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Dermaseptins from *Phyllomedusa oreades* and *Phyllomedusa distincta*: Secondary structure, antimicrobial activity, and mammalian cell toxicity☆

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Abstract

The present study reports the structural characteristics, the biological activities, and preliminary clinical investigations of three synthetic members of the dermaseptin family of antimicrobial peptides. The three peptides showed similar tendencies to form α-helical structures in non-polar media. The antimicrobial activity towards bacteria and fungi was determined in the micromolar concentration and the peptides did not influenced peritoneal cells viability. One of the peptides was intravenously administered in mice at concentrations similar to those of antibiotics employed in bacterial/fungal infections and it did not cause any detectable changes in cells and tissues.

Keywords: Antibacterial; Antifungal peptides; Cell viability; Circular dichroism; Dermaseptins; *Phyllomedusa*

1. Introduction

The presence of a great variety of amphiphilic cationic antimicrobial peptides in the skin secretions of anurans (Batista et al., 1999; Conlon et al., 2006a,b) has a crucial survival selective value associated with innate immunity (Vanhoye et al., 2003). Although these peptides have a broad range of activities, their diversities of primary structure are often coupled with individual activity specificities for different microorganisms encountered in given environments (Mor et al., 1994a,b). Helicities, hydrophobic moments, polar angles, and net charges (Sitaram and Nagaraj, 1999; Dathe et al., 2002; Shai, 2002; Tachi et al., 2002; Yeaman and Yount, 2003) are some structural parameters generally thought to play important roles in both potency and selectivity of these antimicrobial agents. One of the most studied and promising candidates for the designing of new antimicrobial drugs is the dermaseptin peptide family found in the skin secretion of the *Phyllomedusa* genus (Daly et al., 1992; Batista et al., 1999; Brand et al., 2002; Silva et al., 2008). Dermaseptins are cationic molecules composed by 24–34 amino acid residues with high helical propensity in membrane

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mimetic media (Shalev et al., 2002; Lequin et al., 2003). Each molecule has considerable variations in its antimicrobial activity towards Gram-positive and Gram-negative bacteria to fungi, mycobacteria, protozoa, and viruses (Mor et al., 1994a,b; Belaid et al., 2002). Even though dermaseptins share a broad spectrum of action against microorganisms, it has been already shown *in vitro* that they produce irrelevant hemolytic activities to mammalian cells (Helmerhorst et al., 1999). The available data on dermaseptins mode of action suggest that they bind to the acidic components of bacterial membranes disturbing the cells' osmotic balance in a carpet-like manner, promoting membrane disruption by forming several unstable transient pores (Shai, 2002).

In previous studies (Batista et al., 1999; Brand et al., 2002), we described three members of the dermaseptin family, DS 01, DD L and DD K, all active against bacteria and protozoa. In order to complement our earlier findings, the present work deals with the assessment of the differential *in vitro* potencies of these molecules against fungi cell lines *in vitro* and the *in vivo* effects of DS 01, intravenously injected in mice.

2. Materials and methods

2.1. Solid-phase synthesis

Peptides were synthesized on a Pioneer Automatic Peptide Synthesizer (Applied Biosystems, USA) and purified as previously described (Brand et al., 2002).

2.2. Antifungal assay

Three clinically isolated yeast species were assayed: *Candida albicans*, *Candida tropicalis*, and *Candida guilliermondii*. The fourth strain was obtained from the American Type Culture Collection (ATCC): *C. albicans* ATCC 1023. *In vitro* antifungal assays were performed by the broth micro dilution method according to the recommendations of the National Committee for Clinical Laboratory Standards — NCCLS (NCCLS, 1995; Rex et al., 1997; Giacometti et al., 1999).

2.3. Antibacterial activity

Four bacterial strains were used to investigate the DS 01 antimicrobial activity: *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Staphylococcus aureus,* and *Nocardia* spp. The last species were isolated from cows of Brasília, Brazil. Microorganisms were grown in stationary culture at 37 °C and then transferred to the Mueller–Hinton liquid medium (NCCLS-approved standard M100-S9) in which the tests were performed as previously described (Kieffer et al., 2003; NCCLS, 1999).

