Human antimicrobial peptide histatin 5 is a cell-penetrating peptide targeting mitochondrial ATP synthesis in *Leishmania*

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ABSTRACT **Histatin 5 (Hst5) is a human salivary antimicrobial peptide that targets fungal mitochondria. In the human parasitic protozoa *Leishmania*, the mitochondrial ATP production is essential, as it lacks the bioenergetic switch between glycolysis and oxidative phosphorylation described in some yeasts. On these premises, Hst5 activity was assayed on both stages of its life cycle, promastigotes and amastigotes (LC_{50}=7.3 and 14.4 μM, respectively). In a further step, its lethal mechanism was studied. The main conclusions drawn were as follows: *1*) Hst5 causes limited and temporary damage to the plasma membrane of the parasites, as assessed by electron microscopy, depolarization, and entrance of the vital dye SYTOX Green; *2*) Hst5 translocates into the cytoplasm of *Leishmania* in an achiral receptor-independent manner with accumulation into the mitochondrion, as shown by confocal microscopy; and *3*) Hst5 produces a bioenergetic collapse of the parasite, caused essentially by the decrease of mitochondrial ATP synthesis through inhibition of F_1F_0-ATPase, with subsequent fast ATP exhaustion. By using the Hst5 enantiomer, it was found that the key steps of its lethal mechanism involved no chiral recognition. Hst5 thus constitutes the first leishmanicidal peptide with a defined nonstereospecific intracellular target. The prospects of its development, by its own or as a carrier molecule for other leishmanicidal molecules, into a novel anti-*Leishmania* drug with a preferential subcellular accumulation are discussed.—Luque-Ortega, J. R., van't Hof, W., Veerman, E. C. I., Saugar, J. M., Rivas, L. The human antimicrobial peptide histatin 5 is a cell-penetrating peptide targeting mitochondrial ATP synthesis in *Leishmania*. *FASEB J.* 22, 1817–1828 (2008)**

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Eukaryotic antimicrobial peptides (AMPs) act as a deterrent barrier for the initial dissemination of invading pathogens in pluricellular organisms (1). Despite their wide range of primary and secondary structures, most of them share a strong cationic character and the adoption of amphipathic structures on their interaction with the plasma membrane of the pathogen (2). This promotes the initial electrostatic interaction with the anionic phospholipids exposed at the outer leaflet of the plasma membrane. This trait is exclusive of prokaryotes and lower eukaryotes, as in higher eukaryotes this class of phospholipids is confined to the cytoplasmic face of the bilayer, precluding their interaction with AMPs. For most AMPs, this initial interaction is ensued by massive accumulation and insertion into the plasma membrane, disrupting its integrity as a permeability barrier, leading to a fast and lethal disruption of internal homeostasis and eventually to the death of the pathogen (3). This scenario is however being increasingly challenged by other AMPs the lethal action of which is postulated to involve the recognition of intracellular targets (reviewed in ref. 4). These peptides would translocate across the plasma membrane, distorting its structure in a transient, nonlethal manner, and once inside the cell would recognize intracellular targets such as DNA (buforin), DnaK (drosocin and phyrrhocorrycin; ref. 4), mitochondria [histatin 5 (Hst5); ref. 5], and others that remain unknown (6).

The mitochondrion has preserved through evolution its primeval functionality as a potent energy generating organelle, although the molecular machinery in charge of its role shows a broad divergence among different phyla (7). The protozoan Kinetoplastida are, in evolutionary terms, the earliest branch of eukaryotes with a true functional mitochondrion (8). Among its members are the species of the genus *Leishmania,* many of which act as the causative agents of leishmaniasis, a devastating human disease with a wide variety of clinical manifestations (9). *Leishmania* spp. are digenetic protozoa, which cycle between the extracellular promastigote stage in the insect vector and the intracellular amastigote stage, responsible for the pathology in ver-

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tebrates, which dwells inside a parasitophorous vacuole in the mononuclear phagocytes (10). Chemotherapy is the only available method to fight this disease, and it is threatened by the rise of resistant parasites (9).

Aside from the clinical interest in the development of new leishmanicidal drugs, there are three additional reasons to explore the susceptibility of *Leishmania* to Hst5, a histidine-rich AMP present in the saliva of higher primates (11) that displays the highest candidacidal activity among human histatins (12). First, the fact that drugs with both antifungal and leishmanicidal activities, such as some phenalenones, affect mitochondria (13) suggests that the two microorganisms might share this target to a certain extent. Mitochondrial targeting in *Candida* spp., together with low cytotoxicity for mammalian cells, would thus make histatins attractive leishmanicidal drug candidates. Second, despite the above similarities, *Candida* and *Leishmania* have striking differences in bioenergetic metabolism. In *Leishmania*, it is based mainly on oxidative phosphorylation (14, 15), hence lacking the partial switch between fermentative and nonfermentative pathways of *Candida* (16). Finally, the *Leishmania* amastigote is adapted to thrive in acidic pHs (17) where protonation of the histidine residues of Hst5 enhances its cationic properties and, presumably, its antimicrobial action.

To gain some insight on the antimicrobial activity of Hst5 against microorganisms with the most primitive functional mitochondria, as well as to explore the feasibility of Hst5 as a potential leishmanicidal lead, we have studied the activity of Hst5 and its synthetic analog Dhvar4 on the different forms of the parasite. The all-D form of Hst5 (D-Hst5) was also assayed to explore chiral requirements in the key steps of the lethal mechanism of L-Hst5. Our results provide evidence for *1*) a strong leishmanicidal activity for all three peptides, ruling out stereospecific recognition for Hst5; and *2*) a preferential accumulation into the parasite mitochondrion. For Hst5, a faulty oxidative phosphorylation, mainly due to an inhibition of mitochondrial F_1F_0-ATPase, and in a minor extent the extracellular ATP efflux, leads to parasite death.

MATERIALS AND METHODS

Cell lines

Promastigotes from the *Leishmania donovani* strain MHOM/SD/00/1S-2D, their two derived strains, Lucshmania and 3-Luc, expressing the native glycosomal luciferase or a C-terminal mutated form (cytoplasmic luciferase), respectively, and *L. pifanoi* axenic amastigotes (MHOM/VE/60/Ltrod) were grown as described previously (13, 18, 19).

Chemicals

L-Hst5 (DSHAKRHHGYKRKFHEKHHSHRGY), its D-enantiomer, and Dhvar4 (KRLFKKLLFSLRKY) were synthesized by Fmoc-chemistry, purified by preparative reverse phase (RP) -HPLC, and reanalyzed by RP-HPLC plus capillary zone electrophoresis. The purity of the peptides was at least 95%, and the authenticity of the peptides was confirmed by ion trap mass spectrometry (20). Labeling of the peptides with fluorescein-5-isothiocyanate (FITC; Molecular Probes Europe BV, Leiden, NL) was performed as described previously (5), resulting in a molar FITC to peptide ratio of ~0.1.

Unless otherwise stated, the reagents were purchased from Sigma-España (Madrid, Spain). Bisoxonol, [*bis*-(1,3-diethylthiobarbituric) trimethine oxonol], SYTOX Green, rhodamine 123 [2-(6-amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester, chloride], DMNPE-luciferin [D-luciferin, 1-(4,5-dimethoxy-2-nitrophenyl) ethyl ester], MitoTracker Red CMXRos, and H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) were obtained from Invitrogen (Barcelona, Spain).

