

This is the peer reviewed version of the following article:

Enterocin AS-48 as Evidence for the Use of Bacteriocins as New Leishmanicidal Agents.
Abengózar MÁ, Cebrián R, Saugar JM, Gárate T, Valdivia E, Martínez-Bueno M, Maqueda M, Rivas L.
Antimicrob Agents Chemother. 2017 Mar 24;61(4).

.

which has been published in final form at <https://doi.org/10.1128/AAC.02288-16>

TITLE: The enterocin AS-48 as an evidence for the use of bacteriocins as new leishmanicidal agents.

Authors: María Ángeles Abengózar¹; Rubén Cebrián²; José María Saugar^{1,3}; Teresa

Gárate³; Eva Valdivia²; Manuel Martínez-Bueno²; Mercedes Maqueda², and Luis Rivas^{1*}

¹Centro de Investigaciones Biológicas (CSIC), Physico-Chemical Biology Department

Unidad Asociada Interacciones, Metabolismo y Bioanálisis CSIC-CEU, Ramiro de Maeztu 9, 28040, Madrid, Spain.

²Departamento de Microbiología, Facultad de Ciencias, Universidad de Granada. Av. de la Fuente Nueva s/n. 18071 Granada, Spain.

³Helminth Unit, Parasitology Department, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Crtra. Majadahonda-Pozuelo, km 2.2, 28220, Majadahonda, Madrid, Spain.

SHORT TITLE: Leishmanicidal activity of the bacteriocin AS-48.

Keywords: Enterocin AS-48, intracellular parasite, antimicrobial peptide, bioenergetics.

***Corresponding author:**

Luis Rivas

Centro de Investigaciones Biológicas (CSIC) c/ Ramiro de

Maeztu 9, 28040-Madrid (Spain) e-mail:

luis.rivas@cib.csic.es

Phone: +34-918373112(4234)

ABSTRACT:

We report the feasibility of enterocin AS-48, a circular cationic peptide produced by *Enterococcus faecalis*, as a new leishmanicidal agent. AS-48 is lethal on *Leishmania* promastigotes as well as on axenic and intracellular amastigotes at low micromolar concentrations, with scarce cytotoxicity on macrophages. AS-48 induced a fast

bioenergetic collapse of *L. donovani* promastigotes, but only a partial permeation of their plasma membrane with limited entrance of vital dyes, even at concentrations beyond its full lethality. Fluoresceinated AS-48 was visualized inside parasites by confocal microscopy, causing mitochondrial depolarization and reactive oxygen species production. Altogether, AS-48 appeared with a mixed leishmanicidal mechanism that includes both plasma membrane permeabilization as well as additional intracellular targets, being mitochondrial dysfunctionality of special relevance. This complex leishmanicidal mechanism of AS-48 persisted even for the killing of intracellular amastigotes, as evidenced by transmission electron microscopy. We demonstrated the potentiality of AS-48 as a new and safe leishmanicidal agent, expanding the growing repertoire of eukaryotic targets for bacteriocins, and constitutes a proof-of-mechanism for the search of new leishmanicidal bacteriocins, whose diversity constitutes an almost endless source for new structures at moderate production cost and well-established safe use on food preservation.

Keywords: Enterocin AS-48, intracellular parasite, antimicrobial peptide, bioenergetics

INTRODUCTION

The current antibiotic crisis led to a ramping decline of effective drugs against infectious diseases, including those caused by protozoans (1, 2). This shortage of chemotherapeutical resources is of special concern for the so called neglected tropical diseases (NTDs) (3), whose treatment is almost exclusively limited to their already meager chemotherapeutic arsenals, and with a pipeline of new leads barely populated (4). As a result, treatments for NTDs are extremely vulnerable to the loss of effectiveness of just a single drug.

In this regard, leishmaniasis is a case in point. It is a protozoan disease caused by infection with different species of the genus *Leishmania*. Leishmaniasis encompasses a broad spectrum of clinical symptoms with an incidence of 10-12 million people infected worldwide, mostly in tropical or subtropical regions (5). Nowadays, its clinical treatment is based on six drugs, which are threatened by growing resistance, severe side-effects, and implementation cost (6). In the short term, combination therapy (7) and drug repurposing (8) may temporally delay this menace until new leads were approved for their clinical usage.

In the quest for new anti-leishmanial agents, several eukaryotic antimicrobial peptides (EAMPs) were successfully assayed at a preclinical stage on this protozoan (9, 10). Many EAMPs kill the target organisms by disruption of the phospholipid matrix of the membrane, mediated through their privileged interaction with the anionic phospholipids exposed at the external medium, with ensuing loss of the membrane function as permeability barrier (11, 12). Membrane-active EAMPs constitutes an appealing alternative to the shortage of antibiotics, due to their broad spectra of susceptible pathogens and extremely scarce induction of resistance respect to the classical antibiotics (13).

Interestingly, membrane disruption of the targeted cell is also the final lethal outcome for many antibiotic bacterial peptides, either encoded by genes as bacteriocins, or synthesized by the non-ribosomal peptide synthases.

In the last years, there was an increasing awareness of bacteriocins as a new source of anti-infective agents (14-16), even transcending those infections from a bacterial origin (antifungal or antiviral),

substantiated by their staggering structural diversity, feasibility of genetic engineering, and their safe use as food biopreservatives (17, 18), and their safe toxicity profile on higher eukaryotic cells (19). In this trend, the number of reports concerning bacteriocins as antiprotozoal agents (20-23) is surprisingly low when compared with other eukaryotic targets such as fungi (24-26), or even tumoral cells (27-29).

With this background in mind, we sought to investigate the leishmanicidal activity of AS-48, a 70 residue circular bacteriocin (MW= 7.149 kDa), highly cationic (pI = 10.09) and with an amphipatic structure (30, 31). The choice of AS-48 for this goal obeys to its low immunogenicity (32), and to its wide bactericidal activity on Gram positive bacteria (17, 30). AS-48 is a highly compact and stable molecule (31), preserving its activity under harsh environmental conditions, as such suitable for effectiveness either inside the parasitophorous vacuole of the macrophage where the amastigote dwells, or under tropical climate conditions. In addition, AS-48 does not require a cognate receptor at the target membrane, being active even on planar (33) and liposomal model membranes (34). Furthermore, AS-48 has an excellent record to prevent human food spoilage (17). More importantly, a patent was issued for the use of AS-48 against acne and other skin bacterial infections (35), paving the way for a clinical application of this bacteriocin on the ulcers of non-disseminated cutaneous leishmaniasis.

Altogether, AS-48 was lethal on both axenic promastigotes and amastigotes of

Leishmania at low micromolar concentrations with scarce toxicity on the host cell. Furthermore, it was active on intracellular amastigotes without prior encapsulation, a rare feature in bacteriocins. This work is a proof-of-mechanism for the use of membrane-active bacteriocins as a new group of leishmanicidal agents, with poor host cytotoxicity, and affordable costs of production.

MATERIALS AND METHODS

Reagents. All reagents were of the highest quality available and purchased from Sigma-Aldrich (Madrid, Spain), unless otherwise stated. Foetal calf serum was obtained from Gibco-BRL (Paisley, UK). Bisoxonol (bis-(1,3-diethylthiobarbituric) trimethineoxonol), DAPI (4',6-diamidino-2-phenylindole), propidium iodide (PI), Rhodamine123 (Rho123) [2-(6-amino-3-imino-3*H*-xanthen-9-yl) benzoic acid methyl ester, chloride], were purchased from Invitrogen (Carlsbad, CA). DMNPE-luciferin (D-Luciferin, 1-(4,5-dimethoxy-2-nitrophenyl)ethyl ester) were obtained from GoldBio (St. Louis, MO).

Cells. *Leishmania donovani* promastigotes (strain MHOM/SD/00/1S-2D) were grown at 25 °C in RPMI-HIFCS (RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (HIFCS)). Growth of 3-Luc promastigotes, with episomal expression of a cytoplasmic form of *Photinus pyralis* luciferase, was carried as above except for the addition of 30 µg/ml Geneticin (G-418; Gibco-BRL) into the growth medium. *L. pifanoi* axenic amastigotes (strain MHOM/VE/60/Ltrod) were grown at 32 °C in 199 medium (Gibco-BRL) supplemented with 20% HIFCS, as described (36). This cell line was selected due to its well-established record of similarity between axenic- and lesion-amastigotes, as evidenced by a set of morphological, antigenic, biochemical, and metabolic markers, which were maintained through long-term culture (reviewed in (37)). This ensures the homogeneity of the target organisms throughout the whole set of experiments. Quantitation of

intracellular parasites is easily achieved inside the large and distended parasitophorous vacuole in the macrophage, a feature shared by all the *Leishmania* species of the *mexicana* complex, that also encompasses *L. amazonensis* and *L.*

venezuelensis, with close pathological (38) and genetic (39) proximity to *L. pifanoi*.

Cells of the tumoral murine macrophage line RAW 264.7 (Cell Culture Facility, CIB, March, 2015) were grown in the same medium as *L. donovani* promastigotes, at 37 °C in a 5% CO₂ atmosphere.

