Photodiagnosis and Photodynamic Therapy 26 (2019) 261–269

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

Study of the efficacy of N-methyl glucamine antimoniate (Sb^V) associated with photodynamic therapy using liposomal chloroaluminium phthalocyanine in the treatment of cutaneous leishmaniasis caused by *Leishmania (L.) amazonensis* in C57BL6 mice

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ARTICLE INFO

Keywords:
Cutaneous leishmaniasis
N-methyl glucamine antimoniate
Photodynamic therapy
Liposomal chloroaluminium phthalocyanine
Nanodrug

ABSTRACT

Background: Pentavalent antimonials remain first-line drugs in the treatment of cutaneous leishmaniasis (CL); however, adverse effects and drug resistance have led to the search for less toxic and more effective treatments. As an alternative, topical phthalocyanine has been studied and its efficacy and low toxicity demonstrated. We aimed to study the *in vivo* efficacy of N-methyl glucamine antimoniate (NMG) associated with photodynamic therapy (PDT) with topical liposomal chloroaluminium phthalocyanine (AlClPC) in the treatment of experimental CL by *L. amazonensis*.

Methods: Experimental study with 54 C57BL6 isogenic mice divided into 9 groups including uninfected control, untreated control, PDT with AlClPC + NMG at doses of 10 and 20 mgSb^V/Kg/day. The criteria to evaluate the treatment efficacy were: paw diameter, amastigote count, culture, viability test and parasite counts using MTT (3-bromo-4,5-dimethylthiazol-2,5-diphenyl-tetrazolium bromide).

Results: Treatment of CL with the association of NMG20 + PDT with AlClPC showed significant reduction of paw diameter, amastigote count, cultures, viability test and parasite counts. Parasite reduction occurred at the 10th and 20th days of treatment and 60 days after treatment ended, indicating that parasites did not multiply again. The NMG10 + PDT group with AlClPC presented results equivalent to gold-standard treatment (20 mgSb^V/kg/day). Biochemical and histopathological evaluation showed minor changes.

Conclusion: Treatment of CL caused by *L. amazonensis* with NMG20 mgSb^V/kg/day + PDT with AlClPC was more effective than the traditional NMG20 mgSb^V/kg/day.

1. Introduction

Cutaneous leishmaniasis (CL) caused by the protozoan *Leishmania* is a neglected tropical disease and a major public health problem [1,2]. Its control is complex because the cycle, reservoirs, vectors, hosts and species of *Leishmania* vary within any given area, as do the clinical and therapeutic responses [1,3,4].

CL is mainly treated by parenteral administration of pentavalent antimonial (Sb^V), which is highly toxic [1,2,5–8], and whose resistance has been demonstrated in a progressive and worrying way [1,4,9]. The

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https://doi.org/10.1016/j.pdpdt.2019.04.004
Received 14 November 2018; Received in revised form 20 March 2019; Accepted 1 April 2019
Available online 02 April 2019
1572-1000/

occurrence of up to 90% relapse after 1 year of treatment in some forms of leishmaniasis makes it necessary to follow patients' progress for 1–2 years after treatment and remain alert for resistance to Sb^V, a very serious problem in countries like India [1,10–12].

Other options are injectable and toxic, such as amphotericin B (deoxycholate and liposomal) and pentamidine isethionate; all require systemic use and exert cardio-nephro-hepatic and pancreatic adverse effects [1,12,13]. Miltefosine is the first effective oral medication, originally developed to treat cutaneous metastases in mammary carcinomas and later used for the treatment of leishmaniasis. Despite its therapeutic efficacy, miltefosine presents two main limitations that are adherence (and hence potential for selection of drug resistant parasites) and teratogenicity (pregnancy must be avoided during treatment and the following two months). It presents dose-limiting toxicity, commonly inducing gastrointestinal adverse effects such as anorexia, nausea, vomiting and diarrhoea, which may limit adherence to treatment and reduce its efficacy, favouring selection of resistant cell lines and relapse. Although most episodes are brief and are resolved as treatment is continued, the side-effects can occasionally be severe and require interruption of treatment. Skin allergy, elevated hepatic transaminase concentrations and, rarely, renal insufficiency may also be observed [1,14].

Thus, more effective and less toxic therapeutic regimens would represent an advance in disease control, since treatment is currently the first weapon to combat leishmaniasis. Therefore, nanotechnology represents a promising strategy for developing controlled release of promising drugs used in the combat and control of parasites, aiming for effectiveness and the control of adverse effects [15,16].

In the treatment of leishmaniasis, controlled release may be an effective alternative to current treatment modalities, offering lower toxicity, greater adherence to treatment, outpatient application, and therefore lower operational cost [16,17]. This would represent an advance because the disease is more frequent in rural and poor areas where access to healthcare is difficult [1,17]. At this point, nanotechnology has greatly contributed to the development of specific delivery sensitizers, resulting in greater photodynamic efficacy and improved treatment protocols, with an impact on several medical areas [18]. The use of photosensitizers is indicated, with promising results in photodynamic therapy (PDT), especially with phthalocyanines (PC). The inclusion of chloroaluminium phthalocyanine (AlClPC) incorporated into liposomes represents a great advance in the bioavailability of this substance, allowing it to act focused on the cutaneous lesion [15,19,20]. The association between PDT, AlClPC and miltefosine used in the treatment of experimental CL showed a significant reduction in the viability and number of *Leishmania* when compared to other treatments [15].

