Preclinical Studies Evaluating Subacute Toxicity and Therapeutic Efficacy of LQB-118 in Experimental Visceral Leishmaniasis

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Visceral leishmaniasis (VL) is the most severe form of leishmaniasis and is the second major cause of death by parasites, after malaria. The arsenal of drugs against leishmaniasis is small, and each has a disadvantage in terms of toxicity, efficacy, price, or treatment regimen. Our group has focused on studying new drug candidates as alternatives to current treatments. The pterocarpanquinone LQB-118 was designed and synthesized based on molecular hybridization, and it exhibited antiprotozoal and anti-leukemic cell line activities. Our previous work demonstrated that LQB-118 was an effective treatment for experimental cutaneous leishmaniasis. In this study, we observed that treatment with 10 mg/kg of body weight/day LQB-118 orally inhibited the development of hepatosplenomegaly with a 99% reduction in parasite load. An in vivo toxicological analysis showed no change in the clinical, biochemical, or hematological parameters. Histologically, all of the analyzed organs were normal, with the exception of the liver, where focal points of necrosis with leukocytic infiltration were observed at treatment doses 5 times higher than the therapeutic dose; however, these changes were not accompanied by an increase in transaminases. Our findings indicate that LQB-118 is effective at treating different clinical forms of leishmaniasis and presents no relevant signs of toxicity at therapeutic doses; thus, this framework is demonstrated suitable for developing promising drug candidates for the oral treatment of leishmaniasis.

Leishmaniasis is a parasitic disease transmitted through the bite of an infected phlebotomine sand fly and is caused by protozoan parasites of the genus Leishmania. In total, the official number of clinical cutaneous and visceral cases has reached over 278,000 per year, but an incidence of 1.0 to 1.5 million new cases each year in 98 countries is estimated to be more accurate (1). Visceral leishmaniasis (VL) is the most severe form of the disease and is the second major cause of death by parasites after malaria. The main symptoms include fever, weight loss, and swelling of the liver and spleen. Currently, leishmaniasis is treated with a small arsenal of drugs, including pentavalent antimonials, amphotericin B deoxycholate, lipid formulations of amphotericin B, pentamidine, miltefosine, and paromomycin. Unaffordable costs and poor supply of these drugs in countries where leishmaniasis is endemic limit access to the drugs for the majority of affected patients because of the widespread occurrence leishmaniasis predominantly among the poorest social strata. All of these drugs have disadvantages in terms of toxicity, efficacy, price, or treatment regimen (2–4). Given these downsides in the existing treatments for VL, a new orally available and inexpensive antileishmanial drug is needed. With the aim of developing new drugs, a series of compounds were designed and synthesized using the molecular hybridization of naphthoquinones and pterocarps (5–7). The pterocarpanquinone LQB-118, the lead compound from this class, has antiprotozoal and anti-leukemic cell line activities, even in cells with an multidrug resistance phenotype (8–10). We have also demonstrated that LQB-118 is effective in treating experimental cutaneous leishmaniasis via oral delivery (11, 12), and the death of Leishmania amazonensis parasites involved oxidative stress with hallmarks of apoptosis (13). These results suggest that LQB-118 is a promising lead compound and should be further investigated. Here, we report the effectiveness of LQB-118 in a murine model of the most severe form of the disease and describe the results of a detailed subacute toxicity study in mice.

MATERIALS AND METHODS

Chemicals. LQB-118 was synthesized in the Laboratory of Bioorganic Chemistry, Instituto de Pesquisas de Produtos Naturais Walter Mors, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil. The compound was prepared in two steps from commercially available lawsone and ortho-iodophenol, as previously described (7). Meglumine antimoniate (Glucantime; Sanofi-Aventis) was acquired commercially.

Animals. BALB/c and Swiss mice were obtained from the Fundação Oswaldo Cruz (Fiocruz) animal facilities (Rio de Janeiro, Brazil). Mice were housed at a maximum of 5 per cage and kept in a conventional room (23 ± 2°C and relative humidity of 60%, with 12-h light-dark cycles). The animals were provided with sterilized water and chow ad libitum. The
Institutional Animal Ethics Committees approved the use of both mouse types, as indicated below.