Fluorescence and luminescence measurements were recorded in a Polarstar Galaxy microplate reader (BMG Labotechnologies, Offenburg, Germany), fitted with the corresponding optical setting, unless otherwise stated.

Cell proliferation measurements

Parasites were harvested at late exponential phase, washed twice in sorbitol buffer (280 mM D-sorbitol, 4.0 mM Na_2HPO_4, 1.0 mM KCl, 4.8 mM $NaHCO_3$, and 10 mM D-glucose, pH 7.2) at 4°C, and resuspended in the same buffer at a final concentration of 2×10^7 cells/ml. Unless otherwise stated, these conditions were maintained for the rest of the experiments.

Aliquots of 100 μl of the parasite suspension were incubated with the corresponding peptides for 4 h, either at 25 or 32°C for promastigotes or amastigotes, respectively, washed with 1 ml of sorbitol buffer at 4°C, and resuspended in 1 ml of their respective growth medium. Parasites were allowed to proliferate for 48 or 96 h for promastigotes and amastigotes, respectively. Cell proliferation was subsequently measured by reduction of 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/ml), followed by solubilization with 5% (w/v) SDS and readout in a 680 Bio-Rad microplate reader, equipped with a 600 nm filter (Bio-Rad, Madrid, Spain) (18).

The induction of apoptosis by Hst5 was monitored by analysis of the sub-G_1 peak in parasites stained with propidium iodide in a Coulter XL EPICS cytofluorometer (Beckman Coulter, Miami, FL, USA) (21), using 15 μM miltefosine as a positive control.

Changes in plasma membrane potential

Changes in plasma membrane potential were monitored by the increase of bisoxonol fluorescence, a potential-sensitive anionic dye, after its insertion into the parasite membrane, once the cell becomes depolarized. Assays were performed under the standard conditions using as incubation medium 0.2 μM bisoxonol in sorbitol buffer. Fluorescence changes were monitored using excitation and emission filters of 544 and 584 nm, respectively. Maximal depolarization was considered as that achieved with the cecropin A-melittin hybrid peptide CA(1–8)M(1–18) at 2.5 μM (22).

Plasma membrane permeabilization

The procedure described previously (22) was followed, except for the use of sorbitol buffer as incubation medium. Briefly, peptides were added to the parasite suspension (2×10^7 promastigotes/ml) preincubated with 1 μM SYTOX Green. The increase in fluorescence due to binding of the dye to intracellular nucleic acids was monitored using 485 and 520 nm filters for excitation and emission wavelengths,

respectively. Permeation achieved by 0.1% Triton X-100 (TX-100) was considered as maximal.

Electron microscopy

Promastigotes were incubated with the peptides according to the standard procedure, washed twice in PBS, and fixed in 5% (w/v) glutaraldehyde in the same medium. Afterward, parasites were included with 2.5% (w/v) OsO_4 for 1 h, gradually dehydrated in ethanol [30, 50, 70, 90, and 100% (v/v); 30 min each] and propylene oxide (1 h), embedded in Epon 812 resin, and observed in a Jeol-1230 electron microscope (Jeol Ltd., Akishima, Japan) as described (22).

Confocal laser microscopy

The intracellular distribution of fluoresceinated peptides was ascertained by a double-labeling experiment, using MitoTracker Red, a cell-permeable mitochondrion-selective dye. Promastigotes of *L. donovani* and amastigotes of *L. pifanoi* were loaded with 0.05 μM MitoTracker Red for 10 min at 25 and 32°C, respectively, washed twice, and incubated in darkness with the peptides for 4 h at their respective growth temperatures. Finally, unbound peptide was removed by washing twice with 1.5 ml of sorbitol buffer, and parasites were prepared with Dako fluorescence mounting medium (Dako Cytomation, Carpinteria, CA, USA) for observation. Confocal fluorescence images were obtained on a Leica TCS-SP2-AOBS-UV ultraspectral confocal microscope (Leica Microsystems, Heidelberg, Germany) Excitation and emission wavelengths for MitoTracker Red were 543 and 599 nm, respectively, and 488 and 519 nm for the labeled peptides.

Intracellular accumulation of FITC-Hst5

Aliquots of 100 μl of the parasite suspension under the standard conditions were incubated with the fluoresceinated D- or L-Hst5 for 4 h at 25 or 32°C for promastigotes or amastigotes, respectively, washed twice with 1 ml of 10 mg/ml BSA in sorbitol buffer at 4°C, and resuspended in 100 μl of sorbitol buffer. Cells were transferred into a 96-well black microplate and lysed with SDS (1% final concentration), and their fluorescence was measured using excitation and emission filters of 485 and 520 nm, respectively. A standard curve was obtained with the fluoresceinated peptides in sorbitol buffer.

Changes in intracellular ATP levels

The *in vivo* variation of intracellular ATP levels in *L. donovani* promastigotes was measured in parasites expressing firefly luciferase (19), except for the use of sorbitol buffer as incubation medium. Changes in luminescence were recorded for 120 min using a luminescence optics setting. The measurements were averaged every 4 s. *In vitro* inhibition of *Photinus pyralis* luciferase (EC 1.13.12.7) by the peptides was tested as described previously (19) using a commercial purified enzyme (Roche Applied Sciences, Madrid, Spain).

The release of ATP from *L. donovani* promastigotes into the external medium was detected by a luminescence assay. Parasites (2×10^7 cells/ml) were incubated with 6 and 12 μM L-Hst5 for 10 min and centrifuged, and 50 μl of the resulting supernatant was immediately mixed with 750 μl of H_2O and 200 μl of a firefly lantern extract reagent (19). Luminescence was measured in a LKB Bio-Orbit 1250 luminometer (LKB Bio-Orbit, Vantaa, Finland) for 3 min after mixing. The data were normalized relative to a standard curve of ATP. The inhibition of ATP release by the general anion channel inhibitor 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) was assayed in parallel with parasites preincubated with 1 mM of this inhibitor for 1 h before peptide addition.

Effects of Hst5 on mitochondrial activities

Oxygen consumption rates were measured on digitonin-permeabilized promastigotes as described previously (23) in a Clark-oxygen electrode (Hansatech, KingsLynn, UK) at 25°C, using 1 ml of a parasite suspension (10^8 cells/ml) in sorbitol buffer, permeabilized with 60 μM digitonin, and supplemented with 100 μM ADP and 5 mM succinate as substrate. Once a steady rate was reached after peptide addition, a set of inhibitors and substrates specific for the different complexes of the respiratory chain were assayed: 0.1 mM tetramethyl-*p*-phenylendiamine plus 1.7 mM ascorbate (TMPD-ascorbate), 1.9 μM antimycin A, 6.5 μg/ml oligomycin, and 10 μM carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP).

The mitochondrial F_1F_0-ATPase activity was measured in digitonin permeabilized promastigotes (24). Parasites permeabilized with 60 μM digitonin were resuspended at 10^8 cells/ml in a reaction mixture containing 150 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.5 mM EGTA, and 100 μM Na_3VO_4, and they were incubated with different L- or D-Hst5 concentrations for 1 h. L-melittin (GIGAVLKVLTTGLPALISWIKRKRQQ) and its diastereomer [D]-$V^{5,8}$,I^{17},K^{21}-melittin (GIGAvLKvLTTGLPALiSWIkRKRQQ, small caps stand for D-amino acids; ref. 25) were used as controls. Afterward, the reaction was started by the addition of ATP (3 mM final concentration) at 26°C and stopped after 10 min by adding SDS (1% final concentration). The resulting inorganic phosphate was determined by the colorimetric method of Fiske-Subbarow, based on the reduction of the phosphomolybdate complex at 650 nm.

