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Thaulin-1: the first antimicrobial peptide isolated from the skin of a Patagonian frog *Pleurodema thaul* (Anura: Leptodactylidae: Leiuperinae) with activity against *Escherichia coli*

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ABSTRACT

Patagonia's biodiversity has been explored from many points of view, however, skin secretions of native amphibians have not been evaluated for antimicrobial peptide research until now. In this sense, *Pleurodema thaul* is the first amphibian specie to be studied from this large region of South America**.** Analysis of cDNA-encoding peptide in skin samples allowed identification of four new antimicrobial peptides. The predicted mature peptides were synthesized and all of them showed weak or null antimicrobial activity against *Klebsiella pneumonia*, *Staphylococcus aureus* and *Escherichia coli* with the exception of thaulin-1, a cationic 26-residue linear, amphipathic, Gly- and Leu-rich peptide with moderate antimicrobial activity against *E. coli* (MIC of 24.7 µM). AFM and SPR studies suggested a preferential interaction between these peptides and bacterial membranes. Cytotoxicity assays showed that thaulin peptides had minimal effects at MIC concentrations towards human and animal cells. These are the first peptides described for amphibians of the Pleurodema genus. These findings highlight the potential of the Patagonian region's unexplored biodiversity as a source for new molecule discovery.

Key words: Anura; Atomic Force Microscopy; cDNA; Circular Dichroism; Surface Plasmon Resonance.

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INTRODUCCTION

During the last decades there has been a rapid increase in the number of antimicrobial peptides (AMPs) described, with more than 1017 active peptides derived from amphibians (antimicrobial peptide database, APD). Most of these share common characteristics: they are in general, cationic, amphipathic and with an α-helix secondary structure, although some peptides with different characters such as negative charges [Harris et al., 2009] or with different secondary structures [Wang & Zasloff, 2010] have occasionally also been described. All AMPs are expressed as prepropeptides harboring a conserved signal peptide, an acidic propiece and a highly variable domain encoding the AMP itself [Amiche et al., 1999]. This last domain is so variable that the combination of several of these peptides can form a distinctive marker for each amphibian specie [Jackway et al., 2011]. However, within this variability, many peptides share common features such that they have been classified into common peptide families, such as the brevinins [Moricawa et al., 1992], caerin, dermasemptins [Amiche etal., 1999], esculetins [Simmaco et al., 1993], magainins [Zasloff, 1987], ocellatins [Marani et al., 2015], temporins [Mangoni et al., 2005], etc. Meanwhile, some peptides with totally novel structures, which belong to no yet-described family have also been occasionally described (Leu rich peptides).

AMPs are important functional molecules in the innate immune system and they play defensive roles against external risk factors. However, besides antimicrobial activity, multiple potential applications have been identified for AMPs such as anticancer [Oelkrug et al., 2015; Wu et al., 2014; Gaspar et al., 2013; Chu et al., 2015], insecticidal [Smith et

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al., 2011], chemotactic [Craik et al., 2010] antioxidant [Guo et al., 2014], spermicidal [Zairi et al., 2009], wound healing [Mangoni et al. 2016]; protease inhibition [Miller et al., 1989] and neuropeptide function [Pukala et al., 2006] among others. Moreover, even derivatives of AMPs have shown a broad range of different applications [Abdel-Wahab et al., 2008; Chalrabarti et al., 2003; Schulze et al., 2015]. It is worth noting also that several species appear to lack an AMP system or express AMPs with null or weak antimicrobial activity against human pathogenic bacteria, however, when tested, the same peptides showed strong antimicrobial activity against species-specific microorganisms demonstrating adaptation to their living environments [Sun et al., 2015].

This wide natural variation, and the potential industry application of AMPs suggests that bioprospection of new structures and biological functions of AMPs from amphibian skin secretions can become a valuable tool for the discovery of new molecules.

The Patagonian region has a broad biodiversity with more than 50 species of amphibians, all of them unexplored, so it is a promising source of new peptides. *Pleurodema thaul* belongs to the Leptodactylidae family, Leiuperinae (94sp.) subfamily, where only one of the 94 species has been studied to date (Physalaemus nattereri, ex Eupemphix nattereri), in which the presence of only two bradykinin precursors were described (NCBI access number BKL1 KJ955469, BBN1 KJ955468). None of the species of the *Pleurodema* genus has been explored for AMPs until now. *P. thaul* (Schneider, 1799) is widely distributed in Chile and Argentina (Figure 1A) [Veloso et al., 2010] *P. thaul* presents a pair of prominent elevated cutaneous dorso-lateral lumbar glands, just posterior to the sacrum, that may be confused with eyes (Figure 1C) giving predators the impression that is a larger animal, which is why it is also known as “the four eyed frog”.

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These kind of macroglands contains granular alveoli filled with secretions that present toxicity for several microorganisms and vertebrates [de Toledo and Jared, 1989]. Histological analysis of *P. thaul* suggested a defensive role for the lumbar glands given the presence of the granular alveoli and the typical distribution of the dermal chromatophores, although no study regarding its secretion was made until now.

Figure 1. (A) *Pleurodema thaul* distribution from the International Union for Conservation of Nature (IUCN). (B) Collection site area (photo by S. Polcowñuk, used with permission). (C) Photograph of a juvenile *P. thaul* (photo by F. Jara, used with permission)

The objective of this work was the identification and characterization of *P. thaul* skin AMPs by mRNA isolation, cDNA cloning and sequencing. We report the discovery of

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four new peptides identified from the skin of *P. thaul*. The biological activity of the peptides was evaluated and a general characterization of the most active peptide was performed.

RESULTS AND DISCUSSION

Identification of cDNA Encoding Peptides. Isolated *P. thaul*-skin total RNA was reverse-transcribed to AMP cDNA using specific primers. cDNA was cloned and more than 30 colonies were separately grown in liquid media for subsequent plasmid purification. Sixteen purified plasmids were subjected to PCR amplification, and the purified fragments were sequenced (Figure 2A shows nucleic acid and deduced amino acid sequence of cDNA encoding thaulin-1). Four *de novo* sequences were obtained (Figure 2B). The amino acid sequences deduced from the cDNA sequences showed a tripartite structure, as expected [Amiche et al., 1999]: a signal peptide, an acidic sequence and the mature peptide at the C-terminus (Figure 2B). The signal peptide composed of 22 amino acids ended with a Cys residue similarly to other described AMPs [Amiche et al., 1999], except for thaulin-4 were a Ser was present. It is identical to several signal peptides identified in other genera of amphibians, for example dermaseptins B2, of *Phyllomedusa bicolor*; DRP-AA3-6 of *Agalychnis annae*; DRP-PD3-3 of *Pachymedusa dacnicolor*; caerin 1.1 of *Litoria splendida* [Amiche et al., 1999; Jackway et al., 2011; Nicolas et al., 2003]. It has been hypothesized, that this region may be used as a signal for endoplasmic reticulum membrane translocation [Brand et al., 2006]. The acidic region contained between 22 and 29 amino acids residues and, in three of the four peptides, it ends with Lys-Arg as most of reported antimicrobial peptides precursors. However thaulin-1 does not end with Lys-Arg;

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nevertheless, this exception was also seen in other published AMP [Li et al., 2007]. The four mature identified peptides consisted of a 26, 24, 20 and 13 amino acid length. Theoretical molecular weight, estimated pI (http://web.expasy.org/compute_pi/) and total net charge of peptides are presented in Table 1.

