Biomedicine & Pharmacotherapy 134 (2021) 111120

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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

Antileishmanial activity of fullerol and its liposomal formulation in experimental models of visceral leishmaniasis

Guilherme S. Ramos [a], Virgínia M.R. Vallejos [a], Marina S. Ladeira [a], Priscila G. Reis [a,1], Daniel M. Souza [b,2], Yuri A. Machado [a], Luiz O. Ladeira [c], Maurício B.V. Pinheiro [c], Maria N. Melo [b], Ricardo T. Fujiwara [b], Frédéric Frézard [a,*]

[a] Departamento de Fisiologia e Biofísica, Instituto de Ciencias Biologicas, Universidade Federal de Minas Gerais, Avenida Antônio Carlos 6627, Pampulha, 31270-901, Belo Horizonte, Minas Gerais, Brazil
[b] Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Antônio Carlos 6627, Pampulha, 31270-901, Belo Horizonte, Minas Gerais, Brazil
[c] Departamento de Física, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais, Avenida Antônio Carlos 6627, Pampulha, 31270-901, Belo Horizonte, Minas Gerais, Brazil

ARTICLE INFO

Keywords:
Fullerol
Fullerenol
Liposomes
Leishmaniasis
Immunomodulation
Drug delivery

ABSTRACT

Visceral leishmaniasis (VL) is a systemic parasitic disease that leads to high rates of morbidity and mortality in humans worldwide. There is a great need to develop new drugs and novel strategies to make chemotherapy for this disease more efficacious and well tolerated. Recent reports on the immunomodulatory effects and the low toxicity of the spherical carbon nanostructure fullerol led us to investigate *in vitro* and in *vivo* antileishmanial activity in free and encapsulated forms in liposomes. When assayed against intramacrophagic *Leishmania* amastigotes, fullerol showed a dose-dependent reduction of the infection index with IC_{50} of 0.042 mg/mL. When given daily by i.p. route for 20 days (0.05 mg/kg/d) in a murine model of acute VL, fullerol promoted significant reduction in the liver parasite load. To improve the delivery of fullerol to the infection sites, liposomal formulations were prepared by the dehydration-rehydration method. When evaluated in the acute VL model, liposomal fullerol (Lip-Ful) formulations given i.p. at 0.05 and 0.2 mg/kg with 4-days intervals were more effective than the free form, with significant parasite reductions in both liver and spleen. Lip-Ful at 0.2 mg/kg promoted complete parasite elimination in the liver. The antileishmanial activity of Lip-Ful was further confirmed in a chronic model of VL. Lip-Ful was also found to induce secretion of pro-inflammatory TNF-α, IFN-γ and IL-1β cytokines. In conclusion, this work reports for the first time the antileishmanial activity of fullerol and introduces an innovative approach for treatment of VL based on the association of this nanostructure with liposomes.

1. Introduction

Visceral leishmaniasis (VL) is a neglected tropical disease and one of the top parasitic diseases with outbreak and mortality potential. An estimated 50,000–90,000 new cases of VL occur worldwide each year. Most cases occur in Brazil, East Africa and South-East Asia. VL is characterized by prolonged fever, splenomegaly, hepatomegaly, pancytopenia, progressive anemia, and weight loss. The clinical manifestations of VL are attributed to obligatory intracellular protozoa of the *Leishmania donovani* complex and, depending on the etiological agent, the disease presents two distinct forms: anthroponotic VL that is endemic in South Asia and East Africa, caused by *L. donovani*; and zoonotic VL that occurs in countries of the Mediterranean basin, Central Asia, and Americas, caused by *L. infantum*, with dogs being the most important reservoir host [1]. Although less frequent, some cases of VL caused by *L. amazonensis* have also been reported both in humans and dogs [2–4].

VL is fatal if left untreated in over 95 % of cases. Thus, its resolution relies on chemotherapy. The few drugs available for clinical use are pentavalent antimonials, liposomal amphotericin B, miltefosine and paromomycin. Unfortunately, those exhibit severe toxicities, which

* Corresponding author.
E-mail address: frezard@icb.ufmg.br (F. Frézard).
[1] Present address: Departamento de Farmácia/Ensino e Pesquisa, Hospital João XXIII – Fundação Hospitalar do Estado de Minas Gerais, Belo Horizonte, Brazil.
[2] Present address: Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

https://doi.org/10.1016/j.biopha.2020.111120
Received 11 September 2020; Received in revised form 30 November 2020; Accepted 4 December 2020
Available online 17 December 2020

often decrease patient compliance, can result in treatment failure and promote the emergence of drug resistance [5] Undoubtedly, there is a need to develop new drugs or novel strategies to make chemotherapy for this disease more effective, more selective for the parasites and less toxic.

Fullerenes belong to a broad family of spherical carbon nanostructures formed by hexagons interconnected by pentagons with three-dimensional unsaturated bonds and electronic structure [6], conferring unique physical, chemical and biological properties. Fullerol or fullerenol (Ful)[3] is the most abundant and representative soluble form of fullerenes derived from C_{60} formed by the addition of polar groups ($C_{60}(OH)_x$). It retains its unique inherent fullerene properties and achieves reasonable biological availability [7]. Among the pleiotropic biological actions of Ful, its anti-oxidant and immunomodulatory properties have attracted much attention [8]. Ful has been found to be effective for the control of inflammatory responses in some experimental models by reducing the levels of inflammatory cytokines and oxidative stress [9–13]. On the other hand, the anti-tumor activity of Ful in mouse models has been associated with the enhancement of innate immunity, characterized by increased production of T-helper cell type 1 (Th1) cytokines (IL-2, IFN-γ and TNF-α) and decreased production of Th2 cytokines [14,15].

In the present study, we hypothesized that treatment with Ful in VL might lead to the control of parasite development through induction of a pro-inflammatory response. To test this hypothesis, we evaluated the effects of treatment with the Ful on *in vitro* and *in vivo* models of VL. Considering that conventional liposomes passively target the main sites of VL infection, *i.e.* the liver and the spleen [16,17], an innovative approach was also introduced based on the synergistic association of two nanosystems, Ful and liposomes, which resulted in enhanced therapeutic efficacy.

2. Material and methods

2.1. Materials and drugs

Distearoylphosphatidylcholine (DSPC) and dipalmitoylphosphatidylglycerol (DPPG) were obtained from Lipoid (Ludwigshafen, Germany). Tetrabutylammoniun (TBAH), 2,2-diphenyil-picrylhydrazyl (DPPH), cholesterol (CHOL), kanamycin, ampicillin and miltefosine were purchased at Sigma-Aldrich (St. Louis, MO, USA). Meglumine antimoniate or Glucantime® (Glu, Sanofi-Aventis Farmacêutica Ltda., São Paulo, Brazil) was obtained from the Brazilian Ministry of Health. Fullerene (C_{60}, 99+%) was obtained from MER Corporation (Tucson, AZ, USA).

2.2. Sources of fullerol

Ful was obtained from two different sources: i) from MER Corporation, as $[C_{60}(OH)_{24}]$ produced by hydrolysis of $C_{60}Br_{24}$; from synthetic source. Synthetic Ful was prepared through a polyhydroxylation process of fullerene by means of a phase-transfer reaction using TBAH as a catalyst. The process of synthesis has been described in details previously [10,18]. The final synthetic fullerol is a paramagnetic complex salt whose average composition is $Na_n^+[C_{60}O_x(OH)_y]^{n-}$ with $n = 2–3$, $x = 7–9$ and $y = 12–15$, as determined by fourier-transform infrared spectroscopy, electron paramagnetic resonance and thermogravimetry analysis [19,20].

2.3. Preparation and characterization of liposome formulations of fullerol

Ful was encapsulated in dehydration-rehydration vesicles as previously described [21], with the following modifications. Briefly, multilamellar vesicles (MLVs) made from DSPC, CHOL and DPPG (molar ratio of 5:4:1) were prepared in deionized water at final lipid concentration of 90 mM. The MLVs were transformed into unilamellar vesicles through 5 freeze-thaw cycles, followed by repeated extrusions (10 times) at 60 °C across 200-nm polycarbonate membranes [22]. The resulting liposome suspension was mixed with an equal volume of 15 or 60 μg/mL Ful in 0.03 M NaCl aqueous solution (Lip-Ful1 or Lip-Ful2). The mixtures were then immediately frozen in liquid nitrogen and subsequently freeze-dried for 48 h under the protection of light (freeze-dryer L101, Liotop, São Carlos / SP, Brazil). Rehydration of the dried powder was performed as follows: 20 % of the original volume of water was added to the lyophilized powder and the mixture was incubated for 30 min at 60 °C; the same volume of saline (0.15 M NaCl) was then added and the mixture was incubated for 30 min at 60 °C. The volume was completed to the original volume with saline. The liposome suspensions were then repeatedly extruded at 60 °C through two stacked polycarbonate membranes of 200-nm pore size, for calibration of particle size.