2.4. Cell viability

Swiss mice peritoneal cells were collected with 10 mL of PBS (pH 7.2). In order to obtain cells for the assay, a pool from peritoneal exudates of three mice was necessary. After collection, the cell suspensions were centrifuged at 900 *g* at 4 °C for 5 min. The supernatant was removed and discarded, leaving the cell-rich pellet. The cells were suspended in 1 mL of PBS and their viability was evaluated using 0.05% nigrosin in a Neubauer chamber. Data were processed considering the control values, which were always >90% of live cells (Crooke and Mandl, 1947).

2.5. In vivo toxicity assays

Female Swiss mice (*Mus musculus*) weighing around 40 g (São Paulo University—USP, Biotery) were intravenously treated with a single bolus of 50 μL of DS 01 containing 2.5 ($n=4$) or 5.0 ($n=4$) mg/kg mice body mass. The control group ($n=4$) was treated with equal volume of sterile saline solution 0.9%. During the experimental period the animals had a dark/light 12 h cycle, controlled temperature (23 °C), and had free access to balanced chow and water. The mice were killed 7 days after injection (Pacor et al., 2002).

Blood cytotoxicity was evaluated by total and differential leukocyte counts (Feldman et al., 2000). Small fragments from liver, spleen, and kidneys were dissected and processed for histology. The samples were dehydrated through crescent concentrations of ethanol, clarified with xylene, paraffin embedded, and sectioned in 5 μm thickness. The sections were stained by hematoxylin & eosin (H&E), analyzed using a light microscope (AxioSkop Zeiss, Germany), and representative images were acquired using a CCD-camera (Iris, Sony, USA) coupled to a digital capture system.

2.6. Circular dichroism (CD) spectroscopy

CD spectra were recorded on a Jasco J-810 spectrometer (JASCO, Gro-Umstadt, Germany) equipped with a Peltier-type temperature controller and a thermostatted cell holder, and calibrated with ammonium *d*-10-camphorsulfonate. The measurements are carried out using a quartz cell path length of 1 mm at wavelengths ranging from 190 to 260 nm to investigate the secondary structure of DS 01, DD L, and DD K. Samples were prepared by dissolving the peptide to a final concentration of 8 μM in the following solvents: water, TFE/water (5, 10, 15, 20, 25, 30, and 40%, v/v, pH 7.0), and 0.25–2 mM SDS (pH 4.0). Baseline spectra for each solvent were obtained prior to the peptide spectra. The molar ellipticity values were calculated based on a mean molecular mass per residue of 115 Da. The CD spectra were recorded at 25 °C with a 0.5-nm bandwidth and a scan speed of 50 nm/min. The temperature within the sample chamber was kept at 20 °C and continuous nitrogen flow at rate of 5 L/min. Two scans were averaged to improve the signal-to-noise ratio (Chen et al., 1972; Muñoz and Serrano, 1994; Rajan and Balram, 1996; Hemmi et al., 2002).

2.7. Statistical analysis

The Kolmogorov–Smirnov test was used to evaluate the normality of the variables distribution, and the Kruskal–Wallis test to compare multiple unrelated non-normal samples (Glantz, 1992). Differences with a two-tailed value of $p<0.05$ were

Table 1
Comparison of the primary structure and physical–chemical properties of DS 01, DD L and DD K

Peptides	Sequences	Net charge [a]	pI [b]	H [c]
DS 01	GLWSTI-KQKGKEAAIAAAKAAGQAALGAL...-NH_2	+4	10.0	1.8
DD K	GLWK-I-KAAGKEAAKAAAKAAGKAANAVSEAV-NH_2	+5	10.0	0.4
DD L	ALWKTLLKNVGKAAGKAALNAVTDMVNQ...-NH_2	+4	10.0	2.3

[a] Total net charge based on the number of positively and negatively charged amino acids at pH 7.0.
[b] The theoretical pI (isoelectric point), was calculated using the Bjellqvist et al. (1994) scale.
[c] Hydrophobicity (H), was calculated using the Eisenberg et al. (1984) consensus scale for hydrophobicity.

considered statistically significant. The SigmaStat software (Jandel Scientific, San Rafael, CA, USA) was used for statistical tests and the Microcal™ Origin 6.0® software package (Microcal Software Inc. 1999) was employed for the graphic representations.

3. Results

3.1. Amphiphilicity of dermaseptins

The three synthetic dermaseptins investigated here (Table 1) have identical isoelectric points, but they have high and different hydrophobic moments. The mean residue hydrophobicities of DS 01 and DD L are about five-fold higher than DD K hydrophobicity.

