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Novel dermaseptins from *Phyllomedusa hypochondrialis* (Amphibia)

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Abstract

Six new antimicrobial peptides structurally related to the dermaseptin family have been isolated from the skin secretion of the amphibian *Phyllomedusa hypochondrialis*. The primary structures of these molecules named as DShypo 01, 02, 03, 04, 06, and 07 were determined by *de novo* MS/MS experiments, Edman degradation, and cDNA sequencing. The fifth peptide was found to be precisely the same DS 01 from *Phyllomedusa oreades* previously described by our group. The majority of the peptides purified from the crude skin secretion could be directly localized and mapped onto a freshly dissected dorsal skin fragment using mass spectrometry-imaging techniques. Comparisons between peptides and commercial drugs on their antibacterial and anti-*Leishmania amazonensis* efficiencies, associated with peptide lytic effects on mammalian blood cells and surface plasmon resonance interaction studies on immobilized DMPC vesicles, were also performed.

Keywords: Antimicrobial peptide; Frog skin; *Phyllomedusa*; Imaging mass spectrometry; *Leishmania*; Surface plasmon resonance

Antimicrobial peptides represent an essential part of the innate immunity of eukaryotes by preventing the invasion of pathogens and their proliferation in host organisms [1–3]. The expression and accumulation of antimicrobial peptides in frog skin secretions is one of the most conserved features and is shared by anurans from all continents. It is postulated that these molecules have endured a diversifying selection proportional to the variety of microorganisms in the frogs' environments, resulting in the peptide families known nowadays [4,5]. The great majority of these peptides are rather distinct in their primary structures apart from attributes such as high amphiphilicity and net positive charge. Although their precise mechanism of action is not fully understood, those two elements seem to play important roles in the functionality, selectiveness, and their ability to destabilize cell membranes [6–9].

The dermaseptins (DSs) are a family of peptides isolated from the skin secretion of frogs from the *Phyllomedusa* genus [10–12]. They are 24–34 amino acids long cationic molecules that fold into amphiphilic helices when in contact to a hydrophobic media. The DS polypeptide chains are gene encoded as part of larger precursor molecules comprising a signal peptide of 22 residues, followed by an acidic propeptide, a typical prohormone processing

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signal, and a DS progenitor sequence [4,13]. They exert cytolytic action against numerous microorganisms and have been considered promising agents to fight viral diseases [14,15], drug-resistant bacteria [10,11], protozoa [10,16], yeasts, and filamentous fungi [11,17]. These peptides often do not demonstrate significant cytolysis against mammalian blood cells [10].

Skin or visceral leishmaniasis (VL) that can eventually lead to death is caused by protozoa from the *Leishmania* genus and transmitted by phlebotomid sandfly vectors, typical to rainforest harboring countries, such as Brazil and India [18]. According to the World Health Organization, the disease currently infects more than 12 million people and the severe VL form is now endemic in southern Europe due to imported *Leishmania*-infected dogs and climate-induced changes that foster sandfly abundance. Infections are treated with conventional chemotherapy using pentavalent antimonium salts, but these often present drawbacks such as disease rebounds and mucous tissue damage [18,19].

The present study describes the isolation and characterization of six novel dermaseptins (named DShypo 01, 02, 03, 04, 06, and 07) from the skin secretions of *P. hypochondrialis*. Moreover, it reports the occurrence of DS 01, which was previously described by our group in the skin secretion from *P. oreades* [10]. The spatial skin detection, localization, and distribution of the dermaseptins were obtained by imaging mass spectrometry on a freshly dissected skin fragment of *P. hypochondrialis*, also revealing their co-localization profiles as recently demonstrated for *P. hypochondrialis* bradykinins [20]. DShypo 01 appears to be the most abundant among all peptides detected, and further experiments were carried out using it as a model molecule for gene cloning, antimicrobial activity evaluation, and interaction studies with DMPC vesicles. *In vitro* tests using DShypo 01 and DS 01 against *L. amazonensis* and their lytic effects on mammalian blood cells were also compared.

Materials and methods

Amphibian skin secretions. Adult specimens of *P. hypochondrialis* were captured in central Brazil, kept alive accordingly to animal rights procedures under the Instituto Brasileiro do Meio Ambiente e dos Recursos Renováveis (IBAMA) license and process Nos. 240/2005—CGFAU/LIC.

Peptide purification. The secretion was obtained by mild electric stimulation of the granular skin glands of *P. hypochondrialis*; frozen in liquid nitrogen, lyophilized in aliquots of 1.0 mg/mL, resuspended in 0.1% TFA (v/v), and purified by RP-HPLC (10). Fractions containing the DShypo peptides were submitted to further RP-HPLC purification steps using C-18 analytical, SOURCE™, and μRPC columns with optimized gradients of acetonitrile.