The Lip-Ful2-suc formulation was prepared as described for Lip-Ful2, except that prior to freeze-drying the liposome suspension was mixed with a solution of Ful 60 μg/mL in sucrose at 3:1 lipid/sucrose weight ratio, instead of saline. Furthermore, rehydration of the dried powder was performed only with water for 30 min at 60 °C at final Ful concentration of 60 μg/mL. In this case, no additional extrusion step was necessary.

The mean hydrodynamic diameter, polydispersity index and zeta potential of the resulting liposome formulations were determined, after 100x dilution of the suspension in PBS (0.15 M NaCl, phosphate 10 mM, pH 7.2), by photon correlation spectroscopy at 25 °C using a particle size analyzer (Zetasizer S90, Malvern, UK).

The amount of encapsulated Ful was determined in the more concentrated formulation (Lip-Ful2), by exploiting the reaction of Ful with the stable radical DPPH [7] and the induced changes in DPPH absorption spectrum [23]. First, non-encapsulated Ful was removed from the liposome formulation through diafiltration using Biomax500 kDa ultrafiltration disk (Millipore, Billerica, MA, USA) for 12 h at 25 °C. The resulting liposome suspension containing encapsulated Ful was then freeze-dried. Secondly, the lyophilized powder was resuspended in a solution of 50 mg/L DPPH in methanol to promote liposome disruption and, after 30-min incubation at 60 °C, the suspension was centrifuged at 22,000 xg for 30 min at 4 °C. The supernatant was analyzed for the amount of DPPH-Ful complex through absorbance measurement at 435 nm. We checked that the presence of lipids did not induce spectral changes of DPPH or interfere in the reaction of Ful with DPPH. The exact amount of Ful was determined using a calibration curve. On the other hand, due to interference of sucrose in the photometric DPPH assay, it has not been possible to determine the concentration of encapsulated Ful in the Lip-Ful2-suc formulation.

2.4. Animals

BALB/c mice (female, 5–7 weeks old, 20−25 g) were obtained from Centro de Bioterismo (CEBIO) of the Institute of Biological Sciences (ICB) or Biotério Central of Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil,. Free access was allowed to standard diet and tap water was supplied *ad libitum*. The studies involving animals comply with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and were approved by the Ethical Committee for Animal Experimentation of the UFMG (CEUA-UFMG) with protocol numbers 46/2013, 231/2014 and 60/2016.

2.5. Parasites

Leishmania amazonensis (IFLA/BR/67/PH8) and *Leishmania infantum* (MCAN/BR/2002/BH401) parasites were routinely maintained and

[3] Ful: fullerol.

isolated from golden (Syrian) hamsters (*Mesocricetus auratus*) at the Department of Parasitology of UFMG, and were grown as promastigotes at 24 ± 1 °C, pH 7.0, in alpha minimal essential medium (αMEM; Gibco®; Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10 % heat-inactivated bovine fetal serum (FBS; Cultilab, Brazil), 100 μg/mL kanamycin and 50 μg/mL ampicillin in BOD greenhouse (Model: 2005; Johns-VWR Scientific, Toronto, ON, Canada). The promastigotes were grown in cell culture flasks of 25 mL volume (Corning Incorporated, Corning, NY, USA) with an initial inoculum of 1×10^6 cells/mL and transferred to a new medium after reaching the stationary growth phase, twice a week.

2.6. Fullerol cytotoxicity in peritoneal macrophages and in vitro anti-amastigote activity

Macrophages were obtained from the peritoneal cavity of BALB/c mice three days after the injection of 2 mL of 3% brewer thioglycollate medium into peritoneal cavity. Cells were seeded in 96-well plates at 5×10^3 cells per well in RPMI medium supplemented with 10 % FBS, and allowed to adhere for 4 h at 37 °C in 5% CO_2. The non-adherent cells were then removed by washing with RPMI medium. Ful and Glucantime were added to the wells at varying concentrations (0.015−0.15 mg/mL and 0.01−1 mg/mL, respectively) and the cells were further cultured in RPMI supplemented with 10 % FBS at 37 °C for 72 h, under humidified 5% CO_2 atmosphere. Thereafter, the medium was replaced with fresh RPMI containing MTT at 0.5 mg/mL, and the plates were incubated for additional 4 h. Supernatants were aspirated, and the resulting formazan crystals were dissolved in 100 μL of DMSO. After 15 min of incubation at room temperature, the absorbance of the solubilized MTT formazan product was measured by absorbance measurement at 570 nm [24]. Cell viability was calculated from the ratio of the absorbance of the wells treated with the drug to that of the non-treated well.

The anti-amastigote assay was performed as previously described [25]. Briefly, the resident macrophages were obtained from the peritoneum of BALB/c, as described above and allowed to adhere directly on glass microscope coverslips at a cell density of 5×10^3 cells per well. After 2 h, cells were washed gently twice with PBS to remove non-adherent cells. Adherent cells were cultured in αMEM (supplemented with 10 % FBS and 100 units penicillin/streptomycin) at 37 °C under 5% CO_2 atmosphere. Macrophages were infected with late-stationary-phase *L. amazonensis* promastigotes at a ratio of 10:1 (parasite:macrophage). After 4 h, macrophage monolayers were washed with αMEM to remove free parasites.

Treatment of infected cells was carried out after 24 h of infection with different concentrations of Ful (0.00375 to 0.12 mg/mL) for 72 h at 37 °C under 5 % CO_2 atmosphere. The cells were then washed with PBS and stained with panoptic kit (Laborclin, Pinhais, PR, Brazil) according to the manufacturer's instructions. For each coverslip, about 250 macrophages and the average number of parasites per macrophage were counted. Individual amastigotes were clearly visible in the cytoplasm of infected macrophages. The results were expressed as the Infection Index (II), which is obtained by the formula: II = (% infected macrophages) × (amastigotes/infected macrophages/100). The half maximal inhibitory concentration (IC_{50}) was then obtained.

2.7. Antileishmanial activity of free and liposome-encapsulated fullerol in acute visceral leishmaniasis mouse model

The choice of this model was based on previous reports of cases of VL caused by *L. amazonensis* both in humans and dogs [3,4] and the high susceptibility of BALB/c mice to *L. amazonensis* infection. BALB/c mice were inoculated intraperitoneally (i.p.) with 2×10^7 of *L. amazonensis* promastigotes, obtained after 8 days of culture growth. Treatment was initiated 7 days after infection and was maintained during 20 days. The first experiment, designed to evaluate the efficacy of free Ful given once daily, consisted in three groups of infected animals (n = 10): (i) Ful group, free Ful in saline i.p. at 0.05 mg/kg/d; (ii) Glu group, Glucantime i.p. at 120 mg Sb/kg/d; and (iii) Saline group, isotonic saline i.p. given daily. The second experiment, aimed at evaluating the efficacy of Ful-containing liposome formulations given at each 4 days, consisted in the following groups (n = 6): (i) Ful group, free Ful in saline i.p. at 0.05 mg/kg/4d; (ii) Lip-Ful1 group, Lip-Ful1 formulation i.p. at 0.05 mg Ful/kg/4d; (iii) Lip-Ful2 group, Lip-Ful2 formulation i.p. at 0.2 mg Ful/kg/4d; (iv) Glu group, Glucantime i.p. at 120 mg Sb/kg/d; (v) Empty liposomes, given i.p. at the same lipid dose as Lip-Ful groups; (vi) Saline group, isotonic saline i.p. Animals were submitted to euthanasia on day 30 post-infection by cervical dislocation after ketamine-xylazine anesthesia. Liver and spleen were collected and homogenized manually. The DNA was extracted by phenol-chloroform extraction method [26]. The numbers of parasites in liver and spleen was determined using quantitative polymerase chain reaction (qPCR), as described previously [27]. The DNA concentrations were measured by spectrophotometry (Abs at 280/260 nm) and adjusted to 20 ng/μL. One μL of the 2 μM primers, 5 μL of SYBR® Green PCR Master Mix (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and 1 μL of Total DNA (20 ng) were mixed in final volume reaction (10 μL). The assay was performed in duplicate. The baseline threshold values used were adjusted to 3–15 cycles for the baseline from 0.2 to threshold. The parasite load was estimated using primer specific for multicopy *Leishmania* kinetoplast DNA (kDNA, 120 bp) sequence (forward: 5'-CCTATTTTA-CACCAACCCCCA-3'; reverse: 5'-GGGTAGGGGCGTTCTGCGAA-3'). Samples were processed and analyzed on the ABI Prism 7500 Sequence Detection System (SDS Applied Biosystems, Foster City, CA, USA). Thermocycling conditions were: 95 °C, 10 min for initial denaturation step, followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing and extension). The values obtained from cycle threshold (Ct) were used to calculate the parasite load using a standard curve from the genomic DNA of *L. amazonensis* and expressed in number of parasites per nanogram of total DNA.

