Temporins: Small Antimicrobial Peptides with Leishmanicidal Activity*

Maria Luisa Mangoni‡¶, José M. Saugar§, Maria Dellisanti‡, Donatella Barra‡ †, Maurizio Simmaco‡, and Luis Rivas§

From the ‡Istituto Pasteur-Fondazione Cenci Bolognetti, Dipartimento di Scienze Biochimiche ‘A. Rossi Fanelli’, Azienda Ospedaliera S. Andrea, and †CNR Istituto di Biologia e Patologia Molecolari, Università ‘La Sapienza’, Piazzale Aldo Moro 5, 00185 Rome, Italy; §Centro de Investigaciones Biológicas (CSIC), Ramiro de Maeztu 94, E-28040 Madrid (Spain)

¶To whom correspondence should be addressed: Maria Luisa Mangoni; Dipartimento di Scienze Biochimiche, Università La Sapienza, Piazzale Aldo Moro 5, 00185 Rome, Italy. Tel.: +39 06 8034-5457; Fax: +39 06 80345405; E-mail: marialuisa.mangoni@uniroma1.it.

Running Title: Leishmanicidal activity of temporins
SUMMARY

Leishmaniasis encompasses a wide range of infections caused by the human parasitic protozoan species belonging to the *Leishmania* genus. It appears frequently as an opportunistic disease, especially in virus-infected immunodepressed people. Similarly to other pathogens, parasites became resistant to most of the first-line drugs. Therefore, there is an urgent need to develop antiparasitic agents with new modes of action. Gene-encoded antimicrobial peptides are promising candidates, but so far only a few of them have shown anti-protozoa activities. Here we found that temporins A and B, 13-amino acid antimicrobial peptides secreted from the skin of the European red frog *Rana temporaria*, display anti-*Leishmania* activity at micromolar concentrations, with no cytolytic activity against human erythrocytes. To the best of our knowledge, temporins represent the shortest natural peptides having the highest leishmanicidal activity, the lowest number of positively charged amino acids (a single lysine/arginine) and maintaining biological function in serum. Their lethal mechanism involves plasma membrane permeation based on the following data: (i) they induce a rapid collapse of the plasma membrane potential; (ii) they induce the influx of the vital dye SYTOX™ Green; (iii) they reduce intracellular ATP levels; and (iii) they severely damage the parasite’s membrane, as shown by transmission electron microscopy. Besides giving us basic important information, the unique properties of temporins, as well as their membranolytic effect, which should make it difficult for the pathogen to develop resistance, suggest them as potential candidates for the future design of antiparasitic drugs with a new mode of action.
INTRODUCTION

Leishmaniasis includes a wide variety of clinical symptoms caused by infection with different species of the genus *Leishmania*, a human protozoan parasite with a worldwide incidence of 12-14 million people affected. The disease is widespread mostly in tropical and subtropical countries (1). *Leishmania* is a digenetic parasite with two major stages, differing widely in their antigenic pattern, metabolism, and plasma membrane composition (2): (i) the flagellated promastigote, and (ii) the intracellular non-flagellated amastigote. The promastigote dwells in the gut of the insect vector, the sandfly, and is transmitted by bite into the mammalian host. Then, it invades the cells of the mononuclear phagocytic system and transforms into the amastigote, the pathological form of the parasite for vertebrates.

At present, the only available treatment is based on chemotherapy with organic pentavalent antimonials as the first-line drugs (3,4). However, their efficacy is rapidly eroding due to the increasing appearance of resistant clinical isolates (5,6) and the severe side-effects associated with the treatment (7). Despite the availability of alternative drugs, such as amphotericin B or miltefosine, there is a real need for the development of new agents with new modes of action (4). Among them, eukaryotic antimicrobial peptides (AMPs)\(^1\) constitute a promising alternative. They are ubiquitous in pluricellular organisms, where they kill a wide variety of pathogens. Accordingly, they are located at the anatomical sites primarily involved in contact with microorganisms, such as mucosal, epithelial or phagocytic cells (reviewed in ref. 8). Skin secretions from Amphibia represent one of the richest sources for these molecules, because every species synthesizes its own particular set of defence peptides (9-12). Although AMPs differ significantly in their sequences, most of them share some features, such as a high positive
charge and a potential to adopt an amphipathic $\alpha$-helix and/or $\beta$-sheet structures upon their interaction with membranes. To date, there is a compelling evidence that a common step in the microbial killing mechanism is their electrostatic interaction with the negatively charged cell membrane followed by its permeation/disruption (13,14).

During the last few years, a considerable number of studies have been carried out with AMPs on bacteria (15,16), fungi (17,18), viruses (19,20) and tumor cells (21,22), in attempting to understand the parameters responsible for their activity. Nevertheless, reports on the activity and the mode of action of AMPs toward protozoan and metazoan parasites are very scarce (23). For Leishmania, these include dermaseptins (24) and SPYY (25), both isolated from the skin of frogs, gomesin, from the hemocytes of the tarantula spider, Acanthoscurria gomesiana (26), and indolicidin (27), from granules of bovine neutrophils. In 1998, Diaz-Achirica and coworkers (28) and more recently Chicharro (29) and Luque-Ortega and colleagues (30) investigated the leishmanicidal activity of highly positively charged cecropin A-melittin hybrid peptides. Their data suggested that the killing of the parasite was strongly correlated with plasma membrane permeabilization. Conversely, authophagic cell death has been described for indolicidin-treated parasites (27).

