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Parasite burden in *Leishmania (Leishmania) amazonensis*-infected mice: Validation of luciferase as a quantitative tool

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ABSTRACT

Given the lack of effective and safe alternatives to the drugs already in use, considerable efforts are being applied to the search of new therapeutic options to treat leishmaniasis. A necessary step in the discovery of antileishmanial drugs is the validation of drug candidates in mouse models. The standard methods to quantify the parasite burden in animal models, mainly culture-based, are time consuming and expensive. In recent years, in vivo imaging systems have been proposed as a tool to overcome these problems, allowing parasite detection in living organisms. Here we compared different treatment efficacy evaluation approaches. Recombinant *Leishmania (L.) amazonensis* lines expressing the luciferase gene (La-LUC) were obtained and characterized for biological properties as compared with the wild type (WT) parental line. Bioluminescence generated by La-LUC was shown to correlate with the number of promastigotes in vitro. La-LUC promastigotes and intracellular amastigotes were equally sensitive to amphotericin B (AmB) as the WT parasites. The clinical pattern of lesion development upon infection with the transgenic lines was similar to lesions observed after infection with the WT strain. The half maximal effective dose (ED_{50}) of AmB was determined in La-LUC infected mice through quantification of bioluminescence in vivo and ex vivo, by limiting dilution and using clinical parameters. There was agreement in the ED_{50} determined by all methods. Quantification of bioluminescence in vivo and/or ex vivo was elected as the best tool for determining parasite burden to assess drug efficacy in infected mice. Furthermore, the detailed analysis of AmB effectiveness in this model generated useful data to be used in drug combination experiments.

1. Introduction

Species of the *Leishmania* genus are protozoan parasites responsible for cutaneous, mucocutaneous, and visceral leishmaniasis, which occur throughout various parts of the world, mainly in tropical and subtropical areas, representing a major public health problem. *Leishmania* is a digenetic parasite, with extracellular motile promastigotes present in the alimentary tract of their insect vector, and intracellular non-motile amastigotes, which live in the mononuclear phagocytes of mammalian hosts. The etiologic agents of leishmaniasis include many *Leishmania* species. *Leishmania (Leishmania) amazonensis* is responsible for most cases of human cutaneous leishmaniasis in the Amazon region of Brazil and is the most important agent of diffuse cutaneous leishmaniasis (DCL) in South America (Convit et al., 1993). DCL is a rare form of leishmaniasis, characterized by non-ulcerated nodules with diffuse cutaneous infiltration that can spread over most of the exposed areas of the body. DCL is a disease of chronic evolution, being considered refractory to all kinds of treatment used at the moment (Zerpa et al., 2007).

Combination therapy has been put forward as the best strategy to overcome the present problems with leishmaniasis chemotherapy. Drug combinations may involve some of the classical agents already in use and the experimental design for testing a given combination may be greatly facilitated by previous knowledge of the half maximal effective concentration (ED_{50}) of the drugs to be tested. Amphotericin B (AmB) has been used in the treatment of human visceral leishmaniasis since the 1950's (Jha et al., 1995). However, this drug has not been so widely used in the treatment of cutaneous leishmaniasis.

Data in the literature on *L. (L.) amazonensis* sensitivity to AmB in experimental models is scarce. The activity of AmB has been previously determined in vivo in different species of *Leishmania* at a single dose level (Mullen et al., 1997, 1998; Al-Abdely et al., 1999). Previous work reported the dose–response effects of AmB in vivo against *Leishmania (Leishmania) donovani* (Yardley and Croft, 2000). However, to the best of our knowledge, the ED_{50} of AmB in *L. (L.) amazonensis* infected mice has not been reported.

The enzyme luciferase (LUC) represents one of the most efficient biological reporters nowadays. It catalyzes the reaction of the substrate luciferin with adenosine triphosphate (ATP) to generate photons

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(Lang et al., 2005). The expression of the gene encoding *LUC* in *Leishmania* spp., including *L. (L.) amazonensis*, and its use in the evaluation of antileishmanial compounds has been described (Ashutosh et al., 2005; Lang et al., 2005; Ravinder et al., 2012; Roy et al., 2000; Sereno et al., 2001). Of particular interest, is the use of in vivo imaging system (IVIS) which offers several potential advantages over conventional methods of parasite quantification in experimentally infected animals. Because of its non-destructive and non-invasive nature, this technique can be performed repeatedly and permits each animal to be used as its own control over time, overcoming the problem of animal–animal variations (Michel et al., 2011) and reducing the number of animals in each experiment. However, the validation of this novel methodology as a quantitative approach for determining parasite burden is still lacking.

In the present work, we determined the ED_{50} of AmB in BALB/c mice infected with *L. (L.) amazonensis* through different methodologies. We used *L. (L.) amazonensis* parasites expressing *LUC* (La-LUC) to quantify *Leishmania* infection in vivo, in the site of inoculation, and ex vivo, in tissue samples from infected mice, in order to compare the achieved ED_{50} by different approaches. Data presented herein represent an invaluable resource in the design of drug combination experiments with AmB in this model.

