Oral Efficacy of a Diselenide Compound Loaded in Nanostructured Lipid Carriers in a Murine Model of Visceral Leishmaniasis

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ABSTRACT: Leishmaniasis urgently needs new oral treatments, as it is one of the most important neglected tropical diseases that affects people with poor resources. The drug discovery pipeline for oral administration currently discards entities with poor aqueous solubility and permeability (class IV compounds in the Biopharmaceutical Classification System, BCS) such as the diselenide 2m, a trypanothione reductase (TR) inhibitor. This work was assisted by glyceryl palmitostearate and diethylene glycol monoethyl ether-based nanostructured lipid carriers (NLC) to render 2m bioavailable and effective after its oral administration. The loading of 2m in NLC drastically enhanced its intestinal permeability and provided plasmatic levels higher than its effective concentration (IC50). In L. infantum-infected BALB/c mice, 2m-NLC reduced the parasite burden in the spleen, liver, and bone marrow by at least 95% after 5 doses, demonstrating similar efficacy as intravenous Fungizone. Overall, compound 2m and its formulation merit further investigation as an oral treatment for visceral leishmaniasis.

KEYWORDS: diselenide, nanostructured lipid carriers, visceral leishmaniasis, L. infantum, oral treatment

Leishmaniasis comprises a group of parasitic diseases caused by protozoa of the genus Leishmania. Among the different clinical manifestations, visceral leishmaniasis (VL) is the most severe form of the disease, and it is principally caused by the species L. infantum and L. donovani. This parasite resides in host macrophages, mainly from liver, spleen, bone marrow, and lymph nodes, and it causes anemia, leukopenia, hepatosplenomegaly, hypoalbuminemia, weight loss, and ultimately death if untreated. 300,000 out of more than 1 million reported cases of leishmaniasis every year belong to VL, which is responsible for around 40,000 deaths.

The range of drugs available for the treatment of VL is limited, and it includes pentavalent antimonials, amphotericin B deoxycholate, lipid formulations of amphotericin B, miltefosine, and paromomycin. All of them have limitations in terms of toxicity, variable efficacy, price, and inconvenient treatment schedules. Moreover, they are parenteral drugs, with the exception of miltefosine, which is orally administered. Nevertheless, miltefosine includes several limitations such as gastrointestinal toxicity, teratogenicity, high cost, and long elimination half-life, leading to subtherapeutic levels over several weeks and facilitating the appearance of resistances. Due to the geographical distribution of the disease and the poor access to drugs for those people infected in low-middle income countries, it is a priority the search of new drugs and/or formulation strategies to be orally administered.

Although the combination therapy of drugs has improved the VL outcome of current therapeutic arsenal, the long-term goal is to identify new active compounds and develop an entirely new generation of oral drugs. Drug discovery algorithms for oral administration currently recommend discarding entities with poor aqueous solubility and/or permeability because they lead to low bioavailability and suboptimal efficacy. However, lipid-based systems have shown marked increase in the oral absorption of this type of compound. Among the different kinds of lipid systems, nanostructured lipid carriers (NLC) stand out over other types of nanocarriers because they offer many encouraging advantages. Compared with liposomes or nanoemulsions, NLC present higher stability in the physiological conditions found in the gastrointestinal tract. Moreover, they overcome...
the inherent disadvantages of their parent solid lipid nanoparticles (SLN), such as low drug payload and drug expulsion during storage.\(^1\)

In the past years, our group has reported the synthesis and in vitro antileishmanial activity of a series of selenocompounds.\(^2\) Two of these derivatives, \(N,N'-(4,4'-\text{diselanediyllbis}(4,1-\text{phenylene}))\)-bis-furan-2-carboxamide (2\(\text{h}\)) and \(N,N'-(4,4'-\text{diselanediyllbis}(4,1-\text{phenylene}))\)-bisnaphthamide (2\(\text{m}\)) (Figure 1), could be considered a great starting point in order to profile good hit compounds for leishmaniasis treatment, as they presented IC\(_{50}\) values lower than 3 \(\mu\)M against \(L.\) infantum amastigotes and selectivity indexes around 23 and 50, respectively (Table 1).\(^3\) Their mechanism of action has not yet been properly determined, although the increase in the intracellular thiol levels and the inhibition of the trypanothione reductase (TR) activity could implicate alterations in the parasite redox metabolism. TR is a validated target for the designing of antileishmanial drugs.\(^4\) It is essential for parasite survival, but it is absent in the host, in which TR is replaced by glutathione reductase.\(^5\) Moreover, antimonials currently in clinical use inhibit TR and evidence that TR is a druggable target. Although a large number of TR inhibitors have been identified by virtual and high-throughput screening,\(^6\) few of them have further progressed because of the need for strong and irreversible inhibition in the presence of endogenous substrates and suitable drug-like properties.\(^7\)

In this study, compounds 2\(\text{h}\) and 2\(\text{m}\) were put through the screening cascade for VL therapy.\(^8\) The in vitro absorption, distribution, metabolism, and excretion (ADME) properties to predict their oral bioavailability, namely, intestinal permeability, metabolic stability, and in vivo pharmacokinetics, were determined. Besides, to circumvent 2\(\text{h}\) and 2\(\text{m}\) poor water solubility and to enhance their oral permeability and bioavailability, their encapsulation in NLC was carried out. Finally, their efficacy was assayed in a murine model of established VL when administered orally.

**RESULTS**

We recently published the synthesis and in vitro antileishmanial activity of novel selenocompounds whose mechanism of action mainly involved parasite redox processes.\(^9\) Compounds 2\(\text{h}\) and 2\(\text{m}\) were chosen for further profiling because they exhibited the highest selectivity index, calculated as the ratio between the activity against intramacrophage parasites and the cytotoxicity against host macrophages (THP-1 cells) (Table 1), among the different synthesized compounds. Furthermore, both compounds fulfilled hit criteria in drug discovery for VL, as they showed an in vitro IC\(_{50}\) < 10 \(\mu\)M and good selectivity index.\(^10\)

**In Vitro ADME Properties for Compounds 2\(\text{h}\) and 2\(\text{m}\): Acceptable Metabolic Stability but Unsuitable for Oral Bioavailability.** Prior to in vivo studies, the in vitro ADME properties of 2\(\text{h}\) and 2\(\text{m}\) likely to be predictive of their oral bioavailability were measured. Both compounds exhibited low aqueous solubility in fasted simulated intestinal fluid (SSIF) and very poor ex vivo intestinal permeability (Table 2).

| Compound | Microsomal stability \(t_{1/2}\) (h) | \(C_{\text{I}50}\) (\(\mu\)g/ml) | Solubility in SSIF \(\mu\)g/ml | Intestinal permeability \(P_{app}\) \((10^{-6})\) cm/s |
|----------|---------------------------------|-----------------|-----------------|-----------------|
| 2\(\text{h}\) | 2.2 ± 0.8 | >50 | 14.0 ± 0.9 | 5.5 ± 0.1 |
| 2\(\text{m}\) | 1.0 ± 0.2 | >50 | 5.5 ± 0.1 | 5.5 ± 0.1 |

Values are the means ± SD of at least three independent measurements.

