Mechanisms of Action of Substituted β-Amino Alkanols on Leishmania donovani

María Ángeles Abengózar,a Luis A. Busto,b,c Raquel García-Hernández,d Pilar Fernández de Palencia,a Ricardo Escarcena,b Santiago Castanys,b Esther del Olmo,b Francisco Gamarrod, Arturo San Feliciano,a,b Luis Rivasa

Centro de Investigaciones Biológicas (CIB-CSIC), Unidad Metabolómica, Interacciones y Bioanálisis (UMIB), Madrid, Spaina; Departamento de Química Farmacéutica, Facultad de Farmacia, CIETUS, IBSAL, Universidad de Salamanca, Salamanca, Spainb; Departamento de Ciencias Farmacéuticas, Facultad de Ciencias, Universidad Católica del Norte, Antofagasta, Chilec; Instituto de Parasitología y Biomedicina López-Neyra (IPBLN-CSIC), Granada, Spaind

Leishmaniasis is the protozoan disease second in importance for human health, superseded only by malaria; however, the options for chemotherapeutic treatment are increasingly limited due to drug resistance and toxicity. Under this perspective, a quest for new chemical compounds is urgently needed. An N-substituted 2-aminoalkan-1-ol scaffold has been shown to be a versatile scaffold for antiparasitic activity. Knowledge about its mechanism of action is still rather limited. In this work, we endeavored to define the leishmanicidal profile of such β-amino alkanol derivatives using a set of 15 N-mono- and disubstituted surrogates, tested on Leishmania donovani promastigotes and intracellular amastigotes. The best compound (compound 5), 2-ethylamino-dodecan-1-ol, had a 50% effective concentration (EC50) of 0.3 μM and a selectivity index of 72 for infected THP-1 cells and was selected for further elucidation of its leishmanicidal mechanism. It induced fast depletion of intracellular ATP content in promastigotes in the absence of vital dye intracellular entry, ruling out plasma membrane permeabilization as its origin. Confocal and transmission electron microscopy analyses showed that compound 5 induced severe mitochondrial swelling and vesiculation. Polarographic analysis using an oxygen electrode demonstrated that complex II of the respiratory chain (succinate reductase) was strongly inhibited by compound 5, identifying this complex as one of the primary targets. Furthermore, for other β-amino alkanols whose structures differed subtly from that of compound 5, plasma membrane permeabilization or interference with membrane traffic was also observed. In all, N-substituted β-amino alkanols were shown as appealing leishmanicidal candidates deserving further exploration.

The human protozoan Leishmania is the etiological agent of a variety of infections known collectively as leishmaniasis. Its severity depends on the infecting species and the immune status of the patient, ranging from the frequently mild cutaneous forms of the disease to the visceral syndrome, which is fatal if untreated. This disease is endemic in 98 countries; nearly 12 million people are infected worldwide, accounting for 50,000 deaths per year (1). To date, chemotherapy is the only treatment available (2) and is limited by the paucity of available drugs and rising resistance against pentavalent antimonials, the gold standard for treatment (9) constitute the core treatments for confronting this alarming situation. Nevertheless, in the long term, the development of new leads is mandatory in order to hasten their optimization and further implementation. β-Amino alkanols, chemically derived from α-amino acids by reduction, display considerable pharmacological versatility, having been successfully tested as anti-inflammatory, antitumor, antibiotic, or adjuvant agents, among others (10–13). Proof of concept for their use as leishmanicidal agents has been also reported (13–15). Nevertheless, aside from mitochondrial swelling and alterations in endocytic traffic, observed in African trypanosomes treated with some substituted β-amino alkanols (16), their mechanisms of action on trypanosomatids remain mostly unknown.

