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The Antitumoral Depsipeptide IB-01212 Kills *Leishmania* through an Apoptosis-like Process Involving Intracellular Targets

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Abstract: IB-01212, an antitumoral cyclodepsipeptide isolated from the mycelium of the marine fungus *Clonostachys* sp., showed leishmanicidal activity at a low micromolar range of concentrations on promastigote and amastigote forms of the parasite. Despite its cationic and amphipathic character, shared with other membrane active antibiotic peptides, IB-01212 did not cause plasma membrane lesions large enough to allow the entrance of the vital dye SYTOX green (MW = 600), even at concentrations causing full lethality of the parasite. Having ruled out massive disruption of the plasma membrane, we surmised the involvement of intracellular targets. Proof of concept for this assumption was provided by the mitochondrial dysfunction caused by IB-01212, which finally caused the death of the parasite through an apoptotic-like process. The size of the cycle, the preservation of the *C*2 symmetry, and the nature of the bonds linking the two tetrapeptide halves participate in the modulation of the leishmanicidal activity exerted by this compound. Here we discuss the potential of IB-01212 as a lead for new generations of surrogates to be used in chemotherapy treatments against *Leishmania*.

Keywords: Marine depsipeptide; *Leishmania*; apoptosis; antibiotic peptide; mitochondria

Introduction

Leishmaniasis is one of the most important neglected tropical diseases, as recognized by the World Health Organization (http://apps.who.int/gb/ebwha/pdf_files/WHA60/A60_10-en.pdf). In clinical terms, it is a chronic protozoan infection caused by species belonging to the genus *Leishmania*. The disease ranges from self-healing cutaneous lesions to visceral infections, fatal if left untreated. *Leishmania* frequently behaves as an opportunistic pathogen in immunocompromised patients and has special relevance as an HIV co-infection.[1] Affecting approximately 12 million people worldwide, *Leishmania* has an incidence of 2 million cases. A successful vaccine against the disease remains

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elusive.[2] Therefore, chemotherapy is currently the only option available to fight the disease. However, the efficacy of this therapeutic approach is being increasingly eroded by the frequent high toxicity of first-line leishmanicidal drugs, increased incidence of resistant strains, and poor treatment compliance. Furthermore, the cost of treatment is unaffordable for most patients, who are found in underdeveloped areas with poor incomes.[3,4] Therefore, there is an urgent need to develop new and inexpensive therapeutic alternatives. This gap is partially filled by the strong tradition in *Leishmania* chemotherapy to profit from pharmaceutical developments obtained for other applications. In fact, many leishmanicidal drugs in distinct phases of development derived from this strategy, including some under current clinical use, such as the antifungal polyene amphotericin B,[5] the bactericidal aminoglycoside paromomycin,[6] or, more recently, miltefosine, the first successful oral drug against *Leishmania*, formerly developed as an antitumoral drug.[7,8]

In this regard, marine invertebrates and their symbiotic microorganisms provide an almost limitless source of drugs, some of which are under phase I−III clinical trials, while others have attained prescription as analgesic or antitumor agents (reviewed in refs 9 and 10). The repertoire of marine drugs includes a large number of antimicrobial peptides,[11−13] by far the most important components of innate immunity in invertebrates. Profiting from their previous pharmaceutical development programs as antitumoral drugs, some of these antimicrobial peptides, such as kahalalide F, dolastatin, and echinomycin,[14−16] have been further tested as antiprotozoal agents, in order to supply chemotherapeutic leads to the rapidly shrinking antiparasitic armamentarium. In this regard, we recently reported the leishmanicidal activity of kahalalide F, an antitumoral peptide of marine origin under clinical development, and a selected set of its analogues.[14]

Here we expand the repertoire of these new leishmanicidal leads by studying IB-01212, an antitumoral depsipeptide isolated from the mycelium of the marine fungus *Clonostachys* sp.[17] In addition, we studied a set of surrogates, for which key residues of antitumoral activity were substituted, with variation of the cycle size and symmetry. All the compounds resulted showed effective leishmanicidal activity at a micromolar concentration range, and their mechanism of action was associated with the depolarization of the mitochondrial electrochemical gradient ($\Delta\Psi_m$), which, in some cases, led to an apoptotic-like process.

Methods

Chemicals. MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] was purchased from Sigma-España (Madrid, Spain). Bisoxonol [bis-(1,3-diethylthiobarbituric) trimethine oxonol] and rhodamine 123 were from Invitrogen (Barcelona, Spain).