However, 2\(\text{h}\) and 2\(\text{m}\) showed acceptable metabolic stability in mouse liver microsomes, as indicated by their metabolism half-time \(t_{1/2}\) and intrinsic clearance \(C_{\text{I}50}\) values of 1.7 h and 24.9 mL/min−1·kg−1 for 2\(\text{h}\) and 1.2 h and 33.7 mL/min−1·kg−1 for 2\(\text{m}\), respectively (Table 2 and Figure S1). In general, compounds with \(t_{1/2}>1\) h are defined as stable in liver microsomal metabolism, and \(C_{\text{I}50}\) between 15 and 45 mL/min−1·kg−1 are indicators of intermediate clearance rate.\(^11\)

**Loading of Compounds 2\(\text{h}\) and 2\(\text{m}\) into NLC: Preparation and Characterization.** 2\(\text{h}\) (log\(P\) value of 1.19) and especially 2\(\text{m}\) (log\(P\) 5.19) are poorly water-soluble compounds and, thus, a priori suitable candidates for their loading into NLC.\(^12\) Moreover, these types of nanocarriers have widely demonstrated potential to increase solubility, oral bioavailability, and even lymphatic absorption of hydrophobic drugs.\(^13\) The poor solubility of 2\(\text{h}\) and 2\(\text{m}\) compounds in many common organic solvents and lipids restricted NLC fabrication and composition to the hot high-shear homogenization (HHSH) process with glyceryl palmitoesterate (Pecril) and diethylene glycol monoethyl ether (Transcutol) as solid and liquid lipids, respectively. Transcutol was the solvent that presented the highest solubilizing capability for 2\(\text{h}\) and 2\(\text{m}\) compounds, being the only one able to dissolve up to 6 mg of compound in 0.4 g. The composition of the optimized NLC is shown in Table 3.

Homogeneous NLC (polydispersity index, PDI < 0.2) with a mean size around 100 nm and high encapsulation efficiencies (between 85% and 95%) were obtained (Table 4) for both compounds. However, the payload (around 6 mg compound/g

**Table 1. Activity of 2\(\text{h}\) and 2\(\text{m}\) Compounds against L. infantum Amastigotes and TR, and Cytotoxicity on THP-1 Cells**

| Compound | L. infantum amastigotes IC\(_{50}\) (\(\mu\)M) | THP-1 cells CC\(_{50}\) (\(\mu\)M) | Selectivity index | TR inhibition IC\(_{50}\) (\(\mu\)M) |
|----------|---------------------------------|-----------------|-----------------|-----------------|
| 2\(\text{h}\) | 2.2 ± 0.8 | >50 | >20 | 14.0 ± 0.9 |
| 2\(\text{m}\) | 1.0 ± 0.2 | >50 | >50 | 5.5 ± 0.1 |

Values are the means ± SD of at least three independent measurements.

**Table 2. In Vitro ADME Properties Determined for Compounds 2\(\text{h}\) and 2\(\text{m}\)**

| Compound | Microsomal stability \(t_{1/2}\) (h) | \(C_{\text{I}50}\) (\(\mu\)g/ml) | Solubility in SSIF \(\mu\)g/ml | Intestinal permeability \(P_{app}\) \((10^{-6})\) cm/s |
|----------|---------------------------------|-----------------|-----------------|-----------------|
| 2\(\text{h}\) | 2.2 ± 0.8 | >50 | 14.0 ± 0.9 | 5.5 ± 0.1 |
| 2\(\text{m}\) | 1.0 ± 0.2 | >50 | 5.5 ± 0.1 | 5.5 ± 0.1 |

Values are the means ± SD of at least three independent measurements. *Concentration that inhibits parasite growth or TR activity by 50%. *Concentration that kills cells by 50%.

**Figure 1. Structures of compounds 2h and 2m selected for further studies.**

![Figure 1. Structures of compounds 2h and 2m selected for further studies.](https://doi.org/10.1021/acsinfecdis.1c00394)
Table 3. Composition of Aqueous NLC Dispersions Containing Compounds 2h and 2m

|        | Composition (% w/v) | 2h-NLC | 2m-NLC |
|--------|---------------------|---------|---------|
| Solid lipid | Precirol AT05      | 12      | 12      |
| Liquid lipid | Transcutol HP    | 5       | 5       |
| Surfactant   | Tween 80          | 20      | 20      |
| Compound 2h |                     | 0.093   | -       |
| Compound 2m |                     | -       | 0.089   |
| Water      |                     | 63      | 63      |

Table 4. Physicochemical Properties of Unloaded Aqueous NLC and 2h and 2m Loaded NLC Dispersions (2h-NLC and 2m-NLC)\(^a\)

| formulation | size (nm) | PDI\(^a\) | zeta potential (mV) | EE\(^c\) (\%) | DL\(^d\) (\mu g/mg lipids) |
|-------------|-----------|-----------|---------------------|---------------|-----------------------------|
| NLC         | 120 ± 12  | 0.2       | −17 ± 1             | −             | −                           |
| 2h-NLC      | 110 ± 21  | 0.2       | −14 ± 1             | 93 ± 4        | 0.56 ± 0.02                 |
| 2m-NLC      | 125 ± 33  | 0.2       | −16 ± 1             | 89 ± 4        | 0.54 ± 0.02                 |

\(^a\)Values are the means ± SD of at least three independent measurements. \(^b\)Polydispersity index. \(^c\)Encapsulation efficiency. \(^d\)Drug loading.

lipids, Table 4) could be considered as low. Negative zeta potential values of −14 mV and −16 mV were observed for 2h-NLC and 2m-NLC, respectively. Although they are below the critical zeta potential for stability in terms of purely electrostatic repulsions, the sterically stabilizing effect of polysorbate 80 (TWEEN 80) could explain the low PDI of the NLC suspensions. In fact, a relatively high concentration of TWEEN 80 was required to obtain monodisperse NLC of around 100 nm by the HHSH method with a total lipid concentration of 17%. In general, NLC sizes and PDI tended to increase with lipid concentration.