Subacute toxicity studies. (i) Ethics statement. Toxicological studies in Swiss mice were performed in accordance with protocols approved by the Ethics Committee for Animal Use of the Universidade do Estado do Rio de Janeiro (license number 007/2013, IBRAM-UEIR). (ii) Treatment protocol. The subacute toxicity study protocol was adapted from Guideline 407 of the Organization for Economic Cooperation and Development (OECD) (14). For this study, outbred Swiss mice were used because they have genetic variation similar to that found in human populations (15, 16).

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Six female Swiss mice per group (6 to 8 weeks old; 25 to 30 g body weight) were orally administered vehicle (2% dimethyl sulfoxide [DMSO] in saline) or 50 mg/kg of body weight/day LQB-118 for 23 consecutive days. Food and water intake during the treatment was measured by determining changes in amounts/volumes. A known amount of chow and water was freely offered. After 3 or 4 days, the chow and water were reassured, and the consumption levels of each experimental group was inferred based on the differences. The average of all measurements conducted during the entire period was considered for the toxicology analysis. The welfare and body weights of the mice were monitored daily during the treatment. At the end of the treatment, blood was collected to analyze biochemical and hematochemical parameters, and some organs were obtained for histopathological analysis.  

(iii) Biochemical parameters. The biochemical parameter testing was performed using Vitros 250 equipment (Ortho Clinical, Johnson & Johnson, Raritan, NJ, USA) using the dry chemistry methodology (17). The parameters evaluated were alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, total protein, albumin, globulin, alkaline phosphatase, creatinine, urea, uric acid, sodium, potassium, creatine kinase, calcium, iron, glucose, and cholesterol.  

(iv) Hematological parameters. Red blood cells (RBC), white blood cells (WBC), hematocrit (HCT), mean corpuscular volume (MCV), mean hemoglobin concentration (MHC), and mean corpuscular hemoglobin concentration (MCHC) were determined manually by routine hematological methods (18). The hemoglobin (Hb) concentration was determined as described by Van Kampen and Zijlstra (19). White blood cell differential counting was performed with May-Grunwald-Giemsa staining using light microscopy.  

(v) Anatomical and histopathological organ evaluation. Selected organs (e.g., spleen, liver, kidney, lung, esophagus, stomach, small intestine, heart, and thymus) were excised, trimmed of connective tissue and fat, weighed, fixed in 10% formalin, and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin and eosin (20).  

Parasites. For in vitro and in vivo experiments, parasites were isolated from female BALB/c mice infected with Leishmania infantum (strain MHOM/MA67ITMAP263) and cultured at 26°C in Schneider’s insect medium (Sigma-Aldrich) supplemented with 20% heat-inactivated fetal calf serum (HIFCS), 100 µg/ml of streptomycin, and 100 U/ml of penicillin.  

In vitro assays. (i) Antipromastigote assay. L. infantum promastigotes were cultivated in 96-well plates (Nunc, Roskilde, Denmark) in either the absence or presence of different LQB-118 concentrations (1.25, 2.5, 5, and 10 µM). The cultures were initiated with 1.0 × 10⁶ cells/ml and maintained at 26°C for 72 h. Inhibition of parasitic growth was determined with a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.  

