT reactant 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Paraformaldehyde, Poly-L-Lysine, and TritonX-100 was purchased from Sigma, St Louis, USA. LysoTracker Red DND-99 and chloroquine was obtained from Molecular probes, Invitrogen. Magainin-I analog peptide (MIAP) (sequence: GIGKFLKSKGKFGKA) peptide was synthesized using Fmoc solid phase synthesis method. Amino acid resins were procured from Novabiochem, India. Dichoromethane, Acetone, Methanol, and other chemicals and solvents used in the experiments were obtained from Sigma Aldrich, India. Purified water from a Milli-Q (Millipore corp. Massachusetts, USA) water purification system was used in studies. Virulent Mycobacterium TB (*M.tb* H37Rv Code: PN7) was procured from All India Institute of Medical Science (AIIMS), India.

2.2. Peptide synthesis and characterization

Synthetic Magainin-I analog peptide (MIAP) peptide (sequence: GIGKFLKSKGKFGKA) was synthesized on a Liberty-blue™ automated microwave peptide synthesizer (CEM Corporation, NC, USA) using standard cycles of f-MOC chemistry on rink amide resin. The side chains protecting group were removed and peptides were cleaved from the support resin with a mixture of trifluoroacetic acid (90%), thioanisole (5%), ethanediol (3%), and anisole (2%) for 3 h. Crude peptide was purified by semi-preparative gradient RP-HPLC [(Sunfire preparative column C18, 5 μm (10 × 250 mm)] on a Water HPLC system and lyophilized. The mobile phase consisted of acetonitrile (ACN) and de-ionised water containing 0.1% TFA which was used as a gradient program as follows: Flow rate of 5 ml/min was linearly increased from 10 to 45% of Solvent-A (Acetonitrile); while 90–55% Of Solvent B (water containing 0.1% TFA) was used. Purity of designed MIAP was confirmed by analytical RP-HPLC [Waters Spherisorb ODS1, C18, (250 × 4.6 mm, 5 μm)] and validated by MALDI-TOF mass spectrometer (Applied biosystems 4700 proteomics analyzer, MA USA).

2.3. Design of PNAP dry powder inhalation

2.3.1. MIAP nanoparticles

MIAP loaded PLGA-NP were prepared by w/o/w double emulsion–solvent evaporation method with minor modifications (Golub et al., 2010). Briefly, 200 mg of PLGA (50:50) polymer was dissolved in 5 ml dichloromethane, while 100 mg of MIAP (peptide sequence GIGKFLKSKGKFGKA, molecular mass 1567 g/mol) in 2 ml of PBS. Primary emulsion (w/o) was prepared by adding aqueous peptide solution to organic polymer phase with concurrent sonication for 20 s at 40 W of energy output, in ice bath. Further, primary emulsion was added dropwise to 40 ml of 0.5% (*w/v*) PVA, which was further stirred at 300 rpm for 4 h at room temperature. The nanoparticles formed were separated by centrifugation at 20,000 × *g* for 20 min at 4 °C, and washed twice in 0.22 μm filtered-water to remove surplus PVA. Supernatants were collected to evaluate encapsulation efficiency of MIAP. Blank-NP were prepared with the same procedure except the addition of peptide during the preparation of formulation. All the particles were then lyophilized for 48 h and stored at −20 °C until further use.

2.3.2. Porous nanoparticle aggregate particles (PNAP)

To obtain optimal aerodynamic properties and maximum deposition into lungs, MIAP-NP (~300 nm) were further processed into porous micron-sized inhalable nano-assemblies *i.e.* "Porous Nanoparticle Aggregate Particles" (PNAP) (~1–6 μm) using spray freeze dying (SFD) technique. Freshly prepared MIAP-NP (20 mg/ml) were suspended in mannitol (10 mg/ml) solution and sprayed into liquid nitrogen using 0.7 mm spray dryer nozzle (Techno Search instruments, Mumbai,