



Parasitological and immunological evaluation of a quinoline derivative salt incorporated into a polymeric micelle formulation against *Leishmania infantum* infection

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Abstract

Leishmaniasis is a parasitic disease caused by *Leishmania* protozoa, which presents a large spectrum of clinical manifestations. In the present study, a quinoline derivative salt named N-(2-((7-chloroquinolin-4-yl)amino)ethyl)-N-(prop-2-yn-1-yl) prop-2-yn-1-aminium chloride or QDS3 was *in vitro* and *in vivo* tested against *L. infantum* by means of its incorporation in Poloxamer 407-based polymeric micelles (QDS3/M). The *in vitro* antileishmanial activity of QDS3 and QDS3/M was investigated in *L. infantum* promastigotes, axenic amastigotes and infected macrophages. BALB/c mice were infected with *L. infantum*, and parasitological parameters were evaluated 1 and 15 days post-treatment by determining the parasite load by a limiting dilution assay, besides a quantitative PCR (qPCR) method. Immunological response was assessed based on production of cellular cytokines, as well as by quantification of nitrite levels and specific antibodies. *In vitro* results showed that QDS3 free or in micelles presented effective antileishmanial action against both parasite stages, being more effective in amastigotes. *In vivo* data showed that treatment using QDS3 or QDS3/M reduced the parasite load in the livers, spleens, draining lymph nodes (dLN) and bone marrows of the treated animals, 1 and 15 days after treatment, when compared to values found in the control groups. Additionally, treated mice developed a polarized Th1-type immune response, with higher levels of IL-12, IFN- γ , GM-CSF and nitrite, besides high production of specific IgG2a antibodies, when compared to the controls. Parasitological and immunological data obtained using the micellar composition were better than the others. In conclusion, QDS3, mainly when applied in a delivery adjuvant system, could be considered for future studies as therapeutic candidate against VL.

Keywords Visceral leishmaniasis · *Leishmania infantum* · BALB/c mice · Quinoline derivative salts · Micelles · QDS3 · Treatment

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Luciana M. Ribeiro Antinarelli and Nicolás Glanzmann contributed equally to this work.

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Introduction

Leishmaniasis represents a disease complex caused by a variety of protozoan parasites from *Leishmania* genus, which present great impact in public health worldwide. There are about 12 million people clinically affected, with more than 20,000 deaths and 2.0 million new cases registered annually (WHO 2020). The spectrum of clinical manifestations caused by leishmaniasis depends mainly on the infective parasite species and host immunological response (Kaye et al. 2020). Amongst the clinical forms, the most common is cutaneous leishmaniasis (CL), which is able to cause self-healing skin lesions. Uncontrolled parasite dissemination leading to disfiguring lesions represents mucosal leishmaniasis (ML) and can cause morbidity of patients (Scorza et al. 2017). A more severe form of the disease is visceral leishmaniasis (VL), which results from parasite disseminating by mononuclear phagocytes in distinct organs, such as spleen, liver, bone marrow (BM) and draining lymph nodes (dLNs) of the infected hosts. The acute disease causes hepatosplenomegaly, fever, anemia, hemorrhage and secondary infections. The major responsible species are *L. donovani*, which occurs in countries, such as Ethiopia, South Sudan, India and Bangladesh, and *L. infantum*, which causes VL in Brazil and other American countries (Burza et al. 2018; Lindoso et al. 2018).

Treatment against VL presents limitations. The pentavalent antimonials (Glucantime® and Pentostan®) are the first-line drugs in many endemic countries. However, toxicity and increasing cases of parasite resistance have been registered. As second line options, amphotericin B (AmpB) and pentamidine display good efficacy, but also have drawbacks, such as nephro and hepatotoxicity, which are associated to long-term administration time, leading to complications for the patients and frequent interruption of treatment. In recent years, AmpB-based liposomal formulations have been developed, but in spite of the advantages of increase the efficacy associated with lower toxicity; the high cost remains impeditive to their use in developing countries (Ghorbani and Farhoudi, 2017; Roatt et al. 2020). Miltefosine has been used to the oral treatment against VL, but the emergence of resistant strains and teratogenicity are limiting factors (Dorlo et al., 2012; Van Bockstal et al. 2020). There are also reports of nausea, vomiting, diarrhea and abdominal pain in the patients as secondary effects after use of drug (Monge-Maillo and López-Vélez 2015).

In this context, efforts have been made to identify new and less toxic antileishmanial agents, and an *in vivo* promising action has been attributed for different classes of quinoline derivatives, such as 8-hydroxyquinoline, fluoroquinoline, chloroquinoline, 8-aminoquinoline, among

others (Carvalho et al. 2011; Duarte et al. 2016; Manzano et al. 2019; Tavares et al. 2019; Herrera et al. 2020). In a previous study, a new series of quinoline derivative salts (QDS) was synthesized by our group and it showed *in vitro* antileishmanial activity against *L. amazonensis* and *L. braziliensis* (Calixto et al. 2018). From this work, the compound N-(2-((7-chloroquinolin-4-yl)amino)ethyl)-N-(prop-2-yn-1-yl)prop-2-yn-1-aminium chloride or QDS3 (Fig. 1) presented antileishmanial activity against *L. amazonensis* amastigotes ($IC_{50} = 5.48 \mu M$), with similar results to miltefosine. Moreover, QDS3 showed low toxicity in murine macrophages, with an activity 40 times higher in the parasites, and the mechanism of action was related to the parasite bioenergetic system, inducing programmed cell death by an apoptosis-like action (Calixto et al. 2018).

Considering the results obtained in this previous work, QDS3 was herein evaluated *in vitro* and *in vivo* as antileishmanial agent against *L. infantum*. The molecule was used alone or associated in polymeric micelles (QDS3/M) as adjuvant. For the *in vivo* studies, BALB/c mice were infected with *L. infantum* promastigotes, and 45 days post-infection, they received treatment using QDS3 or QDS3/M. Miltefosine was used as drug control. Distinct parasitological and immunological evaluations were performed in two endpoints after therapy, aiming to verify the therapeutic efficacy of the compound against *L. infantum* infection.