2.8. Antileishmanial activity of liposome-encapsulated fullerol in chronic visceral leishmaniasis mouse model

BALB/c mice were inoculated i.p. with 2×10^7 of *L. infantum* promastigotes. After two months of infection, treatment was initiated in four experimental groups (n = 5–8): i) Lip-Ful2 group, Lip-Ful2 formulation prepared in saline, given i.p. at 0.2 mg Ful/kg/4d during 24 days; ii) Lip-Ful2-Suc group, Lip-Ful2 formulation prepared with sucrose, given i.p. at 0.2 mg Ful/kg/4d during 24 days; iii) miltefosine oral, miltefosine in saline given by gavage at 10 mg/kg/d during 24 days; iv) Saline, isotonic saline given i.p. each four days for 24 days. Animals were submitted to euthanasia three days after the end of treatment by cervical dislocation after ketamine-xylazine anesthesia. Liver and spleen were then collected and homogenized manually. Aliquots of the homogenates were added to microtube containing lysis buffer and Proteinase K, vortexed and incubated at 56 °C overnight. After incubation, DNA was extracted with NucleoSpin® Tissue Kit (MN, Macherey-Nagel GmbH & Co. KG, Dürin, Germany), according to the manufacturer's instructions. The DNA concentrations were measured by spectrophotometry (Absorbance at 280/260 nm) and adjusted to 20 ng/μL. The number of parasites was determined by qPCR, as described previously [28]. One microliter of each sample was used to a final volume of 20 μL per reaction that included ultrapure water, 5.0 μL of SYBR® Green PCR Master Mix (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), 10 pmol of each oligonucleotide, as sense (forward, 5′-CGTGGGGGA GGGCGTTCT-3′) and antisense primers (reverse, 5′-CCGAAGCAGCCG CCCCTATT-3′) constructed for amplification of the kDNA. The standard curve was constructed with serial dilutions of the known concentrations of DNA of *Leishmania infantum*, extracted from promastigote culture. Ultrapure water was used as negative control. The amplification protocol included annealing temperature and extension of 60 °C, with melting curve construction, on the ABI Prism 7500 Sequence Detection

System, and the analysis was made using the 7500 System Software. Data are presented in number of parasites per nanogram of total DNA.

2.9. Determination of cytokine production

The levels of IL-4, IL-10, TNF-α and IFN-γ cytokines were determined in the liver of animals of the second *in vivo* experiment (acute VL model), following euthanasia. Tissues were individually weighed and homogenized with a tissue homogenizer (Power Gen 125 – Fisher Scientific Pennsylvania, USA) in PBS, supplemented with protease inhibitors (0.1 mM phenylmethilsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KI aprotinin A) and 0.05 % Tween 20. Following centrifugation at 8000 × *g* for 10 min at 4 °C, the supernatant was used to determine the level of cytokines by ELISA (commercially kit, R&D Systems, USA) according to the manufacturer's instruction.

The level of Interleukin-1β (IL-1β) was determined post-treatment in the serum of mice of the chronic VL model, using sandwich ELISA kit according to the instructions of the manufacturer (DuoSet® ELISA, R&D Systems™, USA). Optical density (O.D.) values were measured at 492 nm in a microplate spectrophotometer reader (Molecular Devices, Spectra Max Plus, Canada).

2.10. Statistical analysis

Comparison of the results obtained between different experimental groups was performed using Kruskal-Wallis test, followed by Dunn's multiple comparison test for data with no Gaussian distribution or One-way ANOVA followed by Dunnett's post-test for data with Gaussian distribution. Differences with P values ≤ 0.05 were considered statistically significant. The concentration that decreases the viability of peritoneal macrophages by 50 % (CC_{50}) and that which reduces the Infection Index by 50 % (IC_{50}) were calculated by non-linear regression using the GraphPad Prism software version 8.4.3 (San Diego, CA, USA).

2.11. Data availability

Raw data will be made available upon request for peer review and publicly available upon publication on a data repository [29].

3. Results

3.1. In vitro cytotoxicity and antileishmanial activity of fullerol

Evaluation of Ful cytotoxicity against murine peritoneal macrophages using the MTT assay showed no significant cytotoxicity at concentration up to 0.12 mg/mL. At 0.15 mg/mL Ful, a 40 % decrease of cell viability was observed (data not shown). When evaluated for its ability to inhibit the growth of *Leishmania* promastigotes, Ful produced no significant effect at concentration up to 0.2 mg/mL (data not shown).

On the other hand, when assayed against intramacrophagic *L. anazonensis* amastigotes, Ful exhibited a dose-dependent reduction of the infection index, with IC_{50} of 0.042 mg/mL (Fig. 1), evidencing Ful antileishmanial activity. In the same model, the IC_{50} of standard drug Glucantime was 0.020 ± 0.012 mg/mL of Sb.

3.2. Therapeutic efficacy of free and liposome-encapsulated synthetic fullerol in acute VL mouse model

The *in vitro* antileishmanial activity of Ful led us to evaluate its therapeutic efficacy in a murine model of VL. Fig. 2 shows the effect of treatment with Ful given i.p. on a daily basis on the parasite burdens in the liver and spleen of BALB/c mice infected i.p. with *L. amazonensis*. As treatment was initiated only 7 days after infection, this model could be considered as an acute VL model. Ful was found to significantly reduce the parasite burden in the liver, in comparison to saline, to the same extent as the standard antimonial drug (Glucantime). On the other hand,

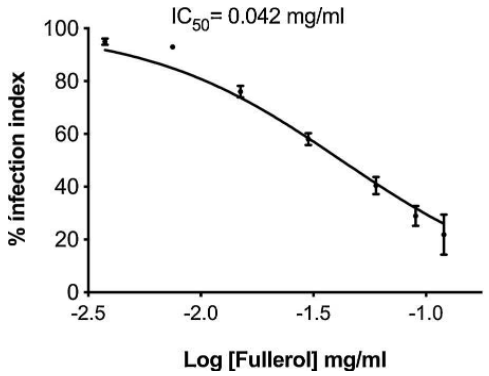

Fig. 1. Antileishmanial activity of fullerol in the intramacrophagic *Leishmania* model. After infection with *L. amazonensis* parasite, murine peritoneal macrophages were exposed for 72 h to Ful at concentration varying from 0.00375 to 0.12 mg/mL. The infection index was calculated as follows: (% infected macrophages) × (amastigotes/infected macrophages/100). Data are shown as means ± SD from triplicates.

Ful did not promote significant reduction of parasite load in the spleen.

As an attempt to enhance the therapeutic efficacy of Ful through improved targeting to the liver and spleen, liposome formulations were developed. The liposome formulations of Ful were obtained by the dehydration-rehydration method, followed by extrusion through 200-nm polycarbonate membrane. Different loads of Ful were used in Lip-Ful1 and Lip-Ful2. As shown in Table 1, the resulting liposome suspensions showed an average vesicle diameter in the range of 192−211 nm and were monodisperse (PDI < 0.2). The negative zeta potential of the vesicles is consistent with the presence of the negatively-charged phospholipid (DPPG). Ful had no significant influence on the size or potential zeta of the vesicles.