EDMAN degradation and de novo sequencing. Monoisotopic molecular masses and purity of the peptides were determined by ESI/MS and MALDI-TOF/MS using a Q-TOF Ultima (Waters-Micromass, Manchester, UK) and an UltraFlex II MALDI-TOF/TOF (Bruker Daltonics, Billerica, MA). Peptide fragmentation was obtained by combined LIFT/CID experiments on the UltraFlex II and the resulting data were analyzed manually using both Pepseq running under MassLynx 4.0 (Waters) and FlexAnalysis 2.4 (Bruker Daltonics) programs. Automatic N-terminal sequencing of DSs was performed on a Protein and Peptide Sequencer PPSQ-23. Sequence similarity searches and alignments were performed using the FASTA 3 (http://ca.expasy.org/) and Clustal W multiple alignment tool available on Bioedit v. 7.0.4.1 software.

Gene cloning cDNA. The frog skin was frozen in liquid nitrogen and pulverized in a mortar, and four aliquots of about 100 mg of the resulting powder were used for total RNA extraction using the Trizol reagent (Invitrogen). The quality and quantity of the RNA were checked by electrophoresis in an agarose gel and by spectrophotometer analysis, respectively. One microgram of total RNA was used as template for 1st strand cDNA synthesis, using the Superscript Reverse Transcriptase kit (Invitrogen). After 2 h, the reaction was terminated by addition of 100 μl of a Tricine buffer (10 mM Tricine–KOH, pH 8.5, 1 mM EDTA). Reactions of 3′RACE were performed for the second strand cDNA synthesis using as primers, PPS-2 (5′-ATGGCTTTCCTGAARAARTCBCTTTTY CTTGTATTATTTCTCGG-3′), designed on a previously described conserved signal peptide, and a reverse primer NOT-1-REV (5′-TCGCGAG CGGCCGCCCTTTTT-3′), present in the oligo(dt) primer used in the first strand cDNA synthesis. The cycling parameters were as follows: four cycles of 94 °C/30 s and 72 °C/120 s; 25 cycles of 94 °C/30 s and 68 °C/180 s. PCRs were loaded on an agarose gel and after electrophoresis, expected fragments ranging from 300 to 400 bp were gel purified using the Wizard SV Gel Clean-up System (Promega). The purified fragments were used in a ligation reaction with the pGEM-T Easy vector system (Promega), following the manufacturer instructions. After dialysis of the ligation mixture, aliquots were used for *Escherichia coli* (DH5α) transfection. PCRs were performed using a MJ PTC-100 thermal cycler (MJ Research Inc.). Sequencing was carried out by the dideoxy chain-termination method [21], using an ABI Prism® 3700 DNA Analyzer system with BigDye™ terminator and POP-5™ Polymer (Applied Biosystems). Sequences were analyzed using the Lasergene® sequence analysis software (DNASTAR, Inc.).

MALDI-imaging analysis. The dorsal skin of *P. hypochondrialis* was dissected and its fragments (1–2 mm^2) were air-dried under a glass slide for 10 min prior to IMS experiments. Tissue pieces were positioned in such a manner that the gland lumens could be visualized with no sectioning. Light microscopic images were captured, and by using a forceps the tissue pieces were directly fixed to a MALDI plate by a double-sided adhesive tape. After the tissue was placed on the plate, a drop (0.5 μl) of α-cyano-4-hydroxy-cinnamic acid was applied and air-dried; molecular ion profiles were obtained using a Voyager DE STR MALDI-TOF/MS spectrometer (ABI, Framingham, MA). Automatic scanning steps were accomplished using MALDI MS Imaging Tool 2.2.1 software. The pulsed nitrogen laser was rastered across the sample surface in 50 μm single steps. A global spectrum was generated for one image after 10–15 laser shots at each position. BioMAP 3x Functional Image Analysis software was used to generate intensity-based ion maps of the molecular components ranging from 2300 to 4000 Da. Co-localization profiles of pairs of components were displayed by using the BioMAP software.

Antimicrobial activity. Antimicrobial activity was investigated against *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 43300. Microorganisms were grown in stationary culture at 37 °C and then transferred to the Mueller–Hinton liquid medium, according to the National Committee for Clinical Laboratory Standards [23]. The bacterial growth inhibition assay was performed as described previously [10]. The peptide was dissolved and diluted 8-fold in Mueller–Hinton broth. The highest peptide concentration used for the assay was 128 μg/mL. The initial inoculum was 2.5×10^8 CFU/mL [25]. Conventional antibiotics (amoxicillin, imipenem, ceftazidime, and trimethoprim) had their MICs determined against the two experimental bacterial strains by automated biochemical analysis (Vitek, bioMeriuex Inc.).

Antileishmanial assays were performed with a strain of *L. amazonensis* routinely isolated from mouse lesions and maintained as promastigotes at 26 °C in RPMI medium and adjusted to 2×10^7/mL [22]. The experiment was performed in triplicate and glucantime was used as a control.