TG-DTA and X-ray diffraction studies were performed to analyze the degree of crystallinity, the possible polymorphic modifications, and the drug incorporation into the lipid matrix, as these parameters could modify their release profile and affect the stability of the system over time.\(^{29,30}\) Thermal curves and X-ray diffraction patterns of pure 2h, pure 2m, empty NLC, 2h-NLC, 2m-NLC, and their physical mixtures in equivalent concentration (0.3% w/w) as in the final optimized formulations are shown in Figure 2 and Figure 3. Thermal curves of pure 2h and 2m compounds showed melting endothermic peaks at approximately 222 and 252 °C, respectively. Empty NLC showed a sharp melting peak at 60 °C corresponding to Precirol. 2h-NLC (Figure 2a) and 2m-NLC (Figure 2b) formulations presented a similar profile to that of empty NLC. They did not show the characteristic melting peaks of the pure compounds. However, these endothermic peaks could be detected in their physical mixture with empty NLC, although with an increase in the case of compound 2h (from 222 °C for 2h to 245 °C in 2h + NLC physical blend).

Regarding X-ray diffraction studies, compound 2h exhibited two sharp peaks at 2θ of 20.5° and 24.8°, respectively. These peaks were still discernible in its physical mixture with empty NLC, whereas they were not present in the 2h-NLC formulation (Figure 3a). Besides, 2m displayed a crystalline structure with multiple peaks in its X-ray diffraction pattern. However, although these peaks were not found in the 2m-NLC formulation, peaks at 2θ of 12.5° and 20.5°, respectively, were still detected in the 2m and NLC physical mixture (Figure 3b).

Overall, the absence of peaks corresponding to the melting transitions of pure compounds or XRD patterns in 2m-NLC and 2m-NLC formulations (whereas some signals could be still visible in the corresponding physical mixtures) probably reflect the incorporation of the compounds into an amorphous or molecularly dispersed state inside the lipid matrix.

In order to prolong their storage, aqueous dispersions of NLC were lyophilized. Trehalose (at a ratio 1:1.5 to lipid) was suitable for protecting NLC of aggregation, as lyophilized NLC with this cryoprotector showed similar size, drug loading, and acceptable PDI (between 0.2 and 0.3).

**Slow Release Profile of Compounds 2h and 2m from NLC.** Figure 4 represents the release profile of 2h and 2m compounds from NLC as a function of time when incubated in simulated gastric fluid (SGF, during 2 h) and later in simulated intestinal fluid (SIF, during at least 4 h further) in order to mimic the oral administration. Both formulations exhibited a slow and incomplete release profile. 2h was released from NLC slightly faster than 2m, although the differences were not significant. About 5% of the compounds (5.5 ± 3.1% for 2h and 3.9 ± 0.3 for 2m) was released in the first 2 h of incubation in SGF (mean residence time in the stomach). Then, the slow release continued in SIF. After 4 h of incubation in SIF, NLC released around 13% and 10% of 2h and 2m, respectively. The rapid degradation of several types of
Precirol-based NLC has been previously described, although the correlation between degradation and drug release has not been properly evaluated. We could hypothesize that the degradation of NLC into mixed micelles solubilizing the drug and unable to cross the dialysis membrane may be responsible for the low amount of compound (around 15%), expressed as selenium (Se) content, released at the end of the study (24 h).

### Loading of compounds 2h and 2m into NLC Enhances Their Oral Permeability and Oral Bioavailability

Figure 5 represents the percentage of the dose of Se absorbed through jejunum portions in the Ussing chambers plotted against time. Either free or encapsulated into NLC, the absorption of compounds through the intestine was characterized by an average lag time of 1 h. However, the loading of 2h and 2m into NLC produced a higher increase in the intestinal permeability compared to free drugs. At the end of the study, the amount of Se in the receptor compartment (RC) for the nanoparticle formulations was 20 times higher than for the free compounds, with apparent permeability (P_app) values of $(7.67 \pm 1.85) \times 10^{-5}$ and $(6.44 \pm 1.08) \times 10^{-5}$ cm/s for 2h-NLC and 2m-NLC, respectively. Estimated P_app values for the free compounds were 10-fold lower, around $(7.17 \pm 2.62) \times 10^{-7}$ and $(5.91 \pm 1.08) \times 10^{-7}$ cm/s for 2h and 2m, respectively (Table 2). It is generally accepted that Caco-2 cell permeability coefficients higher than $>10^{-6}$ cm/s represent high permeability.$^{32}$

A single i.p. or p.o. dose of compounds 2h and 2m, either free or encapsulated in NLC, was administered to mice to estimate the oral bioavailability and, thus, the potential of these compounds as p.o. candidates for the treatment of VL. The dose of 4 mg/kg was selected after determining a LD50 value of 17.5 mg/kg for 2h and 2m, respectively, by the i.p. route. The main pharmacokinetic (PK) parameters, calculated by non-compartmental analysis, are summarized in Table 5. No Se above basal level was quantified after the p.o. administration of free selenocompounds. However, after the oral administration of NLC, Se plasma concentration was significantly higher than the values obtained after the i.p. administration of the solubilized free compounds. This increase was accompanied by a delay of the time of maximum concentration ($t_{max}$) and higher values of elimination half-times ($t_{1/2}$). Apparent clearance (Cl) values, after the p.o. administration, were referred to total administered dose because mice immediately died after i.v. bolus of the compounds. These Cl values allowed the determination of hepatic clearance rates (<0.3) suitable for further biological evaluation, except for i.p. 2m compound that presented an extraction ratio (ER) higher than 0.7 (Table 5). However, we must be very cautious with these PK parameters, because they are probably estimated from parent compounds and metabolites, as Se plasma levels and not the compounds were measured. Metabolism probably also explains the lack of correlation between predicted hepatic clearance from in vitro liver microsomal stability studies and in vivo data. In fact, both compounds either free (i.p.) or loaded into NLC (p.o.) displayed several peaks in the plasma Se concentration—time curve (Figure 6). After oral administration of NLC, the first
The peak concentration of Se in plasma was reached at 0.5 h. After the initial absorption phase, the mean plasma concentration of Se displayed the highest peak at 8 h for 2h-NLC and 2 h for 2m-NLC, followed by a slower decline. In any case, only following oral administration of 2m-NLC did the Se plasma concentration reach values above its in vitro intramacrophage amastigote activity (IC50 of 1 μM) (Table 1).

