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Host-mediated *Leishmania donovani* treatment using AR-12 encapsulated in acetalated dextran microparticles

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Abstract

Leishmaniasis is a disease caused by parasites of *Leishmania* sp., which effects nearly 12 million people worldwide and is associated with treatment complications due to widespread parasite resistance toward pathogen-directed therapeutics. The current treatments for visceral leishmaniasis (VL), the systemic form of the disease, involve pathogen-mediated drugs and have long treatment regimens, increasing the risk of forming resistant strains. One way to limit emergence of resistant pathogens is through the use of host-mediated therapeutics. The host-mediated therapeutic AR-12, which is FDA IND-approved for cancer treatment, has shown activity against a broad spectrum of intracellular pathogens; however, due to hydrophobicity and toxicity, it is difficult to reach therapeutic doses. We have formulated AR-12 into microparticles (AR-12/MPs) using the novel biodegradable polymer acetalated dextran (Ace-DEX) and used this formulation for the systemic treatment of VL. Treatment with AR-12/MPs significantly reduced liver, spleen, and bone marrow parasite loads in infected mice, while combinatorial therapies with amphotericin B had an even more significant effect. Overall, AR-12/MPs offer a unique, host-mediated therapy that could significantly reduce the emergence of drug resistance in the treatment of VL.

Keywords

AR-12; Visceral leishmaniasis; Dose sparing; Drug delivery; Microparticles

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1. Introduction

Leishmaniasis is a neglected tropical disease caused by protozoan parasites of the species *Leishmania.* It is a major global health concern, with approximately a tenth of the world's population at risk of infection (Handman, 2001). Leishmaniasis predominantly manifests itself as either a cutaneous (CL) or visceral (VL) disease, and mortality rates of untreated VL reach upwards of 90%. No vaccine currently exists, and current therapies for VL include systemic delivery of amphotericin B (ampB), orally delivered miltefosine (Impavido, Miltex), or antimonials, like sodium stibogluconate (SSG). Although these therapies are effective, they do not clear pathogens through host-mediated mechanisms and thus, they have an increased risk of cultivating drug resistance. In fact, over 60% of newly diagnosed human cases in Bihar, India are SSG-resistant (Rijal et al., 2003). Additionally, arsenic contaminated drinking water, prevalent throughout many regions of India, has been shown to increase the cross-resistance of *L. donovani* toward antimonials (McConville and Ralph, 2013). Furthermore, clinical isolates from the Bihar region have begun showing signs of ampB resistance (Purkait et al., 2012) and the elongated half-life of miltefosine increases chances of parasite-acquired resistance (Mishra and Singh, 2013). As cases of therapeutic resistance increase, it is necessary to find alternative therapeutic approaches for the treatment of VL. Modulation of the host's immune response is an emerging method of combating pathogen resistance (Collier et al., 2013). Host-mediated therapies have been evaluated in the clearance of *Salmonella enterica* (Ma et al., 2009), *Mycobacterium tuberculosis* (Guo and Zhao, 2012), *Francisella tularensis* (Pyles et al., 2010), *Escherichia coli* (Vuopio-Varkila et al., 1988), as well as fungal (Parameswaran and Segal, 2007) and viral infections (Halperin et al., 2006). In addition, combining host-mediated with pathogen-mediated therapies (such as ampB) have shown synergy in the treatment of CL. Previously, the treatment of CL with Aldara®, an FDA-approved therapy to treat genital warts, has been shown to work effectively while limiting the chances for resistant strains to form. Additionally, combining Aldara® with antimonials resulted in an improved therapy which efficiently cleared CL lesions (Arevalo et al., 2007). The synergistic responses required lower concentrations of each therapeutic to clear infection; a phenomenon known as dose sparing (Arevalo et al., 2001). The combinatorial therapy also correlated with a higher percentage of patients being lesion free at 3 months, and a higher sustained treatment response than Aldara® or antimony alone. Furthermore, the combinatorial therapy eliminated the need for a second dosage of antimony, saving money and reducing the toxic side effects that occur with antimonial treatments. Recombinant human IFN-γ immunotherapy with pentavalent antimonials has shown promising results against VL, compared with individual drugs (Squires et al., 1993; Sundar and Murray, 1995; Sundar et al., 1997). Murray et al. have also used a combinatorial therapy of low-dose ampB and IL-12 to clear VL parasites at similar levels to an optimal ampB treatment (Murray et al., 2003). While extremely promising, due to the cost of recombinant protein production and storage conditions required, cytokine therapies are not fiscally practical in developing nations. With the plethora of treatment complications that have arisen, the paradigm shift toward heightening host defense mechanisms is essential in combating resistant strains of pathogens. One novel host-mediated compound with the potential to clear pathogens is AR-12 (Arno Therapeutics; formerly known as OSU-03012); an IND-approved derivative of

Celecoxib that lacks Cyclooxygenase-2 (COX-2) inhibitor activity. AR-12 has been reported to enhance host-mediated eradication of *S. enterica* serovar Typhimurium (Chiu et al., 2009a), *F. tularensis* (strains: Schu S4, LVS) (Chiu et al., 2009c), *F. novicida* (Chiu et al., 2009b), *Cryptococcus neoformans* (Baxter et al., 2011; Chabrier-Rosello et al., 2013) through up regulation of autophagy and the Akt kinase pathway. AR-12 has even inhibited the entry of several viruses including Lassa, Ebola, Marburg, and Nipah (Mohr et al., 2015). Also, in combination with the FDA approved drug Sildenafil (Viagra®), it knocked down several receptors that play roles in infection in multiple viruses (Booth et al., 2015). In vivo, soluble AR-12 administration resulted in a 10-fold reduction of *Salmonella enterica* serovar Typhimurium bacterial loads in multiple organs, along with a significant increase in survival time of infected mice; however, the soluble AR-12 treated mice ultimately succumbed to infection (Chiu et al., 2009a). AR-12 is noted as being fairly hydrophobic with a solubility of <1 mg/mL in water and former studies have shown that cellular internalization of soluble AR-12 is not an efficient process (Hoang et al., 2014). Additionally, soluble AR-12 is fairly cytotoxic showing greater than 50% cell death at concentrations of 5 μM, limiting the therapeutic range which can be explored (Hoang et al., 2014). While soluble AR-12 showed promising initial results, it is clear that the hydrophobicity and toxicity concerns severely limit its use both in vivo and in vitro.