AS-48 purification. AS-48 was purified from cultures of the *Enterococcus faecalis* UGRA10 strain (40) grown on Esprion-300 (E-300; DMV Int., Veghel, Netherlands) medium supplemented with 1% glucose (E-300-G) under a controlled pH of 6.5 (41). The resulting supernatant was concentrated, and AS-48 purified to homogeneity by cationic exchange chromatography followed by RP-HPLC, as described (42). Protein concentration of the purified AS-48 was determined by UV absorption at 280 nm.

Assessment of leishmanicidal activities. Parasites were harvested at late exponential phase of growth, washed twice in Hanks' balanced salt solution supplemented with 10 mM D-glucose, pH=7.2 (HBSS-Glc) at 4 °C, and resuspended in the same medium. Assays were carried out at 2×10^7 cells/ml in HBSS-Glc medium. Parasites were incubated for 4 h with the corresponding AS-48 concentration at 26 °C or 32 °C for promastigotes or amastigotes, respectively. These conditions were defined as the standard assay, unless otherwise stated.

Parasites resuspended in HBSS-Glc were aliquoted into a 96 microwell plate (120 µl/well) and incubated with AS-48 under the standard conditions described above. After the incubation with AS-48, 20 µl of each well were transferred into a replica microplate containing 180 µl/ well of the corresponding growth medium. The surviving parasites were allowed to proliferate (promastigotes: 72 h, 25 °C; axenic amastigotes: 120 h, 32 °C), and evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction. To this end, MTT was added to each well (0.5 mg/ml final concentration), and its reduction by the cells was allowed to proceed for 2 h. The resulting formazan was solubilized by addition of SDS (0.5 % final concentration), and read at 595 nm in a 680 microplate reader (Bio-Rad). Short-term effects of the bacteriocin on the parasites were evaluated by adding MTT into each well of the plate containing the remaining parasites

(100 µl) immediately after the 4 h incubation with AS-48. Cytotoxicity of AS-48 on RAW

264 cells were assayed by MTT reduction. Cells were seeded in a 96 microwell plate (5×10^4 cells/well); once adhered, medium was replaced by HBSS-Glc, and AS-48 added. After incubation with the bacteriocin for 4 h, the cells were washed and inhibition of MTT reduction measured as above.

Leishmanicidal activity of AS-48 on RAW 264.7 macrophages infected with *L.*

***pifanoi* amastigotes.** Cells of the tumoral murine cell line RAW 264.7 were resuspended in

RPMI-HIFCS and seeded into sterile coverglasses at the bottom of a 24-well microplate

(2.5×10^5 cells in 500 µl/well). Cells were allowed to attach overnight (37 °C, 5% CO₂).

Afterwards, *L. pifanoi* axenic amastigotes infection was carried out at a 2:1 amastigote:macrophage ratio. Once phagocytosed (4 h, 32 °C, 5% CO₂) the not internalized amastigotes were removed by washing. Finally, 1 ml of RPMI + 2% HIFCS was added to each well and intracellular amastigotes were allowed to proliferate for 72 h at 32 °C. Then, AS-48 was added into the plate and incubated for 48 h. Finally, coverglasses were removed and stained with Giemsa. Percentage of infected macrophages and average number of amastigotes per macrophage, were calculated by counting at least 500 macrophages per sample in four different fields of the preparation in an optical microscope.

Real-time variation of intracellular ATP in living *L. donovani* promastigotes by AS-48. The procedure described previously (36) was followed. Briefly, promastigotes from the 3-Luc strain, expressing a cytoplasmic firefly luciferase, were incubated (2×10^7 cells/ml) with AS-48 under the standard conditions at 25 °C in the presence of the 25 μ M DMNPE-luciferin, a membrane-permeable caged substrate of luciferase. Under these conditions, the limiting substrate for luminescence output of living parasites is their free cytoplasmic ATP. Once the luminescence reached a steady value, AS-48 was added and variation of luminescence was followed in a Polarstar Microwell plate reader (Galaxy, Offenburg, DE) using the luminescence setting for reading.

Permeabilization of the plasma membrane of *L. donovani* promastigotes by AS48. Two complementary parameters were measured (36), plasma membrane depolarization and entrance of vital dyes.

Variation of the plasma membrane potential was monitored using the anionic dye bisoxonol. The fluorescence of this dye increases when inserted into the hydrophobic matrix of the membrane, precluded in parasites with a polarized membrane. Assays were performed under standard conditions, except for the presence of 0.1 μ M bisoxonol in the incubation medium. Once the fluorescence readout became stable, AS-48 was added and fluorescence changes registered in a Polarstar Galaxy microplate spectrofluorometer (BGM Labotechnologies, Offenburg, Germany) ($\lambda_{\text{exc}} = 544\text{nm}/\lambda_{\text{em}} = 584 \text{ nm}$). Maximal depolarization was considered as that obtained with 10 μ M CA(1-8)M(1-18), a membraneactive leishmanicidal peptide (13).

The entrance of the cell impermeable dye SYTOX Green (MW = 600) into *L.*

donovani promastigotes was assayed under the standard assay conditions. Briefly, parasites were resuspended at 2×10^7 cells/ml in HBSS-Glc containing 1 μ M SYTOX Green. Once a stable readout of the fluorescence was reached, AS-48 was added, and the increase in fluorescence due to binding of the dye into intracellular nucleic acids, measured in a Polarstar Galaxy microplate spectrofluorometer ($\lambda_{\text{exc}} = 485\text{nm}/\lambda_{\text{em}} = 520 \text{ nm}$). Maximal permeabilization (100%) was considered as that obtained in the presence of 0.1% Triton X100.

In addition, a simultaneous assessment of the end-point variation for both parameters in *Leishmania* was carried out by cytofluorometry. Promastigotes were incubated with AS-48 under the standard conditions for 4 h. Afterwards, parasites were washed in HBSS-Glc, diluted in the same medium at 10^6 cells/ml, and incubated with 1 μ g/ml PI plus 0.1 μ M bisoxonol for 5 min. Then, samples were immediately analyzed in a FC500 Coulter cytofluorometer (fluorescence settings: bisoxonol, $\lambda_{\text{exc}} = 488\text{nm}/\lambda_{\text{em}} = 525 \text{ nm}$; PI, $\lambda_{\text{exc}} = 488 \text{ nm}/\lambda_{\text{em}} = 620 \text{ nm}$).

Mitochondrial dysfunction in *L. donovani* promastigotes caused by AS-48. The variation of $\Delta\Psi_m$ caused by AS-48 was monitored by the intracellular accumulation of Rho123. After parasite incubation with

AS-48 under the standard conditions, cells were diluted to 10^6 cells/ml in HBSS-Glc, loaded with Rho123 (0.3 $\mu\text{g/ml}$, 10 min, 26 °C) and analyzed by cytofluorometry as described (43). Parasites with fully depolarized mitochondrion were obtained by incubation with KCN (10 mM, 30 min) prior to Rho123 loading.

Oxygen consumption rates were measured using a Clark oxygen electrode (Hansatech, King's Lynn, UK) as described (43). Briefly, promastigotes were resuspended at 10^8 cells/ml in respiration buffer containing 2 mM succinate and 10 mM glucose. Experiments were carried out at 26 °C. Once the respiration of the promastigotes reached a steady value, AS-48 was added at the corresponding concentration and variation in the respiration rate recorded.

The production of reactive oxygen species (ROS) in the mitochondrion was evaluated with Mitosox Red (44). Parasites were loaded with Mitosox Red (0.5 μM , 30 min, 26 °C). AS-48 was added at 5 μM , and samples were removed at different times (3 min to 90 min) for fluorescence measurement by flow cytometry in a FC500 Coulter cytofluorometer (Becton-Dickinson, San Jose, CA) ($\lambda_{\text{exc}}=488\text{ nm}/\lambda_{\text{em}}=520\text{ nm}$). Antimycin A (0.3 $\mu\text{g/ml}$) was used as positive control.

Confocal microscopy of *L. donovani* promastigotes treated with fluoresceinated AS-48. Fluorescein-labelled AS-48 (FI-AS-48) was obtained by reaction of AS-48 with fluorescein isothiocyanate (FITC) in 50 mM carbonate buffer (pH 9.2). Final concentration of AS-48 in the reaction was adjusted to 35 μM ; FITC was added at equimolar concentration, and conjugation allowed to proceed at 4 °C overnight. Afterwards, nonconjugated FITC was removed by successive cycles of HBSS addition and centrifugation on a BIOMAX-5K 0.5 ml (Millipore). The absorbance ratio of the resulting FI-AS-48 at 495 nm and 280 nm was used to calculate the degree of fluorescein dye conjugation. Only a single FI-AS-48 batch was used for the whole set of experiments, with an average conjugation of 0.39 fluorescein molecules per AS-48 monomer.