Here we report the biological function and the mode of action of temporin A (FLPLIGRVLSGIL-NH$_2$) and temporin B (LLPIVGNLLKSLL-NH$_2$), both isolated from the frog Rana temporaria, against Leishmania promastigotes and amastigotes. Temporins are short (13-residues) peptides, with an amidated C-terminus and with only one positively-charged amino acid. They were first identified in R. temporaria skin secretions (31) and further detected in several North American Rana species, such as R. clamitans (32), R. luteiventris (33), R. pipiens (33), and R. grylio (34). Temporins, together with indolicidin, are among the smallest
AMPs isolated so far from animal sources. However, in contrast with indolicidin, temporins are non-hemolytic (35,36). Previous studies indicated that these molecules are active mainly against Gram-positive bacteria, Candida albicans, and some human tumor cell lines (12). Our data show that temporins A and B have leishmanicidal activity at concentrations that are not toxic to human red blood cells. In addition, in contrast to most AMPs, temporins preserve biological function in serum. Furthermore, studies on their mode of action suggest that they act directly on the membrane of the parasite and destroy its integrity; therefore, it should make it difficult for the pathogen to develop resistance. Besides providing important basic information, the small size of temporins, their destructive mode of action and ability to maintain activity in serum, suggest them as attractive templates for further development of new antiparasitic drugs.

EXPERIMENTAL PROCEDURES

**Reagents**—Bis-(1,3-diethylthiobarbituric) trimethine oxonol (bisoxonol), SYTOX\textsuperscript{TM} Green, D-luciferin, 1-(4,5-dimethoxy-2-nitrophenyl) ethyl ester (DMNPE-luciferin) were obtained from Molecular Probes (Leiden, The Netherlands). Other reagents of the highest quality were purchased from Sigma (St. Louis, MO) or Merck (Darmstadt, Germany).

**Parasites**—Promastigotes of Leishmania donovani (MHOM/SD/00/1S-2D) were grown in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (HIFCS), antibiotics, and 2 mM glutamine in a Bellco roller device (Ace Glass, Vineland, NJ, USA) at 26 °C. The mutant R2D2 strain, defective in the biosynthesis of the repetitive phosphorylated disaccharide unit of lipophosphoglycan (LPG), was kindly provided by S. J. Turco (School of Medicine, Kentucky University, USA). For this strain, 5 µg/ml of lectin ricin
agglutinin (Ricinus communis) was added to the growth medium (37). Promastigotes of the 3-Luc L. donovani strain were obtained from the afore-mentioned parental strain by transfection with the pX63NEO-3Luc expression vector encoding the gene for a cytoplasmic form of Photinus pyralis luciferase (30). These parasites were grown under the same conditions as the parental strain, except for the additional supplement of 50 µg/ml geneticin to the growth medium. L. pifanoi axenic amastigotes were grown at 32 °C in M199 medium (Gibco-BRL 31100) supplemented with 20% HIFCS, 5% tripticase and 50 µg/ml hemin, pH 7.2 (38).

Peptide Synthesis—Temporin A and temporin B were synthesized by the standard Nα-Fmoc amino acid derivatives with a PAL-resin on an automated peptide synthesizer (Pioneer, Applied Biosystems, Foster City, CA, USA). The purity of the peptides was confirmed by HPLC analysis, and their sequence was determined by both automated Edman degradation using a protein sequencer (Applied Biosystems, model AB476A), and mass spectral analysis with a MALDI-TOF Voyager DE (Applied Biosystems). The concentration of the peptides was determined by quantitative amino acid analysis after acid hydrolysis using a Beckman System Gold instrument, equipped with an ion-exchange column and ninhydrin derivatization.

Antibacterial Activity—The antibacterial activity of the peptides was tested using an inhibition zone assay on agarose plates seeded with viable bacteria, according to Hultmark et al. (39). Two-fold serial dilution of the peptides in 20% ethanol (v/v) as well as in 33% heat-inactivated human serum were used (40).

Activity of the Peptides on Leishmania Promastigotes and Amastigotes—Procyclic promastigotes and amastigotes were harvested at a late exponential phase, whereas metacyclic promastigotes from cultures at a stationary phase. Parasites were then washed twice with Hanks medium (136 mM NaCl; 4.2 mM Na₂HPO₄; 4.4 mM KH₂PO₄; 5.4 mM KCl; 4.1 mM NaHCO₃),
pH 7.2, supplemented with 20 mM D-glucose (Hanks-Glc) (41) and resuspended in the same buffer at a final concentration of \(2 \times 10^7\) parasites/ml. Aliquots of this suspension (120 \(\mu\)l) were incubated with the peptides for 60 min at 26 °C and 32 °C for promastigotes and amastigotes, respectively, and subsequently divided into two further aliquots (100 and 10 \(\mu\)l), which were used in the following two assays (29).

(i) Inhibition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to insoluble formazan by mitochondrial reductases was used as a short-term viability parameter of the parasites, and assayed immediately after peptide incubation (28). To the 100-\(\mu\)l aliquot of parasite suspension, 1 ml of Hanks-Glc was added to slow down peptide activity. The parasites were then collected by centrifugation, resuspended in 100 \(\mu\)l of a 0.5 mg/ml MTT solution in Hanks-Glc, transferred into a 96-well culture microplate, and incubated for 2 h at 26 °C or 32 °C, for promastigotes or amastigotes, respectively. The reduced formazan was solubilized by the addition of an equal volume of 10% (w/v) sodium dodecyl sulfate, incubated overnight at 26 °C and measured in a 450 Bio-Rad Microplate Reader equipped with a 595-nm filter.

(ii) Measuring the inhibition of parasite proliferation. To measure inhibition of promastigotes proliferation, the 10-\(\mu\)l aliquot was added to 100 \(\mu\)l of RPMI medium, devoid of phenol red, and supplemented with 10% HIFCS. The surviving parasites were allowed to proliferate for 72 h; then, 100 \(\mu\)l of MTT solution (1 mg/ml in Hanks-Glc) was added and its reduction was measured as described above. For amastigotes, the inhibition of proliferation was performed in M199 growth medium. After 5 days, the cells were centrifuged, washed with Hanks, and resuspended in 0.5 mg/ml MTT solution. All assays were performed in triplicate and the
experiments were repeated twice. The results were normalized to those of the corresponding controls in the absence of the peptide.

