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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

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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

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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).

2.5. In vitro uptake by macrophages

Uptake of particles was assessed in J774 A.1 mouse macrophages using flow cytometry (FACS Aria, BD Biosciences, Heidelberg, Germany). Cells were maintained in DMEM supplemented with 10% FBS and 1% antimycotic-antibiotic mixture at 37 °C, 5% CO_2, and 95% relative humidity. One million cells were plated per well in six-well plates, incubated overnight and washed with incomplete medium to remove non-adherent and dead cells. Particles containing FITC alone (blank particles), or FITC in addition to AMB, DETA/NO alone, or both drugs in the same particle were added to the designated wells at drug concentrations of 50 μg/mL or FITC concentration 0.1 μg/mL. After 3 h of incubation, the culture medium was aspirated and wells rinsed three times with incomplete medium to remove extracellular particles. Cell-associated fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of 525 nm. Histograms of fluorescence intensity versus counts were plotted.

2.6. NO production

J774 cells were cultured as above in 96-well plates at a density of 0.5×10^6 cells/well. Solutions of AMB and DETA/NO, or a suspension of particles in DMEM without FBS or supplements were added to a final concentration of 50 μM of either drug in wells assigned. After 2 h, the wells were washed three times and complete medium was replaced. NO_2^- accumulation in the supernatant of cultured cells was used as an indicator of NO production by the culture. Supernatants from duplicate wells were recovered sequentially at 5, 15, and 30 min, followed by 1, 3, 6, 12 and 24 h. NO in supernatant was analyzed using Griess' reagent (0.2% naphthylenediamine dihydrochloride, and 2% sulphanilamide in 5% phosphoric acid) against a sodium nitrite standard curve at 540 nm (Powerwave-XS, Bio-Tek Instruments).

2.7. Hemolysis

Hemolysis induced by the particles in fresh citrated mouse blood was determined (Wang et al., 2006). With permission from the Institutional Animal Ethics Committee of CSIR-CDRI, (IAEC/2008/25/Renew 06-65/13), blood was collected from the tail vein of mice. Particles were incubated with citrated blood at 37 °C for 2 h, and then centrifuged at 3000 rpm for 5 min. The hemoglobin released in the supernatant was measured spectrophotometrically at 550 nm. The positive control comprized RBC suspension in 1% Triton-X solution in PBS for surfactant lysis, and the negative control was RBC in PBS.

2.8. Cytotoxicity towards macrophages

The in-vitro cytotoxicity of formulations towards J774 cells was measured by an MTT assay. Cells were seeded in 96 well plates at a density of 0.2×10^6 and were allowed to adhere overnight. The medium was replaced and cells incubated for different time intervals (6, 48 and 72 h) with different formulations at equivalent doses of particles. These doses generated a concentration of 50 μM of AMB or DETA/NO in wells exposed to particles containing single drugs and 25 μM in respect of particles containing both. Cells were then washed twice and incubated in the presence of 5 mg/mL MTT for 4 h at 37 °C. DMSO (50 μl) was added to each well to solubilize formazan crystals. The optical density was measured using a multiwell scanning spectrophotometer (Biotek Microplate Reader, Dynatech Laboratories Inc., Chantilly, VA, US) at a wavelength of 550 nm.

2.9. Parasites

The strain MHOM/IN/1980/DD8 (henceforth, DD8) strain were grown and maintained at 25 °C in M199 medium supplemented 10% FBS and 1% antimycotic-antibiotic mixture.

2.10. In vitro activity against promastigotes

Promastigotes of late log phase were seeded at a concentration of 5×10^5 cells/well in a 96-well flat-bottomed micro-titre plate (Cellstar, NC, USA) and different concentrations of AMB and/or DETA/NO (12.5, 25 and 50 μg/mL of either drug) were generated by adding free drugs or particles. Untreated cells served as the negative control. Each assay was performed in triplicate. The cells were incubated for 48 h at 25 °C after treatment. The promastigotes were counted using a haemocytometer under a light microscope at 48 h and 72 h. The % inhibition of parasite growth of treated cultures were compared to that of untreated control.