(ii) Antiamastigote assay. Resident peritoneal macrophages were plated in RPMI medium (Sigma-Aldrich) at 2 × 10⁶/ml (0.4 ml/well) in Lab-Tek eight-chamber slides (Nunc) and incubated at 37°C in 5% CO₂ for 1 h. Stationary-phase L. infantum promastigotes were added at a 3:1 parasite/macrophage ratio for 4 h. After three washes, LQB-118 at various concentrations was added for 72 h. Next, the slides were stained using the Instant Prov hematological dye system (Newprov, Curitiba, Brazil). The number of amastigotes was determined by counting at least 100 macrophages per sample. The experiment was considered valid when at least 80% of the control macrophages were infected. The results were expressed as an infection index (II), calculated as follows: II = (percentage of infected cells) × (number of amastigotes/total number of macrophages). The control group had an index of infection of 245, with 81.2% ± 2.0% of macrophages infected. Concentration-effect curves were fitted for the antipromastigote and antiamastigote tests by using nonlinear regression analysis within the GraphPad program Prism 5.0, and the 50% inhibitory concentrations (IC₅₀s) were determined.  

In vivo assay. (i) Ethics statement. Studies in L. infantum-infected BALB/c mice were performed in accordance with protocols approved by the Ethics Committee for Animal Use of the Fiocruz (LW07/2010). (ii) Study design. To determine the course of infection of our model, 20 female BALB/c mice, 6 to 8 weeks old, were infected intraperitoneally with 1.0 × 10⁷ stationary-phase L. infantum promastigotes (21–24). At days 7, 14, 21, and 30, five animals were euthanized in a CO₂ chamber for parasite load determinations, as detailed below. For the efficacy study, female BALB/c mice, 6 to 8 weeks old, were infected as described above. After 7 days, the mice were randomly sorted into groups of five, with one control group of three uninfected animals (CNI). Treatment was administered through an orogastric tube (gavage) using 2% DMSO in saline as the vehicle (control infected group [CI]) or 2.5, 5, or 10 mg/kg/day LQB-118 in suspension. Another group was intraperitoneally treated with N-methylglucamine antimoniate (100 mg/kg/day of Sb⁺⁻) and was considered the drug control group. After 23 days of treatment, the animals were euthanized in a CO₂ chamber, and the organs of interest were aseptically removed, weighed, and homogenized in Schneider’s medium supplemented with 20% HIFCS. The parasitic load was estimated using a parasite-limiting dilution assay (25). Briefly, the resulting cell suspensions were serially diluted and evaluated with a limiting dilution analysis after 7 days. The number of parasites per organ was calculated as follows: (geometric mean of titer from triplicate cultures) × (reciprocal fraction of the homogenized organ added to the first well) × (organ weight [in milligrams]).  

Statistical analysis. Antipromastigote assays were repeated three times in triplicates. Antiamastigote assays were repeated three times in duplicates. Following the guidelines for reduction of animal use, in vivo assays were conducted once, with five or six mice per group, as indicated in each case. Significant differences between pairs of groups were assessed using Student’s t test with a significance level set at a P value of <0.05.  

RESULTS

Subacute toxicity. Repeated-dose toxicity studies in animals are an obligatory step to support the progression to clinical trials and the eventual marketing of drug candidates (26, 27). The procedure applied in this study was adapted from Protocol 407 of the OECD guidelines for the testing of chemicals (14).  

Clinical signs. The observation period was 23 days, with clinical evaluations conducted once per day, always at the same time, 30 min after administration, when a higher probability of side effects was expected. An observation was carried out after moving the cage to a suitable enclosure to ensure that variations in assay conditions were minimal. The evaluated signals included the following criteria: changes in the skin, hair appearance, eyes, mucous membranes, occurrence of secretions or autonomous activity (e.g., lacrimation and abnormal breathing), stool consistency, and urine color. Changes in gait, posture, and response to handling were also assessed. All of these parameters were compared with the control group. Mortality was assessed twice daily during the entire treatment period. All of the animals survived the treatment without presenting any of the following symptoms: hyper- or hypoactivity, aggression, unusual locomotion, catalepsy (i.e., the animal remains in any position in which it is placed), prostration, skin...
edema or redness, loss of body hair, piloerection, apnea, cyanosis, convulsions, salivation, lacrimation, corneal opacity, conjunctivitis, diarrhea, constipation, tremor, hypotonia, hypertonia, or red urine. Weight gain as well as food and water intake were also monitored. A slight but statistically insignificant deceleration in weight gain was observed following the 50-mg/kg treatment (Fig. 1), while no reduction in water or food consumption was observed (data not shown). A macroscopic analysis of the organs revealed an increase in the relative weights (organ weight/animal weight) of the spleen, lungs, and liver, as detailed in Table 1; however, the histological analysis indicated only minor differences in the liver.