PDT is a topical and promising therapy of current interest for the treatment of localized diseases. It associates the triad of oxygen, light source and photosensitising agent, factors that in combination produce lethal cytotoxic substances that can selectively destroy cells and parasites including *Leishmania* [2,18,21–23]. The use of aluminium phthalocyanine and zinc phthalocyanine under the action of visible light at 670 nm inhibited the *in vitro* growth of *L. chagasi* and *L. panamensis* promastigotes [20]. Studies of PDT in the treatment of experimental CL in rodents have been promising, with a significant reduction in the parasite burden and a diminished lesion [15,24,25], besides the occurrence of modulation of the inflammatory process [25]. In humans with CL, the use of PDT has obtained good results, although data are still limited [26,27].

The objective of this study was to verify the efficacy of N-methyl glucamine (NMG) associated with PDT using AlClPC in the treatment of CL caused by *L. amazonensis*.

2. Materials and methods

2.1. Synthesis and characterization of AlClPC

All experiments were carried out with an AlClPC formulation at 5 μM, in a liposomal medium. The synthesis of AlClPC was performed at the Laboratory of Photobiology and Photomedicine-CNET-Dep. Chemistry, USP, Ribeirão Preto, São Paulo state, Brazil, as previously reported [15]. Briefly, a small unilamellar liposome (0.7 mM) based on L-α-dipalmitoyl phosphatidylcholine (DPPC) and cholesterol was prepared on the basis of the injection method, where 360 μL of an ethanolic solution (0.525 mM in DPPC, 0.14 mM in cholesterol, and an appropriate volume of AlClPC to reach a final concentration of 5 μM) was injected with a syringe into 5 mL phosphate buffer pH 7.4 at 56 °C, under magnetic stirring and at a flow rate of 1 μL/s. The amount of AlClPC incorporated was analysed by absorption and fluorescence spectra [15,28].

Samples of liposomal chloroaluminium phthalocyanine (AlClPC) were analysed by transmission electronic microscopy (TEM), using a JEOL JEM 1011 electron microscope (Jeol, Tokyo, Japan); a scanning electron microscope – SEM/EDS (energy dispersive x-ray detector, EDX or EDS) JEOL JSM-7001 F (JEOL, Tokyo, Japan/SDS system); and a Zetasizer Nano ZS equipment (Malvern Instruments, Malvern, UK), to evaluate the polydispersity index (PDI), the hydrodynamic diameter by dynamic light scattering (DLS) and the surface charge of liposome particles by measuring the zeta potential [15].

2.2. Light source

A continuous low power (80 mW) diode laser (BWF light source – Tech in), operating at 670 nm, with wavelength of maximum optical absorption, fibre-optic adapted, was used in order to excite AlClPC solution. The laser device used was a manufactured one, which was developed by the Physics Department of Brasilia University. As this experiment was developed *in vivo*, the energy applied was 22.5 J with a light focus of 4 cm^2, covering the entire paw of mice at a distance of 5 cm from the base. So the energy density corresponded to 5.62 J/cm^2 [15].

2.3. Experimental animals and ethics

Fifty-four female mice (*Mus musculus*), C57BL6 inbred strain were used, aged 3 to 4 months and weighing 23.47 ± 0.35 g, with sanitary certificate from the CMIB Animal Science Area, Laboratory of the University of Campinas (Brazil). Animals were housed in polypropylene cages (6/cage) at room temperature (20 ± 2 °C) under a 12 h light/dark cycle with lights on at 6 a.m., with water and feed *ad libitum*.

All procedures were reviewed and approved by the Animal Use Ethics Committee of the Faculty of Medicine of the University of Brasilia (UnBDoc 83795/2012).

2.4. Anaesthesia and euthanasia of animals

To carry out infection, lymph collection and PDT, animals were intramuscularly (im) anaesthetized with a combination of 10 mg/kg of xylazine and 90 mg/kg of ketamine. For euthanasia, mice previously anaesthetized for lymph and/or blood collection were individually taken to an over-70%-CO_2 saturation chamber, until cardiorespiratory arrest [15].