2. Methods

2.1. Parasites

The *L. (L.) amazonensis* strain MHOM/BR/1973/M2269 was a kind gift from Dr. Jeffrey Shaw (University of São Paulo). Wild-type *L. (L.) amazonensis* (La-WT) promastigotes were grown in 25 cm^2 tissue culture flasks containing M199 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco™ Invitrogen Corporation, NY, USA) and 0.25% hemin (Sigma-Aldrich) at 25 °C.

2.2. Transfection and selection of mutants

The 1.7 kb *Photinus pyralis* (firefly) *LUC* open reading frame was excised from the plasmid pT7 (Promega Corporation, Madison, USA) with *Bam* HI and *Sac* I and cloned into the vector pCITE-2a(+) (Novagen, Merck Millipore, Darmstadt, Germany). Transformants were selected and the insert was removed with *Bam* HI and *Bgl* II to be cloned into the *Bam* HI site of the shuttle *Leishmania* vector pXG1 (kindly provided by Dr. Stephen Beverley, Washington University, St. Louis, USA). Cloning was confirmed by restriction digest and sequencing. DNA electroporation in *L. (L.) amazonensis* was done as described (Coburn et al., 1991) and the cells were then plated in M199 medium containing 1% of agar (Gibco™, Invitrogen Corporation) and 20 μg/mL of Geneticin (G418, Sigma-Aldrich). After 2 weeks, colonies were picked, expanded in liquid media and drug concentrations were increased to 32 μg/mL of G418.

2.3. Luciferase in vitro assay

Promastigotes of drug resistant *L. (L.) amazonensis* transfected with the *LUC* construct (La-LUC) were harvested at the late log-phase of growth, washed twice in phosphate-buffered saline (PBS) (pH 7.2) and resuspended in M199. Parasites were serially diluted and the LUC assay was performed according to the manufacturer's instructions. Briefly, One Glo™ Luciferase Assay System (Promega Corporation) and parasites were mixed at 1:5 proportions, respectively. The units of luminescence (UL) were registered in a microplate reader (POLARstar Omega, BMG Labtech, Ortenberg, Germany). Alternatively, plates were read using an in vivo imaging system (IVIS Spectrum, Caliper Life Sciences, Hopkinton, MA, USA). Photons were collected by automatic acquisition with a charge-coupled device (CCD) camera using the high resolution mode (medium binning) (Ploemen et al., 2009) and values were expressed as photons per second (Ph/s). Each point was tested in duplicate or triplicate in at least two independent experiments.

2.4. Determination of half maximal effective concentration (EC_{50}) in promastigotes and intracellular amastigotes

Stock solutions of amphotericin B deoxycholate (AmB) (5.4 mM) (Cristália, Itapira, SP, Brazil) were prepared in sterile distilled water and diluted in M199 medium. Promastigotes were counted in a Neubauer hemocytometer (400× magnification) and seeded at 2×10^7 per mL in a final volume of 200 μL. Parasites were incubated in the presence of increasing drug concentrations for 24 h in 96-well microplates (Costar®, Corning Incorporated, Corning, NY, USA) in triplicate. Viability of promastigotes was assessed by MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide; Sigma-Aldrich) cleavage as described previously (Zauli-Nascimento et al., 2010). Briefly, MTT (5 mg/mL) was dissolved in PBS, sterilized through 0.22 μm membranes and added, 20 μL/well, for 4 h at 25 °C. The reaction was stopped by the addition of 80 μL 10% sodium dodecyl sulfate (SDS, Bio-Rad Laboratories, Hercules, CA, USA) to each well. Optical density (OD) was determined in a plate reader (BMG Labtech) at 550 nm. Results were expressed as the mean percentage reduction of parasite number compared with untreated control wells calculated for at least three independent experiments. The EC_{50} was determined by sigmoidal regression curves using Graph Pad Prism 5.0 software.

To determine the EC_{50} against *L. (L.) amazonensis* intracellular amastigotes, bone marrow-derived macrophages (BMDM) were obtained from BALB/c mice as previously described (Zamboni and Rabinovitch, 2003). BMDM were counted and distributed in 16-well chamber Glass Lab-Tek® slide™ (NUNC, Rochester, NY, USA) at 4×10^5 per mL with a final volume of 200 μL in RPMI 1640 medium (Gibco™, Invitrogen Corporation) supplemented with 10% FCS (Gibco™, Invitrogen Corporation) and allowed to adhere after being kept in a 5% CO_2 atmosphere for 24 h at 37 °C. Macrophages were infected with stationary-phase promastigotes (20:1 parasites/macrophage) for 3 h at 33 °C. Non-internalized parasites were removed by washing, followed by the addition of fresh medium containing increasing drug concentrations. After 48 h, cells were fixed and stained. For the evaluation of parasite burden under light microscopy, 16-well chamber slides were fixed in methanol and stained with the Instant Prov kit (Newprov, Pinhais, PR, Brazil). The percentage of infected cells was determined by counting 200 cells in each of the replicates.

EC_{50} values were determined from sigmoidal regression of the concentration–response curves using GraphPad Prism 5 software. Each point was tested in duplicate or triplicate and the experiments were repeated at least twice.

