Chemotherapeutic Potential of 17-AAG against Cutaneous Leishmaniasis Caused by Leishmania (Vianna) braziliensis

Diego M. Santos1,2*, Antonio L. O. A. Petersen1, Fabiana S. Celes1, Valeria M. Borges1,2, Patricia S. T. Veras1, Camila I. de Oliveira1,2*

1 Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), Salvador, Bahia, Brazil, 2 Instituto Nacional de Ciência e Tecnologia de Investigação em Imunologia (iii-INCT), Salvador, Bahia, Brazil

Abstract

**Background:** Leishmaniasis remains a worldwide public health problem. The limited therapeutic options, drug toxicity and reports of resistance, reinforce the need for the development of new treatment options. Previously, we showed that 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), a Heat Shock Protein 90 (HSP90)-specific inhibitor, reduces *L. (L.) amazonensis* infection *in vitro*. Herein, we expand the current knowledge on the leishmanicidal activity of 17-AAG against cutaneous leishmaniasis, employing an experimental model of infection with *L. (V.) braziliensis*.

**Methodology/Principal findings:** Exposure of axenic *L. (V.) braziliensis* promastigotes to 17-AAG resulted in direct dose-dependent parasite killing. These results were extended to *L. (V.) braziliensis*-infected macrophages, an effect that was dissociated from the production of nitric oxide (NO), superoxide (O\(_2^-\)) or inflammatory mediators such as TNF-α, IL-6 and MCP-1. The leishmanicidal effect was then demonstrated *in vivo*, employing BALB/c mice infected with *L. braziliensis*. In this model, 17-AAG treatment resulted in smaller skin lesions and parasite counts were also significantly reduced. Lastly, 17-AAG showed a similar effect to amphotericin B regarding the ability to reduce parasite viability.

**Conclusion/Significance:** 17-AAG effectively inhibited the growth of *L. braziliensis*, both *in vitro* and *in vivo*. Given the chronicity of *L. (V.) braziliensis* infection and its association with mucocutaneous leishmaniasis, 17-AAG can be envisaged as a new chemotherapeutic alternative for cutaneous Leishmaniasis.

Citation: Santos DM, Petersen ALOA, Celes FS, Borges VM, Veras PST, et al. (2014) Chemotherapeutic Potential of 17-AAG against Cutaneous Leishmaniasis Caused by Leishmania (Vianna) braziliensis. PLoS Negl Trop Dis 8(10): e3275. doi:10.1371/journal.pntd.0003275

Editor: Ben L. Kelly, Louisiana State University, United States of America

Received: March 11, 2014; Accepted: September 16, 2014; Published: October 23, 2014

Copyright: © 2014 Santos et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data are included within the manuscript.

Funding: This work was funded by grants from CNPq and FIOCRUZ. DMS and ALOAP received CAPES fellowships. FSC received a FIBIC/FIOCRUZ fellowship. VMB, PSTV and CiGo are senior investigators from CNPq. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: camila@bahia.fiocruz.br

* Current address: Universidade Federal de Sergipe, Departamento de Educação em Saúde, Lagarto, Sergipe, Brazil

Introduction

Leishmaniasis is a widespread group of parasitic diseases caused by protozoa of the genus *Leishmania*, that is transmitted by the bite of female sand flies. Currently, about 12 million people are at risk of leishmaniasis and there are an estimated 1.5–2 million new cases each year [1]. There are two main clinical manifestations: visceral leishmaniasis, affecting mainly the spleen and liver and cutaneous leishmaniasis, affecting the skin. CL caused by *Leishmania (V.) braziliensis* is particularly distinguished from other leishmaniasis by its chronicity, latency and tendency to metastasize in the human host [2]. In 1–5% of patients, mucocutaneous leishmaniasis may develop due to the ability of *L. (V.) braziliensis* to persist within lesion scars after spontaneous or chemotherapy-mediated healing and to its ability to metastasize to the nasal mucosal [3,4]. In this case, extensive tissue destruction is observed, resulting from the potent cell-mediated immune response triggered by parasite replication [5]. More rarely, parasite invasion of the bloodstream results in disseminated skin lesions [6]. Brazil along with nine other countries account for 70–75% of the global estimated CL incidence [7].

The drugs of first choice for leishmaniasis chemotherapy are Pentavalent Antimonials (Sb\(_5^3\)) [8], which interfere with the oxidative metabolism of intracellular *Leishmania* [5,9,10]. These compounds are significantly toxic and have been associated with drug resistance [11,12]. Amphotericin B and Paramomycin, two other drugs available [13–15], also display limitations with regards to toxicity, cost and/or duration of treatment [16]. In the current scenario, the identification of new chemotherapeutic compounds is urgently needed, especially since vaccines against leishmaniasis are not yet available.

Heat Shock Proteins (HSPs) form complexes that act as chaperones, binding other proteins, denominated client proteins. These multimeric complexes are involved in regulating
Author Summary
Antimony-containing compounds are the main drugs used to treat leishmaniasis but the severe associated side effects pose the need for alternative chemotherapeutic options. Herein, we evaluated the ability of 17-AAG (a Heat Shock Protein 90 inhibitor) to kill Leishmania (Viannia) braziliensis parasites, a species that causes both cutaneous and mucocutaneous Leishmaniasis in Brazil. Heat Shock Protein 90 (HSP90) is associated with important biological processes; inhibition of this molecule interferes with parasite survival and, hence, it can be exploited as a chemotherapeutic target. We show that exposure to 17-AAG induced killing of L. braziliensis parasites in both its extracellular and intracellular forms. This effect was not dependent on the activation of the host cell. More importantly, treatment of mice infected with L. (V.) braziliensis also modulated lesion development and decreased parasite growth at the infection site. Collectively, our results show that targeting HSP90 is a promising alternative for development of novel chemotherapeutic options for leishmaniasis.

protein folding, intracellular protein transport and repair or degradation of proteins partially denatured due to stress, for example [17,18]. Among the HSPs, HSP90 is one of the most abundant cellular chaperones and many of its client proteins are involved in cell signaling, proliferation and survival [19]. It is essential for oncogenic transformation and exploited by malignant cells to support cancer-associated kinases and transcription factors [20]. HSP90 also plays an important role in protozoans such as Leishmania and Trypanosoma, which critically rely on HSP90 for survival in alternating environments associated with their complex life cycles [21]. Therefore, HSP90-inhibitors become interesting candidates for leishmaniasis chemotherapy.