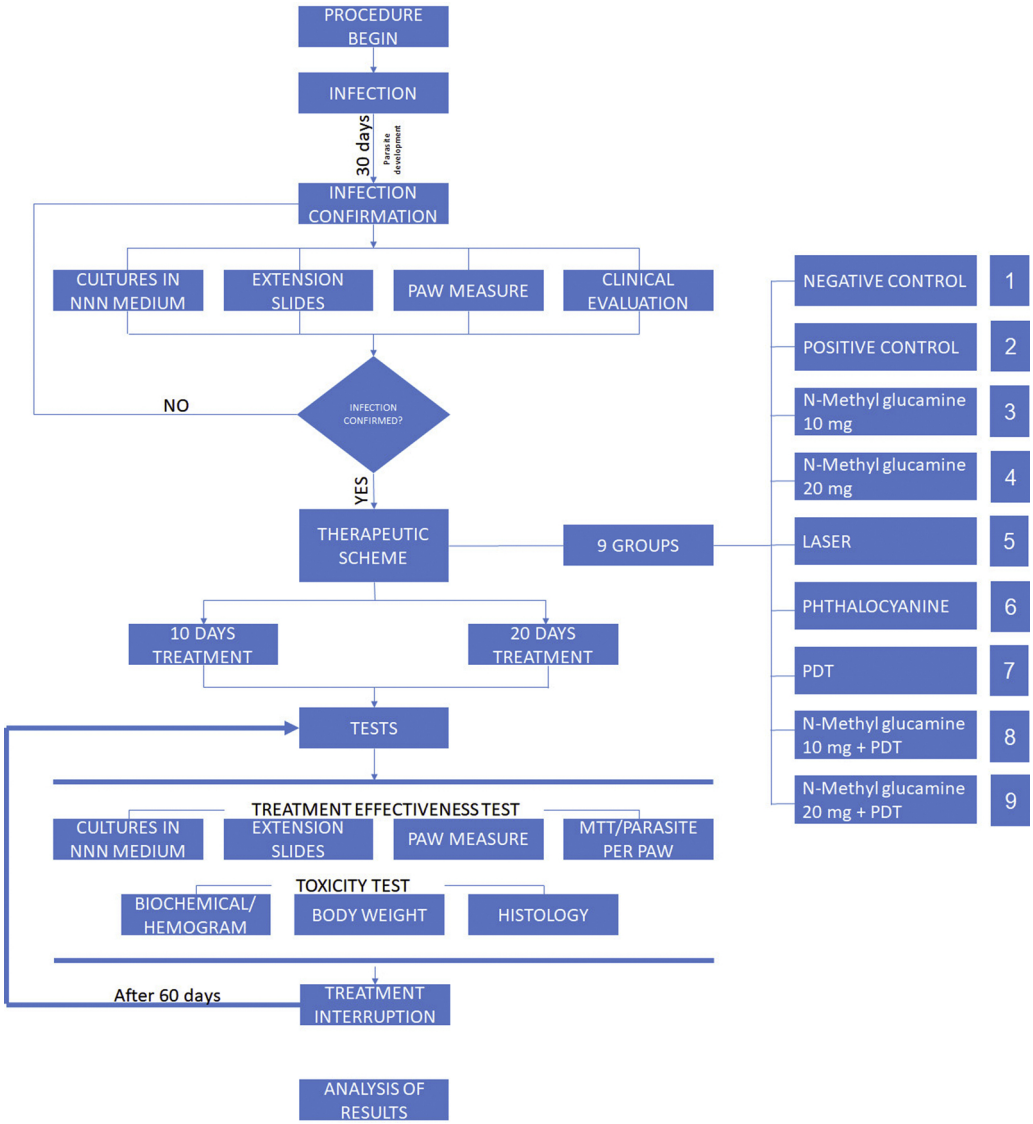

Fig. 1. *In vivo* experimental design flow.

2.5. Inoculum

3×10^6 of metacyclic promastigotes of *L. amazonensis* (MHOM/BR/PH8) [15].

2.6. Infection and its confirmation

Infection was carried out intradermally in the hind paw with 100 µL of the inoculum. After 30 days, the infection was confirmed by paw diameter, amastigote count and cultures (Fig. 1) [15].

2.7. Treatment and therapeutic scheme

Animals were divided into 9 groups (N = 6 per group) (Fig. 1):

1 Negative Control Group: uninfected control; animals received only physiological solution applied identically to that for animals that received infection (Negative Control or NC).
2 Positive Control Group: infected and untreated control; animals received saline daily (Positive Control (PBS) or PC).
3 N-methyl glucamine 10 mgSb^V/kg/day for 20 consecutive days (N-methyl glucamine 10 mg or NMG10).
4 N-methyl glucamine 20 mg Sb^V/kg/day for 20 consecutive days (N-methyl glucamine 20 mg or NMG20).
5 Laser -10 alternate days (Laser or L).
6 AlClPCL-10 alternate days (Phthalocyanine or AlClPCL)
7 PDT with AlClPCL - 10 alternate days (PDT).
8 N-methylglucamine 10 mgSb^V/kg/day + PDT (N-methyl glucamine 10 mg + PDT or NMG10 + PDT).
9 N-methylglucamine 20 mg Sb^V/kg/day + PDT (N-methyl glucamine 20 mg + PDT or NMG20 + PDT).

The choice of the doses used in the experiment followed the recommendations by the World Health Organization (WHO) and the Pan American Health Organization (PAHO): the gold-standard treatment for leishmaniasis is 20 mgSb^V/kg/day for 20 days, as established by the WHO since 1984; 10–20 mg/kg/day of pentavalent antimony in a single daily dose for 20 days is the recommendation by the PAHO for the treatment of leishmaniasis in the Americas [4]. So, SB^V at dose of 10 mg/kg day for 20 days was also used to verify the possibility of reduction of the gold-standard treatment concentration (20 mgSb^V/kg/day) recommended by the WHO, in view of the association with PDT.

2.8. Route and manner of application of drugs

The treatment started 30 days after infection of the mice by the parasite. By intraperitoneal route, the animals received 1 daily dose of 10 or 20 mgSb^V/day for 20 consecutive days. After anaesthesia, 500 μL of gel containing AlClPCL was applied to the right hind paw, keeping it wrapped with aluminium foil in a dark environment. After 15 min, the paw was exposed to visible light irradiation for another 15 min at 5 cm from the source (Fig. 1).