One way to overcome the limitations of AR-12 is through drug delivery vehicles. Drug delivery vehicles, like liposomes and polymeric microparticles (MPs), are known to mitigate drug hydrophobicity, reduce toxicity, and increase the therapeutics' area under the curve (AUC) (Collier et al., 2013). For the treatment of VL, a liposomal delivery system for ampB (AmBisome©) is given, as well as lipid complexes of ampB and ampB deoxycholate. The longer and more toxic 21-day treatment, ampB deoxycholate, is more economically feasible than the up to 10 day treatment required for AmBisome© and lipid-complexes (Sundar et al., 2004). The longer duration of treatment equates to more opportunity for patient compliance to lapse, thereby creating an environment that can lead to increased drug resistance. Another drawback of AmBisome© and other AmpB lipid complexes is they requires cold-chain storage, which can limit the availability within resource limited locations (Burza et al., 2014).

Our group uses a newly developed novel biodegradable polymer known as acetalated dextran (Ace-DEX) that can overcome the need for cold-chain storage. We have demonstrated that enzyme encapsulated within Ace-DEX MPs, illustrated stable activity across a broad range of temperatures inside and outside the cold chain, indicating enhanced cargo stability (Kanthamneni et al., 2012) which is needed in the desert and tropical regions where *Leishmania* is endemic (McDowell et al., 2011). Polyesters such as poly-lactic (co-glycolic) acid (PLGA) and polycaprolactone (PCL) do not retain their stability outside of the cold chain and thus have additional treatment costs for VL (Oyewumi et al., 2010). Ace-DEX is derived from FDA-approved dextran and is created by forming acetal groups in place of hydroxyl groups along the glucose rings of the parent dextran molecules. By extending the reaction time of Ace-DEX, the polymer resists hydrolytic cleavage longer and a more sustained polymer degradation is observed allowing for a large degree of tunability. In the phagosomal compartments, Ace-DEX can have degradation half-lives of minutes to hours allowing for quick intracellular delivery of therapeutics whereas degradation half-lives

in pH neutral environments can range from days to weeks (Broaders et al., 2009; Kauffman et al., 2012). PCL and PLGA have much longer degradation half-lives and drug release has been noted to take place over 13 days post phagocytosis (Kalluru et al., 2013). Kupffer cells and splenic macrophages have lifetimes of less than a week and thus delivery of therapeutic for clearance of an intracellular pathogen in which not all of the drug is utilized would be less than ideal (Takahashi, 1994; Takahashi et al., 1996).

Ace-DEX has also been shown to increase efficacy and reduce toxicity when encapsulating and delivering other host-mediated compounds (Bachelder et al., 2010; Borteh et al., 2013; Peine et al., 2013). Our group has recently shown treatment of intracellular *S. enterica* serovar Typhimurium using AR-12 encapsulated within Ace-DEX MPs (AR-12/MPs) (Hoang et al., 2014). Here, we show AR-12/MPs have the potential to control *L. donovani* both in vitro and in vivo. Furthermore, AR-12/MPs showed synergistic potential with commonly used VL therapies.

2. Material and methodology

2.1. Chemicals

All reagents were purchased and used unmodified from Sigma–Aldrich (St. Louis, MO, USA), except where indicated. Water (H_2O) was purified using a Millipore (Billerica, MA, USA) Milli-Q Integral water purification system. Fluorescence and absorbance measurements were obtained on a Spectra Max M2 Molecular Devices plate reader (Sunnyvale, CA, USA).

2.2. Production of AR-12/MPs

Ace-DEX AR-12/MPs were formulated as previously described (Hoang et al., 2014). To prevent endotoxin contamination, all dishes and glassware were soaked in 0.5 M sodium hydroxide overnight, washed with isopropanol, and dried before use. Ace-DEX was combined with AR-12 (2% wt/wt) and dissolved in dichloromethane (DCM). To this solution, polyvinyl alcohol (87– 89% hydrolyzed) (PVA) (3% wt/wt) in phosphate buffer saline (PBS, pH 7.4) was added and the mixture sonicated on ice using a Misonix Ultrasonic Liquid Processor (Farmingdale, NY; 60 W, duty cycle 50%). The emulsion was placed in 0.3% PVA in PBS and stirred for 3 h to evaporate the organic solvent. To remove un-encapsulated AR-12, the solution was filtered using a sterile Midi Kros tangential flow filtration system (Spectrum Labs., Rancho Dominguez, CA). The particles were collected, frozen and lyophilized. To create empty MPs, the same procedure was used without the addition of AR-12. Prior to use, both AR-12/MPs and empty MPs were confirmed to have endotoxin levels below FDA guidelines (0.25 endotoxin units per milliliter) by a ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript USA Inc, Piscataway, NJ), performed according to the manufacturer's instructions.

2.3. Size, imaging and encapsulation of AR-12/MPs

AR-12/MPs were imaged using an FEI NOVA NanoSEM 400. MPs were suspended in basic water and placed on a SEM stub and allowed to air-dry. Size was determined using an NICOMP Submicron Particle Sizer Model 370 (Santa Barbara, California). Encapsulation

efficiency was determined by preparing a 1 mg/mL solution of AR-12/MPs in DMSO and a standard curve of un-encapsulated AR-12. The samples were read on a plate reader at λ_{ex} 280 nm/λ_{em} 380 nm, and the amount of AR-12 encapsulated within the MPs was calculated.