Treatment of L. donovani parasites with geldanamycin (GA), a HSP90-specific inhibitor, arrested promastigote growth and differentiation into amastigotes [22]. It also reduced glutathione levels, increasing the production of reactive oxygen species (ROS) and promoting apoptosis [23]. Recently, we reported on the effects of 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) on L. (L.) amazonensis [24]. 17-AAG is a HSP90-specific inhibitor analogous to geldanamycin (GA) [25]. Macrophages infected with L. (L.) amazonensis and treated with a low dose of 17-AAG displayed significantly smaller parasite loads, an effect that was not mediated by activation of the macrophage inflammatory response [24].

In the present work, we expanded our previous observations to the effects of 17-AAG on L. (V.) braziliensis, the etiological agent of both cutaneous and mucocutaneous leishmaniasis in Brazil. Experiments were performed in vitro and in vivo, employing an experimental model [26]. 17-AAG was efficient at reducing L. (V.) braziliensis promastigote growth and macrophage infection. More importantly, 17-AAG was equally efficient in vivo, highlighting its potential as a novel chemotherapeutic agent against CL caused by L. (V.) braziliensis.

Methods
Ethics statement
Female BALB/c mice, 6–8 weeks of age, were obtained from CPqGM/FIOCRUZ animal facility where they were maintained under pathogen-free conditions. All animal work was conducted according to the Guidelines for Animal Experimentation of the Colégio Brasileiro de Experimentação Animal and of the Conselho Nacional de Controle de Experimentação Animal. The local Ethics Committee on Animal Care and Utilization (CEUA) approved all procedures involving animals (CEUA-L001/12-CpQGM/FIOCRUZ).

17-AAG and amphotericin B
17-AAG (17-(allylamino)-17-demethoxygeldanamycin) (Invivo-gen) was dissolved in Dimethyl sulfoxide (DMSO) (SIGMA) to a 5 mM stock solution, stored at -20°C in aliquots. For in vitro use, the stock solution was diluted in cell culture medium to the desired concentration at the time of use. For in vivo treatments, a stock solution was prepared at 100 mg/ml and diluted to 20 mg/kg at the time of use. Amphotericin B (Fungizone, Life Technologies) was dissolved in DMEM medium to a 250 ug/ml stock solution. The stock solution was diluted in cell culture medium to the desired concentration at the time of use.

Parasite culture
L. (V.) braziliensis (MHOM/BR/01/BA788) [26] was cultured at 26°C in Schneider’s insect medium (Invitrogen) supplemented with 10% inactive Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (all from Invitrogen).

L. (V.) braziliensis promastigotes viability assay
Axenic L. (V.) braziliensis promastigotes (1×10⁶ parasites/ml), cultivated in supplemented Schneider medium, were treated with increasing concentrations of 17-AAG (25, 75, 250, 500, 100 U/ml penicillin, and 100 mg/ml streptomycin (all from Invitrogen). For some experiments, promastigotes were treated with the half maximal inhibitory concentration (IC₅₀) (65 nM). After 48 h, parasites viability was evaluated by direct counting of live motile parasites using a Neubauer chamber. In some experiments, promastigotes were washed three times with PBS and were further cultured for 24 and 48 h in supplemented Schneider medium, devoid of 17-AAG. The number of viable promastigotes was determined by direct counting.

Macrophage infection with L. (V.) braziliensis and treatment with 17-AAG
BALB/c mice were injected i.p. with 3% thioglycolate. Five days after injection, peritoneal lavage was performed using 8 ml DMEM medium supplemented with 10% Fetal Calf Serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen). To obtain monolayers, cells (6×10⁵ cells/ml) were placed into glass coverslips within the wells of a 24-well plate and were left to adhere for 2 h, at 37°C and 5% CO₂. Non-adherent cells were removed by gentle and extensive washing with PBS; purity was routinely above 99%. Remaining cells (3×10⁵ cells/ml) received 5×10⁵ cells/ml of stationary-phase L. (V.) braziliensis promastigotes and were incubated at 37°C in supplemented DMEM medium. After 24 h of infection, glass coverslips containing infected macrophages were washed to remove non-internalized parasites and cells were treated with different concentrations of 17-AAG (25, 100, 250 and 500 nM) for 12–72 h. Control groups were incubated in supplemented DMEM medium containing DMSO only. Glass coverslips were washed and stained with H&E and the intracellular amastigotes were counted by light microscopy. The results are shown as the percentage of infected cells and the number of intracellular amastigotes was counted in 400 macrophages. Cultures were performed in quintuplicate. Alternatively, infected macrophages were washed extensively and the medium was replaced with...
0.5 ml of supplemented Schneider medium, devoid of 17-AAG. Cells were cultured at 26 °C for an additional 5 days and the number of viable parasites was determined by direct counting. In some experiments, infected macrophages were treated with the half maximal inhibitory concentration (IC_{50}) (220 nM) of 17-AAG or with amphotericin B (0.25 μg/ml; 0.27 μM) for 24 h. Parasite viability was determined by direct counting.

**Viability of 17-AAG-treated murine macrophages**

Macrophages (2 × 10^6 cells/ml), obtained as above, were treated increasing concentrations of 17-AAG (39-20,000 nM) or with DMSO for 72 h. Next, cultures were washed twice cells were incubated with supplemented DMEM containing 10% Alamar-Blue (Invitrogen). Cells were incubated for another 4 h and reagent absorbance was measured at the wavelengths of 570 nm and 600 nm using a spectrophotometer (SPECTRA Max 340 PC). Ethanol-fixed cells were used as positive controls.