2.9. Treatment efficacy (healing criteria)

2.9.1. Paw measurements

Measurement was performed in duplicate, using two Mitutoyo® millimetric pachymeters (São Paulo, Brazil), at six moments: before; 2 and 30 days after infection; after 10 and 20 days of treatment and 60 days after the end of treatment (Fig. 1).

2.9.2. Evaluation of efficacy by parasitological criteria

When measuring diameters, paws were submitted to lymph collection to search for amastigotes in a smear stained with Giemsa and to perform traditional cultures (biphasic NNN medium) [15].

2.9.3. Limiting dilution

Two animals from each group were euthanized and subjected to asepsis and skin dissection of one paw, which was ground in liquid culture medium plus 10% foetal bovine serum and 0.2% gentamycin and seeded in ELISA plate cultures (96 wells) in successive 10-fold dilutions. Limiting dilution cultures were followed by colorimetric method using MTT, 3(4,5 dimethylthiazol-2,5-diphenyl-tetrazolium bromide), which estimates viable amastigotes in paws [15].

2.9.4. Evaluation of the toxicity of the therapeutic schemes

The body weight of the animals was evaluated before, during and after treatments. Additionally, biochemical dosages of total bilirubin, direct bilirubin, indirect bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), alkaline phosphatase, creatinine, urea and albumin, as well as histopathological analyses of liver and kidneys were performed at three evaluation times: 1) after 10 and 2) 20 days of treatment and 3) 60 days after the end of treatment. For the respective times, two animals from each group were used.

For biochemical dosages, animals were anaesthetized and submitted to blood collection without anticoagulant by cardiac puncture. Samples collected in appropriate vacutainer tubes were centrifuged at 3000 rpm for 5 min. Serum samples were run on the automated chemistry analyser ADVIA 2400 (Siemens), using appropriate Advia chemistry reagents, protocols and controls. Total bilirubin, bilirubin fractions and creatinine were measured by colorimetric assays; GGT by a colorimetric kinetic method; urea by an enzymatic colorimetric method; AST, ALT and alkaline phosphatase by optimized kinetic methods; and albumin by turbidimetry.

For the histopathological analyses of liver and kidneys, organs were surgically removed after euthanasia, fixed with 10% formalin for 24 h, transferred to 70% ethanol, embedded in paraffin and submitted to microtome cutting. Histological sections were examined by a pathologist. Interpretation was blind for all exams. For the liver, five criteria were used: architectural alteration (AA), lobular inflammatory infiltrate (IL), portal inflammatory infiltrate (PI), hydropic degeneration (HD) and fatty degeneration (FD). For kidney, six criteria were used: glomerulosclerosis (GE), inflammatory infiltrate interstitial tubule (IIT), glomerular inflammatory infiltrate (GI), tubular degeneration (TD), acute tubular necrosis (ATN) and presence of protein cylinders (PC). Grades from 0 to 4+ were considered: 0 = absence; + = low frequency; ++ = frequent; +++ = very common; ++++ = high frequency.

2.9.5. Statistical analysis

Statistical analysis was performed using IBM SPSS statistics version 18.0. Quantitative variables were tested for normal distribution with Shapiro-Wilk test. For the variable “paw diameter”, possible differences among the groups were investigated by ANOVA, followed by the Tukey post-test; for the time differences within each group, the *t*-test for paired samples was used. The number of parasites per paw was determined by ELIDA, which statistically infers the limiting dilution assay. For analyses of body weight, MTT, number of parasites per paw and % viable cells, possible differences among the groups were investigated by Kruskal-Wallis test (data not normally distributed). The Mann-Whitney *U* test was used to verify differences among treatments (paired comparisons). Possible differences inside each group were tested by Friedman test followed by Wilcoxon test for paired comparisons. To assess the association among the variables “extension slides” and “cultures in NNN medium” with the group at distinct moments, Fisher's exact test was applied, which when significant indicates that there is an association among them [29]. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Liposome particles

The mean diameters measured by the two different techniques showed average size values were 52.9 ± 12.4 nm (modal average diameter) by transmission electronic microscopy (TEM); and 266.9 ± 5.2 nm (hydrodynamic average diameter) by dynamic light scattering (DLS). The average polydispersion index (PDI) and surface charge measured by the zeta potential were 0.55 ± 0.04 and −8.2 ± 3.50 mV, respectively. The liposome gel solution presented

Table 1
Negative and positive cultures and extension slides 30 days after infection (confirmation of infection).

Fisher exact test	Cultures			Extension Slides		
	Negative	Positive	p-value	Negative	Positive	p-value
30 Days After Infection						
Negative control	6	0		6	0	
Positive control (PBS)	1	5		4	2	
N-methyl glucamine 10 mg	0	6		4	2	
N-methyl glucamine 20 mg	0	6		4	2	
Laser	1	5		4	2	
Phthalocyanine	0	6	0.000	5	1	0.811
PDT	0	6		4	2	
N-methyl glucamine 10 mg + PDT	0	6		3	3	
N-methyl glucamine 20 mg + PDT	0	6		5	1	
Total	8	46		39	15	