**p.o. Administration of 2m-NLC Showed Similar Efficacy as i.v. Fungizone in L. infantum-Infected Mice.** The short-term efficacy of the formulations was tested in a mice model of established VL infection 4 weeks after L. infantum stationary-parasites inoculation. Mice received 5 p.o. administrations of compounds loaded into NLC (2h-NLC and 2m-NLC) at a dose of 4 mg/kg every 2 days. Fungizone was used as positive control i.v. administered at 1 mg/kg during 10 consecutive days. As shown in Figure 7, 2m-NLC but not 2h-NLC p.o. reduced the parasite burden similarly to Fungizone. Healing rates of 96.9% in the liver (not significant, ns), 79.1% in the spleen, and 86.4% (p < 0.01) in the bone marrow for 2h-NLC treated mice and cure rates of 99.9% (p < 0.001) in the

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**Table 5. Pharmacokinetic Parameters of Compounds in BALB/c Mice after a Single i.p. (2h and 2m) or p.o. (2h-NLC and 2m-NLC) Administration at 4 mg/kg**

| parameter          | 2h i.p.            | 2h-NLC p.o.        | 2m i.p.            | 2m-NLC p.o.        |
|---------------------|--------------------|--------------------|--------------------|--------------------|
| AUC0–t (ng·h/mL)    | 1935.7 ± 737.2     | 14,915.0 ± 4325.8**| 353.7 ± 179.6      | 14,382.7 ± 2806.2***|
| Cmax (ng/mL)        | 443.0 ± 50.6       | 649.7 ± 146.7      | 115.3 ± 15.3       | 700.7 ± 262.2**    |
| tmax (h)            | 0.3 ± 0.0          | 3.9 ± 0.0          | 0.2 ± 0.0          | 0.3 ± 0.0          |
| t½ (h)              | 7.9 ± 2.9          | 29.1 ± 7.8*        | 3.2 ± 0.2          | 20.7 ± 11.9        |
| CI (mL/min/kg)      | 24.5 ± 10.9        | 3.1 ± 0.7          | 123.7 ± 42.8       | 4.1 ± 0.9***       |
| MRT (h)             | 10.9 ± 3.9         | 43.6 ± 10.2*       | 5.1 ± 0.3          | 23.6 ± 17.1        |
| ER (%)              | <0.3               | <0.3               | >0.7               | <0.3               |

**Data expressed as mean ± SD, n = 3. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0005, using a nonparametric U-Mann–Whitney test. bArea under the concentration–time curve. cMaximum plasma concentration. dTime of maximum concentration. eHalf-life. fClearance rate. Cl was apparent clearance estimated from the administered dose. gMean residence time. hExtraction ratio. Results analyzed by unpaired t test between free compounds and compounds loaded into NLC.**

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**Figure 6.** Plasma concentration–time curve of Se in BALB/c mice after a single i.p. or p.o. administration of compounds at 4 mg/kg either in their free form (2h and 2m, i.p.) or loaded into NLC (2h-NLC and 2m-NLC: p.o.). Data represents mean ± SD, n = 3.

**Figure 7.** Parasite burden in the liver (a), spleen (b), and bone marrow (BM, c) of BALB/c mice infected with L. infantum parasites and measured by the limiting dilution assay. Five p.o. administrations of unloaded NLC or compounds 2h and 2m loaded into NLC (2h-NLC and 2m-NLC) at a dose of 4 mg/kg were administered every other day for 10 days. Fungizone, used as positive control, was administered during 10 consecutive days by the i.v. route at 1 mg/kg. Parasite burden was also determined at the beginning of the treatment (4 weeks after infection, Control 4w) and in untreated mice run in parallel with treated ones (6 weeks after infection, Control 6w). *p < 0.05, **p < 0.01, ***p < 0.001 vs untreated mice (Control 6w), using a nonparametric Kruskal–Wallis test followed by Dunn’s multiple comparison.
liver, 98.5% (p < 0.001) in the spleen, and 94.8% (p < 0.05) in the bone marrow for 2m-NLC treated mice were found. On the other hand, unloaded NLC (around 12 mg of lipids/mice) did not shown any effect, probably because their principal ingredients were either nonabsorbed or digested to compounds without activity after their oral administration.31

Finally, it has been previously described that BALB/c mice are able to control the parasite burden in the liver by the formation of granulomas, whereas they succumb to parasite infection in the spleen,33 although the evolution time seemed to be affected by the experimental conditions.34 Four or six weeks after i.v. administration of 10⁸ stationary-phase promastigotes, untreated mice showed similar parasite burden in the liver (tendency to decrease, ns), spleen and bone marrow (Control 4w vs Control 6w in Figure 7). Thus, we outlined similar efficacy for the different treatments regardless of the control mice group used for comparison (4 or 6 weeks postinfection).

Preclinical Safety Profile. Compounds 2h and 2m Did Not Show Genotoxicity. The genotoxicity of compounds 2h and 2m was tested by the SOS/UMU test. Either with or without metabolic activation, compounds did not produce cytotoxicity and DNA damage at the concentrations evaluated (highest concentration of 1 mg/mL). The detailed results are summarized in the Supporting Information (Figure S2).

Repeated Dose Toxicity Study of Mice. Histopathological examination of liver, kidneys, stomach, and intestine was performed after 5 p.o. administrations every other day of 2m-NLC at 4 mg/kg. No signs of toxicity were found in the stomach and intestines of treated mice (Figure 8a,b,c). Kidneys of mice showed mild interstitial nephritis (Figure 8e). On the contrary, liver histopathology of 2m-NLC treated mice was normal (Figure 8d). Moreover, biochemical parameters after 5 p.o. administrations every other day at 25 mg/kg of 2m-NLC were normal with no differences when comparing to control mice (Table 6). These findings confirmed that 2m-NLC had a safety margin of at least 6-fold.

Table 6. Serum Biochemistry Evaluation for the Repeated-Dose Toxicity Study

|       | control | 2m-NLC |
|-------|---------|--------|
| ALB   | 3.6 ± 0.2 | 2.7 ± 0.4 |
| GLU   | 179.8 ± 15.8 | 237.9 ± 44.4 |
| AST   | 105.6 ± 14.5 | 116.7 ± 24.1 |
| ALT   | 77.5 ± 18.5 | 98.8 ± 67.2 |
| CHO   | 118 ± 2.1 | 128.7 ± 11.1 |
| CREA  | 0.17 ± 0.01 | 0.12 ± 0.06 |
| TP    | 4.8 ± 0.1 | 4.9 ± 0.4 |
| BUN   | 33.5 ± 8.5 | 36.7 ± 3.5 |

Mice were p.o. administered with 2m-NLC every other day for 10 consecutive days at 25 mg/kg. Values are the mean ± SD, n = 5.

