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Full Length Research Paper

Antiviral and antifungal activity of some dermaseptin S4 analogues

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Dermaseptins are peptides found in skin secretions of Phyllomedusinae frogs. These peptides exert lytic action on various microorganisms, and do not have a considerable haemolytic effect apart from dermaseptin S4 (DS-S4) that presents a potent cytotoxic effect. This investigation was an attempt to synthesize several biochemically modified, shorter bioactive analogues of DRS-S4, and to improve its biological profile with respect to that of the parent peptide. Peptides were synthesized and then tested on fungi (*Cryptococcus neoformans* and *Aspergillus fumigatus*) and viruses (HSV-1). Our results show that the N-terminus is necessary for the antifungal activity of peptide, but antiviral effect is determined by C-terminal domain and/or entire peptide sequence.

Key words: Antimicrobial peptides, dermaseptin, structure-activity relationship, peptide synthesis.

INTRODUCTION

Infectious diseases are the second leading cause of death world-wide. Accordingly, there is an increasing need to identify new antimicrobial agents with a particular prominence on multi-drug-resistant microbes and newly emerging pathogens. New hopes, to delay the emergence and subsequent dissemination of resistant microorganisms or resistant genes, are taking birth from the discovery of natural products that may act as efficient leads in the development of novel therapeutic agents (Newman and Cragg, 2007; Gullo et al., 2006). Prominent amongst these natural products are the members of the cationic host defense peptide family, which are widely distributed in nature as a component of the immediate non-specific defense against infections. This defense peptide system exists in species of all kingdoms; especially in plants, insects and vertebrates including mammals (Brown and Hancock, 2006), and its effectiveness has been assessed by demonstrating direct antimicrobial activity against bacteria (Rotem et al., 2006), fungi (De Lucca et al., 1998), parasites (Dagan et al., 2002; Pérez-Cordero et al., 2011), protozoa (Brand et al. 2002) and viruses (Belaid et al., 2002a).

The advantages of these peptides in clinical application include their potential for broad-spectrum activity. We have focused on dermaseptins, a family of related broad spectrum antimicrobial peptides of 27 to 34 amino acids derived from the skin of frogs belonging to the Phyllomedusinae subfamily (Mor et al., 1991, 1994; Mor and Nicolas 1994). The peptide chain is rich with lysine residues (3 to 6) and contains highly conserved tryptophan residue in the third position from the N-terminus (Nicolas and Amiche 2006; Nicolas and El Amri 2009).

These peptides that differ in net charge and hydrophobicity exert a selective lytic action on Gram +ve and -ve bacteria, yeast, filamentous fungi, protozoa and viruses such as HSV-1 and HIV-1 (Belaid et al., 2002a; Lorin et al., 2005).

In this structure- activity relationship study , we chose dermaseptin S4 (DS-S4) because this peptide is

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characterized by the highest hemolytic activity. In an attempt to determine derivatives that display high antimicrobial activity *in vitro* with a more acceptable toxicity profile against human erythrocytes, we synthesized a series of analogues of S4.

MATERIALS AND METHODS

Peptides synthesis

Peptides were prepared by step-wise solid phase synthesis using Fmoc polyamide-active ester chemistry on Milligen 9050 pepsynthetiser (Milligen/Bioresearch, Millipore, Watford,UK). All Fmoc amino acids were from Milligen-Waters (France). 4-(Hydroxymethyl) phenoacetic acid-linked polyamide/kieselguhr resin (pepsin kA), Fmoc amino acid pentafluorophenyl (Pfp) and 3-hydroxy-2, 3-dehydro-4-oxobenzotriazine (Dhbt) esters were from Milligen/Bioresearch. Cleavage of peptidyl-resin and side chain deprotection were carried out at a concentration of 5 mg of peptidyl-resin in 1 ml mixture composed of trifluoroacetic acid, paracresol, thioanisole, water and ethyl methyl sulfide (82.5, 5, 5, 5 and 2.5% v/v) for 2 h at room temperature. After filtration of the resin, the filtered peptide-TFA was precipitated in ice cold diethyl ether, then dissolved in 0.08% TFA and lyophilized. The crude peptide was purified to chromatographic homogeneity by reverse-phase high performance liquid chromatography (RP-HPLC) (Waters modèle 510). HPLC runs were performed on preparative C18 columns (RCM deltapak C18, Waters) with a linear gradient of acetonitrile in water (1%/min); both solvents contained 0.08% TFA. Therefore, to confirm their composition, the purified peptides were subjected to amino acid analysis and mass spectrometry (Nermag R10-10). Peptides were stored as lyophilized powder at -20°C. Prior to experimentation, fresh solutions were prepared in water and diluted in the appropriate medium.