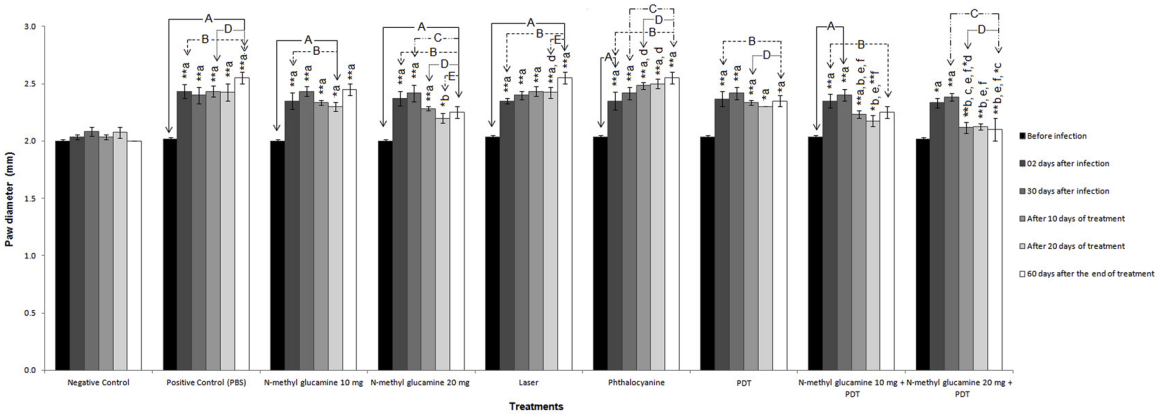

Fig. 2. Paw diameter before infection, 2 and 30 days post infection (confirmation of infection and start of treatment), 10 and 20 days after treatment, and 60 days after the end of treatment.
Data correspond to the mean and standard error of mean (SEM). Lowercase letters indicate significant differences detected by Tukey's multiple comparisons test in the vertical comparisons (among the analysed groups), where: a = significant compared to negative control; b = significant compared to positive control; c = significant compared to N-methyl glucamine 10 mg treated group; d = significant compared to N-methyl glucamine 20 mg treated group; e = significant compared to laser treated group; f = significant compared to liposomal chloroaluminium phthalocyanine treated group. Asterisks indicate significant differences at *p < 0.05 and **p < 0.01. Capital letters indicate significant differences (p < 0.05) in the horizontal comparisons (within each group) detected by the Paired Samples T-test, where: A (arrows with solid line:__) = significant compared to before infection; B (arrow with dashed line:- - -) = significant compared to 02 days after infection; C (arrows with dashed and dotted lines:_.._) = significant compared to 30 days after infection (confirmation of infection and start of treatment); D (arrows with dotted line:.....) = significant compared to after 10 days of treatment; E (arrows with dashed and dotted lines:-.-.-.) = significant compared to after 20 days of treatment.

several layers, including the presence of liposomes probably deformed by the technical processing; it was possible to observe the presence of smooth-surfaced cubic aluminium fragments more freely (outside gel layers). The EDS system confirmed the strong presence of Si, Na, Mg, Al, K and Ca ions in the whole analysed sample [15].

3.2. Infection confirmation

After 30 days of infection, infected animals were confirmed by amastigote count and cultures (Table 1).

3.3. Paw diameter measurements

The PC group maintained a progressive increase in paw diameter at all times, compared to the NC group. At the 2nd and 30th days after infection, all infected animals had similar results to the PC group. On the 10th day after treatment, the NMG20 + PDT was the only group that had a significant reduction in the paw diameter in relation to the PC, similar to NC group measurements. On the 20th day after treatment, this occurred with groups treated with NMG20, NMG10 + PDT, and NMG20 + PDT, but 60 days after treatment ended, the only group which maintained this reduction in paw diameter, approaching that of the NC group, was NMG20 + PDT (Fig. 2).

3.4. Amastigote count and cultures

The NMG20 + PDT group had 100% of the negative slides in the investigations of amastigotes and cultures after 20 days of treatment and 60 days after treatment ended. Followed by this group, groups NMG20 and NMG10 + PDT were the most effective, with 50% of negative cultures 60 days after treatment ended (groups PC, NMG10, Laser, AlClPCL and TFD showed 100% positive cultures 60 days after treatment ceased) (Table 2).

3.5. Percentage of viable cells and number of parasites per paw

Viability: after 10 and 20 days of treatment, groups NMG10, NMG20, PDT, NMG10 + PDT and NMG20 + PDT showed a significant percentage reduction in viable cells compared to PC group. PDT, NMG10 + PDT groups showed a significant percentage reduction in viable cells compared to all other groups, except the NMG20 + PDT group. At 60 days after the end of treatment, Laser and AlClPCL groups had similar results to the PC group. NMG20 + PDT group demonstrated a significant reduction in cell viability at all times compared to all groups, including NMG10 and NMG20 (gold standard) (Fig. 3a).

Parasites: after 10 days of treatment there was no significant difference between the PC, Laser, AlClPCL and PDT groups, while treatments NMG10, NMG20, NMG10 + PDT and NMG20 + PDT significantly reduced the number of *Leishmania*. The largest reduction was observed in the NMG20 + PDT group. After 20 days of treatment, all groups showed a significant reduction in the parasites in comparison to the PC group, including Laser and AlClPCL. However, on the 60th day after treatment ended, Laser and AlClPCL again had similar results to the PC group, while NMG10 + PDT was similar to NMG20. The NMG20 + PDT group also showed a significant reduction in the number of parasites per paw at all times compared to all groups, including NMG10 and NMG20 (gold standard) (Fig. 3b).