Modification of Bioenergetic Parameters—We carried out two different assays.

(i) Collapse of membrane potential. Membrane potential was estimated by the potential-sensitive anionic dye bisoxonol, as its fluorescence increases after its insertion into the membrane, once the cell is depolarized. Assays were performed under standard conditions, in the presence of 0.1 µM bisoxonol. Peptides were added at different concentrations, and changes in fluorescence were recorded continuously for about 30 min using a Fluorostat Galaxy microplate reader (BMG Labotechnologies, Offenburg, Germany), equipped with excitation and emission filters at 540 and 580 nm, respectively. Maximal depolarization was considered as that obtained with 2.5 µM CA(1-8)M(1-18) cecropin A-melittin hybrid (28). All assays were done in triplicate.

(ii) In-vivo monitoring of intracellular ATP variation. The variation of intracellular ATP level was monitored as previously described (30). Briefly, promastigotes transfected with the pX63NEO-3Luc expression vector were incubated for 15 min at 26 °C with 25 µM DMNPE-luciferin, a free membrane-permeable caged luciferase substrate. Changes in luminescence were measured in the Fluorostat Galaxy microplate reader fitted with luminescence optics, with readings averaged every 10 s. Peptides were added once the luminescence reached a plateau. This point was considered as time zero and its luminescence value was taken as 100%. The decrease in luminescence was monitored continuously for 60 min. All assays were performed in triplicate and the luminescence values were normalized to those of the corresponding controls in the absence of the peptide.
Permeation of the Plasma Membrane of the Promastigotes and Amastigotes—We adapted the procedure described by Thevissen and coworkers (42) to assess the permeability of *Leishmania* membrane. Briefly, the parasites were incubated with 1 μM SYTOX™ Green in Hanks-Glc for 15 min in the darkness. After peptide addition, the increase in fluorescence, due to the binding of the dye to intracellular DNA, was measured in the same microplate reader described above, using 485 nm and 520 nm filters for excitation and emission wavelengths, respectively. Maximal membrane permeation was defined as that obtained after the addition of 0.1% Triton X-100 (29).

Electron Microscopy—Parasites were incubated with the peptides for 1 h and washed twice with phosphate buffered saline (PBS). Afterwards, parasites were fixed for 1 h with 5% (w/v) glutaraldehyde in PBS, included with 2.5% (w/v) osmium tetraoxide, gradually dehydrated in ethanol (30, 50, 70, 90, and 100% (v/v), 30 min each) and propylene oxide (1 h). Finally, they were embedded in Epon 812 resin, and observed with a Philips 2200 electron microscope as described previously (28).

Other Methods—Hydrophobicity was calculated according to the normalized scale of Eisenberg (43). LC₅₀ (the concentration of peptide required to inhibit half of the maximum MTT reduction) and its 95% confidence limits were determined by the Litchfield and Wilcoxon procedure using the PHARM/PCS Version 4 software package for PCs. The number of experiments analyzed was indicated in the legend of each figure.

RESULTS

Leishmania Killing by Temporins A and B—Temporins were tested for their activity and plausible mode of action on both promastigote and amastigote stages of *Leishmania*. The ability
of a drug to kill the mammalian intracellular stage of the parasite is crucial for its potential to be developed as a therapeutic agent. Note that this form has been shown to be more resistant to other cationic AMPs than the corresponding form present in the insect host (29).

**Killing of Promastigotes**

*Inhibition of MTT Reduction in Promastigotes by Temporins A and B*—Temporins A and B were tested for their ability to inhibit MTT reduction in *Leishmania* promastigotes at a concentration range of 5-25 µM. The assay was done either after 1-h incubation of the parasites with the peptides (short-term effect) or after proliferation of the remaining viable protozoa at 72-h incubation, as described in the Experimental Procedures section. The results are given in Fig. 1. The data shown in panel A reveal that, at a given peptide concentration, the percentage of inhibition for the short-time effect was practically similar to the inhibition of proliferation, without taking into account the S.D. Moreover, both peptides exhibited a similar antiparasitic effect as indicated also by their corresponding LC$_{50}$ values (Table I). The lethal concentrations against three bacterial strains (the Gram-negative *Escherichia coli* D21 and the Gram-positive *Bacillus megaterium* Bm11 and *Staphylococcus aureus* Cowan I), as well as human erythrocytes were included in Table I for comparison. These results indicate that whereas the antibacterial potency of temporin A was approximately 2-fold higher than that of temporin B, both peptides were similarly active toward the promastigotes, and at least 5-fold more efficient than cecropin A, a potent 36-residue AMP (44). Interestingly, we found that, in contrast to most natural AMPs (40), temporins maintained high activity in serum (Table I). Furthermore, temporins inhibited MTT reduction of the metacyclic promastigotes (Fig. 1B), the circulating form of the parasite in the blood of an infected mammal for about 24 h before becoming amastigote, to a slightly lower extent than that found for the procyclic promastigotes (Fig. 1A).
The Influence of External Parameters on the Activity of Temporins on Leishmania

Promastigotes—To better understand the interaction of these peptides with the surface of the parasite, we carried out a systematic variation of the conditions used in the short-term standard assay for determining the ability of temporins to inhibit MTT reduction.