Peptide Synthesis. IB-01212 and the derived depsipeptides (Figure 1) were prepared by solid-phase synthesis (SPS) on CTC-resin using a Fmoc chemistry strategy, following our previously published methods.[18,19] All peptides were purified by semipreparative RP-HPLC with a linear gradient of 5−60% CH_3CN (+0.1% TFA) in H_2O (+0.1% TFA) over 30 min at a flow rate of 20 mL/min (Symmetry column, C_{18} reverse-phase column, 5 μm, 30 × 100 mm). Peptides were

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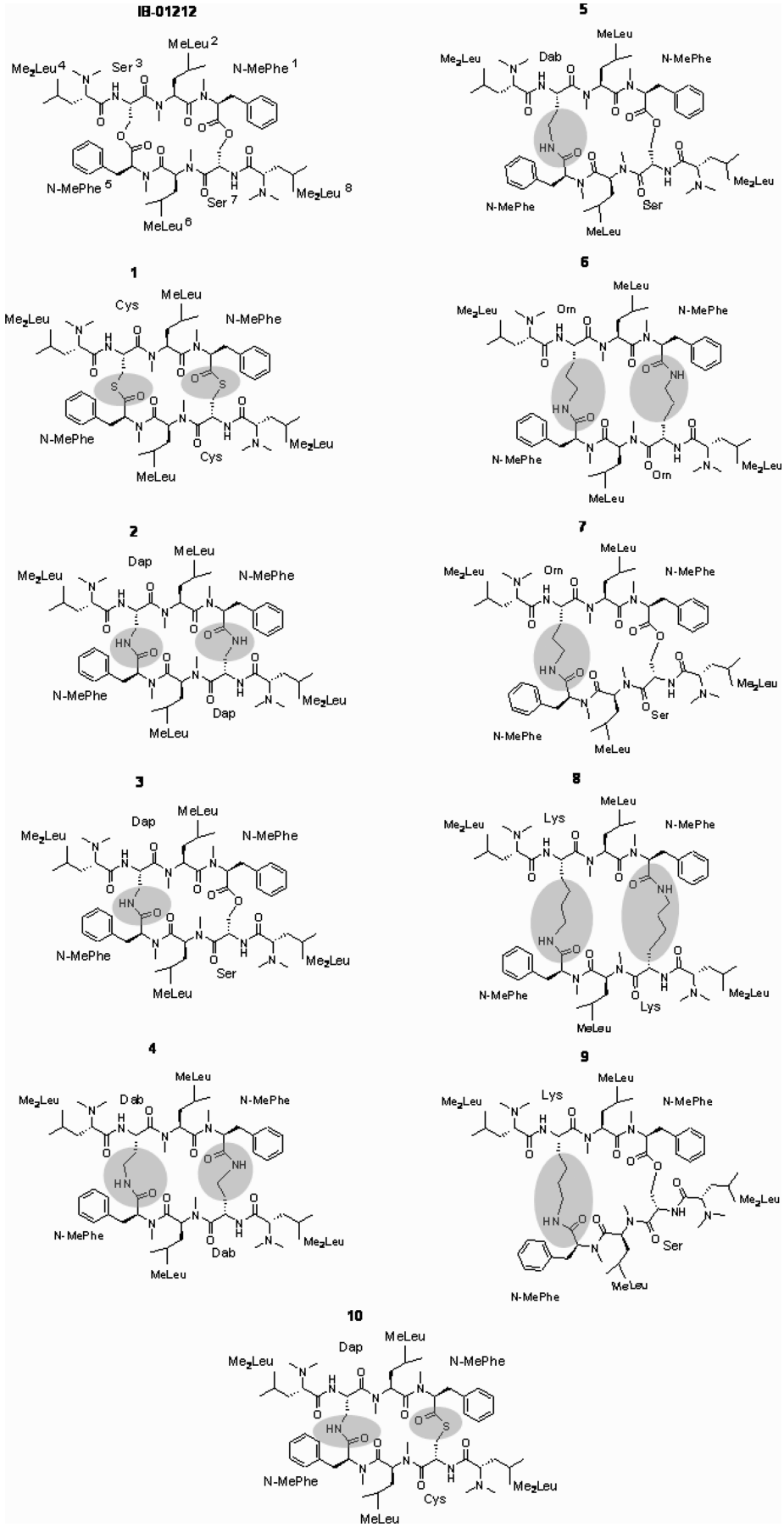

Figure 1. Structures of IB-01212 and its analogues.

Table 1. Characterization of IB-01212 and Its Analogues

code	t_R (min)	purity (RP-HPLC) (%)	mass calcd[a]	Mass exp (*m*/*z*) $[M + H]^+$, $[M + Na]^+$, and $[M + K]^+$
IB-01212	8.90	98%	1032.66	1033.32/1056.28/1072.24
1	7.21	92%	1064.62	1065.87/1087.88
2	8.32	91%	1030.69	1031.83/1053.81/1069.81
3	8.30	90%	1032.36	1033.27/1054.25/1070.00
4	7.40	95%	1058.73	1061.80/1083.10/1100.4
5	7.47	92%	1045.69	1046.69/1068.90/1084.88
6	7.59	95%	1086.76	1087.98/1109.94/1125.92
7	7.47	98%	1059.71	1061.00/1082.84
8	7.39	96%	1114.79	1115.92/1137.90/1153.88
9	7.62	92%	1073.73	1075.65/1098.69
10	7.39	95%	1047.66	1049.21/1072.20/1087.13

[a] The theoretical mass was calculated by ChemDraw.

analyzed by RP-HPLC on Symmetry C18 columns (4.6 × 150 mm, 5 μm) (Waters, Ireland), using a linear gradient of 5−100% of CH_3CN (+0.036% TFA) to H_2O (+0.045% TFA) run over 15 min at a flow rate of 1.0 mL/min, and detected at 220 nm on a Waters instrument 996 photodiode array detector equipped with a Waters 2695 separation module and Millennium software. MALDI-TOF analyses of IB-01212 and its analogues were performed on an Applied Biosystems Voyager DE RP instrument using 2,5-dihydroxybenzoic acid (DHB) matrix. Peptide characterizations are shown in Table 1. Peptide concentrations were determined by careful gravimetry.