2.4. AR-12/MP release profile

AR-12/MPs and empty MPs ($n = 3$) were suspended at a concentration of 1 mg/mL in either PBS (pH 7.4) or sodium acetate (pH 5.0). Once suspended, these particles were placed on a Multi-Blok Heater (Thermo Fisher Scientific) shaker hot plate at 37 °C. Before adding to the hotplate, 150 μL was removed from the neutral pH solution and centrifuged down at 21,000 × *g* for 15 min in a Legend Micro 21 Centrifuge (Thermo Fisher Scientific) benchtop centrifuge at 4 °C. The supernatant was removed and the particle pellet was frozen. At each time point, 150 μL was removed from each sample, centrifuged down, supernatant was removed and the pellet was frozen. Following all time points being collected, the samples were placed under N_2 to dry completely and were then dissolved in methanol. Each sample was placed into a 96-well plate and read on a plate reader at λ_{ex} 280 nm/λ_{em} 380 nm, and the percent of AR-12 retained within the MPs at each time point was calculated.

2.5. Drug tolerance assay on promastigotes

DsRED-expressing *L. donovani* (Lv82) were grown and maintained in Stat 4−/− BALB/c mice and promastigotes (log phase) were seeded at 1×10^6/1 mL in complete RPMI-1640 medium. Soluble AR-12 dissolved in DMSO and AR-12/MPs in media were added at varying concentrations. DMSO, media and empty MPs in media were added as negative controls. AmpB and Quillaja saponaria saponin (Saponin) were added as positive controls. Parasite viability was measured at 48 h by flow cytometry as previously described (Delgado et al., 2001).

2.6. Host cell-mediated leishmanicidal activity

Bone marrow-derived macrophages (BMDMs) (5×10^5) were grown on top of glass rounded cover slips, deposited in wells of a 24-well tissue culture plate (Corning, Inc., Tewksbury, MA). After adherence, macrophages were infected overnight with 3.5×10^6 stationary phase *L. donovani* promastigotes (ratio 1:7). Cells were washed with Hank's balanced salt solution (HBSS) three times to eliminate non-phagocytosed parasites. Test compounds were added to wells and allowed to incubate for 24 and 72 h. The cells were stained with Giemsa stain (Sigma) and for each time point, infection rates were calculated by counting the number of parasites per 200 macrophages on each slide, in triplicate, in a blinded fashion.

2.7. Infection and treatment

Six to eight week old BALB/c mice were purchased from Jackson Labs (Bar Harbor, ME, USA). All animal studies were approved by the Institutional Animal Care and Use Committee of The Ohio State University. All animals were housed in a sterile facility following Institutional Guidelines. *L. donovani* (Lv82) were grown and maintained in Syrian Golden Hamsters. Female BALB/c mice were infected with 1×10^7 *L. donovani* amastigotes by tail vein injection. Mice were treated with a 100 μl tail vein injection of PBS, empty MPs,

PEG 400:0.9% saline:ethanol (50:35:15) with and without AR-12, or 5 mg AR-12/MPs (31.5 μg AR-12/mouse/injection) on days 14 and 21 post infection. The PBS and PEG 400 groups contained 5 mice while all other groups contained 6 mice. Mice were sacrificed and analyzed on Day 28 post infection.

2.8. Estimation of parasite burden

Livers and spleens were harvested and weighed. Organs were sectioned to prepare impression smears, followed by Giemsa staining to enumerate the number of amastigotes per thousand nucleated cells. The parasite loads were calculated as Leishman-Donovan Units (LDU) (Number of amastigotes per 500 nucleated cells × organ weight (grams)). Bone marrow parasite burden was estimated as previously described (Peine et al., 2014). Bone marrow was extracted from the femur and used to generate smears. These bone marrow smears were stained with Giemsa and LDUs were generated similarly to spleen and liver.

2.9. Anti-leishmanial activity of AR-12/MPs in vivo using combinatorial therapies

To confirm efficacy in vivo, female BALB/c mice infected with *L. donovani* were treated at day 14 and 21 post-infection, with either PBS, AR-12/MPs, ampB deoxycholate alone, or AR-12/MPs in combination with sub-optimal or optimal doses of ampB deoxycholate. AR-12/MPs were administered via tail vein injection at a dose of 31.5 μg AR-12/mouse/injection, while ampB deoxy-cholate was injected intraperitoneally (IP) at indicated dose. SSG (500 mg/kg) was used as control and given in a single IP injection at day 14 post infection. The PBS group contained 5 mice and all other groups contained 4 mice. Organs were harvested at day 28 and tissue sections were used to make smears. LDU was determined as previously mentioned.

2.10. Histopathology

Tissue sections from the liver of *L. donovani* infected mice were collected on the day of harvesting and were stained with H&E for histopathological analysis. Hepatic granulomas were evaluated by a veterinarian pathologist, and enumerated and scored as follows: (1) no cellular response; (2) developing granuloma; or (3) mature granuloma as previously described (Murray, 2000). For every group, livers from at least 2–3 individual mice were analyzed.

2.11. Statistical analysis

Student's unpaired *t* test was used to determine statistical significance in the values. A value of $p < 0.05$ was considered significant. All values are represented in mean ± SD.

3. Results

3.1. AR-12/MP preparation

AR-12/MPs were created by emulsion chemistry via sonication. As measured by DLS, the size of the particles was determined to be 255 ± 45 nanometers (nm) (Fig. 1). Following formulation, AR-12/MPs were filtered tangential by flow filtration to remove un-encapsulated drug, then frozen and lyophilized. The particle yield was 71.3% and the

encapsulation efficiency was 31.5%, leading to a final weight loading of 0.0063 mg AR-12 per mg of particles (0.63% w/w).