In this work, a selected set of 15 racemic 2-amino-1-alkanol compounds, varying in the main chain and substituent size with respect to those in previous works, were assayed for leishmanicidal activity against Leishmania donovani parasites and for toxicity on mammalian cells. In addition, we described the mechanism of lethality for compound 5, which had the highest selectivity index. This amino alkanol impaired the bioenergetic metabolism of the parasite through inhibition of the succinate reductase complex of the mitochondrial respiratory chain. Most interestingly, other analogs with slight structural modifications had different effects, such as membrane traffic impairment or severe structural damage. Thus, unexpectedly, the leishmanicidal mechanisms of these compounds are dependent on subtle variations of the alkyl substituent attached to the β-amino alkanol structure.

TABLE 1 Structure, leishmanicidal activity, and toxicity of the β-amino alkanol compounds

<table>
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<tr>
<th>Compound no. or name</th>
<th>β-Amino alkanol*</th>
<th>Structure</th>
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<th>No.</th>
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<th>Promastigotes</th>
<th>EC50 b,c</th>
<th>SI d</th>
<th>IC90c</th>
<th>Amastigotes</th>
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* Abbreviations: AmB, amphotericin B; Bn, benzyl; Et, ethyl; Bu, butyl; n-Hex, n-hexyl; No., the number of methylene groups of the alkyl substituent at C-1 of amino alkanols.

b Data are expressed as μM (mean ± SD).

c Calculated from three independent determinations.

d SI, selectivity index (EC50 THP-1 cells/EC50 parasites).

e IC90, concentration of the compound causing 90% inhibition of MTT reduction, determined in defined medium (HBSS-Glc) after 4 h of incubation.

f Values in bold correspond to the compounds selected for study of their leishmanicidal mechanism.

MATERIALS AND METHODS
β-Amino alkanol synthesis and characterization. The chemical structures of the β-amino alkanols used in this work are compiled in Table 1. Compounds were prepared according to a previously reported method (13) and confirmed through 1H and 13C nuclear magnetic resonance (NMR), mass spectrometry (MS), and infrared (IR) spectral analysis. The procedures for synthesizing compounds 5, 8, and 9, their physical and spectroscopic parameters, and their deduced pharmacological properties are included in the supplemental material.

Parasite strains. Leishmania donovani strain MHOM/SD/00/1S-2D (kindly provided by S. Turco, School of Medicine, University of Kentucky, Lexington) and its derived 3-Luc strain, with episomal expression of a cytoplasmic form of luciferase mutated at its C-terminal tripeptide to avoid import into the glycosome, were grown as described previously (17). L. donovani strain MHOM/ET/67/HU3, with a luciferase gene integrated into the parasite genome, was grown at 28°C in modified RPMI 1640 medium (Invitrogen), supplemented with 20% heat-inactivated fetal bovine serum (HIFBS) and 100 μg/ml of hygromycin B to prevent spontaneous gene deletion (Invitrogen) (R. García-Hernández, V. Gómez-Pérez, S. Castany, and F. Gamarro, unpublished data).

Human myelomonocytic cell line THP-1 culture and determination of cellular toxicity. THP-1 cells were grown at 37°C and 5% CO2 in RPMI 1640 medium supplemented with 10% HIFBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. When required, cells were seeded in 96-microwell plates (3 × 105 cells/well) and differentiated to macrophages in the presence of 20 ng/ml phorbol myristate acetate for 48 h, followed by 24 h of culture in fresh medium (18). The cellular toxicity of the compounds was determined by inhibition of the colorimetric reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (19).

Leishmanicidal activity of β-amino alkanols. The leishmanicidal activity of the compounds on promastigotes was determined as described previously (20). Briefly, parasites were harvested at the late exponential phase of growth, washed, and resuspended in fresh growth medium (2 × 106 cells/ml) with the respective drug. The parasite proliferation was measured by MTT reduction. Promastigotes were allowed to proliferate for 72 h at 26°C, MTT (0.5 mg/ml, final concentration) was added, and the mixture was incubated for 2 h. Then, formazan was solubilized in SDS (5% [wt/vol], final concentration) and absorbance at 590 nm was determined with a 610 Bio-Rad plate reader as described previously (20). The 50% effective concentration (EC50) values were used to evaluate this parameter. Samples were tested in triplicate, and experiments were repeated at least twice.