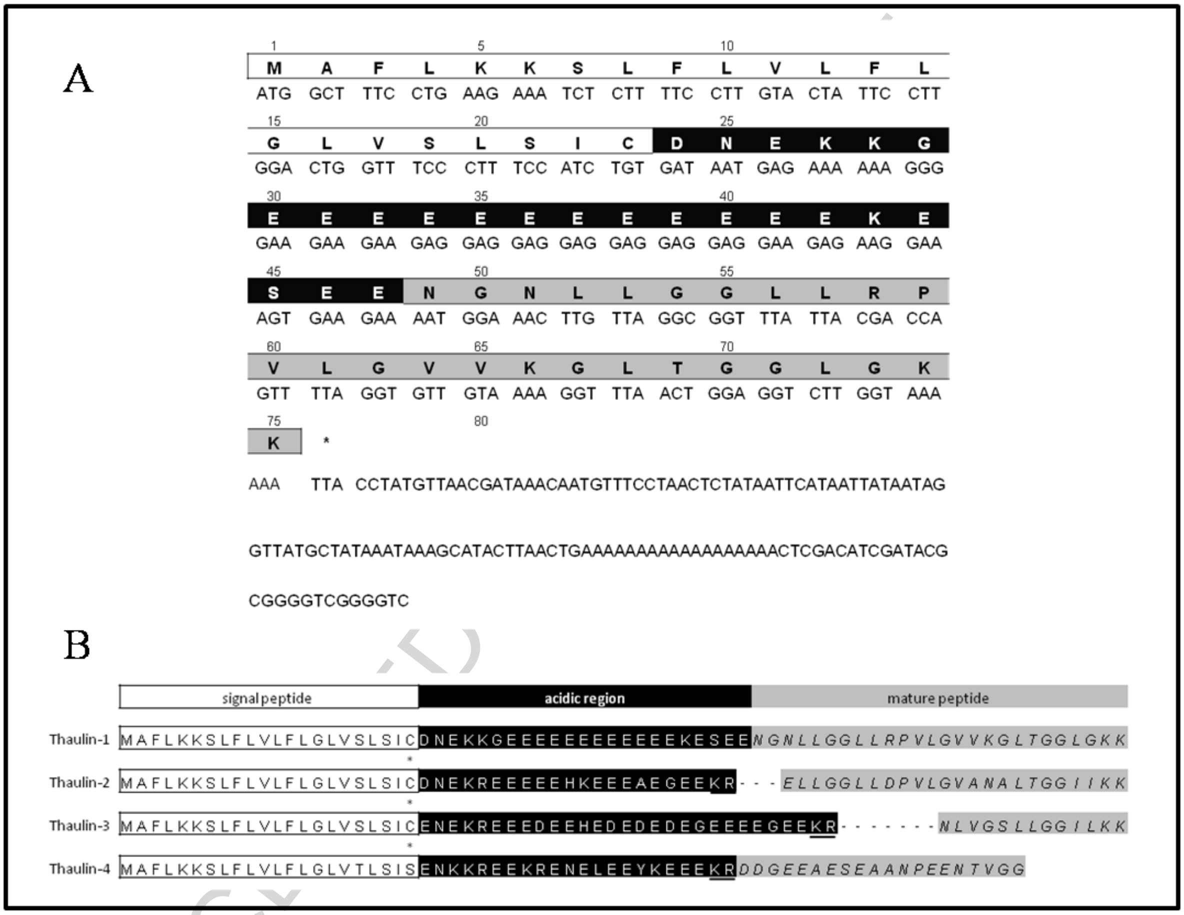

Figure 2. (A) Nucleic acid and deduced amino acid sequence of cDNA encoding thaulin-1 from the skin of *P. thaul*. Signal peptide, acidic region and mature peptide are signaled in boxed white, black and gray letters respectively and stop codon is indicated with an asterisk. (B) Alignment of the deduced amino acid sequences of the precursors of thaulin peptides. Preproregions (signal peptide and acidic region boxed white and black respectively) and variable domain (boxed gray) that correspond to mature peptide are

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signaled. Cys indicating the end of the signal peptide are indicated with an asterisk. Processing sites Lys-Arg present in the acidic region are underlined. Gaps (-) have been introduced to maximize sequence similarities.

The new peptides were named thaulins following the nomenclature of antimicrobial peptides proposed by Amiche et al. [Amiche et al., 2008] and Conlon [Conlon, 2008 (a); Conlon, 2008 (b)]. The most common approach for peptide nomenclature is to derive the peptide name from the name of the species. The numbers 1 to 4 were incorporated to indicate that these are the first AMPs found in this species, as suggested by some authors [Wang and Zasloff, 2010, Amiche et al., 2008¸ Conlon, 2008 (a); Conlon, 2008 (b)]

Preliminary Activity Test. The four thaulin peptides were manually synthesized and submitted to a preliminary activity test against *E. coli* and *S. aureus*. Only thaulin-1 showed activity against the two strains while thaulin-3 presented a weaker activity only against *E. coli*. This could be related with peptide net charge. Thaulin-1 and thaulin-3 were the only two peptides with positive net charge, while thaulin-2 has no net charge and thaulin-4 a negative net charge (Table 1). Based on these results the following analyses were performed only for thaulin-1 peptide, as this presented the highest antimicrobial action.

Sequence Analysis. Although antibacterial peptides have very low sequence similarity, recent studies demonstrate that certain residues are preferred over others in specific spots, particularly at the N and C terminus. Analysis of the C-terminus of thaulin-1 showed that eleven of the last fifteen residues match with the first three most likely residues observed for antibacterial peptides in each position [Lata et al., 2009] Also, the large

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percentage of Leu and Gly in the composition of thaulin-1 agrees with an observation made by Wang & Zasloff about AMP amino acid sequences, which is that Leu, Gly and Lys are the most frequently occurring amino acids in AMPs from animals [Wang and Zasloff, 2010]

The thaulin-1 sequence was compared with the APD database to look for the most similar peptides. Table 2 summarizes the top five results. Multiple alignment analysis revealed structural similarity with two Gly-rich peptides and 3 peptides of the bombinin subfamily (Table 2).

Thaulin-1 has 30% of Gly and 26% of Leu composition, making thaulin-1 a Glycine/Leucine-rich peptide. In common with bombinin, thaulin-1 harbours a PVLGxV motif in the middle of the sequence (where x- position Val-for-Leu change is a conserved substitution) and the GGLxKK motif at the C-terminus (where x is a Gly, Ile or Leu).

Further comparisons through other protein databases revealed that thaulin-1 presents structural similarity with parts of sequences of bacterial transmembrane proteins. Six of the most similar proteins found by Blastp are depicted in Table 3. It is noteworthy that the highest similarity belongs to membrane proteins described in Gram-negative microorganisms. The shared region involves a similar sequence that repeats throughout the molecule a few times. This pattern suggests that the similar region behaves as a transmembrane helix spanning the lipid bilayer, and also supports a potential role of thaulin-1 as a membrane disruptor. This observation is sustained by secondary structure predictions and atomic force microscope observations .

Secondary Structure Prediction. Wang and Zasloff hypothesized that abundance of residues in an AMP sequence may contain structural information. That is that we can

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"predict" secondary structure based on amino acid residues content. They made a statistical analysis of AMPs annotated as "helix" in the APD (based on NMR and circular dichroism) and found that Leu, Ala, Gly and Lys residues are the most abundant in contrast with peptides with known β or αβ structures where Cys, Gly and Arg or Cys and Gly are abundant residues [Wang and Zasloff, 2010]

Thaulin-1 presents 6 Leu, 8 Gly and 3 Lys residues which represent 65% of abundance in their amino acid content.

Three-Dimensional structure prediction of thaulin-1 and Gly-thaulin-1 showed a central alpha helix structure with ends in random coil form (Figure 3, C and D). Helix wheel projection demonstrated an aliphatic structure; especially when we only considered the residues that integrate the α-helix in the predicted 3D structure (Figure 3, E and F).

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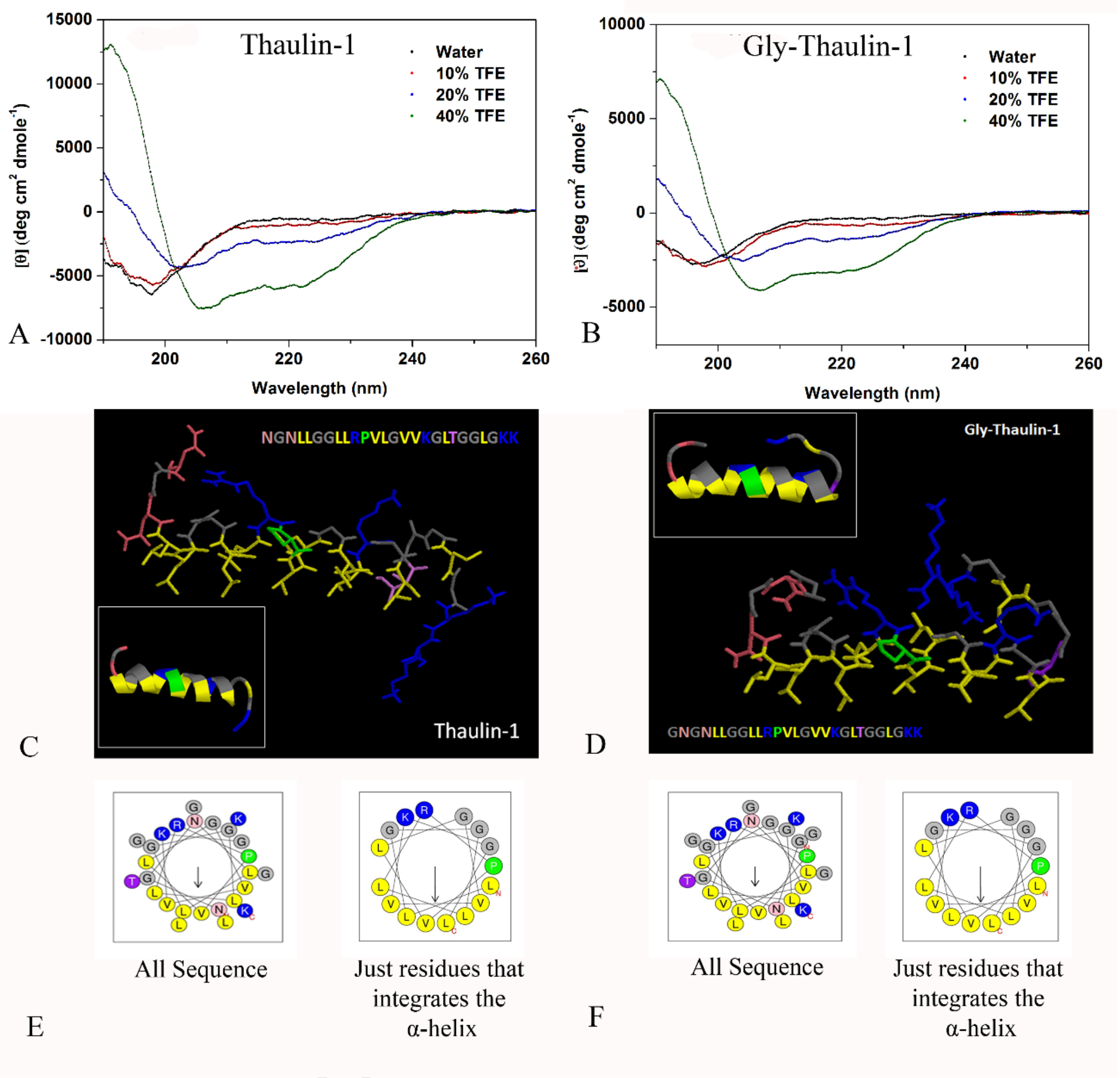

All Sequence

Just residues that integrates the α-helix

All Sequence

Just residues that integrates the α-helix

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Figure 3. Graphical analysis of thaulin-1 and Gly-thaulin-1 peptide structures. Circular dichroism of peptides (A) thaulin-1 and (B) Gly-thaulin-1 in aqueous (black) and 10, 20 and 40% of 2,2,2-trifluoethanol (TFE) solutions (red, blue and green respectively). (C) thaulin-1 and (D) Gly-thaulin-1 3D structure prediction. Schiffer and Edmundson wheel projection diagrams of complete and α-helix fragment of (E) thaulin-1 and (F) Gly-thaulin-1 sequences. Amino acid colour code: yellow= unpolar/hydrophobic (Leu, Val), gray= Gly, blue= basic (Lys,

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Arg), purple= polar without charge (Thr), pink= polar without charge (Asn), green= Pro.

