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Kahalalide F, an Antitumor Depsipeptide in Clinical Trials, and Its Analogues as Effective Antileishmanial Agents

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Abstract: Leishmaniasis is a human parasitic disease caused by infection by the protozoan *Leishmania* spp. Chemotherapy is currently the only treatment available, but its efficacy is increasingly challenged by the rising incidence of resistance and the frequent severe side effects associated with first-line drugs. Thus the development of leads with distinct mechanisms of action is urgently needed. A strategy often used for this purpose consists of assaying for leishmanicidal activity drugs formerly developed for other applications, such as amphotericin B (antifungal) or miltefosine (antitumor), among others, to profit from previous pharmacological and toxicological studies. Kahalalide F (KF) is a tumoricidal cyclic depsipeptide currently under phase II clinical trials for several types of cancer and psoriasis. Its mechanism of action has not been fully elucidated. Here we report the leishmanicidal activity of KF and its synthetic analogues at a micromolar range of concentrations. Its lethality is strongly linked to the alteration of the plasma membrane (PM) of the parasite based on (i) a rapid depolarization of the PM and uptake of the vital dye SYTOX Green upon its addition; (ii) evidence of severe morphological damage to the membrane of the parasite, as shown by transmission electron microscopy; and (iii) a rapid drop in the intracellular ATP levels, which correlates significantly with the leishmanicidal activity for active analogues, some of them with significant improvement of their therapeutic index with respect to the parental molecule. In addition to the basic knowledge obtained, this class of lethal mechanism is considerably less prone to the induction of resistance than classical drugs. All together, these observations foster further studies for the optimization of KF and its analogues as new anti-*Leishmania* leads with a new mode of action.

Keywords: Kahalalide F; *Leishmania*; membrane permeabilization; combinatorial chemistry; structure−activity relationship; marine compound; solid-phase synthesis

Introduction

Leishmaniasis is a human protozoan infection caused by several species belonging to the genus *Leishmania*. It encompasses a wide range of clinical manifestations from the cutaneous form, with frequent self-healing, to the visceral one, which is fatal when untreated.[1] Leishmaniasis affects around 12 million people worldwide.[2] The only treatment currently available is chemotherapy, with organic pentavalent antimonials as the main first-line drugs.[3] However, the efficacy of these agents is rapidly eroding because of the increasing appearance of resistant clinical isolates.[4] These,

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together with the severe side effects associated with many of the first line-drugs, have fostered an intense search for drugs with new mechanisms of action.

To reduce the economical burden of developing new leishmanicidal drugs from scratch, a current strategy is to study drugs previously developed for other purposes and to test them for leishmanicidal activity, thereby saving on most of their pharmaceutical development, as they are already available for the former application. Many leishmanicidal drugs in distinct phases of development derived from this approach have been described, including some in current clinical use such as the antifungal amphotericin B,[65] or more recently miltefosine, the first successful oral drug against *Leishmania*,[7] formerly developed as an antitumor drug.

Eukaryotic antimicrobial peptides (AMPs) and their synthetic analogues are promising chemotherapeutical alternatives against multiresistant infections.[8] A number of these peptides from varied sources have been successfully tested as antileishmanial agents, both *in vitro*[9−11] and *in vivo*.[12] The major hurdles for their clinical use are their short half-lives *in vivo,* mainly because of proteolytic degradation, and the cost of synthesis, compared with other classical antibiotics.

Depsipeptides with antibiotic properties overcome these two limitations.[13−16] First, a considerable number of these compounds are cyclic and contain a large proportion of non-natural amino acids, a feature that precludes, or at least severely impairs, proteolytic degradation. Second, a significant number of them are natural products produced by microbes, which opens the door to mass production through fermentation.

Kahalalide F (KF) is a cyclic depsipeptide derived from the Hawaiian herbivorous marine species of mollusc, *Elysia rufescens*, and its diet, the green alga *Bryopsis* sp. KF,[17] is the most promising compound of the kahalalide family currently described. It shows antitumor activities[18] both on transformed cell lines and on tumor specimens derived from a variety of solid human tumors.[14,19] Moreover, KF is effective against human prostate cancer xenografts in mouse models, and it is currently under distinct phases of clinical trials for the treatment of prostate cancer[19] and in patients with severe psoriasis. Although the molecular bases of the tumoricidal activity of KF are not fully established, the permeabilization of the plasma membrane leading to oncosis,[20,21] alterations in lysosome morphology,[22,23] and inhibition of the ErbB3

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signaling pathways[23−25] are involved in the mechanisms of this activity. Furthermore, KF is also endowed with antimicrobial activity against fungi.[23]

The biosynthesis pathway of KF by the ascidian-algae symbiotic system is known.[26] Nevertheless, the complexity of this system favors chemical synthesis over the modification of biosynthesis as the best approach to obtain faster structure−activity relationship (SAR) studies for this depsipeptide, fostered by the optimization of its synthesis in solid phase.[27,28]

Here we report the leishmanicidal activity of a set of KF analogues in which we substituted residues of key relevance for tumoricidal activity, as shown by a massive SAR study.[23,29] These modifications affected the overall charge and hydrophobicity of the peptide, although the internal cycle and the N-terminal aliphatic acid were preserved throughout the whole set. All analogues but one contained one positively charged amino acid. Our data show that KF and several of its analogues have leishmanicidal activity at low concentrations (about 10 μM). These depsipeptides cause membrane permeabilization by a mechanism that remains to be elucidated. Unlike the tumoricidal activity of these compounds, a net cationic character is necessary for their leishmanicidal activity, but both activities required the maintenance of a fixed configuration of selected residues.