3.2. AR-12/MP release profile

The ability of AR-12/MPs to retain AR-12 in neutral and acidic environments were analyzed (Fig. 4). At a pH of 7.4, AR-12 was slowly released from MPs with less than 15 and 30% released after 2 h and one day respectively. After one week in pH 7.4 conditions, roughly 60% of AR-12 was released from the MPs. Conversely, in a pH 5 environment, AR-12 was released quickly from MPs with close to 50 and 92% released after 2 h and one day respectively. After one week in pH 5 conditions, all of the AR-12 cargo had been released from the MPs.

3.3. *L. donovani* promastigote killing by soluble AR-12 and AR-12/MPs

The direct anti-leishmanial activity of soluble AR-12 and AR-12/MPs was screened in vitro at concentrations of 0.1, 0.5, 1.0 and 2.5 μM. Direct efficacy of both soluble AR-12 and AR-12/MPs was evaluated using dsRED *L. donovani* promastigotes (Fig. 2). Flow analysis showed both soluble AR-12 and AR-12/MPs did not show direct toxicity to *Leishmania* promastigotes. Soluble AR-12 showed parasite inhibition to be <15%, even at the highest drug concentration. AR-12/MPs showed <25% direct sensitivity at the highest drug concentration.

3.4. Amastigote killing in bone marrow derived macrophages with soluble AR-12 or AR-12/MPs

Both soluble AR-12 and AR-12/MPs showed dose-dependent and time-dependent anti-leishmanial activity. After 24 h of treatment with 0.5, 1.0 or 2.5 μM soluble AR-12, approximately 68%, 52% and 80% of intracellular *L. donovani* amastigotes were cleared, respectively. AR-12/MPs showed enhanced amastigote clearance, compared with soluble AR-12, (70%, 83%, and 90%) at 0.5, 1.0 or 2.5 μM, respectively (Fig. 3a). AR-12/MPs, at concentrations of 1 and 2.5 μM, showed significantly lower intracellular parasites in BMDMs, compared with soluble AR-12 ($p < 0.005$). Similarly, 72 h treatment with 0.5, 1.0 or 2.5 μM AR-12 showed amastigote clearance of 80%, 82% and 88% by soluble drug and 85%, 90% and 92% by AR-12/MPs, respectively (Fig. 3b).

3.5. Treatment of *L. donovani* with soluble AR-12 and AR-12/MPs in vivo

Mice treated with AR-12/MPs and soluble AR-12 on day 14 and 21 post infection showed significantly lower liver LDU compared to PBS ($p < 0.005$) (Fig. 5a). In addition to decreasing liver LDU, mice treated with AR-12/MPs showed significantly lower bone marrow parasite levels compared to PBS ($p < 0.005$) (Fig. 5b).

3.6. AR-12/MPs in synergy with current therapy results in parasite clearance in vivo

Mice were treated on Days 14 and 21 post-infection with either AR-12/MPs, ampB deoxycholate, or AR-12/MPs in combination with sub-optimal (1 mg/kg) or clinically effective (2.5 mg/kg) doses of ampB. Mice treated with AR-12/MPs + ampB 1.0 mg/kg showed significantly lower liver LDU than PBS ($p < 0.005$), AR-12/MPs ($p < 0.01$) and

ampB 1 mg/kg ($p < 0.005$) (Fig. 6a). Mice treated with AR-12/MPs + ampB 1.0 mg/kg showed significantly lower spleen LDU than PBS ($p < 0.005$), AR-12/MPs ($p < 0.01$), ampB 1.0 mg/kg ($p < 0.01$), amp 2.5 mg/kg ($p < 0.01$) and AR-12/MPs + ampB 2.5 mg/kg ($p < 0.001$) (Fig. 6b).

3.7. Effect of AR-12/MP combination with anti-leishmanial drugs on the formation of liver granulomas in *L. donovani* infected mice

Livers isolated from mice receiving both singular and combinatorial therapies were stained for histopathological analysis (Fig. 7). Mice treated with AR-12/MPs + ampB 1.0 mg/kg showed increased levels of developing granulomas than all other therapies ($p < 0.005$). Additionally, AR-12/MPs + ampB 1.0 mg/kg had a significantly lower incidence of no cellular response compared to the infected control ($p < 0.005$) and ampB 2.5 mg/kg ($p < 0.01$). Moreover, AR-12/MPs + ampB 1.0 mg/kg had significantly more mature granulomas than the infected control ($p < 0.005$) and ampB 2.5 mg/kg ($p < 0.05$).

4. Discussion

Leishmaniasis remains a significant public health concern with approximately 1.6 million cases occurring each year (CDC, 2013). Moreover, a lack of an effective vaccine, emergence of drug resistant strains, increasing rates of co-infection with human immunodeficiency virus and/or *Mycobacterium tuberculosis*, toxicity and cost of current chemotherapeutics are complicating the control of leishmaniasis (Burza et al., 2014). The development of new approaches to treat leishmaniasis is vital and host-mediated therapies are one avenue for drug development. We propose the use of the host-mediated therapeutic, AR-12 encapsulated within Ace-DEX MPs, for treatment of VL.

AR-12 has previously been shown to amplify the host response toward clearance of many infectious diseases; however, its therapeutic efficacy is limited by its relatively high toxicity and poor solubility (Hoang et al., 2014; Arno Therapeutics, 2009). To limit the toxicity and increase efficacy of AR-12, we have encapsulated it in the acid sensitive polymer Ace-DEX. The average size of AR-12/MPs was 255 nm (Fig. 1) which is beneficial for VL treatment, because prior research has shown that particles under 400 nm have increased accumulation in the bone marrow, which may serve as an important reservoir for *Leishmania* infection (Nagayasu et al., 1996; Tropia de Abreu et al., 2011). Additionally, AR-12/MPs are too large for non-phagocytic cells to internalize allowing them to passively target phagocytic cells which *Leishmania* sp. preferentially infect (Kima, 2007; Rejman et al., 2004).