3.6. Toxicity of the therapeutic schemes

The body weight of the animals before, during and after treatments decreased only on the 20th day of treatment in the PDT group. In the biochemical evaluations, higher values of bilirubin and its fractions were observed in the groups treated with NMG + PDT; the NMG20 + PDT group also presented higher values of AST and ALT (data not shown). However, due to the small number of animals evaluated, it was not possible to perform statistical analysis to evaluate if these differences were significant. Histopathological changes in liver and kidney were found in all groups, including controls (data not shown), where morphological alterations did not correspond to the biochemical alterations.

4. Discussion

Available treatments for leishmaniasis have not yet presented desirable clinical results because of their low efficacy, toxicity, relapse (not necessarily representing parasite resistance), and parasite resistance. Additionally, there is no vaccine [1,15], and these facts justify

Table 2
Negative and positive cultures and extension slides after 10 and 20 days of treatment, and 60 days after the end of treatment.

Fisher exact test	Cultures			Extension Slides		
	Negative	Positive	p-value	Negative	Positive	p-value
10 days after treatment						
Negative control	6	0		6	0	
Positive control (PBS)	0	6		4	2	
N-methyl glucamine 10 mg	0	6		4	2	
N-methyl glucamine 20 mg	1	5		4	2	
Laser	0	6		3	3	
Phthalocyanine	1	5	0.000	4	2	0.507
PDT	1	5		4	2	
N-methyl glucamine 10 mg + PDT	2	4		5	1	
N-methyl glucamine 20 mg + PDT	3	3		6	0	
Total	14	40		40	14	
20 days after treatment						
Negative control	4	0		4	0	
Positive control (PBS)	0	4		2	2	
N-methyl glucamine 10 mg	1	3		2	2	
N-methyl glucamine 20 mg	2	2		3	1	
Laser	0	4		2	2	
Phthalocyanine	0	4	0.000	1	3	0.484
PDT	1	3		3	1	
N-methyl glucamine 10 mg + PDT	3	1		3	1	
N-methyl glucamine 20 mg + PDT	4	0		4	0	
Total	15	21		24	12	
60 days after the end of the treatment						
Negative control	2	0		2	0	
Positive control (PBS)	0	2		1	1	
N-methyl glucamine 10 mg	0	2		1	1	
N-methyl glucamine 20 mg	1	1		2	0	
Laser	0	2		1	1	
Phthalocyanine	0	2	0.167	1	1	1.000
PDT	0	2		1	1	
N-methyl glucamine 10 mg + PDT	1	1		2	0	
N-methyl glucamine 20 mg + PDT	2	0		2	0	
Total	6	12		13	5	

the need for new therapies. The significant benefits of drug combinations have already been well established, and these combinations have the potential advantages of: shortening the duration of treatment, thereby increasing compliance; reducing the overall dose of medicines, thus reducing their toxic effects and cost; and reducing the probability of selection of drug-resistant parasites, consequently prolonging the effective life of the available medicines [1]. At this point, both our previous [15] and present studies demonstrated that associating leishmaniasis treatments with PDT mediated by liposomes containing chloroaluminium phthalocyanine (AlClPC) could be an effective therapeutic option for the treatment of CL caused by *Leishmania amazonensis*. In a previous study [15], the combination of Miltefosine 200 mg/kg/day plus PDT after 20 days of treatment promoted a decrease in the number of viable *Leishmania* cells by approximately 93%. In the present study, with the association of SB^V at 10 and 20 mg/kg/day + PDT, the reduction was of about 91% and 97%, respectively. From these, we could infer, without statistical analysis, that the order of efficacy of the combined treatments could be: NMG 20 mg/kg/day + PDT > Miltefosine 200 mg/kg/day + PDT > NMG 10 mg/kg/day + PDT, at least aiming to treat CL caused by *L. amazonensis*. However, while the present study lasted up to 60 days after the end of treatment, this was not assessed in the previous report [15]. Thus, the inferred order of efficacy cannot be extrapolated as regards possible relapse. With this in mind and thinking about conjugated therapy with SBV or Miltefosine with PDT, the combined treatment of NMG 20 mg/kg/day + PDT with AlClPC would be the best therapeutic option for the treatment of CL caused by *L. amazonensis*, as discussed below.

AlClPC has been developed to mediate PDT as a new approach to treat CL, based on experiences from 50 years of treating cancer [15,20]. PDT employs the combination of non-toxic photosensitizers together with harmless visible light from the appropriate wavelength to produce reactive oxygen species that kill undesirable cells [30]. Because many photosensitizers are hydrophobic molecules prone to aggregation (including most phthalocyanines) [28,30], numerous drug nanoparticulate delivery vehicles, such as liposomes and micelles, have been tested to solubilize these molecules [30]. From this angle, liposomes are an interesting drug delivery system (DDS) because their properties can be regulated according to lipid composition, size, surface charge and preparation method [18]. Also, liposomes also have the potential to supply good targeting and provide a safe, basic formulation into which hydrophilic and lipophilic immunomodulators can be incorporated [31], such as the aluminium chloride used in the formulation of AlClPC. With this in mind, although results obtained in the Zetasizer equipment indicate some aggregation for liposome formulation, this was already expected and is in agreement with the behaviour expected for phthalocyanines incorporated into liposomes [28]. Moreover, our liposomal formulation presented an appropriate size, which is important in that liposome sizes of more than 500 nm could compromise their use as a drug delivery vehicle, due to their quick removal from the bloodstream by phagocytes [15,32]. While we could not present the mechanism that seems to be involved in the uptake of liposome by the infected cells, because no study was performed in this respect (and this was not the aim of our study), it has been reported that the main interaction of liposomes with cells is either simple adsorption (by specific interactions with cell-surface components, electrostatic forces, or by non-specific weak hydrophobicity) or following endocytosis (by phagocytic cells of the reticuloendothelial system, for example macrophages and neutrophils) [33]. Nevertheless, due to the efficacy of combined treatment with SB^V + PDT and through the fact that the amastigotes form of *Leishmania* has its development in the phagolysosomal vacuole of the