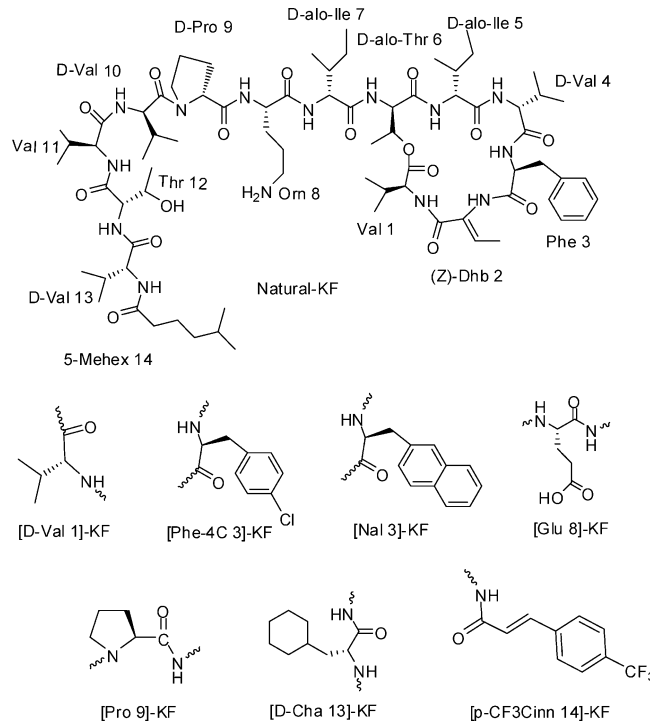

Figure 1. Coded structure of kahalalide F and its analogues.

Methods

Cell Lines. Promastigotes from *Leishmania pifanoi* (MHOM/VE/60/Ltrod), *Leishmania donovani* (MHOM/SD/00/1S-2D) and its derived line 3-Luc and axenic amastigotes of *L. pifanoi* were grown as described.[10] The *L. donovani* promastigote line 3-Luc was obtained by transfection with pX63NEO-3Luc, encoding for a *Photinus pyralis* luciferase form mutated in its C-terminal tripeptide, which locates the enzyme in the cytoplasm.[30]

Peritoneal macrophages from BALB/c mice were obtained by previous elicitation of the mouse by a single intraperitoneal injection of 1 mL of 3% sodium thioglycollate, four days previous to macrophage harvesting by peritoneal lavage. After extraction, the macrophages were seeded in a 96-well microplate and maintained in RPMI 1640 plus 10% heat-inactivated fetal calf serum.

Bovine aortic endothelial cells (BAEC) (passages 4−8), kindly provided by the Santiago Lamas group ("Centro de Investigaciones Biologicas", Madrid, Spain), were cultured in RPMI 1640 plus 10% heat-inactivated fetal calf serum.

Peptide Synthesis. KF and its derived depsipeptides (Figure 1) were prepared by solid-phase synthesis (SPS) using an Fmoc chemistry strategy on CTC-resin, following our previously published methods.[27−29] CTC-resin and protected Fmoc-amino acid derivatives [Nal, 3-(2-naphthyl)-alanine; Phe-4 Cl, 3-(*p*-chlorophenyl)alanine; *p*-CF3Cinn, *p*-(trifluoromethyl)cinnamic acid; D-Cha, 3-(cyclohexane) D-alanine] were purchased from Luxembourg Industries (Tel Aviv, Israel). TFA (trifluoroacetic acid) and CH_3CN (HPLC grade) were purchased from Scharlau (Barcelona, Spain) and

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Panreac (Barcelona, Spain). DMF, DCM and methanol were obtained from SDS (Peypin, France). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was from Sigma Chemical Co. (St. Louis, MO). Bisoxonol [bis-(1,3-diethylthiobarbituric) trimethine oxonol], SYTOX Green, rhodamine 123, DMNPE-luciferin [D-luciferin, 1-(4,5-dimethoxy-2-nitrophenyl) ethyl ester] and BCECF-AM [2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein, triacetoxymethyl ester] were obtained from Molecular Probes (Leiden, The Netherlands). KF and its analogues were verified by analytical HPLC (high pressure liquid chromatography) on a Waters instrument 996 photodiode array detectors, equipped with a Waters 2695 separation module and Millennium software. The depsipeptides were purified by semipreparative RP-HPLC with a linear gradient of CH_3CN. MALDI-TOF (matrix-assisted laser desorption ionization with time-of-flight analysis) analyses of KF and its analogues were performed on an Applied Biosystems Voyager DE RP using 2,5-dihydroxybenzoic acid (DHB) matrix (peptide characterization is available in the Supporting Information).

Cell Proliferation Measurements. Promastigotes and amastigotes were harvested at late exponential phase, washed twice in Hanks' buffer, supplemented with 10 mM D-glucose (pH 7.2) (Hanks-Glc) at 4 °C, and resuspended in the same buffer at 2×10^7 cells/mL. These standard conditions were maintained for the rest of the experiments unless otherwise stated. Parasites (120 μL) were incubated with the drugs for 4 h at 25 or 32 °C for promastigotes and amastigotes, respectively. Afterward, 20 μL aliquots of this suspension were added to 1 mL of Hanks-Glc and centrifuged to remove unbound reagent. Washed parasites were resuspended in 100 μL of their respective growth medium, transferred into another 96-well microplate, and allowed to proliferate (48 h, 25 °C for promastigotes; 96 h, 32 °C for amastigotes). Finally, cells were washed with Hanks-Glc and resuspend in 100 μL of MTT solution (0.5 mg/mL) in Hanks-Glc. Substrate reduction was allowed to proceed for 2 h at 25 or 32 °C for promastigotes and amastigotes, respectively. Precipitated formazan was solubilized by addition of 100 μL of 10% (w/v) SDS solution and read in a 450 Bio-Rad ELISA microplate reader equipped with a 600 nm filter.[10] To measure the short-term effect, 80 μL (1.6×10^6 promastigotes) of the remaining parasites incubated with the peptide was washed with 1 mL of Hanks-Glc, resuspended and assayed for MTT reduction as above.[31]

Assay for Cytotoxic Activity against Murine Macrophages and BAEC. BALB/c peritoneal macrophages and BAEC were resuspended in their culture medium at a final density of 10^6 cells/mL, plated in a 96-well culture microplate (100 μL/well), and incubated with the distinct drugs for 24 h at 37 °C. Cytotoxicity was assessed using the colorimetric MTT reduction assay and expressed as the percentage of the value obtained for control cells.

Monitoring Changes in Plasma Membrane Potential. Changes in plasma membrane potential were monitored by the increase of bisoxonol fluorescence, an anionic potential-sensitive dye, after its insertion into the hydrophobic phospholipid matrix of the plasma membrane, once the cell became depolarized. The assay was performed under standard conditions, except for the inclusion of 0.1 μM bisoxonol in the incubation medium. Fluorescence changes were monitored in a Polarstar Galaxy microplate reader ($\lambda_{ex} = 540$ nm, $\lambda_{em} = 580$ nm). The results were normalized with respect to the depolarization achieved by 2.5 μM CA(1-8)M(1-18), a membrane-active peptide,[31] taken as 100%.