Antifungal assay

Antifungal activity was tested against two reference strains, *Cryptococcus neoformans* (IP 960-67) and *Aspergillus fumigatus* (IP 1025-70), cultured in Sabouraud broth medium. Inocula of 10^6 spores/ml were used. 50 µl of suspension was added to 50 µl of culture medium containing no peptide or various peptide concentrations (serial 2-fold dilutions) in 96-well plates (Iwaki, Japan). Inhibition of growth was determined by measuring optical density at 492 nm (multiskan Titertek MCC) in comparison with the controls (without peptide, medium alone) after incubation time of 24 h (to 36 h) at 30°C. The minimal inhibitory concentration (MIC) was defined as the dose at 100% inhibition of growth (Belaid et al., 2002b).

Anti-HSV assay

Antiviral activity of peptides against HSV-1 (KOS) was determined on monolayers of Hep-2 cells in 96-well tissue culture plates. Inocula constituted of mixtures of 50 µl of virus suspension containing 10^6 $TCID_{50}$/ml with 50 µl of serial peptide dilution in maintenance medium. Sham-treated virus was prepared similarly using maintenance medium without peptides. Assay was realized in duplicates (50 µl of each mixture per well). The virus was allowed to adsorb for 2 h at 37°C, then the virus inoculums was replaced with 0.2 ml of fresh medium. The cultures were incubated at 37°C for 2 days. Inhibition of antiviral effect of HSV-1 was evaluated by the reduction of viral production assay (Belaid et al., 2002a).

Haemolytic activity

To determine the haemolytic activity, human blood was rinsed three times in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7) by centrifugation for 15 min at 900 g, and then 10^8 human red blood cells (RBC) suspended in 250 µl of PBS was added to Eppendorf test tubes containing 250 µl of peptide solutions at desired concentrations, PBS alone (for base-line values) or distilled water (for 100% haemolysis). After incubation (1 h under mild agitation at 37°C), samples were centrifuged, and the haemolytic activity was assessed as a function of haemoglobin leakage by measuring the absorbance of 400 µl of supernatant at 540 nm. The peptide concentration causing 100% lysis (HC_{100}) was obtained from at least two independents experiments performed in triplicate.

RESULTS

Biochemical modifications

The main aim of our study was to identify a shorter bioactive analogue of DRS-S4, and then attempt to improve its biological profile with respect to that of the parent peptide. In order to accomplish this objective, we used the following criteria: (i) C-terminal or N-terminal deletion, (ii) substitution of methionine in position 4 with lysine, and (iii) amidation (Table 1). These modifications were chosen because they affect the positive charge and hydrophobicity of native peptide and it has been previously shown that increasing the net-positive charge and reducing the hydrophobicity of DRS-S4 affected dramatically the peptide's activity and resulted in various analogs that displayed potent antibacterial activity but reduced hemolytic activity (Efron et al., 2002; Feder et al., 2000; Kustanovich et al., 2002).

Cytotoxic and antiviral activity of peptide analogs

In this study, both antiviral activity and cytotoxicity of DS-S4 analogues were evaluated using Hep-2 target cells (Figure 1). Peptide concentrations causing 100% inhibition of HSV-1 infectivity (IC) and concentrations causing 100% cytotoxicity (CC) after 24 h of exposure were measured. The selectivity index (SI), and the ratio CC/IC was calculated and the results for all analogs are summarized in Table 1. The C-terminal amidation native

Table 1. Biological and structural proprieties of DS-S4 and its analogs.