**Detection of NO, reactive oxygen species, cytokines and chemokines**

Macrophages (3 × 10^6 cells/ml), obtained as above, were stimulated with IFN-γ (100 U/ml) (Sigma) and were infected with *L. (V.) braziliensis* (3 × 10^5 cells/ml) for 24 h. Macrophage cultures were then washed to remove non-internalized parasites and fresh culture medium containing IFN-γ and 220 nM of 17-AAG was added. Cultures supernatants were collected 48 h later. Griess reaction was used to measure nitric oxide (NO) production by determining concentration of its stable reaction product nitrite (NO_2^-) [27]. Superoxide (SO) production was determined by adding hydroxyamine (Sigma) (0.5 mM) [28,29] to infected macrophages. Hydroxyamine converts superoxide into nitrite, which is then be quantitated by the Griess reaction, as described above. Background levels of nitrite generated by the release of NO were determined in parallel, without the addition of hydroxylamine. Production of TNF-α, IL-6, IL-10 and CCL2/MCP-1 was determined in parallel, without the addition of hydroxylamine, which is then be quantitated by the Griess reaction, as described above. Background levels of nitrite generated by the release of NO were determined in parallel, without the addition of hydroxylamine. Production of TNF-α, IL-6, IL-10 and CCL2/MCP-1 was determined in parallel, without the addition of hydroxylamine, which is then be quantitated by the Griess reaction, as described above. Background levels of nitrite generated by the release of NO were determined in parallel, without the addition of hydroxylamine. Production of TNF-α, IL-6, IL-10 and CCL2/MCP-1 was determined in parallel, without the addition of hydroxylamine, which is then be quantitated by the Griess reaction, as described above. Background levels of nitrite generated by the release of NO were determined in parallel, without the addition of hydroxylamine. Production of TNF-α, IL-6, IL-10 and CCL2/MCP-1 was determined in parallel, without the addition of hydroxylamine. Production of TNF-α, IL-6, IL-10 and CCL2/MCP-1 was determined in parallel, without the addition of hydroxylamine.

**Intradermal infection with *L. (V.) braziliensis* and in vivo treatment with 17-AAG**

BALB/c mice were inoculated with stationary-phase *L. (V.) braziliensis* promastigotes (10^6 parasites in 10 μl of saline) in the left ear dermis using a 27.5-gauge needle. Four weeks post-infection, mice (n = 10) were treated 3 times/wk for 3 weeks with 17-AAG (20 mg/kg of 17-AAG diluted in DMSO i.p.). The control group (n = 10) received i.p. injections of DMSO in the same concentrations used in 17-AAG treated animals (n = 10). Lesion size was monitored weekly for 10 weeks using a digital caliper (Thomas Scientific). Parasite load was determined using a quantitative limiting-dilution assay as described elsewhere [26]. Briefly, infected ears and lymph nodes draining the infection site were aseptically excised six weeks post-infection and homogenized in Schneider medium. Homogenates were serially diluted in supplemented Schneider complete and seeded into 96-well plates. The number of viable parasites was determined from the highest dilution at which the promastigotes could be grown after up to 2 weeks of incubation at 26 °C.

**Statistical analysis**

The half maximal inhibitory concentration (IC_{50}) of 17-AAG on *L. braziliensis* promastigotes and on intracellular *L. braziliensis* amastigotes were determined from sigmoidal regression of the concentration–responses curves, respectively, using Prism (GraphPad Prism V. 6.0). The selectivity index of 17-AAG was calculated as the ratio between the CC_{50} for murine macrophages and the IC_{50} for intracellular *L. braziliensis* amastigotes.

Data are presented as the mean ± standard error of the mean. Kolmogorov-Smirnov was used for normality analysis. Parametric (One-way ANOVA followed by Dunnett’s Multiple Comparison Test or post-test for linear trend or by Bonferroni) or non-parametric analysis (Mann-Whitney) tests were also performed using Prism (GraphPad software, V. 6.0). To evaluate disease burden in mice, ear thickness of mice treated with 17-AAG or DMSO was recorded weekly for each individual mouse. The course of disease for 17-AAG-treated and control mice was plotted individually, and the area under each resulting curve was calculated using a non-parametric test (Mann-Whitney). p-values ≤ 0.05 were considered significant.

**Results**

**Exposure to 17-AAG reduces the viability of *L. (V.) braziliensis* promastigotes**

Initially, we investigated the effects of 17-AAG on axenic *L. (V.) braziliensis* promastigotes. Parasites were incubated with increasing concentrations of 17-AAG for 48 h and viability was quantified by direct counting. All 17-AAG-treated cultures showed a significantly lower number of parasites in comparison to the control, treated with vehicle (DMSO) alone (Figure 1A) (One-way ANOVA, p < 0.001). After 48 h of treatment, 17-AAG (25 nM) reduced parasite viability by 13% (Figure 1A) (compared to DMSO-treated cultures) and increasing concentrations of 17-AAG (75–625 nM), maximized killing effects. At the highest concentration tested (625 nM) parasite viability was reduced by 98% when compared to DMSO-treated cultures (Figure 1A). These results also indicate that parasite viability was reduced in a dose-dependent effect (One-way ANOVA, p < 0.001 followed by test for linear trend p < 0.001). The DMSO concentration used was not toxic as parasite viability was similar in cultures left untreated (Lb) or treated with DMSO only (Figure 1A). Based on these results, IC_{50} after 48 h of 17-AAG treatment, was established at 65 nM (Figure S1). To evaluate whether the effect of 17-AAG on *L. (V.) braziliensis* promastigotes was reversible, parasites were treated with 17-AAG (65 nM) for 48 h, washed and subsequently re-incubated in 17-AAG-free medium, for an additional 24 and 48 h. Parasite numbers were significantly reduced (p < 0.01) in cultures kept for both 24 h (Figure 1B) and 48 h (Figure 1C). These results show that the effect of 17-AAG on *L. (V.) braziliensis* promastigotes is irreversible.