Preparation of large unilamellar vesicles (LUVs). 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC) was purchased from Avanti Polar Lipids. In brief, 5 mg were gravimetrically determined, dissolved in chloroform at 1 mg/mL, and dried in a rotary evaporator until the formation of a lipid film, which was later left under vacuum for 4 h. The film was

dissolved in 5 mL of the running buffer (20 mM sodium phosphate buffer, 150 mM NaCl, filtered and degassed) and vortexed. After 30 min, the suspension was passed 19 times through a 100 nm polycarbonate membrane using an Avanti mini-extruder kit [23].

Surface plasmon resonance measurements and data analysis. An L1 sensor chip was docked in a Biacore 3000 biosensor and left to hydrate in buffer overnight. Prior to use, the chip surface was cleaned by the injection of 10 μL of 100 mM HCl/Isopropanol (50:50 v/v) at a flow-rate of 20 μL/min. The flow was then set to 2 μL/min and the DMPC LUVs were injected for 600 s. Later, the flow-rate was set to 50 μL/min and 25 μL of 10 mM NaOH was injected for the removal of weakly bound vesicles. Flow-rate was then set to 10 μL/min and concentration series of DShypo 01 and DS 01, from 0.625 to 10 μM, were injected for 300 s and their dissociation was monitored for 600 s. For monitoring non-specific binding, the DMPC LUVs solution was replaced by the running buffer. Data were plotted using BIAEvaluation v3.1 and CLAMP [24].

Leukocyte counts and hematologic parameter analyses. Throughout this study, unless otherwise indicated, we followed the International Council for Standardization in Haematology guidelines for blood cell analyzer evaluation and the National Committee for Clinical Laboratory Standards (NCCLS) for reference leukocyte parameters [25]. White blood cells (WBCs) differential counts and hematologic parameters were performed as described elsewhere [26]. Aliquots of fresh human blood were incubated with DShypo 01 at 37 °C during 30 and 60 min. Total parameter counts were done using Cell-Dyn 3500 SC/SL flow cytometry automated hematology analyzer. The data obtained were the results of three independent measurements.

Results and discussion

Peptide purification

Fractionation of the *P. hypochondrialis* crude skin secretion by RP-HPLC yielded several peptide-rich fractions (Fig. 1). Components eluted at 48.3, 48.9, 56.4, and 58.3% of the acetonitrile/0.1% TFA gradient were further purified by analytical RP-HPLC (data not shown) and their molecular masses determined as follows: DShypo 01 ($[M+H]^+ = 2409.41$ Da), DShypo 02 ($[M+H]^+ = 2868.36$ Da), DShypo 03 ($[M+H]^+ = 3352.96$ Da), DShypo 04 ($[M+H]^+ = 3236.84$ Da), DS 01 ($[M+H]^+ = 2793.76$ Da), and DShypo 06 ($[M+H]^+ = 3252.96$ Da).

Amino acid sequencing and alignment

Peptides $[M+H]^+ = 2793.78$ Da and $[M+H]^+ = 3208.63$ Da purified with a single purification step were MS/MS fragmented and identified as DS 01, previously described [10], and the novel Dshypo 07, respectively. All the other molecules were submitted to similar procedures and they were found to be new members of the Dermaseptin family. Complementary amino acid information to determine Leu, Ile, Lys, and Gln residues on the DS peptides' primary structures was obtained by Edman degradation. Calculated monoisotopic masses for DShypo 01, DS 01, DShypo 04, and DShypo 06, after N-terminal sequencing revealed a −0.98 Da mass discrepancy between experimental and calculated mass values, indicating the presence of carboxyamidated C-terminal residues on these molecules.

FASTA searches revealed high sequence identity among DSs from *P. hypochondrialis* and other DSs previously identified from *Phyllomedusa bicolor* (Table 1). DShypo 01, DS 01, DShypo 04, DShypo 06, and DShypo 07 show 50% identity, calculated by consensus sequence, to the adenoregulin precursor; DShypo 02 is 75.8% identical to DS B IV, whereas DShypo 03 is 82.3% identical to DS B I (Table 1). On the basis of these results one could infer that *P. hypochondrialis* and *P. bicolor* DSs may share a recent common ancestor [27]. Indeed, the genes that encode DShypo 01, DS 01, DShypo 04, DShypo 06, and DShypo 07 in *P. hypochondrialis* are probably orthologous to the adenoregulin precursor in *P. bicolor,* and the same seems to apply to DShypo 02 and the dermaseptins B IV precursor, and to DShypo 03 and the dermaseptins B I precursor [27]. These molecules were deposited in the Swiss-prot under the following codes: DShypo 01 (P84596), DShypo 02 (P84597), Dshypo 03 (P84598), DShypo 04 (P84599), DS 01 (P84600), DShypo 06 (P84601), and DShypo 07 (P84880).