(i) To study the role of electrostatic interactions, we tested temporins in the absence and presence of 50 µg/ml heparin, a strongly anionic polysaccharide. Temporin A, at 15 µM, caused 90.8 ± 2.3% inhibition of MTT reduction in the absence of heparin (Fig. 1A), whereas after a 15-min incubation with heparin, prior to the addition of the peptide to the promastigotes, the inhibition was 78.4 ± 3.2%. This effect was clearly weaker than that obtained for other peptides having a higher cationic character such as CA(1-8)M(1-18) (28). In another assay, temporin A was assayed at 10 µM for its ability to inhibit the MTT reduction using two isosmotic incubation media: the standard Hanks medium (140 mM NaCl) and the same medium but containing 280 mM D-sorbitol instead of NaCl. Only a small difference in the activity was detected under the two conditions: the percentage of inhibition increased from 59.4 ± 1.7 in the standard assay, to 66.7 ± 2.9 in D-sorbitol medium.

(ii) Temperature is a main factor that affects membrane fluidity. We noticed that the leishmanicidal activity of temporins, tested at 15 µM peptide concentration, decreased from 90.8 ± 2.3 (Fig. 1A) under standard conditions (26 ºC) to 32.7 ± 4.1% when assayed at 4 ºC.

Permeabilization of the Plasma Membrane of Leishmania Promastigotes—Previous studies have shown that temporins A and B alter the permeability of bacterial and artificial membranes in a dose-dependent manner (45). To determine whether the promastigote membrane is a potential target for temporins, we performed a peptide-induced membrane depolarization assay using the membrane potential-sensitive dye bisoxonol. Both temporins caused rapid dissipation
of the membrane potential in a concentration-dependent manner (Fig. 2). In the case of temporin A, which displayed a slightly stronger effect than temporin B, the depolarization induced at 20 μM, a concentration that inhibited more than 97% of the parasite proliferation, was identical to that found for CA(1-8)M(1-18) when tested at 2.5 μM. Under these conditions, the hybrid peptide killed all the parasites through a membrane-permeabilization mechanism (28). Thus, our findings suggest a similar mode of action for temporins.

The data revealed also that the membrane perturbation induced by temporins leads to a strong collapse of the bioenergetic metabolism of the parasite. This was monitored by the fast and dose-dependent decrease of luminescence in parasites expressing a cytoplasmic luciferase, when incubated with DMNPE-luciferin (Fig. 3). This means that the intracellular level of ATP diminishes concomitantly with the decrease in luminescence, since the intracellular ATP concentration is the limiting step for the bioluminescence reaction in 3-Luc promastigotes (30,46). Also under these conditions, temporin B displayed a slightly lower efficacy compared with temporin A at the same peptide concentration.

To get a better insight into the extent of membrane damage caused by temporins, we measured fluorimetrically the influx of SYTOX™ Green (MW = 900) into the parasites, as its fluorescence is enhanced when bound to intracellular nucleic acids. The influx of the dye was prevented in intact membranes but not in those parasites having lesions with a size large enough to allow the entrance of the dye (42). Here, the effect caused by temporin A was significantly higher than that for temporin B (Fig. 4, panels A and B).

Transmission Electron Microscopy—To detect morphological changes in peptide-treated parasites, we carried out transmission electron microscopy experiments on promastigotes after their incubation with temporins. As illustrated in Fig. 5 (panels A-C), a severe disruption of the
membrane structure, accompanied by a loss of electron dense intracytoplasmic material, was observed for both peptides at 20 µM.

Killing of Amastigotes

Inhibition of MTT Reduction and Plasma Membrane Permeabilization of Leishmania

Amastigotes by Temporins A and B—The ability of temporins to kill Leishmania amastigotes was tested by measuring the inhibition of MTT reduction after incubation with the peptides, similarly to what we performed with promastigotes. The results are shown in Fig. 6. The data reveal that both temporins preserve their activity against the mammalian intracellular form of the parasite.

We further investigated whether the mode of action of temporins against Leishmania amastigotes was similar to that found for promastigotes. To this end, we tested the peptide ability to induce the permeation of the amastigote membrane by using three assays and found that both temporins caused (i) the plasma-membrane depolarisation, as measured with bisoxonol (data not shown); (ii) the intracellular influx of SYTOX™ Green (measured fluorimetrically) in a dose-dependent manner and in agreement with the potencies of the peptides; temporin B was more active than temporin A (Fig. 4, panels C and D); and (iii) the damage to the cell membrane, visualized as blebs and local disruptions at concentrations causing ~80-90% killing (Fig. 5, panels D-F). Altogether, the results obtained with these three assays revealed that both temporins induced the permeation of the membrane of amastigotes similarly to the promastigotes.
DISCUSSION

Temporins A and B differ from most AMPs isolated from Amphibia by being very short (13-residues) and having only one basic amino acid (lysine or arginine). They adopt an amphipatic \( \alpha \)-helical structure in hydrophobic environments and increase the permeability of bacterial membranes and liposomes (47,48). Here we show that temporins A and B are active on both the insect and the mammalian stages of *Leishmania* with complete inhibition of parasite viability at 15-25 \( \mu \)M. Note that indolicidin, another 13-amino acid antimicrobial peptide, completely inhibits promastigotes proliferation at a micromolar range higher than that for temporins. However, the \( LC_{50} \) of indolicidin is much lower than that of temporins, i.e. 3.5 x 10\(^{-5} \) \( \mu \)M and \(~8.5 \mu\)M, respectively (27). Furthermore, here we demonstrate that temporins function at physiological salt concentrations and in 33% human serum, whereas many AMPs are inactive in these conditions. In addition, they preserve their activity on the mammalian stage of the parasite. Preliminary experiments have also shown that both temporins are devoid of cytotoxic effects on murine macrophages (RAW 264,7 line), which are capable to act as host cells for *Leishmania*, at the maximal concentration tested (80 \( \mu \)M). In addition, treatment of the infected macrophages with 15 \( \mu \)M temporin B also resulted in a lethal effect on the parasites, as the macrophage population remained apparently unharmed and fully viable whereas the ratio amastigote:macrophage, assessed by Giemsa staining, was reduced by a half (data not shown).