**17-AAG reduces parasite load in *L. (V.) braziliensis*-infected macrophages**

Next, we investigated the effect of 17-AAG on intracellular *L. (V.) braziliensis* amastigotes. BALB/c macrophages were infected with *L. (V.) braziliensis* and cells were treated with a range of 17-AAG concentrations (25–500 nM) for 12–72 h. At each time point, cells were fixed and the parasite load was assessed by light microscopy. At the initial time points (12 and 24 h), we did not detect significant alterations in treated cultures versus control cultures (DMSO-treated) (Figure 2A and B). After 48 h, 17-AAG (25 nM) reduced the infection rate to 85% (Figure 2A) and intramacrophage amastigotes were reduced to 82% (compared to the percentages obtained in DMSO-treated cultures) (Figure 2B). With increasing concentrations of 17-AAG (100–500 nM), these effects became more pronounced and, at 500 nM, the percentage of infected cells was reduced to 63% (Figure 2A) and of...
intracellular amastigotes to 43% (Figure 2B) (One-way ANOVA, \( p < 0.001 \)) (again compared to the percentages obtained in DMSO-treated cultures). After 72 h of treatment, these effects were maximal: 500 nM of 17-AAG decreased the infection rate to 20% (Figure 2A) whereas intracellular amastigotes were reduced to 11% (Figure 2B). The absolute percentages of infection, following treatment with different concentrations of 17-AAG and the absolute numbers of amastigotes/100 cells over time are shown in Figures S2A and S2B, respectively. As with promastigotes (Figure 1A), effects observed with 17-AAG on intracellular macrophages were dose-dependent (One-way ANOVA, \( p < 0.001 \) followed by test for linear trend \( p < 0.001 \)). IC\(_{50}\), after 72 h of 17-AAG treatment, was determined as 220 nM (Figure S3); additionally, 17-AAG employed at different concentrations (125, 220 and 500 nM) did not compromise macrophage viability as assayed by MTT (Figure S4). Cytotoxicity against murine macrophages was determined upon treatment of non-infected macrophage cultures with 17-AAG with a calculated CC\(_{50}\) of 3.6 nM. The selectivity index of 17-AAG was established at 16.6.

17-AAG reduces the viability of intracellular \( L. (V.) \) braziliensis amastigotes

Although 17-AAG treatment of \( L. (V.) \) braziliensis-infected macrophages for 24 h did not significantly modify the infection rate (Figure 2), we asked whether it would alter amastigote viability. Cells were infected and treated with a range of 17-AAG concentrations (25–500 nM). Intracellular parasite survival was determined following the replacement of DMEM for Schneider culture medium and direct counting of surviving \( L. (V.) \) braziliensis. Five days after medium replacement, treatment with increasing concentrations of 17-AAG for 24 h significantly reduced the number of viable \( L. (V.) \) braziliensis parasites (Figure 3A) (One-way ANOVA, \( p < 0.001 \)), indicating once more a dose-dependent effect (test for linear trend \( p < 0.001 \)). This effect was also time-dependent as exposure to 220 nM (IC\(_{50}\)) of 17-AAG for longer periods (48 and 72 h) also significantly decreased the number of \( L. (V.) \) braziliensis promastigotes (Figure 3B). Therefore, exposure of infected macrophages to 17-AAG negatively impacted on the survival of \( L. (V.) \) braziliensis.
17-AAG down-modulates the production of inflammatory mediators by *L. (V.) braziliensis*-infected macrophages

Macrophage activation and production of nitric oxide and superoxide are key steps towards elimination of intracellular *Leishmania* [30]. In macrophages infected with *L. (V.) braziliensis* and treated with 220 nM 17-AAG (IC₅₀), production of nitric oxide (Figure 4A) and superoxide (Figure 4B) were lower compared to cells exposed to DMSO. 17-AAG-treatment also down-modulated the production of TNF-α (Figure 4C), IL-6 (Figure 4D) and CCL2/MCP-1 (Figure 4E) by *L. (V.) braziliensis*-infected macrophages.

Figure 3. Treatment with 17-AAG reduces intracellular *L. (V.) braziliensis* viability. *L. (V.) braziliensis*-infected macrophages were treated for 24 h with increasing concentrations of 17-AAG or with vehicle alone (DMSO). The number of viable parasites was evaluated by further culture (5 days) in Schneider medium, free of 17-AAG (A). Infected macrophages were treated with 220 nM (IC₅₀) of 17-AAG for 24–72 h. The number of viable parasites was evaluated by further culture for five days in Schneider medium, free of 17-AAG (B). Data, shown as mean ± SEM, are from one of two independent repeats (**p<0.01 and ***p<0.001).

doi:10.1371/journal.pntd.0003275.g003

Figure 4. Treatment with 17-AAG down regulates ROS and cytokine production in *L. (V.) braziliensis*-infected cells. *L. (V.) braziliensis*-infected macrophages were treated with 17-AAG (220nM) + IFN-γ. After 48h, supernatants were assayed for nitrite production (A) and for presence of (B) superoxide, following addition of hydroxylamine. The presence of secreted (C) TNF-α, (D) IL-6 and (E) CCL2 was determined in culture supernatants by Cytometric Bead Array, after 24 h of treatment. Data, shown as mean ± SEM, are from one of two independent repeats (**p<0.01; *p<0.05).

doi:10.1371/journal.pntd.0003275.g004
Therefore, the leishmanicidal effect of 17-AAG is uncoupled from the production of microbicidal molecules and from the production of pro-inflammatory cytokines.

In vivo control of CL caused by L. (V.) braziliensis

Next, we tested the in vivo effect of 17-AAG against CL caused by L. (V.) braziliensis. These experiments were performed in an mouse model that reproduces aspects of the natural infection such as the presence of an ulcerated lesion, parasite dissemination to lymphoid areas and the development of a Th-1 type immune response [26]. BALB/c mice were inoculated with L. (V.) braziliensis in the ear dermis and lesion development was monitored weekly. Four weeks after infection, mice were treated with 17-AAG or with vehicle (DMSO) alone, three times a week, for three weeks. The ear thickness of mice treated with 17-AAG was significantly smaller compared to controls (Figure 5A). Disease burden, calculated by the area under the curve (AUC) obtained for the two experimental groups, was also significantly ($p<0.05$) different (Figure 5B), demonstrating the ability of 17-AAG to control lesion development, in vivo. 17-AAG treatment significantly ($p<0.05$) reduced the parasite load at the infection site, six weeks post inoculation (Figure 5C). However, treatment with 17-AAG was not able to reduce the parasite load within draining lymph nodes (dLN) (Figure 5D).

