In Vitro and *In Vivo* Evaluation of an Adamantyl-Based Phenyl Sulfonyl Acetamide against Cutaneous Leishmaniasis Models of *Leishmania amazonensis*

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ABSTRACT Phenotypic assay against *Leishmania amazonensis in vitro* and *in vivo* led to identification of an adamantyl-based phenyl sulfonyl acetamide (compound 1) as a promising antileishmanial agent. Compound 1 inhibited the growth of intracellular forms of *L. amazonensis* (50% inhibitory concentration [IC_{50}] = 4 μM) and exhibited low toxicity to host cells, with a selectivity index (SI) of >125. However, in a cutaneous leishmaniasis (CL) mouse model, compound 1 did not reduce lesions and parasite load when administered as monotherapy or when given simultaneously with a suboptimal dose of miltefosine.

KEYWORDS cutaneous leishmaniasis, adamantyl, phenyl sulfonyl acetamide, experimental chemotherapy

utaneous leishmaniasis (CL) belongs to the leishmaniasis complex of diseases caused by over 20 different species of the kinetoplastid protozoan parasite *Leishmania*, which is transmitted through the bite of infected female sandflies. CL infections mostly lead to self-healing localized skin lesions (ulcers) but can also trigger disseminated ulcers and mucosa lesions (https://www.who.int/en/news-room/fact-sheets/detail/leishmaniasis). Approximately 1.2 million new cases of CL occur annually, leaving permanent scars, stigmas, and even serious disability, with huge social and public health impact (1). The current treatments for CL, namely pentavalent antimonials, amphotericin B, and miltefosine (Mt), have several drawbacks in terms of safety, drug resistance, cost, and efficacy, especially when used as monotherapies (2). CL is one of the neglected tropical diseases (NTDs), and its drug discovery and development pipeline remains sparse (3). There is a need for safer and more efficient oral and/or topical treatments for CL.

A series of natural product-inspired phenyl sulfonyl acetamides and acetates, as well as two pentacyclic triterpenoids (Fig. 1) previously investigated against *Trypanosoma brucei*, the causative agent of sleeping sickness (4, 5), were evaluated against *L. amazonensis*. Thus, the promising data on *T. brucei* prompted the present *in vitro* and *in vivo* investigation of compounds (numbered 1 to 23) on experimental models of *Leishmania amazonensis* infection (LTB0016 strain), one of the causative agents of CL. Statistical analysis was conducted in GraphPad Prism v.8.4.3 by ordinary one-way analysis of variance (ANOVA), Fisher's least significant difference (LSD) test, and unpaired *t* test; significance was set at a *P* value of <0.05 (95% confidence interval).

Initially, the compounds were screened using a colorimetric phenotypic assay (6) on extracellular amastigote forms (EF) of *L. amazonensis*. EF were purified from the skin foot paw lesions of male infected BALBc mice (7, 8). Compound 1 (Fig. 1) was active

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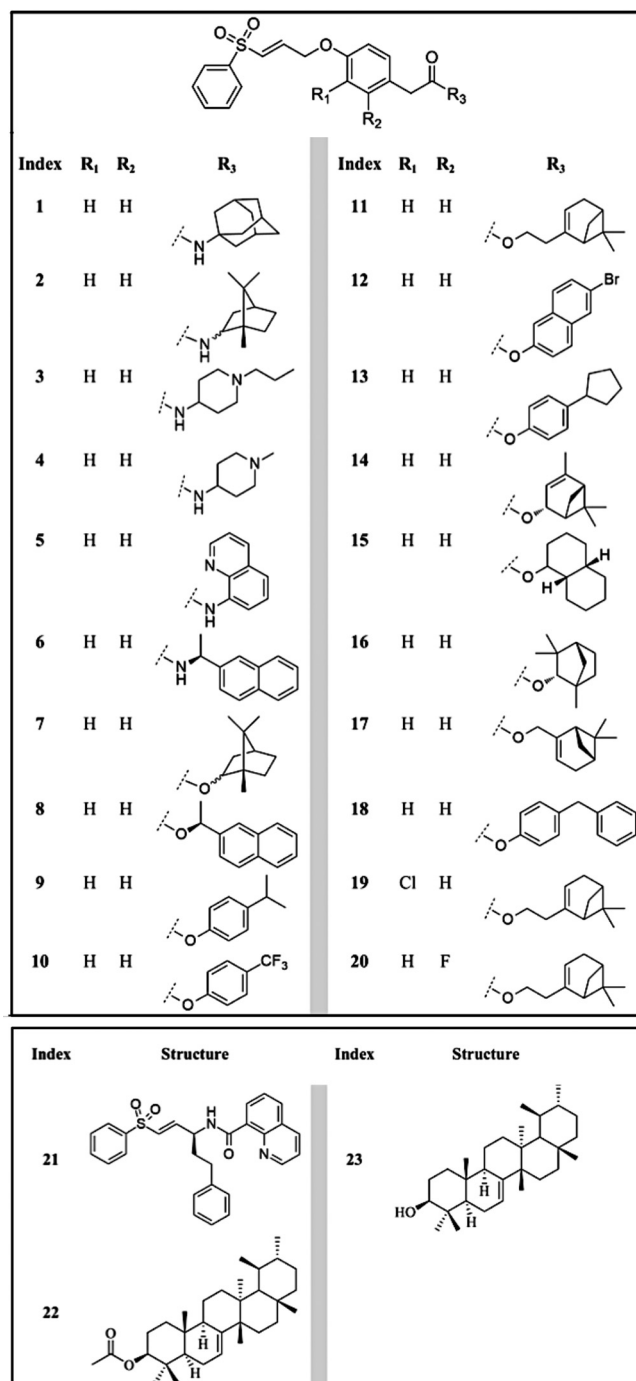
FIG 1 Chemical structures of tested compounds.