Previous studies reported that short cecropin-melittin hybrid peptides possess potent leishmanicidal activity (30). Nevertheless, in contrast with temporins, they are high positively charged molecules (4 basic amino acids).

Our data envisage that the lethal concentrations of temporins against *Leishmania* are similar to those found against *Candida* (12). However, they are higher than those obtained against
Gram-positive bacteria, but much lower than the values obtained against Gram-negative ones (31,49). This suggests that the outer membrane of Gram-negative bacteria, which is composed predominantly of hydrophobic and negatively charged moieties, namely lipopolysaccharides (LPS), acts as a barrier against temporins. The finding that *E. coli* strains deficient in the number of LPS or with a reduced amount of sugar residues of their LPS chain are more sensitive to these peptides (47) supports this notion.

*Leishmania* promastigotes are surrounded by a glycocalix layer, formed mainly by LPG, a highly anionic molecule bound to the membrane through a glycosylphosphoinositol anchor, which cover more than 40% of the total surface of the parasite (50). We found that temporins A and B do not differ significantly (p>0.1) in their activity against the parental strain of *L. donovani* and its R2D2 mutant (data not shown). This is despite the fact that the mutant strain is defective in the biosynthesis of the repetitive units of LPG (37). Conversely, R2D2 is at least two-fold more susceptible to other AMPs, such as CA(1-8)M(1-18) (28), compared with its parental strain. The small size and the low net positive charge of temporins could make it easier for these peptides to diffuse through the glycocalix of the promastigotes and permeate the cytoplasmic membrane, compared with other AMPs that have a higher net positive charge, and therefore can stick easily to the negatively charged components of the glycocalix. This indicates that electrostatic interactions between the peptide and the parasite do not contribute significantly to the activity of temporins. This argument is also supported by: (i) the similar activity obtained against the procyclic and the metacyclic *L. donovani* promastigotes, despite the fact that the number of repetitive units of LPG was double on the metacyclic stage of the parasite (51); (ii) the marginal decrease in their potency in the presence of heparin; (iii) the invariant peptide activity under marked changes in the ionic strength of the incubation media; (iv) the strong
activity on the amastigote form of the parasite where the LPG layer is replaced by glycosylphosphoinositol of neutral or zwitterionic character (52).

Interestingly, in contrast with temporins and other AMPs (29,53), indolicidin, exhibits a consistently reduced activity against parasites defective for LPG biosynthesis, such as the R2D2 mutant (27). However, as reported by Bera and colleagues (27), since indolicidin produces an autophagic cell death, we cannot exclude the possibility of a receptor-mediated pathway for the induction of autophagy, via LPG. Nevertheless, if the killing mechanism of indolicidin is associated to the interaction with a receptor, this might limit its antiparasitic potency compared with that of temporins.

Overall, our results indicate the Leishmania membrane as being the major target for temporins A and B, based on the following data: (i) the rapid induction of the collapse of the membrane potential as well as the lowering of the intracellular ATP levels and the permeation of the membrane simultaneously with the inhibition of parasite proliferation. All of these findings also correlate with the extent of this inhibition and are in contrast with the transient and slow effects caused by peptides that traverse the membrane and act intracellularly. Since maintenance of the trans-membrane potential depends mainly on the gradient of K⁺ and Na⁺ across the membrane, temporins probably promote plasma membrane permeability to these ions; (ii) the fast kinetics of the drop in the intracellular ATP level and the irreversibility of the process. The hypothesis that the decrease in the intracellular ATP level is due to its release from the parasite is reasonable; indeed, if it happened because of the inhibition of the oxidative phosphorylation, which represents the major source for ATP production in Leishmania (54), we should expect a slower initial kinetic; (iii) the increase in SYTOX™ Green fluorescence, which reflects the membrane perturbation; (iv) the pattern of injured parasites as visualized by
transmission electron microscopy. The photographs show damage in the plasma membrane of the parasites in the form of blebs and breakages, as well as a partial depletion in the intracytoplasmic content. A similar effect on the *Leishmania* morphology was also detected with other membrane-active leishmanicidal peptides, such as dermaseptins (55), cecropin A-melittin hybrids (28-30) or magainins (56). In addition, temporin-treated parasites were devoid of the extensive cytoplasmic vacuolation as found with indolicidin, which does not affect the integrity of the plasma membrane (27); (v) the gap between the concentration values for a short-term effect (MTT reduction assayed after 1h of incubation of the parasites with peptides) and those for inhibition of proliferation, which are significantly lower than those achieved for peptides with an intracellular target (57,58); note also (vi) the previously observed alteration of model membrane structure, by forming transient peptide-phospholipid membrane-spanning pores, as described by the two-states model (12,45,47,48) and (vii) the fact that all-D temporins A and B enantiomers preserved the antibacterial activity of the natural peptide, indicating that chiral targets are not involved (49,59).

Interestingly, although against promastigotes both temporins are almost equally efficient in the inhibition of proliferation, in their capacity to depolarize the membrane potential, and their ability to reduce the intracellular ATP levels, they have distinct effects on the influx of vital dyes such as the SYTOX\textsuperscript{TM} Green. For example, at a given concentration, temporin B was substantially less active than temporin A in promoting the intracellular influx of the dye. Conversely, a reversal in potency was manifested against amastigotes (Figs. 4C, 4D and 6) and in the calcein-leakage assay from liposomes (47), where temporin B was the most active peptide. This suggests that the size of the membrane breakages and the kinetics of their formation differ for the two molecules, and that both phenomena depend on the type of the
phospholipid of the target system. Indeed, the calcein-leakage experiments were done with liposomes containing phosphatidylglycerol (PG) and phosphatidylserine (PS) (47). However, PG is almost absent in the plasma membrane of *L. donovani* promastigotes (60), whereas PS is mainly located at its cytoplasmic leaflet. Furthermore, promastigotes contain a LPG layer that is absent in amastigotes as well as in the liposomes, and therefore, the ability to traverse this layer is also a crucial parameter and might be different for the two peptides.