Peptide	Peptide sequence	Charge[b]	IH[c]	CI[d]	CC[e]	SI[f]	HC[g]
DS-S4(1-28)	ALWMTLLKKVLKAAAKAALNAVLVGANA	4	28.9	8	16	2	16
DS-S4(1-28)a	----------NH_2	5	28.9	8	16	2	62.5
K4DS-S4(1-28)	---K----------	5	23.1	4	16	4	125
K4DS-S4(1-16)a	---K----------NH_2	6	5.5	-	16	-	32
DS-S4(1-16)a	----------NH_2	5	11.3	-	4	-	>250
DS-S4(1-12)a	----------NH_2	4	9.8	-	4	-	64
DS-S4(1-9)a	---------NH_2	3	5.7	-	16	-	>250
DS-S4(6-28)	----------	4	23	>16	32	>2	>250

a, Amide sequence; b, net charge (Feder et al., 2000); c, hydropathic index (Kyte and Doolittle,1982); d, peptide concentration (µg/ml) that induces 100% infection inhibition on Hep-2 cells by HSV-1; e, peptide concentration(µg/ml) that causes 100% cytotoxicity on Hep-2 cells; f, selectivity index : ratio CC_{100}/IC_{100}; g, haemolytic concentration of DS-S4 and its derivatives (µg/ml).

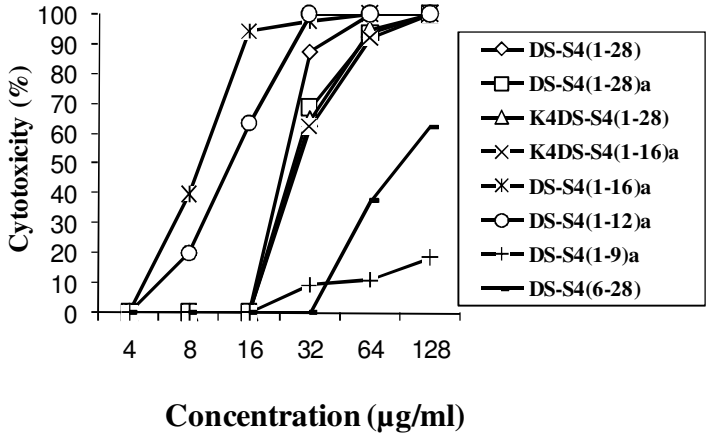

Figure 1. Effect of DS-S4 and its analogs on viability of Hep-2 cell monolayers. The data are mean values from three representative assays performed in triplicates.

S4 had no significant effect on the peptide toxicity for Hep-2 cells or on its anti-HSV1- activity. Deletion of the C-terminal region had variable effect on cytotoxicity (Figure 1) but it dramatically affected the anti-HSV-1 activity: K4DS-S4(1-16)a, DS-S4(1-16)a, DS-S4(1-12)a and DS-S4(1-9)a were inefficient as shown in Figure 2. Likewise, N-terminal deletion reduced the cytotoxicity of the peptide but weakened its anti-HSV-1 activity [DS-S4(6-28) was > 2-fold less active]. On the contrary, a positive charge mono-substitution without shortening the length of the peptide (Met → Lys in position 4) did not affect the cytotoxicity but increased anti-HSV-1 activity. K4DS-S4(1-28) had the highest selectivity index among all analogues tested. Taken together, these results show that a positive charge substitution in K4DS-S4(1-28) increase antiviral activity without affecting the cytotoxicity and that making shorter DS-S4 can decrease its cytotoxicity but certainly reduce its anti-HSV-1 activity.

Antifungal activity of dermaseptin derivatives

As shown in Table 2, S4 was more potent against *C. neoformans* than against *A. fumigates*. DS-S4(1-16)a was approximately 8 and 2-fold more potent, respectively, against *C. neoformans* and *A. fumigates*, than the native peptide. Similarly, DS-S4(1-12)a presented the highest antifungal effect on the two strains

