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Research paper

Th-1 biased immunomodulation and synergistic antileishmanial activity of stable cationic lipid–polymer hybrid nanoparticle: Biodistribution and toxicity assessment of encapsulated amphotericin B

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ABSTRACT

To address issues related to Amphotericin B (AmpB) clinical applications, we developed macrophage targeted cationic stearylamine lipid–polymer hybrid nanoparticles (LPNPs) with complementary characteristics of both polymeric nanoparticles and liposomes, for enhancement of therapeutic efficacy and diminishing toxic effect of encapsulated AmpB. The LPNPs (size 198.3 ± 3.52 nm, PDI 0.135 ± 0.03, zeta potential +31.6 ± 1.91 mV) provide core-shell type structure which has the ability to encapsulate amphiphilic AmpB in higher amount (Encapsulation efficiency 96.1 ± 2.01%), sustain drug release and stabilize formulation tremendously. Attenuated erythrocytes and J774A.1 toxicity of LPNPs demonstrated safe applicability for parenteral administration. Elevated macrophage uptake of LPNPs, rapid plasma clearance and higher drug allocation in macrophage abundant liver and spleen illustrated admirable antileishmanial efficacy of AmpB-LPNPs *in vitro* (IC_{50}, 0.16 ± 0.04 μg AmpB/ml) and *in vivo* (89.41 ± 3.58% parasite inhibition) against visceral leishmaniasis models. Augmentation in antileishmanial activity due to Th-1 biased immune-alteration mediated by drug-free LPNPs which elevated microbicidal mediators of macrophages. Moreover, minimal distribution to kidney tissues and low level of nephrotoxicity markers (creatinine and BUN) demonstrated the safety profile of AmpB-LPNPs. Conclusively, reliable safety and macrophage directed therapeutic performance of AmpB-LPNPs suggest it as promising alternative to commercial AmpB-formulations for the eradication of intra-macrophage diseases.

1. Introduction

Mononuclear phagocytes (macrophages/dendritic cells) are the professional antigen presenting cells (APCs) of the immune system that play central role in bridging innate and adaptive immune responses to act against microbial pathogens in a coordinated manner. As indicated by designation, primary role of these cells is phagocytosis causing destruction of ingested pathogen. Internalization within APCs is mediated through recognition of pathogen-associated molecular patterns (PAMPs) particular for every pathogen, by cellular pattern recognition receptors (PRRs) such as stearylamine receptors, toll-like receptors, lectin-receptors, CD14 or various classes of scavenger receptors, located on APCs [1,2]. Engagement of PRRs by their PAMPs activates APCs, stimulating cytokine secretion that regulates the adaptive immune response, and promotes upregulation of co-stimulatory molecules in order to improve antigen presentation to T cells.

Mononuclear phagocytic system (MPS) plays a pivotal role in the pathophysiology of certain specific diseases including chronic

Abbreviations: AmpB, Amphotericin B; LPNPs, lipid–polymer hybrid nanoparticles; APCs, antigen presenting cells; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; MPS, mononuclear phagocytic system; TNF-α, tumor necrosis factor alpha; NO, nitric oxide; IL-12, interleukin-12; dAmpB, AmpB deoxycholate; LAmpB, AmpB liposomal formulation; PLGA, poly (D,L-lactide-co-glycolide); Sta, stearylamine; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; TPGS, D-alpha-tocopheryl polyethylene glycol 1000 succinate; PNPs, PLGA nanoparticles; FCS, fetal calf serum; DLS, dynamic light scattering; EE, encapsulation efficiency; iNOS, inducible NO synthase; HPRT, hypoxanthine phosphoribosyltransferase; BUN, blood urea nitrogen; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; MRT, mean residence time; AUC, area under curve; IFN-γ, interferon gamma; TGF-β, transforming growth factor beta.

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obstructive pulmonary disease (COPD), asthma [3] and cancer [4,5] and in parasitic infections including leishmaniasis [6], tuberculosis [7] and human immunodeficiency virus (HIV) infection [8] and is a major target for future therapy of mentioned MPS mediated diseases. As a result, drug delivery systems that can target APCs are crucial and could usher in an unorthodox but highly effective therapeutic paradigm for a wide range of diseases.

Cure of intra-APCs parasitic diseases including leishmaniasis, even during chemotherapy, depends upon the development of an effective immune response that activates APCs to kill the intracellular parasites. APCs do this by increasing production of proinflammatory cytokines, tumor necrosis factor (TNF)-α, interleukin (IL)-12 and mediators such as nitric oxide (NO) and reactive oxygen intermediates (ROI) [9–11]. However, this expected immune response is suppressed by the infection which interferes in the mandatory signaling between APCs and T cells, such as IL-12 production and MHC presentation [12,13]. Hence, synergistic chemotherapy with prospective immunomodulatory role may provide a strengthened therapeutic strategy capable of shortening the duration of treatment as well as ensuring the persistent therapeutic effect of drug.

