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Supplementation of host response by targeting nitric oxide to the macrophage cytosol is efficacious in the hamster model of visceral leishmaniasis and adds to efficacy of amphotericin B

Sanketkumar Pandya [a, b], Rahul Kumar Verma [a, 1], Prashant Khare [a, 2], Brajendra Tiwari [a], Dadi A. Srinivasarao [a, c, 3], Anuradha Dube [a], Neena Goyal [a], Amit Misra [a, *]

[a] CSIR-Central Drug Research Institute, Sector 10, Jankipuram Extension, Lucknow 226031, UP, India
[b] Academy of Scientific & Innovative Research (AcSIR), Anusandhan Bhawan, Rafi Marg, New Delhi 110001, India
[c] National Institute of Pharmaceutical Education and Research, ITI Compound, Rae Bareli 229010, UP, India

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ABSTRACT

We investigated efficacy of nitric oxide (NO) against *Leishmania donovani*. NO is a mediator of host response to infection, with direct parasiticidal activity in addition to its role in signalling to evoke innate macrophage responses. However, it is short-lived and volatile, and is therefore difficult to introduce into infected cells and maintain inracellular concentrations for meaningful periods of time. We incorporated diethylenetriamine NO adduct (DETA/NO), a prodrug, into poly(lactide-co-glycolide) particles of ~200 nm, with or without amphotericin B (AMB). These particles sustained NO levels in mouse macrophage culture supernatants, generating an area under curve ($AUC_{0.08\text{-}24h}$) of 591.2 ± 95.1 mM × h. Free DETA/NO resulted in NO peaking at 3 h and declining rapidly to yield an AUC of 462.5 ± 193.4. Particles containing AMB and DETA/NO were able to kill ~98% of promastigotes and ~76% of amastigotes in 12 h when tested *in vitro*. Promastigotes and amastigotes were killed less efficiently by particles containing a single drug– either DETA/NO (~42%, 35%) or AMB (~90%, 50%) alone, or by equivalent concentrations of drugs in solution. In a pre-clinical efficacy study of power >0.95 in the hamster model, DETA/NO particles were non-inferior to Fungizone® but not Ambisome®, resulting in significant (~73%) reduction in spleen parasites in 7 days. Particles containing both DETA/NO and AMB were superior (~93% reduction) to Ambisome®. We conclude that NO delivered to the cytosol of macrophages infected with *Leishmania* possesses intrinsic activity and adds significantly to the efficacy of AMB.

1. Introduction

Oral chemotherapy of leishmaniasis consists of administration of antimonials, miltefosine and paromomycin for about three weeks (Sundar and Chakravarty, 2013). As a standard anti-leishmanial chemotherapy, AMB is currently administered in an inpatient setting, requiring infusion over several hours because of its potential for generating acute nephrotoxicity and haemolysis (Brajtburg et al., 1985; Deray, 2002). AMB, an antifungal drug, primarily acts on membrane ergosterol and kills the parasite by disrupting the surface membrane (Matlashewski et al., 2011). Drug resistance to antimonials is spreading, and inclusion of liposomal amphotericin B (AMB) in the treatment is increasingly recommended (Balasegaram et al., 2012). Even in liposomal form, AMB is administered by slow intravenous infusion, because of its toxicity (Gahart et al., 2016). This is an inpatient procedure, and represents additional burden in the resource-poor settings where visceral leishmaniasis is prevalent.

To our knowledge, there are no new drugs for visceral leishmaniasis in the drug discovery and development pipeline, but several reports are available on anti-leishmanial agents incorporated in particulate delivery systems for use against experimental

* Corresponding author. PCS 011, Pharmaceutics Division, CSIR-Central Drug Research Institute, Sector 10A, Jankipuram Extension, Lucknow 226031, India.
E-mail address: amit_misra@cdri.res.in (A. Misra).
[1] Present address: Institute of Nano Science and Technology (INST), Phase-10, Sector-64, Mohali, Punjab 160062, India.
[2] Present address: Baylor Institute for Immunology Research, Baylor Research Institute, Dallas, TX 75204, USA.
[3] Present address: Department of Biosciences and Bioengineering, Indian Institute of Technology, Kalyanpur, Kanpur 208016, UP, India.

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visceral leishmaniasis (Costa Lima et al., 2012; Kansal et al., 2012; Lopes et al., 2012; Asthana et al., 2013, 2015). There is scope to improve upon such delivery systems to further ameliorate adverse drug effects, prevent emergence of drug resistance, adapt systems to outpatient use, and reduce cost to patients (Jha, 2006). We therefore asked whether anti-leishmanial therapy may be augmented with a novel pharmacophore, employed in unrelated diseases, but relevant to the objective of killing the parasite within the macrophage. NO is one such molecule and its role in innate immune responses of mammalian macrophages is well known (Ascenzi et al., 2003; Klink and Sulowska, 2007; Brune, 2010; Mills, 2012; Bogdan, 2015). The differences in NO cytotoxicity across evolutionarily distinct genera suggest that it can effectively kill bacteria, fungi and protozoa at concentrations that can be tolerated by mammalian cells (Dzik, 2014). Bactericidal activity of NO against a number of human pathogens has been extensively reported, and our group has demonstrated the activity of NO prodrugs against the macrophage-borne pathogen *Mycobacterium tuberculosis* (Verma et al., 2012, 2013).