Due to the acid sensitive nature of Ace-DEX, AR-12/MPs will efficiently retain AR-12 within the MP for extended periods of time in pH neutral environments. When AR-12/MPs are internalized through phagocytosis, acid sensitive AR-12/MPs will rapidly release AR-12 allowing for efficient intracellular delivery of AR-12. According to Fig. 4, after a week in a pH neutral environment (pH 7.4), roughly 40% of the AR-12 cargo is retained within AR-12/MPs. In an acidic environment (pH 5.0), representative of a phagosomal compartment, almost all of the AR-12 within AR-12/MPs is released in 24 h. The rapid intracellular release of a therapeutic which otherwise has a difficult time being internalized (Hoang et al., 2014), coupled with the localized delivery to cells which are preferentially

infected by *Leishmania* sp. (Kima, 2007; Rejman et al., 2004) highlight the advantages of using Ace-DEX as a delivery vehicle for intracellularly active therapeutics. In fact, the National Institute of Biomedical Imaging and Bioengineering (NIBIB) claim that an important area for future research in drug delivery involves enhancing intracellular release and response specific payload release (National Institute of Biomedical Imaging and Bioengineering, 2015) which Ace-DEX can provide.

Current therapeutics including ampB work effectively at killing *Leishmania* however, the specificity of action is also the cause of widespread resistance. AmpB works by targeting membrane sterols on the surface of the parasite resulting in enhanced membrane permeability (Saha et al., 1986). As seen in Fig. 2, ampB kills a majority of the promastigotes, whereas AR-12 has minimal effects. Conversely, amastigotes are cleared quite efficiently when treated with AR-12 and AR-12/MPs due to interaction with the host cells (Fig. 3). Although drug-pathogen interactions have not previously been reported with the usage of AR-12, there is a dose dependent increase in the percent inhibition with promastigotes in Fig. 2. Future studies will broach this concept to understand how AR-12 interacts with the promastigote form of the parasite and if this interaction has any clinical effect.

AR-12 is known to modulate host cell activity through a number of mechanisms including the inhibition of Akt pathway activation (Lo et al., 2014). In contrast, *Leishmania* has been shown to activate Akt signaling conferring host cell resistance to apoptosis, creating a favorable environment for the parasite to replicate and survive (Neves et al., 2010; Ruhland et al., 2007). There remains the potential that through inhibition of the Akt signaling pathway, AR-12 can effectively counteract the parasites survival mechanisms and assist in parasite clearance. AR-12 is also known to induce host cells to perform autophagy, a cellular recycling mechanism. Autophagy has recently been categorized as a central player within the innate immune system and was shown to be crucial against a number of intracellular and extracellular bacteria (Mitroulis et al., 2009). Conversely, autophagy has assisted survival in a number of parasites including *L. amazonensis* (Pinheiro et al., 2009). To date, there are no studies linking autophagy with assisting parasite clearance or parasite survivability in *L. donovani*; however, Ritis et al. have shown that autophagy is induced upon infection, indicating its role in the infection process (Pinheiro et al., 2009). Future work should focus on identifying the role of autophagy in AR-12 mediated clearance of *Leishmania*.

To further characterize the effect of AR-12 and AR-12/MPs on *L. donovani* infection, we intravenously injected mice 2 weeks post-infection. We observed that in comparison with soluble AR-12 treatment, therapy with AR-12/MPs resulted in a significantly lower parasite burden in the liver and bone marrow, showing dose sparing. Dose sparing is said to be one of the most beneficial and desirable traits of new therapies for VL due to a reduction in costs and toxic side effects (Singh and Sundar, 2014). AmBisome© is known to show dose sparing over soluble ampB, limiting the toxicity after each dose, which allows for more consistent dosing (Bellocchio et al., 2005). Another factor which could lead to the enhanced pharmacological effects of AR-12/MPs over the soluble drug is that the solubility of AR-12 is <1 mg/mL in water. Low drug solubility is known to limit the absorption and subsequently limit the pharmacological effects of the drug. While the AR-12/MPs were effective at

clearing the parasite by themselves, we wanted to explore a more advanced treatment using a combination of pathogen-mediated drugs and AR-12/MPs.

With the emergence of drug-resistant parasites, combinatorial therapies have become increasingly attractive therapeutic options. SSG, ampB, and miltefosine have all been evaluated as combinatorial therapies for VL in the clinic (Musa et al., 2012; Omollo et al., 2011). Sundar et al. have shown that combinations of sub-optimal anti-leishmanial compounds have the potential to be a more efficacious treatment with fewer side effects, however, combinatorial therapies using compounds that clear infections through non-host-mediated mechanisms are still at risk for cultivating resistant strains (Sundar et al., 2011). To determine if AR-12 has the potential to be used in combinatorial therapies, we evaluated parasite burden after treatment with AR-12/MPs and sub-optimal doses of ampB deoxycholate. In our study, mice infected with *L. donovani* were treated with either sub-optimal (1.0 mg/kg) or optimal (2.5 mg/kg) doses of ampB alone or in combination with AR-12/MPs. These therapeutic doses were compared to PBS or a high dose of SSG (~5 × clinical dose) as a positive control. In the combinatorial study, AR-12/MPs by themselves showed minimal parasitic load reduction within the liver, which is in contrast to our findings presented in Fig. 5. The overall liver parasite load in Fig. 6, as noted in the PBS control mice, is roughly five times that of Fig. 5 to ensure that we had adequate measurement of effects of the combination therapy on treatment. These data indicate that AR-12/MPs alone cannot control high parasite loads in the liver, but sub-optimal dosing of ampB with AR-12/MPs showed a more effective parasite clearance than either treatment alone, suggesting AR-12 has potential to be used in a combinatorial therapeutic protocol for severe or even highly-drug resistant VL cases. While AR-12/MPs alone were unable to reduce high parasite burdens within the liver, they were capable of lowering high parasite burdens within the spleen. Encouragingly, there was no statistical difference between sub-optimal ampB + AR-12/MPs and the positive control within the spleen. This is important because *Leishmania* has the ability to secrete suppressive cytokines, limiting the Th-1 inflammatory response within the spleen and thus, clearance of the parasite becomes more difficult. (Singh and Sundar, 2014). Interestingly, the dose dependent response seen in the liver when administering AR-12/MPs in combination with ampB was not seen within the spleen. This inconsistency could stem from a differential organ uptake of AR-12/MPs, leading to different concentrations of AR-12 released within the organs. Further studies utilizing trafficking of particles in vivo and using a wider range of doses are needed to elucidate the effectiveness of AR-12/MPs and the optimal concentrations for AR-12 synergy.

