Leishmania donovani: Thionins, plant antimicrobial peptides with leishmanicidal activity

Marta Berrocal-Lobo , Antonio Molina , Pablo Rodríguez-Palenzuela , Francisco García-Olmedo , Luis Rivas

Centro de Investigaciones Biológicas, Ramiro de Maeztu 9, E-28040 Madrid, Spain
Centro de Biotecnología y Genómica de Plantas (UPM-INIA), Departmento de Biotecnología E. T. S. I. Montes, Campus de Montegancedo, Universidad Politécnica de Madrid, Autopista M40 (Km. 38), 28223 Pozuelo de Alarcón, Madrid, Spain

A B S T R A C T

The leishmanicidal activity of plant antibiotic peptides (PAPs) from the principal families, such wheat thionins, a barley lipid transfer protein and potato defensins and snakins were tested *in vitro* against *Leishmania donovani*. Only thionins and defensins were active against this human pathogen at a low micromolar range of concentrations. Thionins resulted as the most active peptides tested until now. They collapsed ionic and pH gradients across the parasite plasma membrane together with a rapid depletion of intracellular ATP without affecting mitochondrial potential. Hence the lethal effect of thionins was mostly associated to permeabilization of the plasma membrane leading to an immediate death of the parasite. The present work is the first evidence for leishmanicidal activity in plant peptides. Future prospects for their development as new antiparasite agents on human diseases are considered.

Keywords:
Leishmania
Plant antimicrobial peptide
Thionin
Defensin
Snakin
Lipid transfer protein

1. Introduction

Plants and animals produce antimicrobial peptides against their pathogens, either expressed constitutively or induced after pathogen contact (Jenssen et al., 2006). Most of these peptides, polycationic and amphipathic, cause cell lysis upon interaction with the phospholipid matrix of the plasma membrane (Shai, 2002). Animal linear antimicrobial peptides have been extensively studied on prokaryotes using chemically synthesized analogs (Jenssen et al., 2006). Peptide specificity on prokaryotes is fairly understood and mainly based on the exposure of acidic phospholipids at the outer leaflet of their plasma membrane (Shai, 2002). However, most PAPs[1] that are active on phytopathogens such as thionins, defensins, lipid transfer proteins or snakins (Castro and Fontes, 2005; Berrocal-Lobo et al., 2002) are not linear, but possess multiple disulfide bonds which impose a more rigid structure and afford a higher stability of the peptide in the plant (Giudici et al., 2006). Recently a *Pyrularia pubera* thionin was synthesized chemically showing identical bactericidal activity *in vitro* as the native peptide (Vila-Perello et al., 2003). Approaches like that open new possibilities to the production of plant peptides to treat human infections. Surprisingly, the *in vitro* activity of PAPs has not been tested until now against Protozoa. For that reason the principal objective of present work was to test the activity of selected PAPs on *Leishmania donovani* promastigotes, the causative agent of human visceral Leishmaniasis. *Leishmania* promastigotes imposes important restrictions to the peptide activity. Their surface shows a high metalloproteinase activity, a thick anionic glycocalix with lipophosphoglycan, and a restrictive membrane repairing mechanism confined to the flagellar pocket (Handman, 1999). Different isoforms of thionins from wheat (*Triticum aestivum*), a barley (*Hordeum vulgare*) LTP2 (lipid transfer protein), a potato (*Solanum tuberosum*) defensin PTH1 and a potato snakin1, were tested against *Leishmania* promastigotes *in vitro*. In a further step, the mechanism of action of thionins, as the peptides with highest leishmanicidal activity, was studied. Some parameters related to the bioenergetic of the parasite and the membrane permeabilization, such as pH, ATP levels, the mitochondrial membrane potential and the H^+/OH^- gradients were measured. Our work shows that PAPs previously active against phytopathogens are also effective against

the human pathogen *Leishmania*. These results may foster the use of plant antimicrobial compounds as a new source of leishmanicidal agents.