2.11. In vitro activity against amastigotes

J774A.1 cells (10^5 cells/well) were taken in 16-well chamber slides (Nunc, IL, USA) and infected with late log phase DD8 promastigotes at a multiplicity of infection of 10. Prior to infection, the cells were washed with medium to remove the non-adherent macrophages. After 12 h, the chamber slides were washed three times with PBS (pH 7.2) to remove extracellular promastigotes and complete medium replaced. Different concentrations of drugs (12.5, 25 and 50 μg/mL) were added to the wells in triplicate and incubated for 48 h. Cells were fixed in absolute methanol, stained with Giemsa stain and examined under oil immersion objective (Zeiss Axiovert 25). At least 100 macrophages were counted per well. Untreated infected macrophages were used for comparing numbers of infected cells and amastigotes per cell.

2.12. In vivo evaluation in L. donovani-infected hamsters

All experiments were conducted under approval/supervision of the Institutional Animal Ethics Committee of CSIR-CDRI (Reference number IAEC/2008/25/Renew 06 (65/13)). The anti-leishmanial activity of particles containing AMB, DETA/NO or both drugs was determined using Fungizone® and Ambisome® as comparators, at drug-equivalent doses of 1 mg/kg body weight. The Syrian golden hamster model was employed (Gupta et al., 2007). Hamsters (n = 3 per group) were infected with *Leishmania donovani* Dd8 promastigotes and after 30 days of established infection, the animals were injected 1 mg/kg equivalent doses of particles or comparators for 5 consecutive days by the intraperitoneal route. Infected untreated hamsters served as positive controls. After 7 days of treatment, the animals were sacrificed. Splenic dab smears obtained by necropsy were microscopically examined using Giemsa-stained imprints wherein the parasite burden was expressed in terms of Leishman-Donovan units (LDU), according to the stauber's formula (Stauber et al., 1958).

LDU or Stauber's count = (Number of amastigotes per 1000 nucleated cells × weight of organ in grams) × 2×10^5

The percent parasite suppression (PS) was calculated using the following formula.

$$PS = 100 \times [1 - (PT/PC)]$$

where P_T and P_C denote the mean stauber's count of treated group and control group respectively.

2.13. Statistics

All results are given as means ± SD. Differences between formulations were compared using one-way analysis of variance (ANOVA) on ranks. $P < 0.05$ denotes significance in all cases. All comparisons of AMB and DETA/NO individually were at equal concentrations, regardless of whether particles or free drug were used. Amounts of AMB exposed to groups of animals or cells receiving two-drug particles were lower than those receiving AMB particles or free drug, since these doses were normalized for 50 μM-equivalents of DETA/NO. For evaluation of efficacy, the power of the study was first calculated using the primary outcome measure of reduction in spleen parasite burden from 400 to 150 amastigotes/100 macrophages after 7 days of stopping treatment. $N = 3$ was chosen to estimate differences by non-parametric analysis or by one-way ANOVA on ranks at a power >0.95.

3. Results

3.1. Characterization of particles

The particle size and size distribution, zeta potential, AMB and/or DETA/NO content, and encapsulation efficiency in respect of different formulations is shown in Table 1. These results indicate that the particles are adequate for preparing an intravenously injectable formulation.

The particles appeared as spheres or deformed spheres in scanning electron microscopy (Fig 1A) and displayed Gaussian size distribution in suspension (Fig 1B). Particles released AMB in a diffusion-controlled fashion (Fig 1C). An initial burst was followed by sustained release. The *in vitro* drug release data were tested against several kinetic models of drug release to elucidate the mechanism, and it was concluded that diffusion controlled release conforming to the Higuchi model supervened (Costa and Sousa Lobo, 2001). A linear relationship was observed between the amount of drug released and square root of time, with correlation coefficients (R^2) of 0.82 and 0.93 respectively for AMB and 2-drug particles. These results suggest a role of DETA/NO as a release modifier for AMB in 2-drug particles.

3.2. Macrophage uptake and elicitation of NO in culture

Particles containing traces of FITC alone (blank particles) were taken up as avidly as those containing AMB within 2 h of exposure to J774 mouse macrophages in culture, as shown in Fig 2A. More than 90% of cultured cells took up particles containing the fluorescent marker. Particles containing DETA/NO alone or in combination with AMB were indistinguishable in terms of uptake, but differed from AMB or blank particles in extent of fluorescence imparted to the cells. Brighter staining by blank and AMB particles as compared to DETA/NO or two-drug particles is attributable to the polydispersity of the different preparations.