Peptide Synthesis and Characterization. Residue composition analysis of thaulin-1 was performed to evaluate potential difficulties during peptide synthesis as well as to increase the yields of the process for possible large-scale production. We estimated that peptides with asparagine (Asn) at the N-terminal position would present difficulties when removing the N-terminal protecting group [http://www.sigmaaldrich.com/life-science/custom-oligos/custom-peptides/learning-center/sequence-analysis.html; http://www.biomatik.com/services/custom-peptide-service/peptide-synthesis.htlm]. It is highly recommended to remove the Asn or substitute it with another amino acid at the N-terminus; therefore, a thaulin-1 derivative with an N-terminal Gly named Gly-thaulin-1 was synthesized to characterize and compare it with thaulin-1. Thaulin-1 and its derivative Gly-thaulin-1 were both manually synthesized and purified by reversed-phase high performance liquid chromatography (RP-HPLC). To evaluate purity and molecular mass of thaulin-1 and Gly-thaulin-1, a MALDI-TOF/TOF analysis was performed resulting in $[M+H]^+$ of 2531.10 Da and 2588.17 Da, respectively (Figures S1 and S2). Tandem mass spectrometry (MS/MS) analysis of purified thaulin-1 and Gly-thaulin-1 corroborates the obtained peptides sequences. Spectrum measurements are available in the supporting information (Figures S3 and S4).

Circular Dichroism (CD). To study the conformational behaviour of thaulin-1 and Gly-thaulin-1, CD analyses were performed in water and TFE solutions of increasing concentrations. CD measurements in water and 10% of TFE revealed a random

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conformation with a minimum close to 198 nm. As TFE concentration increases, the shape of CD spectra suggests the propensity to form defined secondary structures. All 40% TFE spectra showed two minima around 209 and 222 nm, characteristic of α–helix structures (Fig. 3A). This result could indicate that these peptides are unstructured (unfolded/linear) in aqueous solution and that they fold into alpha-helices upon interaction with microorganisms, as was previously demonstrated for other antimicrobial peptides including aurein 1.2 and magainin 2 and cecropin A using different tools such as Fourier-transform infrared (FTIR) spectroscopy [Seto et al., 2007] and CD [Avitabile et al., 2014] respectively. The role of peptide self-assembly is considered as another important property that influences on the activity and mode of action of peptides such as particular responsiveness, sustained release, improved selectivity and stability [Tian et al., 2015].

Antimicrobial Assays. Synthetic purified and quantified thaulin-1 and Gly-thaulin-1 peptides showed identical minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values (Table 4), demonstrating that the mechanism of action is mainly bactericide, not involving a significant inhibitory stage. Both Gram-negative and Gram-positive strains were tested, and the former were found to be more susceptible, showing MIC/MBC values of 62.5 µg/mL and 125 µg/mL for *E. coli* and *K. pneumoniae*, respectively. These results are consistent with the weak antimicrobial activity described for Leptoglycin, the peptide that present 53.84% sequence similarity with thaulin-1 (Table 2). Leptoglycin, is Gly/Leu rich AMP isolated from the skin secretion of the South American frog *Leptodactylus pentadactilus* (Table 2) and it was active only against Gram-negative strains with MIC value for *E. coli* of 50 µM (Sousa et al., 2009), the double of the value found for thaulin-1 (MIC 24.7 µM). According to the Shai-Matsuzaki-

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Huang (SMH) model of antimicrobial peptide action [Matsuzaki, 1999; Shai, 1999], a positive net charge is needed to interact with the bacterial membrane, so that the presence of negatively charged lipopolysaccharides (LPS) in the outer membrane of Gram-negative bacteria oriented towards the exterior can favor the interaction of peptides. No difference between the activity, as measured by MIC and MBC, of thaulin-1 and its analogue Gly-thaulin-1 were observed, indicating that the presence of N-terminal Gly did not alter antimicrobial performance of thaulin-1.

Antileishmania Assays. Thaulin-1 and Gly-thaulin-1 were incubated with amastigote and promastigote forms of *L. infantum* to evaluate whether they possess inhibitory activity or not. We found no activity in the range of 1 to 128 µg/mL (Table S1, Figure S5).

Cytotoxicity. Hemolytic Assays. Thaulin-1 and Gly-thaulin1 peptides were tested in a hemolysis assay. They showed little activity on human red blood cells (RBCs) when assessed at concentrations corresponding to *E. coli-K. pneumonia* MIC/MBC (less than 3.5%). These data indicate that thaulin-1 and Gly-thaulin-1 may have a selective effect towards Gram-negative bacteria cells with respect to human RBCs. However, at higher peptide concentrations (500 µg/mL) both peptides present approximately 85% hemolysis, which may hamper their application against *S. aureus* (Figure S6). Once again, no significant difference was seen between the wild type peptide, and the Gly-substituted variant.

Cytotoxicity. Cell Culture and Proliferation Assay. Thaulin-1 and Gly-thaulin-1 peptides were tested with BMDM to evaluate peptide cytotoxicity in the range of 32 to 1024 µg/mL. After incubation for 24 h and as depicted in Table S1, the two AMPs tested

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showed low cytotoxicity against BMDM. In fact, both peptides presented 50% cytotoxicity concentrations (CC50) superior to 256µg/ml (Figure S7).

Atomic Force Microscopy (AFM). Analysis of images obtained by AFM demonstrated how thaulin-1 affects *E. coli* cell morphology on treatment. Figure 4 shows representative images of the thaulin-1 morphological alterations on *E. coli* ATCC 25922 cells, at the 62.5 µg/mL (25 µM) MIC concentration, compared with untreated cells. Upon treatment we saw considerable changes in the membrane texture that may be due to a membrane destabilization, which could induce cell inhibition and death. Unfortunately, this work does not provide direct evidence that this is the mechanism. Cell surface roughness is a measure of changes in cell texture. Since native cell membranes tend to be very smooth, increased roughness would be expected for significant changes in surface texture, providing a way to quantify such textural changes. An increase in roughness of the treated membranes (mean roughness = 5.05 ± 1.3 nm) was observed when comparing treated membranes with controls (mean roughness = 1.66 ± 0.3 nm) as illustrated in Figure S8. This difference in roughness was also found to be statistically significant, by measuring it over a number of different cells.

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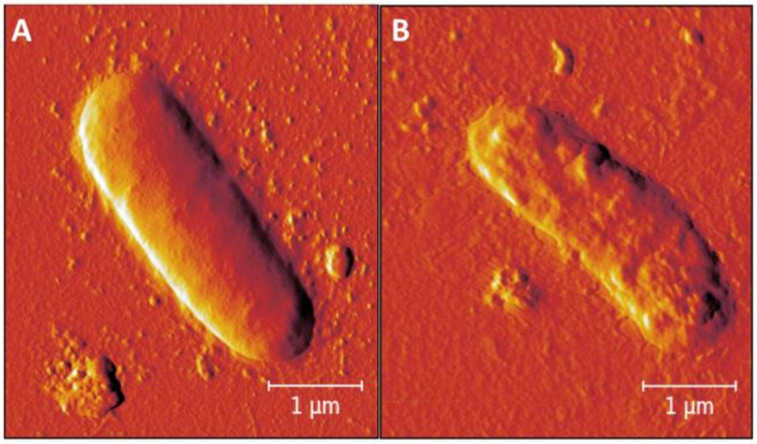

Figure 4. Representative AFM image of the antimicrobial effect of thaulin-1 on E. coli ATCC 25922: (A) untreated; (B) after 24 h of thaulin-1 treatment at the MIC.

Surface Plasmon Resonance (SPR). SPR analysis was performed to evaluate thaulin-1 and Gly-thaulin-1 affinity for membrane models that differ in lipid composition (Figure 5). This and other similar methodologies based in the affinity for different biomembrane models were successfully explored for other peptides.[Salay et al., 2011; Yu et al., 2015; Bechinger, 2010] Two membrane models, consisting of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE): 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG) (3:1) lipids were used to simulate mammalian cell and bacterial membranes, respectively. Phosphatidylcholine (PC) is commonly present in membranes of eukaryotic organisms [de Kroon, 2007] while, despite variations among species, bacterial membranes tend to contain more lipids with anionic head groups [Epand and Epand, 2009]

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The latter group is effectively mimicked by use of phosphatidylglycerol (PG) [Fernandez et al., 2012]