2.5. Experimental studies with L. (L.) amazonensis-LUC infected mice

In vivo experiments were approved by the Ethics Committee for Animal Experimentation of the Instituto de Ciências Biomédicas, Universidade de São Paulo (ICB/USP). BALB/c mice were obtained from the ICB/USP, kept in mini-isolators (dimensions: 32 cm × 20 cm × 21 cm, 6 animals per cage) with absorbent material in ventilated racks (Alesko Industry, Monte Mor, SP, Brazil), and received unlimited food and water. Each animal cage was enriched with a red igloo (Alesco Industry), which is an environmental enrichment item. This item allows the animal to jump, climb, slide and sleep during the day. The red color is perceived as black by mice thus providing shelter and a secluded place for them to build a nest.

Female BALB/c mice (3 to 5 weeks-old) were infected in the footpad or at the base of the tail with 10^6 stationary phase promastigotes (6th day of culture) of La-WT or La-LUC in a final volume of 20 μL, using a 300 μL syringe fitted with an ultra-fine needle (30 G) (Becton Dickinson and Company, Franklin Lanes, NJ, USA). In order to evaluate

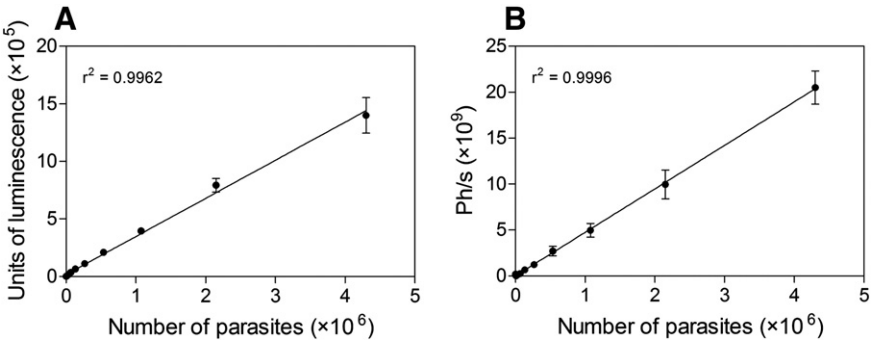

Fig. 1. Correlation between luciferase activity and number of *Leishmania* promastigotes. *L. (L.) amazonensis*-LUC promastigotes were serially diluted and luminescence was measured using a microplate reader (A) or an IVIS (B). Results are the mean and standard deviation of triplicate or duplicate determinations. Figures are representative of one of two experiments with similar results. Ph/s: photons per second.

disease progression, mice were monitored weekly by measuring the difference in the thickness between the infected and contralateral uninfected footpads using a caliper (Mitutoyo Corporation, Kawasaki, Kanagawa, Japan). Tail measurements were registered as the mean of tail base horizontal and vertical diameters and lesion size was estimated by subtracting the tail thickness in the first day of treatment. Ulcer area was expressed in mm^2. For in vivo half maximal effective dose (ED_{50}) determination, mice were randomly assigned into experimental groups ($n = 6$) five weeks post-infection. Treated animals received 1.2, 2 or 4 mg/kg/day AmB intraperitoneally (i.p.). The treatment was administered for four weeks comprising a total of 20 doses, with two day interval each five consecutive days. Drugs were prepared daily and disease progression was evaluated once a week. After the end of the treatment, mice were monitored during four weeks to evaluate drug effectiveness.

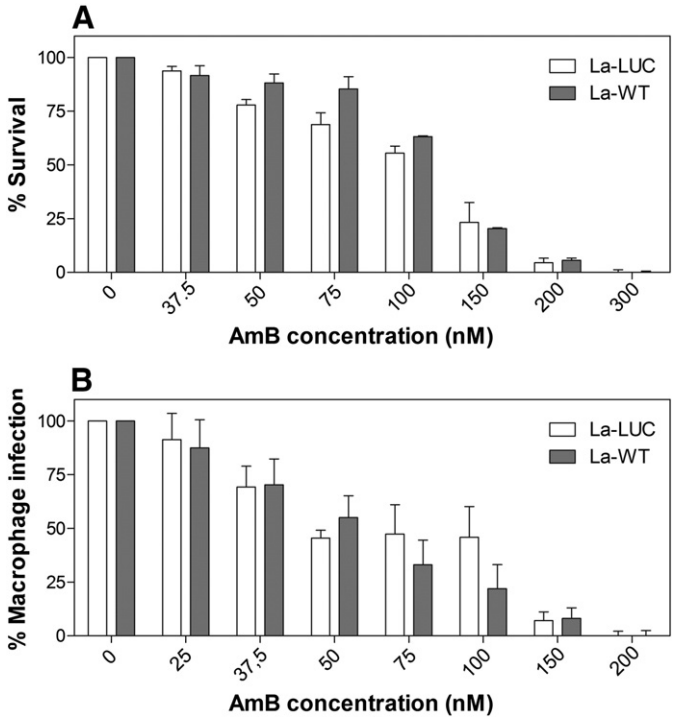

Fig. 2. AmB activity against wild type *L. (L.) amazonensis* (La-WT) and *L. (L.) amazonensis* expressing luciferase (La-LUC) in vitro. The in vitro activity of AmB against promastigotes (A) and intracellular amastigotes (B) was determined by MTT and by microscopic counting, respectively, as described in the Determination of half maximal effective concentration (EC_{50}) in promastigotes and intracellular amastigotes section. Results are the mean and standard deviation of triplicate samples. Figures are representative of one of three experiments with similar results.