**Hematological parameters.** With regard to hematological parameters (Table 2), slight leukopenia was observed after the 50-mg/kg/day treatment. For the blood differential counts, a reduction in neutrophils and an increase in lymphocytes were observed. No immature cells were observed. We did not identify any changes in the red blood cell count or in the hematimetric parameters.

**Biochemical parameters.** From the analysis of biochemical parameters (Table 2), it is important to note that the increase in the relative liver weight observed in the LQB-118-treated animals did not affect the following hepatic functional tests: ALT, AST, total bilirubin, total protein, albumin, globulin, or alkaline phosphatase. Similarly, there was no change in renal function, creatinine, blood urea nitrogen (BUN), uric acid, sodium, or potassium. Of the remaining parameters, which included creatinine kinase, calcium, iron, glucose, and cholesterol, only cholesterol levels were increased with the treatment.

**Histopathology.** When a compound is administered orally, the histopathological analysis of the different digestive tract segments is essential for a critical toxicology evaluation. Figure 2A and B show esophagus sections from the control and LQB-118-treated animals, respectively. The squamous keratinized epithelium, submucosa, and external muscle layer appeared normal in both groups. The gastric mucosa (Fig. 2C and D) and small intestine (Fig. 2E and F) were also assessed. Normal stomach corpus fundic glands (Fig. 2C) and small intestinal villi with intact microvilli membranes (Fig. 2E) were observed in the control group, and the LQB-118 treatment did not alter the gastric (Fig. 2D) or small intestinal (Fig. 2E) mucosal structure. Figures 2G and H depict the kidney histology of the control and the LQB-118-treated groups, respectively. Representative animals from both groups exhibited normal kidney histology with intact glomeruli and renal tubules. Figure 3 depicts the histology of vital organs, such as the lung and heart, and the immune system tissues. No histological differences were observed in these organs between the control and LQB-118-treated groups. Figures 3A and B show the alveoli structure, which consists of a single layer of epithelial cells.

### Table 1: Subacute toxicity based on organ weights

<table>
<thead>
<tr>
<th>Organ</th>
<th>Vehicle</th>
<th>LQB-118*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>4.03 ± 0.18</td>
<td>4.36 ± 0.08</td>
</tr>
<tr>
<td>Kidney</td>
<td>10.91 ± 0.34</td>
<td>11.41 ± 0.31</td>
</tr>
<tr>
<td>Liver</td>
<td>45.68 ± 1.11</td>
<td>51.83 ± 0.78**</td>
</tr>
<tr>
<td>Lung</td>
<td>4.75 ± 0.10</td>
<td>5.07 ± 0.09*</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.26 ± 0.28</td>
<td>4.99 ± 0.16*</td>
</tr>
<tr>
<td>Stomach</td>
<td>19.24 ± 1.01</td>
<td>18.84 ± 1.59</td>
</tr>
<tr>
<td>Thymus</td>
<td>2.51 ± 0.14</td>
<td>2.47 ± 0.26</td>
</tr>
</tbody>
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*Relative organ weight was calculated as the organ weight divided by the body weight (reported in milligrams [organ weight] per gram [body weight]). Data shown are means ± standard errors. *, P < 0.05; **, P < 0.01 (Student’s t test).

**Figures 1** Subacute toxicity based on body weight evaluation. Female Swiss mice (six per group) were treated for 23 days with vehicle or 50 mg/kg/day LQB-118. The weight gain of the animals and average consumption of water and food (insert) were monitored before and during treatment once a week. ns, not significant.