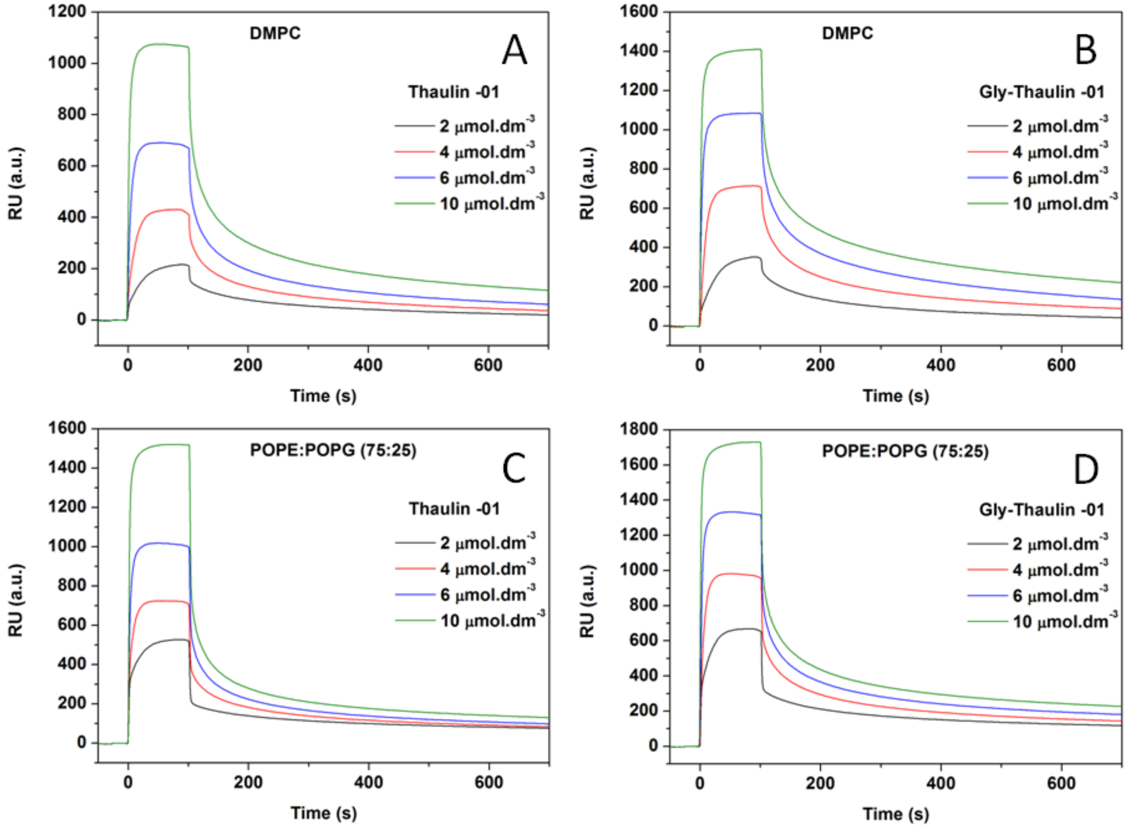

Figure 5. Representative Surface Plasmon Resonance sensograms obtained for thaulin-1 and Gly-thaulin-1 binding to mimetic membrane models. (A) and (B) DMPC supported unilamellar bilayers. (C) and (D) POPE:POPG (75:25) supported unilamellar bilayers.

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Representative SPR sensorgrams are shown in Figure 5, illustrating results found using these membranes as the stationary phase, upon injection into the flow cell of the SPR of the two peptides. These were further analyzed by fitting of this data as described in the

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methods section, and the results are reported in Table 5. Thaulin-1 and Gly-thaulin-1 show an increased affinity for anionic membrane systems, which is an important fact in considering their use as antimicrobial agents (Table 5).

Thaulin-1 has an affinity constant (K_A) approximately three thousand times greater for POPE:POPG than it has for DMPC, indicating a higher molecular binding strength for this bacterial membrane model than for the model for eukaryotic membranes. In addition, the “on rate” constant (ka_1) is four orders of magnitude greater for POPE:POPG than for DMPC demonstrating higher speed in interacting with anionic membranes.

In the case of Gly-thaulin, while it has the same order of magnitude of “on rate” constant for DMPC and POPE:POPG, the affinity constant is again approximately three thousand times higher for POPE:POPG, suggesting a stronger molecular binding for bacterial membranes than for eukaryotic membranes. It is interesting that the difference found in ka_1 between Gly-thaulin, and the wildtype thaulin was the only significant difference in the results we noted between them, indicating that this substitution affects the kinetics of initial interaction with the membranes. However, the affinity itself was unaffected by this substitution, and all other results point to the two peptides being functionally identical. Thus we can state that the addition of Gly at the N-terminal do not impair its antibacterial action, as far as we could determine, while it simplifies considerably the synthesis of the AMP.

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CONCLUSIONS

This work reports the first four peptides identified from the skin of the Patagonian frog *P. thaul*. This represents the first reported study of AMPs from amphibians from this

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large region of South America. A meticulous characterization of thaulin-1 was performed. Thaulin-1 is a new amphipathic, cationic 26-residue Gly- and Leu-rich peptide, with an α-helix secondary structure. Its main antimicrobial activity is against *E. coli*. It is noteworthy that thaulin-1 not only showed structural similarity with other AMPs, but also with parts of the sequence of diverse transmembrane proteins. This observation together with CD results that demonstrate the influence of the environment in the secondary structure could be related with a mechanism of action of thaulin-1 in bacteria death. Environmental conditions could trigger self-aggregation of thaulin-1 in solution through hydrophobic interactions and this could be part of the mechanism of action of the peptide, perhaps a direct interaction with membranes by pore forming or structure destabilization. This conjecture of a mechanism of bactericide action based on alteration of membrane structures is also supported with the AFM evidence collected from *E. coli* and SPR affinity constants calculated for bacteria model membranes. In addition, our cytotoxicity studies demonstrated that thaulin AMPs present acceptable levels of tolerance at MIC concentrations on eukaryotic cells, which corresponds with the small affinity constants found for the thaulins with eukaryotic model membranes. Even though these results do not suggest suitability study for many human applications, alternative uses in areas where light/low antimicrobial activity is required such as food control, wound healing or antibiofilm agents should not be disregarded. The latter activity was predicted using the dPABBs web server [Gupta et al., 2016] where three of the four thaulins peptides were predicted to be biofilm-active. Further work will be performed to explore this application.

MATERIALS AND METHODS

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Amphibian Collection. One juvenile specimen of *P. thaul* was collected in a wetland meadow in Llao Llao Municipal Park (41°2’S; 71°33’W; 821 msnl), San Carlos de Bariloche, Río Negro, Argentina (Figure 1B) under the license SADS RN° 006/14. The authors state that all animal manipulation were carried out in accordance with the ARRIVE guidelines.

cDNA Gene Cloning. Total RNA from the dorsal region skin (previously frozen in liquid nitrogen and pulverized in a mortar) of a single sample of an anesthetized-with-lidocaine hydrochloride *P. thaul* was extracted using the reagent trizol (Invitrogen). Agarose gel and spectrophotometer analysis techniques were used for quality and quantity analysis of the RNA, respectively. Reactions of 3′RACE were performed for cDNA synthesis using the M-MLV Reverse Transcriptase (Promega). The first round was performed by using primer 008-EDdTAP (5′-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTT-3′). The second round of PCR used PPS-1A (5′-ATGGCTTTCCTGAAGAAATCTCTTTTCCTTGTACTATTCCTTGG-3′) as forward primer and 009-EDAP (5´-GACCACGCGTATCGATGTCGAC-3′) as reverse primer. The cycling parameters were as follow: one cycle of 94 °C/120 s, four cycles of 94 °C/60 s, 53 °C/60 s and 72 °C/60 s; 35 cycles of 94 °C/30 s, 53 °C/30 s and 72 °C/60 s and 72 °C/420 s. PCR products were purified using ADN PuriPrep-GP Kit (Highway).

Purified fragments were used in a ligation reaction with the pCR^{TM}4-TOPO$^{®}$ of the TOPO$^{®}$ TA cloning kit for sequencing (invitrogen), following the manufacturer´s instructions. Two microliter aliquots were used for *E. coli* DH5α competent cells transfection. Competent *E. coli* DH5α cells were prepared and transformed using calcium

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chloride as described by Sambrook & Russel.[48] Following the selection and growth of bacterial colonies, the resulting plasmids were purified (Highway) and subjected to sequencing by the BigDye® terminator reaction (Applied Biosystems), using an ABI 3130 DNA analyzer system (Applied Biosystems) and universal M13 forward and reverse primers. Sequences were analyzed using the Lasergene sequence analysis software (DNASTAR, Inc.).

Sequence Analysis. The peptide sequences obtained were compared with the 2696 antimicrobial peptides in the antimicrobial peptide database [Wang and Wang, 2016]. The program ProtParam[49] was used for computation of physical and chemical parameters (MW, theoretical pI, instability index, aliphatic index, and grand average of hydropathicity, GRAVY). The HeliQuest program was used to calculate hydrophobic moments (μH) and helix wheel projections [Gautier et al., 2008; Schiffer and Edmundson, 1967] Three-dimensional structural model predictions and renderings of the new peptide were obtained using the internet resources PEP-FOLD [Shen et al., 2014] and RasMol [Bernstein, 2000] respectively.

The thaulin-1 sequence was also compared against global protein databases (GenBank CDS, Protein DataBase, SwissProt, PIR and PR), under the Blastp algorithm adjusted to short protein sequences. For this analysis, we dismissed hypothetical proteins. Alignment of best hits and distance trees were performed using the ClustalX 2.1 software [Larkin et al., 2007] and visualized by NJPlot.

DNA sequence alignment was performed by Basic Local Alignment Search Tool (BLAST) engine. Nucleic acid search involved the entire sequence of thaulin-1 mRNA as a target query and GenBank, EMBL, DDBJ, PDB and RefSeq as databases. Scores for best

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alignments were calculated as the sum of the reward/mismatch penalty, set to 0.66, and the gap parameters, imposed at the highest value for gap extension. Thirty sequences with higher scores were used to construct a distance tree between sequences using the Neighbour Joining algorithm. Distance between sequences used for tree generation predicted expected fraction of base substitutions per site given the fraction of mismatched bases in the aligned region [Saitou and Nei, 1987].