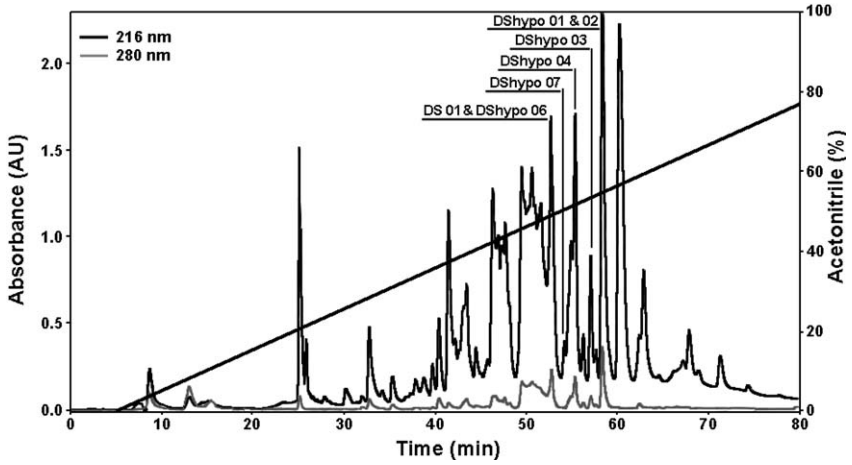

Fig. 1. Chromatographic profile of the total skin secretion from *P. hypochondrialis*. The fractions of interest (DS 01 and DShypo 06, DShypo 04, DShypo 03, and DShypo 02 and 01) were collected at 52.3, 55.4, 57.7, and 58.9 min, respectively, under a linear gradient of acetonitrile.

Table 1
Sequence alignment of dermaseptins from the skin secretion of *P. hypochondrialis*

Peptide	Amino acid sequence
DShypo 01	GLWSTIKNVGKEAAIA----AGKAALG----AL-NH_2
DS 01	GLWSTIKQKGKEAAIAAAKAAGQAALG----AL-NH_2
ADR Prec.	GLWSKIKEVGKEAAKAAAKAAGKAALG----AV
DShypo 04	GLWSTIKQKGKEAAIAAAKAAGKAVLNAASEAL-NH_2
DShypo 06	GLWSTIKQKGKEAAIAAAKAAGQAVLNSASEAL-NH_2
DShypo 07	GLWSTIKQKGKEAAIAAAKAAGQAALNAASEAL-NH_2
DShypo 02	GLWKSLLKNVGVAAGKAALNAVTDMVNQ
DS B IV Prec.	ALWKDILKNVGKAAGKAVLNTVTDMVNQ
DShypo 03	ALWKDVLKKIGTVALHAGKAAFGAAADTISQGGS
DS B I Prec.	AMWKDVLKKIGTVALHAGKAALGAVADTISQGEQ

MALDI-imaging analysis

Fig. 2A shows an adult specimen of *P. hypochondrialis* from which a dorsal skin fragment was dissected and imaged in light microscope (Fig. 2B). *In situ*-imaging mass spectrometry of the animal tissue represented by the dashed-rectangle area is shown in Fig. 2B and the ions corresponding to five DSs (Figs. 2C–G) were concomitantly detected in the dorsal region of a single specimen of *P. hypochondrialis*. There is a discrete co-localization profile between pairs of detected ions on the spectral images (in yellow) as shown in Figs. 2H–Q. Considering the DSs identified in the present study, only DS 01 and DShypo 07 were not detected using the mass spectral scanning approach (Fig. 2R).

Although DSs were isolated from the skin secretion of Phyllomedusinae frogs in the last decade, their expression patterns in tissue sections are still largely unknown. Immunohistochemical and *in situ* hybridization experiments were employed for the detection of a small number of compounds, but the simultaneous co-localization of several antimicrobial peptides had never been reported [28]. Mass spectral images from the skin of *P. hypochondrialis* demonstrate that DSs are not restricted to specific skin sites, even though the present data suggest their spatial compartmentalization. From these findings, one can hypothesize that specific subtypes of serous glands produce and store specific DS members.