The La-LUC light emission in the tail of infected animals was recorded by bioimaging (IVIS Spectrum, Caliper Life Sciences), 9 and 13 weeks post-infection. Previous to the imaging, mice received 75 mg/kg VivoGlo™ Luciferin (Promega Corporation) (i.p.) and were anesthetized in a 2.5% isoflurane atmosphere (Cristália). Animals were then transferred to the imaging chamber and kept in a 1.5% isoflurane atmosphere. Emitted photons were collected using the high resolution (medium binning) mode. Total photon emission from a defined region of interest (ROI) corresponding to the lesion in the tail was registered. The same ROI was applied to all animals. The images were acquired 20 min after luciferin injection. Total photon emission from the dorsal image of each mouse tail was quantified with Living Image software version 4.3.1 (Caliper Life Sciences), and results were expressed as the number of photons/s/ROI. The photon signal from the tail was presented as a pseudocolor image representing light intensity (red = most intense and blue = least intense) and superimposed on the gray scale reference image (Lecoeur et al., 2007).

At the end of the experiment, mice were euthanized and the lesions were removed using scissors and scalpel blades, and then macerated in PBS using an automatic homogenizer (OMNI TH International, Kennesaw, GA, USA). An aliquot of this macerate was utilized to quantify the number of parasites ex vivo, using LUC assay according to the manufacturer's instructions. Briefly, One Glo™ Luciferase Assay System (Promega Corporation), tissue macerate and PBS were mixed at 1:1:3 proportions, respectively. The UL were registered in a microplate reader (POLARstar Omega, BMG Labtech) and the recorded emission was multiplied by the dilution factor. Each point was tested in triplicate. Another aliquot of this macerate was used to perform the limiting dilution method as previously described (Lima et al., 1997).

Drug inhibition curves were drawn from each method and effective dose 50% (ED_{50}) was determined from sigmoidal regression of the dose–response curves.

Table 1
Half maximal effective concentration (EC_{50}) of AmB against *L. (L.) amazonensis* wild type (La-WT) and *L. (L.) amazonensis* expressing luciferase (La-LUC).

Form	EC_{50} (nM) (95% CI)[c]	
	La-WT	La-LUC
Promastigotes[a]	111.0 (104.5–117.8)	98.11 (90.21–106.7)
Amastigotes[b]	69.22 (65.42–73.23)	65.02 (55.15–76.65)

[a] In vitro activity of AmB against promastigotes was determined by MTT. The results are expressed as the mean of duplicate experiments, each one performed with triplicate samples.

[b] Activity against intracellular amastigotes was determined in infected macrophages by microscopic counting. The results are expressed as the mean of duplicate experiments, each one performed with triplicate samples.

[c] 95% CI: 95% confidence interval of the EC_{50} values, given in nM.

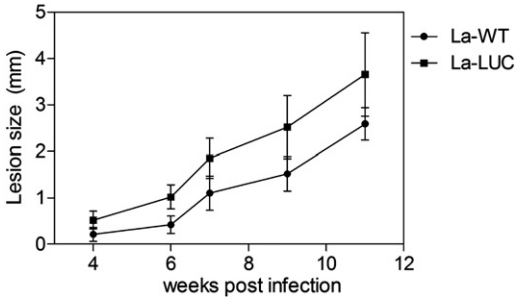

Fig. 3. Infectivity of *L. (L.) amazonensis* expressing luciferase. BALB/c mice were inoculated in the left hind footpad with 10^6 stationary phase promastigotes of *L. (L.) amazonensis* wild type (La-WT) or luciferase expressing (La-LUC). Lesion size represents the average difference between infected and contralateral non-infected hind footpads (n = 6 per group). Statistical analysis was performed with the Student's *t* test and did not show any significant difference between groups.

2.6. Statistical analysis

Data on lesion/ulcer progression, limiting dilution, and in vivo and ex vivo LUC quantification was analyzed for statistical significance by One Way ANOVA, followed by the Tukey post-test. Statistical analyses were performed using GraphPad Prism 5 software.

3. Results

3.1. Characterization of LUC transformed L. (L.) amazonensis lines

LUC expression was evaluated in independent lines selected after transfection of the pXG1[*LUC*] construct. A clear correlation was observed between the number of parasites and luminescence (Fig. 1). The lower limit of detection when promastigotes were analyzed in a luminometer was 10 with luminescence values of 73.5 ± 6.5 units. In the same standard curve, discrete values were obtained in the range of 10 to 10^6 parasites (Fig. 1A). Standard curves with increasing number of parasites were also evaluated by direct imaging of plates using IVIS (Fig. 1B). A linear correlation between parasite number and photons registered was also observed. However, in this case the range of detection was narrower, varying from 10^3 to 10^6 promastigotes.

