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An amphotericin B-based drug for treating experimental *Leishmania major* infection[☆]

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Abstract

There is an urgent need for a non-toxic and low-cost treatment for cutaneous leishmaniasis. We synthesised and tested in vivo an amphotericin B-poly(methacrylic acid) drug (AmB-PMA) that had previously shown in-vitro activity against *Leishmania major* and *L. donovani* parasites. Efficacy was determined using *L. major* footpad infection in 30 non-healing BALB/c mice. Three subcutaneous injections of AmB-PMA at days 7, 14 and 21 post-infection resulted in a reduction of ~80% in lesion size by day 35 post-infection in 18 treated mice compared with six untreated controls and complete healing of lesions by day 50 with no lesion relapse seen at day 80 post-infection in six treated mice. Healing was associated with decreased IL-10 ($P = 0.002$) and increased IFN- γ ($P = 0.005$) in the footpad.

Keywords

Leishmania major; Cutaneous leishmaniasis; Pharmacological therapy; Immunomodulatory therapy; Amphotericin B; Poly(methacrylic acid)

Introduction

Cutaneous leishmaniasis (CL) is caused by protozoan parasites of the species *Leishmania*. Transmitted through the bite of female phlebotomine sand flies, *Leishmania* preferentially infect, multiply and persist in host macrophages. This neglected parasitic disease is endemic in over 70 tropical countries and presents as disfiguring and deforming skin lesions. To date, there is no effective vaccine, and drug therapy is limited by toxicity, increasing resistance, and

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Authors' contributions: SS and IM conceived the study; KDC, MR, IT and IM performed the experiments and analysed the data; All authors contributed to data interpretation and manuscript preparation and revision. All authors read and approved the final manuscript. SS is guarantor of the paper.

Conflicts of interest: None declared.

Ethical approval: All animal studies were approved by the UK Home Office.

long treatment regimens that increase costs to patients. Although deoxycholate-amphotericin B (Fungizone, Bristol-Myers Squibb) and liposomal amphotericin B (AmBisome, Gilead) are effective at treating visceral leishmaniasis (VL), they are ineffective against *L. major*-induced CL.^{1,2}

Resolution of a leishmanial infection is dependent upon: (i) the development of an adequate Th1 immune response; and (ii) production of IFN- γ to activate antiparasiticidal pathways in infected macrophages. BALB/c mice are genetically susceptible to leishmanial infection and cannot control parasite growth. This is due to a dominant Th2 response that involves upregulation of IL-10, and an inability to activate infected macrophages to kill intracellular parasites. The result is persistent and progressive non-healing disease.³

We synthesised and tested a new amphotericin B-poly(methacrylic acid) drug (AmB-PMA) and determined its efficacy against *L. major* footpad infection in BALB/c mice. In this animal model, polarisation and establishment of parasite-specific T-helper cell responses leads to a non-healing disease phenotype.

Methods

Thirty BALB/c mice were infected in the footpad with 2×10^6 *L. major* LV39 (MRHO/SU/59/P-strain) stationary phase promastigotes on day 0. Twenty-four mice were injected subcutaneously with AmB-PMA (reconstituted in water) on days 7, 14 and 21 post-infection. A total of 400 μ g AmB (6 mg AmB/kg) was administered to the site of infection. Six control mice received water injections. Lesion size was monitored twice weekly using a vernier calliper. Eighteen treated and six control mice were culled at day 35 and the lesion's parasite load determined by ex-vivo counting; footpads were homogenised through a metal sieve in PBS and parasites counted using a haemocytometer. Another six mice continued to be monitored twice weekly until day 80 and were then culled. Cytokine mRNA levels were determined by quantitative real-time PCR. RNA was extracted from homogenised footpads with Tri Reagent (Sigma, Dorset, UK) and reverse transcribed with a Qiagen RT kit (Qiagen, Crawley, UK). Quantitative RT-PCR was performed in triplicate using Sybr Green Jumpstart PCR mix (Sigma) on a Corbett thermocycler. A multigene plasmid containing cloned gene sequences of IL-10, IFN- γ and HPRT (housekeeping gene) was used to determine the absolute mRNA copy number, and the results normalised for HPRT. The primer sequences were: IL-10 (forward) GGTTGCCAAGCCTTATCGGA and (reverse) ACCTGCTCCACTGCCTTGCT; IFN- γ (forward) TCAAGTTCAAGTGGCATAGATGTGG and (reverse) TGGCTCTGCAGGTTTTTCATG; HPRT (forward) GTAATGATCCAGTCAACGGGGGAC and (reverse) GCAAGCTTGCAACCTTAACCA.

Results are given as the mean $\pm$ SEM. *P*-values were determined using a Mann-Whitney *U* test (GraphPad Prism, San Diego, CA, US).

Results

The three injections of AmB-PMA on days 7, 14 and 21 led to an $82 \pm 2\%$ reduction in CL lesion size by day 35 post-infection in 18 treated mice compared with the six untreated mice. This was accompanied by a $99.85 \pm 0.03\%$ reduction in the number of viable parasites. In another six treated mice, there was complete lesion healing by day 50 with no lesion relapse seen at day 80 post-infection. Quantitative mRNA PCR-based analysis of the footpads at day 35 post-infection showed a 30-fold decrease in IL-10 ($P = 0.002$) and a 6-fold increase in IFN- γ ($P = 0.005$) in treated, infected footpads compared with untreated, infected footpads (Figure 1).

Discussion

Our results show that AmB-PMA administered subcutaneously induced complete healing in genetically susceptible BALB/c mice. There was a substantial reduction in lesion size and parasite elimination in this mouse footpad model of *L. major* infection. Cure was associated with reduced IL-10 and increased IFN- γ levels in treated, infected footpads compared with untreated, infected footpads.

A Th1 response enhances the healing of cutaneous lesions because it promotes macrophage activation via production of TNF- α and IFN- γ . It also increases iNOS production. Collectively, this leads to parasite killing.^{3,4,5} Cytokine analysis of the lesion site showed reduced IL-10 and increased IFN- γ .

Our results show that the pharmacologically mediated killing of amastigotes by AmB-PMA was associated with additional host-mediated immune responses that facilitated cure of *L. major*-induced CL lesions.^{4,5} This cost-effective AmB-PMA drug has the potential to be suitable for treating CL in low-income countries and further studies are needed.

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Figure 1.

Quantitative real-time mRNA RT-PCR for IL-10 and IFN- γ in the *Leishmania major*-infected footpads of six untreated and eighteen amphotericin B-poly(methacrylic acid) drug-treated mice at day 35 post-infection.