**Figures 2** Relative organ weight was calculated as the organ weight divided by the body weight (reported in milligrams [organ weight] per gram [body weight]). Data shown are means ± standard errors. *, P < 0.05; **, P < 0.01 (Student’s t test).

**Figures 3** The LQB-118 dose was 50 mg/kg. Asterisks indicate statistically significant differences between treatment and controls (*, P < 0.05; **, P < 0.01 [Student’s t test]).
in direct contact with capillaries in the control and LQB-118-treated animals, respectively. The heart histology of the control group animal (Fig. 3C) showed that the cardiac muscle cells were arranged in fibers that were similar in the treated animals (Fig. 3D). Figures 3E and F depict the thymus histology of the control and LQB-118-treated groups, respectively. Female Swiss mice (six per group) were treated for 23 days with vehicle or 50 mg/kg/day LQB-118. Magnification, ×200. Hematoxylin-eosin stain was used.

Preclinical Studies of LQB-118.

In vitro activity against *Leishmania infantum*. *L. infantum* promastigotes were incubated for 72 h with different LQB-118 concentrations. This compound demonstrated activity by inhibiting parasitic growth, with an IC$_{50}$ of 4.08 μM (see Fig. S1 in the supplemental material). Trivalent antimony (Sb$^{3+}$) was used as the reference drug for the promastigotes, as pentavalent derivatives are known to have poor activity on this form of the parasite, and this treatment produced an IC$_{50}$ of 8.2 μM. After demonstrating activity against the parasite found in the insect vector, we tested this compound against intracellular amastigotes, which are involved in the more clinically relevant form of leishmaniasis. For
this test, peritoneal macrophages from BALB/c mice were infected with *L. infantum* and incubated for 72 h with different concentrations of the compound. LQB-118 showed selective activity against intracellular amastigotes and a concentration-dependent decrease in the infection rate, with an IC₅₀ of 3.25 μM (9.98 μg/ml of Sb⁵⁺).

**Efficacy of LQB-118 against experimental visceral leishmaniasis.** Initially, to validate our model, we infected BALB/c mice and determined the parasite load in the spleen and liver after 7, 14, 21, and 30 days. We confirmed that the infection was already well established after 7 days, with parasites in the spleen and liver. The parasite burden increased and sustained the infection until the last determination at day 30 (see Fig. S2 in the supplemental material). Therefore, BALB/c mice were infected with *L. infantum* and treated as described in Materials and Methods. Regarding the organs from the infected and uninfected animals, we observed a significant increase in the relative weights (organ weight [in milligrams]/animal weight [in grams]) of the livers (Fig. 5A) and spleens (Fig. 5B) from the infected (CI) compared with uninfected animals (CNI), as expected. Treatment with 5 or 10 mg/kg/day LQB-118 inhibited the development of typical hepatomegaly, and the liver weights of infected animals were not significantly different from those of the uninfected animals (Fig. 5A). A similar splenomegaly effect was observed in the 10-mg/kg/day treated group (Fig. 5B). As expected, the effects of LQB-118 in controlling hepatomegaly and splenomegaly were accompanied by a dose-dependent reduction in the parasitic load of the liver (67, 88, and 100%) and spleen (38, 83, and 99%) at doses of 2.5, 5, or 10 mg/kg (Fig. 5C and D), respectively. From the suppression of the parasite load, it was possible to estimate the 50% effective dose (ED₅₀) and the ED₉₀, which are the doses capable of suppressing the parasitic load of amastigotes by 50% and 90%, respectively.

**FIG 4** Subacute toxicity based on histopathology of the liver of healthy mice treated with LQB-118. Images are of stained sections from the vehicle group (A and B) and LQB-118-treated group (C and D). Magnification, ×200 (A and B) or ×400 (C and D). Female Swiss mice (six per group) were treated for 23 days with vehicle or 50 mg/kg/day LQB-118. Hematoxylin-eosin stain was used.