3.2. Circular dichroism

DS 01, DD L, and DD K are composed of almost 70% non-polar amino acids. Accordingly it is likely that they can only be studied in media that reproduce, at least in part, apolar environments, *e.g.* amphiphilic micelles or organic solvents. Despite several other tested solutions (data not shown), the most structuring conditions were found to be aqueous micelles of sodium dodecyl sulfate (SDS) and aqueous solutions of trifluorethanol (10, 20, 30 and 40%). Fig. 1 shows the Far-UV CD spectra of DS 01(A), DD L (B), and DD K (C) at 20 °C in TFE/water mixtures at various ratios (v/v). Fig. 2 shows the Far-UV CD spectra of DS 01(A), DD L (B), and DD K (C) dispersed in aqueous micelles of SDS in a concentration range from 0 to 2 mM. In water (0 mM SDS) the spectra of all three peptides are typical of unordered polypeptides. Upon the addition of SDS above the critical micellar concentration (CMC), the shape of the curves is increasingly characteristic of α-helical secondary structure. The minimum absorption peak (pp*-transition) shifts from 198 to 206 nm and the negative ellipticity at 220 nm (nπ*-transition) increases with the SDS concentration. In water the spectra of all three peptides present a negative band around 200 nm, commonly associated to the amide pp* electronic transition of unordered polypeptides, that is compatible with the presence in solutions of some extended conformations together with a significant population of partially ordered conformations. Upon TFE addition, the shape of the curves was increasingly characteristic of α-helical secondary structure in a concentration-dependent manner. The minimum absorption peak (pp*-transition) shifts from 198 to 206 nm and the negative ellipticity at 220 nm (nπ*-transition) increases with the TFE concentration. There is also a clear increase in positive ellipticity below 200 nm upon TFE addition.

3.3. Antifungal assay

The fungistatic and fungicidal activities of the dermaseptins against *C. tropicalis*, *C. guilliermondii*, *C. albicans* (clinical

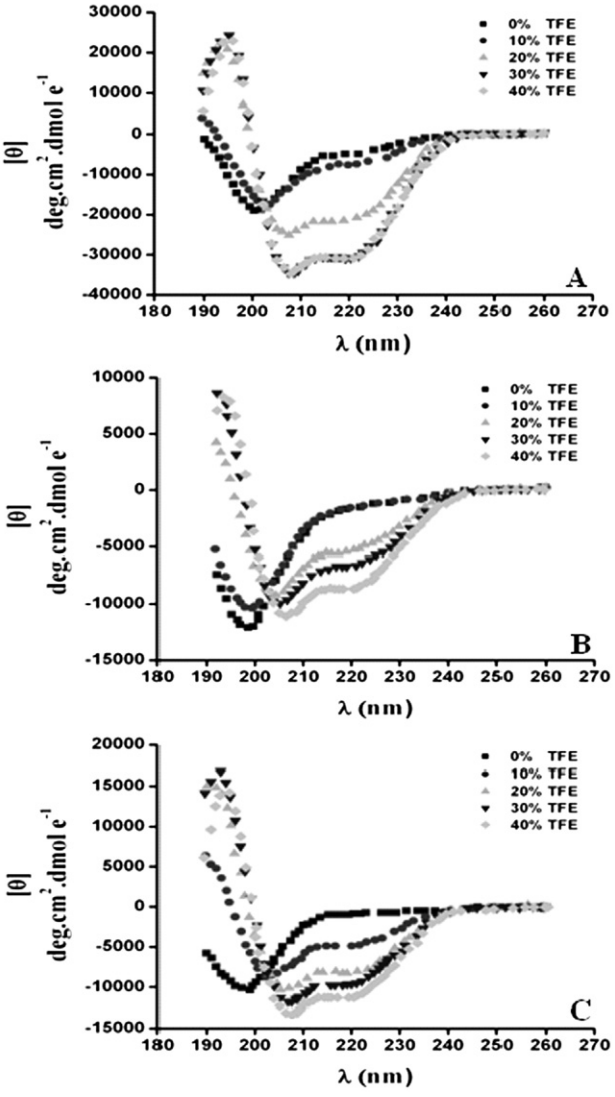

Fig. 1. Influence of 2-trifluoroethanol (TFE) on the secondary structure of the dermaseptins. Far-UV CD spectra of DS 01(A), DD L (B) and DD K (C) at room temperature in TFE/water mixtures at various concentrations of TFE in the range of 0%–40% (v:v). Peptide concentration is 8 μM.