Variation of the mitochondrial membrane potential ($\Delta\Psi_m$) in intact promastigotes was monitored by rhodamine 123 accumulation as described previously (26) after peptide incubation in sorbitol buffer and being measured in a Coulter XL EPICS cytofluorometer (excitation and emission wavelengths 488 and 525 nm, respectively). Parasites either intact or depolarized with 10 μM FCCP were taken as controls.

Assay for cytotoxic activity against murine macrophages and intracellular amastigotes

BALB/c peritoneal macrophages were resuspended in sorbitol buffer at a final concentration of 10^6 cells/ml, plated in a 96-well culture microplate (100 μl/well), and incubated with Hst5 for 4 h at 37°C. Cytotoxicity was assessed using the colorimetric MTT reduction assay and was expressed as the percentage of the value obtained for control cells.

For infection experiments, the macrophages were seeded at a 10^5 cells/well in a Lab-Tek 16-well chamber slide system in RPMI 1640 plus 10% heat inactivated fetal calf serum and incubated with *L. pifanoi* axenic amastigotes at a 1:2 macrophage:amastigote ratio for 4 h. Infection was allowed to progress for 96 h at 32°C, followed by treatment with 25 μM Hst5 for 4 h in sorbitol buffer. After peptide removal, the chambers were additionally incubated for 48 h in culture medium. Finally, slides were air dried, fixed in methanol, and stained with Giemsa. The parasite burden was considered as the average number of amastigotes in 100 macrophages measured by triplicate and referred to as percentage of the untreated macrophages.

TABLE 1. *Inhibition of parasite proliferation by D-Hst5, L-Hst5, and Dhvar4*

	Promastigotes			Amastigotes	
Peptide	pH 7.2	pH 5.5	+1 mM KCN, pH 7.2	pH 7.2	pH 5.5
L-Hst5	7.3 ± 0.2	6.3 ± 0.2	40.7 ± 0.1	14.4 ± 0.1	8.2 ± 0.3
D-Hst5	2.5 ± 0.3	2.4 ± 0.1	—	2.1 ± 0.1	1.4 ± 0.2
Dhvar4	1.8 ± 0.1	1.8 ± 0.1	4.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1

Data are expressed as LC_{50} ± SD (μM). LC_{50} values were calculated using SigmaPlot software.

RESULTS

Leishmanicidal activity of Hst5 and Dhvar4

All the peptides used in this study inhibited parasite proliferation at a micromolar concentration range (**Table 1**). At neutral pH, Dhvar 4 showed the highest activity on promastigotes (LC_{50}=1.8 μM) followed by D-Hst5 and L-Hst5. The same order resulted for the activity on amastigotes. Nevertheless, whereas D-Hst5 and Dhvar4 were equally potent on both forms of the parasite, the LC_{50} of L-Hst5 for amastigotes was twice as high as for promastigotes. Regardless of the assay conditions and stage of the parasite, D-Hst5 was consistently more active than L-Hst5. The fact that both enantiomers were active on *Leishmania* ruled out a critical role for chirality in the lethal process.

At acidic pH, the increase in positive charge due to histidine protonation enhanced the activity of both Hst5 enantiomers, except for D-Hst5 on promastigotes. In sharp contrast, Dhvar4, devoid of histidine residues, did not undergo any significant variation.

It was found that the leishmanicidal activity of the peptides was severely inhibited when assayed on cells previously preincubated with 1 mM KCN for 1 h, a condition that causes a reversible inhibition of cytochrome *c* oxidase, hence of mitochondrial ATP production, without compromising the parasite viability in a time frame of several hours (14).

Plasma membrane permeabilization assays

The permeabilization of the plasma membrane of *L. donovani* promastigotes by the peptides was monitored by two complementary approaches: *1*) membrane depolarization, directly linked to the dissipation of ionic gradients across the membrane; and *2*) access of the vital dye SYTOX Green (molecular weight=600) to the cytoplasm, requiring large size lesions and precluded in intact cells.

As depicted in **Fig. 1*A–C***, promastigotes underwent a fast and dose-dependent increase in bisoxonol fluorescence after peptide addition, typical of a depolarization process. This was followed by a dose-dependent steady recovery for >1 h. At a given concentration, Dhvar4 caused a much larger depolarization than any Hst5 enantiomer.

The differences in plasma membrane permeabilization between L-Hst5 and Dhvar4 were emphasized when the entrance of the vital dye SYTOX Green was measured. Figure 1*D–F* shows the intracellular accumulation of SYTOX Green after peptide addition. The lesions caused by Dhvar4 and both Hst5 enantiomers at equipotent concentrations differed widely. At its LC_{50}, the fluorescence value for Dhvar4 reached 80% of the maximal permeabilization (Fig. 1*F*), defined as that achieved by the detergent TX-100 at 0.1%. In contrast, the increase produced by both Hst5 enantiomers at their respective LC_{50} hardly reached 10% (Fig. 1*D, E*), underscoring the different nature of the lesions for Dhvar4.

Electron microscopy

In a further step, the morphological damage inflicted to the promastigotes by the different peptides was visualized by transmission electron microscopy. L-Hst5 and Dhvar4 were assayed on promastigotes at an equipotent concentration causing ~80% inhibition of parasite proliferation (12 and 3 μM, respectively). Promastigotes incubated with L-Hst5 showed a dramatically distorted mitochondrion, its matrix strongly swollen and, in most parasites, with a bulging-out appearance, while the plasma membrane appeared without substantial damage (**Fig. 2*B***). On the contrary, Dhvar4 caused extensive blebbing of the plasma membrane of which large areas were detached from the subpellicular layer of microtubules (Fig. 2*C*), an effect previously reported for other typical membrane-seeking AMPs in *Leishmania* (22, 26). These different morphological patterns are in tune with the divergences observed for these peptides in membrane permeabilization experiments.

Intracellular localization of the peptides

The electron microscopy experiments were complemented with the intracellular localization of fluoresceinated peptides at their respective LC_{50} by confocal fluorescence microscopy. Previously, it was confirmed that peptide labeling did not affect leishmanicidal activity >5% (data not shown). The intracellular distribution of both dyes, the fluoresceinated peptides and the mitochondrion-selective dye MitoTracker Red, was largely but not exclusively overlapping, as shown in **Fig. 3**. This points out mitochondrion as an organelle