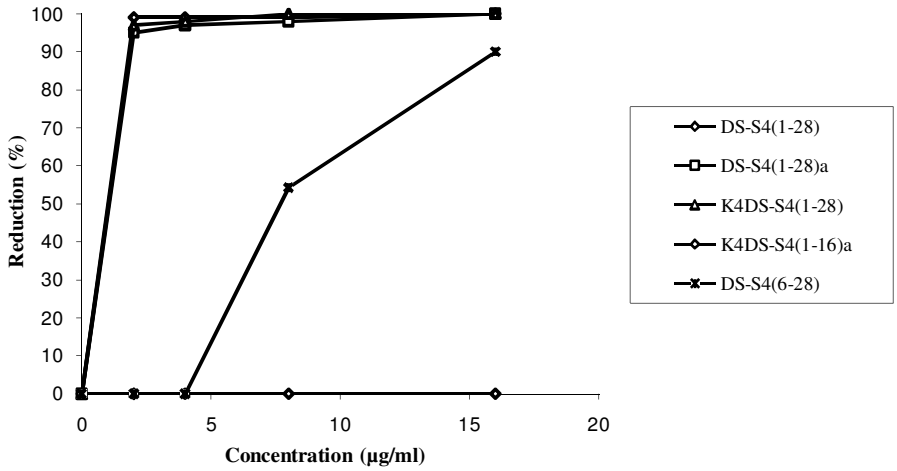

Figure 2. Antiviral activity of DS-S4 and its derivatives against HSV-1 (KOS) as measured by reduction in virus yield. The data are mean values from three representative assays performed in triplicates.

Table 2. Antifungal activity of DS-S4 and its analogs.

Peptide	*C. neoformans* (µg/ml)	*A. fimugatus* (µg/ml)
DS-S4(1-28)	16	>250
DS-S4(1-16)a	<2	125
DS-S4(1-12)a	<2	62
DS-S4(1-9)a	32	>250

a, Amide sequence.

with MIC values of 2 and 62 µg/ml, respectively, against *C. neoformans* and *A. fumigates*. These results suggest that except for DS-S4(1-9)a, the shorter analogues had enhanced antifungal activity as compared to the parent molecule.

Haemolytic activity of DS4 analogs

In the presence of DS-S4, incubation of erythrocytes for 1 h resulted in massive haemoglobin release, corresponding to 100% cell lysis at a peptide concentration of 16 µl/ml (Table 1). All analogues, however, were less effective than the native peptide. Thus, a reduced length, a positive charge and hydrophobicity appeared in reduced haemolytic potency.

DISCUSSION

Amongst the dermaseptins type S, DS-S3 and DS-S4 are particularly interesting, because the polar surface of the helix is the same for both peptides (Feder et al., 2000; Ghosh et al., 1997; Cootte et al., 1998). Therefore, S4 is significantly more hydrophobic than DS-S3 (hydropathic index is respectively of +28.9 and +10.5) (Kyte and Doolittle,1982). In addition, the positive charges of DS-S4 are concentrated in the middle (Feder et al., 2000), while those of the S3 are distributed along the helix and are separated by regular intervals (Mor et al., 1994; Hani et al., 1994). These observations led us to use DS-S4 to study the aspects of the structure-function relationship of dermaseptins.

To identify peptides which were derived from S4 with a reduced haemolytic activity and with an improved antimicrobial activity, and to determine the factors, such as charge and length, that influence these characteristics in solution, peptide analogues were synthesized with various biochemical modifications in peptide structure in Comparison with the native molecule. In order to

characterize the antimicrobial properties of DS-S4 and its analogues, we selected three models of peptide interactions, such as viruses, fungi and cells.

The antiviral activity of DS-S4 was evaluated against HSV-1 (KOS, sensitive strain). These experiments show that K4DS-S4(1-28) was more potent than all tested peptides. Recently, we found that this peptide could be a potential anti-HIV-1 microbicide candidate able to disrupt viral particles before infection (Lorin et al., 2005). This indicates that a positive charge mono-substitution in position 4 without shortening the length of the peptide increased its antiviral activity when compared with the native molecule. The C-terminal amidation in DS-S4a had the same efficiency as the native peptide but the deletions on the N or C-terminal side diminished the antiviral activity of the native peptide. These results suggest that the antiviral activity of the DS-S4 does not depend only on the net charge of the molecule, but also on the hydrophobicity and integrity of the helix.