A photometric method based on the interaction of Ful with the stable radical DPPH was developed to demonstrate the encapsulation of Ful and estimate the amount of encapsulated Ful. The sensitivity of the method was sufficient to allow quantification of encapsulated Ful in Lip-Ful2 formulation. An encapsulation efficiency of 25.3 ± 3.2 % was determined.

The next step was to evaluate the therapeutic efficacy of liposomal Ful formulations in our VL model. In this experiment, mice received only 5 doses given with 4-days intervals. To guarantee the control of the exact dose of Ful applied, the liposome formulations of Ful were administered with both non-entrapped and entrapped Ful (without removing non-encapsulated Ful). Fig. 3 shows the effect of treatment with the liposome formulations of Ful on the liver and spleen parasite loads of mice. Both formulations significantly reduced parasite burdens in both the spleen and the liver, in comparison to saline. The greatest therapeutic efficacy was achieved with the formulation containing the highest amount of Ful (Lip-Ful2), which eliminated liver parasites in 100 % of animals. Free Ful, given at the same dose regimen as Lip-Ful1 (0.05 mg/kg/4d), did not promote significant parasite suppression. As also expected, empty liposomes given at the same lipid dose as Lip-Ful1 and Lip-Ful2 were inactive. This study establishes for the first time the marked therapeutic efficacy of liposomal Ful.

3.3. Cytokine production profile in the liver of infected mice treated with liposomal fullerol

To get insight into the mechanism responsible for the strong antileishmanial efficacy of liposomal Ful, regarding its ability to eliminate parasites from the liver, the profile of production of IL-4, IL-10, TNF-α and IFN-γ was evaluated in this organ at the end of treatment of mice

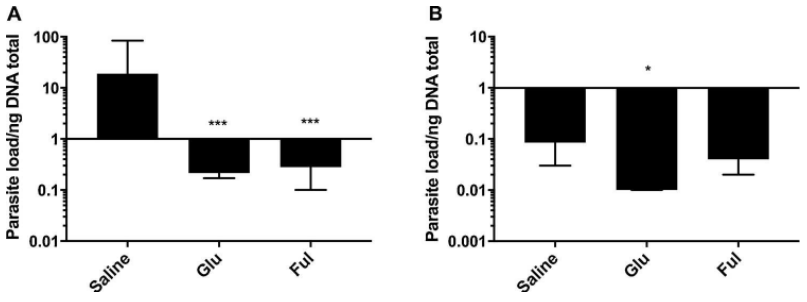

Fig. 2. Effect of treatment with fullerol on the parasite burdens in the liver (A) and spleen (B) of BALB/c mice infected with *L. amazonensis*. Seven days after infection with *L. amazonensis*, animals were treated daily for 20 days i.p. with either fullerol at 0.05 mg/kg/d (Ful), Glucantime at 120 mg Sb/kg/d (Glu) or saline. After euthanasia of the mice at 30-days post-infection, the parasite burden was determined by qPCR. Bars show parasite burden medians with 95 % confidence interval (n = 9-10). *p < 0.05; ***p < 0.001 Kruskal-Wallis with Dunn's multiple comparison post-test, compared to control group (saline).

Table 1
Size distribution and zeta potential of fullerol-containing liposome formulations[a].

Formulation	Initial lipid/ Ful mass ratio	Average Size (nm)	[b]PDI	Zeta Potential (mV)
Lip-Ful1	3667	192 ± 15	0.05 ± 0.03	−22.6 ± 1.5
Lip-Ful2	917	211 ± 21	0.06 ± 0.10	−23.0 ± 1.0
Lip-Ful2-suc	917	160 ± 20	0.13 ± 0.05	−22.0 ± 2.0
Empty liposomes	–	196 ± 2	0.05 ± 0.02	−24.3 ± 1.0

[a] The values are represented by mean and respective standard deviation (n = 3).
[b] PDI: polydispersity index.

infected with *L. amazonensis*.

As shown in Fig. 4, the comparative analysis between treated groups and the control group indicates that Lip-Ful-2 formulation and Glucantime promoted in the liver increased levels of both pro-inflammatory (TNF-α and IFN-γ) and anti-inflammatory (IL-4) cytokines. A significant increase of TNF-α and IL-4 levels was also observed following treatment with empty liposomes.

3.4. Therapeutic efficacy of free and liposome-encapsulated fullerol from commercial source in chronic VL mouse model

The strategy adopted so far raised two questions: 1) whether the results obtained in the *L. amazonensis* – BALB/c mice model may be extrapolated to the classical *L. infantum*– BALB/c mice model of VL; 2) whether the results obtained with a sample of Ful synthesized in our laboratory may be reproduced with a Ful sample from commercial source. To address these points, a new assay of Lip-Ful2 was performed in BALB/c mice infected with *L. infantum*. In this case, treatment was initiated 2 months after infection, indicating a chronic VL model. The Ful sample used in this experiment was obtained from MER Corporation. Miltefosine given orally on a daily basis was used as a positive control. Two different Lip-Ful2 formulations were tested: one prepared as described above; and the other one called Lip-Ful2-Suc, obtained by freeze-drying of the Ful-liposome mixture in the presence of sucrose to control membrane fusion and vesicle size, in order to simplify the preparation process by omitting the final extrusion step. As shown in Table 1, particle size analysis of Lip-Ful2-Suc indicates a monodisperse suspension and an average vesicle diameter slightly lower than that of Lip-Ful2.

As illustrated in Fig. 5A, treatment with Lip-Ful2 and Lip-Ful2-Suc promoted significant reduction of parasite burden in the liver in comparison to saline control. The level of parasite suppression was at least as pronounced as that achieved with miltefosine treatment given on a daily basis. The parasite load was also evaluated in the spleen, but neither liposomal Ful formulations nor miltefosine showed significant parasite suppression (Fig. 5B).

To investigate the possible induction of secretion of pro-inflammatory IL-1β by liposomal Ful, this cytokine was determined post-treatment in the serum of mice. As shown in Fig. 6, Lip-Ful2 group showed significantly greater IL-1β levels, when compared to Saline and the oral miltefosine group. The Lip-Ful2-Suc group displayed significantly higher level of IL-1β, in comparison with miltefosine group. This data supports a possible role of IL-1β in the mechanism of action of Ful in this VL model.

4. Discussion

The present study reports for the first time the antileishmanial activity of Ful, as demonstrated by assays on *in vitro* and *in vivo* models of VL.

The *in vitro* study establishes the activity of Ful against intramacrophagic amastigotes and indicates that either Ful specifically acts against the *Leishmania* amastigote form or activates macrophages to kill intracellular parasite. Previous reports that *in vitro* exposition of macrophages to Ful enhanced the phagocytosis and increased the production

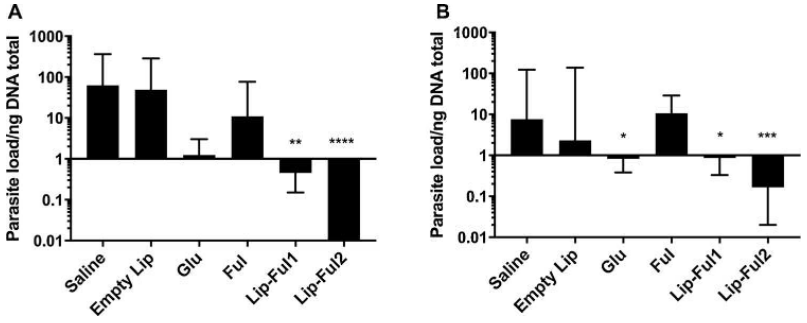

Fig. 3. Antileishmanial efficacy of liposomal formulations of Ful in the liver (A) and spleen (B) of BALB/c mice infected with *L. amazonensis*. Seven days after infection with *L. amazonensis*, animals were treated for 20 days i.p. with Lip-Ful2 at 0.2 mg/kg/4d, free Ful at 0.05 mg/kg/4d (Ful), Glucantime at 120 mg Sb/kg/d (Glu), empty liposomes or saline. After euthanasia of the mice at 30-days post-infection, the parasite burden was determined by qPCR. Bars show parasite burden medians with 95 % confidence interval (n = 6). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 Kruskal-Wallis with Dunn's multiple comparison post-test, compared to control group (saline).