2. Materials and methods

Promastigotes from *L. donovani* strain (MHOM/SD/00/1S-2D) were grown at 25 °C in RPMI at pH 7.2 as previously described (Luque-Ortega et al., 2003). A mixture of equimolar concentrations of the α-1, α-2 and β type I thionins from wheat (*Triticum aestivum*) isoforms, a barley (*Hordeum vulgare*) LTP2 (lipid transfer protein) α-1 isoform, and a PTH1 defensin and snakin1 from potato (*Solanum tuberosum*), were purified by HPLC as previously described (Caaveiro et al., 1997). Reagents were obtained from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO). Antimicrobial activity was measured by inhibition on MTT reduction by *L. donovani* promastigotes as described [8]. Briefly, parasites at 2×10^7 cells/ml were incubated at 24 °C with different peptide concentrations for 2 h. Afterwards, parasites were washed, resuspended in 100 μl Hanks + Glc containing MTT (0.5 mg/mL), and incubated for 2 h at 24 °C. The resulting precipitated formazan was solubilized by SDS addition and read in a 450 Bio-Rad ELISA microplate with a 600 nm filter as previously described (Luque-Ortega et al., 2003). For proliferation experiments, promastigotes (2×10^7 cells/ml) were incubated 2 h with the peptides, washed and resuspended (2×10^6 cells/ml) in their culture medium. Proliferation was then estimated after 48 h by MTT reduction as previously shown (Luque-Ortega et al., 2003). Experiments were done at least twice and samples were run in triplicate. LC_{50} and IC_{50} values were calculated by Litchfield and Wilcoxon procedure as previously described (Luque-Ortega et al., 2003). Effect of thionins on membrane permeation and intracellular pH changes were reached using a pH-sensitive fluorescence dye BCECF-AM, *Leishmania* promastigotes were preloaded with the BCECF-AM at 25 °C in the dark and later washed. Drugs were added after fluorescence stabilization. Afterwards fluorescence was measured in a spectrofluorometer at 500-nm (excitation) and 525-nm (emission) as previously described (Luque-Ortega et al., 2003). Full permeabilization was reached with Triton X-100. The decrease in intracellular ATP drop caused by thionins was assessed by *in vivo* luminescence of promastigotes transfected with *Photinus pyralis* luciferase in presence of the membrane-permeable substrate DMNPE-luciferin. Luminescence was recorded in a Polarstar Galaxy microplate reader (Luque-Ortega et al., 2003).

3. Results and discussion

Thionins showed the highest activity among the different set of PAPs assayed showing $IC_{50} = 0.2$ (0.1–0.5) μM and an $LC_{50} = 1.1$ (0.9–1.3) μM for parasite proliferation (Fig. 1a). Activity of thionin α-1 isoform was almost identical to that of the type I mixture (data not shown). Amastigotes, the intracellular form of the parasite, were more resistant to thionins, with $LC_{50} = 46.3$ (36.6–59.2) μM, than promastigotes. As the expression of LPG and Gp63 is close to nil in amastigotes, the change in phospholipid composition rather than peptide structure, likely underlie this difference between those *Leishmania* stages. In terms of concentration the leishmanicidal activity of thionins is very similar to those described for a broad spectrum of bacteria, fungi or eukaryotic cells (Castro and Fontes, 2005). Defensin (PTH1) kills *Leishmania* at higher concentrations than thionins with $IC_{50} = 33.4$ (21.5–52.4) μM and $LC_{50} = 5.9$ (3.2–10.8) μM (Fig. 1a). The fungal activity of radish defensin involved a specific membrane receptor, Ca^{2+} influx, and G protein activity (Castro and Fontes, 2005). Whereas these or similar features exist in *Leishmania*, this would require further study.