Total protein. ²Blood urea nitrogen.

DISCUSSION

In spite of the great progress in proteomics, metabolism, and any omics technologies, phenotypic drug screening (PDS) still remains the most successful strategy for the identification of antileishmanial compounds,35 mostly due to the limited number of fully validated targets. This strategy allowed us to identify two selenocompounds (2h and 2m) with IC₅₀ values of 2 and 1 μM, respectively, against intramacrophage amastigotes and selectivity index higher than 20 and 50 vs THP-1 macrophages. In the current study, they were progressed for ADMET profiling. According to ADMET prediction,19 neither compound 2h nor 2m fulfilled Lipinski’s rule of 5. However, there are many approved drugs that are outside the cutoff,36 and this issue is especially clear for antimicrobials, probably due to their intracellular target.37 The ex vivo intestinal permeation assay (Figure 5) and in vivo PK study confirmed the null oral bioavailability of both selenocompounds. However, 2h and 2m can be categorized as stable and with an intermediate clearance according with experimentally determined t₁/₂ and calculated Cl₄₅⁸ respectively.
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every other day at a dose 6-fold higher than that administered
sign of toxicity after a regimen of 5 administrations of NLC
topical administration.46,47 In depth, it is frequently used in
samples of gastrointestinal tract, kidneys (Figures 8a
NLC (Table 5) con
(under the limit of detection). Therefore, the
detected after the administration of the free compounds
to allow their oral absorption, whereas no Se levels were
the enhancement of
permeation study (Figure 5) would indicate that, apart from
composition used in the current study also included a relatively
(no killing activity) was about 850
10 days, whereas the no observed adverse e
highest purity, can also be found in a number of oral
prescription drugs, although the Scientific Committee on
Consumer Products (SCCP) did not advise its use because
recent studies indicated side effects by this administration
route.49 However, the toxicity could be produced by impurities and
only at higher exposure levels than the ones used in the
in the current study.56 In fact, the amount of administered NLC
corresponded to 200 mg/kg of Transcutol every other day for
10 days, whereas the no observed adverse effect level
(NOAEL) was about 850–1000 mg/kg/day after a daily oral
administration for 90 days. On the other hand, the NLC
composition used in the current study also included a relatively
high proportion (20%, Table 3) of Tween 80 that would correlate with the administration of 0.8 g/kg per dose, which is
far from the NOAEL value of 10 g/kg/day, previously reported
in mice after its administration for 13 weeks.51 Histological samples of gastrointestinal tract, kidneys (Figures 8a–c), and
biochemistry of serum samples (Table 6) did not evidence any
sign of toxicity after a regimen of 5 administrations of NLC
every other day at a dose 6-fold higher than that administered in
the efficacy study.

The PK parameters obtained after the oral administration of
NLC (Table 5) confirmed the availability of these nanocarriers
to allow their oral absorption, whereas no Se levels were
detected after the administration of the free compounds
(under the limit of detection). Therefore, the ex vivo intestinal
permeation study (Figure 5) would indicate that, apart from the
enhancement of 2h and 2m solubility, NLC improved their oral absorption by the increase in the permeation rates.
Currently, the role of Transcutol as an oral absorption
promoter has not been investigated in detail yet. However, neither the in vivo release nor the ex vivo permeability methods
used in the current study were useful to accurately predict the
in vivo behavior and PK profile of NLC, which was higher and
faster. One hour after their administration, 50% of the administered dose was detected in plasma, whereas the
percentage of Se released did not reach 20% at short incubation times. Actually, there is lack of robust and biorelevant in vivo drug release methods, especially for
nanoparticle systems.52 Sink conditions are generally recom-
manded to mimic the removal of released or absorbed drug by
the systemic circulation; thus, a mixture of organic solvents
(2% v/v PEG 400, 5% w/v d-α-tocopherol polyethylene glycol
1000 succinate and 2.5% v/v DMSO) were used for this
purpose. However, permeability limitations of 2h and 2m
compounds and their probable enterohepatic circulation
complicated their PK profile and their correlation with the in vitro release studies.

Next, as the plasmatic Se level obtained after a single p.o.
administration of 2m-NLC formulation was higher than its
leishmanicidal IC50 value, an in vivo evaluation of its efficacy
was performed in a mice model of established VL after the
administration of 4 mg/kg. 2h-NLC p.o. administered were
also tested for comparison. The reduction of parasite burden in
the liver, spleen, and bone marrow of mice receiving 2m-NLC
was similar to that obtained after the i.v. administration of
Fungizone, whereas 2h-NLC significantly reduced the parasite
load in the liver and bone marrow but not in the spleen (Figure 7).

The higher efficacy of 2m-NLC vs 2h-NLC would be closely
associated with its higher in vitro intrinsic antileishmanial
activity (IC50 of 2 μM vs 1 μM for 2h and 2m, respectively,
Table 1) and better TR inhibition (Table 1), as their
formulation into NLC presented similar effects in the
permeability and oral bioavailability for both of them (Figure 5 and Figure 6). In depth, whereas Se plasmatic levels after the administration of 2h-NLC never reached the IC50 levels
(should be superior to 1000 μg/L), 2m-NLC reached its IC50
at around 8 h (Figure 6). However, it should be pointed out
that the quantification of the Se plasmatic levels by atomic
absorption spectroscopy did not allow the discrimination of
parent compounds and their metabolites. Moreover, plasma
concentration–time curves with several peaks were obtained for
all treatments (2h or 2m either free or loaded into NLC).
Although multiple peaking can occur as a consequence of
different mechanisms, the characteristic enterohepatic circu-
lation shown for organic Se compounds has been previously
described as a way to control their body levels53 and the most
common cause of several peaks in blood.54 However, a slower
lymphatic absorption, especially for 2m compound and its
formulation, cannot be discarded in view of its highly lipophilic character (logP of 5). Furthermore, we should quantify the
compounds (and/or their metabolites) in the main VL affected
organs (spleen, liver, and bone marrow), as the antileishmanial
efficacy of common drugs has been more closely correlated
with their organ accumulation than with their plasmatic
levels.55

Overall, the loading of the selenocompound 2m into NLC
was performed, and this formulation given orally showed
similar efficacy to i.v. Fungizone. Further studies are necessary
to elucidate its mechanism of hepatic clearance and perform
the identification of metabolites in order to optimize the
compound. Finally, we would also consider the formulation of
2m in simpler and cheaper lipid-based delivery systems (i.e.,
self-emulsifying drug delivery systems) for better translation
of an accessible medicine to low-resources countries.