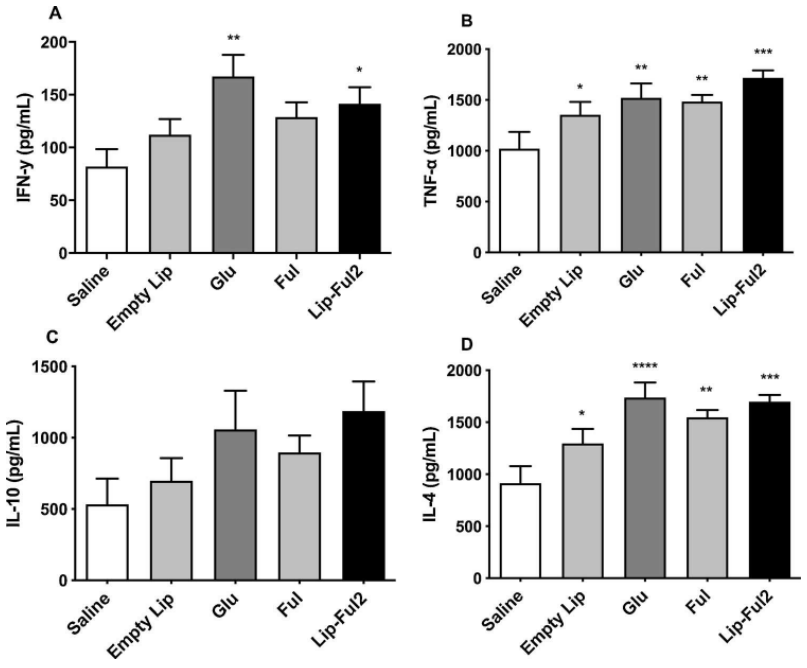

Fig. 4. Cytokines profile in the liver of *L. amazonensis*-infected mice after treatment with free and liposome-encapsulated Ful. Seven days after infection with *L. amazonensis*, BALB/c mice were treated for 20 days i.p. with either liposomal formulations of Ful (Lip-Ful2 at 0.2 mg/kg/4d), free Ful at 0.05 mg/kg/4d (Ful), Glucantime at 120 mg Sb/kg/d (Glu), empty liposomes or saline. After euthanasia of the mice at 30-days post-infection, the levels of IFN-γ (A), TNF-α (B), IL-10 (C) and IL-4 (D) produced by the liver were evaluated. Bars show mean cytokine concentrations ± SEM (n = 6).*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ for comparison with Saline group, using One-way ANOVA followed by Dunnett's post test.

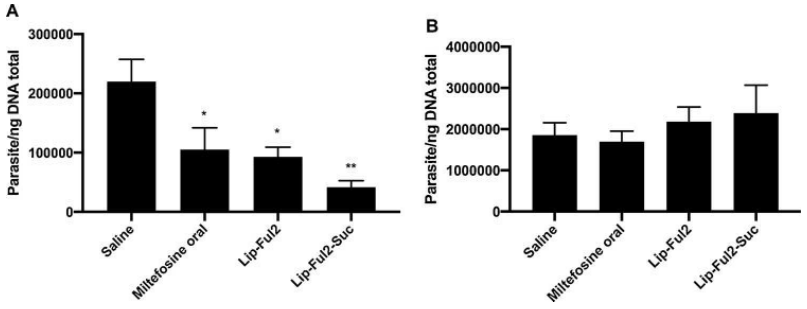

Fig. 5. Antileishmanial efficacy of liposomal formulations of Ful in the liver (A) and spleen (B) of BALB/c mice infected with *L. infantum*. Two months after infection with *L. infantum*, animals were treated for 24 days i.p. with Lip-Ful2, either prepared in saline or sucrose, at 0.2 mg/kg/4d. Animals receiving miltefosine at 10 mg/kg/d by oral route during 24 days and saline i.p. were used as positive and negative control, respectively. After euthanasia of the mice 3 days after the end of treatment, the parasite burden was determined by qPCR. Bars show parasite burden means ± SEM (n = 5-8). *$p < 0.05$ and **$p < 0.01$, for comparison with Saline group by One-way ANOVA followed by Dunnett's post test.

of TNF-α [9,14,15] support the model of a macrophage-mediated antileishmanial activity of Ful. But further studies are needed to confirm this mechanism. The IC_{50} value of Ful (42 μg/mL) is close to that determined for Glucantime but is much higher than that determined previously for miltefosine and amphotericin B standard drugs on *L. amazonensis*-infected murine peritoneal macrophages ($IC_{50} = 3.7$ μg/mL for miltefosine; $IC_{50} = 0.4$ μg/mL for amphotericin B) [30,31]. This comparison reveals a moderate *in vitro* efficacy of Ful. However, the fact that the effective dose of Ful in *L. amazonensis*-infected mice (0.05 mg/kg) is much lower than that of the standard drugs suggests that an additional mechanism probably contributes to the *in vivo* efficacy of Ful.

Ful alone, when given to *L. amazonensis*-infected mice on a daily basis, promoted a significant reduction of parasite load in the liver, but not in the spleen. This profile is consistent with the finding that Ful accumulates to a higher extent in the liver than in the spleen, following i.v. administration [32].

The hydroxylated Ful is actually a polyacidic molecule, and each proton of the hydroxyl groups (C—OH) can dissociate in aqueous solution, thus yielding a conjugated base C–O–. [33]. When dissolved in aqueous solutions, Ful was found to form polyanion nanoaggregates, whose size is mostly between 20 and 100 nm. These nanoaggregates may favor the uptake of Ful by macrophages and their accumulation in the liver.

As an attempt to further improve the distribution of Ful into all tissues of the mononuclear phagocyte system where *Leishmania* parasites reside [16], liposomal formulations of Ful have been developed. Assuming values of 55 $Å^2$ and 38 $Å^2$ per polar head of DSPC (or DPPG) and CHOL [34], respectively, and considering the Ful encapsulation efficiency of 25 %, the amount of lipid per vesicle and the number of encapsulated Ful molecules per vesicle could be estimated in the Lip-Ful2 formulation. A ratio of about 60 Ful molecules per vesicle was determined. The process of preparation of Lip-Ful2 formulation was also simplified, by freeze-drying the Ful/liposome mixture in the presence of cryoprotective sucrose, to control the final size of liposomes after reconstitution. Accordingly, the lyophilized liposome formulation may

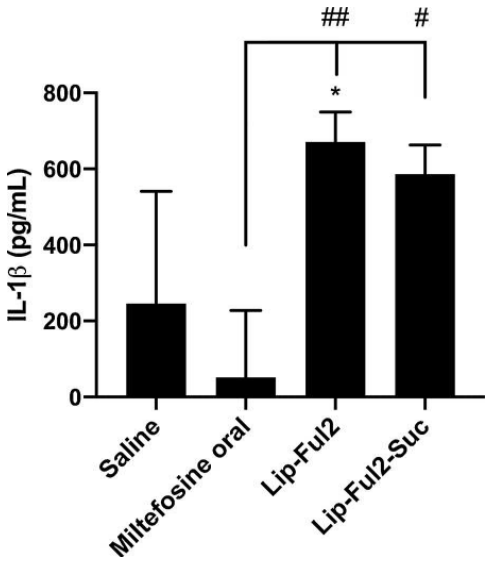

Fig. 6. Levels of IL-1β in the serum of *L. infantum*-infected mice after treatment with liposomal formulations of Ful. Two months after infection with *L. infantum*, BALB/c mice were treated for 24 days i.p. with Lip-Ful2, either prepared in saline or sucrose, at 0.2 mg/kg/4d. Animals receiving miltefosine at 10 mg/kg/d by oral route during 24 days and saline i.p. were used as positive and negative control, respectively. After euthanasia, 3 days after the end of treatment, the sera were collected for determination of IL-1β by ELISA. Bars show the medians of IL-1β level with 95 % confidence interval (n = 6-7). $^{*}p < 0.05$ for comparison with Saline group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ for comparison with Miltefosine oral group; Kruskal-Wallis with Dunn's multiple comparison post-test.

be reconstituted with water just before administration.

The administration of Ful in the encapsulated form in liposomes produced even more pronounced therapeutic effects in *L. amazonensis*-infected mice, with the elimination of parasite in the liver and significant reduction of the parasite burden in the spleen. Importantly, the liposomal Ful formulations were given on each 4 days and, under this regimen, Ful alone did not show therapeutic efficacy.