Solid-Phase Peptide Synthesis. Peptides were manually synthesized using a solid-phase approach using Fmoc/tert-butyl chemistry as previously described [Marani et al., 2015]. Rink amide MBHA resin (Peptides International) was used for the synthesis of N-terminal amidated peptides. Removal of the protecting groups and cleavage of peptides from the resin was carried out with trifluoroacetic acid/triisopropylsilane/water (TFA/TIS/H_2O) (95:2.5:2.5). Cleaved peptides were submitted to ether precipitation washes and were lyophilized three times prior to purification.

Reversed-phase high performance liquid chromatography (RP-HPLC) peptide purification. Peptide purification was performed by preparative RP-HPLC (Phenomenex columns Kinetex 5 μm C18 50 × 21.20 mm) using a Shimadzu Prominence instrument. Each peptide was dissolved in H_2O/CH_3CN (6:4) and submitted to an RP-HPLC system using a gradient of CH_3CN, starting with H_2O/0.1% TFA and rising to 100% CH_3CN over 15 min [Marani et al., 2015]. The formula (A215−A225) × 144 (μg/mL) was applied for peptide quantification [Wolf, 1983].

Peptide Characterization. Mass Spectrometry Analysis. Purity and molecular mass determination of synthetic peptides were performed by using a MALDI-TOF/TOF (UltrafleXtreme, Bruker Daltonics). The instrument was operated in the positive ion mode

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and controlled by the Compass for Flex software, version 1.3 (FlexControl 3.3, FlexAnalysis 3.3, Bruker Daltonics). Five thousand laser shots were accumulated per spectrum in the mass spectrometry (MS) and tandem mass spectrometry (MS/MS) modes. One-microliter aliquots of the chromatographic fractions, dissolved in α-cyano-4-hydroxycinamic acid matrix solution (1:3, v/v), were applied on a stainless steel plate and dried at room temperature for 30 min. The peptide monoisotopic mass was obtained in reflector mode with external calibration, using the Peptide Calibration Standard for Mass Spectrometry mixture (up to 4000 Da mass range, Bruker Daltonics). Isolated peptides were submitted to an automatic sequencer for *de novo* sequencing using LIFT mode.

Peptide Characterization. Circular Dichroism Studies. Secondary structure content was studied by circular dichroism spectroscopy in the far UV wavelengths, using a JASCO J 815 instrument (Jasco Corp., Tokyo, Japan). The measurements were performed under nitrogen gas flow of 8 L/h at 20 ºC temperature, controlled by a Peltier system (JASCO). Spectra were recorded between 190 and 260 nm, using a 100 mm cell path length. The peptides were dissolved in either Milli-Q water or 2,2,2-trifluoethanol (TFE) (10, 20 or 40 %) at 100 µM final concentration. The instrument was set at 50 nm/min scan speed, 1 s response time and 1 nm bandwidth. The spectra were converted to molar ellipticity residue half by using the relationship: [θ]/ θ (10×c×n×d), where [θ] is the molar ellipticity (in degrees×cm^2×dmol^{-1}), θ the ellipticity in mill degrees, n is the number of peptide bonds, c is the molar concentration, d is the length of the cell in centimeters.

Antimicrobial Activity. Assays were performed using the following bacteria strains: *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *K. pneumoniae* ATCC 700603. All bacterial strains were grown at 37 °C in Mueller-Hinton broth until the logarithmic

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phase was reached (1 × 10^8 colony forming units (CFU) per mL). Minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide that inhibits the visible bacterial growth after incubation for 24 h at 37 °C in aerobic conditions (absorbance lower than 0.05 at 600 nm, in a Spectra Max-190 513 spectrophotometer, Molecular Devices). Peptide concentrations ranged from 15.62 to 500 µg/mL and final inoculum concentration was 5 × 10^5 CFU/mL. After determining the MIC, Minimal bactericidal concentration (MBC) was verified seeding 10 µl of the wells that showed results equal to or greater than the MIC onto Mueller-Hinton Agar (MHA) with the assistance of the Drigalski spatula. After 24 h of incubation at 37 °C, the MBC was considered the lowest concentration that inhibited growth of bacterial colonies on the agar. All tests were performed in triplicate and according to CLSI (2012). Standard antibiotics were used as controls (meropenem for *E. coli* and *K. pneumoniae* and oxacillin for *S. aureus*).

Antileishmania Assays. Cytotoxicity of thaulin-1 and Gly-thaulin-1 was tested against *L. infantum* in promastigotes and axenic amastigotes forms. *L. infantum* promastigotes (MHOM MA67ITMAP263) were cultured at 25 °C in RPMI 1640 Glutamax (Gibco) supplemented with 10% (v/v) heat-inactivatedd foetal bovine serum (FBS), 50 U mL^{-1} penicillin, 50 µg ml^{-1} streptomycin, and 20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid hemisodium salt (HEPES) pH 7.4 (Sigma). Axenic amastigotes of the same strain were grown at 37 °C and 5% CO_2, in MAA medium supplemented with 20 % (v/v) iFBS, 2 mM Glutamax (GibcoBRL), and 0.023 mM hemin (Sigma), as described previously [Sereno and Lemesre, 1997]. Parasites were incubated for 24 h in the absence (control) or presence of 1-128 µg/mL of the peptide. Parasite viability was then determined by the resazurin assay, adapted from Vale-Costa et al., [Vale-Costa et al., 2013] and

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calculated as the percentage in relation to control cultures. Data were analyzed with GraphPad Prism 5 software and IC50 (50% inhibitory concentration) values determined.

Cytotoxic Analysis. Generation of Bone-Marrow Derived Macrophages (BMDM). Bone-marrow (BM) cells were isolated by flushing femurs and tibia of BALB/c mouse with Hank's Balanced Salt Solution (HBSS, Gibco), and differentiated into BM derived macrophages (BMDM) adapted from a previously described protocol [Gomes et al., 2008] Briefly, BM cells were collected, centrifuged and suspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% iFBS, 1% of Minimum Essential Media / Non-Essential Amino Acids Solution (MEM), 50 U mL^{-1} penicillin, 50 µg mL^{-1} streptomycin (all from Gibco) (complete DMEM medium or cDMEM), and 10% L929 cell conditioned medium (LCCM) as source of Macrophage-Colony Stimulating Factor (M-CSF). BM cells were cultured in Petri dishes for 24h at 37°C in a 7% CO_2 atmosphere in order to remove fibroblasts. Nonadherent cells were then counted, plated in 96-well plates (2.5-3x10^4 cells per well) and incubated at 37°C in a 7% CO_2 atmosphere. On the 4th and 7th day, cDMEM+10%LCCM medium was renewed. After 10 days the culture was composed by differentiated macrophages.

Assessment of Peptide Cytotoxicity Over BMDM. Cytotoxicity of peptides was determined in BMDM using a standard resazurin assay, adapted from Vale-Costa et al.[59] Cultures of BMDM were supplemented with the different concentrations of the test compounds for 24h. BMDM cytotoxicity was then determined by the resazurin assay, as in Vale-Costa et al. [Vale-Costa et al., 2013], and calculated as the percentage in relation to control cultures to which no peptides were added. Data were analyzed with GraphPad Prism 5 software and 50% cytotoxic concentration (CC50) values determined.

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Hemolytic Activity. The hemolytic activity of the peptides was tested using human red blood cells (RBCs), collected in EDTA (1.8 mg/mL), washed three times, and resuspended with sterile saline solution (0.85%). Peptides were tested at different concentrations (15.62–500 µg/mL) according to Bignami [Bignami, 1993] with modifications. Triton-X (0.1% v/v) and saline solution were used as positive and negative haemolysis controls, respectively. The mixtures were incubated for 1 h at 37°C and centrifuged at 10,000*g* for 1 min. The value of absorbance of the supernatant (A) was measured at 492 nm. The hemolysis percentage was then calculated as follows:

$[(A_{peptide} - A_{saline})/(A_{Triton} - A_{saline})] \times 100$

Atomic Force Microscopy. *E. coli* cells, untreated and treated with thaulin-1 at MIC concentration (62.5 µg/mL), were tested to evaluate the morphological effects of thaulin-1 on cell morphology. After incubation for 24 h, samples were prepared according to Araujo [Araujo et al., 2015]. Thirty-microlitres of the culture media, containing the MIC-treated or untreated *E. coli*, was deposited onto a clean glass surface followed by drying in a bacteriological incubator at 35 °C for 10 min. The samples were then gently rinsed twice with 1 mL of deionized water to remove salt crystals and dried again under the same conditions, before AFM analysis. All samples were prepared at the same time, exposed to the same conditions, and examined within 8 h of deposition [Eaton et al., 2008]. The AFM analysis was carried out in vibrating mode using a TT-AFM from AFMWorkshop, equipped with a 50 µm scanner. NSG10 cantilevers (NT-MDT) with a resonant frequency of approximately 320 kHz were used. Images were analyzed using Gwyddion software 2.40. Representative images were shown after examination of multiple areas of each sample and from obtained images we calculated the mean roughness (Rq) with the assistance of

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software. To compare the means T-test was applied using the GraphPad Prism ® 5.0 software (GraphPad Sofware Inc.), a $p<0.01$ was considered statistically significant.

Surface Plasmon Resonance (SPR) Analysis. SPR experiments were carried out using a Biacore X100 analytical system with a L1 sensor chip (Biacore, Uppsala, Sweden). Prior to use, the L1 chip surface was washed with a conditioning cycle consisting on the injection of 3-[(3-cholamidopropyl) dimethylamonio]-1-propanesulfonate (CHAPS), 20 mmol.dm^{-3} followed by running buffer (HEPES). The interaction of thaulin-1 and Gly-thaulin-1 with the lipid model membranes was examined at peptide concentrations of 0, 2, 4, 6 and 10 μmol.dm^{-3} and a distinct SPR cycle was performed for each peptide concentration tested. Each SPR cycle consisted of the immobilization of the vesicles of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE): 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG) (3:1) on the L1 chip surface with an injection of the vesicles suspension, 1 mmol.dm^{-3}, at a flow rate of 2 μL/min for 45 min. This was followed by successive injections of running buffer (50μl/min for 100s) and NaOH (1 mmol.dm^{-3}) (50μL/min for 60s). Stabilization finished with an injection of running buffer at 5 μL/min for 500s. After that, peptide solution was injected at a flow rate of 10μl/min during 100s followed by a dissociation time of 10 min. Then the lipid membrane was removed by a double injection of CHAPS and a double injection of running buffer, both at a flow rate of 10 μL/min for 2.5 min.