Promastigote Membrane Permeabilization. The procedure to assess the uptake of the vital dye SYTOX Green, adapted for *Leishmania* promastigotes, was used.[9] Briefly, parasites were incubated following standard conditions, except for the inclusion of 1 μM SYTOX Green in the incubation assay, 5 min prior to being placed (100 μL aliquots) into 96-well microplates. The increase in fluorescence, caused by the binding of dye to intracellular nucleic acids, was measured in a Polarstar Galaxy microplate reader using 485 and 520 nm filters for excitation and emission wavelengths, respectively. Full permeabilization (100%) was considered as that achieved after addition of 0.1% Triton X-100.

Bioluminescence Assays. Promastigotes from the *L. donovani* 3-Luc strain were resuspended in Hanks-Glc at 4×10^7 cells/mL; 50 μL aliquots of this suspension were transferred into 96-well microplates. An equal volume of a fresh solution of 50 μM DMNPE-luciferin was then added to the same medium. Once the luminescence reached a steady state, peptide was added and changes in luminescence, proportional to the intracellular ATP content, were monitored in a Polarstar Galaxy microplate reader fitted with luminescence optics, with the measurements averaged for every 4 readings.[10]

Electron Microscopy. After incubation with KF analogues for 4 h, promastigotes were washed twice in PBS, fixed in 5% (w/v) glutaraldehyde in PBS, included with 2.5% (w/v) OsO_4 for 1 h, and gradually dehydrated in ethanol [30, 50, 70, 90 and 100% (v/v); 30 min each], and propylene oxide (1 h). They were then embedded in Epon 812 resin and observed in a Philips 2200 electron microscope.[31]

Statistical Analysis. Data represent the mean of triplicate samples ± SD. LC_{50} values were calculated by SigmaPlot software version 9.0.

Results

Leishmanicidal Activity of KF and Its Analogues. The depsipeptide KF contains a lateral chain and cycled region (Figure 1) with modified amino acids such as the didehydroamino acid (Z)-Dhb 2, or with distinct stereoisomerism (D-Val 4, D-*allo*-Ile 7, *allo*-Thr 6). Overall, KF is a hydrophobic peptide with only two hydrophilic amino acids, Orn 8 and Thr 12, and a blocked N-terminal amino by amidation with the aliphatic acid 5-Mehex 14 (Figure 1).

The seven new KF analogues synthesized for this study preserved the basic KF structure, except replacement of specific positions by other amino acid analogues in order to explore their respective contribution to the biological activity.

Table 1. Lethal Activity of KF and Its Analogues on *Leishmania* Parasites and Mammalian Cells

	$LC_{50} \pm SD$ (μM)[a]				
	promastigotes		amastigotes		
drug	*L. donovani*	*L. pifanoi*	*L. pifanoi*	peritoneal macrophage[b]	BAEC[c]
natural KF	6.13 ± 0.16	8.31 ± 0.40	29.53 ± 1.07	10.23 ± 1.02	25.80 ± 0.11
[Phe-4Cl 3]-KF	3.02 ± 0.09	7.82 ± 0.07	11.04 ± 0.55	5.22 ± 0.18	14.01 ± 0.70
[Nal 3]-KF	3.04 ± 0.32	5.82 ± 0.07	5.01 ± 0.27	10.30 ± 0.38	10.02 ± 0.02
[D-Cha 13]-KF	3.12 ± 0.60	7.90 ± 0.10	5.56 ± 1.43	62.8 ± 0.01	36.4 ± 3.70
[*p*-CF3Cinn 14]-KF	2.53 ± 0.08	12.20 ± 1.10	8.59 ± 0.65	29.70 ± 6.60	31.71 ± 4.21
[D-Val 1]-KF	>50	>50	>50		
[Pro-9]-KF	>50	>50	>50		
[Glu-8]-KF	>50	>50	>50		
miltefosine	12.5		26.3	>100	
amphotericin B	0.08		0.2	>250	

[a] LC_{50}: the concentration that caused 50% inhibition of proliferation compared with control parasites. [b] From Balb/c mice. [c] BAEC: bovine aortic endothelial cells.

KF and its analogues were tested for their activity and plausible mode of action on promastigote and amastigote stages of *Leishmania*. Their respective LC_{50} (concentration at which the proliferation of the parasites was inhibited by 50%) values are shown in Table 1.

The two replacements in which the configuration of the residues was modified (L-Val and D-Pro at positions 1 and 9) resulted in loss of activity, thereby highlighting the relevance of conformation on activity. Our results stressed the importance of maintaining the positive charge (Orn 8), as its replacement by an anionic residue (L-Glu) led to the abrogation of the activity.

In general, amastigotes were more resistant to KF and its analogues than promastigotes. However, for the latter form in *L. donovani,* full inhibition was obtained at concentrations below 10 μM. At this concentration, none of the active analogues achieved complete lethality on *L. pifanoi* amastigotes, which required concentrations higher than 25 μM. Even at a concentration of 50 μM, parasites incubated with analogue [D-Cha 13]-KF still preserved 20% of viability. However, these differences were blurred or even reversed when the two stages of *L. pifanoi* were compared (Table 1).

To ascertain whether KF killed *Leishmania* through a cytostatic or cytocidal mechanism, the inhibition of MTT reduction was measured immediately after the 4 h incubation with the peptide (short-term effects), and compared with the inhibition of proliferation of the surviving parasites when grown in the absence of the depsipeptides, as described in Methods. The concentration dependence of both effects was similar in both forms of the parasite, as shown in Figure 2 for KF, as representative of the whole set of depsipeptides. This observation supports a leishmanicidal mechanism rather than transitory damage to the parasites.

Cytotoxicity on Mammalian Cells. Natural KF showed cytotoxic activity at the micromolar range against both peritoneal macrophages and BAEC (Table 1). Nevertheless, most of the KF analogues showed an improved therapeutic index, with LC_{50} for these cells types, ranging from 2- to 10-fold higher than those for *Leishmania*, except for [Phe-4Cl 3]-KF. [D-Cha13]-KF and [*p*-CF3Cinn 14]-KF showed the best performance with a therapeutic index close to 11 in amastigotes and their host cell, the macrophage.