Cell Lines. Promastigotes from *Leishmania donovani* (MHOM/SD/00/1S-2D), its derived line 3-Luc, and axenic amastigotes of *L. pifanoi* (MHOM/VE/60/Ltrod) were grown at 24 and 32 °C, as described previously.[20,21]

Peritoneal macrophages from BALB/c mice were obtained by previous elicitation by a single intraperitoneal injection of 1 mL of 3% (w/v) sodium thioglycollate, 4 days prior to macrophage harvesting by peritoneal lavage. After extraction, the macrophages were seeded in a 96-well microplate (10^5 cells/well) and maintained in RPMI 1640 plus 10% heat-inactivated fetal calf serum.

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 (HBSS-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. Aliquots (120 μL) of these suspensions were incubated with the drugs for 4 h at 25 and 32 °C for promastigotes and amastigotes, respectively. Afterward, 20 μL aliquots of these suspensions were added to 1 mL of HBSS-Glc and centrifuged to remove unbound peptide. Washed parasites were resuspended in 100 μL of their respective growth medium devoid of phenol red, transferred into another 96-well microplate, and allowed to proliferate (72 h, 25 °C for promastigotes; 96 h, 32 °C for amastigotes). Finally, 100 μL of MTT solution (1 mg/mL) in HBSS-Glc was added, and substrate reduction was allowed to proceed for 2 h at 25 and 32 °C for promastigotes and amastigotes, respectively. Precipitated formazan was solubilized by addition of 100 μL of 10% (w/v) SDS (sodium dodecyl sulfate) solution and read in a 450 Bio-Rad ELISA microplate reader equipped with a 600 nm filter.[21] To measure the short-term effect of IB-01212 and its analogues, 100 μL aliquots (2×10^6 promastigotes) of the same parasite suspension were taken after incubation with the peptide. They were then washed with 1 mL of HBSS-Glc, resuspended, and assayed for MTT reduction as above.

The induction of apoptosis by IB-01212 and its analogues was monitored by analysis of the sub-G_1 peak in these parasites. After 4 h of incubation with a range of concentrations of the peptides, parasites were transferred to a 24-well microplate and incubated overnight in their growth medium. Afterward, the promastigotes were stained with 50 μg/mL propidium iodide (PI), and individual cellular fluorescence was measured in a Beckman Coulter FC500 MPL cytofluorometer. As a positive control for apoptosis induction, parasites were treated under identical conditions with 15 μM miltefosine (hexadecylphosphocholine).[22]

Monitoring Changes in Plasma Membrane Potential. After cell depolarization, bisoxonol fluorescence, an anionic potential-sensitive dye, was inserted into the hydrophobic phospholipid matrix of the plasma membrane, and changes in plasma membrane potential were monitored. The assay was performed under standard conditions, except for the inclusion of 0.1 μM bisoxonol in the incubation medium. Variations in fluorescence were measured in a Polarstar

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Table 2. Leishmanicidal and Cytotoxic Activities of IB-01212 and Its Analogues

			$LC_{50} \pm SD$ (μM)		
compd	modification	bond	amastigotes *L. pifanoi*	promastigotes *L. donovani*	peritoneal macrophages
IB-01212	natural	ester	7.1 ± 0.4	10.5 ± 1.3	18.3 ± 0.1
1	2[Cys^3,Cys^7]	thioester	25.9 ± 2.8	49.1 ± 1.2	
2	2[Dap^3,Dap^7]	amide	9.0 ± 1.5	21.5 ± 0.9	39.6 ± 3.7
3	[Dap^3]	amide, ester	6.8 ± 1.3	21.8 ± 3.7	23.4 ± 0.3
4	2[Dab^3,Dab^7]	amide	22.8 ± 4.4	27.1 ± 1.2	
5	[Dab^3]	amide, ester	14.1 ± 5.1	30.7 ± 7.3	48.6 ± 5.0
6	2[Orn^3,Orn^7]	amide	9.1 ± 0.6	20.0 ± 0.1	
7	[Orn^3]	amide, ester	5.9 ± 0.7	19.8 ± 0.1	19.8 ± 0.1
8	2[Lys^3,Lys^7]	amide	>50	>50	
9	[Lys^3]	amide, ester	10.3 ± 1.3	26.8 ± 4.9	
10	2[Dap^3,Cys^7]	amide, thioester	6.4 ± 1.1	24.2 ± 2.4	

Galaxy microplate reader (λ_{ex} = 540 nm; λ_{em} = 580 nm). The results were normalized taking as reference for full depolarization (100%) that achieved by 5 μM CA(1-8)M(1-18), a membrane-active peptide.[23]