The running buffer contained 20 mM HEPES and 150 mM NaCl pH 7.4. Lipid vesicles (100 nm) were prepared by extrusion above the lipid transition temperature [Sousa

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et al, 2015]. Kinetic analyses of the sensorgrams were performed using the Langmuir, the parallel and the two-state curve fitting models.

Associated content

Supporting Information

This material includes: a distance tree of the most similar mRNA sequences using thaulin-1 as a query, MALDI-TOF/TOF MS spectra and MS/MS spectra analysis to corroborate thaulin-1 and Gly-thaulin-1 sequences, cytotoxicity of thaulin-1 and Gly-thaulin-1 to *L. infantum* and BMDM cells, hemolysis assays results and mean roughness of the untreated and treated with thaulin-1 *E. coli* cell membranes.

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TABLES

Table 1. Theoretical molecular weight, estimated pI and total net charge of thaulin peptides

Name	**Sequence**	**Mw**	**pI**	**Net charge**
Thaulin-1	NGNLLGGLLRPVLGVVKGLTGGLGKK	2531.08	11.26	+4
Thaulin-2	ELLGGLLDPVLGVANALTGGIIKK	2361.85	6.17	0
Thaulin-3	NLVGSLLGGILKK	1311.63	10.00	+2
Thaulin-4	DDGEEAESEAANPEENTVGG	2019.92	3.27	-8

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Table 2. Thaulin-1 structural similarity with peptides. Boxed black letters represent identical amino acid position, and boxed gray letters represent conservative substitutions.

Name	Sequence																													Similarity percentage (%)	species	Reference
Thaulin-1	N	G	N	L	L	G	G	L	L	R	P	V	L	G	V	V	K	G	-	-	L	T	G	G	L	G	K	K	-		*Pleurodema thaul*	This work
Leptoglycin	-	G	-	L	L	G	G	L	L	G	P	L	L	G	-	-	G	G	-	-	G	G	G	G	G	G	L	L	-	53,84	*Leptodactylus pentadactylus*	Sousa et al. 2009
Maximin H39	-	-	-	I	L	-	G	-	-	-	P	V	L	G	L	V	-	G	N	A	L	-	G	G	L	I	K	K	L	48,27	*Bombina maxima*	Liu et al. 2011
Maximin H3	-	-	-	I	L	-	G	-	-	-	P	V	L	G	L	V	-	G	N	A	L	-	G	G	L	I	K	K	I	48,27	*Bombina maxima*	Lai et al. 2002
Bombinin H4	-	-	-	-	L	I	G	-	-	-	P	V	L	G	L	V	-	G	S	A	L	-	G	G	L	L	K	K	I	48,27	*Bombina variegata*	Mignogna et al. 1993
Plasticin-S1	-	G	-	L	V	S	D	L	L	S	T	V	T	G		L	L	G	N	L	-	G	G	G	G	L	K	K	I	44,82	*Phyllomedusa sauvagei*	Amiche et al. 2008

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Table 3. Thaulin-1 structural similarity with internal sequences of proteins. Boxed black letters represent identical amino acid position, and boxed gray letters represent conservative substitutions.

Protein	Sequence	species	Similarity percentage (%)
Thaulin-1	N G N L L G G L L R P - - V L G V V K G L T G G L G K K	*Pleurodema thaul*	
Hemagglutinin-related transmembrane protein	T G N G E G G L L S P - - V T G L L G G L T G G L G G -	*Alcaligenes sp.*	62
Uncharacterized protein	T G N G E G G L L S P - - V T G L L G G L T G G L G G -	*Achromobacter sp.*	62
Membrane protein	T G N G E G G L L S P - - V T G L L G G L T G G L G G -	*Alcaligenes sp.*	62
Membrane protein	N G S S S G S P A S - N P L T G L L G S L G G A T G G -	*Burkholderia gladioli*	27
bPA24NtDFP	W G N L L G G V L G V - S V I G A I V L L T G G M G E -	*Clostridium sp.*	54
Lipoprotein	T G N G D G G L L S P - - V T G L L G G L T G G L A G -	*Alcaligenes faecalis*	58

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Table 4. Antimicrobial activity determinations (MIC and MBC) of thaulin-1, from *P. thaul*, and its Gly-thaulin-1 analogue[#].

Peptide Name	***S. aureus*** **ATCC 29213**		***E. coli*** **ATCC 25922**		***K. pneumoniae*** **ATCC 700603**	
	MIC* (µg/mL)	MBC[a] (µg/mL)	MIC* (µg/mL)	MBC[a] (µg/mL)	MIC* (µg/mL)	MBC[a] (µg/mL)
Thaulin-1	500	500	62.5	62.5	125	125
Gly-thaulin-1	500	500	62.5	62.5	125	125

[#] Experiments performed in triplicate.
*Minimal Inhibitory Concentration.
[a] Minimal Bactericidal Concentration.

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Table 5. Association (ka_1 and ka_2) and dissociation (kd_1 and kd_2) rate constants obtained from the SPR data, for interaction of thaulin-1 and Gly-thaulin-1 by two membrane models, and affinity constant (K) determined by numerical integration using the two-state reaction model.

Peptide Name	**Lipid type**	**ka_1 (1/Ms)**	**kd_1 (1/s)**	**ka_2 (1/s)**	**kd_2 (1/s)**	**K_D (M)**	**K_A (1/M)**
thaulin-1	DMPC	6.83×10^{3}	0.100	0.0070	0.0021	3.53×10^{-3}	2.84×10^{2}
	POPE:POPG(3:1)	1.891×10^{7}	0.081	0.0056	0.0020	1.15×10^{-6}	8.71×10^{5}
Gly-thaulin-1	DMPC	1.099×10^{4}	0.130	0.0082	0.0022	2.59×10^{-3}	3.86×10^{2}
	POPE:POPG(3:1)	1.841×10^{4}	0.074	0.0063	0.0018	9.02×10^{-7}	1.11×10^{6}

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SUPPORTING INFORMATION

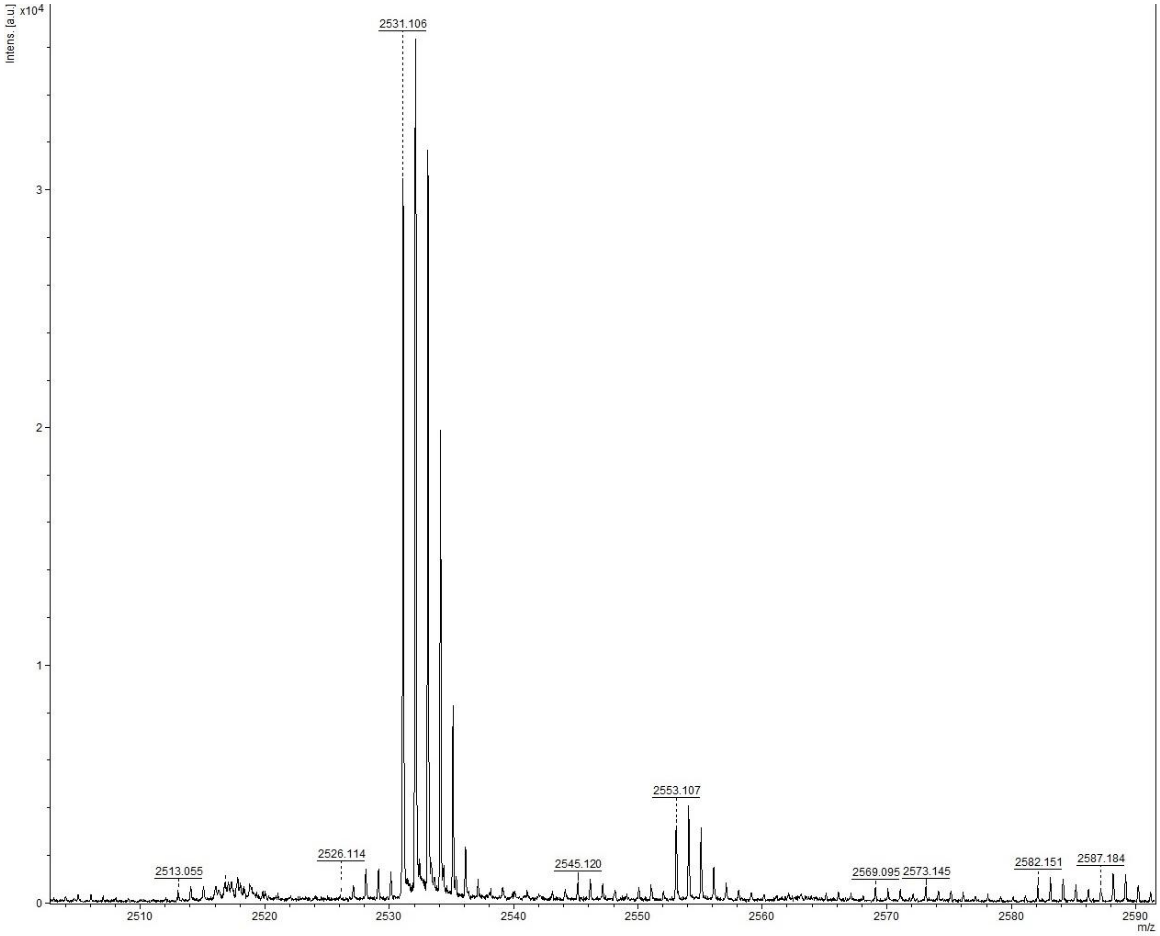

Figure S1. MS spectrum of the peptide thaulin-1 MALDI TOF/TOF optics.