Promastigotes were incubated under the standard conditions with 5 μM FI-AS-48 for 4 h, at 26 °C and 4 °C. Confocal microscopy of RAW 264.7 macrophages infected *L. pifanoi* amastigotes was carried out according to the protocol for leishmanicidal activity

(see above). FI-AS-48 in full growth medium was added to the infected cells at 5 μM , and incubated at 32 °C for 14 h.

Regardless of the *Leishmania* stage analysed, all samples were stained with 10 $\mu\text{g/ml}$ DAPI (5 min, 26 °C for promastigotes; 30 min, 32 °C for infected macrophages) prior to their microscopic observation. Non-incorporated dye was removed by washing, and cells were observed unfixed in a laser confocal microscope Leica TCS SP2 ($\lambda_{\text{exc}}=488\text{ nm}/\lambda_{\text{em}}=519\text{ nm}$, $\lambda_{\text{exc}}=358\text{ nm}/\lambda_{\text{em}}=461\text{ nm}$, for FI-AS-48 and DAPI, respectively).

Transmission electron microscopy of *L. pifanoi* infected macrophages treated with AS-48. Macrophages were infected as described for the measurement of intracellular leishmanicidal activity, except that AS-48 was added at 5 μM and the incubation time shortened to 14 h. Afterwards, the samples were processed as described (36). Briefly, parasites were fixed with OsO_4 (2.5 %, w/v), gradually dehydrated in increasing concentrations of ethanol, included in propylene oxide and embedded in Epon 812 resin.

Samples were observed and photographed under a Philips 2200 electron microscope.

DNA content analysis in *L. donovani* promastigotes. Aliquots of 100 μl of *L.*

donovani promastigotes suspension were incubated with AS-48 according to the standard assay conditions. Afterwards, parasites were collected by centrifugation and resuspended in full growth medium at 4×10^6 cells/ml. Promastigotes were transferred into 1 ml of growth medium and incubated for 18 h. Parasites were fixed in ice-cold ethanol overnight at 4 °C, washed twice with 1 ml of HBSS-Glc, resuspended in 500 μ l of the same medium containing 20 μ g/ml propidium iodide (PI) plus 3 mg/ml bovine RNase A, and incubated for 30 min at 26 °C. DNA content was analyzed by flow cytometry in a FC500 Coulter cytofluorometer ($\lambda_{\text{exc}} = 488$ nm / $\lambda_{\text{em}} = 620$ nm) (36).

Statistical analysis. Data were represented as the mean \pm SD from triplicate samples. Experiments were repeated at least twice. The inhibitory concentrations (IC_x ; x stands for the percentage of inhibition) were calculated by the four parameter logistic curve fitting using Sigma Plot vs 11.0. Statistical comparisons were assessed by the Student's ttest. Differences were considered significant at $p < 0.05$.

RESULTS

Leishmanicidal and cytotoxic activities of AS-48. The leishmanicidal activities of AS-48 were assessed by the inhibition of MTT reduction. When measured immediately after a 4 h incubation with AS-48, the resulting IC_{50} s and IC_{90} s were 3.9 ± 1.1 μ M and 9.4 ± 1.2 μ M, respectively, for *L. donovani* promastigotes; whereas for *L. pifanoi* axenic amastigotes these values were 10.2 ± 1.2 μ M and 19.5 ± 2.1 μ M, respectively. To assess inhibition of proliferation, the surviving parasites after the 4 h incubation with AS-48 were allowed to grow in absence of AS-48; IC_{50} s and IC_{90} s values for promastigotes decreased to 1.3 ± 0.2 μ M and 2.7 ± 0.4 μ M, and for axenic amastigotes 7.5 ± 0.7 μ M and 15.5 ± 2.1 μ M, respectively. Thus, axenic amastigotes were almost six-fold higher resistant than promastigotes to AS-48. In addition, the deleterious effect of AS-48 on *Leishmania* was not fully completed after 4 h incubation.

Variation of intracellular ATP levels in *L. donovani* promastigotes caused by AS-48. The content of intracellular ATP is an excellent parameter to assess the viability of the parasites. Real-time variation of cytoplasmic free-ATP was monitored on living *L. donovani* promastigotes of the 3-Luc strain that express a cytoplasmic form of firefly luciferase. In the presence of DMNPE-luciferin, a free-membrane caged substrate of luciferase, ATP was the limiting substrate for the luminescence output. The luminescence of 3-Luc promastigotes underwent a concentration-dependent decrease of luminescence after AS-48 addition. At concentrations close to the IC_{50} for AS-48 (3.1 μ M), the luminescence decreased by nearly 50 % respect to untreated promastigotes (Figure 1). To rule out inhibition of firefly luciferase, a commercial luciferase was assayed in vitro in the presence of AS-48. At 50 μ M, the highest AS-48 concentration tested, reduction of luciferase activity was 4% (data not shown).

Permeabilization of the plasma membrane of *L. donovani* promastigotes by AS-48. The two more feasible alternatives to account for the decrease of intracellular ATP were either plasma membrane permeabilization or inhibition of ATP synthesis. Membrane permeabilization was assessed by the entrance of vital dyes impermeable to organisms with an intact plasma membrane, as well as by membrane depolarization, accounting for the dissipation of ionic gradients across the membrane (Figure 2).

After addition of AS-48 to the promastigotes suspension, SYTOX green fluorescence increased in a concentration dependent manner (Figure 2, panel A). Nevertheless, even at 25 μ M AS-48, the highest

concentration tested, fluorescence never reached the value obtained for full permeabilized parasites, obtained by treatment with 0.1% TX-100.

To assess subtler membrane damage by AS-48, plasma membrane depolarization was next assayed. After AS-48 addition, the fluorescence of the potential-sensitive dye bisoxonol raised rapidly, due to its insertion into the hydrophobic matrix of the membrane, precluded in polarized parasites (Figure 2, panel B). This effect occurred even at 1.5 μM

AS-48, the lowest concentration tested. Only at 25 μM AS-48, fluorescence matched that obtained with 10 μM CA(1-8)M(1-18), a membrane-active leishmanicidal peptide causing full depolarization of promastigotes (36). Nevertheless, for AS-48 concentrations under 3.1 μM the promastigotes experimented a slow re-polarization, suggesting a partially reversible membrane injury by the bacteriocin at this range of concentrations.

Additionally, both parameters were simultaneously assayed for end-point values by two-channel cytofluorometry. SYTOX Green was replaced by PI as vital dye to avoid fluorescence overlapping with bisoxonol. Both dyes were added to the parasite suspension after the end of the incubation (Figure 2 panel C). Parasites treated with increasing AS-48 concentrations showed a progressive shift towards higher fluorescence values of bisoxonol. Only a single parasite population was conspicuous throughout the range of concentrations assayed. In contrast, two clearly differentiated parasite populations were discerned for PI fluorescence at the highest AS-48 concentration tested. Both populations were characterized by a characteristic mean fluorescence value. The increase in AS-48 concentration led to higher percentages of parasites into the upper fluorescence population, without shift in the fluorescence position, assimilated to an all-or-none process for PI entry.

Internalization of AS-48 into *L. donovani* promastigotes. The previous results suggested the involvement of additional intracellular targets to the membrane permeabilization damage in the final leishmanicidal outcome of AS-48. For this, access of AS-48 into the intracellular milieu, were required.

Thus, the FI-AS-48 accumulated inside the promastigotes after 4 h incubation, as assessed by confocal microscopy was followed (Figure 3). Bacteriocin uptake was perceptible even at 2 μM , a concentration lower than its IC_{50} under these conditions. The intracellular accumulation of FI-AS-48 increased with higher concentrations (5 μM and 10 μM). Nevertheless, severely distorted morphology was only observed at 10 μM AS-48, but not at lower concentrations.

The intracellular accumulation of AS-48 into *L. donovani* promastigotes was severely inhibited at 4 °C (Supplemental Figure 1). This supports an AS-48 uptake mediated by endocytosis rather than through its direct translocation across the membrane.

Damage to the mitochondrion of *L. donovani* promastigotes caused by AS-48. The study of a dysfunctional mitochondrion in *Leishmania* induced by AS-48 was prompted by two results: i) Fast and severe bioenergetic collapse in AS-48-treated *Leishmania* parasites in the absence of a substantial plasma membrane permeabilization, and ii) The capacity of AS-48 to access the intracellular space of *Leishmania*.

AS-48 induced the depolarization of the single mitochondrion of *Leishmania*, as assessed by Rh123 accumulation. AS-48 at 2.5 μM (lower than its IC_{50} under the standard assay conditions) decreased the

intracellular accumulation of Rh123 by 40% (Figure 4, panel A), in the absence of SYTOX green entrance (Figure 2, panel A).

The mitochondrial accumulation of Rh123 is driven by the mitochondrial electrochemical potential ($\Delta\Psi_m$), a parameter governing the respiration rate. AS-48 decreased the oxygen consumption rate of promastigotes in a concentration dependent manner (Figure 4 panel A, inset). At 12.5 and 50 μ M AS-48, promastigote respiration was inhibited by 30% and 75%, respectively. The higher cellular density required by this technique (five-fold higher than the standard value) accounted for the higher AS-48 concentrations required.