against EF (Fig. 2A). After 48 h of treatment, 5 μM compound 1 led to a 44% reduction in viable EF, and the 50% inhibitory concentration (IC_{50}) was determined to be 12.07 $\pm$ 1.78 μM (Fig. 2A). Due to this promising profile, compound 1 was further evaluated by light microscopy against intracellular forms (IF) localized in primary cultures of peritoneal macrophages (PMM) (9). The intracellular forms are the relevant forms in mammalian infections. Compound 1 (IC_{50} = 4.00 $\pm$ 1.79 μM) was about 3-fold more potent (P = 0.0367) against IF than miltefosine (IC_{50} = 13.3 $\pm$ 3.03 μM), with no observable toxicity against PMM up to 500 μM and with a selectivity index (SI) of >125 (Fig. 2A). The relatively high SI of compound 1 makes it a good hit compound for CL

A

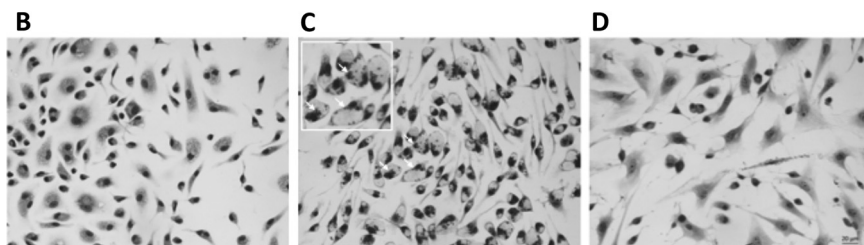| Compound	IC$_{50}$ EF	IC$_{50}$ IF	IC$_{50}$ PPM	SI EF	SI IF
1	12.07±1.78	4.00±1.79	>500	>41.42	>125
Miltefosine	4.37±0.11	13.3±3.03	169.69±5.07	38.83	12.76

FIG 2 (A) Compound 1 and miltefosine activity expressed as 50% inhibitory concentration (IC$_{50}$) on *L. amazonensis* (LTB0016 strain) after 48 h of drug exposure against extracellular amastigote forms purified from animal lesion (EF) and intracellular forms (IF) within primary cultures of peritoneal macrophages (PMM), host cell toxicity, and respective selectivity indexes (SI). (B to D) Light microscopy images of Giemsa-stained uninfected (B) and infected PMM (C) subjected or not to 10 μM compound 1 (D), demonstrating parasite sterilization due to drug exposure. Arrows indicate intracellular parasites.

(10). Moreover, analysis by light microscopy clearly demonstrate that compound 1 has a cidal rather than a static effect upon the intracellular forms, since it reduced not only the number of parasites per host cell but also the percentage of infected PMM, leading to cell culture sterilization (0% infection) at 10 μM (Fig. 2B and C).