In summary, our results demonstrate that temporins A and B are among the smallest highly active natural antiparasitic peptides reported so far that act directly on membranes. This should make it difficult for the parasite to develop resistance. In addition, the small size of temporins and the finding that, in contrast to many natural AMPs, they maintain activity in physiological salt concentration as well as in serum makes them as attractive lead compounds for the future development of antiparasitic drugs with a new mode of action.
FOOTNOTES

*This work was supported by grants from the Italian Ministero dell'Istruzione, dell’Università e della Ricerca and Università La Sapienza, and the Spanish Ministry of Science and Technology BIO2003-09056-CO2-2, the Comunidad Autonoma de Madrid (08.2/0054/2001.02) and Plan Estratégico de Grupos en Biotecnología. CIB belongs to the Red Española de Investigación en Patología Infecciosa, Fondo de Investigaciones Sanitarias, grant C03/14.

The abbreviations used are: Antimicrobial peptide, AMP; bisoxonol, bis-(1,3-diethylthiobarbituric) trimethine oxonol; CA(1-8)M(1-18), cecropin A-melittin hybrid; DMNPE-luciferin, D-luciferin-(1-(4,5-dimethoxy-2-nitrophenyl)ethyl ester); Hanks-Glc, Hanks’s balanced salt medium plus 20 mM D-glucose; HIFCS, heat-inactivated fetal calf serum; LPS, lipopolysaccharide; LPG, lipophosphoglycan; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PG, phosphatidylglycerol; PS, phosphatidylserine.
REFERENCES

1. Herwaldt, B. L. (1999) *Lancet* **354**, 1191-1199
2. Handman, E. (1999) *Adv. Parasitol.* **44**, 1-39
3. Croft, S. L., and Yardley, V. (2002) *Curr. Pharm. Des.* **8**, 319-342
4. Croft, S. L., and Coombs, G. H. (2003) *Trends Parasitol.* **19**, 502-558
5. Mbongo, N., Loiseau, P. M., Billion, M. A., and Robert-Gero, M. (1998) *Antimicrob. Agents Chemother.* **42**, 352-357
6. Seifert, K., Matu, S., Javier Perez-Victoria, F., Castanys, S., Gamarro, F., and Croft, S. L. (2003) *Int. J. Antimicrob. Agents* **22**, 380-387
7. Guerin, P. J., Olliaro, P., Sundar, S., Boelaert, M., Croft, S. L., Desjeux, P., Wasunna, M. K., and Bryceson, A. D. (2002) *Lancet Infect. Dis.* **2**, 494-501
8. Boman, H. G. (2003) *J. Intern. Med.* **254**, 197-215
9. Simmaco, M., Mignogna, G., and Barra, D. (1998) *Biopolymers* **47**, 435-450
10. Mor, A. (2000) *Drug Development Research* **50**, 440-447
11. Nicolas, P., and Mor, A. (1995) *Annu. Rev. Microbiol.* **49**, 277-304
12. Rinaldi, A. C., Mangoni, M. L., Rufo, A., Luzi, C., Barra, D., Zhao, H., Kinnunen, P. K., Bozzi, A., Di Giulio, A., and Simmaco, M. (2002) *Biochem. J.* **368**, 91-100
13. Shai, Y. (1999) *Biochim. Biophys. Acta.* **1462**, 55-70
14. Shai, Y. (2002) *Biopolymers* **66**, 236-248
15. Friedrich, C. L., Moyle, D., Beveridge, T. J., and Hancock, R. E. (2000) *Antimicrob. Agents Chemother.* **44**, 2086-2092
16. Wu, M., Maier, E., Benz, R., and Hancock, R. E. (1999) *Biochemistry* **38**, 7235-7242
17. De Lucca, A. J., Bland, J. M., Jacks, T. J., Grimm, C., and Walsh, T. J. (1998) Med. Mycol. 36, 291-298
18. De Lucca, A. J. (2000) Expert. Opin. Investig. Drugs 9, 273-299
19. Wachinger, M., Kleinschmidt, A., Winder, D., von Pechmann, N., Ludvigsen, A., Neumann, M., Holle, R., Salmons, B., Erfle, V., and Brack-Werner, R. (1998) J. Gen. Virol. 79, 731-740
20. Robinson, W. E., Jr., McDougall, B., Tran, D., and Selsted, M. E. (1998) J. Leukoc. Biol. 63, 94-100
21. Baker, M. A., Maloy, W. L., Zasloff, M., and Jacob, L. S. (1993) Cancer Res. 53, 3052-3057
22. Papo, N., and Shai, Y. (2003) Biochemistry 42, 9346-9354
23. Vizioli, J., and Salzet, M. (2002) Trends Parasitol. 18, 475-476
24. Feder, R., Dagan, A., and Mor, A. (2000) J. Biol. Chem. 275, 4230-4238
25. Vouldoukis, I., Shai, Y., Nicolas, P., and Mor, A. (1996) FEBS Lett. 380, 237-240
26. Silva, P. I., Jr., Daffre, S., and Bulet, P. (2000) J. Biol. Chem. 275, 33464-33470
27. Bera, A., Singh, S., Nagaraj, R., and Vaidya, T. (2003) Mol. Biochem. Parasitol. 127, 23-35
28. Diaz-Achirica, P., Ubach, J., Guinea, A., Andreu, D., and Rivas, L. (1998) Biochim. J. 330, 453-460
29. Chicharro, C., Granata, C., Lozano, R., Andreu, D., and Rivas, L. (2001) Antimicrob. Agents Chemother. 45, 2441-2449
30. Luque-Ortega, J. R., Saugar, J. M., Chiva, C., Andreu, D., and Rivas, L. (2003) Biochim. J. 375, 221-230
31. Simmaco, M., Mignogna, G., Canofeni, S., Miele, R., Mangoni, M. L., and Barra, D. (1996) *Eur. J. Biochem.* **242**, 788-792