Variation of the Mitochondrial Membrane Potential ($\Delta\Psi_m$). Differential accumulation of rhodamine 123, a parameter directly related to the electrochemical potential of the mitochondrion, was used to monitor the variation of $\Delta\Psi_m$ in intact promastigotes after incubation with peptide.[23] To this end, promastigotes were incubated with the cyclodepsipeptides at a range of concentrations in HBSS-Glc for 4 h. Parasites, either untreated or fully depolarized by incubation with 10 μM FCCP, were used as controls. Dye uptake was measured in a Beckman Coulter FC500 MPL cytofluorometer (λ_{ex} = 488 nm; λ_{em} = 525 nm).

Bioluminescence Assays. Promastigotes from *L. donovani* 3-Luc were resuspended in HBSS-Glc at 2×10^7 cells/mL and incubated for 4 h with a range of concentrations of IB-01212, **2**, and **7**, selected because they showed the highest leishmanicidal activity on promastigotes. Afterward, 100 μL aliquots of these suspensions were transferred into black polystyrene 96-well microplates. An equal volume of a fresh solution of 50 μM DMNPE-luciferin in HBSS-Glc was then added to each well, and luminescence, proportional to the intracellular ATP content, was monitored in a Polarstar Galaxy microplate reader fitted with luminescence optics.[21]

Electron Microscopy. After incubation with IB-01212 or its 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 Jeol-1230 electron microscope (Jeol Ltd. Akishima, Japan).[21]

Cytotoxicity against Murine Macrophages. BALB/c peritoneal macrophages were resuspended at a final density of 10^6 cells/mL in culture medium devoid of phenol red. They were then plated in a 96-well culture microplate (100 μL/well) and incubated with the different peptides for 4 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.

Statistical Analysis. Data represent the mean of triplicate samples ± SD. LC_{50} values were calculated from the sigmoideal inhibition curves using the SigmaPlot 11.0 statistical package.

Results

Cytotoxicity against *Leishmania* Parasites. The capacity of IB-01212 and its analogues to inhibit MTT reduction by *Leishmania* parasites was used to assess the potential leishmanicidal activity of these compounds (Table 2). All peptides, except **8**, showed leishmanicidal activity on both parasite forms at low micromolar concentrations, being more active on the amastigote, the pathological form in vertebrates of *Leishmania*. IB-01212 showed the highest activity on promastigotes, with an LC_{50} value half that shown by the other analogues. However, when assayed on amastigotes, the pattern changed significantly. In general, for this parasite stage, monosubstituted analogues (**2**, **3**, and **7**) were more active than bisubstituted ones (Table 2), thereby suggesting that perturbation of *C*2 symmetry improves leishmanicidal activity. The nature of the chemical bond between residues *N*-MePhe-Ser (positions 1 and 7) and Ser-*N*-MePhe (positions 3 and 5) (Figure 1), forming the original depsipeptide linkages of the two tetrapeptide moieties, was relevant for modulating activity. The combination of amide and ester bonds to close the cycle (**3**, **5**, **7**, and **9**) resulted in a higher leishmanicidal effect than cycling exclusively through amide bonds (**2**, **4**, **6**, and **8**). Furthermore, within the whole set of compounds **7**, and to a lesser extent, **3** and **10** showed higher activity on amastigotes than IB-01212 (Table 2). This observation indicates that ring size affects the cidal effect, but with an upper limit represented by **8**, where substitution of Ser (positions 3 and 7) by two Lys residues abrogated the leishmanicidal effect. The combination of a 22-atom

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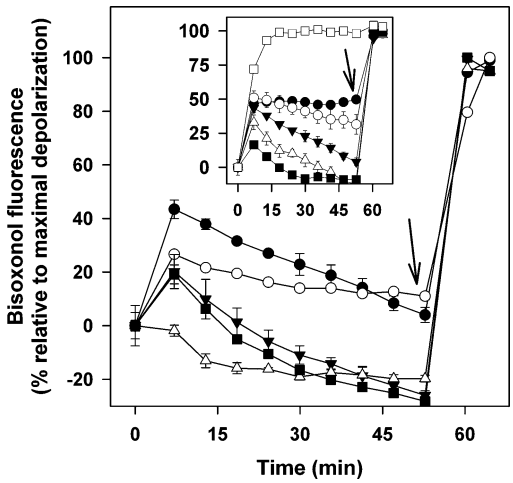

Figure 2. Depolarization of the plasma membrane of *L. donovani* promastigotes by IB-01212 and its analogues. Parasites (2×10^7 cells/mL) were equilibrated with 0.1 μM bisoxonol at 25 °C. The cyclodepsipeptides IB01212 (■), **2** (○), **3** (●), **5** (△), and **7** (▼) were then added (t = 0 min) at their corresponding LC_{50}, and changes in fluorescence were monitored continuously (λ_{ex} = 540 nm; λ_{em} = 580 nm). Fluorescence data were normalized relative to maximal depolarization achieved by addition of 5 μM CA(1-8)M(1-18) (arrow). Values represent the mean of triplicate samples from a single experiment, representative of three separate experiments. Inset shows the concentration-dependent variation of bisoxonol fluorescence after addition of **3**. Compound **3** concentrations (μM): 5 (■), 15 (△), 20 (▼), 40 (○), and 50 (●). Positive control of full depolarization: CA(1-8)M(1-18) 5 μM (□).

cycle, amide and ester bonds, and *C*2 asymmetry, displayed by **7**, resulted the most active structure to kill the amastigote (Table 2).