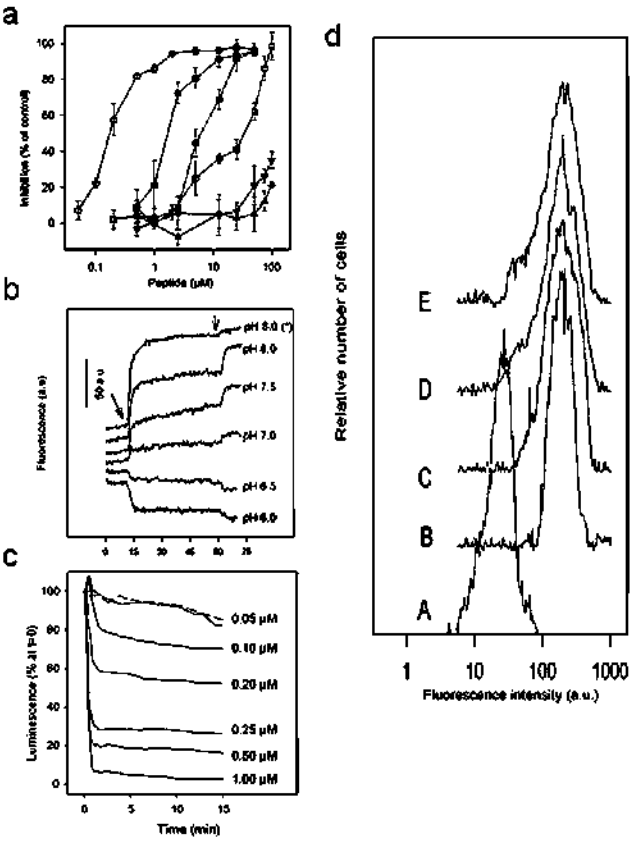

Fig. 1. (a). Inhibition of proliferation and MTT reduction of *L. donovani* promastigotes. Proliferation (solid symbols), MTT reduction (empty symbols). Type I thionins (circle), defensin PTH1 (square), LTP2 (triangle down) and snakin 1 (triangle up). (b) Dissipation of H^+/OH^- gradients across plasma membrane of *L. donovani* promastigotes preloaded with BCECF-AM. The external pH is shown at the left side of the trace. Left and right arrows stand for addition of 1 μM thionin and Triton X-100, respectively. (+) Thionins 2 μM. (c) Intracellular ATP decrease after thionin addition assessed by luminescence *in vivo* on *L. donovani* promastigotes. Traces corresponding to the peptide concentrations are continuous. The luminescence of untreated parasites is shown as dotted trace. (d) Mitochondrial membrane potential of *L. donovani* measured by rhodamine 123 accumulation after thionin treatment. Traces: (A) Uncoupled parasites with 7.5 μM FCCP; (B) Untreated parasites; (C–E) 0.5, 1.0 and 2.5 μM thionins, respectively.

LTP2 and snakin1 were virtually inactive at the conditions tested; showing LC_{50} higher than 100 μM (Fig. 1a).

Due to their highest activity, thionins were selected for a detailed study. This activity was dependent on temperature and pH, decreasing dramatically below 25 °C and pH7, respectively, and was inhibited by Ca^{2+} (94% inhibition at 1 mM), heparin and seroalbumin (Table 1), both known quenchers of hydrophobic compounds. Nevertheless, these conditions are far to be physiological for cell survival. However, thionin activity increased with ionic strength which, in some way, may promote higher hydrophobic interactions with parasitic membrane (Table 1). Depolarization by valinomycin only increased modestly the parasite survival, discarding membrane potential as an essential factor for thionin effectiveness (Table 1). At 1 μM thionins dissipated H^+/OH^- gradients across the plasma membrane of *Leishmania* promastigotes, inducing a rapid equilibration between internal and external pH. Total permeation was reached with Triton X-100 (right arrow at Fig. 1b). Furthermore, this permeation lead to drop in free intracellular ATP levels with 80% of their effect reached in just 2 min even at concentrations lower than their corresponding IC_{50} or LC_{50} (Fig. 1c). This quick kinetics supports the membrane permeability as an essential step in the lethal mechanism of thionins on *Leish-*

Table 1
MTT reduction inhibition by thionins on *L. donovani* promastigotes tested.

Changed condition respect standard[a]	% Inhibition (MTT reduction)
4 °C	6.3 (3.5)
20 °C	32.6 (5.6)
37 °C	85.3 (8.7)
pH 5.0	56.5 (3.5)
pH 6.0	54.8 (8.3)
pH 8.0	80.3 (6.1)
0.5 mM Ca^{2+}	40.5 (3.7)
1.0 mM Ca^{2+}	6.2 (4.6)
0.2 mg/ml Heparin	5.1 (7.4)
0.05 μM Valinomycin	80.3 (4.5)
0.10 μM Valinomycin	65.7 (5.1)
10 mg/ml BSA	3.5 (2.6)
NaCl 140 mM, 0.2 μM thionin	63.7 (4.5)
NaCl 120 mM, 0.2 μM thionin	52.5 (6.5)
NaCl 80 mM, 0.2 μM thionin	6.7 (4.5)
NaCl 5 mM, 0.2 μM thionin	8.4 (2.3)

[a] Standard conditions: 24 °C, pH 7.0, 140 mM NaCl, 0.5 μM thionins; % Inhibition (MTT reduction): 76.3 (6.7).