Comparison of the in vitro killing effects of 17-AAG and amphotericin B

In order to further characterize 17-AAG as an anti-leishmanial, its effect on infected macrophages was compared to that exerted by amphotericin B. Macrophages were infected with L. (V.) braziliensis and treated with 220 nM 17-AAG (IC$_{50}$) or with amphotericin B (AMB) (0.27 μM). Forty-eight hours after medium replacement (Figure 6), cultures treated with either 17-AAG or amphotericin B displayed significantly lower parasite numbers ($p<0.01$) in comparison with controls, treated with vehicle (DMSO) alone.

Figure 5. In vivo treatment with 17-AAG decreases L. (V.) braziliensis infection. Mice were infected with L. (V.) braziliensis and four weeks later, mice were treated with 17-AAG, 3x a week for 3 weeks (boxed area) or with vehicle (DMSO) alone. (A) The course of lesion development was monitored weekly. (B) Disease burden [shown as Area Under the Curves (AUC) depicted in (A)] in mice treated with 17-AAG or injected with DMSO. Parasite load was determined at the infection site (C) and at the dLN (D), 6 weeks later, by limiting dilution analysis. Data, shown as mean ±SEM, are from one of two independent repeats, each performed with 10 mice in each group (**$p<0.01$; *$p<0.05$).

doi:10.1371/journal.pntd.0003275.g005
Geldanamycin (GA), a HSP90 inhibitor analogous to 17-AAG, induces an anti-oxidative and attenuated inflammatory response in sepsis [43], autoimmune encephalitis [44], experimental atherosclerosis [45] and endotoxin-induced uveitis [46]. The proposed mechanism for these effects is the reduced nuclear translocation of NF-κB, reflecting in decreased production of IL-6, TNF-α and NO [41]. Although we cannot extrapolate the complexities of in vivo situations cited above, L. (L.) amazonensis-infected macrophages treated with 17-AAG displayed parasite killing, in spite of a diminished production of inflammatory mediators [24]. It has been shown that in BALB/c mice infected with L. (V.) braziliensis, the density of INOS+ cells was higher when compared to L. (L.) amazonensis-infected mice [47]. So, different responses to NO between L. (V.) braziliensis and L. (L.) amazonensis could also impact on the killing effect exerted by 17-AAG. In our previous work, [24], we showed that L. (L.) amazonensis amastigotes displayed structural alterations following exposure of infected macrophages to 17-AAG. Visible alterations in the cytoplasm of parasites such as the presence of myelin figures, vesicles with double-layered membranes and mitochondrial segments inside membrane-bounded structures were the suggestive indications of autophagy, a process that naturally occurs in Leishmania and which plays an important role in the transition from promastigote to amastigote [40]. It is possible that inhibition of HSP90 activity interferes with cell cycle progression, blocking differentiation or expression of stage specific protein and, consequently, affecting survival in the intracellular environment.

17-AAG was also effective in vivo: mice infected with L. (V.) braziliensis and treated with 17-AAG showed a significantly smaller disease burden in parallel to a smaller parasite load at the infection site. However, 17-AAG was not able to alter parasite load at the draining lymph nodes (dLN), a site where L. (V.) braziliensis parasites persist following lesion healing [26]. In this experimental model, parasite persistence is associated with the presence of regulatory T cells (Tregs) that accumulate within dLNs of L. (V.) braziliensis-infected mice [49] and these Tregs control Th1 responses by IL-10-dependent mechanisms [50]. Although 17-AAG treatment controlled parasite replication at the infection site and promoted lesion healing, parasite persistence within distal sites such as the dLNs may have important effects with regards to maintenance of immunity to Leishmania [51] and/or development of mucocutaneous leishmaniasis, deserving further investigation.

Currently, the drugs available for the treatment of CL are limited and among them, pentavalent antimonials have been the choice for over 60 years. However, treatment is long (20-30 days), patients develop several side effects and, in the recent years, the number of cases refractory to treatment has increased [52,53]. In the case of therapeutic failure, second-line drugs such as amphotericin B can be employed as well as combination of two available drugs [34]. Advantages of a combination treatment include increased efficacy, less drug resistance, lower drug dosage and a general decrease in side effects [53]. Herein, 17-AAG was as effective as amphotericin B at decreasing the parasite load within infected macrophages. Experimentally, the treatment of L. infantum and L. panamensis promastigotes with 17-AAG plus edelfosine improved the anti-leshmanical activity of the latter [56]. In vitro synergism was also observed for the combinations of paramomycin and amphotericin B against L. (V.) braziliensis [57]. In vivo, association of tamoxifen with amphotericin B yielded an additive effect in mice infected with L. (L.) amazonensis [58]. The combination of GA with fluconazole showed synergistic activity against Candida albicans isolates resistant to fluconazole alone [59]. Thus, we propose that combinations of 17-AAG and

![Figure 6. Comparison of the effects of 17-AAG and amphotericin B on the growth of intracellular L. (V.) braziliensis. L. (V.) braziliensis-infected macrophages were treated with 17-AAG or with amphotericin B (AMB) for 48 h. The number of viable parasites was evaluated by further culture in Schneider medium, free of 17-AAG. Data, shown as mean ± SEM, are from one of two independent repeats (**p<0.001). (ND, not detected).](image)

doi:10.1371/journal.pntd.0003275.g006

**Discussion**

HSP90 is a molecular chaperone fundamental for the life cycle of a variety of protozoa [31] and, as such, inhibitors of HSP90 have been suggested as novel chemotherapeutic agents against malaria [32], filariasis [33,34] and schistosomiasis [34]. Recently, we showed that 17-AAG, a HSP90 inhibitor, reduced L. (L.) amazonensis infection in vitro [24]. Herein, we investigated the potential of 17-AAG as a chemotherapeutic agent against L. (V.) braziliensis, the main etiological agent of CL and MCL in Brazil [35]. We confirmed the effects of 17-AAG against this L. (V.) braziliensis promastigotes and we extended these findings to a pre-clinical model of CL.