Preservation of Plasma Membrane Integrity. As IB-01212 shares cationicity and amphipathicity with typical membrane-active peptides, the integrity of the parasite plasma membrane after peptide addition was studied. With this aim, the variation of the plasma membrane potential was monitored by bisoxonol fluorescence. Figure 2 shows the increase in fluorescence, hence in plasma membrane depolarization, caused by depsipeptides at their respective LC_{50}. Compound **3** increased fluorescence up to 40% in a reversible manner, while the remaining compounds peaked at 20% at best. In all cases, depolarization was reversible, and initial values were recovered after 40 min and for some of them, such as **2** and **3**, with a moderate hyperpolarization, thereby reflecting a slight and transitory depolarization caused by discrete alterations in plasma membrane integrity. Compounds without any depolarizing effect were not represented. The effect and reversibility was concentration-dependent, as observed for compound **3** (Figure 3, inset). The possibility that an intrinsic drift of the system was responsible for this variation was ruled out, as the membrane-active leishmanicidal peptide CA(1-8)M(1-18)[23] induced full and stable depolarization for the time lag of the experiment.

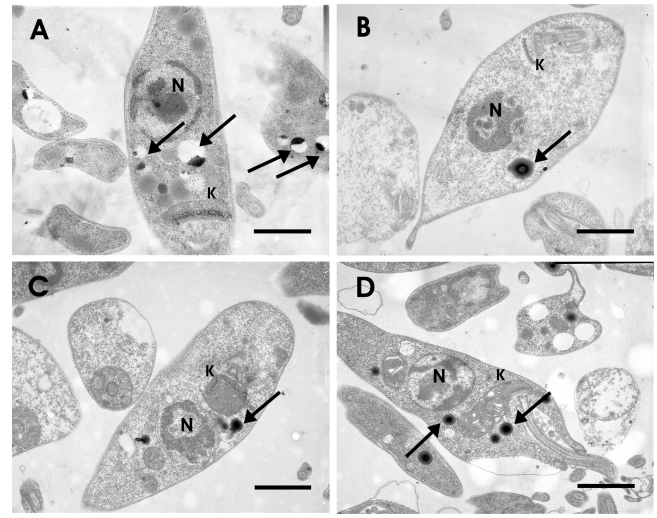

Figure 3. Transmission electron microscopy images of *L. donovani* promastigotes treated with cyclodepsipeptides. Parasites were incubated for 4 h with IB-01212 and its surrogates at their respective LC_{50} prior to sample processing. Panels: (A) Control parasites; (B**)** IB-01212; (C) **2**; (D) **7**. Bar =1 μm. (N) Nucleus, (K) kinetoplast. Arrows indicate acidocalcisomes.

Morphological Alterations Induced by IB-01212. In a further step, the morphological damage to promastigotes caused by IB-01212 and two of its analogues was visualized by transmission electron microscopy. Electron micrographs showed an apparently intact plasma membrane for promastigotes treated with IB-01212 and **2** at their respective LC_{50}, while this membrane was partially detached from its subpellicular layer of microtubules in parasites treated with **7** (Figure 3D). However, promastigotes treated with IB-01212, **2**, or **7** (Figure 3) showed an unstructured cytoplasm, with a slight, but not significant, reduction in the number of acidocalcisomes, which in turn showed an altered internal electron-dense core. In these parasites, a more irregular distribution of chromatin within the nucleus was also observed. Although IB-01212 and some of its analogues caused mitochondrial dysfunction in *Leishmania* (see below), no significant morphological alteration was observed in the first 4 h of incubation.

Alteration of Mitochondrial Functionality. In order to gain insight into the effects of cyclodepsipeptides on the parasite, we assessed the variation of the intracellular concentration of free ATP. To this end, the use of the 3-Luc strain allows the real-time monitoring of intracellular ATP concentrations in living promastigotes. IB-01212 and its active analogues at their respective LC_{50} did not reduce luminescence of the 3-Luc strain in the first hour after peptide addition, a time interval far longer than that required to observe changes with typical membrane-active peptides. However, after 4 h, cyclodepsipeptides caused a substantial decrease up to 80% of the luminescence level shown in untreated parasites (data not shown). This observation reflects a decrease in the intracellular ATP concentration, pinning down mitochondrial dysfunction as its most likely origin,