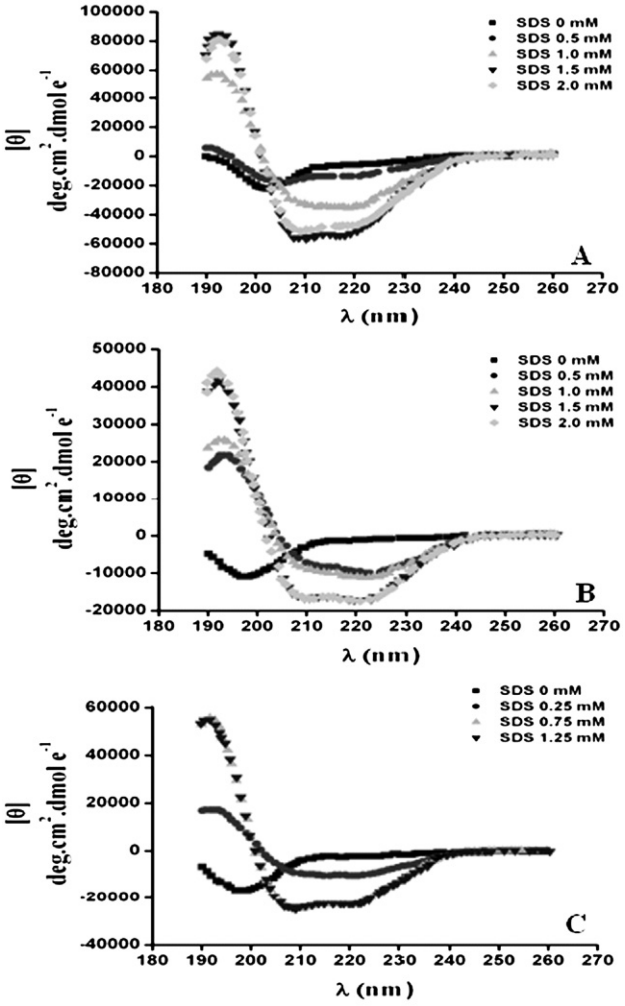

Fig. 2. Influence of the sodium dodecyl sulfate (SDS) on the secondary structure arrangement of dermaseptins. Far-UV CD spectra of DS 01(A), DD L (B) and DD K (C) at room temperature in aqueous micelles in the concentration range of 0 mM to 2 mM SDS. Peptide concentration is 8 μM.

isolates), and *C. albicans* ATCC 1023 are shown in Table 2. Despite the significant differences in the growth inhibition among the different strains, all peptides displayed fungicidal activities in accordance to the literature published for other DSs. The DSs effects were compared with those obtained by testing three conventional antifungal antibiotics (fluconazole, ketoconazole, and nystatin). DS 01 MICs (Minimal Inhibition Concentration) and MFCs (Minimal Fungicidal Concentration) were smaller than DD K and DD L for almost all tested microorganisms. In general, the MFC displayed for DS 01, DD K, and DD L showed to be more effective than conventional antibiotics.

3.4. Antibacterial assays

DS 01 was assessed for its antibacterial activity against four (Table 3 and Fig. 3). As it is shown in Table 3, MICs for DS 01 were 5.7 μM for *S. aureus*, *S. dysgalactiae*, *S. uberis* and 22.9 μM for *Nocardia* spp. Sensitive strains were not capable of resuming growth on agar plates after a 12 h treatment with concentrations above their corresponding MICs (Fig. 3).

Table 2
Comparison of the activity of antifungal dermaseptins (MICs and MFCs values) and the other conventional fungicides (fluconazole, ketoconazole and nystatin) against *Candida* genus[a]

Peptides and drugs	MIC/MFC[b] (μM)			
	C. tropicalis[c]	*C. guilliermondii*	*C. albicans*[a]	*C. albicans* ATCC 1023
DS 01	0.37/0.37	22.9/22.9	5.7/5.7	5.7/5.7
DD K	10.1/10.1	20.3/20.3	20.3/20.3	10.1/10.1
DD L	10.9/10.9	21.8/21.8	21.8/21.8	10.9/10.9
Fluconazole	6.5/_[d]	61.1/_	209.0/_	209.0/_
Ketoconazole	8.7/>50	75.0/75.0	4.0/22.2	3.1/16.4
Nystatin	5.1/15.4	25.0/75.0	1.3/11.1	1.3/10.0

[a] MIC (Minimal Inhibition Concentration) and MFC values measured by the fungal testing laboratory SABIN Clinical Analyses, Brasília, Brazil. Analysis according to the recommendation of the National Committee for Clinical Laboratory Standards MIC and MFC values was measured for 48 h.
[b] Minimal Fungicidal Concentration.
[c] Resistant to amphotericin B.
[d] The MFC of fluconazole was not determined because fluconazole was known as fungistatic.