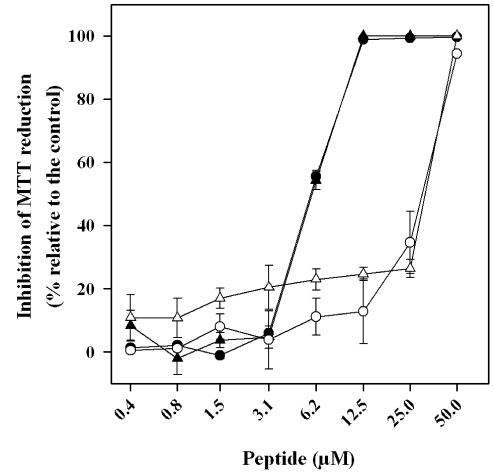

Figure 2. Activity of kahalalide F on *Leishmania* parasites. *L. donovani* promastigotes (filled symbols) or *L. pifanoi* axenic amastigotes (empty symbols) (2×10^7 cells/mL) were incubated with a range of peptide concentrations at 25 or 32 °C respectively. Inhibition of MTT reduction was assayed at 4 h (circles), or after proliferation of the survival parasites (triangle up) for 48 h (promastigotes) or 96 h (amastigotes). Values represent the mean of triplicate samples ± SD from a single experiment, representative of three separate experiments.

KF and Its Analogues Caused Permeation of the Plasma Membrane of *Leishmania donovani* Promastigotes. To determine whether the plasma membrane of the promastigotes was a major target for KF and its analogues, we performed two distinct but complementary experiments: depolarization and entrance of vital dyes. Depolarization is associated with the dissipation of ionic gradients across the plasma membrane, caused by the structural distortion of its phospholipid matrix by the depsipeptides, or, alternatively,

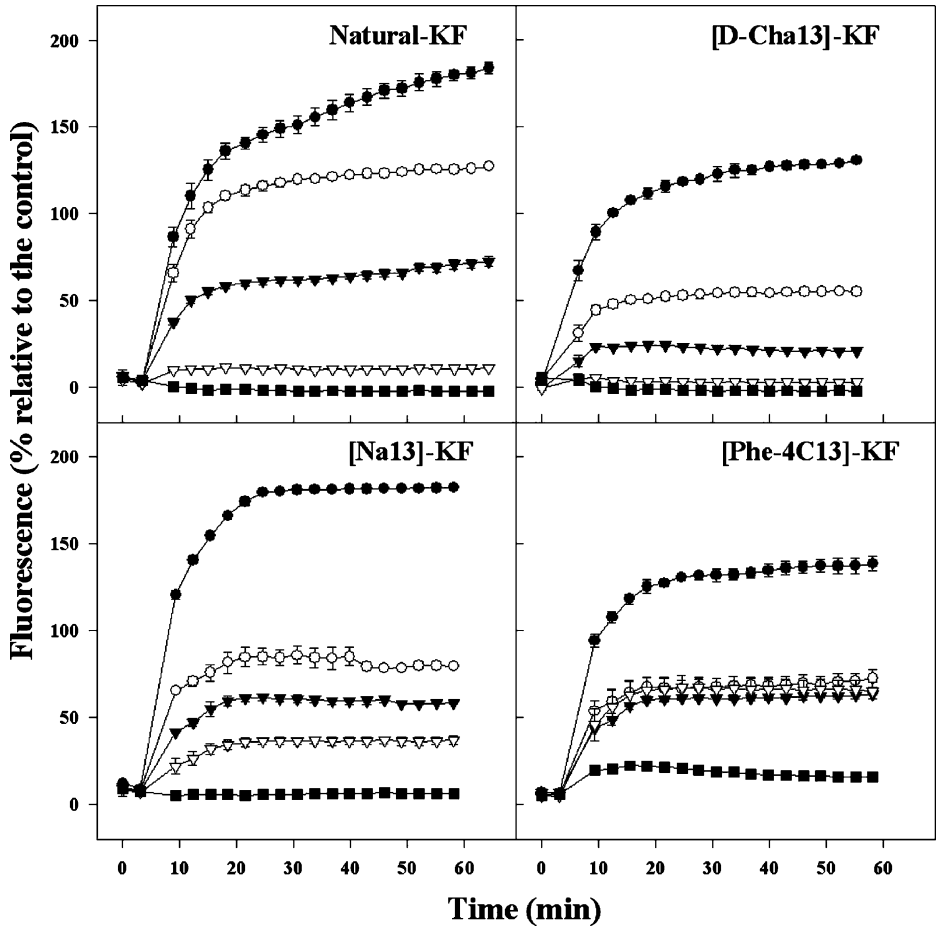

Figure 3. Depolarization of the plasma membrane of *L. donovani* promastigotes by KF and its analogues. Parasites (2×10^7 cells/mL) were equilibrated with 0.1 μM bisoxonol at 25 °C. The peptides were then added ($t = 0$ min) at the corresponding concentrations, and changes in fluorescence were monitored continuously ($\lambda_{ex} = 540$ nm, $\lambda_{em} = 580$ nm). Fluorescence data were normalized by comparison with the increase in fluorescence measured on parasites incubated with 2.5 μM CA(1-8)M(1-18) taken as 100%. Peptide concentrations: 3.12 μM (■), 6.25 μM (▽), 12.5 μM (▼), 25 μM (○) and 50 μM (●). Values represent the mean of triplicate samples from a single experiment, representative of three separate experiments.

by inhibition of pumps or channels involved in ionic transport and permeability.

All the active compounds caused a rapid decrease of the membrane potential in a concentration-dependent manner (Figure 3), as measured in *L. donovani* promastigotes. In this system, the threshold value concentration was 12.5 μM for KF, whereas for its active analogues this concentration was 2- to 4-fold lower. Analogue [*p*-CF3Cinn 14]-KF was the most active, with an onset at 3.12 μM. Interestingly, both natural KF and [Nal 3]-KF achieved a greater increase in fluorescence (>100%) than that obtained with 2.5 μM CA(1-8)M(1-18), a membrane-seeking peptide with high leishmanicidal activity.[30] Nevertheless, this perception is deceptive, as potential, and hence fluorescence, is related to the Nernst equation and thus to the logarithm of the ratio for extracellular and intracellular K^+ concentrations. Therefore, tiny variations at lower K^+ concentrations caused large changes in fluorescence.