Materials and methods

Chemicals

Previously synthesized by our research group, the QDS3 compound was characterized by spectroscopic and spectrometric methods. Procedures and results of purification were previously described (Calixto et al. 2018). Additional data

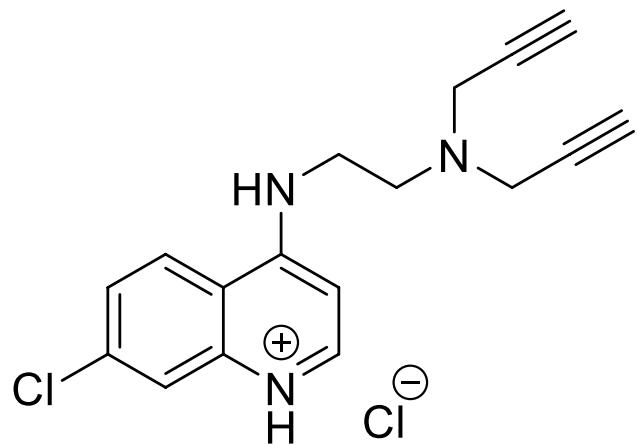


Fig. 1 Chemical structure of the QDS3 molecule

such as NMR spectra are presented in the supplementary material.

QDS3/M preparation

Micelles were prepared by dilution of Poloxamer P407 (18% w/w) in 1x phosphate buffer pH 7.4 (PBS), moderately stirred with a magnetic agitator for 18 h at 4 °C. QDS3 (8.0 mg) was dissolved in absolute dichloromethane (500 µL) by vortex agitation, for better solubilization, and added to the Poloxamer P407 containing solution, under vigorous stirring using a magnetic agitator and submitted to an ice bath, until forming an emulsion. The dichloromethane solution was removed by vacuum evaporation, and the composition was filtered using a 0.45-µm microfiltration membrane and conserved at 4 °C. QDS3 content was evaluated spectrophotometrically by using the ultraviolet method (Barichello et al., 1999). Briefly, QDS3/M samples were collected and diluted in absolute methanol, and absorbance was measured in a UV/Vis spectrophotometer (Double beam AJX-6100 PC; Micronal, São Paulo, Brazil), at 380 nm. The concentration of QDS3 was calculated using a standard curve (0 to 18 µM), which was previously prepared in methanol. The analyses were carried out using three replicates. To perform the physical–chemical characterization of the micellar composition, dynamic light scattering (DLS) was employed to measure the average particle size and zeta potential using the Zetasizer Nano ZSP system (Malvern Instruments, UK). The micellar morphology was evaluated by transmission electron microscopy (TEM; Tecnai G20, FEI Company, USA), and the stability of the preparation was evaluated by measuring the particle size and zeta potential during seven days (Liu et al. 2017). Empty micelles (18% w/w) were prepared using the same protocol, but without addition of QDS3.

Mice

Female BALB/c mice (6–8 weeks old) were maintained under specific conditions: water and food ad libitum, temperature at 22 °C and 12/12 h light/dark cycle. The procedures using the animals were conducted in accordance with the protocol approved by the Ethical Committee for Research Animal Handling of Federal University of Minas Gerais (UFMG) (protocol number 085/2017 approved in 05/06/2017).

Parasites

Leishmania infantum (MHOM/BR/1970/BH46) strain was used. Parasites were cultured in complete Schneider's medium (Sigma-Aldrich) pH 7.4 with addition of 20% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), 20 mM L-glutamine and a mixture of antibiotics (200 U/mL

penicillin, 100 mg/mL streptomycin, and 50 mg/mL gentamicin). They were maintained at 25 °C in a BOD incubator (Mendonça et al. 2018). To obtain axenic amastigotes, 2×10^8 stationary promastigotes were washed three times in PBS pH 7.4, resuspended in 5 mL FBS and incubated for 72 h at 37 °C. Parasites were then washed three times in PBS, and their morphology was evaluated by Giemsa staining and visualization using an optical microscope.

In vitro antileishmanial activity

The 50% *Leishmania* inhibitory concentration (IC₅₀) was evaluated by incubating *L. infantum* stationary promastigotes or axenic amastigotes (2×10^6 cells, each) in the presence of QDS3 or QDS3/M (6.25 to 100.0 µM, each) in 96-well culture plates for 48 h at 25 °C. Miltefosine (0.94 to 30.0 µM; Cayman Chemical Company, Michigan, USA) was used as control. The (3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) method was used to assess parasite viability. The optical density (OD) values were read in a microplate spectrophotometer (Molecular Devices, Spectra Max Plus, San Jose, CA, USA) at 570 nm. Results were entered into Microsoft Excel (version 10.0) spreadsheets, and IC₅₀ values were calculated by sigmoidal regression of the dose–response curves (Reis et al. 2021).

In vitro cytotoxicity assay

The 50% macrophage inhibitory concentration (CC₅₀) was evaluated by incubating murine macrophages (5×10^5 cells) in 24-well plates containing sterile glass coverslips for 24 h at 37 °C in 5% CO₂. Macrophages were obtained from female BALB/c mice, by means of peritoneal lavage using 5 mL cold PBS. Peritoneal exudate cells were centrifuged at 1,000×g for 10 min and resuspended in RPMI 1640 medium. After adherence, macrophages were incubated with varied concentrations of QDS3 or QDS3/M (6.25 to 200.0 µM, each) in RPMI 1640 medium and in 96-well plates (Nunc) for 48 h at 37 °C and 5% CO₂. Miltefosine (0.94 to 50.0 µM) was used as control. Macrophage viability was also assessed by MTT method. The CC₅₀ values were calculated by applying a sigmoidal regression of the dose–response curve (Mendonça et al. 2018). The selectivity index (SI) was calculated by the ratio between CC₅₀ and IC₅₀ values.