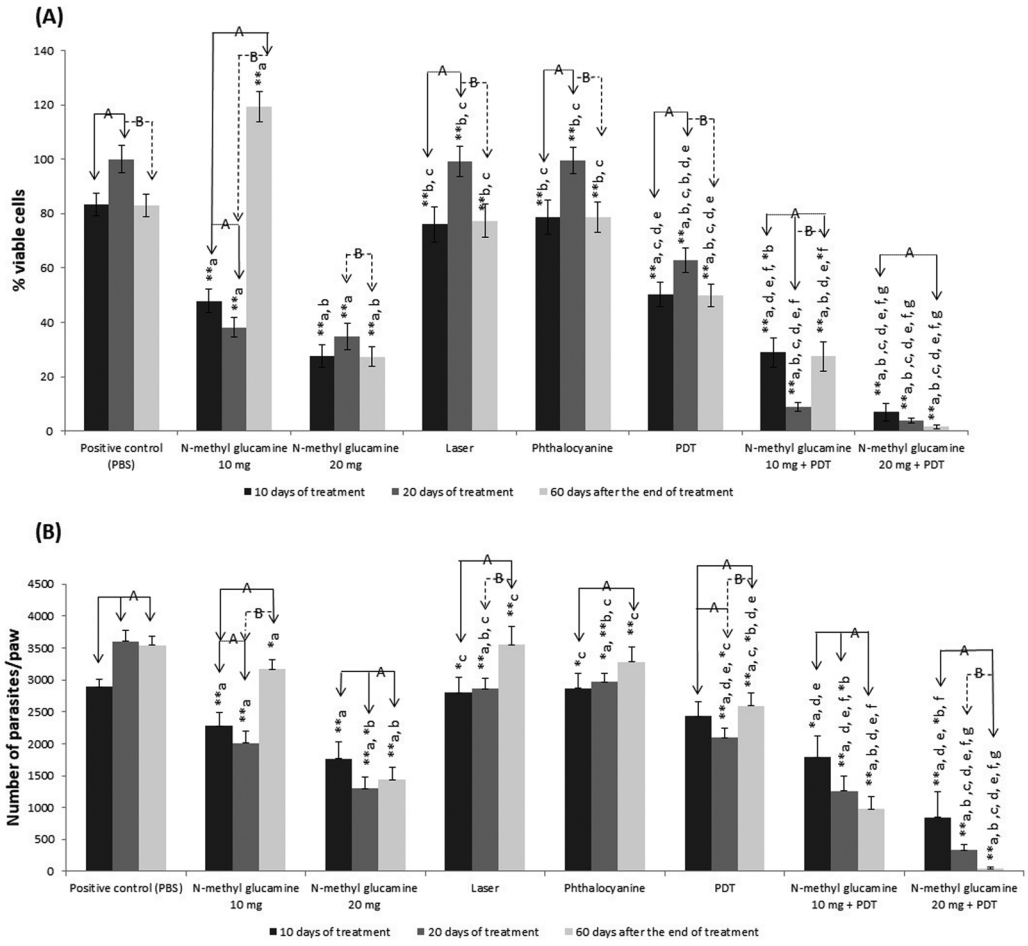

Fig. 3. Distribution of percentage of viable cells (A) and number of parasites per paw (B) in C57BL/6 mice infected with *Leishmania (L.) amazonensis*.
The lowercase letters indicate significant differences detected by the Mann-Whitney test in the vertical comparisons (2-to-2; between the groups), a = significant compared to the positive control (PBS); b = significant compared to the group treated with N-methyl glucamine 10 mg; c = significant compared to the group treated with N-methyl glucamine 20 mg; d = significant compared to the laser treated group; e = significant compared to group treated only with the liposomal chloroaluminium phthalocyanine (AlClPC); f = significant compared to the group treated with PDT; g = significant compared to the group treated with N-methyl glucamine 10 mg + PDT. The asterisks indicate significant differences at $*p < 0.05$ and $**p < 0.01$. The upper-case letters indicate significant differences ($p < 0.05$) in the horizontal comparisons (within each group) detected by the paired Wilcoxon test, being: A (solid line) = significant compared to 10 days of treatment; B (dashed line) = significant compared to 20 days of treatment.

macrophages when infective metacyclic promastigote forms are inoculated in the mammals by the hematophagus vector [34], endocytosis of the liposomal formulation of AlClPC would be the most plausible hypothesis. In fact, *in vitro* investigations have shown that some nano drug carriers induced a selective destruction of intracellular amastigotes [20,35]. The use of liposomes as drug carriers alters the pharmacokinetics and biodistribution of intracellular amastigotes in order to selectively accumulate them in the injured tissue [36]. The liposomes potentiate the photodynamic effect due to the cellular internalization of the photosensitizer, minimizing side effects [37].