32. Halverson, T., Basir, Y. J., Knoop, F. C., and Conlon, J. M. (2000) *Peptides* **21**, 469-476

33. Goraya, J., Wang, Y., Li, Z., O'Flaherty, M., Knoop, F. C., Platz, J. E., and Conlon, J. M. (2000) *Eur. J. Biochem.* **267**, 894-900

34. Kim, J. B., Halverson, T., Basir, Y. J., Dulka, J., Knoop, F. C., Abel, P. W., and Conlon, J. M. (2000) *Regul. Pept.* **90**, 53-60

35. Selsted, M. E., Novotny, M. J., Morris, W. L., Tang, Y. Q., Smith, W., and Cullor, J. S. (1992) *J. Biol. Chem.* **267**, 4292-4295

36. Subbalakshmi, C., Krishnakumari, V., Nagaraj, R., and Sitaram, N. (1996) *FEBS Lett.* **395**, 48-52

37. King, D. L., and Turco, S. J. (1988) *Mol. Biochem. Parasitol.* **28**, 285-293

38. Pan, A. A., McMahon-Pratt, D., and Honigberg, B. M. (1984) *J. Parasitol.* **70**, 834-835

39. Hultmark, D., Engstrom, A., Andersson, K., Steiner, H., Bennich, H., and Boman, H. G. (1983) *EMBO J.* **2**, 571-576

40. Oren, Z., Hong, J., and Shai, Y. (1997) *J. Biol. Chem.* **272**, 14643-14649

41. Hanks JH, W. J. (1958) *Proc. Soc. Biol. Med.* **98**, 188-192

42. Thevissen, K., Terras, F. R., and Broekaert, W. F. (1999) *Appl. Environ. Microbiol.* **65**, 5451-5458

43. Eisenberg, D. (1984) *Annu. Rev. Biochem.* **53**, 595-623

44. Steiner, H., Hultmark, D., Engstrom, A., Bennich, H., and Boman, H. G. (1981) *Nature* **292**, 246-248
45. Rinaldi, A. C., Di Giulio, A., Liberi, M., Gualtieri, G., Oratore, A., Bozzi, A., Schinina, M. E., and Simmaco, M. (2001) *J. Pept. Res.* **58**, 213-220

46. Luque-Ortega, J. R., Rivero-Lezcano, O. M., Croft, S. L., and Rivas, L. (2001) *Antimicrob. Agents Chemother.* **45**, 1121-1125

47. Mangoni, M. L., Rinaldi, A. C., Di Giulio, A., Mignogna, G., Bozzi, A., Barra, D., and Simmaco, M. (2000) *Eur. J. Biochem.* **267**, 1447-1454

48. Zhao, H., Rinaldi, A. C., Di Giulio, A., Simmaco, M., and Kinnunen, P. K. (2002) *Biochemistry* **41**, 4425-4436

49. Wade, D., Silberring, J., Soliymani, R., Heikkinen, S., Kilpelainen, I., Lankinen, H., and Kuusela, P. (2000) *FEBS Lett.* **479**, 6-9

50. Turco, S. J., Hull, S. R., Orlandi, P. A., Jr., Shepherd, S. D., Homans, S. W., Dwek, R. A., and Rademacher, T. W. (1987) *Biochemistry* **26**, 6233-6238

51. Sacks, D. L., Pimenta, P. F., McConville, M. J., Schneider, P., and Turco, S. J. (1995) *J. Exp. Med.* **181**, 685-697

52. McConville, M. J., Mullin, K. A., Ilgoutz, S. C., and Teasdale, R. D. (2002) *Microbiol. Mol. Biol. Rev.* **66**, 122-154

53. Diaz-Achirica, P., Ubach, J., Guinea, A., Andreu, D., and L., R. (1996) *Mechanisms of action of cecropin A-melittin hybrid peptides on Leishmania sp parasites*. Peptides: Chemistry, Structure and Biology (Kaumaya, P., and Hodges, R.S, Eds, Ed.), Mayflower Scientific, Kingswinford, UK

54. Van Hellemond, J., Van der Meer, P. Tielens, A.G.M. (1997) *Parasitology* **114**, 351-360

55. Hernandez, C., Mor, A., Dagger, F., Nicolas, P., Hernandez, A., Benedetti, E. L., and Dunia, I. (1992) *Eur. J. Cell Biol.* **59**, 414-424
56. Guerrero, E., Saugar, J. M., Matsuzaki, K., and Rivas, L. (2004) *Antimicrob. Agents Chemother.* **48**, 2980-2986

57. Takeshima, K., Chikushi, A., Lee, K. K., Yonehara, S., and Matsuzaki, K. (2003) *J. Biol. Chem.* **278**, 1310-1315

58. Park, C. B., Kim, H. S., and Kim, S. C. (1998) *Biochem. Biophys. Res. Commun.* **244**, 253-257

59. Oh, H., Hedberg, M., Wade, D., and Edlund, C. (2000) *Antimicrob. Agents Chemother.* **44**, 68-72

60. Wassef, M. K., Fioretti, T. B., and Dwyer, D. M. (1985) *Lipids* **20**, 108-115
FIGURE LEGENDS

FIG. 1. Activity of temporins A and B on promastigotes. *L. donovani* promastigotes (2 x 10^7 parasites/ml), were incubated with different concentrations of temporins at 26 °C for 60 min and inhibition of MTT reduction was assayed immediately (solid symbols), or after proliferation for 72 h of the surviving promastigotes (empty symbols). Panel A, procyclic promastigotes; panel B, metacyclic promastigotes. Peptide designation: Temporin A, circle; temporin B, triangles. Data points represent the mean of triplicate samples ± S.D. from a single experiment, representative of three different experiments.