The inhibition of MTT reduction by β-amino alkanols was also determined for parasites in defined medium as required for the determination of parameters associated to their mechanisms of action. Briefly, parasites harvested at the late exponential phase were washed in Hanks’ buffer supplemented with glucose (HBSS-Glc) (137 mM NaCl, 5.3 mM KCl, 0.4 mM KH2PO4, 4.2 mM NaHCO3, 0.4 mM Na2HPO4, 10 mM d-glucose [pH 7.2]), resuspended in the same medium at 2 × 107 cells/ml (final concentration), and dispensed into a 96-microwell plate (100 μl/well, final volume). Compounds were added, and parasites were incubated for 4 h and immediately assayed for MTT reduction as described above. These conditions were considered standard for the rest of the experiments and maintained unless otherwise stated. The 90% inhibitory concentration (IC90) for the reduction of MTT was determined for this purpose.
To assess the susceptibility of intracellular *L. donovani* amastigotes, THP-1 cells differentiated into macrophages were infected with stationary promastigotes of *L. donovani* MHOM/ET/67/HU3, a strain with a luciferase gene incorporated into its genome, at a macropage/parasite ratio of 1:10. The infected cells were maintained at 37°C with 5% CO₂ and challenged with the respective concentration of the compounds in RPMI 1640 medium plus 10% HIFBS. After 72 h of incubation, the luminescence was measured using the Promega kit luciferase assay system.

The statistical parameters, EC₅₀ and ICₙ₀, were calculated using the statistical utilities of SigmaPlot software, version 11.0.

**Plasma membrane permeabilization by β-amino alkanols on promastigotes.** The permeabilization of the plasma membrane of *L. donovani* promastigotes by selected compounds was measured by the entrance of the vital dye Sytox Green (molecular weight [MW] of 600) (Invitrogen). Briefly, promastigotes were resuspended in HBSS-Glc buffer at standard conditions, except for the additional presence of Sytox Green (1.0 μM, final concentration), and dispensed into a black 96-microwell plate (100 μl/well). Once a stable readout was obtained, the drug was added and the increase in fluorescence (excitation wavelength [λex] of 504 nm and emission wavelength [λem] of 524 nm), caused by the binding of the dye to the intracellular nucleic acids, was measured in a BMG POLARstar Galaxy microwell reader (Öffenburg, Germany). Results are expressed as the percentage of fluorescence relative to that of full permeabilized cells, achieved by the addition of 0.1% Triton X-100 (TX-100) (20). The same procedure was applied to measure plasma membrane permeabilization in THP-1 cells, once differentiated into macrophages. To assess the parasite permeability after longer incubation times, promastigotes were incubated with the drug for 4 h, and Sytox Green was added at the end of the experiment (1 μM, final concentration). The stable fluorescence readout, reached in 5 min, was taken for the calculations as above.

**Variation of ATP levels in 3-Luc *L. donovani* promastigotes.** Changes in the level of free cytoplasmic ATP in the living parasites caused by the compounds was monitored in the *L. donovani* 3-Luc strain, expressing an episomal cytoplasmic form of *Photinus pyralis* luciferase with a nonfunctional mutated glycoside import C-terminal tripeptide (21). Briefly, N-luciferin, 1-(4,5-dimethoxy-2-nitrophenyl) ethyl ester (DMNP-luciferin), a free membrane-permeable caged substrate for luciferase, was added to a 3-Luc promastigote suspension in HBSS-Glc (standard conditions) at a final concentration of 25 μM. The parasite suspension was immediately aliquoted (100 μl/well) into a black 96-well microplate. When the luminescence reached a plateau, compounds were added (t = 0) at their respective IC₉₀, and luminescence was monitored in a BMG POLARstar Galaxy microwell reader. Data are reported as the percentage of luminescence at the addition of the drug (t = 0), considered 100% luminescence. When required, total ATP content was measured using the CellTiter Glo luminescence assay (Promega) as described previously (22).