3.5. Cell viability

After 4 h of incubation, peritoneal cells treated with DS 01, DD K, and DD L had their viability lowered in about 20%, 35%, and 55%, respectively (Fig. 4) when compared to the control.

3.6. In vivo blood cytotoxicity assays

Leukocyte counts (total and differential) had no significant difference between control and DS 01-treated groups ($p > 0.05$) (Fig. 5).

3.7. Morphology

Light microscopy qualitative evaluation of livers, spleens, and kidneys showed no detectable morphological changes in

Table 3
Antimicrobial activity of DS 01 against cow pathogenic microorganisms

Bacteria	MIC[a] (μM)			
	DS 01	Amoxicillim	Imipenem	Cephalosporine
S. dysgalactiae	5.7	<18.3	205.0	>40.0
S. uberis	5.7	>15.0	>35.0	>38.5
Nocardia spp.	22.9	<20.3	67.3	<34.6
S. aureus	5.7	15.7	78.7	56.5

Comparison of minimal inhibitory concentrations (MICs) with conventional antibiotics used from human and veterinary clinical.
[a] MIC minimal peptide concentration for total inhibition of cell growth in liquid medium. These analyses were performed according to the recommendations of NCCLS. Experiments performed in triplicates.

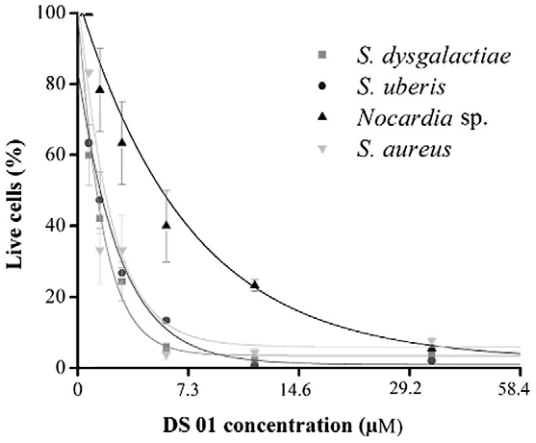

Fig. 3. Concentration–response curves of the DS 01 on the growth inhibition of four bacteria strains. The initial inoculum was 2.5×10^8 CFU/mL (Colony Forming Units per milliliter). The assay was performed in triplicate and the bars indicate the standard deviation.

DS 01-treated animals when compared to the controls for both evaluated concentrations (Fig. 6).

4. Discussion

Cationic peptides such as magainins (Tachi et al., 2002), cecropins (Daly et al., 1992; Yeaman and Yount, 2003), and dermaseptins (Brand et al., 2002) are known to disrupt cell membranes of microorganisms through their association with phospholipids (Sitaram and Nagaraj, 1999; Shai, 2002). The disruption of membrane bilayer by dermaseptins has been proposed to occur via barrel-stave pore formation, whereby a threshold concentration of peptides arranged on the cell membrane surface can lead to the fragmentation of the pathogen membrane (carpet-like model) (Biggin and Sansom, 1999; Shai and Oren, 2001). However, it is largely accepted that a preliminary step of helical/secondary structure organization is necessary prior to cell lysis (Rocca et al., 1999).

The Far-UV CD spectra of DS 01, DD K, and DD L indicated that these peptides exist in an unstructured conformation in water. TFE and SDS micelles have been commonly used to provide a membrane-mimicking environment for peptides and promote secondary structure formation. Helical configurations of the peptides were observed in the presence of 20–30% TFE and 0.75–1.0 mM of SDS strongly suggesting their formation in a hydrophobic environment like cell membranes (Lai et al., 2005; Sun et al., 2005).