CONCLUSION

Despite its good antileishmanial activity and selectivity index,
the BCS type IV compound 2m (low solubility and low
permeability) would be currently discarded for further
evaluation according with the most common decision
algorithm for oral drug discovery against leishmaniasis. NLC
drastically modified its drug-likeness properties and rendered it
bioavailable and effective after oral administration. This work is
a good example of the importance of introducing delivery
approaches in early drug discovery.
METHODS

Chemicals. Compounds 2h (N,N’-(4,4′-diselanediyldibis-(4,1-phenylene))bis-furan-2-carboxamide) and 2m (N,N’-(4,4′-diselanediyldibis(4,1-phenylene))bisnaphthalamide) were synthesized as previously described.19 Brieﬂy, compound 2h was obtained by the reduction of N-(4-selenocyanatophenyl)-furan-2-carboxamide with sodium borohydride in ethanol and stirring for 2 h. Compound 2m was synthesized from bis(4-amino phenyl)diselenediyldiethane and naphthyl chloride in dry chloroform with stirring for 24 h. For their puriﬁcation, 2h was recrystallized from ethanol, whereas 2m was only washed with diethyl ether. Both compounds were obtained with high yields (NADPH), glycerol, sodium phosphate dibasic (Na2HPO4), phosphate reduced tretra(cyclohexylammonium) salt (Transcutol HP) were kindly gifted by Gattefosse (Massachusetts, USA). Glycerol palmitoesterate (Precol ATOs) and high purity diethylene glycol monoethyl ether (Transcutol HP) were kindly gifted by Gattefosse (Madrid, Spain), Polysorbate 80 (Tween 80) was purchased from Panreac (Illinois, USA). L-Glutamine was purchased from Panreac (Illinois, USA). d-Glucose was purchased from VWR prolabo (Llinars del Vallés, Spain). D-α-Tocopherol polyethylene glycol 1000 (α-Tocopherol polyethylene glycol 1000 (ONPG) as a substrate. Genotoxicity activity was measured in terms of β-galactosidase activity calculating an induction factor (IF) relative to the negative control (i.e., bacteria exposed to the solvent) and positive controls (4-nitroquino line for normal treatments and 2-aminoantrenac for S9 metabolic activated treatments). A compound was considered genotoxic for IF values ≥ 2 at nontoxic concentration (i.e., bacterial survival ≥80%). The experiment was considered valid for positive controls if IF ≥ 2.

In Vitro Hepatic Microsomal Metabolism Studies. Liver microsomes were isolated as previously described with minor modiﬁcations.57 Mouse livers were removed and rinsed with 0.9% (w/v) NaCl solution. Then, livers were homogenized in ice-cold 0.25 M sucrose in 0.1 M PBS (pH 7.5). After 20 min of centrifugation at 9000 × g, the supernatant was ultracentrifuged at 100,000 × g for 1 h at 4 °C. The obtained microsome pellet was washed with 0.05 M PBS (pH 7.5) followed by another ultracentrifugation at 100,000 × g for 1 h at 4 °C. Microsomes were resuspended in 20% glycerol–0.1 M PBS and stored at −80 °C until use. Protein concentration was measured by microBCA kit (Thermo Scientiﬁc). For the metabolic assay, compounds were incubated with liver microsomes (0.5 mg protein/mL) for several time points. Stock solutions were prepared in DMSO, and the ﬁnal concentration in the mixtures did not exceed 1% (v/v). The tested compounds 2h and 2m, at a ﬁnal concentration of 500 μM, along with the microsomes were mixed in 0.05 M potassium phosphate buffer and incubated at 37 °C with continuous shaking in a ﬁnal volume of 300 μL. Reaction was initiated by adding NADPH (1 mM). Controls without hepatic cofactor and blanks without compounds were also analyzed. Metabolism was determined at 0, 0.1, 0.25, 0.5, 1, 1.5, and 2 h by the addition of an equal volume of ice-cold ACN, and samples were centrifuged at 9000 × g for 10 min. Supernatants were stored at −20 °C until high liquid performance chromatography (HPLC) quantitation analysis at 290 nm, as described in the Supporting Information. Results are expressed as mean ± SD of at least three replicates. In vitro half-time (t1/2, h) was established from the slope of linear regression of the percentage of remaining parent compound against time. The rate of microsomal intrinsic clearance (Clint,micr, mL·min−1·kg−1) was then calculated following eq 1:

\[
Cl_{int,micr} = \frac{\ln 2}{t_{1/2}} \times \frac{vol \text{ incubation}}{mg \text{ protein}}
\]

(1)

where \(t_{1/2}\) was the half-life of the tested compound (h), vol was the volume of incubation (0.3 mL), and mg was the amount of protein (0.15 mg).

The calculated Clint,micr was used for the prediction of in vivo intrinsic hepatic clearance (Clint) using eq 2 and suitable scaling factors:

\[
Cl_{int} = Cl_{int,micr} \times \left( \frac{mg \text{ microsome}}{g \text{ liver}} \times \left( \frac{\text{ liver mass (g)}}{\text{ body mass (kg)}} \right) \right)
\]

(2)

where the scaling factors were 45 mg of microsomal protein per gram of liver tissue and 40 g of liver tissue per kilogram of body weight (mice).

In Vitro Genotoxicity Studies. Salmonella typhimurium (TA1535/pSK1002 strain) (DSMZ, Germany) was employed to determine the genotoxic potential of the compounds 2h and 2m and some of their possible metabolites, as previously described.58 Bacteria were exposed to different concentrations of compounds either with or without metabolic activation (S9 mix, Mutazyme) to determine the bacterial survival percentage, calculated by measuring the absorbance at 600 nm. Afterward, the β-galactosidase activity was conducted using a colorimetric method employing o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. Genotoxicity activity was measured by the reduction of β-galactosidase activity calculating an induction factor (IF) relative to the negative control (i.e., bacteria exposed to the solvent) and positive controls (4-nitroquino line for normal treatments and 2-aminoantrenac for S9 metabolic activated treatments). A compound was considered genotoxic for IF values ≥ 2 at nontoxic concentration (i.e., bacterial survival ≥80%). The experiment was considered valid for positive controls if IF ≥ 2.