Additionally, the fact that aluminium fragments were found on liposome surfaces cannot be ignored. Aluminium salts are widely used as an adjuvant in the composition of human and veterinary vaccines due to their immunopotentiation and safety records since the 1930s [31,38,39]. Although the way in which these mineral agents influence the immune response to vaccination remains elusive, many hypotheses exist as to the mode of action of these adjuvants, such as depot formation, antigen (Ag) targeting, and the induction of inflammation [39]. Aluminium salts have been reported as inducing strong Th2 and IgE responses, good targeting (if the immunogen is adsorbed), resulting in a moderate depot effect but inducing minimal CD8+ cytotoxic T-lymphocyte (CTL) responses or cell mediated immunity (CMI) induction [31]. Therefore, even though our study was not designed to support immunological inferences (and this was not the objective of the present study), we cannot rule out the possibility that aluminium chloride used in the formulation of AlClPC may have contributed to the efficacy of the results in this study, since aluminium adjuvants have also been reported to enhance the antigen uptake capacity of macrophages and dendritic cells *in vivo* and human peripheral blood mononuclear cells [39].

The evaluation of the paw diameters of the animals before and after treatment is a criterion that closely approximates what is done in curative clinical practice with the patient when following lesion progress during treatment. Moreover, the limiting dilution for the quantification of *Leishmania* is an established and more commonly used method [15,40–43]. Results of limiting dilution corroborate the findings of paw diameter measurements, where the group treated with NMG20 + PDT outperformed all other groups, including those treated only with NMG, maintaining the reduction in paw diameter, percentage of viable cells and number of parasites per paw, even 60 days after the end of treatment. It was closest to the negative control (healthy animals), indicating that AlClPC was effective as a photosensitiser agent. Results of amastigote count and cultures confirmed these findings.

The finding of positive cultures and increased numbers of parasites 60 days after treatment ended in most groups, including NMG10 and NMG20, appeared to make these groups vulnerable to recurrence. Low and discontinuous doses of Sb^V and the use of a single drug, according to some authors, seem to be the major causes of relapses and resistance in leishmaniasis [11,12]. The better results in NMG10 + PDT than in NMG10 and its similarity with NMG20 at 60 days after treatment end could indicate possible resistance to the isolated drug and reinforce the findings of reduced cell viability with PDT with AlClPC, corroborating other studies [15,19,20].

As regards results of the extension slides, the low sensitivity of the test may be associated with the small amount of material collected from the mouse paw. Because the mice foot is very small, it is difficult to collect large amounts of material, and the sensitivities of direct examination may be low, varying from 52% to 98% [44].

Regarding evaluations of the toxicity of the therapeutic scheme, biochemical alterations did not correspond to morphological alterations. It seems that the changes were random, a consequence more of

captive stress than of the therapies themselves, and, thus, that the therapeutic schemes were safe. However, due to the small number of animals evaluated, it was not possible to perform statistical analysis to confirm this, suggesting a study with larger groups. Although a greater number of animals would be desirable to avoid bias in the study of adverse effects, the total number met the standards of the Animal Ethics Committee. Moreover, during the experiment there was no loss of animals. At the time of testing, all animals were alive. The tests were divided into three stages. Thus, it was important to evaluate the long-term efficacy of the treatment (the main objective of the study), so animals could not be euthanized all at once for the evaluation of efficacy and adverse effects criteria.

In conclusion, the association of PDT with AlClPC and NMG appeared to be effective in the treatment of experimental CL caused by *L. amazonensis*. The therapeutic scheme NMG20 + PDT showed greater efficacy than the scheme now standardized for CL (20 mgSb^V/Kg/day) and all other therapeutic schemes tested. The NMG10 + PDT treatment with AlClPC showed similar results to the NMG20 group, indicating that the combination may reduce the antimonial dose by decreasing the adverse effects of the drug. The therapeutic scheme tested with the NMG + PDT association with AlClPC did not provoke significant changes in renal function, hepatic function and histopathological lesions, but the analysis of a larger sample of tests would be desirable. The effectiveness of Sb^V + PDT with AlClPC could be tested in other species of *Leishmania*, to evaluate the topical solution by intralesional route, to allow greater and more appropriate delivery of the substance.

Funding

This work was supported by the Coordination of Superior Level Staff Improvement (CAPES), grant number 1515969; and the Foundation to Support Research in the Federal District (FAPDF), process number 0193-001.447/2016.

Conflicts of interest

There is no conflict of interest.

Transparency declarations

None to declare.

Acknowledgments

The authors gratefully acknowledge the Post-Graduate Program in Medical Sciences of the University of Brasília; Sabin Institute/Sabin Laboratories for technical support in the biochemical dosages; Professor José de Souza Filho (*in memoriam*), for the contribution with the characterisation of the liposome sample through TEM and SEM/EDS; Professor Antonio Cláudio Tedesco, for the supply of phthalocyanine; and the technicians from the Dermatomycology Laboratory, Tercio Rodrigues Pereira and Viviane da Silva Medeiros, for technical support.

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