The cytotoxicity of DS-S4 was measured on the Hep-2 cells permissive for HSV-1. Results in Figure 1 and Table 1, showed that deletions made on both sides of the molecule allows the obtaining of structural analogues devoid of cytotoxicity, except in the case of DS-S4 (1-16)a and the DS-S4 (1-12)a, which exhibited higher toxicity on Hep-2 cells in comparison with the native DS-S4. The peptide truncated at the C-ter (DS-S4(1-9)a was devoid of cytotoxicity as well as antimicrobial activity. This reflects the importance of size, specifically, the importance of structure in the biological function of the molecule.

K4DS-S4(1-28), however, presented the same effect on Hep-2 cells than DS-S4. On the other hand, the same peptide on P4-CCR5 and HIV-1 target cells, had reduced cytotoxicity at high concentrations (Lorin et al., 2005). Concerning the antifungal activity, the results summarized in Table 2 showed that C-terminal deleted molecules DS-S4(1-16)a and DS-S4(1-12)a; were remarkably more active than the parent peptide.

In a preliminary study concerning DS-S1 (Mor and Nicolas, 1994), it was shown that the mere deletion of 2 residues from its N-terminus lowered its lytic potency. On the other hand, DS-S1(1-18) displayed a comparable potency than the native peptide, and DS-S1(16-34) was devoid of antimicrobial activity (Mor and Nicolas, 1994). This suggest that the α-helical N-terminal domain of DS-S1 contains the essential features responsible for its activity. In addition, neutralization of the negative charge at the C-terminal end of DS-S1(1-18) improved its antifungal potency (Mor et al., 1994; Hani et al., 1994). These observations suggest that unlike the antiviral activity, antifungal activity of dermaseptines was improved by the deletion performed on the C-ter of the molecule. This is probably due to the difference between the viral envelope and the cytoplasmic membrane of other microorganisms. Besides, in previous studies, it was reported that the minimum bioactive sequence is the DS-S1(1-15)a and that a further Lys residue in the N-terminal improves the antimicrobial activity against *Escherichia coli* (Savoia et al., 2008).

Taken together, these results suggest that the N-ter segment is necessary for the antifungal activity of dermaseptin like antibacterial activity (Lequin et al., 2003), while the C-ter region is important for antiviral activity.

The high efficiency of amide peptide against microorganisms can be attributed to the increase in positive charges and also to the stabilization of the conformation of the helix. This is due to the fact that the amidation can provide more rigidity to the molecule, when compared with similar non-amide analogues (Shalev et al., 2002).

In this study, we report that all the newly synthesized molecules were less hemolytic than the native peptide (Table 1). This effect is well illustrated in the case of the mono-substituted peptides, K4DS-S4(1-16)a, DS-S4(1-9)a and DS-S4(6-28).

So, we suggest that reducing the length of the molecule decreases its hemolytic activity. Likewise, the amidation or the increase in the net charge of the molecule diminishes its toxicity against red blood cells. In fact, it was previously shown that increasing the number of positive charges of DS-S4 and reducing its hydrophobicity index resulted in the reduction of heamolytic activity (Feder et al., 2000)

The antimicrobial activity observed by some of the synthetic compounds tested are similar to those obtained by other authors with defensins (Ericksen et al., 2005) and with peptides purified from *Phyllomedusa distincta* (Batista et al., 1999).

Conclusion

The study of the structure-function performed on the DS-S4 revealed that the neutralization by amidation of the negative charge of the carboxylic group in C-ter of the native peptide improves its biological activity. Similarly, increasing the net positive charge of the molecule or reducing its hydrophobiciy results in analogs that display potent antimicrobial activity and decreased cytotoxicity as compared to native peptide.

A deletion made on the molecule shows that N-terminal is necessary for the antifungal activity of the DS-S4. However, C-terminal is essential for the antiviral activity of the peptide.

We showed that synthesis of peptide analogues which are more active and less toxic when compared with the native molecule is possible and we gave the example of K4DS-S4 in the case of antiviral activity and example of DS-S4(1-16)a and DS-S4(1-12)a in the case of antifungal activity.

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