**Transmission electron microscopy of β-amino alkanol-treated *L. donovani* promastigotes.** Parasites were incubated with selected compounds for 4 h at their corresponding IC₉₀ in defined medium as above, collected by centrifugation, washed twice with 1 ml of HBSS, fixed in 5% (wt/vol) glutaraldehyde in the same medium, and included with 2.5% (wt/vol) OsO₄ for 1 h. Next, cells underwent a graded dehydration in ethanol (30, 50, 70, 90, and 100% [vol/vol]; 30 min each) with addition of propylene oxide (1 h), embedded in Epon 812 resin, and observed in a Jeol-1230 electron microscope (23).

**Changes in mitochondrial morphology by confocal microscopy imaging.** *L. donovani* promastigotes were loaded with 0.05 μM Mito-Tracker Red for 10 min at 26°C, washed twice, and incubated in the dark with compound 5 at 12 μM (IC₉₀) for 4 h at 26°C. Confocal fluorescence images were obtained on a Leica TCS-SP2-AOBS-UV ultraspectral confocal microscope (Leica Microsystems, Heidelberg, Germany). The fluorescence settings for MitoTracker Red were λex of 543 nm and λem of 599 nm.

**Determination of oxygen consumption rates in promastigotes.** Oxygen consumption rates were measured with a Clark oxygen electrode (Hansatech, Kings Lynn, United Kingdom) at 26°C, using 600 μl of promastigote suspension (10⁶ cells/ml) in respiration buffer (10 mM Tris-HCl, 125 mM sucrose, 65 mM KCl, 1 mM MgCl₂, 2.5 mM NaH₂PO₄, 0.3 mM EGTA [pH 7.2]) supplemented with 5 mM succinate and 1 mg/ml fatty acid-free bovine serum albumin, as described previously (24). Cells were permeabilized with 60 μM digitonin to allow selective permeation of the plasma membrane but not of the inner mitochondrial membrane (25), and then 100 μM ADP was added to restore state 3 respiration, followed by addition of amino alkanol 5 at 50 μM.

The inhibition site inside the respiratory chain for compound 5 was identified by the addition of specific inhibitors and substrates for the different complexes of the respiratory chain to the digitonized parasites (see Fig. 4) (26).

**DNA content analysis.** Parasites (4 × 10⁶ promastigotes/ml) were incubated with or without amino alkanol 5 at 0.1, 2.5, and 5 μM for 24, 48, and 72 h at 26°C in complete culture medium, washed twice with phosphate-buffered saline (PBS), fixed in ice-cold ethanol overnight at 4°C, and washed again twice with PBS. The promastigotes were resuspended in 500 μl of 20 μg/ml propidium iodide (PI) plus 3 mg/ml RNase A in PBS and incubated for 30 min in the dark at room temperature. The DNA content was analyzed by flow cytometry in an FC500 Coulter cytometer. The fluorescence settings were λex of 488 nm and λem of 620 nm (20).

**RESULTS**

Selection of the β-amino alkanols with the highest selectivity index. An initial set of 15 amino alkanols substituted with a variety of alkyl or benzyl groups were tested for inhibition of proliferation on *L. donovani* promastigotes and THP-1 cells. Only 3 out of the 15 compounds assayed (3, 10, and 25) showed EC₅₀ of >20 μM for *L. donovani* promastigotes. However, the EC₅₀ for THP-1 cells was nearly 100 μM for compound 9. The highest selectivity index (SI) values for promastigotes (SI = EC₅₀ for mammalian cells/EC₅₀ for *Leishmania* parasites) were 21.0, 14.2, and 6.7 for compounds 5, 8, and 9, respectively, which were accordingly selected as candidates for the remaining experiments.