mania as reported for other membrane-active compounds (Luque-Ortega et al., 2003). Additionally, it makes unlikely the establishment of mixed disulfides bounds between key metabolic enzymes and thionins previously described *in vitro* as essential for lethality [10]. Furthermore the maintenance of electrochemical potential in mitochondria, at least for a time range where the drop in free ATP takes place, ruled out the inhibition of thionins on mitochondrial ATP synthesis, as described for histatin 5 in *Leishmania* (Luque-Ortega et al., 2008) (Fig. 1d). Cytotoxicity of *Pyrularia* thionin on mammalian cells required Ca^{2+}influx and activation of endogenous PLA_2 activity, likely by enhancing phospholipid accessibility to the enzyme (Evans et al., 1989). However, this is not the case for *Leishmania*, on which EGTA, $NiCl_2$, verapamil (chelator and competitive and non-competitive inhibitors of Ca^{2+} channels, respectively), and dexamethasone (inhibitor of PLA_2), were unable to affect to the thionin activity (data not shown).

Plant thionins are among the highest efficient antimicrobial peptides tested against *L. donovani* promastigotes so far. This work and recent works related to thionin activity against other human pathogens (Vila-Perello et al., 2003; Stec, 2006) open new therapeutic possibilities to PAPs on human infectious diseases. Including that thionin activity, although lower, was still preserved on amastigotes, the effect on promastigote might be enough to stop the infection before happen. Further studies addressing the pharmacological applications of thionins and the therapeutic potential of their shorter synthetic analogs are currently in progress.

Acknowledgments

We thank Dr. Juan Román Luque-Ortega and Dra. Katrina Ramonell for critical reading of the manuscript. Mutated luciferase gene was kindly provided by Toni Aebischer.

This work was supported by research grants from the Consejo Superior de Investigaciones Científicas (CSIC; PIF 80F0171), Fondo de Investigación Sanitaria (FIS PI06115 and RD 06/0021/0006) to L.R., BIO2005-07198 to P.R.-P., BIO2005-07198, BIO2003-4424 and BIO2006-00488 to A.M.

References

Berrocal-Lobo, M., Segura, A., Moreno, M., Lopez, G., Garcia-Olmedo, F., Molina, A., 2002. Snakin-2, an antimicrobial peptide from potato whose gene is locally induced by wounding and responds to pathogen infection. Plant Physiology 128, 951–961.

Caaveiro, J.M.M., Molina, A., González-Mañas, M.J., Rodríguez-Palenzuela, P., García-Olmedo, F., Goñi, F.M., 1997. Differential effects of five types of antipathogenic plant peptides on model membranes. FEBS Letters 410, 338–342.

Castro, M.S., Fontes, W., 2005. Plant defense and antimicrobial peptides. Protein Peptide Letter 12, 13–18.

Evans, J., Wang, Y., Shaw, K.-P., Vernon, L.P., 1989. Cellular responses to *Pyrularia* thionin are mediated by Ca2+ influx and phospholipase A2 activation and are inhibited by thionin tyrosine iodination. Proceedings of the National Academy of Sciences of the United States of America 86, 5849–5853.

Giudici, M., Poveda, J.A., Molina, M.L., de la Canal, L., Gonzalez-Ros, J.M., Pfuller, K., Pfuller, U., Villalain, J., 2006. Antifungal effects and mechanism of action of viscotoxin A3. FEBS Journal 273, 72–83.

Handman, E., 1999. Cell biology of *Leishmania*. Advances in Parasitology 44, 1–39.

Jenssen, H., Hamill, P., Hancock, R.E., 2006. Peptide antimicrobial agents. Clinical Microbiology Reviews 19, 491–511.

Luque-Ortega, J.R., Saugar, J.M., Chiva, C., Andreu, D., Rivas, L., 2003. Identification of new leishmanicidal peptide lead structures by automated real-time monitoring of changes in intracellular ATP. Biochemical Journal 375, 221–230.

Luque-Ortega, J.R., van't Hof, W., Veerman, E.C., Saugar, J.M., Rivas, L., 2008. Human antimicrobial peptide histatin 5 is a cell-penetrating peptide targeting mitochondrial ATP synthesis in *Leishmania*. FASEB Journal 22, 1817–1828.

Shai, Y., 2002. Mode of action of membrane active antimicrobial peptides. Biopolymers 66, 236–248.

Stec, B., 2006. Plant thionins – the structural perspective. Cell Molecular Life Sciences 63, 1370–1385.

Vila-Perello, M., Sanchez-Vallet, A., Garcia-Olmedo, F., Molina, A., Andreu, D., 2003. Synthetic and structural studies on *Pyrularia pubera* thionin: a single-residue mutation enhances activity against Gram-negative bacteria. FEBS Letters 536, 215–219.