Consistently, the use of liposomes here allowed significant benefits, including the improved delivery of Ful to the liver and spleen and the possibility of reducing the number of doses and frequency of dosing. The increase of Ful concentration in the liposomal formulation and resulting higher dose of Ful promoted a significantly higher therapeutic efficacy.

Although the *L. amazonensis*-BALB/c mice model is not a classical VL model, *L. amazonensis* has been shown to cause not only cutaneous leishmaniasis but also VL in both humans and dogs in South America [3–5]. Thus, the results obtained are informative on the capacity of the formulations to control the visceral extension of *L. amazonensis*. Nevertheless, it was important to confirm the activity of liposomal Ful in *L. infantum*-infected mice as a pure VL model. Depending on the *L. infantum* strain and the parasite burden, the disease is not always self-curable in BALB/c mice. Particularly, the infection with viscerotropic species such as *L. infantum* in BALB/c mice can provide a chronic infection that lasts at least 4–8 months and can cause animal death. Since treatment was initiated after two months of infection, our model could be considered as a chronic VL infection that better mimics infection in dogs. Furthermore, a commercial Ful was used in order to ensure that the activity of Ful was not specific of our synthetic compound. Importantly, liposomal Ful was found to be at least as effective in reducing hepatic parasite burden as miltefosine, the drug currently approved in Brazil for treatment of infected dogs [35].

The liver-specific effect of liposomal Ful and miltefosine in the chronic VL model may be explained by tissue-specific immune responses. In contrast to liver, infection in the spleen in BALB/c mice promotes parasite persistence, disruption of splenic microarchitecture and impaired immune responses, including suppression of host protective Th1 responses, generation of defective CD8 T cells and inhibition of dendritic cell functions [36]. Indeed, lower levels of parasite suppression in the spleen, in comparison to the liver, have been reported in chronic VL infection of BALB/c mice for several drugs, including free and liposomal antimonial drugs and miltefosine [37,38]. But a comparison between mouse and hamster models of VL has indicated that the tissue-specific effect of drug observed in BALB/c mice could not be generalized to other species [37].

To explain the surprisingly low efficacy of miltefosine, especially in the spleen, one may also consider the possibility of our *L. infantum* strain being naturally resistant to this drug. Recently, the low efficacy of miltefosine in the treatment of VL in Brazil has been correlated with low *in vitro* drug sensitivity of isolated *Leishmania infantum* strains, suggesting natural drug resistance [39]. Our hypothesis is further supported by the high IC_{50} of miltefosine (14 μM) determined in DH82 macrophage-like cell line infected with the strain used in the present study (data not shown).

Clearly, the antileishmanial action of Ful cannot be explained on the basis of its anti-oxidative (NO and ROS scavenger) properties. Indeed, one may expect the anti-oxidative properties of Ful to prevent the increase of NO production by the macrophage and the subsequent parasite killing. Nevertheless, it is possible that the anti-oxidative property of Ful may be restricted to the extracellular space and may not interfere in the activity of the macrophage against intracellular parasite.

The levels of IL-4, IL-10, TNF-α and IFN-γ cytokines in the liver of mice of the acute VL model and of IL-1β in the serum of mice of the chronic VL model were determined, as a first attempt to get insight into the mechanism responsible for the antileishmanial activity of Lip-Ful2. The increased levels of pro-inflammatory cytokines, TNF-α and IFN-γ, in response to treatment with Lip-Ful2 probably contributed to its antileishmanial activity, even though the liposomal formulation also enhanced the level of anti-inflammatory IL-4. Nevertheless, the lack of significant increase of IL-10 suggests a polarization towards Th1 immune response. As empty liposomes also promoted an increase of TNF-α and IL-4 levels, liposomes themselves may contribute to the immunostimulatory action of the formulation, in accordance with previous reports [40–42]. The increased levels of both pro- and anti-inflammatory cytokines in response to liposomal Ful is not surprising, as some therapeutic approaches for VL have been shown to induce a mixed Th1/Th2 response in both mice and human [43,44].

Interestingly, the serum level of the pro-inflammatory cytokine IL-1β was also enhanced upon treatment with liposomal Ful. This result is consistent with a previous report on the action of Ful on macrophages, consisting in the induction of rapid secretion of IL-1β [45]. This effect was attributed to the activation of at least two signaling pathways. On one hand, Ful primed macrophages to express pro-IL-1β *via* TLRs/MyD88 pathway and activation of NF-κB. On the other hand, Ful activated NLRP3 inflammasome, which is required for processing pro-IL-1β into mature IL-1β, under the assistance of relative factors including K^+ efflux and P2 × 7 receptor activation. The simultaneous activation of both pathways may have important implications *in vivo*, considering the key role of IL-1β in the innate immune response to microbial infection [46] and the recent demonstration that inflammasome-driven IL-1β production participates as a major host resistance factor to *Leishmania* infection, as signaling through IL-1 receptor and MyD88 was necessary and sufficient to trigger inducible nitric oxide synthase (NOS2)-mediated production of NO [47].

Tang et al. (2016) also evaluated the ability Ful to activate macrophages, through assessment of mitochondrial metabolism, phagocytic activity and cytokine secretion, for cancer immunotherapy [48]. This work reported that $C_{60}(OH)_{22}$ reversed the suppression of macrophages

mediated by cancer cells through activation, increased viability and phagocytic activity of these cells. One proposed signaling pathway was the activation of NF-Kβ. In addition to activating the macrophages, Ful was found to antagonize the suppression of macrophages mediated by cancer cells. The ability of tumor cells to restrict metabolism and cytokine secretion by macrophages is similar to the mechanism by which *Leishmania* can survive in cells of the host's mononuclear phagocytic system. In this previous study, Ful promoted immunomodulatory effects through the activation of macrophages, which culminated in the inhibition of cancer cells. Thus, the immunomodulatory effect of liposomal Ful in our study through induction of TNF-α, IFN-γ and IL-1β production could explain its marked *in vivo* antileishmanial action.

An important contribution of this work is the proof of concept of an innovative therapeutic approach for VL based on the association of two nanosytems: Ful as immunomodulating nanostructure; and liposomes as drug carrier system. It also noteworthy that preliminary evaluation of clinical status of animals treated with Ful and their liposome formulation revealed no sign of toxicity, contrary to those treated with the conventional antimonial drug (data not shown), reinforcing their potential as a drug candidate. In the near future, it would be worth investigating the therapeutic efficacy of liposomal Ful in dogs. The liposomal formulation may also incorporate additional leishmanicidal drug to make it even more effective.

5. Conclusions

The present study establishes for the first time the antileishmanial activity of Ful *in vitro* and in murine models of VL. Due to the passive targeting of liposomes to liver and spleen, liposome encapsulation of Ful enhanced its antileishmanial activity *in vivo* and liposomal fullerol reduced the hepatic parasite load in both acute and chronic murine models of VL. Our data suggests that the activity of the nanoformulation is mediated at least in part by the induction of a pro-inflammatory response.

Funding

The sources of funding for this work are the following Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, grant No. 305659/2017-0 and 425332/2018-7), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG, grant number RED-00007-14, APQ-03129-16) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

We are thankful to Nayara Kesia Lima Mendes Moura for technical support during *in vivo* experiments and Cristiano C. P. dos Santos for adding the raw data to a data repository. We thank Profa. Danielle G. Souza from Department of Microbiology (UFMG) for donation of the IL-1β kit. We acknowledge the Brazilian agencies, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, grant No. 305659/2017-0 and 425332/2018-7), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG, grant number RED-00007-14, APQ-03129-16) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