The leishmanicidal activity of compound 1 and with its relatively low IC$_{90}$ (7.16 μM; IF) are desirable features to avoid parasitic relapses and drug resistance (11). According to the international target product profile recommended for novel drug candidates for leishmaniasis (10, 12), the sum of these phenotypic findings encouraged us to move compound 1 to LC mouse experimental models. *In vivo* assays were conducted using a CL mouse model by the left foot paw subcutaneous infection of BALBc male mice (18 to 20 g) with 2×10^5 amastigotes of *L. amazonensis* (BT0016 strain) as described by Feitosa and coworkers in 2019 (7) (full details are provided in the supplemental material). The drug treatment started at 15 days postinfection (dpi), on the onset of the lesions measuring 200 mm³, corresponding to a 3- to 4-mm diameter (3). Compound 1 at 10 mg/kg was given twice a day (b.i.d.), alone or in coadministration with a suboptimal dose of Milteforan (Mt) 4 mg/kg once a day (q.d.), for 14 days. As a positive control, Mt was administered at 40 mg/kg q.d. The animals were monitored daily, and the lesion size was regularly measured until the endpoint of 31 dpi. Thereafter, the animals were euthanized and their skin lesions collected for imprinting Giemsa analysis by light microscopy, as well as for parasite load quantification by quantitative PCR (qPCR), as reported previously (11, 13). All procedures were carried out in accordance with the guidelines established by the Fiocruz Committee of Ethics for the Use of Animals (CEUA L038/2017).

The *in vivo* infection led to a gradual increase in the size of the skin lesions of mice treated with the drug vehicle alone, which reached 438.8 ± 42.05 mm³ at the endpoint (Fig. 3A). Compound 1 given alone (10 mg/kg b.i.d. for 14 days) led to a mild increase (19%; $P = 0.0328$) in lesion sizes, with a mean value of 550.7 ± 23.15 mm³. When compound 1 was coadministered with a suboptimal dose of Mt (10 mg/kg of compound 1 plus 4 mg/kg of Mt, q.d. for 14 days), the lesions decreased by 20% ($P = 0.0934$). Mt alone at the optimal dose (40 mg/kg q.d. for 14 days) led to about a 72% significant reduction ($P < 0.0001$) in the size of the lesions, thereby reverting the clinical condition (Fig. 3A and B).

For qPCR, standard curves were constructed using DNA samples extracted from mouse skin fragments spiked with 10^6 amastigotes of *L. amazonensis*. Parasite load
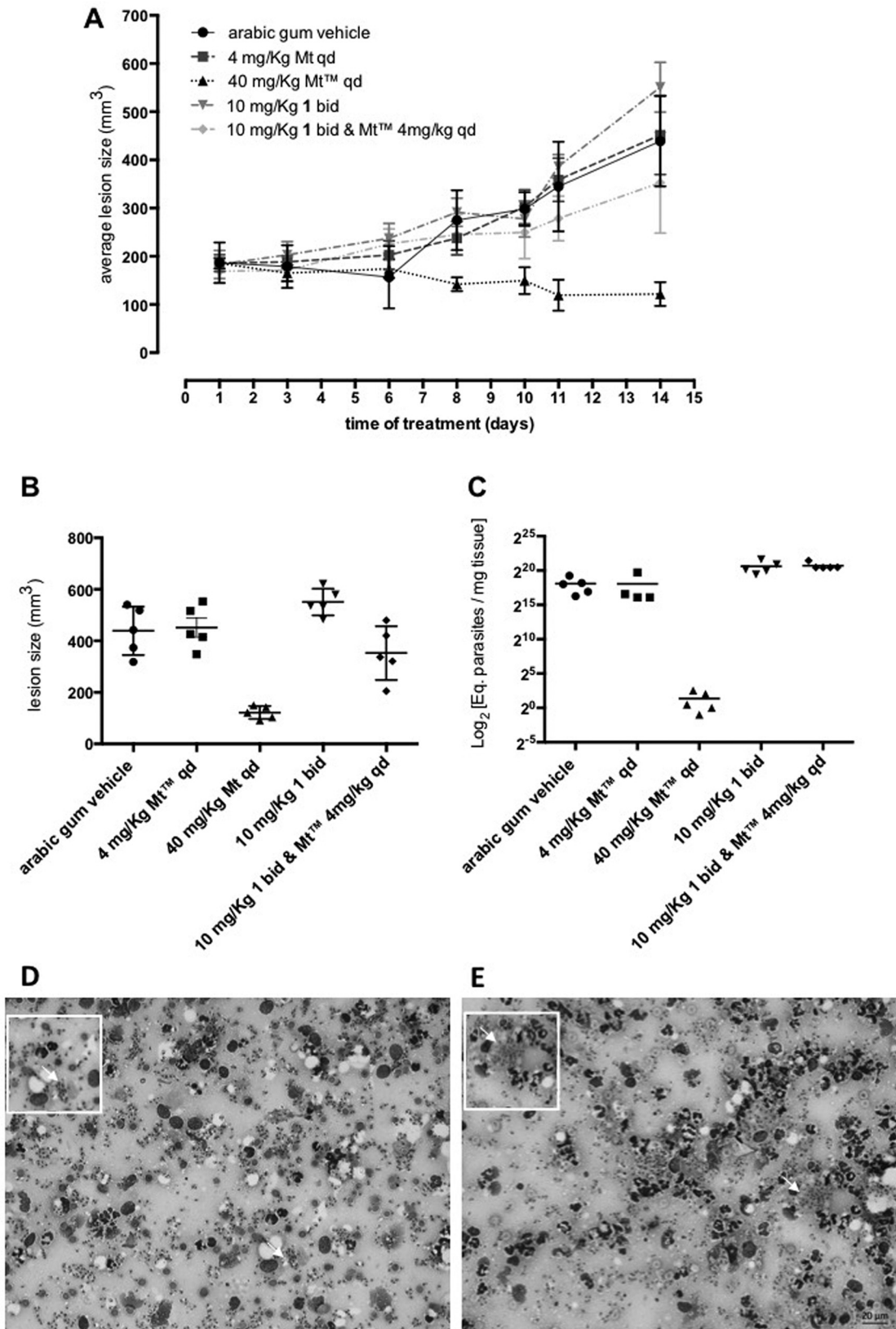
FIG 3 Activity of compound 1 in the *L. amazonensis*-BALBc model of CL. The graphics show average lesion size during treatment (A) and average lesion size (B) and parasite load by quantitative PCR (qPCR) at 31 days postinfection (C), according to each experimental group. Light microscopy of lesion imprints of infected mice treated with vehicle (D) and after oral (p.o.) administration of compound 1 at 10 mg/kg b.i.d. (E). Arrows indicates intracellular parasites.