AS-48 increased local production of ROS, assessed by the increase in fluorescence of Mitosox Red. After AS-48 (5 μ M) addition to the parasites, the accumulated ROS production at 30 min was approximately 40% of that elicited by antimycin A, taken as positive control (Figure 4, panel B).

ROS overproduction in *Leishmania* is frequently associated to programmed-cell death processes through mitochondrial dysfunction (45). After 4 h incubation, AS-48 induced subG₁ appearance at 5 μ M. The percentage of subG₁ at 25 μ M AS-48 closely matched that of Miltefosine (HePC), a typical apoptotic inducer in *Leishmania* sp (46-48) (Supplemental Figure 2).

Leishmanicidal activity of AS-48 on intracellular *L. pifanoi* amastigotes.

The cidal activity of AS-48 was first assayed on cells of the Raw 264.7 murine monocytic cell line, used as host cells for *Leishmania*. The inhibition of MTT reduction by this bacteriocin was 22.0 ± 2.6 % at 50 μ M, the highest concentration tested (data not shown).

At 7.0 μ M, AS-48 decreased the parasitization index of the macrophage from 3.42 ± 0.14 in untreated macrophages to 0.41 ± 0.04 , a reduction close to 90%. Also the percentage of infected macrophages decreased from 55.3 ± 7.0 in untreated macrophages to 18.3 ± 3.2 after AS-48 treatment.

The decrease in intracellular *L. pifanoi* parasites was also confirmed by electron microscopy. Cellular debris from killed intracellular amastigotes were spotted inside AS-48 treated macrophages (Figure 5, lower row), but not in control parasites (Figure 5, upper row). Furthermore, FI-AS-48 accumulated preferentially in intracellular amastigotes respect to the host cell (Supplemental Figure 3). Thus, a direct action of AS-48 on the intracellular amastigotes is feasible, even when AS-48 may trigger additional leishmanicidal effects mediated by the macrophage.

DISCUSSION

In this work we have explored the leishmanicidal activity of AS-48, a bacteriocin produced by *E. faecalis* strains, as feasible candidate for its further pharmacological development. The IC₅₀ of AS-48 on promastigote proliferation is 1.3 μ M, a value similar to those reported for some Gram-positive bacteria (17, 34, 49, 50) but significantly lower than many membrane-active eukaryotic peptides (EAMPs) (9, 13). AS-48

shares with many EAMPs the permeation of the cell membrane as an essential step of its bactericidal mechanism. Furthermore, the entrance of vital dyes, induction of a bioenergetic collapse and release of cytoplasmic material induced by AS-48 in *Leishmania*, also support this mode of action.

AS-48 does not require a cognate macromolecular receptor at the targeted cell for its bactericidal activity, mandatory for other bacteriocins at low concentration (51). Specificity of AS-48 and of most EAMPs toward bacterial membranes is achieved by recognition of acid phospholipids of the target membrane exposed to the external medium (33, 34), a feature present also in *Leishmania*. Thus, the higher efficacy of AS-48 over other EAMPs should rely on its mode of interaction with the membrane. In fact, a very low number of bacteriocin molecules is required to kill bacteria (17). How AS-48 induces membrane permeabilization is not yet fully understood. Both formation of canonical pores (34, 52, 53) and induction of phospholipid discontinuities by insertion of AS-48 molecules (52, 54), have been proposed to account for this process. Regardless of how AS-48 achieved membrane permeabilization, both modes were highly dependent on the local density of AS-48 in the membrane. As AS-48 is oligomeric in aqueous solution (55), mostly as dimers (53), once inserted into the membrane the number of local AS-48 molecules will be already disruptive, or become so once few additional AS-48 molecules were recruited into this incipient permeating structure, as envisaged by coarse graining modeling (52). This mode of insertion and membrane permeation by AS-48 is highly advantageous over other EAMPs, whose monomers insert independently into the membrane, for which a further assembly into a functional membrane permeating structure is mandatory (56).

AS-48 has a lower activity on axenic amastigotes than on promastigotes. This trend was also described for some EAMPs (10, 57), but its underlying molecular rationale has not been yet unveiled. Differences between the two stages on phospholipid composition or its asymmetrical distribution at the two layers of the plasma membrane may be envisaged, but a detailed report on this issue is only available for promastigotes (58). Membrane potential influences the bactericidal activity of AS-48 (49), but not the permeation of artificial vesicles (34). Furthermore, the values reported of membrane potential in amastigotes and in promastigotes were quite similar (59), despite the significant difference between their respective proliferation rates. In contrast, lipophosphoglycan (LPG), the main oligosaccharide constituent of the glycocalyx of *Leishmania* promastigotes, is almost absent in amastigotes (60). The phosphate groups of LPG will create an anionic environment that would facilitate a high density of AS-48 close to the plasma membrane, promoting after AS-48 insertion the formation of the “putative” active permeating structures (55).

Nevertheless, the leishmanicidal activity of AS-48 cannot be solely ascribed to a membrane permeation mechanism, but intracellular targets were envisaged as well. The entrance of SYTOX Green was only partial, even at AS-48 concentrations close to full lethality in promastigotes. The leishmanicidal mechanism of AS-48 was not finished after 4 h, while for a sheer typical membrane permeation, including AS-48 on bacteria, completion is often reached in few minutes. Finally, fluoresceinated AS-48 was spotted inside *Leishmania* below its IC_{50} . The inhibition of AS-48 uptake at 4 °C ruled out its entrance through a direct translocation across the plasma membrane of the parasite. The membrane pores formed by AS-48 were unlikely to be used as a gate for intracellular entrance of new additional bacteriocin molecules in *Leishmania*. The reported dimensions for AS-48 pore (2.1 nm (52) or 0.7 nm (34)) will allow only entrance of monomeric AS-48 forms (20 Å) (31). In addition, even a small molecule as SYTOX Green (MW=600) showed only partial entrance in AS-48-treated parasites. The formation of a mixed phospholipid–peptide toroidal pore, predicted by molecular

dynamics (52), will easily afford the translocation of AS-48 across the membrane, similar to some other cell-penetrating peptides (61).

The single mitochondrion of *Leishmania* is a likely target for AS-48. Aside from its evolutionary resemblance with bacteria, cardiolipin, a typical mitochondrial phospholipid, interacts strongly with AS-48 (50). AS-48 induced a fast depletion of ATP, whose synthesis in *Leishmania* is carried out by oxidative phosphorylation. Damage to the functionality of the respiratory chain by AS-48 is also supported by the decrease in respiration, with a concomitant rise in mitochondrial ROS production measured by MitoSox Red fluorescence (44). Mitochondria as target for bacteriocins was also described for microcin J25 (62), both inside tumoral cells or as an isolated organelle.

To note, AS-48 also reduced the parasite burden of infected macrophages, even at concentrations lower than those required for axenic amastigotes. We may surmise a privileged accumulation of the cationic AS-48 at the parasitophorous vacuole, achieved by its binding to the anionic oligosaccharides of the extracellular matrix of the macrophage. Furthermore, oligomerization of AS-48 is dependent on the concentration of the bacteriocin and on the pH (55).

By electron microscopy, intracellular amastigotes in AS-48-treated macrophages showed a mixed pattern of morphological alterations suitable for a membrane damage scenario, but also with involvement of other intracellular targets. Subtle changes in local concentration of AS-48 may be responsible for this difference. To note, bacteriocins or bacteriocin-like peptides were active on other intracellular pathogens such as *Legionella* (63) or *Mycobacterium* (64), but only on axenic cultures, or after pharmacological vehiculation (65). In this case AS-48 is a rare exception, being able to reach and kill intracellular amastigotes without further manipulation. Even when AS-48 is considered as safe for the preservation of human food (66), we cannot discard an indirect leishmanicidal mechanism of the macrophage, triggered by the bacteriocin. Induction of nitric oxide synthase (NOS2), key for the leishmanicidal activity of the macrophage, was induced by other bacteriocins, such as BacSp222 from *Staphylococcus pseudintermedius* (67), as well by the cecropin A-melittin hybrid peptide CA(1-8)M(1-18), a surrogate of natural EAMPs (68), through subtle and reversible plasma membrane permeation.