The growth curves of WT and La-LUC parasites were indistinguishable. La-LUC promastigotes were infective to macrophages leading to the development of the typically large parasitophorous vacuoles observed when cells are infected with *L. (L.) amazonensis* (data not shown).

To further validate La-LUC parasite as a drug screening tool, in vitro susceptibility to AmB of WT parental strain and La-LUC lines was compared (Fig. 2). Transgenic and WT *L. (L.) amazonensis* promastigotes presented similar sensitivity to AmB, as observed in Fig. 2A. The same reduction in the percentage of macrophage infection was observed upon treatment with increasing concentrations of AmB in both La-LUC and La-WT infected cultures (Fig. 2B). The EC_{50} of AmB against La-LUC promastigotes and amastigotes, measured by MTT and by microscopic examination, respectively, was in accordance with the values obtained for La-WT (Table 1), confirming the suitability of La-LUC for studying the effectiveness of antileishmanial drugs.

In order to compare the infectivity of La-LUC and La-WT in vivo, *LUC*-expressing or WT stationary phase promastigotes were inoculated into BALB/c mice. In both cases, the onset of cutaneous clinical signs was detectable around week four, and progressively increased during the period studied (Fig. 3). No significant difference was observed in the lesion size between the two groups. Thus, La-LUC and La-WT presented the same disease progression in infected mice.

3.2. Evaluation of AmB treatment in L. (L.) amazonensis-LUC infected mice

We then set out to characterize AmB response in the *L. (L.) amazonensis*-BALB/c infection model. Effectiveness of treatment was evaluated by clinical and parasitological criteria. Different approaches were used to determine the parasite burden in an attempt to compare

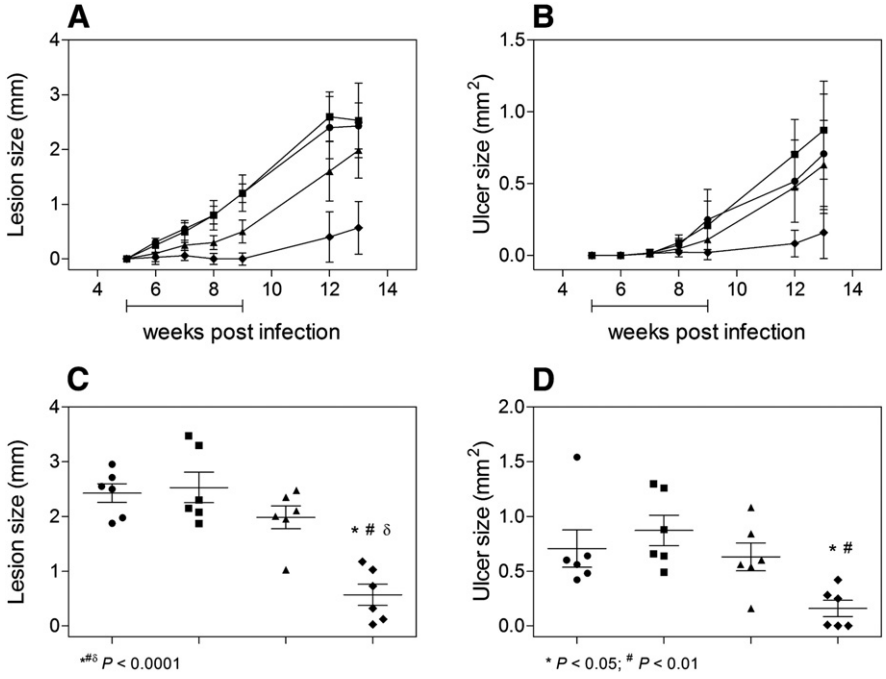

Fig. 4. AmB treatment of BALB/c mice infected with *L. (L.) amazonensis* expressing luciferase. Mice were inoculated in the tail with 10^6 stationary phase promastigotes of La-LUC. Treatment with AmB (1.2, 2 or 4 mg/kg/day) was initiated five weeks post-infection. Lesion size (A) and ulcer size (B) were recorded in AmB treated or untreated (NT) groups (n = 6 per group) during and until 4 weeks after the end of treatment. Horizontal bars indicate the period of AmB administration. (C) and (D) show individual measures of lesion (C) and ulcer sizes (D) at the end of the experiment (four weeks after the end of treatment). Statistical analysis was performed using One-Way ANOVA with the Tukey post-test: statistical results are shown for the group treated with AmB4 as compared with the control group (*), with AmB 1 (#) or with AmB 2 (δ). ● NT; ■ AmB 1.2; ▲ AmB 2; ◆ AmB 4.