To gain a better insight into the extent of membrane damage caused by KF and its analogues, we measured the influx of SYTOX Green ($M_r = 600$) into the parasites. The fluorescence of this dye is enhanced when bound to intracellular nucleic acids, a feature precluded in intact parasites, as the dye is unable to cross membranes. The entrance of SYTOX Green is passive and depends only on the presence of membrane discontinuities large enough to allow its entrance into the parasite cytoplasm. All the peptides tested induced a fast increase in fluorescence (Figure 4). Nevertheless, several differences were observed between the active analogues; full permeation required a concentration of 50 μM for [Na13]- KF, the same effect required a concentration of [Phe-4Cl 3]-KF four times lower. This analogue also displayed the highest kinetics; in contrast, KF was unable to reach full permeabilization even at 50 μM.

Of note, the extent of permeation did not run parallel to peptide concentration; rather there was a sharp increase

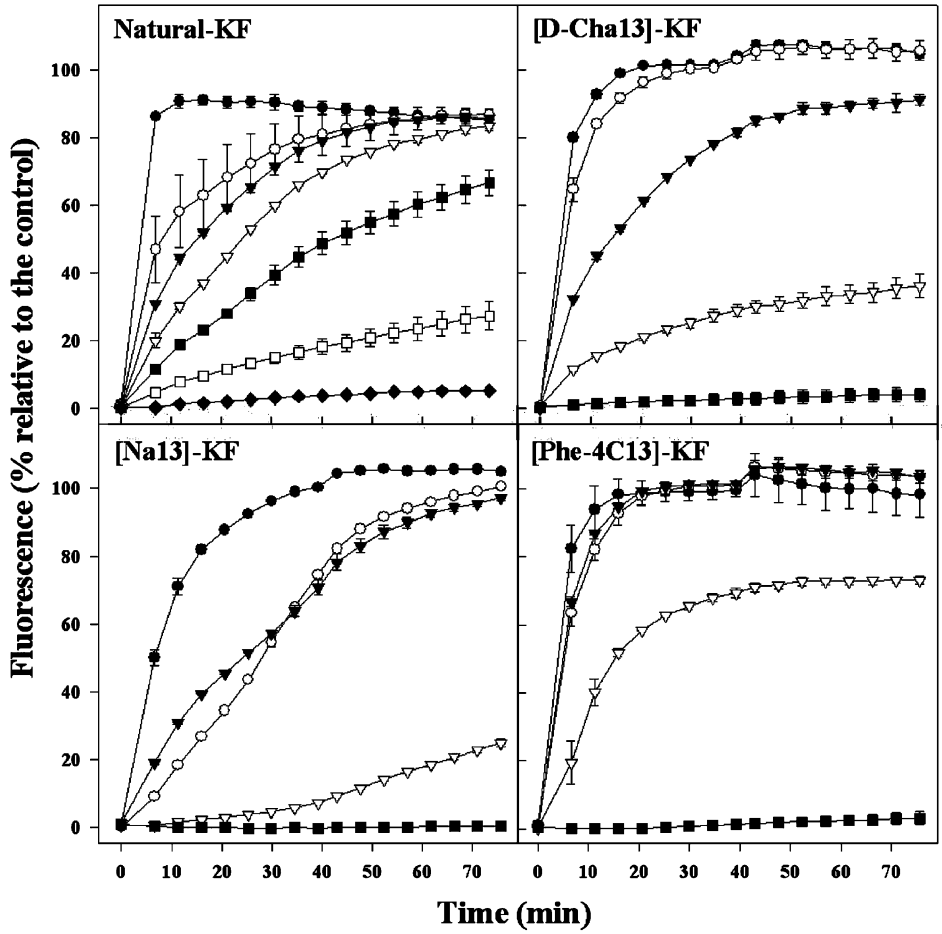

Figure 4. SYTOX Green influx into *L. donovani* promastigotes after addition of KF or its analogues. Parasites (2×10^7 cells/mL) were incubated with 1 μM SYTOX Green in Hanks-Glc. Once basal fluorescence reached a constant value ($t = 0$), the corresponding peptides were added, and the increase in fluorescence (λ_{ex} =485 nm, λ_{em} = 520 nm), due to dye binding to intracellular nucleic acids, was plotted as percentage of fluorescence relative to that of parasites fully permeabilized by 0.1% Triton X-100. Values represent the mean of triplicate samples from a single experiment, representative of three separate experiments. Concentrations were 0.78 μM (◆), 1.56 μM (□), 3.12 μM (■), 6.25 μM (▽), 12.5 μM (▼), 25 μM (○) and 50 μM (●).

at a given concentration, specific for each peptide. For instance, this transition occurred at a range of 6.25−12.5 μM for [Nal 3]-KF and [D-Cha l3], and for 3.12−6.25 μM, whereas for KF this discontinuity occurred in a more gradual manner, from 1.56 to 6.25 μM. On the basis of these results, it can be concluded that the disruption of the plasma membrane is sensitive to small structural variations, and probably involves supramolecular assemblies, triggered once a threshold concentration, specific for each peptide, is reached.

KF and Its Analogues Induce the Bioenergetic Collapse of the Parasite. All the depsipeptides assayed induced an abrupt drop in luminescence of the 3-Luc strain after addition (Figure 5). [Phe-4Cl 3]-KF was the most active, with a decrease in luminescence of ca. 40% at a concentration of only 1.56 μM. In contrast, the decrease in luminescence for [D-Chal3] was asymptotic to 40% at 6.25 μM, a concentration at which the rest of the peptides achieved almost full inhibition of luminescence. These parasites express cytoplasmic luciferase, whose luminescence affords a real-time follow-up of the intracellular ATP levels provided that the concentration of luciferin, the other substrate of the luminescence reaction, is not limiting. This requirement is fulfilled by the use of the free permeable caged substrate DMNPE-luciferin. Altogether these data substantiate a significant impairment of the bioenergetic metabolism of *Leishmania* after depsipeptide addition.