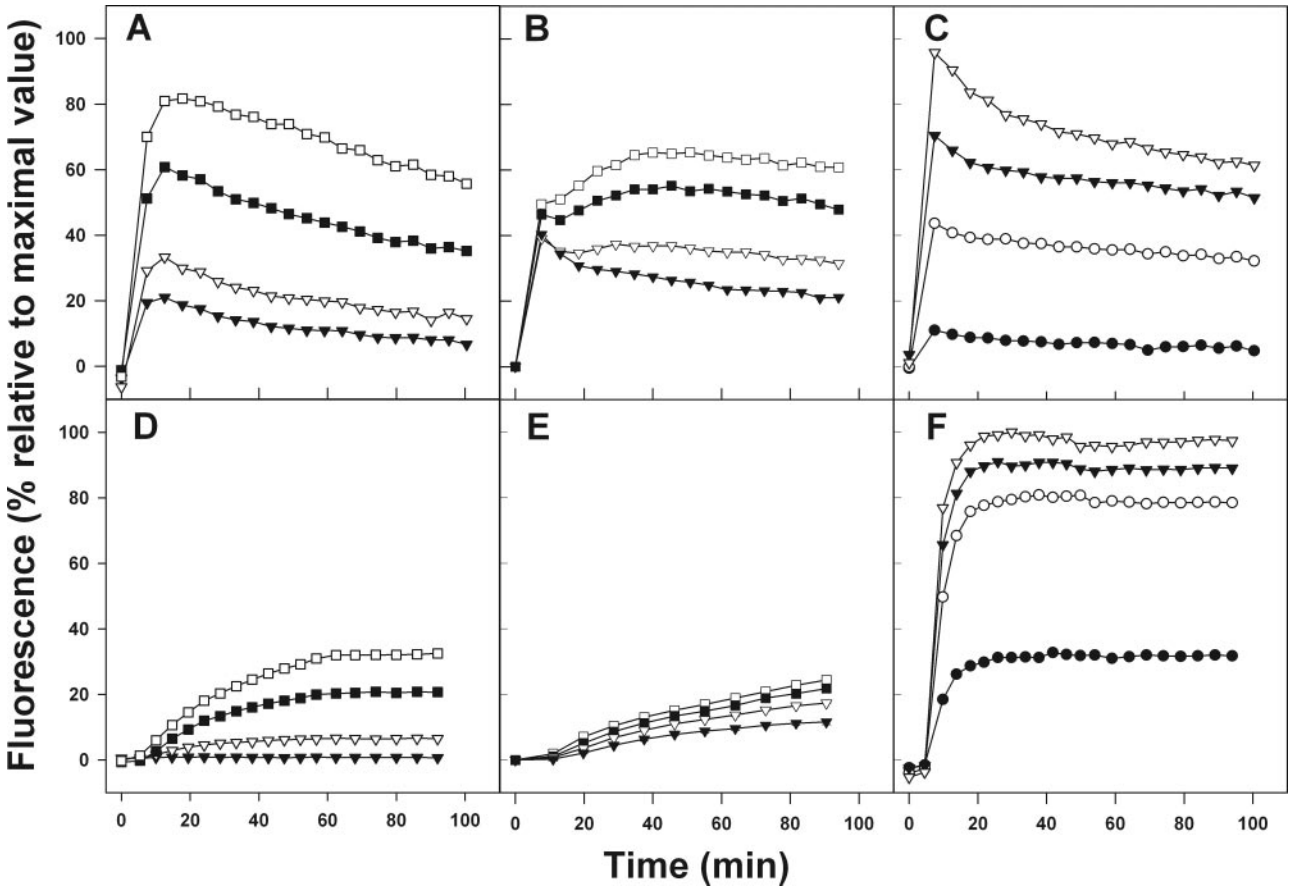

Figure 1. Plasma membrane permeabilization of *L. donovani* promastigotes after addition of L-Hst5, D-Hst5, and Dhvar4. The plasma membrane depolarization achieved by L-Hst5 (*A*), D-Hst5 (*B*), or Dhvar4 (*C*) was monitored by the increase of bisoxonol fluorescence (λ_{EXC}=544 nm; λ_{EM}=584 nm). Results were normalized as percentage of fluorescence relative to the maximal depolarization achieved with 2.5 μM CA(1–8)M (1–18). SYTOX Green entrance after addition of L-Hst5 (*D*), D-Hst5 (*E*), or Dhvar4 (*F*) was followed by the increase in its fluorescence (λ_{EXC}=485 nm, λ_{EM}=520 nm) due to binding to intracellular nucleic acids. Results were normalized as percentage of fluorescence relative to maximal parasite permeabilization, obtained with 0.1% TX-100. Peptide concentrations were as follows: 0.5 μM (●), 1.5 μM (○), 3 μM (▼), 6 μM (▽), 12 μM (■), and 24 μM (□).

with a privileged accumulation of the peptides in contrast with the scarce fluorescence associated to the plasma membrane. The translocation of FITC-D-Hst5 into the intracellular space excludes a key participation of a stereospecific receptor for this process in *Leishmania*. Again, a difference in the fluorescence pattern between FITC-L-Hst5 and its analog FITC-Dhvar4 was observed (Supplemental Fig. 1*B*). For FITC-Dhvar4, the distribution was more diffuse, in contrast with that for FITC-L-Hst5, which appeared mostly confined to specific spots. Preincubation of the parasite with KCN, a condition that significantly decreased both leishmanicidal and candidacidal activity of Hst5 (5), prevented the accumulation of both peptides into the promastigote, being especially noticeable for FITC-L-Hst5 (Supplemental Fig. 1*D*).

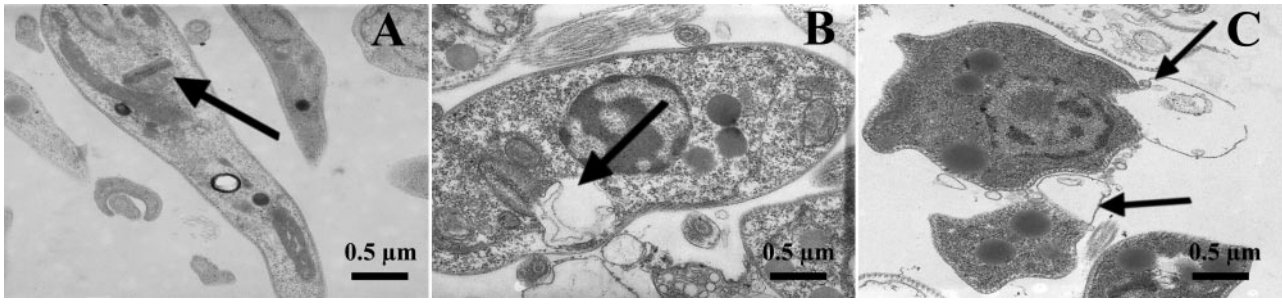

Figure 2. Electron microscopy of *L. donovani* promastigotes treated with Hst5 and its analog Dhvar4. Peptides were added at an equipotent concentration that caused ~80% inhibition of proliferation. *A*) Control parasites. *B*) 12 μM Hst5. *C*) 3 μM Dhvar4. Mitochondrion was identified by the presence of cristae and/or kinetoplast and indicated by arrow in *A* and *B*. Arrows in *C* point to plasma membrane detachment.

