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Short communication

Lactoferrin-modified Betulinic Acid-loaded PLGA nanoparticles are strong anti-leishmanials

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ABSTRACT

Visceral Leishmaniasis (VL), caused by the protozoan parasite *Leishmania donovani*, is a potentially fatal disease. The only orally bioavailable drug miltefosine is toxic and the effective liposomal Amphotericin B (AmBisome) is limited by its prohibitive cost and requirement for parenteral administration. Therefore, finding a new potential drug candidate and an alternative delivery system is imperative. We report that Betulinic acid (BA), a pentacyclic triterpenoid from *Betula alba* bark, was loaded onto uniformly spherical PLGA nanoparticles (BANPs; diameter 187.5 ± 5.60 nm) coated with Lactoferrin (Lf-BANPs). The amastigotes count in macrophages was more effectively reduced by Lf-BANP than BA and BANP. Lf-BANPs reduced the pro-parasitic, anti-inflammatory cytokine IL-10, but increased nitric oxide (NO), production in *L. donovani*-infected macrophages indicating that Lf-BANP possesses a significant anti-leishmanial activity.

1. Introduction

With a prevalence of 12 million cases and an approximate annual incidence of 0.5 million cases of VL, leishmaniasis is a major neglected tropical disease, which is potentially fatal if left untreated. The treatment options are limited and unsatisfactory since the available drugs either require parenteral administration or severely toxic despite being orally bioavailable. Clearly, new approaches for treatment have been a pressing need. Nanoparticles are being increasingly pushed into therapeutics [1] due to their biodegradability, stability, ease of surface modification and facilitatory pharmacokinetics [2]. Here, we used PLGA nanoparticles surface modified with lactoferrin (Lf) – an iron-binding glycoprotein of about 700 amino acids folding into N- and C-terminal globular lobes – loaded with Betulinic acid (BA: 3β, hydroxy-lup-20(29)-en-28-oic acid), a pleiotropic pentacyclic triterpenoid from *Betula alba*, *Eucalyptus camaldulensis*, *Syncarpa glomulifera* and *Tetracentron sinense*. BA is cheap but suffers from poor biopharmaceutics due to low solubility and permeability [3]. The compound is a strong inhibitor of topoisomerase and induces caspase activation, mitochondrial membrane damage and apoptosis [4]. Compared to agents like taxol, camptothecine or vincristine, BA has low toxicity and high degree of selectivity [5]. Betulinic acid is currently enlisted under the 'rapid access to intervention development program' in National Institutes of Health, USA. In the present study, we report nanoparticulation of BA within a narrow Gaussian size range. The US-FDA approved biodegradable and stable biopolymer PLGA [poly (DL-lactide-co-glycolic acid)] carried BA on Lf (Lf-BANP) [6,7]. The whole Lf-BANP is for the first time being tested for its immunomodulatory capacity and for its anti-leishmanial efficacy.

2. Materials and methods

2.1. Materials

BA, PLF-127 surfactant, biopolymer PLGA 50:50, Lf, FITC, Bradford reagent and HEPES buffer were from Sigma-Aldrich (St Louis, MO). HPLC grade solvents (Merck, Mumbai, India) were used.

2.2. Preparation of nanoparticles

BA nanoparticles (BANPs) were prepared following a modified emulsion solvent evaporation technique [8]. Briefly, 50 mg PLGA and

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2.5 mg of BA were dissolved together in 3 ml of chloroform. This phase was emulsified by sonication for 30 sec in 10 ml of 1% w/v aqueous PLF 127 at 20 kHz (Vibra cell VCX 750, Sonics, USA). Resultant emulsion was homogenized at 20,000 rpm (30 min, 4 °C) and evaporated with constant stirring (12 h, RT). The nanoparticles were harvested by ultracentrifugation at 30,000 rpm (25 min, 4 °C), washed with water and preserved in vacuum desiccators at 4 °C. For Lf conjugation, Lf (1.5 mg/mL) and BANP were dispersed in Ringer–Hepes buffer (RH, pH 7.4) on a cyclomixer (4 °C, 12 h). Final nanoparticles were recovered by centrifugation. The conjugated Lf protein was analysed by Bradford protein estimation assay.

2.3. Particle size and zeta potential

The size, the zeta potential and polydispersity index (PDI) of the prepared nanoparticles were measured in Zetasizer Nano ZS (Malvern, USA) against a 4 mw He–Ne laser beam, 633 nm and back scattering angle of 173°. Electrophoretic mobility under an applied electrical field measured Zeta potentials.

2.4. Transmission electron (TEM) and atomic force microscopy (AFM)

Samples were prepared by applying 30 µl of diluted particles onto carbon-coated copper TEM grids (300 mesh, Ted pella, Redding, CA, USA), removal of excess solution and staining with uranyl acetate, followed by air drying and visualization under a TEM (JEOL-JEM 2010, Tokyo, Japan).

The water-suspended samples were deposited onto fused mica substrates. The particles' shape were visualized by an AFM (Nanoscope 3A, Veeco, USA) in tapping mode using RTESP tip with 267–328 kHz resonance frequency, 1.2 Hz scan speed.

2.5. Fourier transform infrared (FTIR) spectroscopy studies

FT-IR (FT/IR-670 plus, Jasco, Tokyo, Japan) vibrational spectroscopy studies were carried out in ground KBr pellets. Samples were diluted separately in IR grade KBr, pelleted in a hydraulic press, scanned over a wave number range of 4000–400 cm^{-1} and the data stacked in Bio-Rad KnowItAll (Bio-Rad, Hercules, CA) software for comparative studies.