Conventional antifungals are commonly associated with different fungicidal and fungistatic concentration ranges. Otherwise, DSs of the present study displayed their fungistatic and fungicidal effects at identical concentrations ranges. This fact may be explained by their distinct mechanisms of action (Gaidukov et al., 2003; Boichot et al., 2004). In this way, while the tested conventional antifungals act only by inhibiting the synthesis of cell wall components (Maebashi et al., 2002), DSs irreversibly disrupt cell membranes killing the microorganisms (Biggin and Sansom, 1999; Rocca et al., 1999). Another singular feature of DSs antifungal effects was the distinct MICs profiles of DS 01 when compared to DD K and DD L (except for similar activities against *C. guilliermondii*). This fact may be due to the aliphatic and uncharged C-terminus of DS 01 that could strongly interact with the aliphatic chains of membrane phospholipids. Otherwise, DD K and DD L have a negatively charged C-terminus that could create some electrostatic repulsion effect when interacting to phosphate groups of fungal membranes. In the case of *C. guilliermondii* species, the similar behavior activities for all DSs may be due to the lowest content of phosphatidylglycerol (PG) associated to the highest content of phosphatidylethanolamine (PE) observed in this species when compared to the other species (Abdi and Drucker, 1996). Such a scenario may be conducted in a less negatively charged membrane environment that made the interactions of positively charged peptides like DSs difficult.

Due to the higher potency of DS 01 against *Candida* strains when compared to the other tested DSs, further

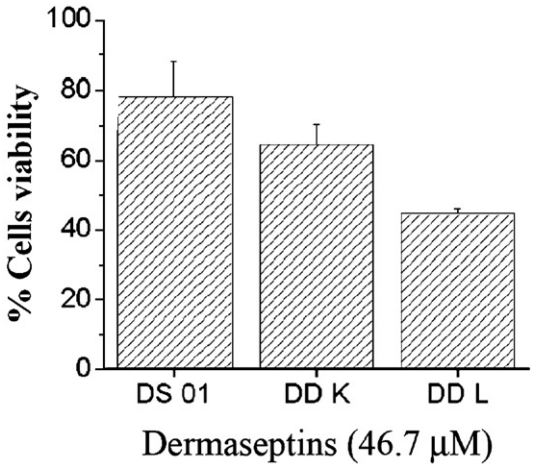

Fig. 4. Cytotoxicity of the dermaseptins to peritoneal cells. The cells (10^5 cells/mL) were exposed for 4 h to dermaseptins (DS 01, DD K, and DD L) with concentration of 46.7 μM. The percentage cell viability was determined using the 0.05% nigrosin exclusion method. The values of the three independent measurements are identified as a mean ± S.E.M. when compared to the control.

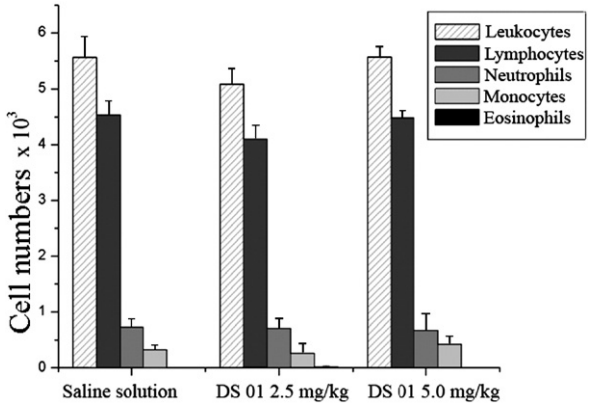

Fig. 5. Effects of DS 01 on blood leukocyte counts. Bar graphs showing leukocyte populations treated with DS 01 at 2.5 and 5.0 mg/kg and corresponding standard deviations. The values corresponded to the three independent measurements.