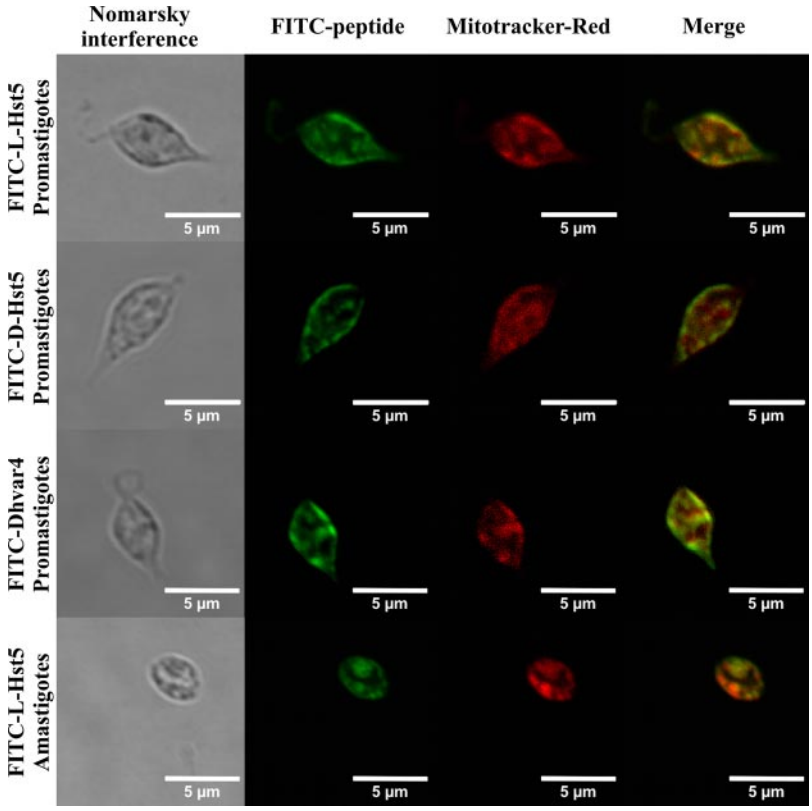

Figure 3. Pattern of fluorescence of *L. donovani* promastigotes and *L. pifanoi* amastigotes labeled with fluoresceinated peptides and MitoTracker Red, assessed by confocal microscopy. Parasites were loaded with 0.05 μM Mitotracker Red before their incubation with equipotent concentrations of the following peptides: 7 μM for FITC-L-Hst5 (top row), 2.5 μM for FITC-D-Hst5 (second row), 1.8 μM FITC-Dhvar4 (third row) for promastigotes, and 15 μM FITC-L-Hst5 for amastigotes (bottom row). Wavelengths used were λ_{EXC} = 489 nm and λ_{EM} = 519 nm for FITC-peptide and λ_{EXC} = 543 nm and λ_{EM} = 599 nm for MitoTracker Red.

Intracellular accumulation of Hst5

Once the internal localization of the peptides was established by confocal microscopy, we proceeded to measure their intracellular concentration. The accumulation of FITC-D-Hst5 at 7.5 μM in promastigotes was 3-fold higher (0.127 nmol/10^6cells) than FITC-L-Hst5 (0.043 nmol/10^6 cells) in accordance with its higher leishmanicidal activity on this form of the parasite (Table 1). The same trend was also found for amastigotes, with an intracellular accumulation of 0.023 nmol/10^6cells for FITC-D-Hst5, nearly 9-fold higher than that for FITC-L-Hst5 (2.5×10^{-3} nmol/10^6cells). These data were further used to calculate the intracellular concentration of FITC-L-Hst5 in parasites. For promastigotes, the values for internal volume (4.3 μl/mg of protein; ref. 27) and 1 mg of protein=3.6×10^8 promastigotes; ref. 28) were taken, giving an intracellular concentration ~3.6 mM. Similar calculations in amastigotes resulted in a Hst5 concentration ~0.7 mM, with the internal volume calculated as by Alberio *et al.* (29).

Effect of the histatins on the bioenergetic metabolism of *L. donovani* promastigotes

Having identified the mitochondria as an organelle with a privileged accumulation for Hst5, we studied the bioenergetic metabolism of the promastigote in greater detail to obtain a deeper understanding of the intracellular targets of L-Hst5 and Dhvar4 in this organism.

To this end, promastigotes of the cytoplasmic luciferase-encoding 3-Luc strain show a luminescence that parallels the concentration of free-cytoplasmic ATP, the limiting substrate for this reaction, when incubated in the presence of the membrane-permeable luciferase substrate DMNPE-luciferin (19, 22). Both L-Hst5 and Dhvar4 inhibited luminescence in a concentration-dependent manner, reaching the final levels after 10 min (**Fig. 4**). Dhvar4 induced a consistently higher inhibition relative to L-Hst5, as observed for the inhibition of proliferation; in addition, the end point for this peptide was reached faster. The specificity of this effect was shown, as the luminescence associated to the glycosomal luciferase was hardly altered after addition of L-Hst5 at the highest concentration tested (Fig. 4).

The release of intracellular ATP induced by L-Hst5 and inhibited by DIDS was very instructive about its candidacidal mechanism (30, 31). In *Leishmania*, the external ATP accumulation caused by L-Hst5 at 6 and 12 μM was of 65 and 166 pmol $\times$ 10^{-6} cells, respectively, accounting for 3.2 and 15.1% of the total intracellular ATP, compared with parasites fully permeabilized with 0.1% TX-100 (887.7 pmol$\times10^{-6}$ cells) after subtracting the spontaneous ATP release (37.2 pmol$\times10^{-6}$ cells; **Fig. 5*B***). These values were obtained 10 min after peptide addition, a time frame at which the drop of luminescence, that is of intracellular ATP, reached its respective end point with a reduction of 17.8 and 47.8% for 6 and 12 μM, respectively (Fig. 5*A*). Furthermore, parasites pretreated with 1 mM DIDS before L-Hst5 addition showed an identical rate of luminescence decay (Fig. 5*A*) and identical accumulation of external ATP. Altogether, these data provide

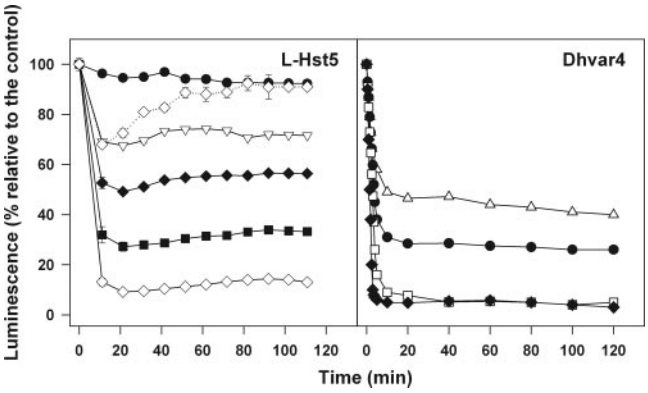

Figure 4. Variation of *in vivo* luminescence of 3-Luc *L. donovani* promastigotes after peptide addition. Promastigotes (2×10^7 cells/ml) were preloaded with 25 μM DMNPE-luciferin; when luminescence reached a plateau, peptides were added (t=0) and luminescence value taken as 100%. Variation in luminescence was normalized relative to the control parasites in absence of peptide. Peptide concentrations were as follows: 3 μM (△), 5 μM (●), 8.5 μM (□), 10 μM (▽), 12.5 μM (♦), 25 μM (■), and 50 μM (◇). Dotted line represents luminescence obtained from *L. donovani* promastigotes expressing glycosomal luciferase.

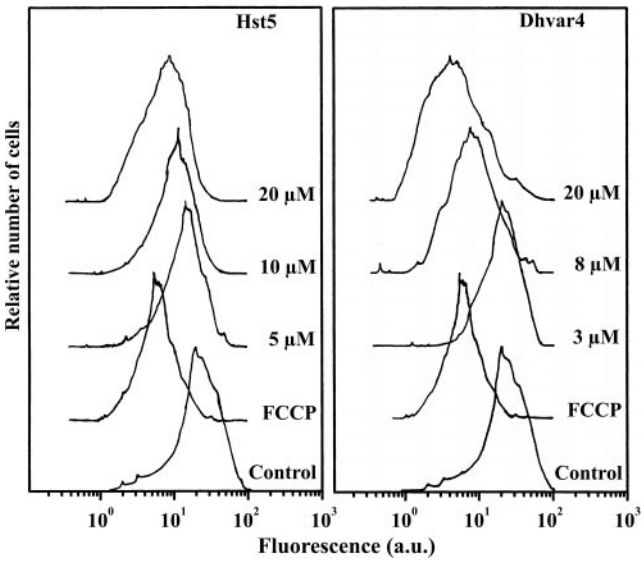

Figure 6. Variation of the mitochondrial membrane potential of *L. donovani* promastigotes by L-Hst5 and Dhvar4, monitored by rhodamine 123 accumulation. Parasites preincubated with the peptides for 4 h were loaded with 0.3 μg/ml rhodamine 123, and fluorescence level was measured by cytofluorometry. Peptide concentration was shown at the side of the corresponding trace. Full depolarized parasites were obtained by incubation with 10 μM FCCP.

evidence that the release of ATP induced by Hst5 cannot fully account for the drop in its intracellular level, hence for a major role in its leishmanicidal activity, in opposition to its candidacidal one.