Culture supernatants of J774 macrophages exposed to a solution of DETA/NO, or to particles containing the prodrug for 2 h were monitored for NO- levels for the next 12 h, as shown in Fig 2B. NO generated by soluble DETA/NO peaked within 15 min and declined, while particle-incorporated DETA/NO resulted in NO levels peaking later and lower. Differences in the areas under the concentration–time curve (AUC) of NO elicited by soluble and particulate DETA/NO were statistically in significant (one-way ANOVA on ranks, $P < 0.05$), even when the dose of DETA/NO was reduced to half in respect of particles containing both DETA/NO and AmB. These results are depicted in Fig 2C.

3.3. Preliminary assessment of biocompatibility

None of the formulations induced significant haemolysis. Haemolysis following incubation with particles is depicted quantitatively in Fig. 3A. The particles induced negligible hemolysis at 50 μM-equivalent doses. Fig. 3B shows the cytotoxicity of different formulations against uninfected J774 cells. Panel 3C shows results obtained when cells were infected with *L. donovani* strain DD8 at MOI of 10, 6 h prior to treatment. The control in Panel 3B represents viability of uninfected cells receiving no treatment, and in Panel 3C, infected cells receiving no treatment. *In vitro* evaluation of cytotoxicity was carried out over 72 h, and yielded intriguing data in respect of DETA/NO. It appears that DETA/NO protected cells from death over the first 6 h of exposure. AMB is a well-known cytotoxic agent, and reduced cell viability to ~20% of untreated controls within 6 h, regardless of whether it was administered as particles or in solution.

3.4. In vitro activity against L. donovani

Anti-leishmanial activity against Dd8 promastigotes was measured by counting method. The results were transformed to display average % inhibition of parasite survival, and are shown in Fig. 4. Dose-dependent inhibition of parasite growth was observed with all particle formulations. Particles containing DETA/NO alone showed little inhibitory activity at 12.5 μM and 25 μM, but at higher concentrations, they led to a significant ($P < 0.05$) inhibition of promastigotes. At 50 μM concentration, free DETA/NO effected 34.9% parasite inhibition, while particle-borne DETA/NO had no significant difference in efficacy (41.71%). AMB in solution and AMB particles showed 81.12 and 89.91 percent inhibition at 50 μM respectively. Particles containing both AMB and DETA/NO inhibited 98.51% of free-living parasites at 50 μM.

Activity against *L. donovani* Dd8 amastigotes was evaluated by staining and counting numbers of parasites/macrophage nucleus. Blank particles were inactive against the resistant strain. DETA/NO and AMB in solution at 50 μM resulted in per cent inhibition of 28.04 ± 8.18 and 61.54 ± 3.28 respectively with respect to infected, untreated controls. Particles containing these two agents individually resulted in 51.13 ± 3.95 and 65.90 ± 5.97% inhibition respectively, which was significantly greater than that observed with blank particles ($P < 0.00001$). When both agents were incorporated together in particles, inhibition was 76.41 ± 3.58%. Two-drug particles induced significantly higher inhibition as compared to particles containing either of the two drugs alone ($P = 0.00012$, 0.046 in respect of DETA/NO and AMB). These results are summarized in Fig. 4.

Blank or drug-free particles also inhibited promastigote survival,

Table 1
Mean particle size, polydispersity and Zeta potential of formulations with indicated entrapment efficiency of process and drug loading; ± SD.

Particles	Size (d_{50}, nm)	Polydispersity index	Zeta potential (mV)	Entrapment efficiency (% w/w)	Drug content (% w/w)
Blank	127.9 ± 3.6	0.07 ± 0.02	−15.5 ± 7.47	–	–
AMB	171.8 ± 6.6	0.086 ± 0.05	−6.75 ± 4.94	61.04 ± 7.49	2.62 ± 0.37
DETA/NO	178.7 ± 10.4	0.406 ± 0.01	−14.8 ± 4.36	48.05 ± 2.60	1.96 ± 0.62
2-Drugs	201.4 ± 13.8	0.316 ± 0.00	−16.6 ± 8.36	76.4 ± 4.34	1.53 ± 0.37