Transmission Electron Microscopy. To gain more complete data on the lethal subcellular effects of KF and its analogues, we carried out electron microscopy of *L. donovani* promastigote and *L. pifanoi* amastigote parasites treated with 5 μM of [Nal 3]-KF (Figure 6), a concentration that caused 90% inhibition of proliferation. Under these conditions, the parasite showed a fully permeabilized plasma membrane, with the cytoplasm full of vesicles translucent to electrons and also large vacuoles (middle panel). In some promastigotes, a huge intracellular vacuole

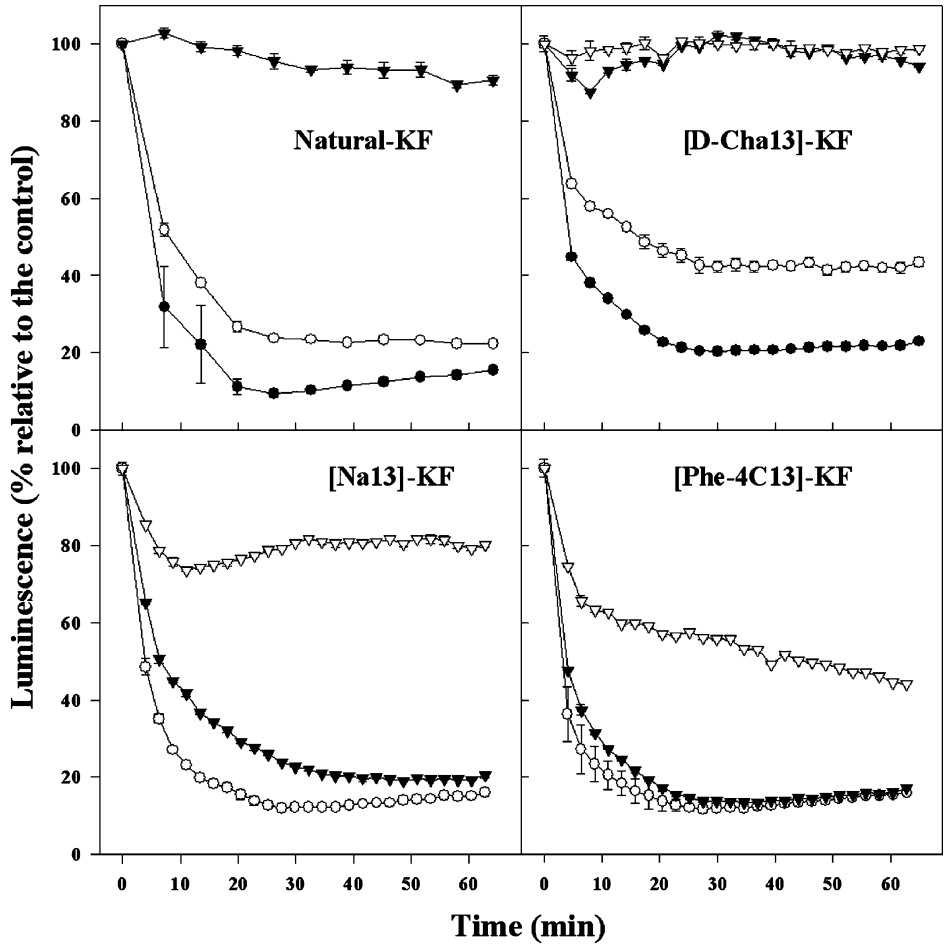

Figure 5. Real-time decrease in luminescence in living *L. donovani* promastigotes from 3-Luc strain after addition of KF or its analogues. Promastigotes (2×10^7 cells/mL) from *L. donovani* 3-Luc strain, expressing a cytoplasmic form of luciferase, were incubated with cell-permeable luciferin ester DMNPE-D-luciferin (25 μM), and luminescence was monitored until it reached a constant value. At this point ($t = 0$), the corresponding peptides were added at the corresponding concentration: 1.56 μM (▽), 3.12 μM (▼), 6.25 μM (○) and 12.5 μM (●). Luminescence decay was measured and corrected for that of control parasites. Values represent the mean of triplicate samples from a single experiment, representative of three separate experiments.

was observed. Interestingly, the acidocalsome, an organelle characterized by its electron-dense appearance as a result of its high concentrations of heavy metals, appeared mostly intact in comparison with the overall degeneration of the rest of the subcellular structures (see arrows in middle and right panels). These observations suggest some kind of membrane discrimination.

Discussion

Many of the leishmanicidal drugs currently in use derive from developments for other clinical applications, thereby profiting either from the existence of a shared target for both biological systems or simply by virtue of the same compound having distinct lethal mechanisms depending on the target cell. The antifungal amphotericin B or the antitumor miltefosine are the best examples of this strategy.[5,7]

KF shows cytotoxicity *in vitro* and antitumor activity *in vivo* in a number of tumor models.[32] Furthermore, this peptide shows antifungal activity.[19,23] Under these premises, we set out to study the leishmanicidal activity of KF and some of its analogues, which for KF was succinctly mentioned in an earlier publication.[23]

In a previous study,[33] 132 KF analogues, encompassing modifications on each position of the lead sequence, were synthesized and tested for antitumor activity on a panel of 14 cell tumor lines. This study prompted us to assay a

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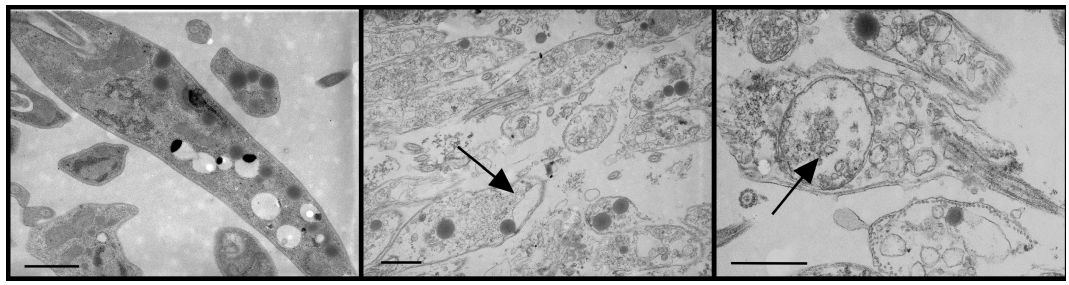

Figure 6. Electron microscopy of *L. donovani* promastigotes treated with the KF analogue [Nal 3]-KF. Parasites were incubated for 4 h with 5 μM of [Nal 3]-KF, a concentration that caused 95% lethality. Left panel: Control parasites. Middle and right panels: Promastigotes treated with 5 μM [Nal 3]-KF. Bar = 1 μm. Arrows in middle and right panels show the formation of large vacuoles inside the parasites treated with [Nal 3]-KF.