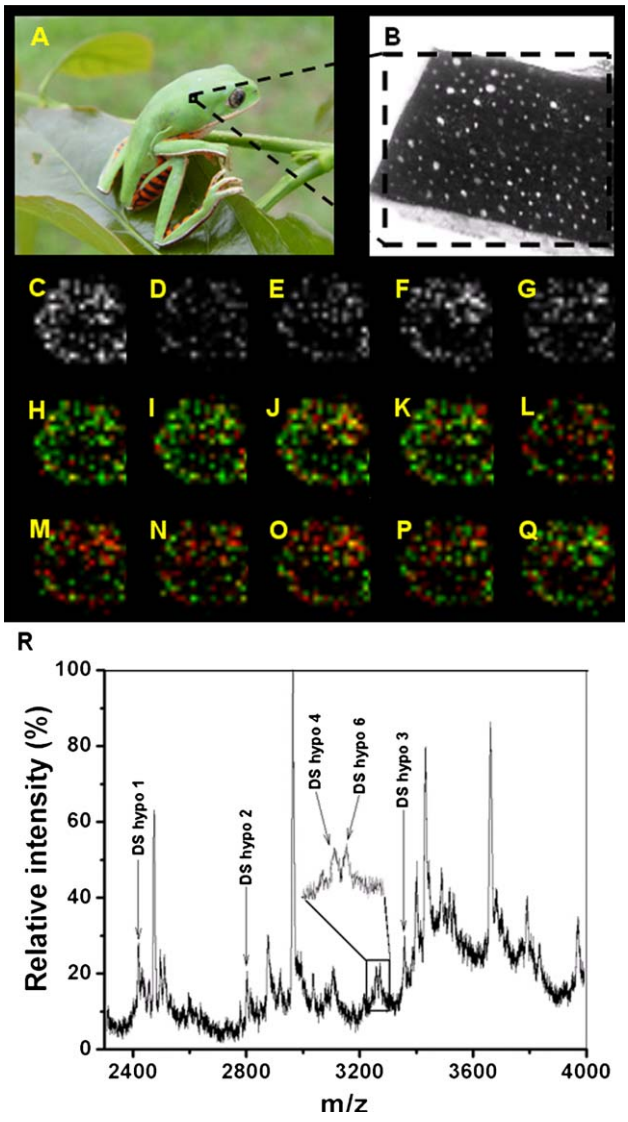

Fig. 2. MALDI-imaging analysis of *P. hypochondrialis* skin. (A) Adult specimen of *P. hypochondrialis*. (B) Light micrograph of a small (1.4 × 1 mm) frog skin fragment. (C–G) Mass spectral images corresponding to ions with the molecular mass (M+H) of DS hypo 01, DS hypo 02, DS hypo 03, DS hypo 04, and DS hypo 05, respectively. (H–Q) Co-localization profile of the DS hypo 01 + DS hypo 02, DS hypo 01 + DS hypo 03, DS hypo 01 + DS hypo 04, DS hypo 01 + DS hypo 06, DS hypo 02 + DS hypo 03, DS hypo 02 + DS hypo 04, DS hypo 02 + DS hypo 06, DS hypo 03 + DS hypo 04, DS hypo 03 + DS hypo 06, and DS hypo 04 + DS hypo 06, respectively. The areas in yellow correspond to the regions of the sample simultaneously displaying both ions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Isolation and identification of cDNA clones encoding DShypo 01

The complete cDNA of DShypo 01 was cloned and sequenced. The deduced amino acid sequence of the precursor shows that it comprises a potential signal peptide for endoplasmic reticulum membrane translocation (residues 1–22) ending with a Cys residue. The putative signal sequence is immediately followed by an acidic proregion of 23 residues with basic residues (Lys22-Arg23) at its carboxyl terminus. Fig. 3 demonstrates that the gene region encompassing the signal peptide contains 22 residues and presents 86% amino acid positional identity, while the acidic prosegment contains 22–23 residues and shows 78% amino acid identity, when both regions are compared to corresponding dermaseptin genes available in Swiss-Prot.

The DShypo 01 polypeptide precursor has the standard tripartite structure of antimicrobial peptides, that is, a signal peptide, an acidic proregion that terminates with two consecutive basic amino acids (Lys-Arg), and a progenitor sequence coding for the mature molecule [4,5].

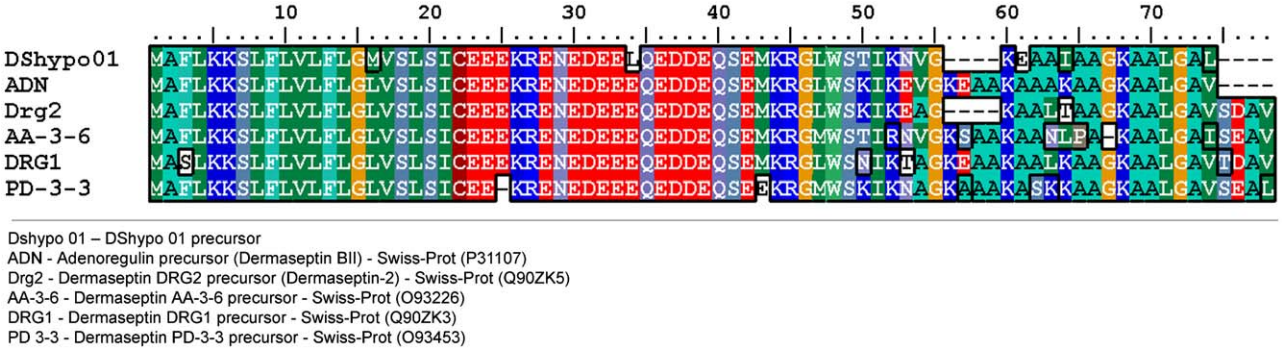

Fig. 3. Alignment of the amino acid sequences of the preproregions of representative members of the preprodermaseptins. Preprodermaseptin DShypo 01 (*P. hypochondrialis*), preproadenoregulin (*P. bicolor*), preprodermaseptin Drg2 (*Phyllomedusa sauvagei*), preprodermaseptin AA-3-6 (*A. annae*), preprodermaseptin Drg1 (*P. bicolor*), and preprodermaseptin PD-3-3 (*Phyllomedusa danicolor*), including the signal peptide (residues 1–22), the acidic propiece, and the processing site Lys-Arg. Gaps (-) have been introduced to maximize sequence similarities.