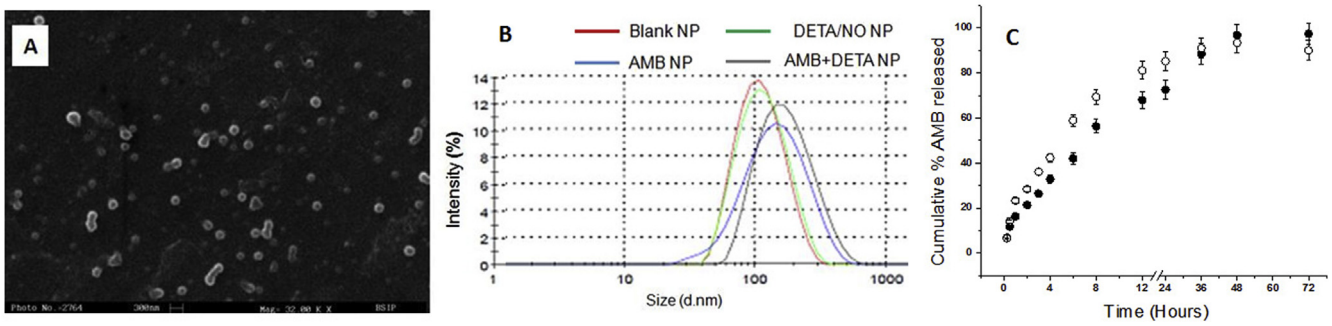

Fig. 1. Particle characteristics. (A): Representative scanning electron micrograph of DETA/NO + AmB particles. The scale bar represents 300 nm. (B): Volume-average size distribution of (1): blank particles, (2): particles containing DETA/NO alone, (3): AMB alone, and (4): containing both agents. (C): Cumulative % AMB released in 72 h from AMB particles (open symbols) and two-drug particles (filled symbols).

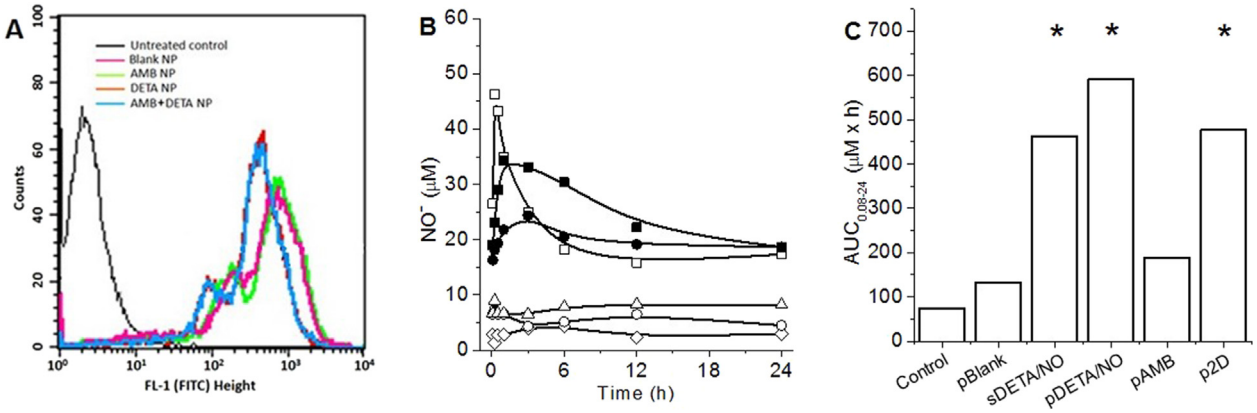

Fig. 2. Uptake of particles and production of NO by J774 cells in 30 min of exposure. (A): Fluorescence acquired by cells exposed to (1): no particles, (2): blank particles, (3): AMB particles, (4): DETA/NO particles and (5): two-drug particles. (B): Nitrite in culture supernatant following exposure to 50 μM DETA/NO in solution (open squares) or particles (filled squares). Particles containing both DETA/NO and AMB (filled circles) yielded a final theoretical concentrationof 25 μM DETA/NO, generating a profile of lower levels of NO secretion. Particles containing AMB alone (open triangles), or no drug (open circles) did not evoke NO release that was significantly different from untreated cells (open diamonds). Means (N = 2) of the data are shown. (C): Areas under the NO concentration–time curve ($AUC_{0.08-24}$) derived by integration. The suffixes 's' and 'p' to x-axis labels indicate 'solution' and 'particles respectively while p2D corresponds to two-drug particles. Means (N = 2) of the data are shown, significance at $P < 0.05$ compared to blank particles is denoted by an asterisk (*).