2.6. BA loading and entrapment efficiency (%)

BA estimation was carried out using C_{18} column (Supelco, USA) in a reverse-phase HPLC system (Waters, USA) with acetonitrile:water mobile phase (86:14, v/v; flow rate 1 ml/min) [9]. The actual BA content was determined by dissolving 2 mg nanoparticles in 1 ml acetonitrile. The solution was filtered and diluted in HPLC grade water. The BA entrapment efficiency (EE) and loading content (LC) was calculated by the following equations:

$$\text{BA entrapment}\,(\%) = \left(\frac{\text{Mass of BA originally taken} - \text{Mass of BA in supernatant}}{\text{Mass of BA originally taken}}\right) \times 100$$

$$\text{BA loading content}\,(\%) = \left(\frac{\text{Mass of BA in nanoparticles}}{\text{Mass of nanoparticles}}\right) \times 100$$

2.7. In vitro release studies

BANPs (~2 mg of BA) in phosphate buffer (1 ml, 100 mM, pH 7.4) were dialyzed in glass vials containing phosphate buffer (10 ml, 37 °C). The BA release kinetics was estimated by HPLC [3,8]. Standard curve was used for analysis with necessary corrections for the dilution factors. To understand the molecular release mechanism, the Korsemeyer-Peppas model was applied and n and K values were calculated using Sigma plot 6.0 software.

2.8. Parasites, mice, infection of peritoneal macrophages

L. donovani (MHOM/IN/1983/AG83) was maintained in RPMI-1640 with 10% FCS (Life Technologies-BRL, Grand Island, NY). BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were maintained in the experimental animal facility of NCCS, Pune and were used following IEC-approved animal use protocol. The thioglycolate-elicited BALB/c-derived peritoneal macrophages (Purity > 98%) were cultured in RPMI-1640, 10% FCS. 6 h post-infection, extracellular parasites were washed out and cells were incubated at 37 °C containing 5% CO_2 in a humidified atmosphere [10]. The *L. donovani*-infected macrophages were treated with BA (2.5 µg/ml), BANP (2.5 µg/ml) and Lf-BANP (2.5 µg/ml) for 72 h, followed by chilled methanol fixation, Giemsa-staining and enumeration of amastigotes/100 macrophages.

2.9. Lf-BANP induced nitrite production by Leishmania-infected macrophages

BALB/c-derived peritoneal macrophages, uninfected (UIM) or *Leishmania-infected* (IM), were treated with the BA (2.5 µg/ml), BANP (2.5 µg/ml) and Lf-BANP (2.5 µg/ml) for 48 h. Nitrite concentration in the culture supernatants were measured by Griess reagent [11].

2.10. iNOS2, IL-12 and IL-10 expression by RT-PCR

BALB/c-derived macrophages, uninfected or *Leishmania*-infected, were treated with BA (2.5 µg/ml), BANP (2.5 µg/ml) and Lf-BANP (2.5 µg/ml) for 8 h. Total RNA was extracted using TRI-Reagent (Sigma-Aldrich, St. Louis, MO), cDNA synthesized, and reverse transcriptase PCR was performed using gene-specific primers (Integrated DNA Technologies, San Diego, CA) as described earlier [12]. Gene-specific primers amplified the mouse GAPDH (forward 5′-GAGCCAAACGGGT CATCATC-3′, reverse 5′-CCTGCTTCACCACCTTCTTG-3′), IL-12 p40 (forward 5′-CACGCCTGAAGAAGATGACA-3′, reverse 5′-GACAGAGAC GCCATTCCACA-3′), iNOS (forward 5′-CAGAGGACCCAGAGACA AGC-3′, reverse 5′-AAGACCAGAGGCAGCACATC-3′), IL-10 (forward 5′-TCACTCTTCACCTGCTCCAC-3′, reverse 5′-CTATGCTGCCTGCTCTTA CTC-3′).

2.11. Statistical analysis

Experimental results were expressed as mean ± SD. Student's *t*-test was used to calculate the statistical difference of mean values.

3. Results and discussion

Despite its therapeutic potential, BA was not considered an anti-leishmanial for its poor solubility and permeability. Therefore, Lf-modified PLGA nanoparticles with BA payload were considered a probable solution. All BANP particles' size, PDI and zeta potential were studied in PCS (Supplementary Table 1). PLF-127 stabilizer had a distinctive role both in nanoparticle size and PDI. Lf-BANP showed positive zeta potential of +27.41 mV due to Lf deposition with average particle size at 187.5 nm. DLS intensity plots, corelogram and zeta potential distribution show the homogeneity in size and charge (Fig. 1A–C). The particle surface topography was studied by TEM and AFM (Fig. 1D). The 2D amplitude data of every BANP preparation exhibited spherical shape with a smooth solid surface and minimal coalescence (Fig. 1D). Molecular interactions between PLGA nanoparticles and BA were studied by FTIR analysis (Fig. 1E). FTIR spectra of BANP – a strong −C═O response – was observed at 1759 cm^{-1} due to PLGA esters and the characteristics −C−H stretching was recorded at 2999 cm^{-1}. In Lf-BANP, characteristics −O−H stretching vibrations at

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