In its precursor form, DShypo 01 is immediately followed by a Gly-Glu-Gln extension (Supplementary data), which is cleaved from the active peptide, exposing an extra Gly residue that may serve as an amide donor for the C-terminal amidation [13,29] deduced from the mass spectrometry experiments. The high similarity encountered between DShypo 01 and dermaseptin B II is also in attendance when both DShypo 01 and adenoregulin precursors are aligned, reinforcing the idea that these arose through duplications from a common and recent ancestor [4,5].

Antimicrobial activities

DShypo 01 was tested against *S. aureus* and *Escherichia coli* and compared to ceftazidine, amoxicillin, imipenen, and trimethopin, antibiotics used in human clinical practice (Table 2). Results show that DShypo 01 acts with distinct efficiency against both Gram-positive and Gram-negative bacteria; *S. aureus* (MIC = 26.5 μM) and *E. coli* (MIC = 6.6 μM). DS01 previously tested under the same conditions against the same bacteria presented MICs of 3.2 μM for *S. aureus* and 6.4 μM for *E. coli* [10]. *L. amazonensis* bioassays revealed that DShypo 01 and DS 01 are efficient anti-*L. amazonensis* promastigote agents. Figs. 4A and B show that when incubated for 2 and 6 h with the protozoan cells in concentrations up to 32 μg/mL, both peptides are capable to lyse most of the cell population. Interestingly, at low concentrations, such as 5–20 μg/mL, differences to glucantime, the drug of choice for the treatment of leishmaniasis, were not significant. On the other hand, in higher concentrations (20–64 μg/mL), the DSs

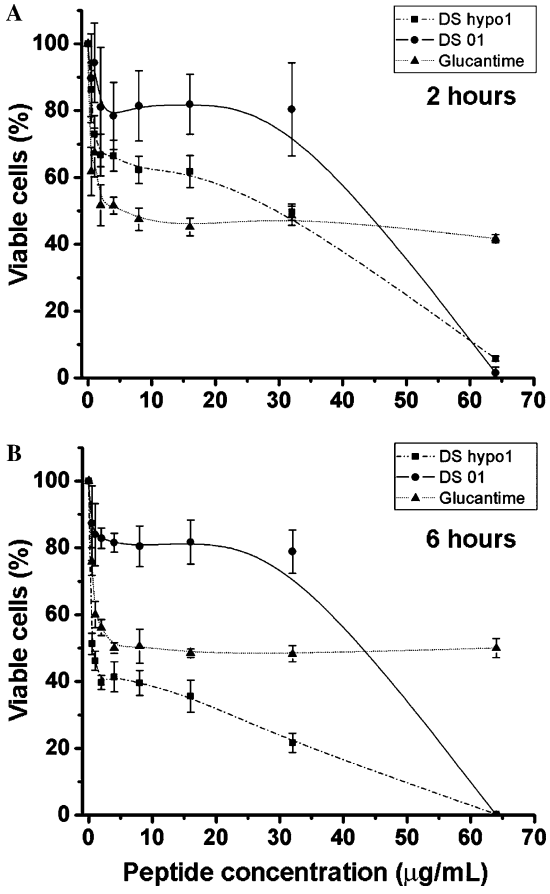

Fig. 4. Anti-*L. amazonensis* bioassays. Lytic activity of DShypo 01, DS 01, and glucantime against *L. amazonensis* promastigote forms grown in RPMI media at different incubation times, 2 (A) and 6 h (B).

Table 2
Antibacterial activity of DShypo 01

Bacteria	MIC (μM)[a]				
	DShypo 01	Amoxicillin	Imipenen	Ceftazidine	Trimethopin
S. aureus ATCC 43300	26.5	NDA[b]	NDA	NDA	175
P. aeruginosa wt MR	6.6	17	35	40	80

[a] MIC, minimal peptides concentrations required for total inhibition of cell growth in liquid medium. These analyses were performed according to the recommendations of NCCLS. Experiments were performed in triplicate.
[b] NDA, no detectable activity.

showed a significant increase in activity when compared to the control drug.