We therefore hypothesized that the bioenergetic collapse produced by Hst5 was due to its effect on oxidative phosphorylation, the major source of ATP synthesis for *L. donovani* (14). In fact, L-Hst5 caused a significant inhibition on two parameters directly related to this process, the mitochondrial electrochemical potential ($\Delta\Psi_m$) and the oxygen consumption rate. Both L-Hst5 and Dhvar4 caused a substantial decrease in the levels of $\Delta\Psi_m$-driven rhodamine 123 uptake (**Fig. 6**). Nevertheless, when compared at their respective LC_{50}, L-Hst5 produced a 30% decrease in $\Delta\Psi_m$, whereas for Dhvar4 the inhibition was hardly perceptible. On living promastigotes, respiration was inhibited by both L-Hst5 and Dhvar4 in a dose-dependent manner. L-Hst5 was the less potent, resulting in an EC_{50} of 23.9 ± 1.2 μM, whereas that for Dhvar4 was 4.6 ± 0.6 μM, not correlating with its mild inhibition on $\Delta\Psi_m$ and suggesting that for this peptide the inhibition of respiration was not a consequence of a specific effect on mitochondria but rather due to the leakage of nutrients and ions from the cytoplasm caused by plasma membrane permeabilization, as described for other membrane-seeking AMPs.

For further identification of the molecular target of Hst5, we explored its effects on mitochondrial ATP synthesis using digitonin-permeabilized promastigotes. Under these conditions, a selective permeation of the plasma membrane, but not of mitochondria, takes place, allowing free access of substrates and inhibitors to the different complexes of the respiratory chain. The integrity of the inner mitochondrial membrane in the assay was preserved, as the respiration increased by recovery of state 3 after ADP addition (**Fig. 7**, left trace). In digitonized parasites, 20 μM L-Hst5 reduced the respiratory activity by ~70% relative to the value in

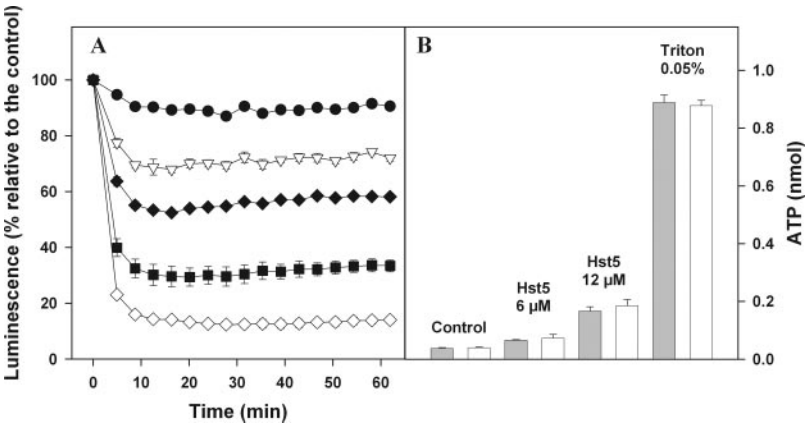

Figure 5. Hst5-induced variation of ATP levels in parasites pretreated or not with DIDS. *A*) Intracellular ATP decay assessed by luminescence of 3-Luc promastigotes pretreated with 1 mM DIDS for 1 h before Hst5 addition. Peptide concentrations were as follows: 5 μM (●), 10 μM (▽), 12.5 μM (♦), 25 μM (■), and 50 μM (◇). *B*) ATP efflux from 10^6 promastigotes, pretreated with DIDS (open bars) or not (filled bars), after a 10 min incubation with Hst5.

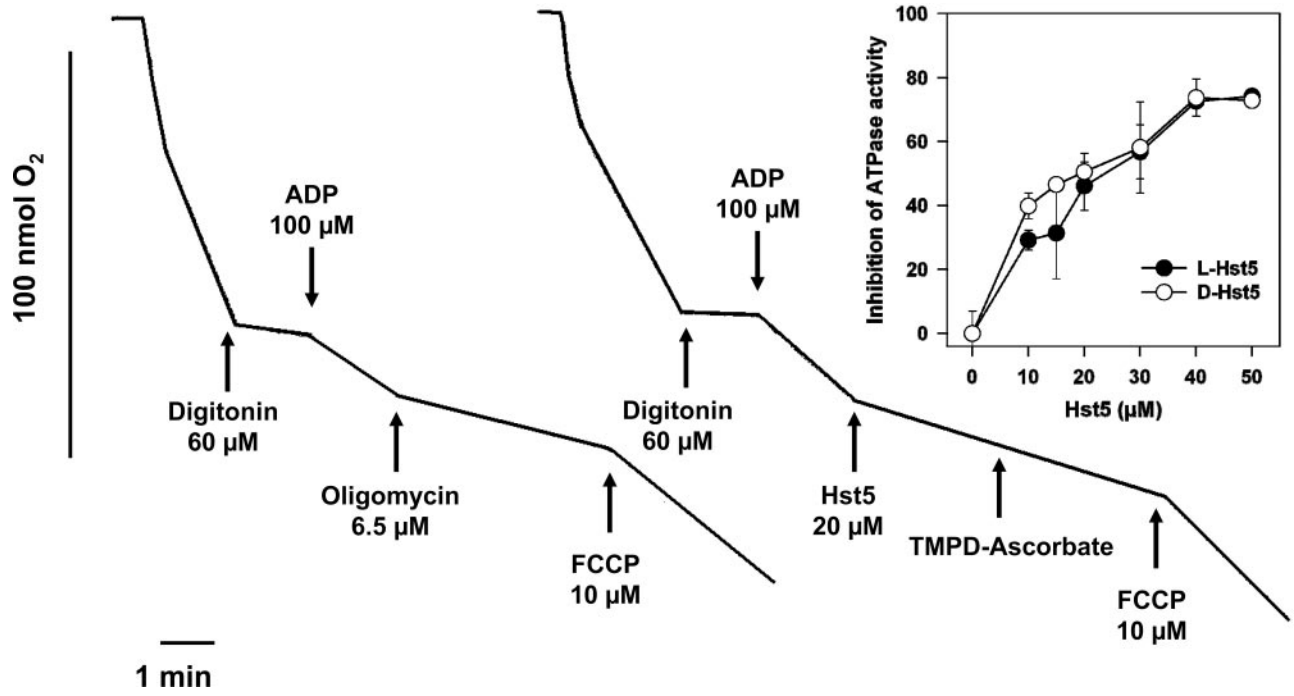

Figure 7. Variation of bioenergetic parameters by Hst5 in digitonin-permeabilized *L. donovani* promastigotes. Traces represent the oxygen consumption rates using 5 mM succinate as substrate. Oxygen consumption rate of digitonin-permeabilized parasites was 11.4 nmol $\times$ min^{-1} $\times$ 10^{-8} cells, after ADP addition. Arrows represent addition of the indicated substrates and inhibitors. Inset shows the inhibition of F_1F_0-ATPase activity in digitonin-permeabilized parasites with increasing D- or L-Hst5 concentrations. Data were normalized relative to the control inhibition achieved by 10 μg/ml oligomycin. ATP hydrolysis was 380 nmol and 184 nmol $\times$ min^{-1} $\times$ mg $protein^{-1}$ for control and oligomycin-treated parasites, respectively.