The leishmanicidal activity of AS-48 paves the way for the use of bacteriocins not only against this parasite, but for other trypanosomatids infections as well. A caveat for wider spectra of protozoan targets came from AS-48 inactivity against *Acanthamoeba* and *Naegleria*, two free-living parasitic amoebae (49). Several characteristics of AS-48 are quite advantageous for its further pharmacological development as a new leishmanicidal agent. AS-48 can be produced in large amounts from industrial fermentation broths with a reasonably cost (69, 70) and possesses a very highly stable and compact structure, resistant to exopeptidase degradation, as well as low immunogenicity (32). Furthermore, AS-48 was recently patented as a topical antibacterial agent for skin infections (35), suggesting a relatively easy extrapolation into the ulcers in non-disseminated cutaneous leishmaniasis, inasmuch as AS-48 maintains its activity on high ionic strength, suitable to the hypertonic microenvironment of the skin immune cells (71). Nowadays, the native form of AS-48 is mandatory for its use. All the cutting-edge strategies aimed to improve the microbicidal activity of AS-48 by mutational analysis, or to get minimalist AS-48 analogues resulted unsuccessful (72), accounting for the dubbing of AS-48 as “close to perfection”. Nevertheless, two new aspects for AS-48 optimization has been recently tackled: the molecular basis for dimer stability may allow the design of new AS-48 mutants using

bioinformatics tools, with improved antimicrobial activity that facilitate its insertion into the membrane (54); secondly, the report of the full chemical synthesis of AS-48 including a final enzymatic circularization (73), opens the modification of residues otherwise essential for the correct biosynthesis and processing of AS-48, as well as the incorporation of non-proteinogenic amino acids into its structure.

Acknowledgments.- We thank Fernando Escolar and Begoña Pou for their technical assistance in electron microscopy, and Teresa Seisdedos and Gema Elvira for their help in confocal microscopy.

References

1. **Caljon G, De Muylder G, Durnez L, Jennes W, Vanaerschot M, Dujardin JC.** 2016. Alice in microbes' land: adaptations and counter-adaptations of vector-borne parasitic protozoa and their hosts. *FEMS Microbiol Rev* **40**:664-685.
2. **Tilley L, Straimer J, Gnädig NF, Ralph SA, Fidock DA.** 2016. Artemisinin Action and Resistance in *Plasmodium falciparum*. *Trends Parasitol* **32**:682-696.
3. **Mackey TK, Liang BA, Cuomo R, Hafen R, Brouwer KC, Lee DE.** 2014. Emerging and reemerging neglected tropical diseases: a review of key characteristics, risk factors, and the policy and innovation environment. *Clin Microbiol Rev* **27**:949979.
4. **Goupil LS, McKerrow JH.** 2014. Introduction: drug discovery and development for neglected diseases. *Chem Rev* **114**:11131-11137.
5. **Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M, Team WHOLC.** 2012. Leishmaniasis worldwide and global estimates of its incidence. *PloS one* **7**:e35671. doi: 10.1371/journal.pone.0035671
6. **Nagle AS, Khare S, Kumar AB, Supek F, Buchynskyy A, Mathison CJ, Chennamaneni NK, Pendem N, Buckner FS, Gelb MH, Molteni V.** 2014. Recent developments in drug discovery for leishmaniasis and human African trypanosomiasis. *Chem Rev* **114**:11305-11347.
7. **van Griensven J, Balasegaram M, Meheus F, Alvar J, Lynen L, Boelaert M.** 2010.

Combination therapy for visceral leishmaniasis. *Lancet Infect Dis* **10**:184-194.

8. **Ferreira LG, Andricopulo AD.** 2016. Drug repositioning approaches to parasitic diseases: a medicinal chemistry perspective. *Drug Discov Today*. **21**:1699-1710
9. **Rivas L, Luque-Ortega JR, Andreu D.** 2009. Amphibian antimicrobial peptides and Protozoa: lessons from parasites. *Biochim Biophys Acta* **1788**:1570-1581.
10. **Mangoni ML, Papo N, Saugar JM, Barra D, Shai Y, Simmaco M, Rivas L.** 2006. Effect of natural L- to D-amino acid conversion on the organization, membrane binding, and biological function of the antimicrobial peptides bombinins H. *Biochemistry* **45**:4266-4276.
11. **Epand RM, Walker C, Epand RF, Magarvey NA.** 2016. Molecular mechanisms of membrane targeting antibiotics. *Biochim Biophys Acta* **1858**:980-987.
12. **Sharma S, Sahoo N, Bhunia A.** 2016. Antimicrobial peptides and their pore/ion channel properties in neutralization of pathogenic microbes. *Curr Top Med Chem* **16**:46-5311.
13. **Torrent M, Pulido D, Rivas L, Andreu D.** 2012. Antimicrobial peptide action on parasites. *Curr Drug Targets* **13**:1138-1147.
14. **Cavera VL, Arthur TD, Kashtanov D, Chikindas ML.** 2015. Bacteriocins and their position in the next wave of conventional antibiotics. *Int J Antimicrob Agents* **46**:494-501.
15. **Drider D, Bendali F, Naghmouchi K, Chikindas ML.** 2016. in press. Bacteriocins: Not only antibacterial agents. *Probiotics Antimicrob Prot*. doi: 10.1007/s12602-016-9223-0.

16. **Montalbán-López M, Sánchez-Hidalgo M, Valdivia E, Martínez-Bueno M, Maqueda M.** 2011. Are bacteriocins underexploited? Novel applications for old antimicrobials. *Curr Pharm Biotechnol* **12**:1205-1220.
17. **Grande Burgos MJ, Pulido RP, López Aguayo MDC, Gálvez A, Lucas R.** 2014. The cyclic antibacterial peptide enterocin AS-48: Isolation, mode of action, and possible food applications. *Int J Mol Sci* **15**:22706-22727.
18. **Woraprayote W, Malila Y, Sorapukdee S, Swetwiwathana A, Benjakul S, Visessanguan W.** 2016. Bacteriocins from lactic acid bacteria and their applications in meat and meat products. *Meat Sci* **120**:118-132.
19. **Belguesmia Y, Madi A, Sperandio D, Merieau A, Feuilleley M, Prevost H, Drider D, Connil N.** 2011. Growing insights into the safety of bacteriocins: the case of enterocin S37. *Res Microbiol* **162**:159-163.
20. **Chaudhari A, Fialho AM, Ratner D, Gupta P, Hong CS, Kahali S, Yamada T, Haldar K, Cho W, Chauhan VS, Gupta TKD, Chakrabarty AM.** 2006. Azurin, *Plasmodium falciparum* malaria and HIV/AIDS: Inhibition of parasitic and viral growth by azurin. *Cell Cycle* **5**:1642-1648.
21. **Amer EI, Mossallam SF, Mahrous H.** 2014. Therapeutic enhancement of newly derived bacteriocins against *Giardia lamblia*. *Exp Parasitol* **146**:52-63.
22. **Mossallam SF, Amer EI, Diab RG.** 2014. Potentiated anti-microsporidial activity of *Lactobacillus acidophilus* CH1 bacteriocin using gold nanoparticles. *Exp Parasitol* **144**:14-21.
23. **Porrini MP, Audisio MC, Sabate DC, Ibarguren C, Medici SK, Sarlo EG, Garrido PM, Eguaras MJ.** 2010. Effect of bacterial metabolites on microsporidian *Nosema ceranae* and on its host *Apis mellifera*. *Parasitol Res* **107**:381-388.
24. **Belguesmia Y, Choiset Y, Rabesona H, Baudy-Floc'h M, Le Blay G, Haertle T, Chobert JM.** 2013. Antifungal properties of durancins isolated from *Enterococcus durans* A5-11 and of its synthetic fragments. *Lett Appl Microbiol* **56**:237-244.
25. **Mohr KI, Volz C, Jansen R, Wray V, Hoffmann J, Bernecker S, Wink J, Gerth K, Stadler M, Müller R.** 2015. Pinensins: the first antifungal lantibiotics. *Angew Chem Int Ed Engl* **54**:11254-11258.

26. **Sharma A, Srivastava S.** 2014. Anti-*Candida* activity of two-peptide bacteriocins, plantaricins (Pln E/F and J/K) and their mode of action. *Fungal Biol* **118**:264-275.
27. **Cornut G, Fortin C, Soulieres D.** 2008. Antineoplastic properties of bacteriocins: revisiting potential active agents. *Am J Clin Oncol* **31**:399-404.
28. **Kaur S, Kaur S.** 2015. Bacteriocins as potential anticancer agents. *Front Pharmacol* **6**:272. doi: 10.3389/fphar.2015.00272.
29. **Lagos R, Tello M, Mercado G, Garcia V, Monasterio O.** 2009. Antibacterial and antitumorigenic properties of microcin E492, a pore-forming bacteriocin. *Curr Pharm Biotechnol* **10**:74-85.
30. **Maqueda M, Gálvez A, Bueno MM, Sánchez-Barrena MJ, González C, Albert A, Rico M, Valdivia E.** 2004. Peptide AS-48: Prototype of a new class of cyclic bacteriocins. *Curr Prot Pept Sci* **5**:399-416.
31. **González C, Langdon GM, Bruix M, Gálvez A, Valdivia E, Maqueda M, Rico M.** 2000. Bacteriocin AS-48, a microbial cyclic polypeptide structurally and functionally related to mammalian NK-lysin. *Proc Natl Acad Sci USA* **97**:11221-11226.
32. **Maqueda M, Gálvez A, Martínez-Bueno M, Guerra I, Valdivia E.** 1993. Neutralizing antibodies against the peptide antibiotic AS-48: immunocytological studies. *Antimicrob Agents Chemother* **37**:148-151.
33. **Abriouel H, Sánchez-González J, Maqueda M, Gálvez A, Valdivia E, GálvezRuíz MJ.** 2001. Monolayer characteristics of bacteriocin AS-48, pH effect and interactions with dipalmitoyl phosphatidic acid at the air-water interface. *J Colloid Interface Sci* **233**:306-312.
34. **Gálvez A, Maqueda M, Martínez-Bueno M, Valdivia E.** 1991. Permeation of bacterial cells, permeation of cytoplasmic and artificial membrane vesicles, and channel formation on lipid bilayers by peptide antibiotic AS-48. *J Bacteriol* **173**:886892.