The antibacterial data in Table 2 demonstrate activity against bacteria comparable to other DSs [10–12], however DShypo 01 was found to be more effective against *E. coli* than against *S. aureus* (MICs of 6.6 and 26.5 μM, respectively) and, in general, less potent than DS 01 [10]. In fact, both seem to present functional differentiation, since DS 01 is more potent against the Gram-positive than the Gram-negative bacteria. Indeed, the hyper-divergence of primary sequences of antimicrobial peptides is considered a consequence of the different types of microorganisms each species co-habits [4,5]. *P. hypochondrialis* has a large geographical range and is an inhabitant of Brazil, Argentina, Colombia, Bolivia, and Paraguay. Therefore, with such a wide range, this species is subjected to a broad spectrum of different microbes that could have produced some variations in its innate defense mechanism.

Anti-*L. amazonensis* assays demonstrated that DShypo 01 and DS 01 are remarkable leishmanicidal agents. The protozoan cell population was reduced to a non-detectable level at concentrations close to 64 μg/mL after 2 and 6 h of incubation. Anti-protozoan activity might be a general feature of DSs, but once again it seems to have specificities, for DS 01, a more effective antibacterial agent, is less potent than DShypo 01. *Leishmania* causes a disease that has been associated with different clinical forms; including cutaneous, hyperergic mucocutaneous, and the anergic diffuse leishmaniasis. *L. amazonensis* used in this work has been isolated from patients with visceral disease or with postkalazar dermal leishmaniasis [30]. The recommended drugs for the treatment of leishmaniasis are pentavalent antimonium salts but they are responsible for side effects such as renal and cardiac failures [31,32]. Several new antileishmanial compounds are under development, but a drug with the capacity to eradicate the infections is yet to be discovered [33,34].

Surface plasmon resonance assays

DMPC LUVs were immobilized in an L1 sensor chip docked into a Biacore 3000. Following that, peptides were injected in concentration series ranging from 0.625 to 10 μM, resulting in the sensorgrams in Fig. 5A for DS 01 and Fig. 5B for DShypo 01. It is worth noting that curves from both sensorgrams show higher signal with increasing peptide concentrations, interactions that tend to a steady-state and very slow dissociation constants. From these data, one can assume that DShypo 01 exhibits a higher affinity for DMPC vesicles when compared to DS 01. In order to rule out any possible interference of unspecific interactions between the cationic peptides and the negatively charged dextran-based surface of the Biacore chip, experiments using the same peptide concentrations were performed over naked sensor chips under the same conditions previously described, and their interaction was evaluated. Dose–response relationships for the interaction of DS 01 and DShypo 01 with both immobilized vesicles and naked chips are described in the Figs. 5C and D, respectively, and demonstrate that both peptides show massive interaction with the dextran layer, which is, in some concentrations, even higher than with vesicle-immobilized surfaces. Further studies varying the capture level of immobilized membranes with subsequent injection of 10 μM DS 01 are depicted as sensorgram and as a dose–response relationship in Figs. 5E and F. They reinforce the notion that raw SPR signals are in fact a sum of specific and non-specific interactions.

Affinity measurements using SPR were performed in order to try to understand the physical–chemical parameters that rule the affinity of both DSs towards model DMPC membranes. Such analysis was not possible due to the fact that interaction signals were indeed a sum of the specific interaction with the vesicles and non-specific interaction with the dextran layer, as demonstrated in Figs. 5C and D. These non-specific interactions are probably the result of electrostatic attraction between the positively charged antimicrobial peptides and the negatively charged carboxymethyldextran layers. It is by no means a particularity of the assayed peptides, for dermaseptin B2 was already reported to strongly interact with the dextran layer of CM5 chips [35]. Since there is no current model to accommodate such complex interaction signals, no numerical data could be extracted from them and this sort of study should be carried out using other techniques.

Effect on leukocytes and hematological parameter analyses

Investigation of the DShypo 01 cytotoxicity against white blood cells revealed no detectable lytic activity, for total cell counts on blood samples incubated with DShypo 01 at 64 μg/mL (53 μM) had no discrepancy from the control (data not shown). There were no significant differences between populations found in the control and the experimental group for any of the analyzed cell types (neutrophils, eosinophils, monocytes, lymphocytes, basophils, and platelets) as evaluated by the *t* test. The analyses of hematologic parameters also revealed no differences for mean platelet volume, hematocrit, free hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, and red blood cells between the control and the experimental group.

The usefulness of DShypo 01-based therapeutical agents depends not only on its leishmanial activity, but also on its lack of toxicity for the blood cells. The hemolysis (RBC counts) test and hematological parameters' analyses reveal that DShypo 01 at concentrations up to 53 μM had no hemolytic effect (data not shown). Although that is clearly an important property for a novel drug candidate, there is also the need to probe peptide interactions with other mammalian cell types and its resistance to host endogenous proteases.