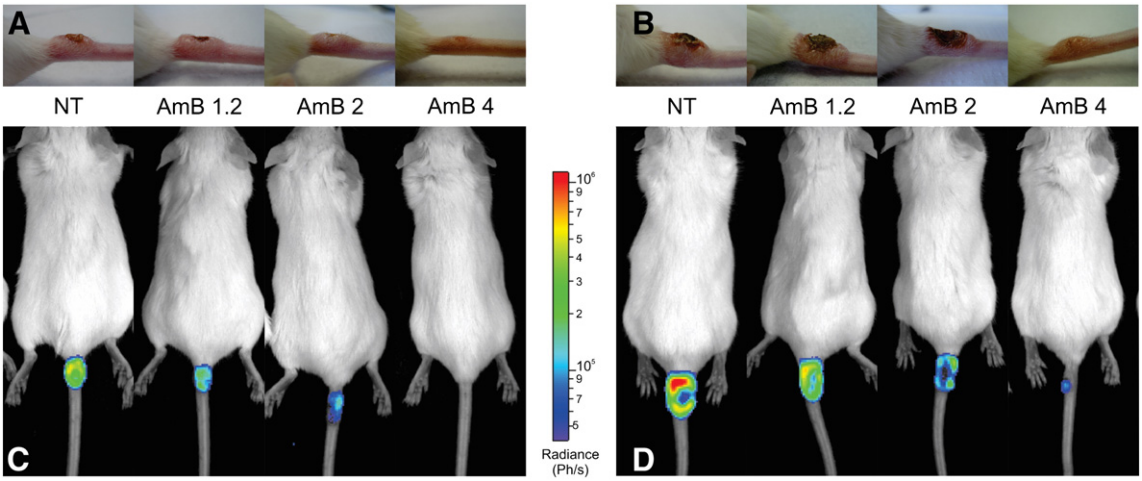

Fig. 5. Bioluminescence of lesions in untreated and AmB treated mice. BALB/c mice were inoculated in the tail with 10^6 La-LUC stationary phase promastigotes and treated with AmB at 1.2, 2 or 4 mg/kg/day for 20 days. Images were acquired at the end of AmB treatment (nine weeks post-infection) (A and C) and in the end of the experiment (13 weeks post-infection) (B and D). Images illustrate the macroscopical aspect of lesions (A and B) and bioluminescence imaging (C and D) from representative mice in each group (n = 6 per group). Ph/s: photons per second.

these methods in the evaluation of drug responses in the mouse model.

The progression of the clinical signs of disease, such as lesion and ulcer sizes (Fig. 4A and B), was followed up for 13 weeks. Treated mice showed reductions in lesion and ulcer sizes in a dose-dependent manner (Fig. 4C and D). At the end of treatment, lesion size was reduced by 100% and 58.3% in groups treated with 4 and 2 mg/kg/day AmB, respectively, in comparison with untreated mice. Lesion size was not reduced in the group treated with 1.2 mg/kg/day AmB (Fig. 4A and C). Similar results were observed for ulcer size (Fig. 4B and D). The group treated with 4 mg/kg/day AmB was significantly different from 1.2 mg/kg/day AmB and untreated groups (Fig. 4C and D).

During follow up after the end of treatment, clinical signs worsened in all groups (Fig. 4A and B) indicating that no treatment scheme was completely effective. The group treated with 4 mg/kg/day AmB was the least unstable of all with only a small increase in lesion and ulcer sizes after the interruption of treatment.

LUC quantification through bioimaging showed correlation with the macroscopical examination of lesions (Fig. 5). Light emission was reduced in a dose-dependent manner in AmB-treated mice at both time points. In each group, light emission increased at the end of the evaluation as compared to the values obtained at the end of treatment (Fig. 5C and D).

To quantitatively validate the data obtained by bioimaging, parasite burden was evaluated by limiting dilution. At the same time, an alternative method, ex vivo quantitation of *LUC* expression in parasites recovered from the lesion, was assessed (Fig. 6). When quantified by limiting dilution, the average reduction in the group treated with 4 mg/kg/day AmB was 93.52% as compared with the untreated group (Fig. 6A and Table 2). Bioimaging and ex vivo LUC quantitation for the same groups showed 87.38 and 79.89% reductions, respectively (Fig. 6B, C and Table 2). Therefore, although there were differences in the quantitative results between the three methods (Table 2), all techniques produced the same profile and indicated that only the group treated with 4 mg/kg/day AmB was significantly different from the untreated group.

Sigmoidal dose–response curves were derived to calculate the ED_{50} of AmB for each of the parameters used to evaluate the response to treatment (Table 3). For lesion size, LUC quantification (in vivo and ex vivo) and limiting dilution, values obtained varied from 1.19 to 2.86 mg/kg/day and the average ED_{50} was 2.09 mg/kg/day. An overlapping of 95% confidence intervals (95% CI) was observed for lesion size and LUC quantification (in vivo and ex vivo) and for LUC quantification in vivo and limiting dilution (Table 3). The differences in ED_{50} values obtained with the data from these different evaluation methods are well within the acceptable range for this type of determination. Therefore, the results of LUC quantification are congruent with the limiting dilution and lesion size results. For ulcer size, the AmB ED_{50} was 0.61 mg/kg/day (95% CI = 0.35–1.05) and was different when compared with all the other parameters.