The same β-amino alkanols (5, 8, and 9) were also assayed for inhibition of proliferation of *L. donovani* intracellular amastigotes in THP-1 cells. SI values (Table 1) ranged from 5.7 (compound 9) to 72.1 (compound 5), highlighting the effectiveness of compound 5 as a drug against *Leishmania*.

**Bioenergetic collapse of *L. donovani* promastigotes by β-amino alkanols.** Compounds 5, 8, and 9, with the highest selectivity for both forms of the parasite, together with other three additional compounds (4, 7, and 14), were assayed for their mechanisms of action. The variation in the cytoplasmatic free ATP level in living parasites, a parameter directly linked to the healthy status of the parasites, was assayed. Promastigotes of the 3-Luc strain were challenged with this set of β-amino alkanols as described in Materials and Methods. Under these conditions, the cytoplasmatic free ATP is the limiting substrate for luminescence output for these parasites (21).

Luminescence was inhibited by the β-amino alkanols selected, except for compounds 4 and 7, at their respective IC₉₀ in HBSS-Glc, only 10 min after their addition (Fig. 1A). Whereas compounds 9 and 14 caused initially a small and reversible decrease, reaching at the most 20% for compound 14, compound 8 fully inhibited luminescence in <10 min, challenging the full permeabilization achieved by TX-100. Compound 5 showed slower kinetics than compound 8, reaching 75% inhibition at the end of the observation period. CA(1-7)M(2-9), a membrane-active peptide on *Leishmania* (21), was in-
Structural damage to mastigotes as assessed by transmission electron microscopy.

Values were corrected for the spontaneous decay undergone by control parasites throughout the incubation (<10% of the initial value for the whole observation period).

To determine whether the origin of the decrease in ATP levels was either the inhibition of ATP synthesis or plasma membrane permeabilization, the latter was checked by the entrance of the vital dye Sytox Green into promastigotes, precluded in parasites with an intact membrane. At their respective IC₉₀s, only compound 8 induced full permeabilization of the parasite after 1 h of incubation (Fig. 1B). For longer times, parasites were incubated with the compounds at their respective IC₉₀s for 4 h; either Sytox Green or DMNP-luciferin was added at the end of the incubation, and the maximal readout was determined (Fig. 1C). Compound 8 showed the highest scores for both the inhibition of luminescence and the entrance of vital dye, similar to those for CA(1-7)M(2-9) and TX-100, two membrane-active compounds (21). Consequently, it was inferred that the loss of intracellular ATP caused by compound 8 was associated to plasma membrane permeabilization, whereas for the rest of the compounds, this parameter can not adequately account for this effect, as the decrease in luminescence was consistently higher than the Sytox Green entrance, especially for compounds 5, 9, and 14.

Additionally, the plasma membrane permeabilization of THP-1 by the three selected compounds was also assayed (see Fig. S1 in the supplemental material). Only compound 8 induced significant permeability at concentrations close to its EC₅₀, whereas for the β-amino alkanols assayed at 100 μM, only modest permeabilization was observed.

Ultrastructural effects of amino alkanols in L. donovani promastigotes as assessed by transmission electron microscopy. Structural damage to L. donovani promastigotes was assessed by transmission electron microscopy (TEM). Parasites were treated with the compounds for 4 h at their respective IC₉₀s and processed for TEM. The electron micrographs of promastigotes treated with β-amino alkanols for 4 h are shown in Fig. 2. While for compound 5 the most remarkable effect was a swollen mitochondrion, easily spotted by the presence of the kinetoplast, the pattern for compound 8 differed entirely in that the parasites appeared with a discontinuous plasma membrane, severe permeabilization, and massive loss of the cytoplasmic content. A strong vacuolization around the area of the flagellar pocket was observed for compound 9. The same morphological alterations caused by the three selected β-amino alkanols on L. donovani promastigotes were reproduced on Leishmania pifanoi axenic amastigotes at their respective IC₉₀s (see Fig. S2 in the supplemental material). These axenic amastigotes were selected because their morphological, antigenic, and metabolic homologies contrasted with those of amastigotes isolated from animal lesions for this strain (26). This allowed a straightforward interpretation of the direct effects of these compounds on the amastigote forms of the parasite, precluding