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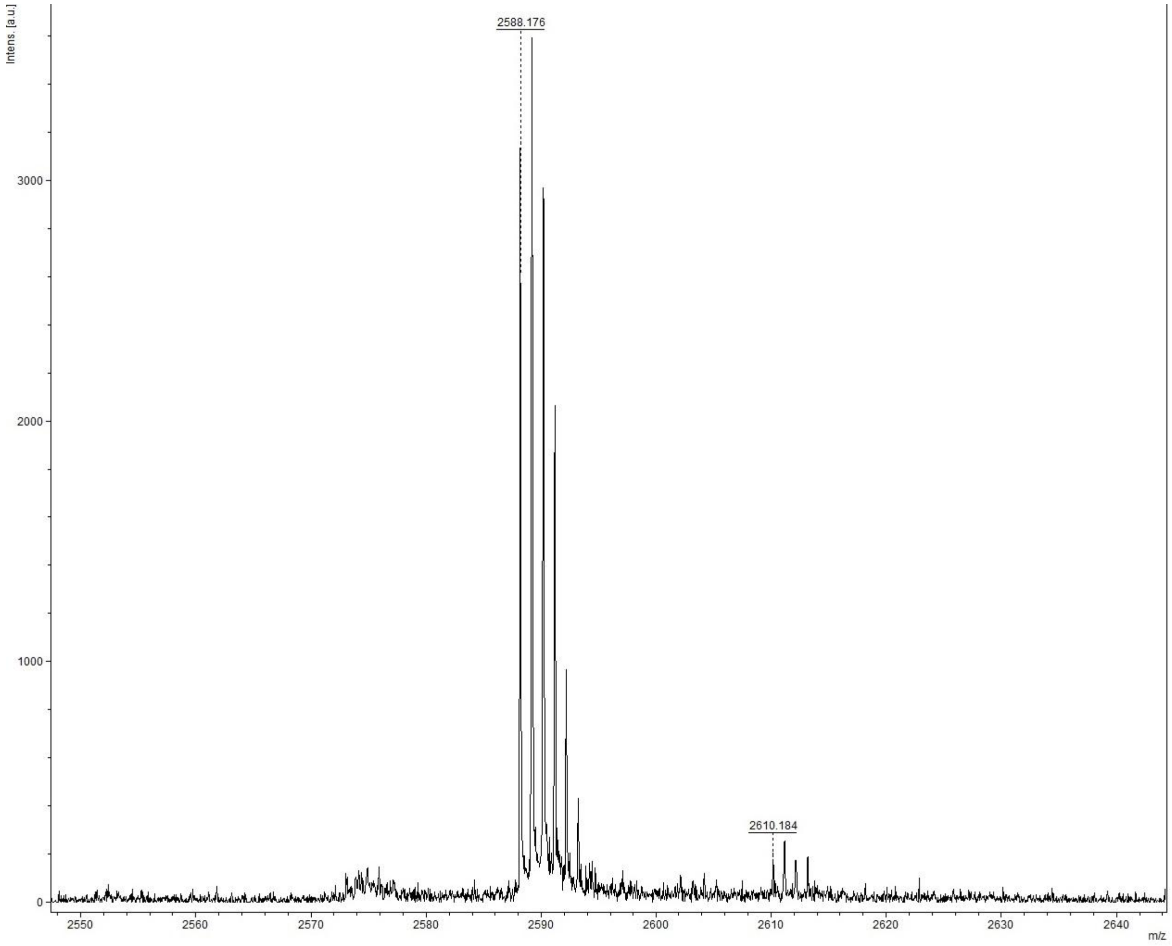

Figure S2. MS spectrum of the peptide Gly-thaulin-1 MALDI TOF/TOF optics.

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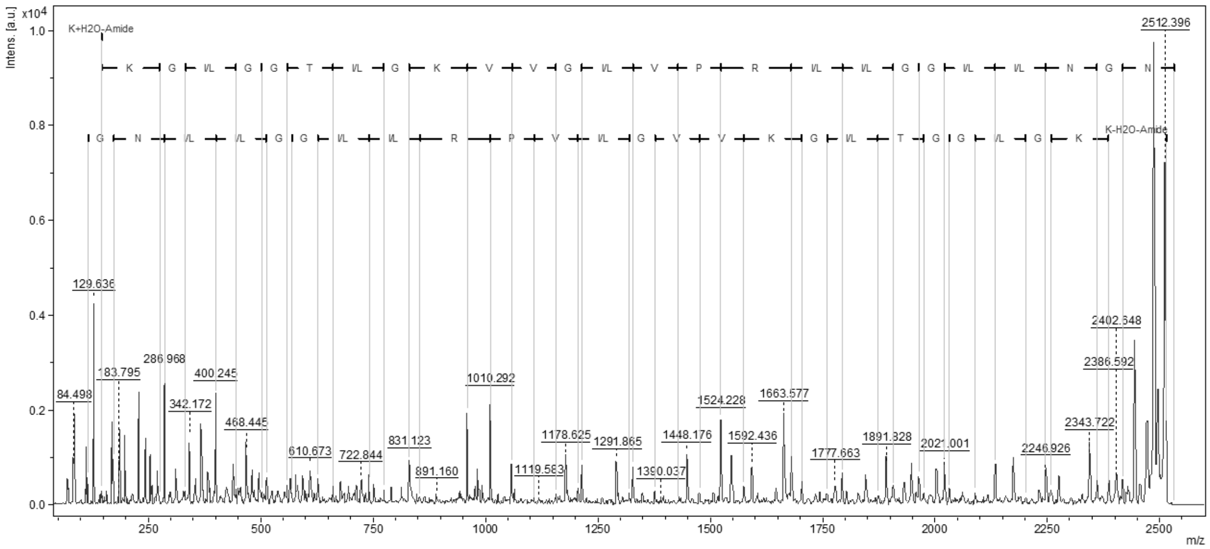

Figure S3. MS/MS spectrum of the peptide thaulin-1 MALDI TOF/TOF optics using N_2 as CID gas. The observed fragments allowed assignment of the major y and b ion series.

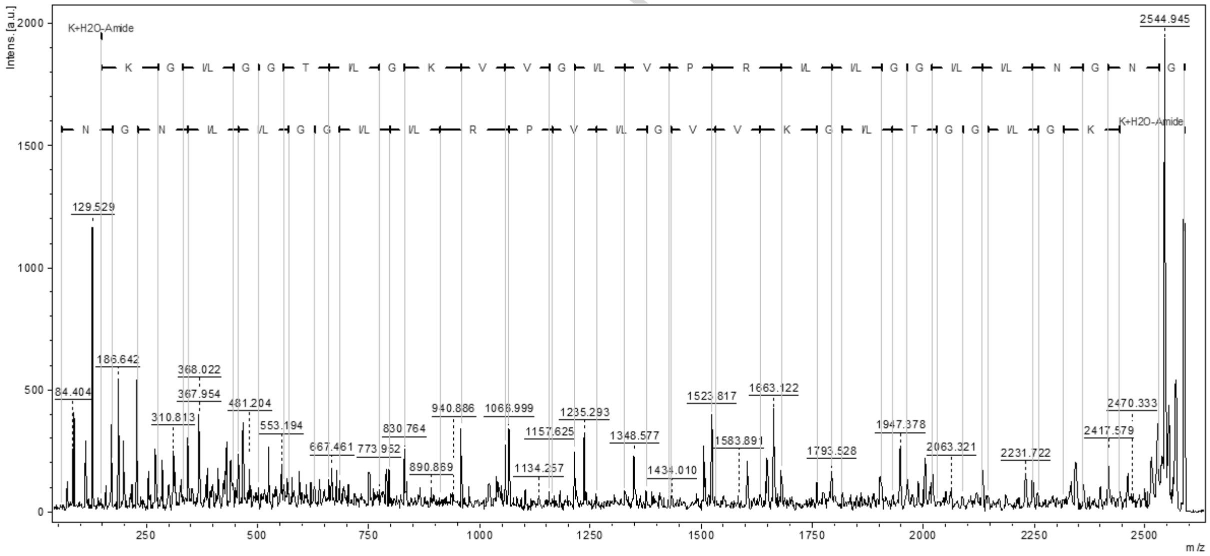

Figure S4. MS/MS spectrum of the peptide Gly-thaulin-1 MALDI TOF/TOF optics using N_2 as CID gas. The observed fragments allowed assignment of the major y and b ion series.

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Table S1. I*n vitro* efficacy of thaulin-1 and Gly-thaulin against *L. infantum* promastigotes and axenic amastigotes and toxicity against BMDM.

	L. infantum		**Toxicity in BMDM**
	Promastigotes IC50^{a} (μg/mL)	Amastigotes IC50^{a} (μg/mL)	CC^{b}50 (μg/mL)
Thaulin-1	>128	>128	>256
Gly-thaulin-1	>128	>128	>256

2 independent experiments performed in duplicate for axenic amastigotes

3 independent experiments performed in duplicate for promastigotes and BMDM.

[a] IC50 – 50% inhibitory concentration.

[b] CC50 – 50% cytotoxic concentration.