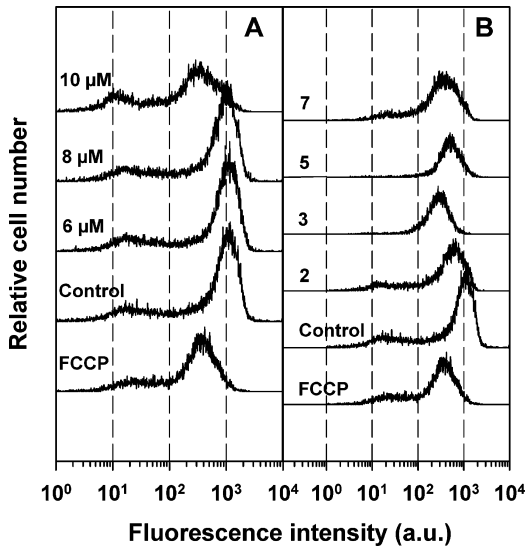

Figure 4. Variation of $\Delta\Psi_m$ of *L. donovani* promastigotes as monitored by rhodamine 123 accumulation. Parasites previously incubated with cyclodepsipeptides for 4 h and untreated cell controls were loaded with 0.3 μg/mL rhodamine 123 and their intracellular accumulation was measured by cytofluorometry. (A) Variation of $\Delta\Psi_m$ with IB-01212 concentration. (B) IB-01212 analogues at their respective LC_{50}. Full depolarized parasites were obtained by incubation with 10 μM FCCP.

as plasma membrane permeation, the other most likely alternative source to account for this effect, was discarded.

As oxidative phosphorylation is the main source of ATP production for both the promastigote and amastigote stages of *Leishmania*, $\Delta\Psi_m$ was measured after 4 h incubation with the cyclodepsipeptides. Figure 4A shows the dose-dependent decrease of rhodamine 123 accumulation, and hence of $\Delta\Psi_m$, after incubation with increasing concentrations of IB-01212. In parasites treated with IB-01212 at its LC_{50}, rhodamine 123 accumulation in promastigotes was almost identical to that of totally depolarized cells treated with 10 μM FCCP. Therefore, this finding points to the mitochondrion as a target for this peptide. The same trend was observed for the set of monosubstituted analogues and also for **2**, the most active cyclodepsipeptide within the bisubstituted group, at their respective LC_{50} (Figure 4B).

Leishmania killing by IB-01212 involves an apoptotic-like process. Given that the mitochondrion is a key component of the onset of apoptosis in *Leishmania*, we studied the induction of apoptosis by IB-01212 and three analogues ranking among those with the highest leishmanicidal activity.

At its LC_{50}, IB-01212 produced an increase in PI fluorescence in the sub-G_1 peak region, which accounts for the parasite population with fragmented chromatin, hence of apoptotic cells. This increase was similar to that obtained with HePC, a well-known apoptosis inducer in *Leishmania*[22] (Figure 5). Analogues **2**, **3**, and **7,** at their respective LC_{50}, also increased the cell percentage at the sub-G_1 peak region. However, **5** did not induce apoptosis (data not shown).

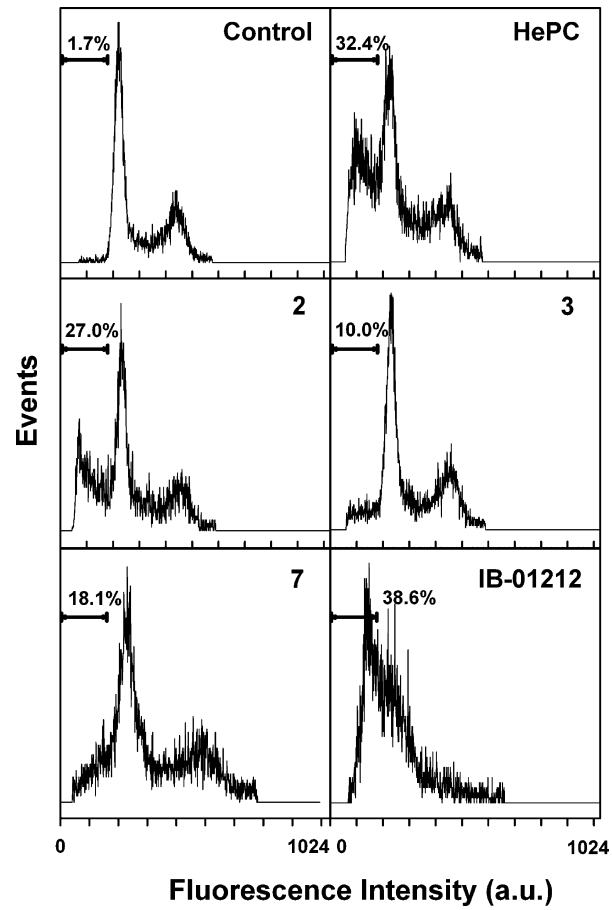

Figure 5. Cytofluorometric assessment of apoptosis in *L. donovani* promastigotes treated with IB-01212 and its analogues. The apoptotic population was identified by the sub-G_1 peak, characterized by chromatin fragmentation, hence with lower PI staining. The percentage of the cells under the sub-G_1 peak is indicated for each histogram. Parasites treated with 15 μM HePC (hexadecylphosphocholine, miltefosine) were used as positive controls of apoptosis.