![FIG 1](http://aac.asm.org/)

**FIG 1** Assessment of plasma membrane permeabilization in L. donovani promastigotes by β-amino alkanols. Promastigotes were incubated under standard conditions with selected amino alkanols at equipotent concentrations corresponding to their IC₉₀s in HBSS-Glc. (A) Inhibition of luminescence (free cytoplasmic ATP) in 3-Luc L. donovani promastigotes after amino alkanol addition. Compounds were added at t = 0, and luminescence at this point was taken as 100%. Values were corrected for the spontaneous decay undergone by control parasites throughout the incubation (<10% of the initial value for the whole observation period). (B) Entrance of the vital dye Sytox Green after amino alkanol addition (t = 0). Fluorescence (λ_exc = 504 nm; λ_em = 524 nm) is expressed as a percentage of the value for parasites treated with 0.1% TX-100. Arrows represent for detergent addition. β-amino alkanols: compound 4; compound 5; compound 7, ∇; compound 8, ▽; compound 9, □; compound 14, ◇; 0.2 μM CA(1-7)M(2-9), ◇; 0.1% TX-100, ◆, control (untreated) parasites. ▲ (C) Assessment of bioenergetic collapse and plasma membrane permeabilization after 4 h of incubation with the amino alkanols. Values are expressed as percentages of fully permeabilized parasites, i.e., treated with 0.1% TX-100: ■, decrease of luminescence in 3-Luc promastigotes; □, increase in Sytox Green fluorescence. Experiments were repeated at least twice. Significant differences were determined using Student’s t test (*P < 0.01; **P < 0.001).

![FIG 2](http://aac.asm.org/)

**FIG 2** TEM of L. donovani promastigotes treated with β-amino alkanols 5, 8, and 9. Parasites were incubated with the amino alkanols at their respective IC₉₀s in defined medium (HBBS-Glc). Bar = 1 μm. Arrows point to the most remarkable feature of the phenotype of parasites treated with the respective β-amino alkanol: swollen mitochondrion (compound 5), severe permeabilization (compound 8), and highly vesiculated flagellar pocket (compound 9).
Addition of compound 5 (50 μM) halved the oxygen consumption rate, which was restored by tetramethyl-p-phenylenediamine (TMPD)-ascorbate addition, whereby electrons are directly fed into cytochrome c oxidase (complex IV) by the maintenance of a pool of reduced cytochrome c (Fig. 4A). Thus, inhibition was upstream of complex IV. Malonate, a competitive inhibitor for succinate at the level of complex II, blocked reduction of ubiquinone by succinate dehydrogenase. Under these conditions, electron flow came exclusively from the activity of the mitochondrial α-glycerophosphate dehydrogenase; the addition of α-glycerophosphate increased the respiratory rate, regardless of the previous treatment with compound 5 or no treatment (Fig. 4B and C). Based on these observations, we conclude that complex III remained functional, whereas complex II was inhibited by compound 5, although not entirely. When this compound was tested at higher concentrations, unspecific inhibition was obtained, probably caused by the detergent activity of the amino alkanols (data not shown).

When the cell cycle of *Leishmania* treated with compound 5 was observed, a modest increase in the sub-G1 population over time was found (see Fig. S4 in the supplemental material), as well as a marked rise in the G2/M population along with changes in ploidy, presumably preceding further evolution into the sub-G1 stage.

**DISCUSSION**

β-Amino alkanols have consistently demonstrated their usefulness as new lead compounds against protozoan diseases (12, 28, 29), including those caused by *Leishmania* spp. (15, 30) and *Trypanosoma brucei brucei* (31). Despite their potential antiprotozoal application, knowledge of their mechanisms of action on these organisms is limited and dispersed. Hypotheses based on their structural homology with other metabolites, e.g., interference of alkyl-substituted amino alkanols on sphingolipid metabolism, have been advanced (16). Clearly, a deeper insight into the targets of β-amino alkanols might boost the further chemotherapeutic development of these compounds.