selection of seven analogues for their leishmanicidal activity on the two main stages of the parasite. The compounds were chosen in order to test the structural traits with greatest influence on tumoricidal activity on the basis of results from two recent studies.[29,33] This rationale includes changes in the residue configuration ([D-Val 1]-KF) and ([Pro 9]-KF), abrogation of the cationic character of KF ([Glu 8]-KF), variation of the side chain ([Phe-4Cl 3], [Nal 3]-KF and [D-Cha 13]-KF), or the hydrophobicity of the N-terminal hydrophobic tail ([*p*-CF3Cinn 14]-KF). The modifications were located in the three structural domains of KF: the macrolactone ring ([D-Val1]-KF, [Phe-4Cl 3], [Nal 3]-KF), the linear N-terminal stretch ([Pro 9]-KF, [Glu 8]-KF, and [D-Cha 13]-KF), or the hydrophobic tail at the N-terminus ([*p*-CF3Cinn 14]-KF).

In an initial test, three analogues ([D-Val1]-KF, [Pro 9]-KF and [Glu 8]-KF) showed no leishmanicidal activity under 50 μM and thus were discarded for further studies. Two major conclusions were drawn from this result. First, the configuration of the residues is critical for activity, as previously reported for tumoricidal activity of KF.[29,34] The enantiomeric substitutions were carried out in L-Val 1 (macrolactone ring) and in D-Pro 9 (linear stretch), that is to say, in the KF domains with a more defined conformation. The six-membered macrolactone ring showed an almost planar orientation, mostly due to the constraints imposed by the presence of (Z)-Dhb, an α-β dehydro amino acid, and strong β-turn inducer, in addition to another turn formed by L-Val 14 with the D-*allo* Thr 6-D-*allo*-Ile 5 and D-Val 4.[33] Accordingly, [D-Val1]-KF may affect the planar orientation of the six-member macrolactone ring, thereby precluding an active conformation. The alteration of the cycle conformation was previously tested in gramicidin S, a cyclic antibiotic peptide, and its analogues. The introduction of single diastereomeric substitutions in gramicidin S caused significant changes in its therapeutic index.[35,36]

The other diasteromeric change, [Pro 9]-KF, occurred inside the linear region of KF. Previous reports on KF structure describe this domain to comprise two distinct stretches, an unstructured N-terminus, spanning the first five components, followed by a tight β-type II turn formed by D-Val-D-Pro-L-Orn-D-*allo*-Ile where the [Pro 9]-KF substitution occurs. The maintenance of this structure imposes a strict configuration for each of its constituent residues,[33] and its modification, even in a single residue, leads to the loss of activity.

In this regard, the behavior of KF differed from the α-helical antibiotic peptides. For these, a single diastereomeric substitution usually entails only a modest increase in membrane specificity, hence of the target, and, as a general rule, two consecutive diastereomeric substitutions inside an α-helical region were required to achieve a significant effect on the cytotoxic activity in mammalian cells, whereas the microbicidal activity was scarcely affected.[37,38] Nevertheless, the epimerization of position 2 of the bombinin H2 (L-Ile for D-*allo*-Ile), a 27-mer antibiotic peptide from Amphibians, produced significant changes in its leishmanicidal activity and interaction with model membranes, despite its location in a fraying

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α-helical area, hence apparently innocuous to the overall architecture of the peptide.[11]

Finally, a major substitution with deactivation of the leishmanicidal activity occurred with [Glu 8]-KF, where the L-Orn at position 8 was replaced by L-Glu, with loss of the only cationic residue of KF at physiological pH. The cationic character of a membrane-seeking antibiotic peptide is essential for the discrimination between the own membranes of the producing organism from those of the pathogen. Prokaryotes, lower eukaryotes such as fungi and *Leishmania*, and tumor cells from higher eukaryotes expose anionic phospholipids at the outer leaflet of their cytoplasmic membrane,[39−41] thereby promoting their interaction with cationic peptides. In contrast, for higher eukaryotes this class of phospholipids is confined to the cytoplasmic leaflet, thereby ruling out their electrostatic interaction with the peptides.[42] In addition, L-Orn 8 was involved in the structure adopted by KF in SDS micelles.[33] Nevertheless, this minimalist electrostatic interpretation was challenged by more subtle traits in KF. Tumoricidal activity was preserved in many KF analogues with the δ-NH_2 group of Orn amidated, with loss of positive charge; in contrast, analogues with other modifications at the same residue, which preserved a net positive charge, were inactive on tumor cell lines.[29] A similar effect was described for reductive alkylation of the Orn δ-amino group for antifungal activity.[23]

In contrast to the full abrogation of leishmanicidal activity, the remaining four analogues showed improvement up to ca. 6-fold, such as [Nal-3]-KF and [D-Cha 13]-KF on amastigotes. All these analogues had increased hydrophobicity of the side chain, which could be explained by an increase in their interaction with the membranes, although changes to the secondary structure of KF cannot be ruled out. It is also well-known that increasing the hydrophobicity of a drug can enhance both its bioavailability (transport through membranes) and its protein/receptor binding. A change almost exclusively related to the increment in hydrophobicity is the substitution of the N-terminal 5-MetHex residue by *p*-(trifluoromethyl)cinnamic acid [pCF3Cinn 14]-KF. Interestingly, the improvement in activity for this modification depends on the stage of the parasite. Modification of leishmanicidal activity by fatty acid acylation has been reported for cecropin A−melittin hybrid peptides[4] and dermaseptins.[43] In both cases, the addition of a hydrophobic tail increases their leishmanicidal activity compared to the respective nonacylated form. Nevertheless, an excessive increase in hydrophobicity causes either aggregation of the peptide in solution, with loss of activity, or an increase in affinity for zwitterionic mammalian membranes with an increase in cytotoxicity.[44]

Regarding resistance to the peptides, amastigotes were more resistant than promastigotes, regardless of the peptide modification involved. This is a repeated trend for many linear antibiotic peptides active on *Leishmania*.[9,10,31,45,46] The basis underlying this effect is currently unknown; the proteolytic activity strongly differs between the two stages of the parasite, although it also depends on the species under study (Table 1). With its high percentage of D-amino acids plus the compactness of its structure, KF will presumably be impervious to proteolytic attack. Thus, either a distinct phospholipid composition or differential expression of a putative dedicated KF receptor/target is an alternative explanation for this effect. This will be also in tune with the differences observed in the activity of the peptide for the promastigotes belonging to two *Leishmania* species.