Dermaseptin's lytic effect appears to be restricted to pathogenic microorganisms, and it does not induce obvious dam-

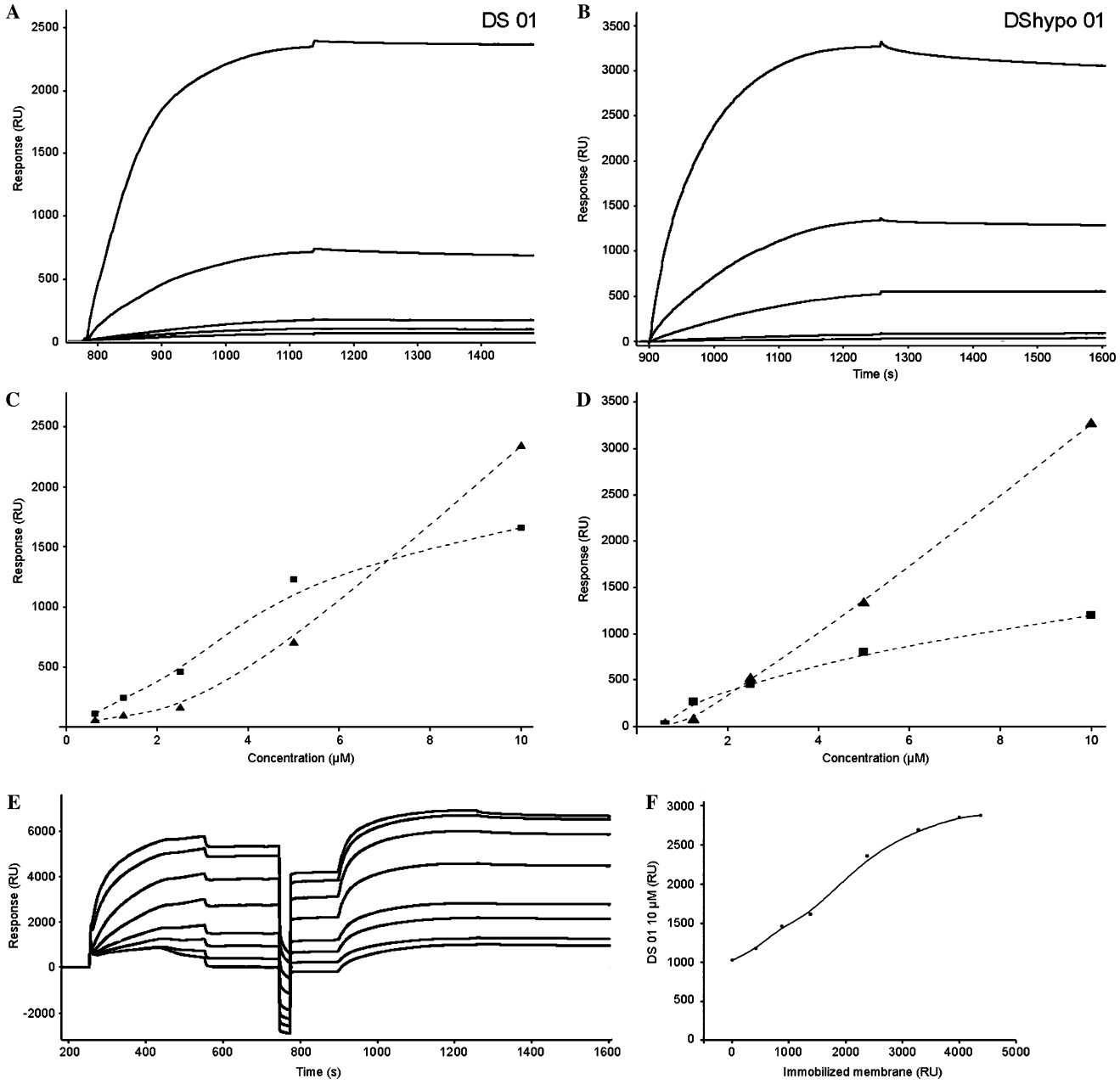

Fig. 5. Surface plasmon resonance measurements on the interaction of DS 01 and DShypo 01 with model DMPC membranes. (A) DS 01 injections from 0.625 to 10 μM over a DMPC-immobilized LUVs surface. (B) DShypo injections from 0.625 to 10 μM over a DMPC-immobilized LUVs surface. (C) Dose–response relationship for the interaction of DS 01 with DMPC LUVs (▲) and naked L1 chips (■). (D) Dose–response relationship for the interaction of DS 01 with DMPC LUVs (▲) and naked L1 chips (■). (E) Injection of 10 μM DS 01 across DMPC LUVs at varying capture levels. (F) DS 01-binding capacity of DMPC surfaces as a function of their lipid capture level.

age to animal cells. Whether this specificity relies on the inherent net charge and conformational features of the lytic peptide or rather on the membrane proper features of the target cell remains unclear. Thus, the likelihood that somewhere in the biosphere the molecular survival machinery of amphibians has solved our problem of treating multidrug-resistant pathogenic infections in humans with an efficacious peptide is greatly increased (e.g., dermaseptins), but remains to be seen in practical terms. Apart from this, the study of such molecules, generated by natural combinatorial chemistry and selection events over millions of years, can provide further insights into structure/activity requirements for therapeutic antimicrobial drug design.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.06.168.

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