Another potential mechanism of AR-12 mediated parasite clearance is through the formation of granulomas. Developing granulomas have been shown to be important in treating VL by helping to wall off the infected cells (Murray, 2001). The addition of AR-12/MPs to the ampB treatments showed an increase in the number of mature granulomas and developing granulomas, with sub-optimal ampB + AR-12/MPs showing the highest number of developing granulomas throughout all of the groups. The creation of a pro-granuloma environment, as seen with AR-12/MPs in these experiments, can effectively increase the efficacy of long term treatments. AR-12 has not previously been shown to increase granuloma formation and thus additional studies should be performed to analyze AR-12′s dose dependent ability to stimulate granuloma formation.

5. Conclusion

Overall, AR-12/MPs show great promise, especially as a combinatorial therapy, for the clearance of VL. We illustrated in a mouse model of VL, using *L. donovani*, that AR-12/MPs given IV, are capable of decreasing liver and spleen LDUs, as well as bone marrow parasite burden. This parasite clearance was enhanced after addition of ampB to the therapeutic regimen, illustrating not only parasite clearance in a mouse model but also increasing numbers of granulomas with AR-12/MPs and co-treatment. Furthermore, preferential clearance of in vitro amasitgotes suggests AR-12 efficacy occurs through host-mediated mechanism, which should limit the emergence of drug resistant strains. Future work should evaluate the mechanism through which AR-12 alone, as well as AR-12 with ampB clear VL in mice and other animal models of disease.

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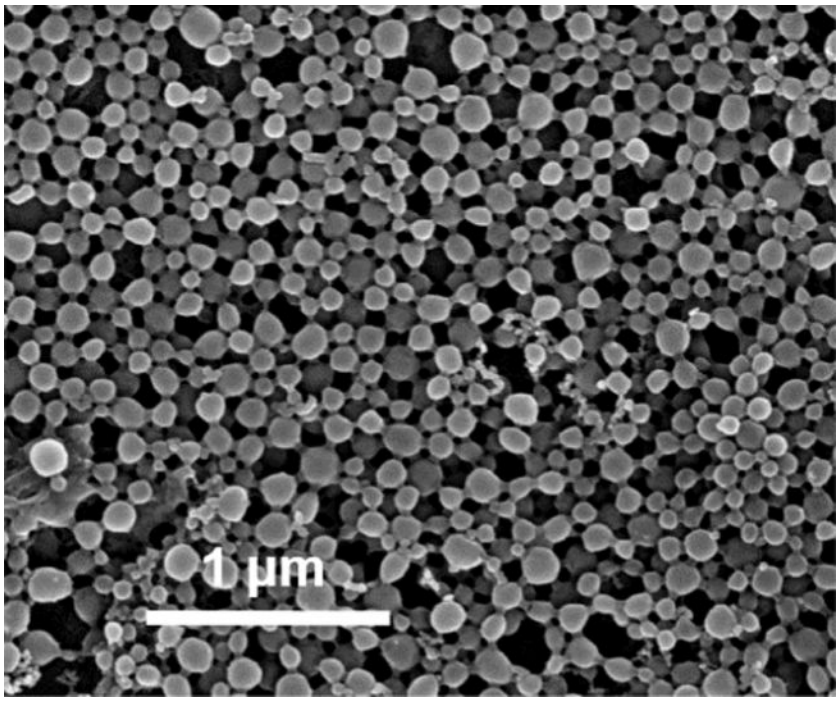

Fig. 1.
Scanning electron micrograph of acetalated dextran microparticles encapsulating AR-12 (AR-12/MPs).

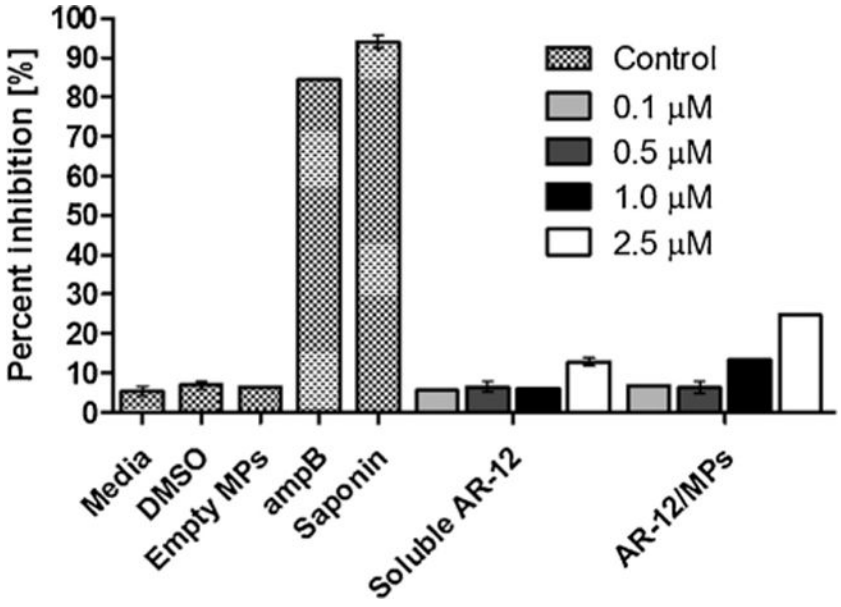

Fig. 2.