Parasites. L. infantum infective promastigotes (strain BCN-150) were maintained at 26 °C in a continuously stirred Schneider’s modiﬁed medium (Sigma) supplemented with 20% FBS and 40 mg/mL of gentamicin (Sigma). Parasites were grown until they reached the stationary phase. Then, they were washed twice with PBS and were harvested for inoculation in BALB/c mice. Parasite infectivity was maintained by constant passages in BALB/c mice.

Animals. Female BALB/c mice weighting between 20 and 25 g and female Wistar rats weighing 200–250 g (Harlan) were kept under normal conditions with free access to food and water. They were housed in groups of four or five in plastic cages in controlled environmental conditions (12/12 h light/dark cycle and 22 ± 2 °C). The study was conducted according to ethical standards approved by the Animal Ethics Committee of the University of Navarre in strict accordance with the European legislation in animal experiments (protocols code number 125-14E1, 127-14E1, and 100-19 approved by the Government of Navarra).

In Vitro Genotoxicity Studies. Salmonella typhimurium (TA1535/pSK1002 strain) (DSMZ, Germany) was employed to determine the genotoxic potential of the compounds 2h and 2m and some of their possible metabolites, as previously described.58 Bacteria were exposed to different concentrations of compounds either with or without metabolic activation (S9 mix, Mutazyme) to determine the bacterial survival percentage, calculated by measuring the absorbance at 600 nm. Afterward, the β-galactosidase activity was conducted using a colorimetric method employing o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. Genotoxicity activity was measured by the reduction of β-galactosidase activity calculating an induction factor (IF) relative to the negative control (i.e., bacteria exposed to the solvent) and positive controls (4-nitroquino line for normal treatments and 2-aminoantrenac for S9 metabolic activated treatments). A compound was considered genotoxic for IF values ≥ 2 at nontoxic concentration (i.e., bacterial survival ≥80%). The experiment was considered valid for positive controls if IF ≥ 2.
Solubility of Compounds 2h and 2m in Fasted Simulated Intestinal Fluid (FaSSIF). This experiment determines the solubility of a solid compound in fasted simulated intestinal fluid (FaSSIF) at pH 6.5 after 4 h of equilibration at r.t.10 In brief, 1 mL of FaSSIF (3 mM sodium taurocholate and 0.75 mM lecithin in sodium phosphate buffer at pH 6.5) was added to 2 mg of the solid compounds. The resulting suspensions were shaken at 900 rpm for 4 h at r.t. Then, they were centrifuged at 12,600 × g for 10 min. The amount of selenocompounds, as selenium (Se) content, remaining in the supernatant was quantified by graphite atomic absorption spectrometry, as described in the Supporting Information. Results are expressed as mean ± SD of at least three replicates.

Preparation of Nanostructured Lipid Carriers (NLC) Formulations. Compounds 2h and 2m were formulated in NLC. The preparation of NLC was made using a modification of a previously described hot high shear homogenization (HHSH) method.12 Briefly, 0.35 g of Precirol ATO5 was melted at a temperature 10 °C above its melting point and then mixed along with 0.15 g of Transcutol HP and 0.6 g of Tween 80. Then, 3 mg of the compounds 2h and 2m were added to the melted lipid mixture until complete solubilization. After that, 12 mL of purified water, previously heated at the same temperature, was added to the lipid phase and the mixture was homogenized using a high-shear laboratory mixer (Silverson SL2, UK) in the heated water bath at 12,300 rpm for 10 min to maintain the melting temperature of the lipids. The NLC dispersions were finally obtained by allowing the hot nanoeumulsion to cool in an ice bath with gentle agitation for 5 min. After 24 h, samples were purified by centrifugation at 1500 × g for 5 min and Sephadex G-25/PD-10 columns and concentrated until 3 mL by dialysis using PEG 20,000. The final dispersions were lyophilized (Lyobeta lyophilizer, Telstar) with 10% (w/v) of trehalose and stored at 4 °C until further use.

NLC Characterization. Particle Size, Polydispersity, and Surface Charge. The mean hydrodynamic diameter of the formulations and the zeta potential were determined before and after freeze-drying by photon correlation spectroscopy and electrophoretic laser Doppler anemometry, respectively, using a Zetasizer analyzer system (Malvern Instruments Ltd., Worcestershire, UK). The resuspension of freeze-dried NLC was performed in type I water with vigorous mechanical agitation. The diameter of the NLC and polydispersity index (PDI) were determined after dispersion in ultrapure water with 2% v/v PEG 400, 5% w/v D-α-tocopherol polyethylene glycol 1000 succinate and 2.5% v/v DMSO added to ensure sink conditions. The dialysis membranes (with 100 kDa pore size) were filled with 30 mg of lyophilized formulations either 2h-NLC or 2m-NLC (corresponding to around 60 μg of the selenocompounds) dispersed in 1 mL of distilled water. The loaded dialysis membranes were placed into a beaker containing 25 mL of SGF. The simulated fluid was kept under magnetic stirring at 150 rpm, and at fixed times 0.250 mL samples were withdrawn and replaced with an equal volume of SGF. Two hours after the incubation in SGF, the device was transferred to another beaker containing 25 mL of SIF magnetically stirred at 150 rpm. As previously, at fixed times, samples were withdrawn and replaced with free SIF. The in vitro release study finished 24 h after the experiment began. The amount of Se released from the nanocarriers was determined by graphite atomic absorption spectrometry, as described in the Supporting Information. Results are expressed as mean ± SD of at least three replicates.