35. **Maqueda AM, Martínez BM, Valdivia ME, Ananou JS, Cebrián CR.** July 2014. WO2014006253 A1.
Composition for treating bacterial infections of the skin and mucous membranes.
36. **Luque-Ortega JR, Rivas L.** 2010. Characterization of the leishmanicidal activity of antimicrobial peptides. *Methods Mol Biol* **618**:393-420.
37. **Pan AA, Duboise SM, Eperon S, Rivas L, Hodgkinson V, Traub-Cseko Y, McMahon-Pratt D.** 1993. Developmental life cycle of *Leishmania*--cultivation and characterization of cultured extracellular amastigotes. *J Eukaryot Microbiol* **40**:213-223
38. **Colmenares M, Constant SL, Kima PE, McMahon-Pratt D.** 2002. *Leishmania pifanoi* pathogenesis: selective lack of a local cutaneous response in the absence of circulating antibody. *Infect Immun* **70**:6597-6605.
39. **Stevenson LG, Fedorko DP, Zelazny AM.** 2010. An enhanced method for the identification of *Leishmania* spp. using real-time polymerase chain reaction and sequence analysis of the 7SL RNA gene region. *Diagn Microbiol Infect Dis* **66**:432-435.
40. **Cebrián R, Banos A, Valdivia E, Pérez-Pulido R, Martínez-Bueno M, Maqueda M.** 2012. Characterization of functional, safety, and probiotic properties of *Enterococcus faecalis* UGRA10, a new AS-48-producer strain. *Food Microbiol* **30**:59-67.
41. **Ananou S, Muñoz A, Gálvez A, Martínez-Bueno M, Maqueda M, Valdivia E.** 2008. Optimization of enterocin AS-48 production on a whey-based substrate. *Int Dairy J* **18**:923-927.
42. **Abriouel H, Valdivia E, Martínez-Bueno M, Maqueda M, Gálvez A.** 2003. A simple method for semi-preparative-scale production and recovery of enterocin AS-48 derived from *Enterococcus faecalis* subsp. *liquefaciens* A-48-32. *J Microbiol Meth*

55:599-605.

43. **Luque-Ortega JR, Reuther P, Rivas L, Dardonville C.** 2010. New benzophenonederived bisphosphonium salts as leishmanicidal leads targeting mitochondria through inhibition of respiratory complex II. *J Med Chem* **53**:1788-1798.
44. **Piacenza L, Irigoin F, Alvarez MN, Peluffo G, Taylor MC, Kelly JM, Wilkinson SR, Radi R.** 2007. Mitochondrial superoxide radicals mediate programmed cell death in *Trypanosoma cruzi*: cytoprotective action of mitochondrial iron superoxide dismutase overexpression. *Biochem J* **403**:323-334.
45. **Roy A, Ganguly A, BoseDasgupta S, Das BB, Pal C, Jaisankar P, Majumder HK.** 2008. Mitochondria-dependent reactive oxygen species-mediated programmed cell death induced by 3,3'-diindolylmethane through inhibition of F_0F_1 -ATP synthase in unicellular protozoan parasite *Leishmania donovani*. *Mol Pharmacol* **74**:1292-1307.
46. **Paris C, Loiseau PM, Bories C, Breard J.** 2004. Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. *Antimicrob Agents Chemother* **48**:852859.
47. **Khademvatan S, Gharavi MJ, Rahim F, Saki J.** 2011. Miltefosine-induced apoptotic cell death on *Leishmania major* and *L. tropica* strains. *Korean J Parasitol* **49**:17-23.
48. **Marinho F de A, Goncalves KC, Oliveira SS, Oliveira AC, Bellio M, d'Avila-Levy CM, Santos AL, Branquinha MH.** 2011. Miltefosine induces programmed cell death in *Leishmania amazonensis* promastigotes. *Mem Inst Oswaldo Cruz* **106**:507-509.
49. **Gálvez A, Maqueda M, Martínez-Bueno M, Valdivia E.** 1989. Bactericidal and bacteriolytic action of peptide antibiotic AS-48 against gram-positive and gramnegative bacteria and other organisms. *Res Microbiol* **140**:57-68.
50. **Gálvez A, Valdivia E, Martínez M, Maqueda M.** 1989. Effect of peptide AS-48 on *Enterococcus faecalis* subsp. *liquefaciens* S-47. *Antimicrob Agents Chemother* **33**:641-645.

51. **Cotter PD.** 2014. An 'Upp'-turn in bacteriocin receptor identification. *Mol Microbiol* **92**:1159-1163.
52. **Cruz VL, Ramos J, Melo MN, Martínez-Salazar J.** 2013. Bacteriocin AS-48 binding to model membranes and pore formation as revealed by coarse-grained simulations. *Biochim Biophys Acta* **1828**:2524-2531.
53. **Sánchez-Barrena MJ, Martínez-Ripoll M, Gálvez A, Valdivia E, Maqueda M, Cruz V, Albert A.** 2003. Structure of bacteriocin AS-48: from soluble state to membrane bound state. *J Mol Biol* **334**:541-549.
54. **Cebrián R, Martínez-Bueno M, Valdivia E, Albert A, Maqueda M, SánchezBarrena MJ.** 2015. The bacteriocin AS-48 requires dimer dissociation followed by hydrophobic interactions with the membrane for antibacterial activity. *J Struct Biol* **190**:162-172.
55. **Abriouel H, Valdivia E, Gálvez A, Maqueda M.** 2001. Influence of physicochemical factors on the oligomerization and biological activity of bacteriocin AS-48. *Curr Microbiol* **42**:89-95.
56. **Sal-Man N, Oren Z, Shai Y.** 2002. Preassembly of membrane-active peptides is an important factor in their selectivity toward target cells. *Biochemistry* **41**:11921-11930.
57. **Silva T, Abengózar MA, Fernández-Reyes M, Andreu D, Nazmi K, Bolscher JG, Bastos M, Rivas L.** 2012. Enhanced leishmanicidal activity of cryptopeptide chimeras from the active N₁ domain of bovine lactoferrin. *Amino acids* **43**:2265-2277.
58. **Wassef MK, Fioretti TB, Dwyer DM.** 1985. Lipid analyses of isolated surface membranes of *Leishmania donovani* promastigotes. *Lipids* **20**:108-115.
59. **Glaser TA, Utz GL, Mukkada AJ.** 1992. The plasma membrane electrical gradient (membrane potential) in *Leishmania donovani* promastigotes and amastigotes. *Mol Biochem Parasitol* **51**:9-15.

60. **Turco SJ, Descoteaux A.** 1992. The lipophosphoglycan of *Leishmania* parasites. *Annu Rev Microbiol* **46**:65-94.
61. **Kauffman WB, Fuselier T, He J, Wimley WC.** 2015. Mechanism matters: A taxonomy of cell penetrating peptides. *Trends Biochem Sci* **40**:749-764.
62. **Niklison-Chirou MV, Dupuy F, Saavedra L, Hebert E, Banchio C, Minahk C, Morero RD.** 2011. Microcin J25-Ga induces apoptosis in mammalian cells by inhibiting mitochondrial RNA-polymerase. *Peptides* **32**:832-834.
63. **Verdon J, Labanowski J, Sahr T, Ferreira T, Lacombe C, Buchrieser C, Berjeaud J-M, Héchard Y.** 2011. Fatty acid composition modulates sensitivity of *Legionella pneumophila* to warnericin RK, an antimicrobial peptide. *Biochim Biophys Acta* **1808**:1146-1153.
64. **Carroll J, Draper LA, O'Connor PM, Coffey A, Hill C, Ross RP, Cotter PD, O'Mahony J.** 2010. Comparison of the activities of the lantibiotics nisin and lacticin 3147 against clinically significant mycobacteria. *Int J Antimicrob Agents* **36**:132-136.
65. **Sosunov V, Mischenko V, Eruslanov B, Svetoch E, Shakina Y, Stern N, Majorov K, Sorokoumova G, Selishcheva A, Apt A.** 2007. Antimycobacterial activity of bacteriocins and their complexes with liposomes. *J Antimicrob Chemother* **59**:919-925.
66. **Abriouel H, Lucas R, Omar NB, Valdivia E, Gálvez A.** 2010. Potential applications of the cyclic peptide enterocin AS-48 in the preservation of vegetable foods and beverages. *Probiotics Antimicrob Proteins* **2**:77-89.
67. **Wladyka B, Piejko M, Bzowska M, Pieta P, Krzysik M, Mazurek L, GuevaraLora I, Bukowski M, Sabat AJ, Friedrich AW, Bonar E, Miedzobrodzki J, Dubin A, Mak P.** 2015. A peptide factor secreted

by *Staphylococcus pseudintermedius* exhibits properties of both bacteriocins and virulence factors. Sci Rep **5**:14569. doi: 10.1038/srep14569