While subtle modification of the peptidic backbone of KF frequently leads to a loss of leishmanicidal activity, modifications performed on the side chain of the depsipeptide were much more tolerated, thereby allowing the modulation of the initial activity of KF, often with enhanced leishmanicidal activity as the final outcome.

Mechanism of Leishmanicidal Activity. There are several reports that associate the basis of the tumoricidal activity shown by KF with perturbation of the membrane, either plasmatic or the membrane of other organelles.[20−22]

In our hands, KF and its four active compounds showed membrane-perturbing activity on promastigotes, as assessed by two complementary techniques, membrane depolarization and entrance of vital dyes. No linear correlation was observed between leishmanicidal activity and membrane activity. As a general rule, short-term membrane-perturbing activity was detected at concentrations higher than their respective LC_{50}, except for natural KF. However, the electron microscopy of promastigotes after incubation with KF analogue [Nal 3]-KF at concentrations close to full lethality showed a massive loss of cytoplasmic material, with an intracellular space filled

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with empty vacuoles, thereby making it difficult to distinguish the original organelles. A possible explanation for this finding would be slow kinetics in the acquisition of a given active conformation of the KF monomers, or the formation of a supramolecular structure by aggregation of several monomers, which may be accelerated at higher peptide concentrations. It is interesting to note that this pattern differs from that of other antibiotic α-helical membrane-seeking peptides active on *Leishmania*.[9,46] For these, permeation showed a linear correlation with membrane disruption parameters, the final effect is usually reached within minutes, and the morphology of the plasma membrane of the promastigote is altered, with the appearance of large blebbing structures, which were absent in the micrographs for the KF analogue.

A landmark of our results is the rapid drop in luminescence after peptide addition, which reached its end-point in the first 10 min. This luminescence is related to free-cytoplasmic ATP; as such, it is a sensitive parameter to assess changes in the bioenergetic capacity of the parasite. As mentioned above, at these concentrations, plasma membrane permeability for SYTOX Green (MW = 600) was scarce or nil, except for natural KF. On the basis of this observation, we can discard the leakage of intracellular ATP through plasma membrane lesions being responsible for this effect. Hence, the inhibition of ATP synthesis by KF is the most likely origin of the decrease in luminescence. In *Leishmania,* this process is performed mostly by oxidative phosphorylation rather than glycolysis.[47] In fact, in the electron microscopy micrographs, mitochondria are difficult to identify, thus substantiating the feasibility of this process. A consequence of this rapid ATP depletion is the impossibility for cells to undergo apoptosis, as this process requires an adequate level of ATP. In fact, the tumoricidal activity of KF was related to oncosis rather than apoptosis.[48] Nevertheless, despite the similarity in their respective LC_{50} for the three KF analogues, the levels of luminescence inhibition at low concentrations differed greatly between them. While at 1.56 and 3.12 μM a visible drop from the initial values was evident for the two Phe 3 analogues, [Nal3]-KF and [Phe-4Cl3]-KF, for [D-Cha13]-KF this was nil at 3.12 μM. These observations mirror the variety of activities caused by subtle structural differences on several tumor cell lines,[29] as well as the feasibility of a multitarget lethal mechanism entailing several processes, each of which may contribute in a distinct manner depending on the structure of the analogue as well as on the cell target. In agreement with this premise, plasma membrane disruption cannot fully account for the leishmanicidal activity of KF. Other targets, such as interaction with specific structures and organelles, cannot be discarded. In tumor cells, the activity of KF has been linked to a number of processes: interference with the EGF signaling pathway through the expression levels of ErbB3, one of its receptors;[24] disruption of plasma membrane[21] and of lysosomes,[22,23] and more recently, to RPS25, a protein belonging to the 40S of the ribosome, as it shows a strong interaction with KF *in vitro*.[25] By genome mining, homologues for ErbB3 are absent in *Leishmania*, whereas a homologue for RPS25 has been described in *Leishmania infantum* (gi|51702155), as expected for the high ribosome conservation. With respect to the lysosomes, their role as targets is quite appealing for *Leishmania*. In fact, the amastigotes of species belonging to the *mexicana* complex, such as *L. pifanoi,* used in this work, possess megasomes (very large lysosomes), which contain a variety of acid hydrolases, the abundance of which is much lower in promastigotes.[49] The amastigotes should be the preferred target for KF rather than promastigotes, in contrast to what the experimental data show.

Another alternative mechanism is difference(s) in target(s) or mechanisms of action for KF in *Leishmania* and tumor cells, as occurs with miltefosine.[5,50] The correlation between tumoricidal and leishmanicidal activity for KF and its analogues assayed in this study is only partial. Thus, remarkable differences may be observed for [Glu 8]-KF, active on tumor cells but not on *Leishmania*, and [D-Cha13]-KF, which showed the opposite behavior.[29]

Finally, the development of KF or its analogues for their putative clinical use requires the adequate balance between their leishmanicidal activity and cytotoxicity on mammalian cells. Although the LC_{50}s of KF for diverse tumor cells span an order of magnitude, the LC_{50}s for leishmanicidal activity are inside the average range of its tumoricidal[13,19] and fungicidal activities.[23] The specificity of KF *in vitro* showed preferential cytotoxicity on tumor cells from 5- to 40-fold higher on transformed vs nontransformed cells. For the leishmanicidal activity of two analogues [D-Cha 13]-KF and [*p*-CF3Cinn 14]-KF, these differences reached values close to 11-fold between macrophages, the host cell for *Leishmania*, and the amastigote stage, responsible for the pathology in vertebrates. Furthermore, the clinical and preclinical trials performed through KF development as an antitumor drug[19,32,51−53] allow savings on the economic burden of the pharmacological and cytotoxicity studies required for

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the development of brand-new drugs from scratch. Although further *in vitro* and *in vivo* experiments are required, our data substantiate the feasibility of KF as a novel leishmanicidal drug with a new mechanism of action.

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Supporting Information Available: Table of characterization data for kahalalide F and its analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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