**FIG 5** Treatment efficacy for experimental visceral leishmaniasis. Mice (five per group) were infected intraperitoneally with 1.0 × 10⁷ *L. infantum* promastigotes at the stationary phase. Seven days after infection, treatment with LQB-118 was initiated daily at three different doses. The drug control group (Sb⁵⁺) was treated with meglumine antimoniate (100 mg/kg/day) via the intraperitoneal route. The vehicle control group (CI) was treated with 2% dimethyl sulfoxide in saline via the oral route. A noninfected control group (CNI) was also evaluated. After 23 days of treatment, the relative weights of the liver (A) and spleen (B) were evaluated, and the parasitic load was evaluated in the liver (C) and spleen (D) via a limiting dilution assay. The suppression of parasite burden (insets in panels C and D) was used to calculate the ED₅₀ and ED₉₀ values. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ns, not significant.
burden by 50% and 90% (Fig. 5C and D, inserts), and the following values were calculated: for liver, ED_{50} of 2.1 ± 0.4 mg/kg/day and ED_{90} of 3.4 ± 0.7 mg/kg/day; for spleen, ED_{50} of 3.2 ± 0.5 mg/kg/day and ED_{90} of 5.6 ± 1.3 mg/kg/day.

**DISCUSSION**

Drug development is a lengthy and costly process that can be further prolonged for diseases, such as leishmaniasis, that affect developing countries. For more than 100 years, antimony has been present in the treatment of all clinical forms of leishmaniasis. Initially, antimony was used as a trivalent salt (28) or, beginning in the 1940s, as pentavalent complexes, such as sodium stiboglucoconate and N-methyl glucamine antimoniate (29). These two pentavalent antimonials remained the first-line treatments in numerous countries. Miltefosine and new formulations of amphotericin B represented a breakthrough for leishmaniasis treatment; however, the risk of emergence of resistant strains and the high cost of these new alternatives raised concerns regarding their widespread use in developing countries (30–33).

In an effort to find new orally active chemotherapeutics for VL, the most severe clinical manifestation of the disease, we describe here some preclinical studies in which we examined LQB-118, including its subacute toxicity, the in vitro antileishmanial activity, and the in vivo efficacy in murine VL. In previous studies, we determined the in vitro activity of LQB-118 against promastigotes and intracellular amastigotes of *L. amazonensis* and its effectiveness in controlling lesions of murine cutaneous leishmaniasis by intraperitoneal, subcutaneous, and oral routes. Furthermore, none of the main serological liver and renal toxicity markers (ALT/AST and creatinine, respectively) were altered after 82 days of LQB-118 oral treatment (11). We also have contributed in the understanding of the cell death mechanism that is induced by LQB-118. We reported that LQB-118 induces ROS production in *L. amazonensis* promastigotes a few minutes after incubation and it is sustained after 24 h, suggesting that this event is one of the parasite death triggers (13). Increased ROS levels were also found in *L. braziliensis* promastigotes treated with LQB-118 (12). This oxidative stress is accompanied by loss of the mitochondrial membrane potential in promastigotes. Furthermore, DNA fragmentation in intracellular amastigotes was observed for both mastigote forms without interfering with the nucleus of the cellular host (13).

To evaluate its potential drug properties, the adherence of *L. braziliensis* to Lipinski’s rule of five (Ro5) (34) and its in silico pharmacokinetic properties (35) were evaluated and compared with two drugs approved by the Food and Drug Administration (FDA) with quinone cores: doxorubicin and atovaquone (see Table S1 in the supplemental material). Theoretical analyses of the absorption, distribution, metabolism, excretion, and toxicity (ADMET) and Ro5 suggested a favorable profile for LQB-118, because it did not infringe the Ro5, in contrast to findings with doxorubicin (three violations) and atovaquone (one violation). Moreover, LQB-118 showed a probability of 100% human intestinal absorption (HIA). One pharmacological drawback is that the inhibition or induction of the enzymatic activity of the cytochrome P450 (CYP450) complex, which is responsible for the metabolism of several drugs, may result in toxicity or subtherapeutic drug concentrations (36).