4. Discussion

L. (L.) amazonensis lines expressing LUC were obtained in order to quantify parasite loads in infected animals. Bioluminescence generated by La-LUC was evaluated either in a microplate reader or in an IVIS. In both cases, luminescence correlated with the number of parasites, confirming that LUC activity is a reliable method to quantify transfected promastigotes in vitro, as previously reported (Gupta, 2005; Lang et al., 2005; Roy et al., 2000). Transfected parasites were equally sensitive to AmB as the WT parasites, as shown by the EC_{50} for promastigotes and intracellular amastigotes of both strains. These values were also in accordance with the previous reports for *L. (L.) amazonensis* (Zauli-Nascimento et al., 2010).

Reports exist on the use of *L. (L.) donovani and Leishmania (Leishmania) major* expressing the *LUC* gene either as part of extra-chromosomal circular molecules or integrated into the parasite genome (Roy et al., 2000). The authors found that for prolonged growth in the absence of drug selection, such as within animal models, quantitation of parasites was more reliable when the *LUC* gene was stably integrated in the parasite genome. In fact, when reporters are part of plasmids, the relative output of the reporter may depend on the copy number of the transfected plasmid, which varies from cell to cell (Kutzleb et al., 1973). However, several observations suggest that episomal DNA is maintained during the amastigote stage for long periods, irrespective of drug pressure (Uliana et al., 1999). In our model, *LUC* expression was maintained after infection in mice, regardless of the absence of antibiotic pressure, since amastigotes recovered from the tail of infected *L. (L.) amazonensis* BALB/c mice 13 weeks post-infection were still expressing luciferase. The clinical pattern of lesion development was also similar when the WT strain was compared to the lines expressing *LUC*, indicating that both strains are driving a similar clinical course, as previously reported (Lang et al., 2005).

Aiming to validate the use of La-LUC infected mice for experimental studies with antileishmanial drugs and to quantitatively evaluate results obtained from IVIS, we tested the efficacy of AmB given at

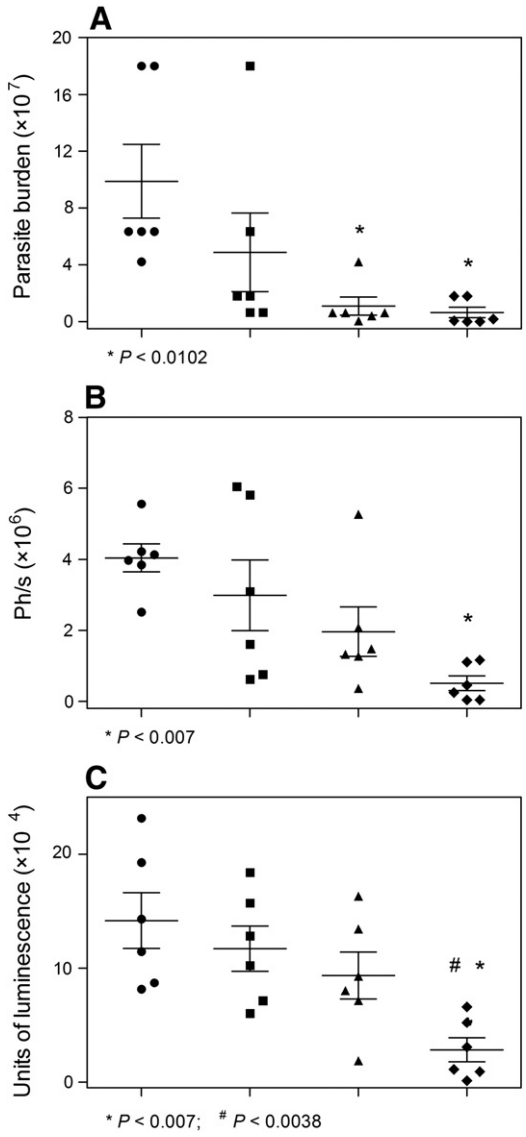

Fig. 6. Evaluation of parasite burden after AmB treatment by different techniques. AmB was administered at 1.2, 2 and 4 mg/kg/day for 20 days and the treatment was evaluated at the end of the experiment (four weeks after the end of treatment) by limiting dilution (A), bioluminescence quantification in vivo (B) and ex vivo (C). Untreated (NT) animals were used as control (n = 6 per group). Statistical analysis was performed using One-Way ANOVA with the Tukey post-test: statistical results are shown for the groups treated with 2 or 4 mg/kg/day (AmB2 or AmB4) as compared with the control group (*), or with AmB 1.2 (#). ● NT; ■ AmB 1.2; ▲ AmB 2; ♦ AmB 4. Each symbol corresponds to one mouse. Ph/s: photons per second.

three different doses. Criteria used to evaluate drug response included lesion and ulcer sizes and parasite burden, determined by three different methods: limiting dilution, IVIS and ex vivo LUC quantification. Evaluation at the end of treatment indicated that all these parameters were in accordance.

To date the most widely used criteria to evaluate effectiveness in cutaneous leishmaniasis models have been lesion size and limiting dilution-determined parasite burden. Limiting dilution is a highly laborious and time consuming technique and is subject to errors due to loss of parasites during cell isolation steps. It can take days or weeks until parasites are observed. Moreover, the cultures may become contaminated (Kobets et al., 2012). Therefore, limiting dilution results are difficult to reproduce. Attempts to overcome these difficulties include the determination of parasite burden based on reverse transcription and real-time PCR, for example (Nicolas et al., 2002) but this alternative is not devoid of difficulties itself. With the availability of bioimaging, new attractive alternatives

Table 2
Effectiveness of AmB in vivo measured by different criteria evaluated four weeks after the interruption of treatment.