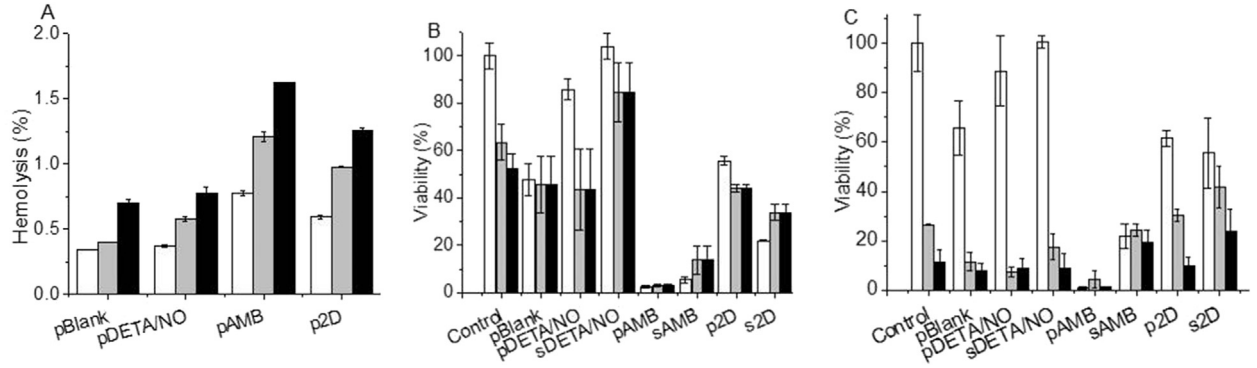

Fig. 3. Preliminary assessment of biocompatibility and differential survival of infected versus infected cells (A): Hemolysis induced by particles at concentrations of 12.5- (open bars), 25- (gray bars) and 50- (filled bars) μM-equivalent drug concentrations of particles. (B): Cell viability after exposure to 50 μM-equivalent drug concentrations of drugs in solution or in particle form in the absence of intracellular *L. donovani* at 6 h (open bars), 48 h (gray bars) and 72 h (filled bars) post exposure. P2D and s2D correspond to particle two-drugs and soluble two-drugs, respectively (C): Viability after cells were infected with *L. donovani*. The prefixes 'p' and 's' refer to particulate and soluble material. Means (n = 3) ± SD are plotted.

presumably by means of non-specific membrane disruption or stress.

3.5. *In vivo* activity in *L. donovani* infected hamsters

Ambisome® and Fungizone® were the comparators employed here and the percent parasite suppression observed by their administration was 88.30 ± 1.76% and 74.02 ± 5.38% respectively