Initially, we investigated the in vitro effects of 17-AAG, against both axenic promastigotes and intracellular amastigotes. Treatment of L. braziliensis promastigotes with the lower dose of 17-AAG (25 nM) already decreased promastigote viability. Herein, the IC_{50} determined for L. (V.) braziliensis was comparable to that described for L. (L.) amazonensis (65 nM) whereas in experiments performed with L. (L.) major, the IC_{50} was established at 80 nM [24]. 17-AAG was equally effective at reducing intracellular amastigote numbers and the viability of surviving L. (V.) braziliensis promastigotes. These effects were not associated with an increase in the microbical functions of macrophages as levels of NO, superoxide and TNF-α were diminished in the presence of 17-AAG. These results are in accordance with our previous report [24]. Additionally, the lack of amastigote replication in control macrophages could be attributed to innate microbical properties of macrophages that allow L. (V.) braziliensis killing, as observed with L. (V.) guyanensis and L. (L.) major [36–38]. In CL patients, an exacerbated inflammatory immune response is associated with the development of mucocutaneous leishmaniasis (rev. in [39]) whereas subclinical patients, who do not develop the disease, have a more controlled immune response [40]. Therefore, the possibility of selectively inducing parasite killing without contributing to overt inflammation is an important advantage for the treatment of CL using 17-AAG. Of note, macrophages treated with 17-DMG alone displayed reduced production of IL-6, TNF-α and NO [41] whereas 17-AAG prevented iNOS expression upon stimulation with LPS or IFN-γ [42].
amphotericin B may be further investigated for the treatment of CL caused by *L. (V.) braziliensis*.

Herein, we reported that 17-AAG reduces *L. (V.) braziliensis* infection in vitro and in vivo. 17-AAG shows excellent bioavailability when given to mice by the i.p. route [60]. At 60 mg/kg, 17-AAG caused no changes in appearance, appetite, weight elimination, or survival of treated animals as compared to vehicle-treated controls. We employed with 20 mg/kg, a dose well below that reported as having any harmful effects, as those described by Solit et al. (equal or above 75 mg/kg) [61]. Given that HS990 inhibitors, analogous to 17-AAG, have entered clinical trials with cancer patients [62], we propose that 17-AAG could be further investigated as a novel target for chemotherapy against cutaneous leishmaniasis.

Supporting Information

Figure S1 Determination of IC50 values for 17-AAG in *L. braziliensis* promastigotes. Cells were treated in sextuplicate with 17-AAG for 48 hours with varying concentrations of 17-AAG. Following treatment, parasite viability was evaluated by direct counting. IC50 values (nM) were determined using GraphPad Prism. (TIF)

Figure S2 Treatment with 17-AAG controls *L. braziliensis* replication inside macrophages. *L. braziliensis*–infected macrophages were treated with increasing concentrations of 17-AAG or with vehicle alone (DMSO). After 12–72 h, glass coverslips were stained with H&E and assessed for the percentage of infected macrophages (A) and the number of amastigotes per 100 macrophages (B) by light microscopy. Data, shown as mean ±SEM, are from one of three independent repeats (*p<0.05; **p<0.01 and ***p<0.001). (TIF)

Figure S3 Determination of IC50 values for 17-AAG in macrophages infected with *L. braziliensis* promastigotes. Infected macrophages were treated in sextuplicate with 17-AAG for 72 hours with varying concentrations of 17-AAG. Following treatment, glass coverslips were stained with H&E and assessed for the presence of amastigotes by light microscopy. IC50 values (nM) were determined using GraphPad Prism. (TIF)

Figure S4 Cell viability following macrophage exposure to 17-AAG. Thioglycolate-elicited macrophages were exposed to different concentrations of 17-AAG or to DMSO (vehicle) alone for 24 h. Cell viability was evaluated by MTT assay. (TIF)

Acknowledgments

The authors are grateful to Lucas Menezes Moura for excellent technical assistance. The authors are also thankful to Diego Menezes and Ricardo Khouri for helpful comments and discussions.

Author Contributions

Conceived and designed the experiments: DMS ALOAP FSC. Analyzed the data: DMS ALOAP PSTV CIdO. Contributed reagents/materials/analysis tools: VMB PSTV CIdO. Wrote the paper: DMS ALOAP VMB CIdO. Performed the experiments: DMS ALOAP FSC. Analyzed the data: DMS ALOAP PSTV CIdO. Wrote the paper: DMS ALOAP.