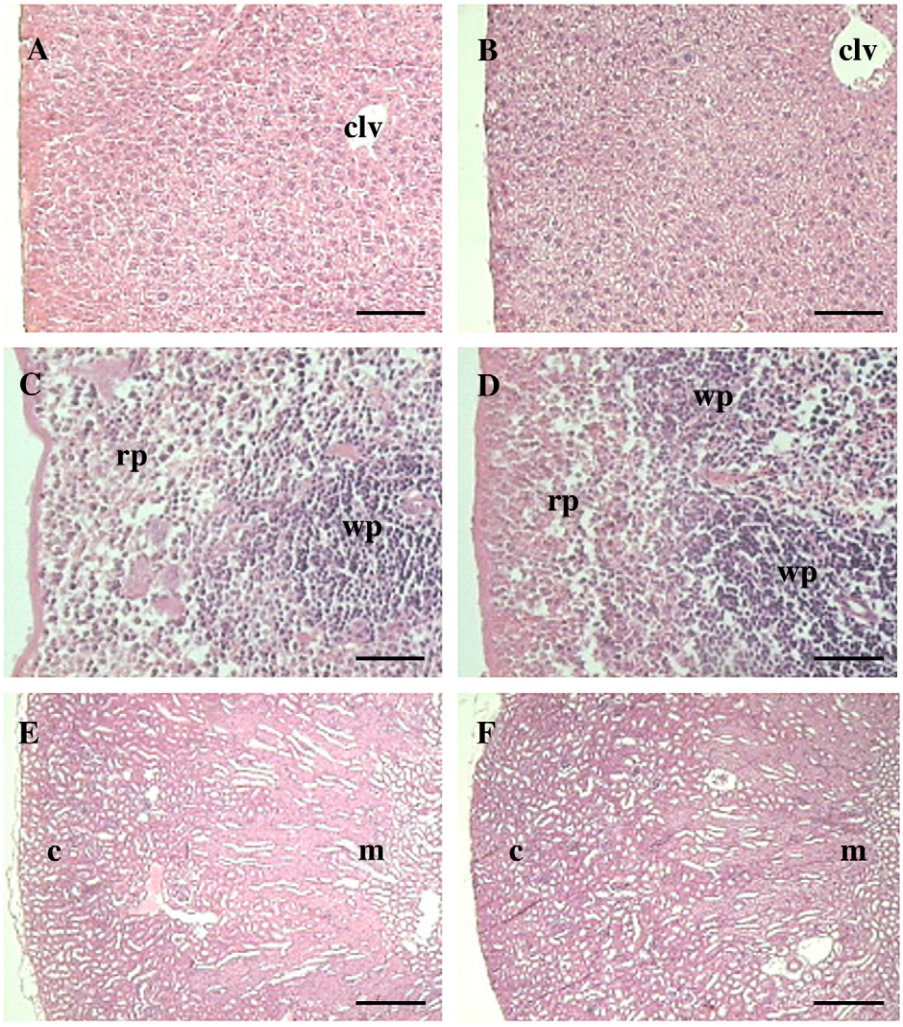

Fig. 6. Representative photomicrographs of liver (A), spleen (C) and kidney (E) of control. DS 01 (5.0 mg/kg) in treated liver (B), spleen (D) and kidney (F) of mice. Bars: A and B=100 μm; C and D=50 μm; and E and F=200 μm. clv = centrolobular vein, rp = red pulp, wp = white pulp, c = cortex and m = medulla.

biological studies were carried out with this peptide. Our previous study (Brand et al., 2002) showed that DS 01 have a wide range of activity towards laboratory-cultured microorganism Gram-positive and Gram-negative bacteria strains. Focusing on some other bacterial pathogens, the present study evaluated the effects of DS 01 on clinical isolates from bovine mastitis. Mastitis reduces milk yield and alters its composition. Mastitis-associated microorganisms produce toxins that can directly damage milk-producing tissue of the mammary gland (Craigmill et al., 1994). *Streptococcus*, *Staphylococcus*, and *Nocardia* are some of the major bacteria responsible by mild and acute mastitis. Indeed, our data showed that DS 01 killed these microorganisms at micromolar concentrations. Therefore, DS 01 is a candidate for a new antimicrobial drug in bovine mastitis control since conventional antibiotics are far from DS 01 activity and also display enhanced toxicity.

Despite the relevance of the *in vitro* tests, aiming future use of such peptides for animal therapeutic approaches, the first step in clinical trial is to test their toxicity towards mammalian cells *in vitro* and *in vivo*. Despite the efficacy of DS 01 killing microorganisms (bacteria and fungi) and in a minor extent decreasing mice peritoneal cells viability *in vitro*, there were not significant mammalian blood cells or tissues (liver, spleen, and kidneys) toxicity *in vivo* at concentrations similar to those commonly used for conventional antibiotics therapy (2.5 and 5.0 mg/kg) by intravenous infusion (Sun et al., 2005).

Small peptides belonging to the DS family of antimicrobial peptides are particularly well suited for production by chemical peptide synthesis. This fact associated with the selective activity against microorganisms points their potential uses on human and animal health. The development of *in vivo* studies treating mammalian infections is fundamental to establish the safety therapeutic strategies and it is one of our major current efforts.

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