absence of the peptide (Fig. 7, right trace). This inhibition was reverted by the addition of the mitochondrial uncoupler FCCP (10 μM), discarding any specific inhibitory effect on the carriers of the electron transport chain. Also, addition of TMPD-ascorbate, an electron donor to cytochrome *c*, did not revert the inhibition (Fig. 7, right trace). These results strongly suggested that L-Hst5 did not act at the level of the mitochondrial respiratory chain, *e.g.*, as an uncoupler or blocker of the electron transfer. Since the electron transfer is obligatorily coupled to mitochondrial ATP-synthesis, inhibition of one process will result in inhibition of the other and *vice versa*. We therefore examined whether the inhibitory effect of Hst5 was due to inhibition of the F_1F_0-ATPase, which carries out mitochondrial ATP synthesis. Both L-Hst5 and D-Hst5 reduced the oligomycin-sensitive ATPase activity by ≅50 and ≅75% at 20 and 50 μM, respectively. This points toward the mitochondrial F_1F_0-ATPase as a likely target for Hst5 (Fig. 7, inset). L-melittin and its diastereomer [D]-$V^{5,8}$,I^{17},K^{21}-melittin, with a partially crippled α-helical structure, were used as controls, inhibiting F_1F_0-ATPase activity with an EC_{50} of 0.8 and 3 μM, respectively.

Leishmania killing does not involve an apoptotic process

The above results have shown that the functionality of the mitochondrion was severely hampered by L-Hst5. Since the mitochondrion plays a pivotal role in the onset of apoptosis, it was investigated whether the effects of L-Hst5 involved induction of apoptotic processes in *Leishmania*. The percentage of promastigotes with a propidium iodide fluorescence in the sub-G_1 peak region typical of apoptotic cells (21) was identical to the untreated cells after L-Hst5 incubation, even for concentrations causing a mortality rate >80%, in oppositition to the 30% observed for those treated with miltefosine (Supplemental Fig. 2).

Cytotoxicity on mammalian cells and intracellular amastigotes

L-Hst5 was not cytotoxic (<5%) when assayed on peritoneal mouse macrophages at 25 μM. At this concentration, the reduction of the parasite burden in *L. pifanoi* infected macrophages was 61.4 ± 4.6%. In comparison, the Hst5 LC_{50} for axenic amastigotes was 14.4 μM (Table 1).

DISCUSSION

The protozoan parasite *Leishmania* constitutes an interesting biological test for histatins, which preferentially target yeast mitochondria; *Leishmania* possesses the most primitive functional mitochondria (8), and it thrives in harsh acidic pH (17), where the cationicity of histatins increases due to histidine protonation. Fur-

thermore, its plasma membrane is specially adapted to provide a partial protection against AMPs, due to the restriction of the membrane traffic to the flagellar pocket and a dense and highly anionic glycocalyx, rich in proteolytic activity (10).

Interaction of L-Hst5 with the plasma membrane of *Leishmania*

The differences reported for the action of L-Hst5 and its analog Dhvar4, endowed with a much higher amphipathicity and membrane-disrupting activity, both on model membranes (20) and on *Candida* (32), were also mirrored on *Leishmania* promastigotes. Dhvar4 induces a fast and irreversible plasma membrane depolarization in *Leishmania* promastigotes and a large entrance of the vital dye SYTOX Green into the parasite, associated to a massive plasma membrane disruption with large blebbings, as visualized by electron microscopy, similar to the effect observed with other membrane-active AMPs on *Leishmania* promastigotes (33). Altogether, these effects are detrimental enough to account for a lethal outcome. In contrast, Hst5 produced a limited and mostly reversible depolarization of the plasma membrane and a much lower rate and final extent of SYTOX Green uptake. In addition, the electron micrographs of promastigotes treated with this peptide showed a plasma membrane that was scarcely damaged. By contrast, Hst5 induced in *Candida* large invaginations of its plasma membrane (5). The high stability of the subpellicular microtubular layer beneath the plasma membrane of *Leishmania* may partially account for this difference. In fact, in *Candida* the increase in membrane rigidity or in cytoskeleton stability leads to a reduced sensitivity to Hst5 (34). Nevertheless, this is a limited effect, as other membrane-seeking AMPs (26), including Dhvar4, even at much lower concentrations than the EC_{50} for L-Hst5, produced a pattern of extensive membrane disruption. This strongly suggested that in the leishmanicidal action of Hst5, membranolysis plays only a minor role; thereby, the involvement of an intracellular target is essential.

Intracellular uptake of Hst5 by *Leishmania*

An important issue is how L-Hst5 translocates across the membrane and gains access to the intracellular space. Several mechanisms are feasible: active uptake though the involvement of a surface receptor, endocytosis, or a passive translocation across the phospholipid matrix plasma membrane. Ssa1/2p, members of Hsp70 family, have been reported to act as cell wall-expressed major receptors for some AMPs, including L-Hst5 (35–37). To the best of our knowledge, the surface expression of *Leishmania* heat shock proteins has not been yet reported. By a BLAST similarity search (Blastp; available at www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) for *L.donovani*, an Hsp70 protein (Swiss-Prot accession number P17804) with a 73% amino acid sequence identity was the closest homologue of Ssa1/2p. Preliminary experiments carried out with an extensive set of monoclonal antibodies raised against *Leishmania* Hsp70 were negative, both for the binding of these antibodies to intact promastigotes and for the inhibition of the leishmanicidal activity of L-Hst5 (data not shown). These results, together with the activity of the all-D-Hst5, make it quite unlikely that chiral recognition by a putative plasma membrane receptor/transporter in *Leishmania* is a key event in Hst5 uptake.

Preliminary experiments of colocalization of rhodamine B dextran, a marker for fluid phase endocytosis, and FITC-L-Hst5, point to the plasma membrane and to a lesser extent the endosomal membrane, as the translocation site for Hst5, discarding endocytosis, a process also inhibited by KCN, as an exclusive pathway for Hst5 uptake (data not shown).

The third option is the translocation of L-Hst5 across the phospholipid matrix of the plasma membrane. L-Hst5 showed a poor translocation capacity in anionic liposomes when compared with living *Candida* (20). This might be due to the presence of a membrane potential in living cells in agreement to the worm-hole model (3). On the other hand, the internalization of Hst5 in *Candida* is strongly dependent on the physical state of the plasma membrane, highlighting its importance in this process (34).

Overall, two major conclusions can be drawn: *1*) damage to the plasma membrane of *Leishmania* promastigotes induced by L-Hst5 is less crucial for its lethal action than for other AMPs, and *2*) regardless of the translocation mechanism, the peptide acts through intracellular targets, similar to its action in *Candida*, where the L-Hst5 lethal hit and many of its associated effects caused by extracellular L-Hst5 or L-Hst3 were reproduced by the peptide synthesized endogenously (38).