expressed as equivalents of parasite DNA/mg tissue (eq par/mg tissue) showed a 93.2% efficiency for the target 18S rRNA gene in *Leishmania*, with a linearity coefficient of 0.98 (Fig. S1A). For the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) target, an efficiency of 91.4% was observed, with a linearity coefficient of 0.99 (Fig. S1B), confirming the sensitivity and accuracy of parasite detection and quantification (13). The molecular readout showed that mice treated with compound 1 alone (160,496 $\pm$ 45,419 eq par/mg tissue) or in coadministration with Mt (170,706 $\pm$ 28,584 eq par/mg tissue) led to an increase in parasite load (6- to 7-fold; $P = 0.0018$ and 0.0010, respectively) compared to that in the vehicle-treated group (27,753 $\pm$ 9,539 eq par/mg tissue) at 31 dpi (Fig. 3C). As expected, Mt given at 40 mg/kg suppressed parasite load (0.256 $\pm$ 0.102 eq par/mg tissue, 99.99%; $P = 0.00196$) in infected mice (8).

Analysis of lesion imprints on Giemsa-stained slides using light microscopy (Fig. 3D and E) showed that vehicle-treated mice (Fig. 3D) have numerous intracellular parasites (arrow) in macrophages besides the inflammatory diffuse infiltrates. Compound 1 lesions (Fig. 3E) also revealed a similar pattern, exhibiting high parasitemia, while the Mt group displayed only a few nonviable parasites. Our findings revealed that compound 1 has leishmanicidal activity *in vitro* but lacks *in vivo* efficacy under the treatment conditions used in this study. The lack of efficacy is potentially tied to compound 1's lipophilicity. Its relatively high lipophilicity (cLogP of 4.3) actually impaired its use at a higher dose such as the one used for the reference drug Mt (40 mg/kg). Drugs in clinical use for leishmaniasis generally have high aqueous solubility. Thus, ongoing work on analogues of compound 1 is aimed at improving aqueous solubility and using absorption, distribution, metabolism, and excretion (ADME) experiments to reduce obvious metabolic liabilities. Analogues of compound 1 with improved bioavailability could potentially contribute to the drug discovery and development pipeline of leishmaniasis.

Finally, although it did not reach successfully *in vivo* outcomes, our present phenotypic study brings novel knowledge to the field of drug discovery for LC, which aims to achieve a future delivery of new therapies for patients suffering from this disregarded neglected tropical disease.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

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