[1] World Health Organization, 2020 (Accessed on august 31, 2020), https://www.who.int/en/newsroom/fact-sheets/detail/leishmaniasis/.
[2] A. Barral, D. Pedral-Sampaio, J.G. Grimaldi, H. Momen, D. McMahon-Pratt, A. Ribeiro de Jesus, R. Almeida, R. Badaro, M. Barral-Netto, E.M. Carvalho, Leishmaniasis in Bahia, Brazil: evidence that *Leishmania amazonensis* produces a wide spectrum of clinical disease, Am. J. Trop. Med. Hyg. 44 (1991) 536–546, https://doi.org/10.4269/ajtmh.1991.44.536.
[3] H.O. Valdivia, L.V. Almeida, B.M. Roatt, J.L. Reis-Cunha, A.A. Pereir, C. Gontijo, R. T. Fujiwara, A.B. Reis, M.J. Sanders, J.A. Cotton, D.C. Bartholomeu, Comparative genomics of canine-isolated *Leishmania (Leishmania) amazonensis* from an endemic focus of visceral leishmaniasis in Governador Valadares, southeastern Brazil, Sci. Rep. 7 (2017) 40804, https://doi.org/10.1038/srep40804.
[4] C.S.F. De Souza, K.S. Calabrese, A.L. Abreu-Silva, L.O.P. Carvalho, F.O. Cardoso, M. E.M.C. Dorval, E.T. Oshiro, P.F. Quaresma, C.M.F. Gontijo, R.S. Pacheco, M.I. D. Rossi, S.C.G. da Costa, T. Zaverucha do Valle, *Leishmania amazonensis* isolated from human visceral leishmaniasis: histopathological analysis and parasitological burden in different inbred mice, Histol. Histopathol. 33 (7) (2018) 705–716, https://doi.org/10.14670/HH-11-965.
[5] S. Sundar, J. Chakravarty, An update on pharmacotherapy for leishmaniasis, Expert Opin. Pharmacother. 16 (2) (2015) 237–252, https://doi.org/10.1517/14656566.2015.973850.
[6] H.W. Kroto, J.R. Heath, S.C. O'Brien, R.F. Curi, R.E. Smalley, C_{60}: buckminsterfullerene, Nature 318 (14) (1985) 162–163, https://doi.org/10.1038/318162a0.
[7] A. Djordjevic, J.M. Canadanovic-Brunet, M. Vojinovic-Miloradov, G. Bogdanovic, Antioxidant properties and hypothetic radical mechanism of fullerenol $C_{60}(OH)_{24}$, Oxid. Commun. 27 (4) (2004) 806–812.
[8] A. Dordevic, G. Bogdanovich, Fullerenol-a new nanopharmaceutic? Arch. Oncol. 16 (3–4) (2008) 42–45, https://doi.org/10.2298/AOO0804042D.
[9] K. Yudoh, R. Karasawa, K. Masuko, T. Kato, Water-soluble fullerene (C_{60}) inhibits the development of arthritis in the rat model of arthritis, Int. J. Nanomed. 4 (2009) 217–225, https://doi.org/10.2147/ijn.s7653.
[10] P.T.T. Bernardes, B.M. Rezende, C.B. Resende, T.P. De Paula, A.C. Reis, W. A. Gonçalves, E.G. Vieira, M.V.B. Pinheiro, D.G. Souza, M.G.M. Castor, M. T. Teixeira, V. Pinho, Nanocomposite treatment reduces disease and lethality in a murine model of acute graft-versus-host disease and preserves anti-tumor effects, PLoS One 10 (4) (2015) e0123004, https://doi.org/10.1371/journal.pone.0123004.
[11] L. Jin, M. Ding, A. Oklopcic, B. Aghdasi, L. Xiao, Z. Li, V. Jevtovic-Todorovic, X. Li, Nanoparticle fullerol alleviates radiculopathy via NLRP3 inflammasome and neuropeptides, Nanomedicine 13 (6) (2017) 2049–2059, https://doi.org/10.1016/j.nano.2017.03.015.
[12] L. Xiao, K. Hong, C. Roberson, M. Ding, A. Fernandez, F. Shen, L. Jin, S. Sonkusare, X. Li, Hydroxylated fullerene: a stellar nanomedicine to treat lumbar radiculopathy via antagonizing TNF-α-induced ion channel activation, calcium signaling, and neuropeptide production, ACS Biomater. Sci. Eng. 4 (1) (2018) 266–277, https://doi.org/10.1021/acsbiomaterials.7b00735.
[13] Y. Pei, F. Cui, X. Du, G. Shang, W. Xiao, X. Yang, Q. Cui, Antioxidative nanofullerol inhibits macrophage activation and development of osteoarthritis in rats, Int. J. Nanomed. 14 (2019) 4145–4155, https://doi.org/10.2147/IJN.S202466.
[14] J. Zhu, Z. Ji, J. Wang, R. Sun, X. Zhang, Y. Gao, H. Sun, Y. Liu, Z. Wang, A. Li, J. Ma, T. Wang, G. Jia, Y. Gu, Tumor-inhibitory effect and immunomodulatory activity of fullerol C_{60}(OH)x, Small 4 (8) (2008) 1168–1175, https://doi.org/10.1002/smll.200701219.
[15] Y. Liu, F. Jiao, Y. Qiu, W. Li, Y. Qu, C. Tian, Y. Li, R. Bai, F. Lao, Y. Zhao, Z. Chai, C. Chen, Immunostimulatory properties and enhanced TNF-α mediated cellular immunity for tumor therapy by $C_{60}(OH)_{20}$ nanoparticles, Nanotechnology 20 (41) (2009) 415102, https://doi.org/10.1088/0957-4484/20/41/415102.
[16] F. Frézard, C. Demicheli, New delivery strategies for the old pentavalent antimonial drugs, Expert Opin. Drug Deliv. 7 (12) (2010) 1343–1358, https://doi.org/10.1517/17425247.2010.529897.
[17] H. Daraee, A. Etemadi, M. Kouhi, S. Alimirzalu, A. Akbarzadeh, Application of liposomes in medicine and drug delivery, Artif. Cells Nanomed. Biotechnol. 44 (2016) 381–391, https://doi.org/10.3109/21691401.2014.953633.
[18] G.C. Alves, L.O. Ladeira, A. Righi, K. Krambrock, H.D. Calado, R.P.F. Gil, M.V. B. Pinheiro, Synthesis of $C_{60}(OH)_{18\text{-}20}$ in aqueous alkaline solution under O_2-atmosphere, J. Braz. Chem. Soc. 17 (6) (2006) 1186–1190, https://doi.org/10.1590/S0103-50532006000600017.
[19] T.H. Goswami, R. Singh, S. Alam, G.N. Mathur, Thermal analysis: a unique method to estimate the number of substituents in fullerene derivatives, Thermochim. Acta 419 (1–2) (2004) 97–104, https://doi.org/10.1016/j.tca.2004.02.001.
[20] L.O. Husebo, B. Sitharaman, K. Furukawa, T. Kato, L.J. Wilson, Fullerenols revisited as stable radical anions, J. Am. Chem. Soc. 126 (38) (2004) 12055–12064, https://doi.org/10.1021/ja047593o.
[21] C. Kirby, G. Gregoriadis, Dehydration-rehydration vesicles: a simple method for high yield drug entrapment in liposomes, Biotechnology 2 (1984) 979–984, https://doi.org/10.1038/nbt1184-979.
[22] R. Nayar, M.J. Hope, P.R. Cullis, Generation of large unilamellar vesicles from long-chain saturated phosphatidylcholines by extrusion technique, Biochim. Biophys. Acta 986 (1989) 200–206, https://doi.org/10.1016/0005-2736(89)90468-9.
[23] W. Brand-Williams, M.E. Cuvelier, C.L.W.T. Berset, Use of a free radical method to evaluate antioxidant activity, Lebensm.-Wiss. U.-Technol. 28 (1995) 25–30, https://doi.org/10.1016/S0023-6438(95)80008-5.