Amphotericin B (AmpB) has long been recognized as the most effective drug for the treatment of mycosis as well as protozoan infections, viz. leishmaniasis, with great commercial success. However, dose-limiting adverse events, particularly renal insufficiency and hematologic toxicity have somewhat dented its clinical relevance [14]. Investigation toward reduction of free AmpB and conventional AmpB deoxycholate (dAmpB) mediated toxicity, has led to development of liposomal formulation (LAmpB); however, its poor scale-up, stability and cost concern [15] still leave a lot to be desired. Consequently, efforts are needed to develop a highly stable and cost effective delivery tool that can subdue toxicity of drug by delivering it directly to parasite residence site (APCs). In this respect, incorporation of PRR specific ligand or immunomodulatory moieties including proteins, antibodies, polysaccharides, glycolipids, glycoproteins and lectins, into polymeric nanoparticles could be a beneficial strategy for improving efficacy of drug targeting and eliminating undesirable side actions. The comparative higher stability profile of polymeric nanoparticles accompanied by the low cost of its components provides economic relevance to the delivery system raising the affordability quotient.

Accordingly in the present study, we architected lipid–polymer hybrid nanoparticles (LPNPs) by utilizing poly (D,L-lactide-co-glycolide) (PLGA) as a biocompatible polymer [16], while stearylamine (Sta) as lipid was selected due to its cationic nature, biocompatibility, benign antiprotozoan activity and immunopotentiation strength [17]. Since stearylamine also acts as a ligand for stearylamine PRR present on macrophage surface and phosphatidylserine biomarker of *Leishmania* parasite ligand [17], its presence on the surface of nanoparticles may serve as targeting moiety as well. Herein, we demonstrate a practical drug delivery system for better management of visceral leishmaniasis.

2. Materials and methods

2.1. Materials and reagents

The authentic sample of AmpB drug was kindly supplied by Intas Pharmaceuticals (Ahmadabad, India). D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS), stearylamine (Sta), bovine serum albumin (BSA), fluorescein isothiocyanate (FITC) and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) were supplied by Sigma–Aldrich (MO, USA). PLGA (50:50, Mw ~ 57 kDa) was from Birmingham Polymers, Inc. (Birmingham, AL). Dialysis membrane (cutoff mol. wt. 12 kDa) and potassium dihydrogen phosphate (KH_2PO_4) were purchased from HiMedia (Mumbai, India). For cell culture, all components were from Sigma–Aldrich. HPLC grade acetonitrile and methanol were purchased from SD Fine Chem Ltd. (Mumbai, India). Ultrahigh pure water produced by three-stage Millipore Milli-Q Plus 185 purification system (Bedford, US) was utilized throughout all experiments. All other chemicals and solvents were of analytical grade.

2.2. Parasite, culture conditions and animals

In the present study, *Leishmania donovani* clinical strain used was MHOM/IN/80/Dd8. Leishmania promastigotes were cultured in RPMI-1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% FCS at 26 °C in a humidified atmosphere with 5% CO_2 [18] and strain has also been maintained in Syrian golden male hamsters (45–50 g) as experimental visceral leishmaniasis model through serial passage [19]. Male Wistar rats (150–200 g) were utilized for pharmacokinetic and biodistribution studies, while *in vivo* toxicity study was conducted in male Swiss mice (18–20 g).

All experimental animals were obtained from the Institute's animal house facility and used for experimental purposes with prior approval of the Institutional Animal Ethics Committee. Studies were conducted in accordance with the guidelines of the Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.3. Preparation of lipid polymer hybrid nanoparticles

Hybrid nanoparticles (AmpB-LPNPs) were fabricated using a modified w/o/w double emulsification method. Firstly, polymer solution (50 mg PLGA in 5 ml acetone) and acidified methanolic AmpB solution (usual amount 10% wt/wt of polymer and zero for the blank formulation) were mixed together. Then obtained organic phase was added dropwise in the internal aqueous phase (1% wt/vol TPGS, w_1 phase, 10 ml) under vigorous stirring (Remi, Mumbai, India). After 15 min, formed w/o coarse emulsion was poured into external aqueous phase containing 20 ml 0.25% w/v TPGS (w_2 phase) and 5 ml Sta lipid solution in methanol (0.5%, 0.75%, 1.0%, 1.25% and 1.5% wt/vol) under stirring and left overnight to evaporate organic solvent. After that colloidal suspension of AmpB-LPNPs was recovered by ultracentrifugation at 50,000*g* for 20 min at 4 °C (Beckman Coulter, Fullerton, CA, US) and washed with water (pH 2) once followed by repeated cycles of deionized water to remove excessive emulsifier, lipid and free AmpB. In addition, the intermediate pro-carrier polymeric PLGA nanoparticles (AmpB-PNPs) were also fabricated in a same manner except the addition of cationic lipid (Sta).

The fluorescently-labeled nanoparticles were prepared with the same procedure replacing AmpB with 0.5 mg/ml FITC.

2.4. Physicochemical characterization

The diameters, PDIs, and zeta (ζ) potential of the nanoparticles in water were analyzed using dynamic light scattering (DLS) with a Zetasizer Nano ZS apparatus (Malvern Instruments, Worcestershire, UK) at 25 °C as our previously reported method [20].

The morphology of nanoparticles was ascertained using high resolution transmission electron microscopy (HR-TEM, Tecnai™ G^2 F20, Eindhoven, the Netherlands) [20].

Drug quantification was achieved using HPLC (LC-10ATvp, Shimadzu, Tokyo, Japan) equipped with a Lichrosphere reverse-phase C_{18} column (250 × 4 mm, pore size 5 μm; Merck, Darmstadt, Germany). The chromatographic conditions were injection volume = 20 μl, flow rate = 1.0 ml/min, mobile phase = 60/40 acetonitrile/KH_2PO_4 buffer (pH 3.5), and UV detection at