68. **Arias C, Guizy M, Luque-Ortega JR, Guerrero E, de la Torre BG, Andreu D, Rivas L, Valenzuela C.** 2006. The induction of NOS2 expression by the hybrid cecropin A-melittin antibiotic peptide CA(1-8)M(1-18) in the monocytic line RAW 264.7 is triggered by a temporary and reversible plasma membrane permeation. Biochim Biophys Acta **1763**:110-119.
69. **Gálvez A, Valdivia E, González-Segura A, Lebbadi M, Martínez-Bueno M, Maqueda M.** 1993. Purification, characterization, and lytic activity against *Naegleria fowleri* of two amoebicins produced by *Bacillus licheniformis* A12. Appl Environ Microbiol **59**:1480-1486.
70. **Bali V, Panesar PS, Bera MB.** 2016. Trends in utilization of agro-industrial byproducts for production of bacteriocins and their biopreservative applications. Crit Rev Biotechnol **36**:204-214.
71. **Jantsch J, Schatz V, Friedrich D, Schroder A, Kopp C, Siegert I, Maronna A, Wendelborn D, Linz P, Binger KJ, Gebhardt M, Heinig M, Neubert P, Fischer F, Teufel S, David JP, Neufert C, Cavallaro A, Rakova N, Kuper C, Beck FX, Neuhofer W, Muller DN, Schuler G, Uder M, Bogdan C, Luft FC, Titze J.** 2015. Cutaneous Na⁺ storage strengthens the antimicrobial barrier function of the skin and boosts macrophage-driven host defense. Cell Metab **21**:493-501.
72. **Sánchez-Hidalgo M, Montalbán-López M, Cebrián R, Valdivia E, MartínezBueno M, Maqueda M.** 2011. AS-48 bacteriocin: close to perfection. Cell Mol Life Sci **68**:2845-2857.
73. **Hemu X, Qiu Y, Nguyen GK, Tam JP.** 2016. Total synthesis of circular bacteriocins by butelase 1. J Am Chem Soc **138**:6968-6971.

FIGURES

FIGURE 1

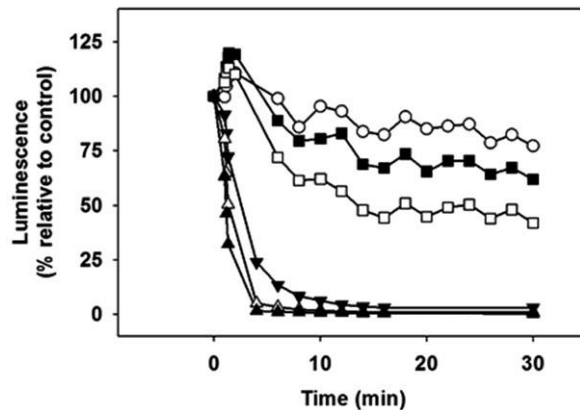
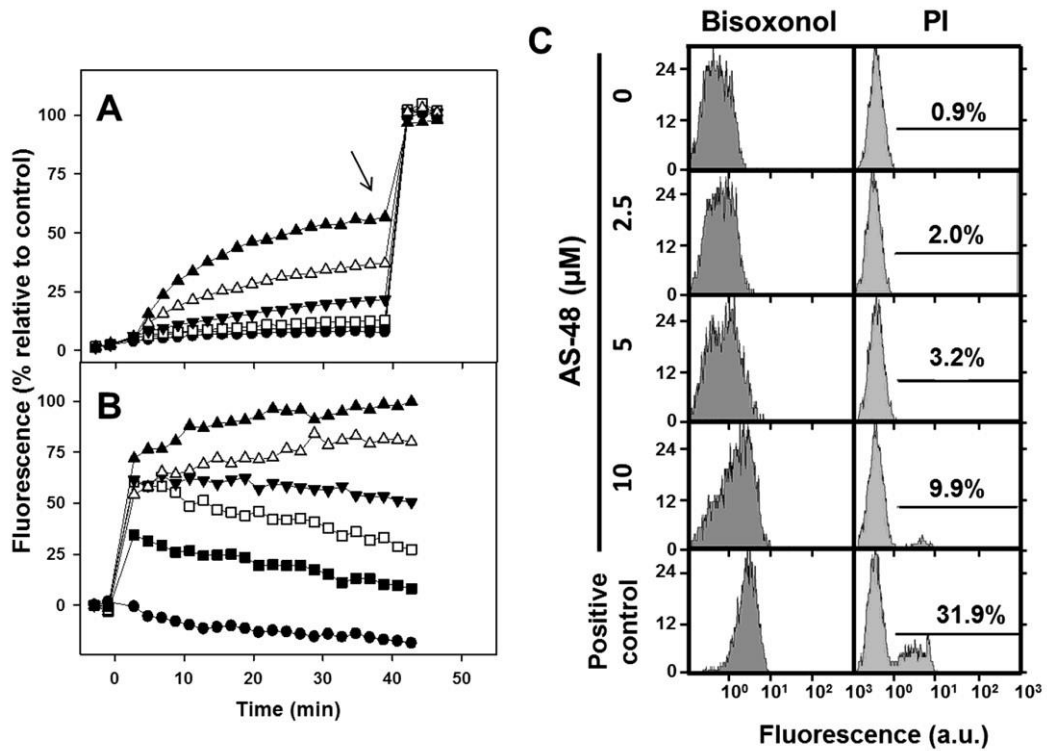


Figure 1.- *In vivo* monitoring of intracellular ATP levels of *L. donovani* 3-Luc promastigotes after AS-48

addition. Promastigotes were resuspended at 2×10^7 cells/ml.

DMNPE-luciferin was added at 25 μ M. Once the readout became stable, AS-48 was added ($t=0$) and variation in luminescence expressed as percentage respect to the luminescence of control, untreated parasites. Legend: AS-48 concentration (μ M): 0.8, (○); 1.6, (■); 3.1, (□); 6.2, (▼); 12.5, (△); 25.0, (▲). Experiment is representative of other three performed independently.

FIGURE 2



See figure 2 legend at the next page

Figure 2.- Permeabilization of the plasma membrane of *L. donovani* promastigotes by AS-48. Parasites were resuspended at 2×10^7 cells/ml in HBSS-Glc with the corresponding probe. AS-48 was added at $t=0$, and

fluorescence referred as the percentage of fully permeabilized parasites. **Panel A.-** Kinetics of intracellular entrance of the vital dye SYTOX green. Promastigotes were resuspended in HBSS-Glc + 1 μ M SYTOX Green.

Fluorescence settings: λ_{EXC} = 485 nm, λ_{EM} = 520 nm. Arrow stands for the addition of TX100 (0.1% final concentration), taken as full permeabilized parasites. **Panel B.-** Kinetics of plasma membrane depolarization.- Parasites were resuspended in HBSS-Glc + 0.1 μ M bisoxonol. Fluorescence settings: λ_{EXC} = 544 nm, λ_{EM} = 584 nm. Full depolarized parasites (100 % fluorescence) were considered as those treated with 10 μ M CA(1-8)M(1-18).

Legend for panels A and B: AS-48 concentration (μ M): 0.78, (●); 1.6, (■); 3.1, (□); 6.2, (▼); 12.5, (△); 25.0, (▲). **Panel C.-** End-point cytofluorometric determination for both parameters after 4 h incubation. Propidium iodide (PI, 1 μ g/ml) plus bisoxonol (0.1 μ M) were added to the parasites 5 min before the cytofluorometric analysis. Fluorescence settings: bisoxonol, λ_{EXC} = 488nm, λ_{EM} = 525 nm; PI, λ_{EXC} = 488 nm/ λ_{EM} = 620 nm. Positive controls for permeabilization were 0.1% TX-100 (PI) and 10 μ M CA(1-8)M(1-18) (bisoxonol). Percentage of the full permeabilized population was shown inside the corresponding histogram.