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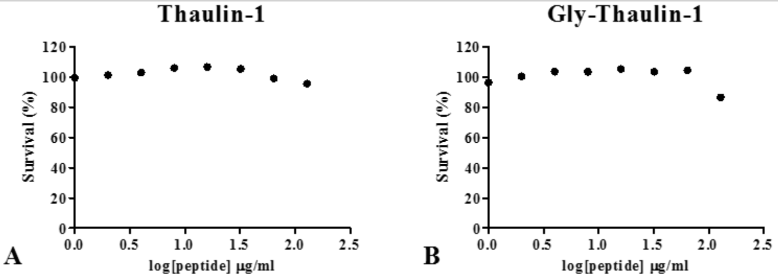

Promastigote form

Axenic amastigote form

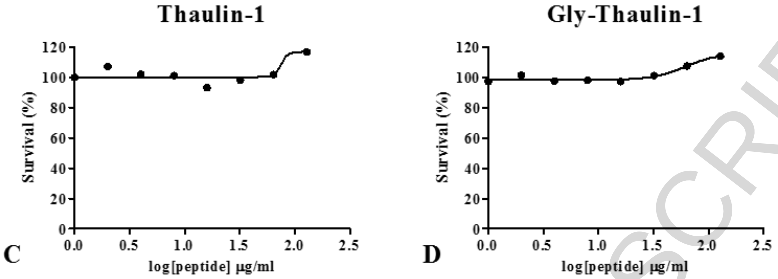

Figure S5. Cytotoxicity of thaulin-1 and Gly-thaulin-1 to *L. infantum* after 24 h. Promastigotes incubated with thaulin-1 (A) and Gly-thaulin-1 (B). Axenic amastigotes incubated with thaulin-1 (C) and Gly thaulin-1 (D).

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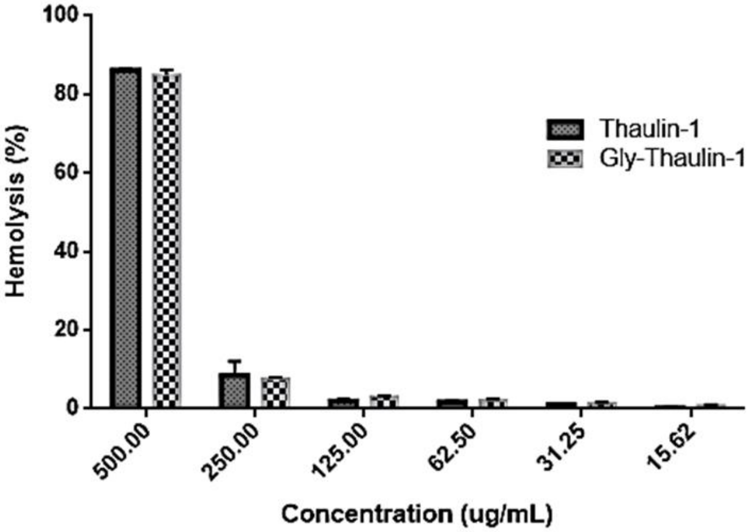

Figure S6. Hemolysis assay results of thaulin-1 and Gly-thaulin1.

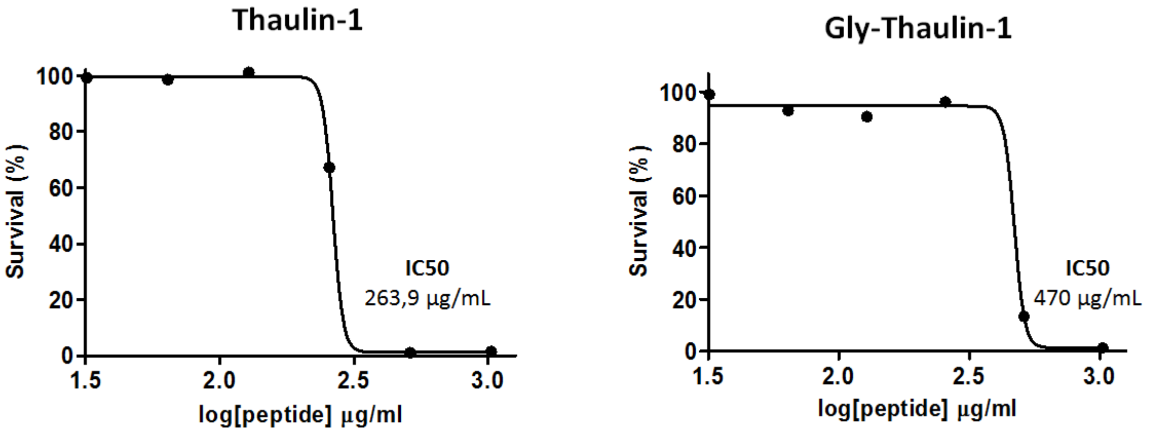

Figure S7. Cytotoxicity of thaulin-1 (Left) and Gly-thaulin-1 (Right) to BMDM cells after 24 h. The graphs illustrate the relationship between time of incubation and fluorescence emitted after the reduction of resazurin, transformed into the % of survival. The inhibitory concentration that caused 50% reduction in cell viability is indicated in each graph as IC50.

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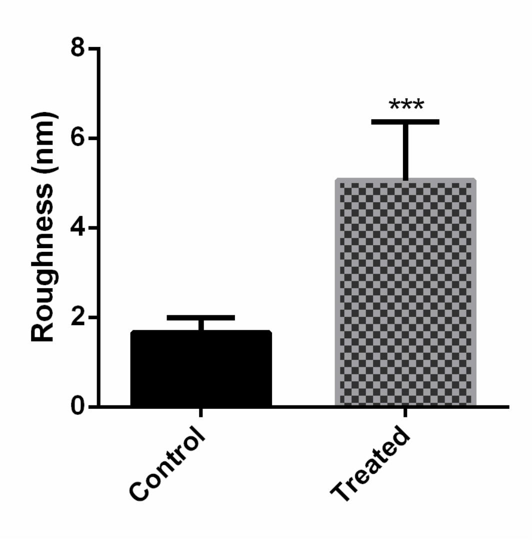

Figure S8. Mean roughness (nm) of the cell membrane untreated and treated with thaulin-1.

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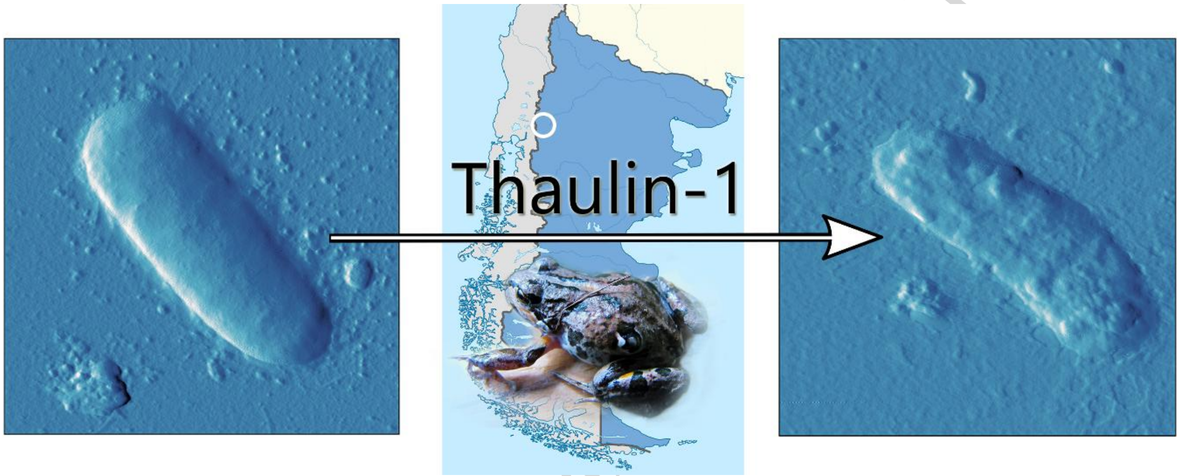
Thaulin-1: the first antimicrobial peptide isolated from the skin of a Patagonian frog *Pleurodema thaul* (Anura: Leptodactylidae: Leiuperinae) with activity against *Escherichia coli*

Graphical abstract



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Abreviations

AFM = Atomic Force Microscopy

AMPs = Antimicrobial Peptides

BMDM Bone-Marrow Derived Macrophages

CC50 = 50% cytotoxicity concentrations

CFU = Colony Forming Units

CD = Circular Dichroism

DMEM = Dulbecco's Modified Eagle's Medium

DMPC = 1,2-dimyristoyl-sn-glycero-3-phosphocholine

FBS = Foetal Bovine Serum

FTIR = Fourier-Transform Infrared Spectroscopy

HBSS = Hank's Balanced Salt Solution

HEPES = 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid hemisodium salt

K_A = Affinity Constant

LPS = Lipopolysaccharides

MBC = Minimal Bactericidal Concentration

M-CSF = Macrophage-Colony Stimulating Factor

MEM = Minimum Essential Media

MHA = Mueller-Hinton Agar

MIC = Minimal Inhibitory Concentration

MS = Mass Spectrometry

MS/MS = Tandem Mass Spectrometry

PC = Phosphatidylcholine

PG = Phosphatidylglycerol

POPE = 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine

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POPG = 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)

RP-HPLC = Reversed-Phase High Performance Liquid Chromatography

SMH = Shai-Matsuzaki-Huang Model

SPR = Surface Plasmon Resonance

TFA = Trifluoroacetic acid

TFE = 2,2,2-trifluoethanol

TIS = Tri isopropyl cylane

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Highlights

1. Thaulin-1 is the first antimicrobial peptide isolated from the skin of *P. thaul*;
2. Thaulin-1 is a Gly/Leu-rich peptide with similarities to bombinin peptide family;
3. In organic media, thaulin-1 showed characteristic CD spectra of α–helix structures;
4. Thaulin-1 presents activity against *E. coli, K. pneumoniae* and *S. aureus* strains;
5. Thaulin-1 showed low cytotoxicity to mammalian cells.

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