In vitro clearance of *L. donovani* promastigotes by soluble AR-12 or AR-12 microparticles (AR-12/MPs). Fluorescently labeled dsRED *L. donovani* promastigotes were cultured with media, dimethylsulfoxide (DMSO), amphotericin B deoxycholate (ampB) (0.15 mg/mL), saponin (positive control), or soluble AR-12 and AR-12/MPs at varying concentrations. Promastigotes were cultured under these conditions for 72 h and inhibition was measured via flow cytometry.

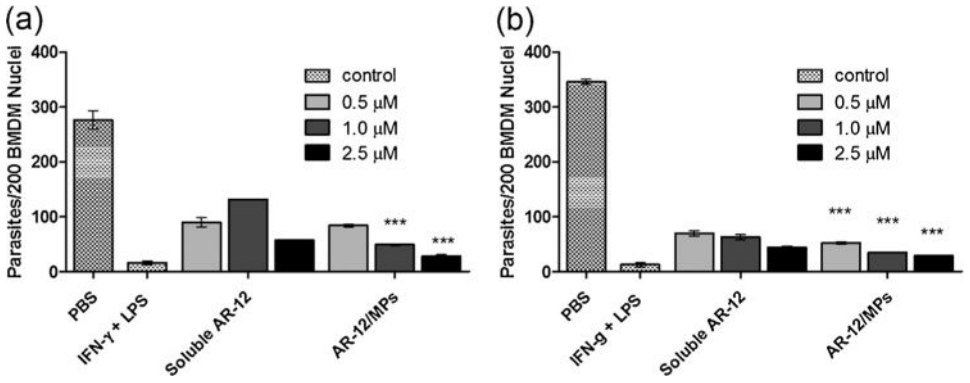

Fig. 3.

In vitro clearance of *L. donovani* amastigotes in bone marrow derived macrophages. Amastigotes were cultured with PBS, interferon γ (IFN-γ) with lipopolysaccharide (LPS), soluble AR-12 or AR-12 microparticles (AR-12/MPs). Amasitgote viability was measured at (a) 24 and (b) 72 h by staining with Giemsa and counting under a light microscope. Three asterisks, $p < 0.005$ comparing soluble AR-12 to AR-12/MPs at their respective concentrations.

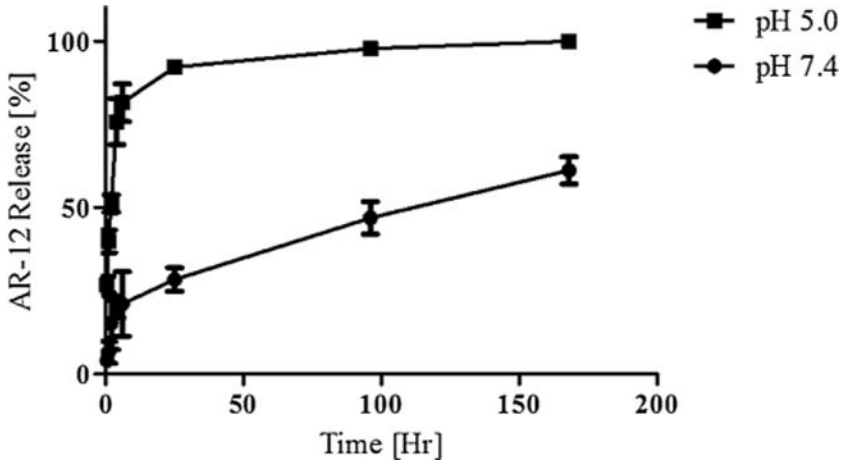

Fig. 4.

Release profile of AR-12 from AR-12 microparticles (AR-12/MPs). Briefly, AR-12/MPs and Empty MPs were suspended in pH 7.4 and pH 5.0 buffers and placed on a shaker hot plate at 37 °C. At each time point, samples were removed from the tube and spun down to pellet the remaining particles. After all time points were collected, remaining particles were degraded and residual AR-12 was detected. Data are plotted as average (n = 3) ± standard deviation.

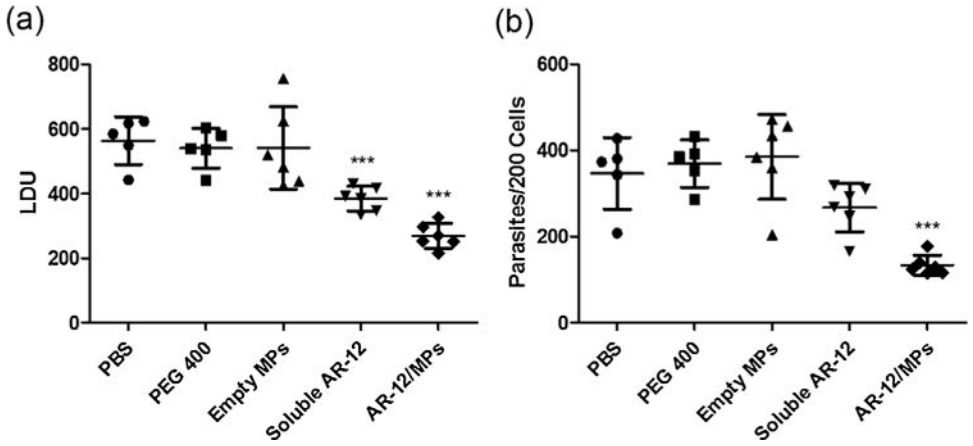

Fig. 5.

Leishman-donovani units (LDUs) of the (a) liver and (b) bone marrow of BALB/c female mice infected with *L. donovani*. Mice were treated 14 and 21 days post-infection with tail vein injections of PBS, polyethylene glycol (PEG) 400 (vehicle control for soluble AR-12), soluble AR-12, or AR-12 microparticles (AR-12/MPs) at a dose of 31.5 μg AR-12/mouse/injection. Mice were sacrificed 28 days post-infection and LDUs were determined using the number of amastigotes/500 nuclei × organ weight (grams). Bone marrow counts were determined with smears stained with Giemsa. $n = 6$ for Empty MPs, soluble AR-12 and AR-12/MPs while $n = 5$ for PBS and PEG 400. Three asterisks, $p < 0.005$ with respect to PBS.