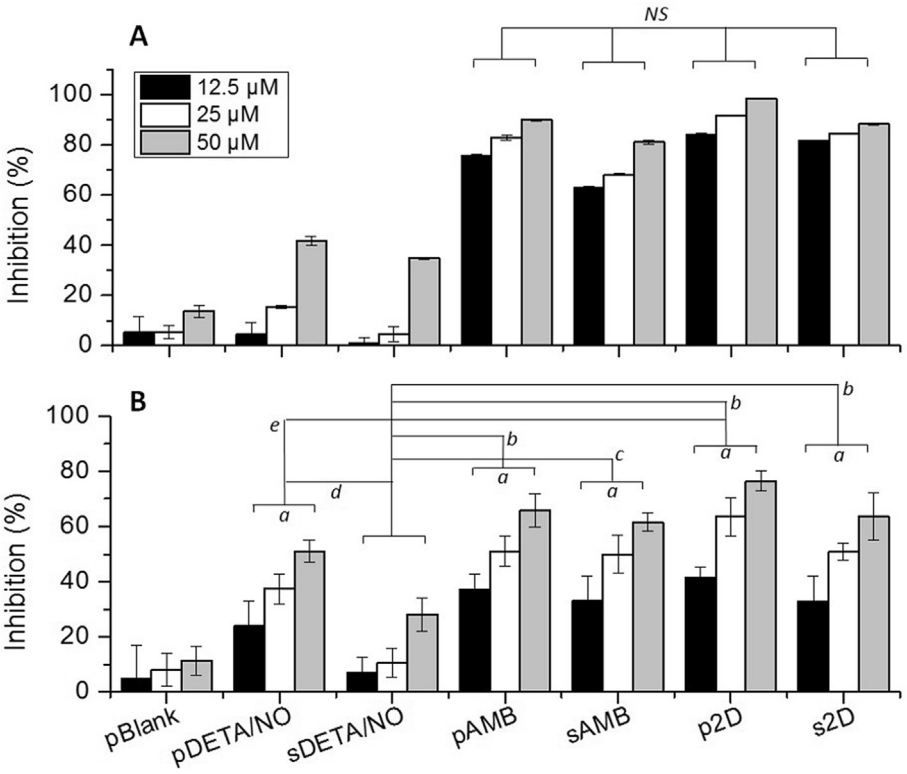

Fig. 4. In vitro activity of AMB ± DETA/NO against Leishmania donovani. (A): Promastigotes and (B): Amastigotes of the laboratory strain Dd8. Soluble (s) or particle-borne (p) DETA/NO displayed no significant ($P > 0.05$) differences in killing promastigotes and amastigotes at 50 μM equivalent concentration; while the presence of AMB imparted significant differences in activity towards the two forms with promastigotes being more susceptible than amastigotes. Means ($N = 3$) ± SD are plotted, and non-significant differences between treatment groups in a single ANOVA are indicated as NS. Markers indicate significant difference at: (a) $p < 0.0001$ compared to blank particles, (b): $p < 0.0001$ between treatment groups, (c): $p < 0.001$ between treatment groups, (d) $p < 0.01$ between treatment groups, and (e) $p < 0.05$ between treatment groups.

(Table 2). Particles containing DETA/NO possessed significant *in vivo* leishmanicidal efficacy (73.10 ± 2.50% suppression) that was comparable to Fungizone®, but not Ambisome®. As expected, 2-drug particles displayed the highest activity (92.82 ± 1.03% suppression) among all formulations. DETA/NO particles effectively reduced the splenic parasite burden at a dose of 1 mg/kg/day under the treatment regimen employed. The significantly higher activity of 2-drug particles is attributable to the combined anti-parasitic effect of both AMB and NO prodrug. There was no significant difference observed in the leishmanicidal activities between particles containing DETA/NO and Fungizone ($P > 0.05$). Significant differences ($P < 0.05$) were observed in activities of other particles and the commercial formulation, having leishmanicidal activity in the rank-order 2-drug particles > Ambisome®>AMB particles > Fungizone®. The mean ranks of treatment groups compared to the untreated control group (mean rank 2.0) in a Kruskal–Wallis ANOVA were 2.0, 6.3, 6.7, 11.0, 14.0 and 17.00 respectively.

4. Discussion

The use of AMB particles in PLGA has been proposed by several researchers over the years (Van de Ven et al., 2012; Jain et al., 2014; Ribeiro et al., 2014; Asthana et al., 2015; Gupta et al., 2015). A particle formulation for targeted delivery of anti-leishmanial agents to macrophages as reported earlier (Pandya et al., 2011), has the potential to facilitate bolus, intravenous injection to outpatients. However, in view of the relatively large dose volume to be administered as a bolus, it would be advantageous if a drug combination regimen could reduce the required dose of AMB (Davidson, 1991). The present results suggest that the NO prodrug DETA/NO possesses intrinsic leishmanicidal activity against promastigotes and amastigotes, especially if incorporated in particles, comparable to the commercial formulation Fungizone®. It can be used alone, or preferably, in combination with AMB for additive effects on parasite inhibition if formulated as particles suitable for intravenous injection.

Particles reported here were prepared by a scalable process

Table 2
In vivo anti-leishmanial activity of particles containing AMB, DETA/NO, or both drugs, compared to Ambisome® and Fungizone®.