We undertook the study of the leishmanicidal mechanism of action of 15 β-amino alkanols. According to Table 1, the best compounds have a free hydroxyl group, and their selectivity index
values were in an order opposite to that of their hydrophobicity values, although a minimal threshold value for the latter is needed, as compound 5 performs better than compound 1. Calculation of clogP, a parameter directly associated to hydrophobicity, gave values of 3.9, 5.8, and 7.6 for compounds 5, 8, and 9, respectively, in an order opposite to the selectivity index values for intracellular parasites (72.1, 38.7, and 5.7, respectively).

Compounds 5 and 9 showed a relatively small cytotoxic profile on THP-1 cells (Table 1). This is remarkable considering the antitumoral activity described for other β-amino alkanols that share some structural similarity with them (32). The smaller hydrophobicity of the former will likely account for this difference, stressing the tight dependence of biological activity on subtle structural variations of the substituents of the β-amino alkanol scaffold.

According to the bioenergetic collapse in parasites and their plasma membrane permeabilization by β-amino alkanols, two different models of leishmanicidal mechanisms were revealed: the decrease in luminescence observed for compound 5 agreed with mitochondrial dysfunction, leading to faulty synthesis of ATP, while for compound 8 membrane permeabilization contributed the most to its leishmanicidal mechanism. Compound 8 also permeabilized THP-1 cells at percentages closely matching those for the inhibition of MTT reduction in these cells, accounting for the cytotoxicity on mammalian cells (see Fig. S1 in the supplemental material). Its selectivity for biological targets will be mostly dependent on this activity; the protonation of its tertiary amino group at physiological pH will favor its interaction with the anionic Leishmania plasma membrane over the zwitterionic membrane from mammalian cells. In contrast, compounds 5 and 9 only induced weak intracellular dye accumulation, even at concentrations far surpassing their respective EC50s.

In this sense, the clogP values for compounds 8 and 5 matched their respective mechanisms; the higher clogP for compound 8 entails greater affinity for the membrane, promoting increased insertion and disruption of phospholipid packing, finally leading to entry of the vital dye. Nonetheless, clogP cannot be considered the sole descriptor for the leishmanicidal effect; compound 9, with a clogP of >8 but with the two alkyl chains of rather different lengths, induced limited uptake of the vital dye and significantly depleted ATP content only with longer incubations. This bell-shaped dependence with amphipathicity can be explained by the fact that the partition of the β-amino alkanol into biological membranes competes with micelle formation. As the critical micellar concentration decreases with longer alkyl chains, it precludes fast and massive insertion of amino alkanol monomers into the membrane, required to promote dye uptake through the lesions. The same dependence of membrane permeabilization on the length of the alkyl chain substituents was also observed for the membrane-active peptides acylated at their N termini with fatty acids of different chain lengths (33).

TEM scans showed that compound 9 differed from the trend expected from its high clogP. The treated parasites showed a strong intracellular vacuolization and the appearance of vesicles inside the flagellar pocket. This can be caused by interference in membrane traffic and mechanistically is intermediate between the two polarized patterns of compounds 5 and 8. Interestingly, other β-amino alkanols induced in T. b. brucei the phenotype known as “big eye” with the formation of a huge vacuole inside the parasite, which is associated to interference in endocytosis and hence in membrane traffic (16).

Mitochondria in trypanosomatids display a number of biochemical and genetic differences that support their druggability (34). This is especially relevant for Leishmania spp., as the largest contribution to their bioenergetic requirements comes from oxidative phosphorylation (ca. 70%) at the expense of glycolysis (35). Inhibition of the respiratory chain underlies the leishmanicidal mechanism for miltefosine (36), for paromomycin (37), and for some repurposed or brand-new drugs (38–40). In this sense, a swollen mitochondrion, as observed for compound 5, was described for leishmanicidal compounds killing through inhibition of the respiratory chain (27, 36, 40, 41). This is in agreement with its higher activity on amastigotes, a stage with a stronger reliance on mitochondrial metabolism than promastigotes (42).