References

1. den Boer M, Argaw D, Jannin J, Avar J (2011) Leishmaniasis impact and treatment access. Clin Microbiol Infect 17: 1471–1477.
2. Bittenauer A, Silva N, Straatmann A, Nunes VL, Follador I, et al. (2003) Post-kala-azar dermal leishmaniasis associated with AIDS. Braz J Infect Dis 7: 229–233.
3. Schubach A, Marzochi MC, Cuzzi-Maya T, Oliveira AV, Araujo ML, et al. (1998) Cutaneous scars in American tegumentary leishmaniasis patients: a site of Leishmania (Viannia) braziliensis persistence and viability eleven years after antimonial therapy and clinical cure. Am J Trop Med Hyg 58: 824–827.
4. Mendonca MG, de Brito ME, Rodrigues EH, Bandeira V, Jardim ML, et al. (2004) Persistence of leishmania parasites in scars after clinical cure of American cutaneous leishmaniasis: is there a sterile cure? J Infect Dis 189: 1018–1023.
5. Marsden PD (1986) Mucosal leishmaniasis ("espundia" Escomel, 1911). Trans R Soc Trop Med Hyg 80: 859–876.
6. Costa JM, Marsden PD, Llanos-Cuentas EA, Neto EM, Carvalho EM, et al. (1986) Disseminated cutaneous leishmaniasis in a field clinic in Bahia, Brazil: a report of eight cases. J Trop Med Hyg 89: 319–325.
7. Avar J, Velez ID, Bern C, Herrera M, Dejean P, et al. (2012) Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7: e35671.
8. Croft SL, Coombs GH (2005) Leishmania–current chemotherapy and recent advances in the search for novel drugs. Trends Parasitol 19: 502–508.
9. Berman JD (1997) Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. Clin Infect Dis 24: 684–705.
10. Chalay JD, Oser CN, McGreevy PB, Hendricks LD, Kreuzer RD (1988) American cutaneous leishmaniasis: presentation and problems of patient management. Rev Soc Bras Med Trop 21: 163–172.
11. Amato VS, Tsou FF, Siqueira AM, Nicrodeno AC, Neto VA (2007) Treatment of mucosal leishmaniasis in Latin America: systematic review. Am J Trop Med Hyg 77: 266–274.
12. Modabber F, Buffet PA, Torreche E, Milton G, Croft SL (2007) Consulative meeting to develop a strategy for treatment of cutaneous leishmaniasis. Institute Pasteur, Paris. 13–15 June, 2006. Kinetoplastid Biol Dis 6: 3.
13. Llanos-Cuentas A, Tulliano G, Araujo-Castillo R, Miranda-Verastegui C, Santamaria-Castrellon G, et al. (2008) Clinical and parasite species risk factors for prevalent/antivalent treatment failure in cutaneous leishmaniasis in Peru. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 46: 223–231.
14. Machado P, Araujo C, Da Silva AT, Almeida RP, D'Oliveira Jr A, et al. (2002) Failure of early treatment of cutaneous leishmaniasis in preventing the development of an ulcer. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 34: E69–73.
15. Oliveira LF, Schubach AO, Martins MM, Passos SL, Oliveira RV, et al. (2011) Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. Acta Tropica 118: 87–96.
16. Mishra J, Saxena A, Singh N (2007) Chemotherapy of leishmaniasis: past, present and future. Curr Med Chem 14: 1153–1169.
17. Morimoto RI, Kline MP, Bimoto DN, Cotto J (1997) The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. Essays Biochem 32: 17–29.
18. Hartl FU, Hayer-Hartl M (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295: 1852–1856.
19. Goetz MP (2003) The Hsp90 chaperone complex as a novel target for cancer therapy. Annals of Oncology 14: 1169–1176.
20. Neckers L, Workman P (2012) Hsp90 molecular chaperone inhibitors: are we there yet? Clinical cancer research: an official journal of the American Association for Cancer Research 18: 64–76.
21. Rochanai AK, Singh M, Tatu U (2013) Heat shock protein 90 inhibitors as broad spectrum anti-infectives. Current pharmaceutical design 19: 377–386.
22. Wiesigl M, Clos J (2001) The heat shock protein 90 of Leishmania donovani. Medical microbiology and immunology 190: 27–31.
23. Li Q, Zhou Y, Yao C, Ma X, Wang L, et al. (2009) Apoptosis caused by Hsp90 inhibitor geldanamycin in Leishmania donovani during promastigote-to-amastigote transformation stage. Parasitology research 105: 1539–1548.
24. Petersen AL, Guedes CE, Versoza CL, Lima JG, de Freitas LA, et al. (2012) 17-AAG kills intracellular Leishmania amazonensis while reducing inflammatory responses in infected macrophages. PLoS One 7: e19496.
25. Schulte TW, Neckers LM (1998) The benzoxazinone ansamycin 17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. Cancer Chemother Pharmacol 42: 273–279.
26. de Moura TR, Novaes FO, Oliveira F, Clavencio J, Noronha A, et al. (2005) Toward a novel experimental model of infection to study American cutaneous leishmaniasis caused by Leishmania braziliensis. Infect Immun 73: 5827–5834.
27. Miranda KM, Espey MG, Wink DA (2003) A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 5: 62–71.
28. Khouri R, Bafica A, Silva Mda P, Noronha A, Kolb JP, et al. (2009) IFN-beta impairs superoxide-dependent parasite killing in human macrophages: evidence for a deleterious role of SOD1 in cutaneous leishmaniasis. J Immunol 182: 2525–2531.
29. Eltze EF, Heupel A (1976) Inhibition of nitrite formation from hydroxylammonium chloride: a simple assay for superoxide dismutase. Anal Biochem 70: 616–620.

30. Liese J, Schleicher U, Bogdan C (2000) The innate immune response against Leishmania parasites. Immunobiology 213: 377–387.

31. Roy N, Nageshan RK, Ranade S, Tatu U (2012) Hsp90 is essential in the filarial nematode Brugia pahangi. International journal for parasitology research 35: 599.

32. Mout R, Xu Z-D, Wolf AKH, Jo Davisson V, Jarori GK (2012) Anti-malarial activity of geldanamycin derivatives in mice infected with Plasmodium yoelii. Malaria journal 11: 54.

33. Devanev E, O’Neill K, Harnett W, Whitesell L, Kinnaird JH (2005) Hsp90 is essential in the filarial nematode Brugia pahangi. International journal for parasitology 35: 627–636.

34. Wenkert D, Ramirez B, Shen Y, Kron MA (2010) In Vitro Activity of Geldanamycin Derivatives against Schistosoma japonicum and Brugia malayi. Journal of parasitology research 2010: 716498–716498.

35. Costa JML (2005) Epidemiologia das Leishmanioses do Brasil. Gazeta Médica 34.

36. Sousa-Franco J, Araujo-Mendes E, Silva-Jardim I, J LS, Faria DR, et al. (2006) Infection-induced respiratory burst in BALB/c macrophages kills Leishmania guyanensis amastigotes through apoptotic possible involvement in resistance to cutaneous leishmaniasis. Microbes Infect 8: 390–400.

37. Matta NP, Cyne-Finkelstein L, Machado GM, Da-Cruz AM, Leon L (2010) Differences in the antigenic profile and infectivity of murine macrophages of Leishmania (Viannia) parasites. J Parasitol 96: 509–515.

38. Carvalho LP, Passos S, Schriefer A, Carvalho EM (2012) Protective and pathologic immune responses in human tegumentary leishmaniasis. Frontiers in immunology 3: 301.

39. Poulaki V, Ikaki E, Misiades N, Misiaides CS, Paulus YN, et al. (2007) Inhibition of Hsp90 attenuates inflammation in endotoxin-induced uveitis. FASEB journal: official publication of the Federation of American Societies for Experimental Biology 21: 2113–2123.