Ex Vivo Intestinal Permeability Studies. Intestinal permeation experiments were performed by using the Ussing chamber method as previously described.39 Wistar female rats were fasted for 16 h with free access to water before the experiment. Rats were anesthetized with isoflurane and sacrificed by cervical dislocation. Jejunum was aseptically extracted, excised in 2 cm segments avoiding Peyer’s patches, and placed in cold 0.9% NaCl solution. Jejunum segments were anchored into the Ussing chambers. All experiments were realized from the apical side or donor compartment (DC) to the basolateral or receptor (RC) direction. For 2h and 2m compounds experiments, 5 mL of deionized water at pH 7.4 containing 4% (w/v) D-α-tocopherol polyethylene glycol 1000 succinate, and 5% (w/v) PEG 400 was added to both compartments to ensure sink conditions. In the case of NLC, 5 mL of PBS was used in both compartments. Chambers were continuously oxygenated and maintained at 37 °C. After 10 min of preincubation, the solution in the DC was replaced by 5 mL of either 2h and 2m solutions (final concentration of 5 mM) or their lyophilized NLC diluted in PBS at a 1 mM (in selenocompound concentration). Samples of 500 μL were
collected from the RC every 20 min up to 2 h and replaced by an equal volume of the RC solution. Samples were frozen at –20 °C until analysis. The membrane integrity was evaluated by the addition of yellow lucifer to the DC and the determination of its fluorescence at the end of the experiment. Experiments were carried out in triplicate for each compound and formulation. Se content in the RC was established by flame atomic absorption spectrometry, as explained in the Supporting Information. The apparent permeability coefficient per unit membrane surface area (P_app (cm/s)), expressed as mean ± SD, was calculated according to eq 3

\[ P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0} \]  

where \( dQ/dt \) was the steady-flux (mg/s), \( C_0 \) was the initial compound concentration in the DC (mg/mL), and \( A \) was the surface area of the membrane (cm²). It has been established that, in general, compounds with \( P_{app} > 1 \times 10^{-6} \text{ cm/s} \) are well-absorbed in the human intestinal tract whereas \( P_{app} < 1 \times 10^{-6} \text{ cm/s} \) are correlated with poor absorption.32

Toxicity Studies. Acute toxicity was carried out following the Up-and-Down procedure proposed by the OECD 425.67 Three mice were treated with a dose of 17.5 mg/kg of each compound, 2h and 2m, administered by i.p. route. If all the three mice died, 1/3 of the dose was administered to other new three mice. If all of them survived, the dose was tripled. If one or two of them died, the same dose was administered to one or two mice more. Both compounds were solubilized in PEG 400:H2O:α-tocopherol polyethylene glycol 1000 succinate (60:38:4:1.6, v/v/w).

For the 5-day repeated dose toxicity experiments, 2m-NLC were p.o. administered at 4 mg/kg every other day for 5 days. Two days after the last dose, animals were sacrificed, and liver and kidneys were fixed in 4% (w/v) paraformaldehyde for 24 h, embedded in paraffin, and stained with hematoxylin-eosin (Merck) for histological observation analysis. Furthermore, body weights of 2m-NLC treated mice were recorded prior to day 1 and prior to necropsy. Serum biochemistry was also analyzed after 2m-NLC p.o. administered at 25 mg/kg every other day for 5 days. Blood samples were collected 2 days after the last dose. For that, blood samples were kept at r.t. for 30 min and then centrifuged at 3500 × g for 10 min. Serum was harvested from each blood sample, and albumin (ALB), glucose (GLU), alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol (CHO), creatinine (CREA), total protein (TP), and blood urea nitrogen (BUN) were measured in a Cobas biochemistry analyzer (Roche, Basel, Switzerland). Serum levels of treated mice were compared to untreated ones. Results were expressed as mean ± SD (n = 5).

Pharmacokinetic Studies. A single-dose pharmacokinetic study of 2h, 2m, 2h-NLC, and 2m-NLC were evaluated in mice after i.p. (free drugs) or oral administration (free drugs and loaded into NLC). The dose (4 mg/kg) was selected taking into account LD50 obtained after i.p. administration of the compounds. Blood was collected from the submandibular plexus at different time points: 0, 0.25, 0.5, 1, 2, 4, 8, 24, and 48 h. Collected whole blood samples were centrifuged at 3000 × g for 10 min at 4 °C. The obtained plasma samples were stored at –80 °C until quantification of the drug by graphite atomic absorption spectroscopy, as described in the Supporting Information. Experiments were carried out in triplicate for each compound and formulation. Pharmacokinetic parameters were estimated by fitting the experimental data to a noncompartamental model (NCA) using PKsolver Excel program.62 The analyzed parameters were as follows: Area under concentration–time curve (AUC), maximum serum concentration (Cmax), clearance (CL), time of maximum concentration (tmax), mean residence time (MRT), and product half-life (t1/2). The extraction rate (ER) was calculated as the ratio between systemic blood clearance and mouse hepatic blood flow (considered 90 mL·min⁻¹·kg⁻¹). Extraction ratio can be generally classified as high (>0.7), intermediate (0.3–0.7), or low (<0.3), and these values are currently used for new chemical entities triaged during drug discovery.63

Efficacy Studies in L. infantum-Infected BALB/c Mice. BALB/c mice were i.v. infected with 10⁸ stationary-phase promastigotes of L. infantum in the tail vein. Four weeks after infection, mice were divided into the following groups: (i) negative control (PBS, p.o.), (ii) unloaded NLC (p.o.), (iii) 2h-NLC (4 mg/kg of 2h, p.o.), (iv) 2m-NLC (4 mg/kg of 2m, p.o.), and (v) positive control (1 mg/kg Fungizone, i.v.). Fungizone was daily administered for 10 consecutive days, whereas oral treatments were given every other day for 10 days (5 treatments).

Levels of parasite burden in liver, spleen, and bone marrow were determined at the beginning and the end of the treatments by limiting the dilution assay (LDA), as previously described.64 Briefly, the liver, spleen, and bone marrow samples perfused from the femur cavities of each mouse were individually homogenized in Schneider medium supplemented with 10% heat-inactivated FBS and antibiotics (100 U/mL of penillin, 100 µg/mL of streptomycin) and filtered through 40 µm cell strainers (Corning GmbH, Germany) to obtain a cell suspension. Cells were serially diluted (1/3) in 96-well flat-bottomed microtiter plates (Thermo Fischer Scientific) containing the same medium employed for homogenization (6 replicates). The number of viable parasites was calculated as the geometric mean of the titer obtained from 6 replicates cultures × reciprocal fraction of the homogenized organ added to the first well. The titer was the reciprocal value of the highest dilution at which promastigotes were observed with an inverted light microscope after 7 days of incubation at 26 °C. Results are indicated per mg of spleen and liver, or parasites per 1 × 10⁶ BM cells.65

Statistical Analysis. Statistical analyses between multiple groups were performed by using Kruskal–Wallis (non-parametric) followed by Dunn’s multiple comparisons tests. Differences between the two groups were analyzed by an unpaired t test. GraphPad Prism7 version (GraphPad Software, Inc., San Diego, CA, USA) was used to perform the analyses. Significance was established for a p value <0.05.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00394.

Detailed methodology of the lyophilization process and quantification of the two selenocompounds as well as results regarding the microsomal metabolism and genotoxicity (PDF)
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Notes
The authors declare no competing financial interest.

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