NO donors, also termed diazeniumdiolates or NONOates, are formed by the chemical reaction of NO with nucleophilic amines (Keefer and Saavedra, 2002) and can release NO in a sustained manner. NO donors are essentially prodrugs, since the active moiety, NO, is a gaseous molecule with a biological half-life ($t_{1/2}$) of not more than 2 min (Liu et al., 1998). NO donors like DETA/NO, isosorbide mononitrate, sodium nitroprusside (SNP), 3-morpholinosydnonimine (SIN-1), S-nitrosoglutathione (GSNO) and S-nitroso- N-acetyl penicillamine (SNAP) are familiar pharmacological agents. DETA/NO has a significantly longer half-life ($t_{1/2}$) of 20 h at pH 7.4 and 37 °C; and releases two moles of NO per mole of parent compound without prior biotransformation (Yamamoto and Bing, 2000; Keefer and Saavedra, 2002; Xu et al., 2011). NO has efficacy against *Leishmania* and the effective use of NO donors in patients of cutaneous Leishmaniasis through topical application of creams containing SNAP has been demonstrated (Lopez-Jaramillo et al., 1998). NO-releasing diazeniumdiolates have also been formulated as particles for topical treatment of cutaneous leishmaniasis (Moreno et al., 2014).

Particulate drug delivery systems are taken up by macrophages, which also represent the ecological niche for amastigotes of *Lesishmania* species that cause visceral leishmaniasis. While the promastigote is killed relatively easily by drugs in blood circulation, the intracellular amastigote survives in phagosomes of tissue-resident macrophages. The phagosome membrane acts as an additional barrier to the entry of parasiticidal drugs circulating in the bloodstream. Phagosome-resident amastigotes also evade innate host defense mechanisms (Sacks and Sher, 2002) and interfere with development of defensive immunity. Cytokines such as gamma-interferon activate macrophages to produce NO which kills intracellular *Leishmania* (Gatto et al., 2015). Mice deficient in inducible NO synthase (*iNOS*) are more susceptible to *Leishmania* infection (Green et al., 1990). Several membrane molecules of *Leishmania* inhibit macrophage iNOS as part of the parasite's survival strategy inside macrophages, and cells expressing high levels of iNOS are resistant to *Leishmania* infection (Proudfoot et al., 1996).

We employed a prodrug-in-particle approach to target NO to the macrophage cytosol through passive internalization. The objective of the present work was to establish 'proof of principle'in respect of the efficacy of NO against visceral leishmaniasis. Our approach also involves incorporating multiple drugs, AMB and DETA/NO in the same particle (Mi et al., 2013). We expected that NO delivered to the macrophage cytosol will have important outcomes, both in terms of killing the parasite as well as stimulating the host to mount defense responses. Our observations suggest that the prodrug-in-particle approach may be of use in targeting NO to infected macrophages, where the molecule exhibits parasite killing *in vitro* and *in vivo*, without undue toxicity to the host cell.

2. Material and methods

2.1. Materials

Biodegradable poly(lactic-co-glycolic acid) (PLGA) of monomer ratio 65:35 was purchased from Birmingham Polymers Inc., (Birmingham, AL, USA). DETA/NO, Cell culture medium (RPMI-1640), fetal bovine serum (FBS), supplements and antibiotics; Pluronic F-68 (Poloxamer 188); methylthiazoletetrazolium (MTT) and dialysis tubing (70 KDa) were purchased from Sigma–Aldrich (St. Louis, MO, USA). AMB was donated by Sun Pharma Advanced Research Centre, Vadodara, India. Acetone and methanol were of spectroscopic grade and all other reagents and chemicals were of analytical grade. The water used in all experiments was prepared in a three-stage Millipore Milli-Q plus 185 purification system (Bedford, MA, USA).

2.2. Preparation of particles

Particles were prepared by a solvent displacement method (Pandya et al., 2011; Verma et al., 2011). PLGA was dissolved in acetone by stirring at 37 °C for 10 min to obtain a 2.5% (w/v) solution. Methanol containing 4 M sodium iodide was used to solubilize AMB to obtain a 0.1% solution (Lopes et al., 2014). Acetone and methanol were used in a ratio of 1.5:1. DETA/NO was added to a final concentration of 0.1% and the pH was adjusted to 8.5. The drug solution was then added to the polymer solution. FITC was also added to the methanol solution to prepare fluorescent particles. Then, 25 mL of the organic solution were added dropwize into 40 mL of distilled water containing 40 mg poloxamer 188 under homogenization (IKA® Ultra TURRAX® model T-25) at room temperature. The preparation was then placed in a rotary evaporator at 55–58 °C under vacuum to evaporate the organic phase and concentrate the aqueous phase to 10 mL. The aqueous preparation was centrifuged at 70,000 rpm for 30 min and the pellet lyophilized to obtain particles in the dry state.

2.3. Characterization

Particle morphology was studied using a Scanning Electron microscope (Quanta 200, FEI, Oregon, USA). Three batches were analyzed for size distribution (mean diameter and polydispersity index) and Zeta potential using a Zetasizer NanoZS (Malvern Instruments, UK). AMB content and encapsulation efficiency were determined by a validated HPLC method, while the estimation of DETA/NO was done by UV photometry of the ultracentrifugation supernatant.

2.4. In vitro drug release

The release of AMB from the particles was determined using a dialysis membrane method. Each sample of drug loaded particles (2 mL) was filled in dialysis bags with a molecular mass cut-off of 70 kDa. The bags were suspended in 200 mL of 0.1 M phosphate buffer (pH 7.4) containing 1% v/v Tween 80, at 37 °C in a USP dissolution apparatus type II (DISSO-2000, LabIndia, Mumbai, India) running at 50 rpm. At predetermined intervals, aliquots of 0.5 mL were withdrawn and the amount of drug released estimated by HPLC (Verma et al., 2011).