Hst5 targets the bioenergetic metabolism of *Leishmania*

Once the plasma membrane was discarded as the major target for L-Hst5 in *Leishmania*, we proceeded to define its intracellular targets in this parasite. A number of pleiotropic effects of Hst5 on *Candida* have been described, but their respective crucial or secondary role in the lethal action is not completely settled yet (16), although the involvement of the Hog1 MAP kinase as a global regulator was recently proposed (39).

Apparently, the mitochondrion is a major target for L-Hst5 in *Leishmania,* as *1*) electron microscopy showed extensive morphological alterations of this organelle, with a swollen matrix and poor cristae definition after Hst5 treatment; *2*) the distribution of the fluoresceinated Hst5 largely, but not exclusively, overlapped with mitochondrial markers, and its uptake was precluded in cells preincubated with metabolic inhibitors; and *3*) both the respiration rate and $\Delta\Psi_m$, two parameters of mitochondrial functionality crucial for an adequate production of ATP, were severely decreased by L-Hst5.

According to the results obtained in intact and

digitonized promastigotes, inhibition of oxidative phosphorylation remained the most feasible option for the action of L-Hst5, as the inhibition of the oxygen consumption by this peptide was not reverted even after feeding the complex IV, the terminal electron donor, with specific artificial substrates. In contrast, addition of FCCP, a typical uncoupler, to L-Hst5-treated parasites, recovered the initial rate. Therefore, it may be concluded that L-Hst5 did not impair the functionality of the respiratory chain, in contrast with *Candida*, as in this pathogen the inhibition by Hst5 was not reverted by the uncoupler CCCP (32). Furthermore, the important role of reactive oxygen species (ROS) described for the candidacidal mechanism of L-Hst5 (16) was also excluded in *Leishmania*, as in our hands, neither the coincubation of *Leishmania* promastigotes with L-Hst5 plus the free radical scavenger TEMPO (2,2,6,6-tetramethylpiperidine-*N*-oxyl) nor production of ROS, assessed by H_2DCFDA, was positive (data not shown). This was not totally unexpected, since *Leishmania* is endowed with a large set of neutralizing mechanisms for ROS (41) to thrive permanently or temporarily on macrophage (10) or neutrophils (42), respectively, two dedicated ROS producer cells.

Our results also exclude L-Hst5 as an uncoupler for oxidative phosphorylation in *Leishmania*, described for other AMPs with high amphipathicity and membrane disrupting characteristics as the cecropin A-melittin peptides on purified mammalian mitochondria (40).

L-Hst5 and its D-enantiomer caused a significant decrease in the oligomycin-sensitive F_1F_0-ATPase activity, pointing toward the inhibition of ATP synthesis as a crucial step in the leishmanicidal mechanism. To our knowledge, this is the first AMP hitherto reported to act on this enzymatic activity in living *Leishmania* parasites. Although a detailed mechanism for this activity is still missing, it is worth noting that in bovine mitochondrial F_1F_0-ATPase inhibition by a variety of amphipathic peptides such as melittin or synthetic analogues of the mitochondrial import presequence of yeast cytochrome oxidase subunit IV has been reported (43); whether a similar assumption may be valid for *Leishmania* remains uncertain. Our results also support an inhibition of the *Leishmania* F_1F_0-ATPase through a promiscuous interaction, since L-melittin was also inhibitory. A certain amphipathicity threshold is critical, as demonstrated by the loss of activity of a melittin diastereomer with a partially crippled secondary structure (44). A similar absence of chirality requirement, the presence of an amphipathic structure but not of chirality, was described for the modulation of calmodulin by amphipathic peptides (45).

In AMPs, the activity of an all-D-enantiomer is taken as a touchstone to rule out intracellular targets involving stereoselective interactions (6, 46–48) in contrast to the receptor-independent killing carried out through disruption of the phospholipid matrix of the plasma membrane (49). To the best of our knowledge, only the all-D-enantiomers of the plant peptide Ib-AMP1 (50) and of the pentadecamer lactoferricin $B_{17–31}$ (51) act through intracellular targets not yet identified and are as active as their all-L counterparts. The stronger leishmanicidal activity of the all-D-Hst5 relative to its natural counterpart is likely due to its higher accumulation inside the parasite due to increased resistance to enzymatic degradation.

Altogether these data show that the release of ATP induced by Hst5 cannot fully account for the drop in its intracellular level, hence for a major role in its leishmanicidal activity, in opposition to its action on *Candida*. A similar conclusion was reported in *Candida* by functional and proteomic analysis of the Hst3-resistant strains (52). The final outcome is the bioenergetic exhaustion and death of the parasite by a nonapoptotic process, in opposition to the apoptosis induced by indolicidin, the only leishmanicidal AMP acting through intracellular targets described so far (53).

Prospects for the pharmacological application of Hst5

The clinical importance of *Leishmania* and the paucity of new leishmanicidal drugs may support the application of histatins as a new chemotherapeutical approach for *Leishmania*. The feasibility of this approach is supported by successful results of histatins in oral and upper airway candidiasis (54, 55), together with the lack of toxicity toward mammalian cells, and by their capability to reduce the parasite burden in infected macrophages. The lower effect of Hst5 in this system might be conveniently increased by encapsulation of Hst5 into liposomes, similar to other leishmanicidal drugs (56), which exploit the high rate of phagocytosis of the macrophage.

In addition, the oxidative phosphorylation in *L. donovani*, the main target for L-Hst5, is crucial to fulfilling the bioenergetic requirement of the parasite (14), and consequently, the bioenergetic flexibility of *Leishmania* to switch from fermentative to nonfermentative metabolism is much lower than in *Candida* (57). Therefore, *Leishmania* will presumably be less prone to induce resistance to Hst5 than *Candida*, which has been already obtained *in vitro* for Hst3 (52).

Finally, an important feature of Hst5 is its behavior as a true cell-penetrating peptide (CPP), with a rather specific toxicity for lower eukaryotes. CPPs gain access to the intracellular milieu through their translocation across the endosomal or plasma membrane of the cell (review in ref. 58 and references therein). A major practical advantage is that they may ferry a wide variety of cargo molecules, such as peptides, proteins, nucleic acids, and even nanoparticles, across the membrane without the requirement of a dedicated membrane receptor (59). In this way, if another leishmanicidal agent is coupled to Hst5, it might gain access to the inside of the parasite, bypassing the low expression or even the absence or faulty receptor for these drugs, converting the conjugate drug-Hst5 into a single molecule with dual leishmanicidal action. Other CPPs have been used for a similar goal in *Leishmania* (60, 61);

however, their selectivity is much lower than that for Hst5. Two additional nanotechnological advantages of Hst5 are its high intracellular concentration inside the parasite, which might allow a high accumulation of the cargo molecule attached to Hst5, and its capacity to target the mitochondria of the pathogen, a scarce trait among the CPPs currently in use (59). According to the inhibition of Hst5 accumulation by KCN, a functional mitochondrion is required for the lethal hit of Hst5. We may envisage that the mitochondrial potential attracts the cytoplasmic monomers of Hst5, promoting their accumulation in this organelle that acts as a sink for this peptide.

Although the lethal mechanisms of Hst5 on *Leishmania* shared some steps with *Candida*, it showed significant differences in the translocation mechanism and site of action, showing the plasticity of these set of peptides to target different pathogens. Work in progress concerns the evaluation of the feasibility of Hst5 as a new template for leishmanicidal drugs and as a cell-specific CPP. FJ

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