Treatment group ($n = 3$)	Untreated	AMB	DETA/NO	AMB + DETA/NO	Ambisome	Fungizone
LDU or Stauber's number ($\times 10^8$)	21.90 ± 1.79	3.55 ± 0.43*#†	5.89 ± 0.73*†	1.57 ± 0.23*#†	2.55 ± 0.36*#	5.62 ± 0.73*†
Percent parasite suppression (%)	–	83.72 ± 2.06*#†	73.10 ± 2.50*†	92.82 ± 1.03*#†	88.30 ± 1.76*#	74.02 ± 5.38*†

The percent parasite suppression was calculated using the LDU/Stauber's count vs. untreated group as discussed in the experimental section. Data are Mean ± SD for 3 hamsters per group. The groups were compared using Kruskal–Wallis non-parametric analysis at 95% confidence intervals.
*Significantly different when compared with untreated ($P < 0.05$), # Significantly different when compared with Fungizone® ($P < 0.05$), †Significantly different when compared with Ambisome® ($P < 0.05$).

(Section 2.2 and Table 1), not requiring the use of ultrasonic energy. All particles in the preparation, however, were not spherical although the preparations were fairly monodisperse (Fig. 1A,B). Matrix-controlled release of AMB was observed from the particles, suggesting that intracellular concentrations of released drug would follow similar temporal profiles when the particles are taken up by macrophages. We did not study the release of the prodrug (DETA/NO) in these experiments, preferring instead to study the time kinetics of the active moiety (NO) in culture supernatants. As evident from Fig. 2B, NO levels were sustained at 20–30 μM for a period of 24 h when mouse macrophages were exposed to particles containing DETA/NO. These concentrations are similar to values reported in respect of macrophages recovered from mice receiving protective immunization against *Leishmania infantum* (Khoshgoo et al., 2008).

Preliminary indicators of biocompatibility of the particles (Fig. 3) are encouraging. It is premature to speculate whether cell death induced by AMB and/or DETA/NO would provide benefit in chemotherapy of leishmaniasis by virtue of denying intracellular 'sanctuary' to the parasite. Results shown in Fig. 4, however, support the view that host cell death could affect inhibition of parasite growth. The findings are encouraging with regard to the safety of proposed formulation, but more detailed investigations are required for a tenable claim of safety.

It has been demonstrated elsewhere that lactide-glycolide particles show leishmanicidal activity (Costa Lima et al., 2014). There was significant enhancement of activity of DETA/NO when incorporated in particles, towards amastigotes. While the differences in the extent of parasite inhibition by soluble versus particle-borne AMB are not statistically significant, it is expected that lower concentrations might reveal clearer differences in efficacy at equivalent dose. Further, the lack of difference between activities of particulate and soluble AMB suggests that the drug is capable of sufficient intracellular accumulation *in vitro*, without the need for targeting.

We did not evaluate host responses of the macrophage in these experiments. However, it is likely that uptake of particles may induce macrophages to mount parasiticidal innate responses, especially if the contents release NO, a known mediator of such responses (Verma et al., 2012). Analogous results from our work on macrophages infected with an unrelated pathogen, *M. tuberculosis*, suggest that the presence of particles in the macrophage's intracellular compartment induces a variety of processes indicative of classical activation (Sharma et al., 2007). Both AMB and DETA/NO can reportedly elicit the expression of iNOS in macrophages (Larabi et al., 2001), presumably leading to enhanced intracellular NO. It is also possible that NO might act directly on the parasite membrane. We submit that these particles might be of interest to researchers investigating host-pathogen dialectics in the context of *Leishmania* where intracellular generation of NO could result in important outcomes. The DETA/NO particles reported here can serve as a biochemical tool to investigate effects of exogenous supplementation of what is essentially an innate host response.

We found compelling evidence of pre-clinical superiority of the two-drug particles in comparison to marketed liposomal formulations of AMB, and non-inferiority of particles containing DETA/NO alone (Table 2). Thus, we submit that the prodrug-in-particle approach to passively target NO to macrophages deserve further investigation, especially to establish appropriate dosing regimens. Finally, we submit that the only way to clinically deploy the invaluable contributions of NO to stimulation of host macrophage responses as well as parasiticidal chemotherapy of *Leishmania* infection is through targeted delivery of NO donors to the macrophage cytosol.

Conflicts of interest

None.

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