Treatment of infected macrophages

Murine macrophages (5×10^5 cells) were incubated in RPMI 1640 medium supplemented with 20% FBS and 20 mM L-glutamine pH 7.4, for 24 h at 37 °C in 5% CO₂. Afterwards, stationary promastigotes were added in the wells

(5×10^6 cells), and plates were incubated for 48 h at 37 °C in 5% CO₂. Free parasites were removed by extensive washing with medium. Infected macrophages were treated with QDS3 or QDS3/M (2.5, 5.0 and 10.0 µM, each) for 48 h at 37 °C in 5% CO₂. Miltefosine (1.0, 2.5 and 5.0 µM) was used as control. After fixation with 4% (w/v) paraformaldehyde, cells were washed and stained with Giemsa. Percentage of infected macrophages, infectiveness reduction and number of amastigotes per macrophage were determined by counting 200 cells, in triplicate, using an optical microscope (Mendonça et al. 2019).

In vivo infection and therapeutic schedules

A subcutaneous route in the right hind footpad was used to infect BALB/c mice ($n = 12$ per group) with 10^7 *L. infantum* stationary promastigotes. After 45 days of infection, animals received treatment every two days in a period of 15 days, through one of the regimens described: (a) control group (saline): mice received 50 µL of PBS; (b) micelle group (B-Mic): mice treated with 50 µL of empty micelles (10 mg/kg body weight), (c) QDS3/M group: mice treated with 50 µL of QDS3/M (5 mg/kg body weight). (d) QDS3 group: mice treated with 50 µL of QDS3 (10 mg/kg body weight), and (e) miltefosine group (Milt): mice treated with miltefosine (2 mg/kg body weight). Treatments were performed by subcutaneous route, while miltefosine was administered by oral route. The concentration of products used for the treatments was based on previous studies, where quinoline derivatives were also evaluated as antileishmanial agents (Tavares et al. 2020; Freitas et al. 2021; Reis et al. 2021; Mendonça et al. 2022). In addition, the parasitological and immunological assays were performed 1 and 15 days after treatment.

Parasite load evaluation

A limiting dilution technique was used to evaluate the parasite load in spleen, liver, dLN and BM of the animals (Mendonça et al. 2018). In order to do so, the weight of the organs was measured and a glass tissue grinder was used for homogenization in sterile PBS. Centrifugation at $150 \times g$ was performed to remove tissue debris and, to concentrate the cells, centrifugation at $2,000 \times g$ was used. In sequence, complete Schneider's medium was used to resuspend the pellets. Then, each resuspended sample (220 µL) was plated in triplicate into 96-well flat-bottom microtiter plates (Nunc), to be serially diluted in log-fold (10^{-1} to 10^{-12} dilution) in complete Schneider's medium. The reading of each sample was performed 7 days after incubation at 24 °C. Results were expressed as the negative log of the titer (i.e., the highest dilution value that promoted parasite growth), adjusted per organ milligram.

Splenic parasite load evaluated by qPCR

The Wizard® Genomic DNA Purification Kit (Promega Corporation) was used to extract the spleen DNA, following the recommendations from the manufacturer. Parasite load was estimated using primers to amplify *L. infantum* kDNA: 5'-CCTATTTTACACCAACCCCAAGT-3' and 5'-GGGTAGGGGCGTTCTGCGAAA-3' (Forward and Reverse, respectively). Primers (Forward 5'-CAGAGCAAGAGAGGTATCC-3' and Reverse 5'-TCATTGTAGAAGGTGTGGTGC-3') to amplify the mouse β-actin gene were used as endogenous control. ABI Prism 7500 Sequence Detection System (96 wells-plate; Applied Biosystems) with 2X SYBR™ Select Master Mix (5 µL; Applied Biosystems) was used to process and analyze the reactions, with 1 µL of each primer (2 mM) and 4 µL of DNA (25 ng/µL). Samples were incubated at 95 °C for 10 min and submitted to 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The fluorescence values were collected during each cycle. The parasitism was determined interpolating from the standard curve, which was the result duplicate experiments, and then, it was estimated the number of parasites per nucleated cells (Duarte et al. 2016).

Cellular response and nitrite production

Splenocytes were obtained from spleens of the animals, 1 and 15 days post-treatment, when cells (5×10^6 per mL) were cultured in DMEM medium supplemented with 20% FBS and 20 mM L-glutamine pH 7.4 and incubated in duplicate in 24-well plates (Nunc). Cultures were stimulated with *L. infantum* SLA (25 µg/mL) for 48 h (37 °C, 5% CO₂) and, subsequently, levels of IFN-γ, GM-CSF, IL-12p70, IL-4 and IL-10 were measured in culture supernatant through a capture ELISA using commercial kits (BD Pharmingen, San Diego, CA, USA). Nitrite levels were also determined in the same supernatant by Griess reaction (Sigma-Aldrich, USA) (Freitas et al. 2021).

Humoral response evaluation

Sera from infected and treated mice were collected 1 and 15 days after treatment and were applied in ELISA experiments to evaluate the antileishmanial IgG1 and IgG2a isotypes production. *Leishmania infantum* SLA (1.0 mg per well) was used as antigen. The dilution of samples was performed at 1:100 in PBS plus 0.05% Tween 20 (PBS-T), and incubation occurred for 1 h at 37 °C. After washing the plates, wells were incubated with a 1:10,000 dilution in PBS-T of anti-mouse IgG1 or IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich). The OD values were

read in ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 492 nm (Freitas et al. 2021).

Statistical analysis

To determine the IC_{50} and CC_{50} values, dose–response curves in the Probit program were used. One-way analysis of variance (ANOVA) followed by Tukey’s test for multiple comparisons between the groups was used to perform the statistical analysis, with level of significance of $P < 0.05$. At least two independent experiments were conducted in duplicate and results were similar.

Results

Characterization of the QDS3/M

QDS3/M composition showed mean particle size of 98.45 ± 5.89 nm and narrow distribution of 0.217 ± 0.046 by DLS. Micelles presented negative zeta potential of -9.02 ± 1.08 mV and, when evaluated by TEM, in the morphology assay they showed homogeneous spherical shapes and smooth surfaces, making it possible to suggest that it will be prone for accumulation in host phagocytes. The zeta potential evaluation showed that QDS3/M was stable at least for seven days. After examining the size distribution by DLS, the polydispersity index of QDS3/M was 0.19.