Method[a]	NT	AmB 1.2[b]	AmB 2	AmB 4
Lesion size (mm)	2.43 (100%)	2.53 (104.12%)	1.98 (81.48%)	0.57 (23.46%)
Ex vivo LUC quantification (UL × 10^4)	14.17 (100%)	11.71 (82.64%)	9.35 (65.98%)	2.85 (20.11%)
In vivo LUC quantification (Ph/s × 10^6)	4.04 (100%)	2.99 (74.01%)	1.96 (48.51%)	0.51 (12.62%)
Number of parasites (×10^7)	9.88 (100%)	4.87 (49.29%)	1.10 (11.13%)	0.64 (6.48%)
Ulcer size (mm^2)	0.71 (100%)	0.87 (122.54%)	0.63 (88.73%)	0.16 (22.54%)

[a] Treatment of BALB/c mice infected with La-LUC was initiated five weeks after the infection. Clinical parameters or parasite burden was determined four weeks after the end of treatment. UL: units of luminescence; Ph/s: photons per second; number of parasites was determined by limiting dilution.
[b] AmB was given at 1.2, 2 or 4 mg/kg/day. Columns show the average value for each group expressed as the absolute measurement and as a percentage as compared with the untreated group (in parenthesis). NT: untreated control group.

to quantify parasites in tissues became apparent but there is still a need to validate bioimaging as a quantitative measure of parasite loads (Thalhofer et al., 2010). Also, no data comparing different methodologies to evaluate drug effectiveness at the ED_{50} level is available.

We found that luminescence measured in vivo and ex vivo was directly correlated to parasite burden estimated by the limiting dilution method and to lesion and ulcer sizes, validating the use of LUC as a quantitative tool in this model.

IVIS possess several advantages, such as the possibility to monitor the disease progression in the same animal and the consequent reduction of the number of animals in each experimental group. There are also some limitations to this methodology, such as the use of expensive substrate. Also, mixing of reagents and injection into animals needs to be timed with anesthesia and image acquisition, as the luminescent read out is transient (Gupta, 2005). On the other hand, we found that the analysis of luminescence ex vivo was a rapid and simple mean for measuring parasite burden in infected mice and was compatible with both lesion size measurements and in vivo bioimaging. The ex vivo method is relatively low-cost and can be performed in most laboratories if bioimaging equipment is unavailable.

As compared to the limiting dilution assay, the ex vivo quantification has the advantage of determining the number of parasites in the crude extract of lesion macerate, eliminating the washing steps, which avoids loss of parasites. Furthermore, limiting dilution will only detect amastigotes capable of transforming into promastigotes and surviving in culture in the presence of antibiotics. High concentrations of toxic products in necrotic tissues, frequently seen in leishmaniasis lesions, may impair the differentiation and growth of parasites when placed in culture. In contrast, ex vivo and in vivo LUC quantification derive from all parasites capable of expressing the *LUC* gene at that particular point in time.

Table 3
AmB half maximal effective dose (ED_{50}) in *L. (L.) amazonensis* infected mice as measured by different techniques.

Method	ED_{50}[a]	95% CI[b]
Lesion size	2.86	2.42–3.37
Ex vivo LUC quantification	2.43	1.82–3.25
In vivo LUC quantification	1.89	1.35–2.65
Limiting dilution	1.19	0.82–1.71
Ulcer size	0.61	0.35–1.05

[a] ED_{50} (mg/kg/day) was calculated based on parameters obtained four weeks after the interruption of treatment.
[b] 95% CI: 95% confidence interval.

Ulcer size was the least reliable numerical parameter, as expected. Ulcer formation is a late effect of disease and results from a complex interplay among the parasite burden, the activated state of the immune system, and the repair system (Oliveira et al., 2011). Nevertheless, we consider ulcer size as a valid parameter to be used in qualitative terms rather than as a quantitation tool.

Only limited data regarding AmB ED_{50} for *L. (L.) amazonensis* infection are available in the literature. In our model, even the highest AmB dose (4 mg/kg/day), did not lead to cure. Parasite load was only reduced and the treatment did not result in long-lasting resolution of the disease. Toxic effects limit the testing of higher doses.

To improve the management of leishmaniasis, new drugs or alternative therapeutic strategies are required. Combination therapy of antileishmanial drugs is currently considered as one of the most rational approaches to decrease treatment failure rates and limit the spread of drug resistance. Results presented herein will be useful in the design of in vivo drug combination experiments including AmB and may contribute to the study of new therapies for leishmaniasis using *LUC* expressing parasites.

In conclusion, the quantification of bioluminescence in vivo and ex vivo seems to be the most effective method for the determination of parasite burden to assess drug efficacy in infected mice. Furthermore, this approach is a reliable model to evaluate drug therapy at the ED_{50} level.

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