However, as shown previously, it depolarized the parasite mitochondrion. Thus, another alternative mechanism of action involving this organelle might be expected.

Cytotoxicity of IB-01212 on Mammalian Cells. Natural IB-01212 at the micromolar range showed cytotoxic activity against peritoneal macrophages; however, its LC_{50} was 2.6-fold higher than that for amastigotes (Table 2). A slightly improved ratio of up to 4.4 was found for **2, 3**, **5**, and **7**. Thus, these compounds showed a lower toxicity than that of the parental peptide.

Discussion

Despite a significant number of marine peptides with antimicrobial activities,[13] those successfully tested as antiprotozoal agents are limited, with kahalalide F, dolastatin, and echinomycin being representative examples.[14−16] The clinical use of antimicrobial peptides is seriously hampered by their higher cost of synthesis compared with classical antibiotics. In this regard, mass production of IB-01212 is

less difficult than that of kahalalide F, as the former is synthesized by an isolated fungus and extracted from its mycelium.[17] In contrast, the natural production of kahalalide F depends on a complex symbiotic system, comprising the marine mollusk *Elysia* sp. and the alga *Bryopsis* sp. From a synthetic perspective, the full *C*2 symmetry of IB-01212, which is shared with two other marine bicyclic cyclodepsipeptides of bacterial origin (thiocoraline and triostin[24,25]), facilitates its synthesis by a stepwise or convergent strategy,[18,19] whereas for kahalalide F only the former is possible.

Although IB-01212 and kahalalide F show high biological stability, the mechanism underlying this feature differs substantially between these compounds. While the imperviousness of kahalalide F to proteolytic attack is due to the presence of strongly modified residues in its sequence, including D-amino acids, that of IB-01212 relies on its near to full N-methylation of peptidic bonds.

In order to expand the number of peptide leads available to fight protozoal infectious diseases, we studied the leishmanicidal activity of IB-01212, a cyclic depsipeptide isolated from the marine fungus *Clonostachys* sp.[17] On the basis of its structure, IB-01212 can be easily assimilated to a membrane-active antibiotic peptide as a result of its amphiphilic character, which is provided by the following features: (i) overall cationicity caused by the protonation of the two dimethylated N-terminal of Me_2Leu, together with the interchain esterification of the C-terminal N-MePhe carboxylic groups, which are engaged in the formation of the cycle; and (ii) strong hydrophobicity, coming from the four side chains of *N*-MeLeu and *N*-MePhe residues. The standard lethal mechanism for most membrane-active peptides relies on their insertion and further structural disruption of the phospholipid matrix of the cell membrane on the target cell, ensued by rapid plasma membrane depolarization and leakage of intracellular metabolites into the external medium,[26] as described for kahalalide F in *Leishmania*.[14] Nevertheless, this pattern did not fit the leishmanicidal mechanism of IB-01212, as this compound did not cause any large or steady plasma membrane depolarization, even at concentrations beyond full lethality [much less entrance of the vital dye SYTOX green (data not shown), for which a larger size of membrane lesion is mandatory]. Moreover, the decrease in intracellular ATP in promastigotes treated with IB-01212 or its analogues was observed only after 4 h of incubation. This observation contrasts with the dramatic decrease in intracellular ATP observed after addition of canonical membrane-active peptides.[27]

Accordingly, the involvement of intracellular targets of IB-01212 in *Leishmania* appears as a plausible option to increase the growing group of anti-infectious peptides acting on intracellular targets (reviewed in ref 28). This notion is consistent with the results obtained with other related IB-01212 analogues, endowed with intrinsic fluorescence because of a quinoline group in their structure, which were massively localized into the cytoplasm of the lung tumor cell line A 549.[29]

Our experimental results highlight the following three major features: (i) strong DNA fragmentation, assessed by cytofluorometry of the DNA content, with a concentration-dependent increase in the cell population in the sub-G_1 region, typical of apoptotic cells; (ii) decrease in $\Delta\Psi_m$; and (iii) progressive depletion of ATP, synthesized mostly by oxidative phosphorylation in *Leishmania*.[30] Altogether, they may be ascribed to type I programmed cell death (PCD), or apoptosis, in trypanosomatids.[31,32] This is a well-ordered, energy demanding process that preserves membrane integrity up to the late steps of this process. The other two PCD types were ruled out by the absence of extensive breakdown of plasma membrane, with subsequent permeabilization, a feature typical of necrosis. Moreover, there were no autophagosomes, double membrane structures surrounding organelles, the mainstay for autophagy.