[24] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63, https://doi.org/10.1016/0022-1759(83)90303-4.
[25] M.L. Gomes, G. De Freitas-Silva, P.G. dos Reis, M.N. Melo, F. Frézard, C. Demicheli, Y.M. Idemori, Synthesis and characterization of bismuth(III) and antimony(V) porphyrins: high antileishmanial activity against antimony-resistant parasite, J. Biol. Inorg. Chem. 20 (5) (2015) 771–779, https://doi.org/10.1007/s00775-015-1264-4.
[26] P. Chomczynski, N. Sacchi, The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on, Nat. Protoc. 1 (2) (2006) 581–585, https://doi.org/10.1038/nprot.2006.83.
[27] V.M. Carregal, J.S. Lanza, D.M. Souza, A. Islam, C. Demicheli, R.T. Fujiwara, L. Rivas, F. Frézard, Combination oral therapy against *Leishmania amazonensis* infection in BALB/c mice using nanoassemblies made from amphiphilic antimony (V) complex incorporating miltefosine, Parasitol. Res. 118 (10) (2019) 3077–3084, https://doi.org/10.1007/s00436-019-06419-2.
[28] S. Bretagne, R. Durand, M. Olivi, J.F. Garin, A. Sulahian, D. Rivollet, M. Vidaud, M. Deniau, Real-time PCR as a new tool for quantifying *Leishmania infantum* in liver in infected mice, Clin. Diagn. Lab. Immunol. 8 (4) (2001) 828–831, https://doi.org/10.1128/CDLI.8.4.828-831.2001.
[29] G.S. Ramos, V.M.R. Vallejos, M.S. Ladeira, P.G. Reis, D.M. Souza, Y.A. Machado, L. O. Ladeira, M.B.V. Pinheiro, M.N. Melo, R.T. Fujiwara, F. Frézard, Raw_Data_ Antileishmanial Activity of Fullerol and its Liposomal Formulation in Experimental Models of Visceral Leishmaniasis, Figshare Database Repository [dataset], 2020, https://doi.org/10.6084/m9.figshare.12934820.
[30] D.C. Ayres, L.A. Pinto, S. Giorgio, Efficacy of pentavalent antimony, amphotericin B, and miltefosine in *Leishmania amazonensis*-infected macrophages under normoxic and hypoxic conditions, J. Parasitol. 94 (6) (2018) 1415–1417, https://doi.org/10.1645/GE-1613.1.
[31] R.M. Santa-Rita, A. Henriques-Pons, H.S. Barbosa, S.L. De Castro, Effect of the lysophospholipid analogues edelfosine, ilmofosine and miltefosine against *Leishmania amazonensis*, J. Antimicrob. Chemother. 54 (2004) 704–710, https://doi.org/10.1093/jac/dkh38.
[32] C. Wang, Y. Bai, H. Li, R. Liao, J. Li, H. Zhang, X. Zhang, S. Zhang, S.-T. Yang, X.-L. Chang, Surface modification-mediated biodistribution of 13C-fullerene C_{60} *in vivo*, Part. Fibre Toxicol. 13 (2016) 14, https://doi.org/10.1186/s12989-016-0126-8.
[33] B. Vileno, P.R. Marcoux, M. Lekka, A. Sienkiewicz, T. Fehre, L. Forro, Spectroscopic and photophysical properties of a highly derivatized C_{60} fullerol, Adv. Funct. Mater. 16 (2006) 120, https://doi.org/10.1002/adfm.200500425.
[34] F. Szoka, F. Olson, T. Heath, W. Vail, E. Mayhew, D. Papahadjopoulos, Preparation of unilamellar liposomes of intermediate size (0.1–0.2 μm) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes, Biochim. Biophys. Acta 601 (1980) 559–567, https://doi.org/10.1016/0005-2736(80)90558-1.
[35] F. Dos Santos Nogueira, V.C. Avino, F. Galvis-Ovallos, V.L. Pereira-Chioccola, M.A. B. Moreira, A.P.P.L. Romariz, L.M. Molla, I. Menz, Use of miltefosine to treat canine visceral leishmaniasis caused by *Leishmania infantum* in Brazil, Parasit. Vectors 12 (1) (2019) 79, https://doi.org/10.1186/s13071-019-3323-0.
[36] R. Bankoti, S. Stager, Differential regulation of the immune response in the spleen and liver of mice infected with *Leishmania donovani*, J. Trop. Med. (2012) 639304, https://doi.org/10.1155/2012/639304.
[37] K.C. Carter, J. O'Grady, T.F. Dolan, A.J. Baillie, J. Alexander, J. Keys, A direct comparison of sodium stibogluconate treatment in two animal models of human visceral leishmaniasis, mouse and hamster, Int. J. Pharm. 53 (2) (1989) 129–137, https://doi.org/10.1016/0378-5173(89)90236-6.
[38] Y. Le Fichoux, D. Rousseau, B. Ferrua, S. Ruette, A. Lelievre, D. Grousson, J. Kubar, Short- and long-term efficacy of hexadecylphosphocholine against established *Leishmania infantum* infection in BALB/c mice, Antimicrob. Agents Chemother. 42 (3) (1998) 654–658, https://doi.org/10.1128/AAC.42.3.654.
[39] J.B.T. Carnielli, R. Monti-Rocha, D.L. Costa, A.M. Sesana, L.N.N. Pansini, M. Segatto, J.C. Mottram, C.H.N. Costa, S.F.G. Carvalho, R. Dietze, Natural resistance of *Leishmania infantum* to miltefosine contributes to the low efficacy in the treatment of visceral leishmaniasis in Brazil, Am. J. Trop. Med. Hyg. 101 (4) (2019) 789–794, https://doi.org/10.4269/ajtmh.18-0949.
[40] S. Yamamoto, T. Ishida, A. Inoue, J. Mikami, M. Muraguchi, Y. Ohmoto, H. Kiwada, HEPC-based liposomes trigger cytokine release from peripheral blood cells: effects of liposomal size, dose and lipid composition, Int. J. Pharm. 236 (2002) 125–133, https://doi.org/10.1016/S0378-5173(02)00026-1.
[41] R.E. Lewis, G. Chamilos, R.A. Prince, D.P. Kontoyiannis, Pretreatment with empty liposomes attenuates the immunopathology of invasive pulmonary aspergillosis in corticosteroid-immunosuppressed mice, Antimicrob. Agents Chemother. 51 (3) (2007) 1078–1081, https://doi.org/10.1128/AAC.01268-06.
[42] C.T. Inglut, A.J. Sorrin, T. Kuruppu, S. Vig, J. Cicalo, H. Ahmad, H.-C. Huang, Immunological and Toxicological Considerations for the design of liposomes, Nanomaterials (Basel) 10 (2) (2020) 190, https://doi.org/10.3390/nano10020190.
[43] M.L. Murphy, S.E.J. Cotterell, P.M.A.C.R. Gorak, C.R. Engwerda, P.M. Kaye, Blockade of CTLA-4 enhances host resistance to the intracellular pathogen, J. Immunol. 161 (1998) 4153–4160. https://www.jimmunol.org/content/161/8/4153.
[44] M. Kemp, J.A.L. Kurtzhals, K. Bendtzen, L.K. Poulsen, M.B. Hansen, D.K. Koech, A. Kharazmi, T.G. Theander, *Leishmania donovani*-reactive Thl- and Th2-Like T-cell clones from individuals who have recovered from visceral leishmaniasis, Infect. Immun. 61 (3) (1993) 1069–1073, https://doi.org/10.1128/IAI.61.3.1069-1073.1993.
[45] Z. Chen, Y. Liu, B. Sun, H. Li, J. Dong, L. Zhang, L. Wang, P. Wang, Y. Zhao, C. Chen, Polyhydroxylated metallofullerenols stimulate IL-1β secretion of macrophage through TLRs/MyD88/NF-κB pathway and NLRP₃inflammasome activation, Small 10 (12) (2014) 2362–2372, https://doi.org/10.1002/smll.201302825.
[46] M. Schenk, M. Fabri, S.R. Krutzik, D.J. Lee, D.M. Vu, P.A. Sieling, D. Montoya, P. T. Liu, R.L. Modlin, Interleukin-1β triggers the differentiation of macrophages with enhanced capacity to present mycobacterial antigen to T cells, Immunology 141 (2) (2014) 174–180, https://doi.org/10.1111/imm.12167.
[47] D.S. Lima-Junior, D.L. Costa, V. Carregaro, L.D. Cunha, A.L.N. Silva, T.W.P. Mineo, F.R.S. Gutierrez, M. Bellio, K.R. Bortoluci, R.A. Flavell, M.C. Bozza, J.S. Silva, D. S. Zamboni, Inflammasome-derived IL-1β production induces nitric oxide–mediated resistance to *Leishmania*, Nat. Med. 19 (2013) 909–915, https://doi.org/10.1038/nm.3221.
[48] J. Tang, Z. Chen, B. Sun, J. Dong, J. Liu, H. Zhou, L. Wang, R. Bai, Q. Miao, Y. Zhao, C. Chen, Y. Liu, Polyhydroxylated fullerenols regulate macrophage for cancer adoptive immunotherapy and greatly inhibit the tumor metastasis, Nanomedicine 12 (4) (2016) 945–954, https://doi.org/10.1016/j.nano.2015.11.021.