FIGURE 3

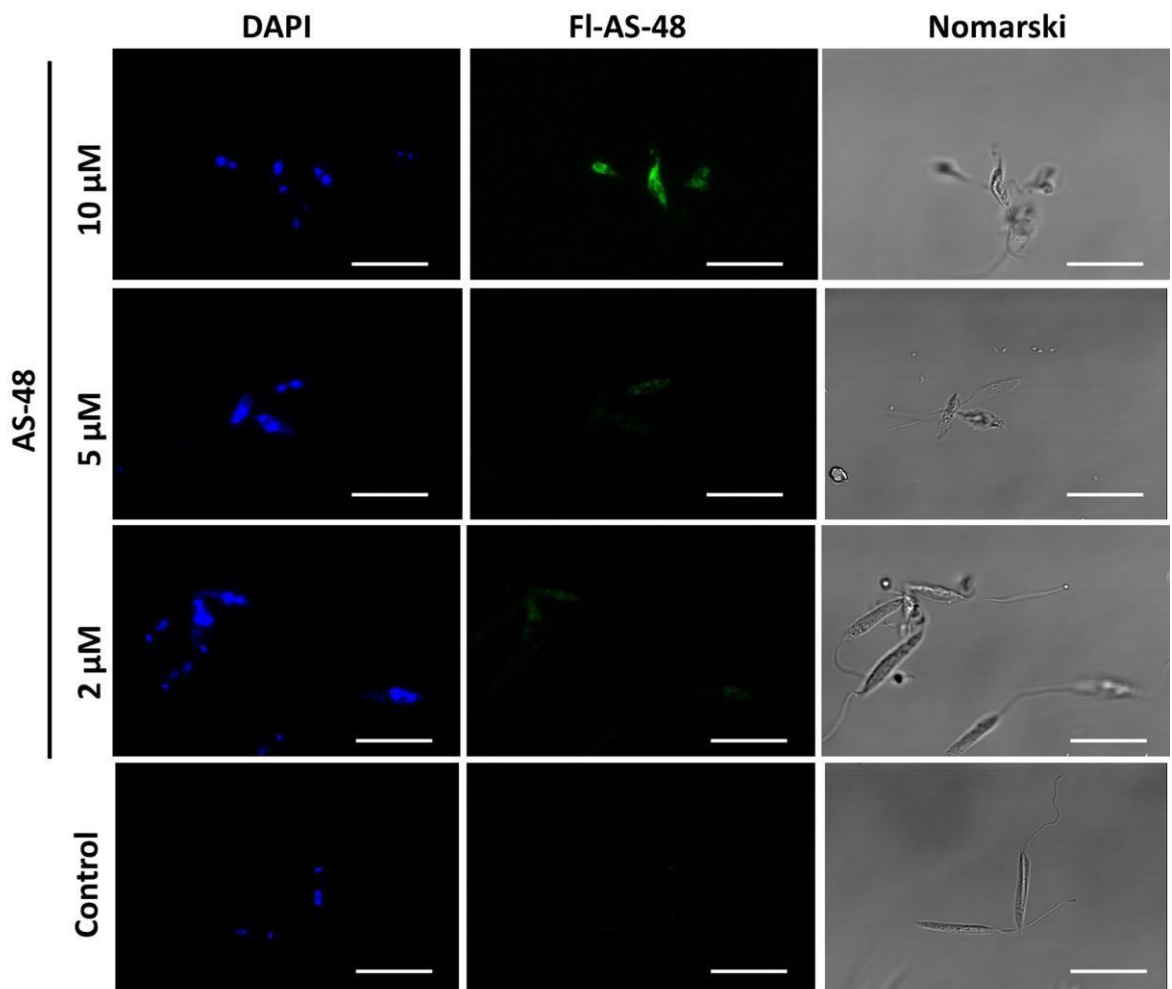


Figure 3.- Confocal microscopy of *L. donovani* promastigotes treated with fluoresceinated AS-48. Promastigotes were incubated with fluoresceinated AS-48 (FI-AS48) at different concentrations. Incubation was carried out at 2×10^7 cells/ml for 4 h in

HBSS-Glc. Promastigotes were stained with DAPI (10 μ g/ml, 5 min), prior to their observation as unfixed parasites in the confocal microscopy. Fluorescence settings: FI-AS-

48, $\lambda_{\text{EXC}} = 488$ nm, $\lambda_{\text{EM}} = 519$ nm; DAPI, $\lambda_{\text{EXC}} = 358$ nm, $\lambda_{\text{EM}} = 461$ nm. Magnification bar

=10 μ m.

FIGURE 4

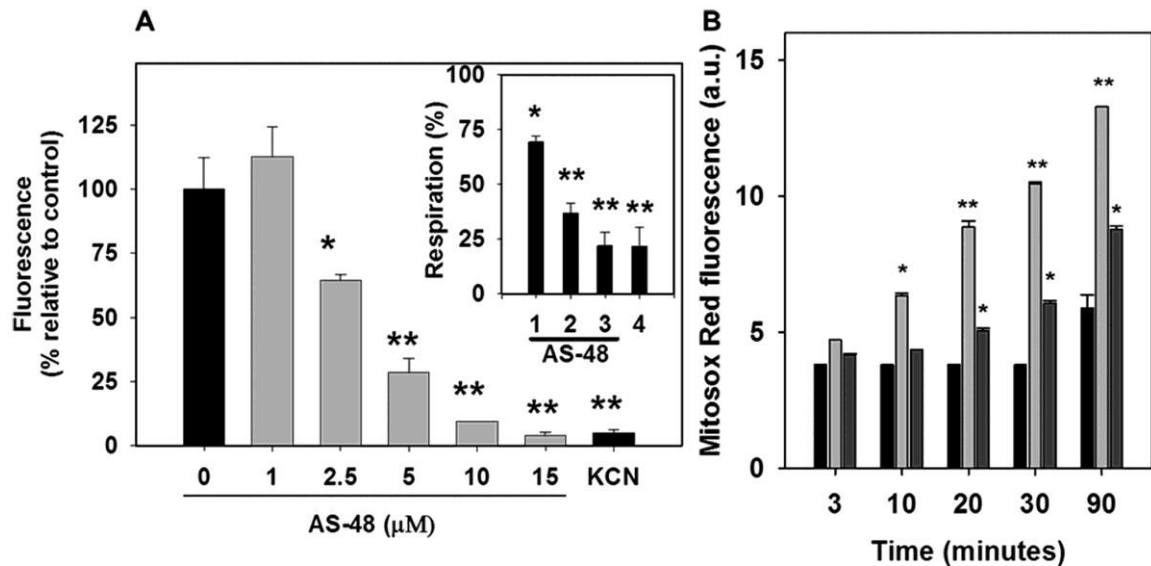


Figure 4.- Assessment of mitochondrial damage to *L. donovani* promastigotes by AS48.- Panel A.- Inhibition of $\Delta\Psi_m$ and respiration (inset). Parasites were incubated with AS-48 for 4 h (HBSS-Glc, 2×10^7 cells/ml), loaded with Rhodamine 123, and analysed by cytofluorometry. Parasites incubated with 10 mM KCN were used for positive control of mitochondrial depolarization. **Panel A, inset.-** Oxygen consumption rate of promastigotes treated with AS-48. Respiration was measured at 10^8 cells/ml in a Clark oxygen electrode and expressed as percentage respect to untreated cells. **Inset legend:** 1, 2, and 3: parasites treated with AS-48 at 12.5, 25, and 50 μ M, respectively. 4: Parasites treated with 10 μ M CA(1-8)M(1-18), as positive internal control. **Panel B.-** Production of mitochondrial ROS induced by AS-48. Parasites were loaded with 0.5 μ M Mitosox Red and treated either with

3 μ g/ml antimycin A (□), or with 5 μ M AS-48 (■), and compared with control parasites (■), as positive control. Samples were taken at different times and analysed by cytofluorometry ($\lambda_{\text{EXC}} = 488$ nm, $\lambda_{\text{EM}} = 520$ nm), $p < 0.05$ (*), $p < 0.01$ (**).

FIGURE 5.

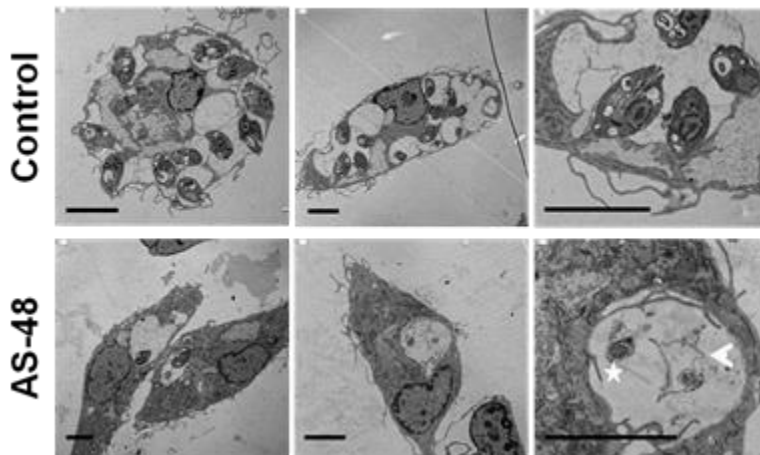


Figure 5.- Electron microscopy of RAW 264.7 murine macrophage cells infected with *L. pifanoi* and treated with AS-48. Raw 264.7 cells were infected with *L. pifanoi* axenic amastigotes as described in Materials and Methods. Once the infection was established, infected macrophages were treated or not, with 7 μ M AS-48 for 24 h, and processed for electron microscopy. White arrow and star indicated the cellular debris from killed amastigotes inside the parasitophorous vacuole of AS-48-treated macrophages. Upper row:

Control parasites. Lower row: AS-48 treated parasites. Magnification bar =10 μ m.