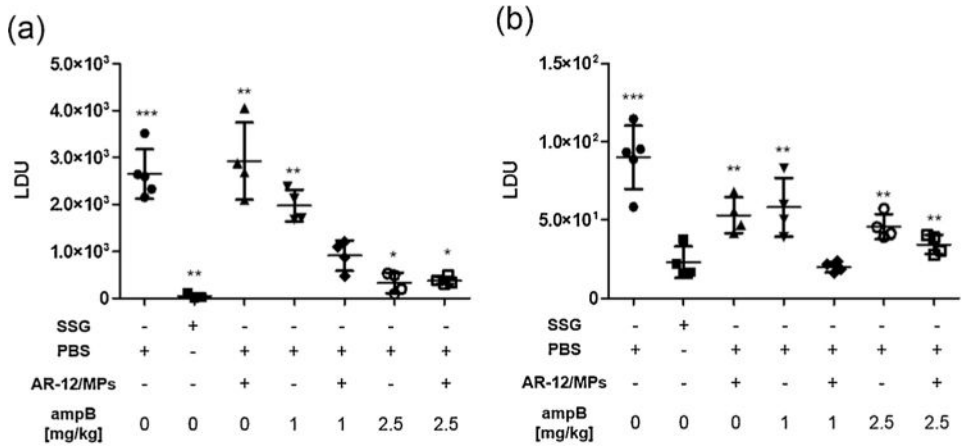

Fig. 6.

Leishman-Donovani Units (LDUs) in the (a) liver and (b) spleen of BALB/c female mice infected with *L. donovani*. Mice were treated 14 and 21 days post-infection with tail vein injections of PBS, sodium stiboglucontate (SSG), AR-12 microparticles (AR-12/MPs), amphotericin B deoxycholate (ampB) 1.0 mg/kg, AR-12/MPs + ampB 1.0 mg/kg, ampB 2.5 mg/kg and AR-12/MPs + ampB 2.5 mg/kg. All AR-12/MPs were administered at a dose of 31.5 μg AR-12/mouse/injection. Mice were sacrificed 28 days post-infection and LDUs were determined using the number of amastigotes/500 nuclei × organ weight (grams). $n = 5$ for PBS and $n = 4$ for SSG, AR-12/MPs, ampB 1.0 mg/kg, AR-12/MPs + ampB 1.0 mg/kg, ampB 2.5 mg/kg and AR-12/MPs + ampB 2.5 mg/kg. One asterisk, $p < 0.05$, two asterisks, $p < 0.01$, three asterisks, $p < 0.005$ with respect to AR-12/MPs + ampB 1.0 mg/kg.

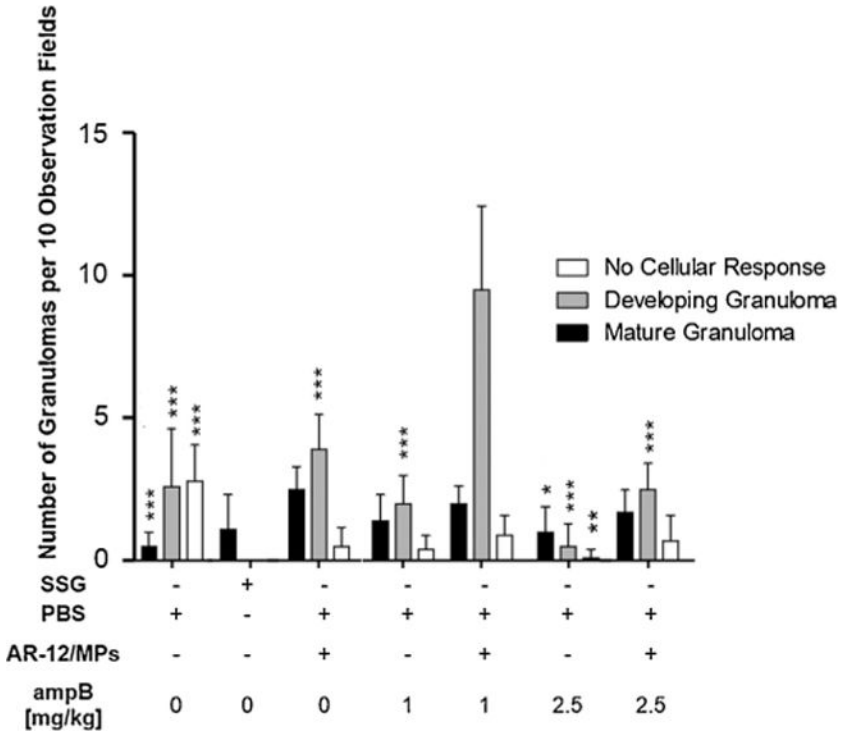

Fig. 7.

Liver granuloma scores from *L. donovani* infected BALB/c females treated 14 and 21 days post infection with PBS, sodium stibogluconate (SSG), AR-12 microparticles (AR-12/MPs), amphotericin B deoxycholate (ampB) 1.0 mg/kg, AR-12/MPs + ampB 1.0 mg/kg, ampB 2.5 mg/kg and AR-12/MPs + ampB 2.5 mg/kg. Mice were scored for granuloma formation. Results were from 2 to 3 individual mouse samples from each group. Data were expressed as mean granuloma number per 10 high-power fields (magnification 400×). 1 asterisk $p < 0.05$, 2 asterisks $p < 0.01$, 3 asterisks $p < 0.005$ compared to the respective granuloma stage for the AR-12/MPs + ampB 1.0 mg/kg treatment group.