40. Carvalho AK, Silveira FT, Pasero LF, Gomes CM, Corbett CE, et al. (2012) Leishmania (V.) braziliensis and L. (L.) amazonensis promote differential expression of dendritic cells and cellular immune response in murine model. Parasite Immunol 34: 395–403.

41. Besteiro S, Williams RA, Morrison LS, Coombs GH, Mottram JC (2006) Endosome sorting and autophagy are essential for differentiation and virulence of Leishmania major. J Biol Chem 281: 11384–11396.

42. Falcao S, de Moura TR, Clarrecio J, Brodkyn C, Barral A, et al. (2012) The presence of Tregs does not preclude immunity to reinfection with Leishmania braziliensis. Int J Parasitol 42: 771–780.

43. Belkaid Y, Hoffmann KF, Mendoza S, Kamhawi S, Udey MC, et al. (2001) The role of interleukin (IL)-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. J Exp Med 194: 1497–1506.

44. Falcao S, de Moura TR, Clarrecio J, Brodkyn C, Barral A, et al. (2012) The presence of Tregs does not preclude immunity to reinfection with Leishmania braziliensis. Int J Parasitol 42: 771–780.

45. Dello Russo C, Polak PE, Mercado PR, Spagnolo A, Sharp A, et al. (2006) The role of interleukin (IL)-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. J Exp Med 194: 1497–1506.

46. Shimp SK, Parson CD, Regna NL, Thomas AN, Chafin CB, et al. (2012) 17-AAG Controls Infection by Leishmania donovani. J Parasitol 98: 616–620.

47. Trinconi CT, Reimao JQ, Yokoyama-Yasunaka JK, Miguel DC, Uliana SR (2011) In vitro interaction between paromomycin sulphate and four drugs with leishmanicidal activity against three New World Leishmania species. J Antimicrob Chemother 67: 150–154.

48. Tuon FF, Amato VS, Graf ME, Siqueira AM, Nicrodeno AC, et al. (2008) Treatment of New World cutaneous leishmaniasis-a systematic review with a meta-analysis. Int J Dermatol 47: 109–124.

49. van Griensven J, Balasegaram M, Meheus F, Alvar J, Lynen L, et al. (2010) Combination therapy for visceral leishmaniasis. Lancet Infect Dis 10: 184–194.

50. Rollier C, Bade M, Kim J, Liau W, Tan J, Sun Y, Wanzo F, et al. (2013) Inhibition of Hsp90 attenuates inflammation in endotoxin-induced uveitis. FASEB journal: official publication of the Federation of American Societies for Experimental Biology 21: 2113–2123.

51. Carvalho AK, Silveira FT, Pasero LF, Gomes CM, Corbett CE, et al. (2012) Leishmania (V.) braziliensis and L. (L.) amazonensis promote differential expression of dendritic cells and cellular immune response in murine model. Parasite Immunol 34: 395–403.

52. Besteiro S, Williams RA, Morrison LS, Coombs GH, Mottram JC (2006) Endosome sorting and autophagy are essential for differentiation and virulence of Leishmania major. J Biol Chem 281: 11384–11396.

53. Falcao S, de Moura TR, Clarrecio J, Brodkyn C, Barral A, et al. (2012) The presence of Tregs does not preclude immunity to reinfection with Leishmania braziliensis. Int J Parasitol 42: 771–780.

54. Luo S, Wang T, Qin H, Lei H, Xia Y (2011) Obligatory role of heat shock protein 90 in iNOS induction. Am J Physiol Cell Physiol 301: C227–233.

55. Chatterjee A, Dimitropoulou C, Drakopanayiotakis F, Antonova G, Snejd C, et al. (2007) Heat shock protein 90 inhibitors prolong survival, attenuate inflammation, and reduce lung injury in murine sepsis. American journal of respiratory and critical care medicine 176: 667–675.

56. Madrigal-Matute J, López-Franco O, Blanco-Colio LM, Muñoz-García B, Ramos-Moza P, et al. (2010) Heat shock protein 90 inhibitors attenuate inflammatory responses in atherosclerosis. Cardiovascular Research 86: 336–347.

57. Usmani SZ, Bona R, Li Z (2009) 17-AAG for HSP90 inhibition in cancer–from bench to bedside. Curr Mol Med 9: 654–664.

58. Varela MR, Mollinedo-Gajate C, Muro A, Mollinedo F (2013) The HSP90 inhibitor 17-AAG potentiates the antileishmanial activity of the other lipid edelfosine. Acta Trop 131C: 32–36.

59. Moraes-Teneura E, Galupo MK, Rodrigues LF, Romanação AJ, Rabello A (2014) In vitro interaction between paromomycin sulphate and four drugs with leishmanicidal activity against three New World Leishmania species. J Antimicrob Chemother 69: 130–154.

60. Tripathi CT, Reimao JQ, Yokoyama-Yasunaka JK, Miguel DC, Uliana SR (2014) Combination therapy with tamofoxifen and amphotericin B in experimental cutaneous leishmaniasis. Antimicrob Agents Chemother.

61. Zhang J, Liu W, Tan J, Sun Y, Wanzo F, et al. (2013) Antifungal activity of geldanamycin alone or in combination with fluconazole against Candida species. Mycopathologia 175: 273–279.

62. Gyorik M, Zuzowski EG, Rosen DM, Sentz DL, Covey JM, et al. (2001) Plasma pharmacokinetics and tissue distribution of 17-allylamino-17-demethoxygeldanamycin in CD2F1 mice. Cancer Chemother Pharmacol 47: 291–302.

63. Solit DB, Zheng FF, Drobnjak M, Munster PN, Higgins B, et al. (2002) 17-Allylamino-17-demethoxygeldanamycin induces the degradation of androgen receptor and HER-2/neu and inhibits the growth of prostate cancer xenografts. Clin Cancer Res 4: 898–903.

64. Usmani SZ, Bona R, Li Z (2009) 17-AAG in cancer—from bench to bedside. Curr Mol Med 9: 654–664.