In vitro antileishmanial activity

The *in vitro* antileishmanial activity of QDS3 and QDS3/M was evaluated against *L. infantum* promastigotes and axenic amastigotes. Results showed IC_{50} values of 32.4 ± 2.5 and 15.6 ± 3.4 μ M, respectively, when QDS3 was used; and of 25.4 ± 4.0 and 10.9 ± 1.6 μ M, respectively, when QDS3/M was employed in the experiments. The CC_{50} values for QDS3 and QDS3/M were 289.6 ± 14.5 and 344.5 ± 19.3 μ M, respectively. With such data, SI was calculated and the result for QDS3 was 9.0 and 18.6 against promastigotes and amastigotes, respectively, while the result for QDS3/M was 13.6 and 31.6, respectively. Miltefosine showed IC_{50} values of 2.5 ± 0.5 and 1.9 ± 0.6 μ M against promastigotes and amastigotes, respectively, and CC_{50} value of 19.8 ± 2.8 μ M. With these results, SI values were of 7.9 and 10.4, respectively.

The treatment of infected macrophages presented infectiveness reduction in the order of 68.0% and 77.6%, when QDS3 and QDS3/M were used at concentrations of 10.0 μ M, respectively, while the number of recovered amastigotes per macrophage was 1.0 ± 0.3 and 0.7 ± 0.4 , respectively (Table 1). Miltefosine showed infectiveness reduction of 56.5%, when used in a concentration of 5.0 μ M, and a number of recovered amastigotes per cell of 1.9 ± 0.5 . Untreated and infected macrophages presented number of recovered amastigotes of 4.8 ± 0.8 (Table 1).

Table 1 Treatment of infected macrophages. Murine macrophages (5×10^5 cells) were incubated in complete RPMI 1640 medium for 24 h at 37 °C in 5% CO_2 , when stationary promastigotes (5×10^6 cells) were added in the wells, and plates were incubated for 48 h at 37 °C in 5% CO_2 . Free parasites were removed by extensive washing with medium. Infected macrophages were treated with QDS3 or

QDS3/M (2.5, 5.0 and 10.0 μ M, each) for 48 h at 37 °C in 5% CO_2 . Miltefosine (1.0, 2.5 and 5.0 μ M) was used as control. Percentage of infected macrophages, infectiveness reduction and number of amastigotes per macrophage were determined by counting 200 cells, in triplicate, using an optical microscope. Results are expressed as mean \pm standard deviation

Compound	Concentration (μ M)	Percentage of infected macrophages after treatment	Infectiveness reduction (%)	Number of amastigotes per macrophage
QDS3	10.0	26.7 ± 3.2	68.0	1.0 ± 0.3
	5.0	38.9 ± 4.0	53.4	1.9 ± 0.6
	2.5	52.2 ± 3.6	37.5	2.9 ± 0.5
	0	83.5 ± 4.7	(-)	4.8 ± 0.8
QDS3/M	10.0	18.7 ± 2.6	77.6	0.7 ± 0.4
	5.0	28.9 ± 3.3	65.4	1.6 ± 0.5
	2.5	45.5 ± 3.6	45.5	2.5 ± 0.7
	0	83.5 ± 4.7	(-)	4.8 ± 0.8
Miltefosine	5.0	36.3 ± 2.8	56.5	1.9 ± 0.5
	2.5	47.8 ± 3.6	42.8	2.9 ± 0.5
	1.0	62.9 ± 4.9	24.7	3.5 ± 0.7
	0	83.5 ± 4.7	(-)	4.8 ± 0.8

In vivo therapeutic evaluation against *L. infantum*

The *in vivo* therapeutic efficacy of QDS3 and QDS3/M against *L. infantum* infection was evaluated in BALB/c mice, 1 and 15 days after therapy. The parasite load was evaluated through the limiting dilution assay (LDA) and by qPCR assay. Regarding evaluation using LDA, results showed that QDS3 and QDS3/M-treated groups significantly reduced the parasite load in all evaluated organs, when compared to control groups (Fig. 2). Moreover, treatment using QDS3/M most significantly reduced parasitism in the liver, spleen, dLNs and bone marrow, in order of 73.0%, 86.0%, 85.0% and 44.0%, 1 day after treatment, and of 77.0%, 68.0%, 72.0% and 58.0%, 15 days after therapy, as compared to B-Mic control group. Treatment using

miltefosine reduced the parasite load in the treated and infected mice, in the order of 42.8% and 43.0% in their spleens and dLNs, 1 day after treatment, and of 37.7% and 45.0%, 15 days post-therapy, when compared to saline control group. Although reduction in the parasite load in the liver and BM of miltefosine-treated mice had been found; none significant difference was reached regarding to the saline and B-Mic groups.

The splenic parasitic burden was also evaluated by qPCR technique, and results showed significant reduction in the parasitism in QDS3 and QDS3/M-treated groups, as compared to controls, both 1 and 15 days post-treatment (Fig. 3). Miltefosine-treated mice presented also reductions in the splenic parasitism, when compared to saline control; however, values were higher as compared to those obtained in the QDS3 and QDS3/M groups.