An unfavorable point for LQB-118 that was indicated by the in silico analysis was a high probability of acting as a promiscuous inhibitor of CYP450 enzymes. If confirmed, this inhibition could increase the concentration of drugs that are metabolized to toxic levels by these enzymes if they are administered concomitantly with LQB-118 (see Table S1). The in silico analysis indicated LQB-118 in category II for acute oral toxicity (compounds with 50% lethal dose values greater than 50 mg/kg but less than 500 mg/kg).

The in vivo subacute toxicity analysis of this compound demonstrated that it did not induce clinical signs of toxicity. A histopathological analysis showed no toxicity in the gastrointestinal tract (e.g., esophagus, stomach, or small intestine). This is a very important finding, because the animals were orally treated for 23 days with LQB-118. Vital organs, such as the lungs, heart, and kidneys, of the LQB-118-treated animals did not exhibit any histological alterations. Previously, interstitial nephritis and severe glomerulosclerosis had been reported in dogs with visceral leishmaniasis treated with *N*-methyl glucamine antimoniate (37). Only the liver showed points of hepatocyte necrosis with leukocytic infiltration; however, because these were only focal changes, they were not accompanied by increased serum transaminase, alkaline phosphatase, or bilirubin levels. The total protein and albumin serum concentrations in the LQB-118-treated animals were also observed to be at normal levels. Different drugs with quinone cores that were approved by FDA, such as doxorubicin, exhibit some liver toxicity (38). However, unlike doxorubicin, which has a characteristic toxic effect on cardiac tissue (39), LQB-118 did not induce any changes in the hearts of the treated animals. Another significant change induced by LQB-118 treatment was a decrease in the peripheral blood total leukocyte levels, possibly due to the enlargement of the spleen, which might retain and store more cells; however, further studies are needed to confirm and assess the relevance of these findings, because the immune system organs (e.g., the thymus and spleen) of these animals did not exhibit any histopathological changes.

Corroborating the previous results for CL, LQB-118 was effective in the VL model because it reduced the parasitic load in the liver and spleen in a dose-dependent manner. Treatment was initiated after the establishment of the parasites in the liver and spleen on the seventh day after infection (see Fig. S2 in the supplemental material). This time of infection corresponds to the beginning of the midpoint of the acute phase of the disease (40, 41). By adopting this methodology, we found that after treatment with 10 mg/kg LQB-118, the spleen culture of one animal (20% of the group) and the liver culture of five animals (100% of the group) were negative, demonstrating a promising activity profile for this compound. The finding that no parasites could be rescued from the organs of some of the treated animals suggests that LQB-118 has the potential to induce a long-term cure; however, additional studies are needed to evaluate this hypothesis. This efficiency was comparable to that of intraperitoneal *N*-methylglucamine antimoniate (100 mg/kg/day of Sb^{III}) treatment, the first-choice drug for leishmaniasis. Interestingly, the reduction in the parasitic load by antimonal treatment was not matched by a reduction of the relative weight of the liver, perhaps because of the well-known hepatotoxicity of this drug (42). The response to the 10-mg/kg/day for 23 days of LQB-118 treatment by mouth (per os [p.o.]) was similar to that obtained with treatments with 20 mg/kg/day for 5 days (43) and 25 mg/kg/day for 5 days (44) miltefosine in a short-term infection model that demonstrated a mean efficiency greater than 85%. In conclusion, our findings indicate that LQB-118 is effective at treating different clinical forms of leishmaniasis and presents no relevant signs of...
toxicity at therapeutic doses; thus, our study demonstrates that this framework is suitable for development of promising drug candidates for oral treatment of leishmaniasis.

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REFERENCES

18. FDA Center for Drug Evaluation and Research and Center for Biologics Evaluation and Research. 1995. Guidance for industry: content and format of investigational new drug applications (INDs) for phase 1 studies of drugs, including well-characterized, therapeutic, biotechnology-derived products. FDA, Rockville, MD.