The involvement of the mitochondrial pathway in the apoptosis of *Leishmania* is mandatory, as this parasite lacks the external pathway described in higher eukaryotes. Mitochondrial dysfunction was evidenced by a dose-dependent decrease of $\Delta\Psi_m$ as well as slow depletion of the intracellular ATP pool. Nevertheless, mitochondrial structure was mostly preserved even after 4 h incubation with IB-01212, the same time range at which the two former parameters were strongly inhibited. This observation is in sharp contrast to the effects observed for other leishmanicidal drugs acting on this organelle under similar conditions, such as loss of mito-

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chondrial cristae definition caused by the human antibiotic peptide histatin 5,[33] and massive swelling of mitochondria produced by benzophenone bisphosphonium compounds.[34]

Although the leishmanicidal mechanism of IB-01212 has been partially ascertained, the basis for its differential activity on the two main forms of the parasite remains unsolved. Amastigotes, the pathological form of the parasite in vertebrates, were more susceptible than promastigotes, the invertebrate vector stage of *Leishmania*, to the effects if this compound. This observation could be attributable to differential peptide accumulation inside the parasite, although the uptake of IB-01212 by *Leishmania* and its putative machinery is, at present, unknown. For tumoral cells, a dedicated receptor was invoked to account for the distinct tumoricidal activity shown by the IB-01212 analogues.[19] Nevertheless, a more unspecific entry cannot be ruled out. Similar to other cationic and amphiphilic antibiotic peptides acting on *Leishmania*, IB-01212 may gain access to the intracellular space by translocation across the plasma membrane, through transient peptide-phospholipid complexes.[26] This process is enhanced by plasma membrane potential, which is higher for *Leishmania* than for many typical mammalian resting cells,[23,35,36] but not between the two stages of the parasite. We also surmise that the significant metabolic and proteomic changes between promastigotes and amastigotes[37−39] may imply stage-specific expression of the IB-01212 target(s), thereby accounting for the differential susceptibility of the two stages to IB-01212 and its analogues.

To gain insight into the main features that modulate the leishmanicidal activity of IB-01212, a set of surrogates, previously tested for tumoricidal activity, were assayed on the parasite. Our data demonstrated that size of the cycle, nature of the chemical bonds involved in the linkage between the two tetrapeptide halves, and preservation of the *C*2 symmetry influenced the leishmanicidal activity of these compounds. Nevertheless, it must be kept in mind that these parameters are not fully independent of each other. The combination of amide and ester bonds closing the ring, together with the loss of *C*2 symmetry found in **7**, improved leishmanicidal activity on amastigotes relative to the parental peptide. Ring size and *C*2 asymmetry are determinant parameters in the cytotoxic potency of gramicidin S and its analogues,[40−42] in which rings with an odd number of carbon molecules perform better than their symmetric counterparts. Moreover, FAJANU, a closely related analogue of IB-01212 lacking *C*2 symmetry, shows greater antitumoral activity than its parental peptide.[29] Unfortunately, this asymmetry implies a more complicated strategy of synthesis.

Although gramicidin S shows a robust conformation based on the formation of a β-sheet,[42] for IB-01212 the structural constraints were dictated by the rigidity imposed on the peptidic bond by N-methylation in 6 out of its 8 residues. In fact, non-N-methylated analogues showed a strong decrease in tumoricidal activity.[19] In our hands, the higher flexibility conferred by a larger cycle size improved the leishmanicidal activity of these cyclodepsipeptides. However, there was an upper limit to this improvement, since replacement of the Ser residues by Lys to obtain the largest cycle (**8**) led to full abrogation of leishmanicidal activity.

In IB-01212, when the original ester linkages between the hydroxyl group of Ser residues at positions 3 and 7 with the C-terminal carboxyl group of N-Me-Phe at positions 1 and 5, respectively, were replaced by a thioester bond through substitution of the two Ser residues by Cys in surrogate **1**, the activity dropped 4 times relative to that of the natural peptide. A similar inhibitory effect was reported for this substitution on the tumoricidal activity of IB-01212 assayed on several carcinoma cell lines.[19] This loss was attributed to the higher reactivity of the thioester vs ester bond. Nevertheless, this explanation cannot fully account for inactivation in *Leishmania*, as the redox system of the parasite is performed by trypanothione,[43] which has a higher

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redox potential than that of glutathione, the corresponding molecule in charge of the redox environment in mammals. Under these premises, a higher inactivation would be expected in *Leishmania*.

In view of the antitumoral and leishmanicidal activities shown by the set of cyclodepsipeptides, the question arises as to whether promastigotes and amastigotes, two substantially different susceptible types of cells, share similar targets. In this regard, a significant number of antitumoral compounds exert leishmanicidal activity.[8] Unfortunately, the answer has been elusive until now because of lack of knowledge regarding the targets of this kind of compound in the two cell types. Nevertheless, structure−activity relationship studies did not show dramatic overlapping between these two cell types.

To sum up, IB-01212 is a new marine cyclodepsipeptide that shows intrinsic leishmanicidal activity; the presence of modified residues, plus methylation of six amino groups, increases the stability of the peptide in biological fluids and also structural rigidity. Several factors were found to have a significant influence on the leishmanicidal activity of IB-01212. This compound induces an apoptosis-like process without significant permeabilization of the plasma membrane, thereby suggesting the involvement of an intracellular target. If this were so, IB-01212 would provide a new lead for a cell-penetrating peptide (CPP) with only eight residues and with greater biological stability than other linear CPPs. Experiments to elucidate the potential targets of IB-01212 and improved analogues are in progress.

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