This mitochondrial distress eventually led to an increase in the sub-G1 population. A similar process was described for drugs such as miltefosine (43) and curcumin (44), known to act on mitochondria. Even so, we cannot rule out completely the contribution of additional targets for compound 5, as other stimuli allegedly not acting directly on mitochondria (45–47) may also induce this phenotype and inhibition of succinate dehydrogenase was not complete under our assay conditions.

Polarographic studies pinpointed complex II as the main target for compound 5 inside the respiratory chain. Of note, the inhibition of complex II was reported to increase the susceptibility of L. donovani to paromomycin (48), in this era when multidrug-resistant parasites have been described (49). Because of the significant differences in the mechanisms of action caused by subtle structural modifications of the pharmaceutical scaffold, we cannot rule out other targets whose specific contributions to the final leishmanicidal outcome may vary.

In this regard, the previously proposed role of β-amino alkanols as pseudoanalogs for sphingosine is appealing (16). The presence of vesicles inside the flagellar pocket of Leishmania treated with compound 9 resembles the pattern observed for parasites defective in key enzymes of sphingolipid metabolism (50, 51). Also, the hypothetical blocking of sphingosine-1 kinase by β-amino alkanols may lead to an intracellular buildup of sphingosine and other sphingoid bases, toxic for the parasites (52), through changes in membrane fluidity and induction of interdomain discontinuities on the membrane, leading finally to membrane permeability (53, 54). Nevertheless, this scenario does not match either the massive membrane destruction carried out by compound 8 or the alteration in the bioenergetic metabolism by compound 5. A detailed lipidomic study of parasites treated with different amino alkanols may resolve some of these uncertainties.

Intracellular amastigotes were more susceptible to the selected β-amino alkanols than promastigotes. It is well documented that a straightforward extrapolation of the mechanism of drug action from one stage to the other is not always feasible. Metabolic retooling of the parasite to thrive in different environments such as the sandfly gut or the phagolysosome of the vertebrate host, together with the effects of the drug on the macrophage, underlies these differences, which may be highlighted in vivo, due to the plethora of immune mediators acting on the macrophage, including other β-amino alkanols, that enhanced nitric oxide production, an important leishmanicidal mechanism, in primed macrophages (13). As mentioned above, in terms of bioenergetics, amastigotes rely more on mitochondrial metabolism than promastigotes (42). From our results, we may surmise that the mechanisms described for the promastigote are rather preserved in-
tracellular amastigotes of this species. The selected compounds followed an identical ranking for activity on both stages in *L. donovani*, and TEM scans of axenic *L. pifanoi* amastigotes treated with the same compounds produced the same morphological alteration patterns as those observed for promastigotes.

In all, compound 5 showed the highest potential as a leishmanicidal agent. It fulfills the Lipinski rule of five (55) for considering a compound as a putative hit: clogP of 3.9 (<5), MW of 229.4 (<500), 2 (<10) hydrogen bond acceptors, 2 (<5) hydrogen bond donors, and the conditions used by the Drugs for Neglected Disease Initiative (DNDi) (EC₅₀ of < 1 µM, SI of >10) (16). Other theoretical, structure-derived parameters for skin absorption and toxicity (see the supplemental material) are encouraging enough to proceed with animal studies where the real potential of these compounds and their possible synergisms with immune mechanisms of the host might be revealed. Aside from its excellent *in vitro* selectivity index, the amphiphatic nature of compound 5 may favor its encapsulation and direct delivery into the site of infection, given the characteristics of *Leishmania* as an obligate macrophage intracellular parasite. Further studies along these lines are currently in progress.

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