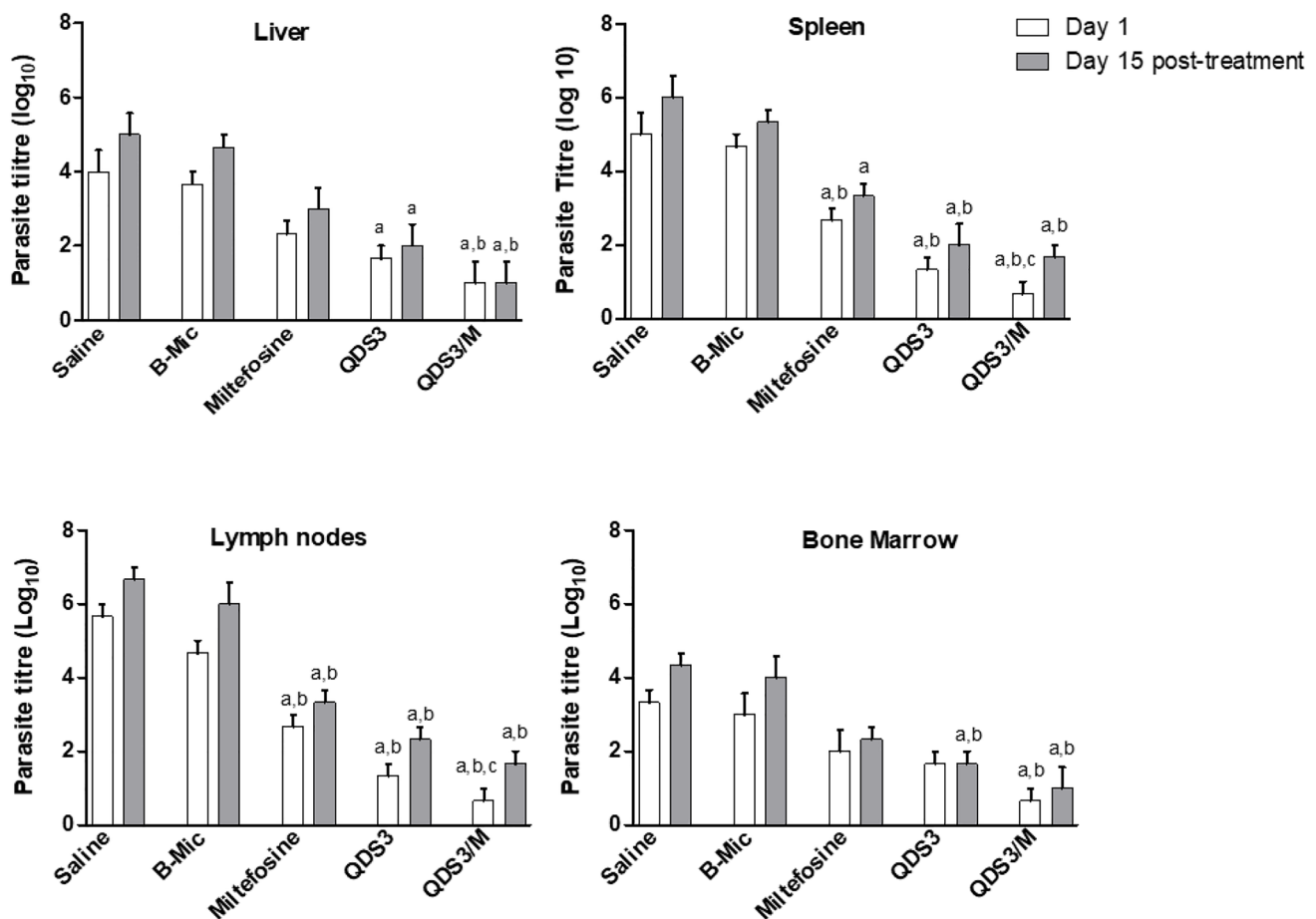


Fig. 2 Parasite load evaluation by limiting dilution technique. For *in vivo* assay, 10^7 *L. infantum* stationary promastigotes were used to infect the BALB/c mice. After 45 days from the infection, the mice were grouped ($n = 12$ per group) and treated as follows: saline, empty micelle (B-Mic), miltefosine, QDS3 or QDS3-containing micelles (QDS3/M). Treatments were performed every two days and during 15 days. Parasite load evaluation was performed 1 and 15 days ($n = 6$

per group, each) after treatment of the animals, through limiting dilution assay in their liver, spleen, draining lymph nodes and bone marrow. Data are presented as the mean value plus standard deviation of the groups. The letters “a”, “b” and “c” indicate significant statistical differences in relation to the saline, B-Mic and miltefosine groups, respectively ($P < 0.05$)

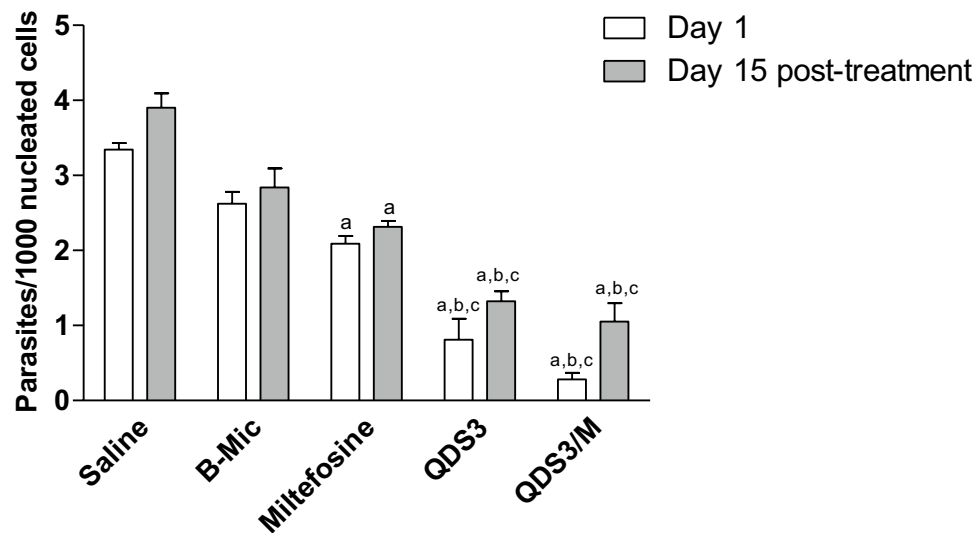


Fig. 3 Splenic parasite load evaluated by qPCR technique. To investigate the splenic parasitism by qPCR method, 10^7 *L. infantum* stationary promastigotes were used to infect BALB/c mice. After 45 days from infection, animals were grouped in: saline, empty micelle (B-Mic), free QDS3 (QDS3), QDS3-containing micelle (QDS3/M) and miltefosine groups. Treatments were performed, and the splenic

parasitic load was evaluated 1 and 15 days after treatment ($n=6$ per group, each). Data are presented as the mean plus standard deviation of the groups. The letters “a”, “b” and “c” indicate significant differences in relation to the saline, B-Mic and miltefosine groups, respectively ($P < 0.05$)

Immune response generated after treatment

The immune response developed after treatment was evaluated in the cellular supernatant of splenocyte cultures, after stimulus using SLA. Results showed that QDS3, QDS3/M and miltefosine-treated mice produced significantly higher levels of IFN- γ , IL-12 and GM-CSF, as well as lower antileishmanial IL-4 and IL-10 levels, when compared to values found in the saline group, 1 and 15 days after treatment (Fig. 4). A more polarized Th1-type cellular response was found in QDS3/M-treated mice, when compared to data obtained in the miltefosine and QDS3 groups. A positive effect of the treatment with empty micelles was also observed, in comparison with data described in saline group mice, although the Th1-type cytokines had been produced in lower levels, as compared to those found in miltefosine, QDS3 and QDS3/M groups (Fig. 4).

The nitrite production in SLA-stimulated splenic cultures was also investigated as marker of macrophage activation. Results showed that QDS3, QDS3/M or miltefosine-treated mice produced higher levels of this antileishmanial molecule, both 1 and 15 days after treatment, when compared to values found in the controls (Fig. 5).

Concerning the humoral response, data indicated that miltefosine, QDS3 and QDS3/M-treated mice produced significantly higher levels of parasite-specific IgG2a isotype antibodies, contrasting to the lowest levels of IgG1 isotype (Fig. 6). Ratios between IgG2a and IgG1 levels were

calculated, and the data also indicated the occurrence of a Th1-type humoral response in the treated animals.

Discussion

In a previous study, the *in vitro* antileishmanial activity of a quinoline derivative salt called QDS3 was demonstrated in *Leishmania* species responsible of causing TL (Calixto et al. 2018). The molecule, used alone, was effective against *L. amazonensis* and *L. braziliensis* promastigotes, with IC_{50} values of 43.25 μ M (14.45 μ g/mL) and 39.19 μ M (13.10 μ g/mL), respectively, as well as against *L. amazonensis* amastigotes, with IC_{50} value of 5.48 μ M (1.83 μ g/mL). Following the rationale to identify new antileishmanial targets able to act against distinct *Leishmania* species, in the present study, the antileishmanial activity of QDS3 was *in vitro* and *in vivo* evaluated against the *L. infantum* promastigotes and axenic amastigotes. Results showed that the molecule was effective against both parasite stages, being even more effective in amastigotes than promastigotes. Better *in vitro* results were found when QDS3 was incorporated in polymeric micelles, when compared to data obtained using the lone molecule. The *in vivo* therapeutic effect of QDS3 and QDS3/M was also evaluated in *L. infantum*-infected mice and significant reductions in the parasite load in distinct organs of the animals, as well as the development of a Th1-type cellular and humoral immune response, were found. Two endpoints

Fig. 4 Cellular immune response generated in treated and *L. infantum*-infected animals. Levels of IFN- γ , IL-4, IL-10, IL-12 and GM-CSF were evaluated in the splenocyte culture supernatants of mice groups ($n=6$ per group) by a capture ELISA technique, 1 and 15 days after treatment. Cells were stimulated with *L. infantum* SLA (25 $\mu\text{g/mL}$) for 48 h at 37 $^{\circ}\text{C}$ in 5% CO_2 . Data are presented as the mean plus standard deviation of the groups. The letters “a”, “b”, “c” and “d” indicate significant statistical differences in relation to the saline, B-Mic, miltefosine and QDS3 groups, respectively ($P < 0.05$)

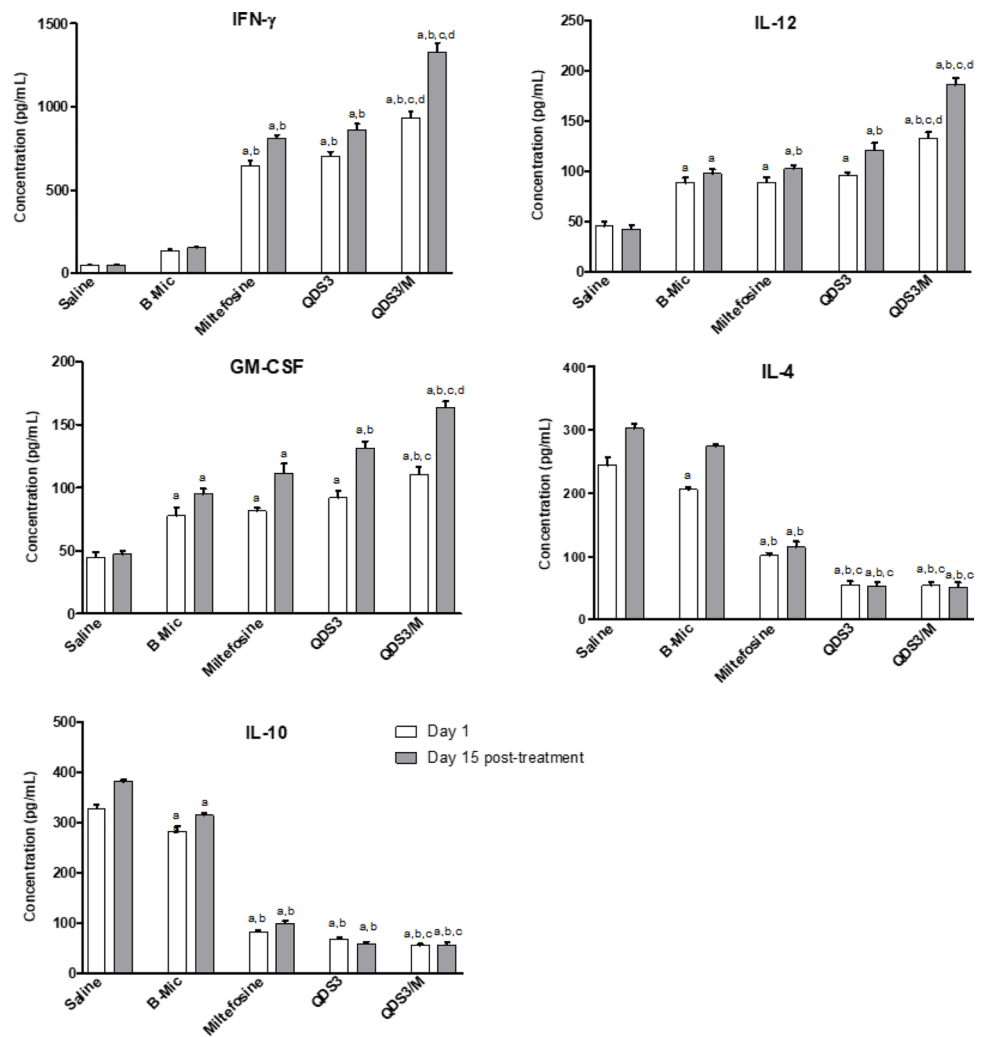
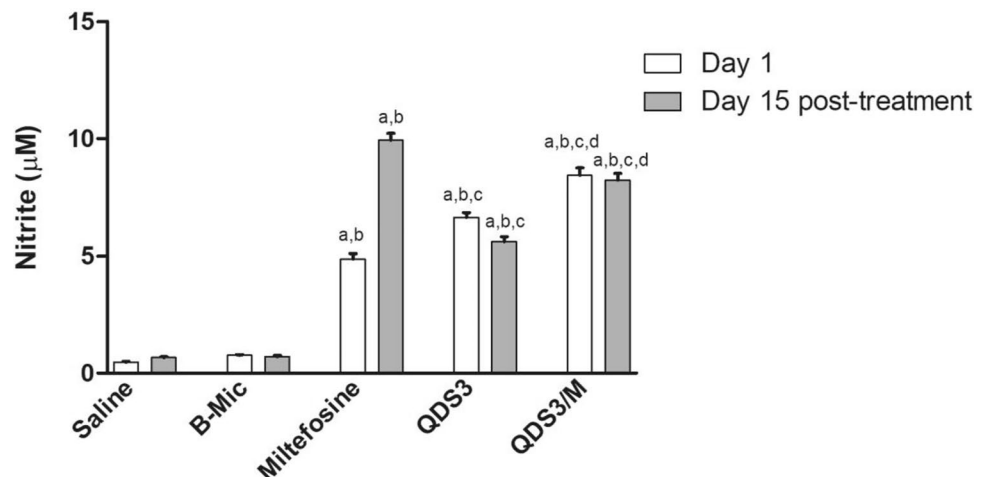


Fig. 5 Evaluation of parasite-specific nitrite production. The nitrite secretion was investigated in parasite-stimulated culture supernatants, 1 and 15 days ($n=6$ per group, each) after therapy. Data are presented as the mean plus standard deviation of the groups. The letters “a”, “b”, “c” and “d” indicate significant statistical differences in relation to the saline, B-Mic, miltefosine and QDS3 groups, respectively ($P < 0.05$)



were evaluated after therapy, and results obtained in both periods of time corroborated with the therapeutic response attributed to QDS3 and QDS3/M. Miltefosine was used as drug control and also showed therapeutic efficacy against

infection; however, data of QDS3/M group were better than those obtained for miltefosine- and QDS3-treated groups.

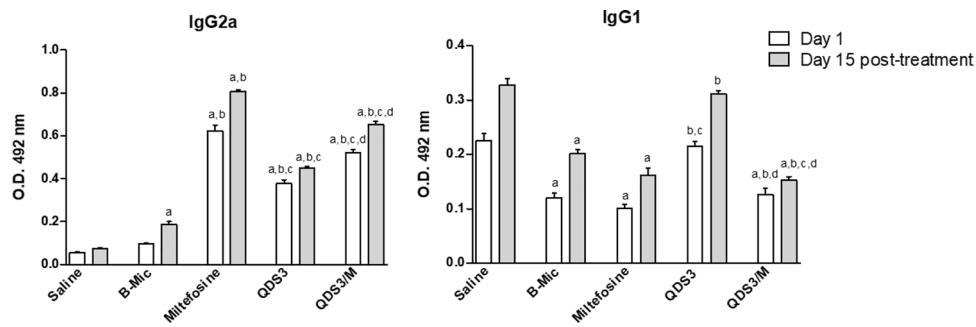


Fig. 6 Production of antileishmanial IgG2a and IgG1 antibodies in treated and infected mice. The parasite-specific IgG2a and IgG1 isotype levels were measured in serum samples collected from treated and infected mice, 1 and 15 days ($n=6$ per group, each) after treat-

ment. Data represent the mean value plus standard deviation of the groups. The letters “a”, “b”, “c” and “d” indicate significant statistical differences in relation to the saline, B-Mic, miltefosine and QDS3 groups, respectively ($P < 0.05$)

In recent years, with the technological advances of drug delivery systems, numerous studies showed that compositions formed by Poloxamer 407-based polymeric micelles present high potential as drug nanocarriers, due to their ability to minimize the toxicity of molecules, besides its ability of targeting the phagocytic system and immune adjuvant properties (Valenzuela-Oses et al. 2017; Oyama et al. 2019). This system is composed by amphiphilic copolymers that present suitable physicochemical characteristics in terms of reduced size, easily of production, biodegradability, good thermodynamic stability under high temperatures and low toxicity (Bruni et al. 2017). In this context, molecules with antileishmanial action have been incorporated in Poloxamer 407-based micelles, and satisfactory results have been obtained in experimental TL and VL models (Duarte et al. 2016; Mendonça et al. 2016; Singh et al. 2017; Tavares et al. 2019; Freitas et al. 2021). Recently, a study showed higher therapeutic efficacy of an AmpB-containing Poloxamer 407 formulation for the murine treatment against *L. infantum* infection, with better results as compared to those obtained using amphotericin B deoxycholate, Ambisome® and Glucantime®, with AmpB-containing micelles, and presenting more satisfactory results than the conventional drugs regarding to the parasitological and toxicological parameters evaluated (Mendonça et al. 2018). Moreover, the treatment using AmpB-containing micelles promoted an effective Th1-type response in infected mice, based on the production of high IFN- γ , GM-CSF, IL-12 and nitrite levels, besides the parasite-specific IgG2a antibody presence (Mendonça et al. 2018). Singh et al. (2017) reported also the efficacy and safety of a formulation composed by AmpB incorporated into Pluronic® F127-polymeric micelles against *L. donovani* infection in hamsters. In this experimental model, treatment with AmpB-loaded micelles showed better therapeutic effects in comparison with liposomal AmpB formulation of in terms of reduction of parasite load, toxicity and development of a specific Th1-type immunity, with elevated

secretion of the TNF- α and IL-12, which were associated to macrophages’ activation (Singh et al. 2017). Promising results were also found when other quinoline derivatives, such as 8-hydroxyquinoline and clioquinol, were incorporated in a delivery system based on Poloxamer 407 micelles and used for the treatment against murine leishmaniasis. Results showed a marked parasitism reduction in the animals’ tissue and organs, without systemic toxicity; demonstrating thus that this drug-carrying polymeric system can be considered suitable as delivery for antileishmanial candidates (Duarte et al. 2016; Lage et al. 2016; Tavares et al. 2020).

Here, considering the high potential of this drug delivery system, QDS3 was incorporated in polymeric micelles and investigated as therapeutic agent in a murine model of VL infection. Treatment using QDS3 or QDS3/M demonstrated to be effective in reducing the parasitism in the liver and spleen, the main organs affected by the infection in both early and chronic stages of the disease, in comparison to control groups. Moreover, QDS3 and QDS3/M were also effective in decreasing the parasite burden in dLN and bone marrow of the treated mice in both endpoints after treatment; suggesting a possible long-term therapeutic efficacy for the drug, mainly when in a micellar composition. Miltefosine also promoted significant reduction in the parasitism in the spleen and dLN, while higher parasite burden was detected in the liver and BM of the animals, at both 1 and 15 days after therapy. These results were similar to others described in the literature, where a partial therapeutic response was attributed to this drug in murine models against *L. infantum* infection (Sousa-Batista et al. 2018; Valdivieso et al. 2018; Rebello et al. 2019). One possible reason for this similarity may be to the low dose of miltefosine and the therapeutic regimen used for the treatment of the infected animals. In addition, this drug was administered by oral route, while QDS3 and QDS3/M were administered by a subcutaneous route. Such fact could also account for a variation in the

therapeutic response to miltefosine; thus, other drug controls also administered by subcutaneous route should be considered to compare the therapeutic efficacy of QDS3 and QDS3/M with more suitable controls, and this fact is a limitation of the study.

The protective immunity required for the control of *Leishmania* infection is characterized by development of specific Th1-type response sustained by activation of both CD4⁺ and CD8⁺ T cells, with increased production of pro-inflammatory cytokines, such as IFN- γ , IL-12, GM-CSF and TNF- α , among others, which contribute to infection control in infected cells by induction of nitric oxide (NO) secretion (Scott 2003). In contrast, disease progression is related with the occurrence of polarized Th2-type response, which results in the production of anti-inflammatory cytokines, such as IL-4, IL-10 and IL-13, among others, which deactivate parasitized macrophages, contributing to the development of disease (Gollob et al. 2014; Srivastava et al. 2016). The evaluation of the immunotherapeutic action in our study showed that the treatment using QDS3 or QDS3/M stimulated to the development of a polarized Th1-type response in the treated animals, in comparison with data obtained in control groups, being evidenced by higher levels of IFN- γ , IL-12 and GM-CSF and associated with significantly lower levels of IL-4 and IL-10, by the spleen cells stimulated with the parasite extract. Furthermore, increased levels of nitrite in response for the treatment with QDS3 and QDS3/M were found and associated with the ability of these products to activate macrophages. Parasitological and immunological data described in this work were similar in two endpoints evaluated after treatment, suggesting a possible long-term therapeutic efficacy of the compounds against infection.

In our study, parasitological evaluations performed in two endpoints after treatment suggested a positive therapeutic response of the products against infection, since significant reductions in the parasite load in distinct organs were found in miltefosine-, QDS3- and QDS3/M-treated mice, when compared to the data obtained in the controls. However, the parasite burden was not eliminated completely in the treated animals. So, the adjustment of the number of doses and/or the increase in the drug concentration should be evaluated, aiming to completely eliminate parasites in the treated animals, then making it possible to produce a sterile cure in these mice. Nonetheless, similar results have been described in other studies, where antileishmanial candidates were also evaluated in murine models, highlighting the need to improve the therapeutic schedules tested to reach the complete elimination of the parasites (Malafaia et al. 2011; Cunha-Júnior et al. 2016; Freitas et al. 2021; Reis et al. 2021). Our purpose was to evaluate treated animals longer than 15 days post-therapy; however, mice receiving saline showed high parasitism

associated with relevant loss of weight and weakness. In this context, all experimental groups were euthanized 15 days after treatment, when parasitological and immunological evaluations were performed, aiming to adhere to the Ethical experimental procedures. Nevertheless, a dose–response curve to evaluate the number of doses and/or concentration of the products should be certainly performed; aiming to reach the complete elimination of organic parasitism.

Taken together, results described here suggest the therapeutic action of QDS3 and QDS3/M to control the parasite load in *L. infantum*-infected mice, where the response was associated with the occurrence of a pronounced Th1-type immune response, as well as by activation of macrophage-mediated mechanisms by a NO-dependent pathway, which played to eliminate parasites. Additional studies are certainly necessary while aiming to obtain a sterile cure for the treated animals, by means of increase in the number of doses and/or concentration of the products in the formulations. In addition, the absence of a drug control administered by subcutaneous route is a limitation of this work, since the comparison of the therapeutic efficacy was performed only with mice receiving miltefosine by oral route and with a short therapeutic schedule. In this context, additional experiments using drug controls also administered by subcutaneous route are certainly necessary to be performed. Alternatively, the administration of QDS3 and QDS3/M could also be evaluated by other routes in the animals, such as by oral route, making their use simpler and without the necessity to perform their administration in hospitals. In conclusion, preliminary data presented here are a proof-of-concept of the antileishmanial activity of QDS3 against an important parasite species able to cause VL in the world.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00436-022-07544-1>.

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Declarations

Conflict of interest The authors confirm that there is no conflict of interest.

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