FIG. 2. Promastigotes plasma membrane depolarization by temporins. Parasites (2 x 10^7 cells/ml) were equilibrated with 0.1 µM bisoxonol at 26 °C. Peptides were then added (t=0 min) at the corresponding concentrations, and changes in fluorescence (arbitrary units, a.u.) were monitored continuously (λ_exc=540 nm, λ_em=580 nm). Panel A, temporin A; panel B, temporin B. Control parasites (●). The peptide concentrations were 10 µM (■), 15 µM (▲), 20 µM (▽). The arrow indicates the addition of peptide. The values represent the mean of triplicate samples from a single experiment, representative of three different experiments.

FIG. 3. Intracellular ATP decrease in *L. donovani* 3-Luc promastigotes following temporin addition. Promastigotes (2 x 10^7 cells /ml) of the 3-Luc *L. donovani* strain, expressing a cytoplasmic form of *P. pyralis* luciferase, were incubated with 25 µM DMNP- luciferin, a free-membrane permeable luciferase substrate, and luminescence was monitored until it reached a constant value. At a certain point (t=0 min), temporins were added at the corresponding concentrations and luminescence decay was measured and normalized to that
of control parasites. Panel A, temporin A; panel B, temporin B. The peptide concentrations were 10 µM (■), 15 µM (▲), 20 µM (▽). The arrow (t=0 min) indicates the addition of peptide. Values represent the mean of triplicate samples from a single experiment, representative of three different experiments.

FIG. 4. Influx of the vital dye SYTOX™ Green into *Leishmania* promastigotes and amastigotes following temporin addition. Parasites (2×10⁷ cells/ml) were incubated according to the standard assay with 1 µM SYTOX™ Green in Hanks-Glc. Once basal fluorescence reached a constant value, temporins at the corresponding concentrations were added (t=0) and the increase in fluorescence (arbitrary units, a.u.) was monitored (λ_exc=485 nm, λ_em=520 nm). Panels A and B, temporins A and B on promastigotes; panels C and D, temporins A and B on amastigotes. Control parasites (●). The peptide concentrations were 7.5 µM (?), 10 µM (■), 15 µM (▲), 20 µM (▽). The first arrow (t=0 min) indicates the addition of peptide; the second arrow (t=45 min and 17 min for promastigotes and amastigotes, respectively) indicates the addition of 0.1% (final concentration) Triton X-100, as a complete permeabilization of the parasites. The values represent the mean of triplicate samples from a single experiment, representative of three different experiments.

FIG. 5. Electron microscopy of *Leishmania* promastigotes and amastigotes treated with temporins. Promastigotes and amastigotes were incubated for 1 h under standard conditions with 20 µM or 15 µM temporin A, respectively (panels A and D), 20 µM or 10 µM temporin B, respectively (panels B and E), or media without peptide (panels C and F). Membrane disruption (indicated by arrows), membrane blebbing and breakages as well as depletion of electron-dense cytoplasmic material can be observed for the two temporins (Bar=0.5 µm).
FIG. 6. Activity of temporins A and B on amastigotes. *L. pifanoi* amastigotes (2 x 10^7 parasites/ml) were incubated with different concentrations of temporins at 32 °C for 60 min and inhibition of MTT reduction was assayed immediately (panel A), or after proliferation for 5 days of the remaining viable amastigotes (panel B). Peptide designation: temporin A, circle; temporin B, triangles. Data points represent the mean of triplicate samples ± S.D. from a single experiment, representative of three different experiments.
### TABLE I

**Antimicrobial and hemolytic activity of temporin A, temporin B and cecropin A**

| Peptide     | *Leishmania donovani* | *E. coli* | *B. megaterium* | *S. aureus* Cowan I | Human erythrocytes |
|-------------|-----------------------|-----------|------------------|---------------------|--------------------|
| Temporin A  | 8.4 (7.5-9.4)         | 11.9      | 1.1 (1.8)        | 2.7 (3.4)           | >120               |
| Temporin B  | 8.6 (7.2-8.9)         | 21.0      | 1.3 (2.5)        | 6.2 (6.7)           | >120               |
| Cecropin A  | >50                   | 0.3       | 0.5 (not active) | >300                | >400               |

**Lethal concentration (μM)**

- *L. donovani* promastigotes were incubated with the corresponding peptides at different concentrations and viability was evaluated by MTT reduction as described in the Experimental Procedures section. Values are expressed as LC$_{50}$. 95% confidence limits determined by the Litchfield and Wilcoxon procedure are included in parentheses.

- Values are taken from (31).

- Numbers in brackets were obtained in 33% human serum. *S. aureus* Cowan I is resistant to 33% human serum, whereas *B. megaterium* Bm11 is only slightly sensitive. Controls were done in 33% human serum without peptide.

- Values are taken from (28).
Figure 1

Inhibition of MTT reduction (% relative to control parasites)

Peptide concentration (µM)

Inhibition of MTT reduction (% relative to control parasites)

Peptide concentration (µM)
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Temporins: Small antimicrobial peptides with leishmanicidal activity
Maria Luisa Mangoni, José M. Saugar, Maria Dellisanti, Donatella Barra, Maurizio Simmaco and Luis Rivas

J. Biol. Chem. published online October 28, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M410795200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts