Antiviral activity of dermaseptin 01 against *Dengue virus* type 2, *Herpes simplex* virus type 1 and *Vaccinia virus*

Atividade antiviral da dermaseptina 01 contra Dengue virus tipo 2, vírus Herpes simplex tipo 1 e Vaccinia virus

João Luiz Macedo de Souza Cardoso[1], Maria José dos Santos Soares[2], José Roberto Souza Almeida Leite[3], Luiz Cosme Cotta Malaquias[4], Luiz Felipe Leomil Coelho[4]

[1] Center for Research on Medicinal Plants, Federal University of Piauí, Teresina, PI, Brazil.
[2] Department of Veterinary Morphophysiology, Federal University of Piauí, Teresina, PI, Brazil.
[3] Biodiversity and Biotechnology Research Group – BioTec, Federal University of Piauí, Parnaíba, PI, Brazil.
[4] Institute of Biomedical Science, Federal University of Alfenas, Alfenas, MG, Brazil.

This study was supported by the Brazilian National Council of Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq) and Rede Nanobiomed Capes/Brasil. J.L.M.S.C and L.C.C.M were research fellows from CNPq.

ABSTRACT

Aims: To determine the *in vitro* antiviral activity of dermaseptin 01, an antimicrobial peptide discovered in the skin secretion of *Phyllomedusa hypochondrialis* frogs, against *Herpes simplex* virus type 1, *Vaccinia virus* and *Dengue virus* type 2.

Methods: The peptide dermaseptin 01 was used for the cytotoxic assays using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay (MTT) in VERO, LLCMK2 and C6/36 cells. The antiviral activity was also evaluated using MTT for *Herpes simplex* virus type 1 and *Vaccinia virus* in VERO cells and for *Dengue virus* type 2 in LLCMK2 cells. The antiviral activity of dermaseptin 01was also evaluated in C6/36 cells infected with *Dengue virus* type 2 by cytopathic effect reduction.

Results: The 50% cytotoxicity concentration of dermaseptin 01 was 105 µg/mL in insect cells (C6/36) and >1000 µg/mL in mammalian cells (VERO and LLCMK2). Dermaseptin 01 displayed antiviral effect only against *Dengue virus* type 2 with a 50% effective concentration of 15 µg/mL in C6/36 cells and 60 µg/mL in LLCMK2 cells.

Conclusions: These data suggested that dermaseptin 01 have an *in vitro* antiviral action against *Dengue virus* type 2 but not against *Herpes simplex* virus type 1 or *Vaccinia* virus.

KEY WORDS: PRODUCTS WITH ANTIMICROBIAL ACTION; ANTIVIRAL AGENTS; *DENGUE VIRUS*; *VACCINIA VIRUS*; *HERPES SIMPLEX*; ANPHIBIANS

RESUMO

Objetivos: Determinar a atividade antiviral *in vitro* da dermaseptina 01, um peptídeo antimicrobiano isolado da secreção de pele de pererecas *Phyllomedusa hypochondrialis*, contra vírus *Herpes simplex* tipo 1, *Vaccinia virus* e *Dengue virus* tipo 2.

Métodos: O peptídeo dermaseptina 01 foi usado em ensaios de citotoxicidade com ensaio colorimétrico utilizando brometo tiazoil azul tetrazólio (MTT) em células VERO, LLCMK2 e C6/36. A atividade antiviral foi determinada também por MTT para *Herpes simplex* tipo 1 e *Vaccinia virus* nas células VERO e para *Dengue virus* tipo 2 nas células LLCMK2. A atividade antiviral da dermaseptina 01 também foi testada nas células C6/36 infectadas com *Dengue virus* tipo 2 através da quantificação da redução do efeito citopático.

Resultados: A concentração citotóxica da dermaseptina 01 para 50% das células foi 105 µg/mL nas células de inseto (C6/36) e >1000 µg/mL nas células de mamíferos (VERO e LLCMK2). A dermaseptina 01 apresentou efeito antiviral somente contra *Dengue virus* tipo 2 com uma concentração efetiva para 50% das células de 15 µg/mL nas células C6/36 e 60 µg/mL nas células LLCMK2.

Conclusões: Os dados sugerem que a dermaseptina 01 possui ação antiviral *in vitro* contra *Dengue virus*, mas não contra *Herpes simplex* ou *Vaccinia virus*.

DESCRITORES: PRODUTOS COM AÇÃO ANTIMICROBIANA; ANTIVIRAIS; VÍRUS DA DENGUE; VÍRUS VACCINIA; HERPES SIMPLES; ANFÍBIOS

Recebido em 06/12/2012; aceito em 15/02/2013.

Endereço para correspondência/Corresponding Author:
Luiz Felipe Leomil Coelho
Instituto de Ciências Biomédicas, Universidade Federal de Alfenas
Rua Gabriel Monteiro da Silva, 700
CEP 37130-000, Alfenas, MG, Brazil
Tel.: (55-35) 3299-1418 – Fax: (55-35) 3299-1067
E-mail: luleomil@yahoo.com

INTRODUCTION

Dermaseptins (DSs) are a family of peptides isolated from the skin secretion of frogs from the *Phyllomedusa* genus. DSs are small, linear cationic peptides with 28–34 amino acid residues. They are able to form α-helices upon association with lipid bilayers and also in hydrophobic milieu.[1-3] A cytolytic activity of DSs against several microorganisms, including bacteria, virus, fungi and protozoa has been described. Despite its antimicrobial activity, DSs do not exhibit significant cytolytic activity in mammalian cells.[1-3] Indeed, the selective toxicity of DSs suggests a potential use for treatment of animal and human infections.

Dermaseptin 01 (DS-01) was discovered in the skin of Brazilian frogs *Phyllomedusa oreades* and *Phyllomedusa hypochondrialis* and has a potent *in vitro* antibacterial activity against Gram-negative and Gram-positive bacteria, *Trypanosoma cruzi*, *Leishmania amazonensis* and *Schistosoma mansoni*.[4-6] However, the potential of DS-01 to interfere with viral replication was not explored. Antiviral activity of some dermaseptins on the replication of Retrovirus (*Human immunodeficiency virus*), Herpesvirus (*Herpes simplex* virus type 1 [HSV-1] and *Channel catfish* virus) and Ranavirus (*Frog* virus 3) has been described.[7-9]

In view of the fact that very few viral diseases have adequate treatment and for some there are not even available vaccines, we investigated in this study the *in vitro* antiviral effect of DS-01 against HSV-1, *Vaccinia virus* (VACV) and *Dengue virus* type 2 (DENV-2), and its potential as a therapeutic agent.

METHODS

Peptide synthesis, cell culture and virus

VERO and LLCMK2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37°C, in 5% CO_2 atmosphere, supplemented with 5% fetal bovine serum, 50 µg/mL gentamicin, 100 U/mL penicillin and 5 µg/mL amphotericin B. C6/36 cells were cultured in L-15 medium at 28°C, supplemented with 10% fetal bovine serum and the same antibiotic concentrations as described for VERO and LLCMK2 cells. Peptide DS-01 were synthesized on a Pioneer Automatic Peptide Synthesizer (Applied Biosystems, Foster City, California) as described.[4] DS-01 was ressuspended in L-15 medium at final concentration of 1 mg/mL (358 µM) and filtered using a 0.22 µm pore filter.

DENV-2 and VACV strain Western Reserve (VACV-WR) were kindly donated by Dr. L. Figueiredo (USP, Ribeirão Preto, Brazil), and Dr. C. Jungwirth (University of Würzburg, Germany), respectively. A clinical isolate of HSV-1 was obtained in the Laboratory of Virus, Minas Gerais Federal University, Belo Horizonte, Brazil. HSV-1 and VACV-WR were propagated in VERO cells and DENV-2 in C6/36 cells. The supernatant containing viruses were utilized for virus titration by Tissue Culture Infectious Dose microculture assay as described elsewere.[10]

Cytotoxicity assay

Viability of VERO, LLCMK2 and C6/36 cells in the presence of DS-01 was tested by the MTT assay as described.[10] The percentage of cytotoxicity was calculated as [(A-B)/Ax100)], where A and B are the absorbances of control and treated cells, respectively. The 50% cytotoxic concentrations (CC_{50}) of DS-01 were estimated from concentration-effect curves after linear regression analysis.

Antiviral assay

Antiviral activity was evaluated using MTT colorimetric assay for HSV-1 and VACV-WR in VERO cells and for DENV-2 in LLCMK2 cells. Briefly, 1x 10^5 cells/well were seeded in 96 well plates and the cell monolayers were infected with 1x 10^3 $TCID_{50}$ of HSV, VACV-WR or DENV-2. DS-01 dilutions [100 (358 µM) to 0.8 (2,8 µM) µg/mL] were added one hour post infection. After 48 hours (HSV-1 and VACV-WR) or five days (DENV-2) the cell viability was assayed by MTT colorimetric method as described above for the cytotoxicity assay and values expressed as effective concentration by 50% (EC_{50}). The EC_{50} is the concentration of DS-01 required to inhibit the cytopathic effect to 50% of the control value (infected cells).[10]

Additionally, antiviral activity of DS-01 was also evaluated in C6/36 cells infected with DENV-2 by cytopathic effect (CPE) reduction. Briefly, monolayers of C6/36 cells in 24 well plates obtained from 1x 10^5 cells/well were infected with 1x10^4 $TCID_{50}$ of DENV-2 for one hour at 28°C to allow viral adsorption. After, the medium containing the non-adsorbed virus was removed and the monolayers rinsed twice with PBS. The dilutions of nontoxic doses of DS-01 were added to the cells. The plates were then incubated at 28°C and observed daily for development of CPE. The number of multinucleated cells (syncicia) was counted under an inverted microscope. The antiviral concentration of 50% effectiveness (EC_{50}) was defined as the concentration that achieved 50% inhibition of

virus-induced cytopathic effects and is calculated from concentration-effect curves after linear regression analysis. The selectivity index was determined as the ratio of the CC_{50} for cell viability and EC_{50} for virus replication.

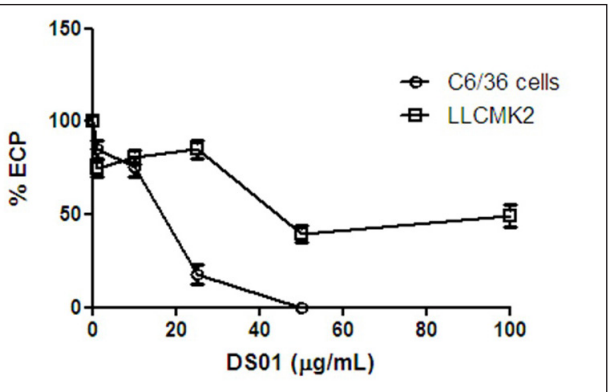
RESULTS

Cytotoxic effect of DS-01 was more intense in insect cell line (C6/36 cells) than in mammalian cells (VERO and LLCMK2) and was characterized by cell lysis and membrane disruption. A dose-dependent toxic effect of DS-01 in C6/36 cells with a CC_{50} value of 105 µg/mL (37.59 µM) was observed. As expected, DS-01 has lower cytotoxic effect in the mammalian cells (CC_{50}>1 mg/mL) (**Table 1**). DS-01 displayed antiviral effect only against DENV-2 with a 50% effective concentration (EC_{50}) value of 60 µg/mL (21.5 µM) in LLCMK2 cells (**Table 1** and **Figure 1**). In C6/36 insect cells, DS-01 also displayed antiviral activity with EC_{50} of 15 µg/mL (5.37 µM) (**Figure 1**).

Table 1. Cytotoxic and antiviral *in vitro* activity of dermaseptin 01 on different types of cell and virus.

Cell type	CC_{50} (µg/mL) (mean±SE)	EC_{50} (µg/mL) (mean±SE)		Selectivity index
C6/36	105±3	DENV-2	15±2	7
LLCMK2	>1000	DENV-2	60±5	>16.6
VERO	>1000	HSV-1	ND	-
VERO	>1000	VACV-WR	ND	-

ND: not determined; CC_{50}: cytototoxic concentration for 50% of cultured cells; EC_{50}: effective concentration able to inhibit the cytopathic effect to 50% of the control value (infected cells); DENV-2: *Dengue virus* type 2; HSV-1: *Herpes simplex* virus type 1; VACV-WR: *Vaccinia virus* strain Western Reserve; SE: standard error



C6/36 and LLCMK2 cells were infected with *Dengue virus* type 2 and one hour post infection, dermaseptin-01 were added to infected cells at different concentrations. The antiviral activity was measured by cell viability using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay (LLCMK2 cells) or by reducing the cytopathic effect of *Dengue virus* in C6/36 cells compared to control virus wells. The graph shows the means ± standard error from at least three independent experiments.
DS01: dermaseptin-01.
% ECP: cytopathic effect percent.

Figure 1. Antiviral effects of dermaseptin-01 in insect and mammalian cells.

DISCUSSION

Cytotoxic activity of DS-01 in C6/36 cells could be attributed to its action on the cell plasma membrane. DS-01 can induce cytotoxic activity in the same way as the oxyopinins, an antimicrobial peptide isolated from the venom of spider *Oxyopes kitabensis* that shows structural similarity with DS1-01. Oxyopinins have an evident toxic effect against insect cells (SP9 cells) by leading to a drastic cell membrane reduction by inducing nonselective ion channels, particularly in membranes rich in phosphatidylcholine and poor in cholesterol.[11]

It is still unclear how DS-01 can discriminate between mammalian and insect cells, but it is known that this distinction could be based on differences in constitution and structure of membranes, such as composition and concentration of phospholipids and cholesterol. [4,12-14] It is known that the dermaseptins peptides could disrupt the cell membrane by forming pores.[14] So, the high cytotoxic activity of DS-01 against insect cells may suggest a new role for amphibian antimicrobial peptides. The antimicrobial peptides not only can fight against microbial pathogens but could also be able to control the amphibian skin infestation by arthropods such as parasitic mites or insect larvae. This feature is highly desirable, since the majority of amphibians live in tropical and subtropical environments with a high density of insects.

Our antiviral assays data suggests a specific inhibitory mechanism of DS-01 on the replication of DENV-2. Interestingly, DS-01 showed no interference with the replication of VACV-WR and HSV-1. The antiviral activity of DS-01 in DENV-2 infected cells *in vitro* permitted to consider this peptide as a possible antiviral drug to treated dengue. Indeed, DS-01 has low toxicity in mammalian cells and presented good antiviral activity with an excellent selectivity index. The differential antiviral activity of DS-01 could be attributed to differences in the replication cycle of the viruses tested. Both VACV-WR and HSV-1 are enveloped viruses that depend on a mechanism of fusion of their viral envelope with the cell membrane to release the nucleocapsid into the cytoplasm.[15,16] *Dengue virus* is also an enveloped virus, but it enters the cell through a process of endocytosis receptor-mediated.[17] DS-01 at non-toxic concentrations may also induce ion channels in cell membranes and as it was added one hour after DENV-2 infection, it could interfere with the early events of viral replication such as viral penetration and/or with release of viral particles from the endosomal vesicle.

Dengue is a major public health problem worldwide, especially in the tropical and subtropical areas.[18] The disease is caused by a positive single strand RNA virus that belongs to *Flavivirus* genus, *Flaviviridae* family. Infection with one of the DENV serotypes can causes a mild, self-limiting febrile illness called dengue fever. However, some patients could develop severe forms of disease called dengue hemorrhagic fever and dengue shock syndrome and the number of patients with severe forms may increase in case of reinfection with a different serotype of *Dengue virus*.[19] Antiviral therapy could potentially reduce morbidity and mortality from dengue infections, but no effective drugs are currently available to treated *Dengue virus* -infected patients.[20] The mechanisms of action of candidate drugs to treated *Dengue virus* involve the inhibition of virus uncoating, inhibition of interference with the genome replication and modulation of the post-translational processing of the viral polyprotein.[21-23]

This study shows the *in vitro* inhibitory potential of DS-01 on DENV-2 replication but not against HSV-1 or VACV. Future experiments should be performed to elucidate the mechanism of action of DS-01 antiviral activity on the DENV-2 replication cycle and to explore its potential use as a therapeutic weapon to combat dengue.

ACKNOWLEDGEMENTS

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Rede Nanobiomed Capes/Brasil. J.L.M.S.C and L.C.C.M were research fellows from CNPq.

REFERENCES

1. Mor A, Hani K, Nicolas P. The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms. J Biol Chem. 1994;269(50): 31635-41.
2. Zairi A, Tangy F, Bouassida K, et al. Dermaseptins and magainins: antimicrobial peptides from frogs' skin-new sources for a promising spermicides microbicides-a mini review. J Biomed Biotechnol. 2009;2009:452567.
3. Nicolas P, El Amri C. The dermaseptin superfamily: a gene-based combinatorial library of antimicrobial peptides. Biochim Biophys Acta. 2009;1788(8):1537-50.
4. Brand GD, Leite JR, Silva LP, et al. Dermaseptins from Phyllomedusa oreades and Phyllomedusa distincta. Anti-Trypanosoma cruzi activity without cytotoxicity to mammalian cells. J Biol Chem. 2002;277(51):49332-40.
5. Zampa MF, Araujo IM, Costa V, et al. Leishmanicidal activity and immobilization of dermaseptin 01 antimicrobial peptides in ultrathin films for nanomedicine applications. Nanomedicine. 2009;5(3):352-8.
6. de Moraes J, Nascimento C, Miura LM, et al. Evaluation of the in vitro activity of dermaseptin 01, a cationic antimicrobial peptide, against Schistosoma mansoni. Chem Biodivers. 2011;8(3):548-58.
7. Lorin C, Saidi H, Belaid A, et al. The antimicrobial peptide dermaseptin S4 inhibits HIV-1 infectivity in vitro. Virology. 2005;334(2):264-75.
8. Chinchar VG, Bryan L, Silphadaung U, et al. Inactivation of viruses infecting ectothermic animals by amphibian and piscine antimicrobial peptides. Virology. 2004;323(2): 268-75.
9. Belaid A, Aouni M, Khelifa R, et al. In vitro antiviral activity of dermaseptins against herpes simplex virus type 1. J Med Virol. 2002;66(2):229-34.
10. Brandao GC, Kroon EG, Duarte MG, et al. Antimicrobial, antiviral and cytotoxic activity of extracts and constituents from Polygonum spectabile Mart. Phytomedicine. 2010;17(12):926-9.
11. Corzo G, Villegas E, Gomez-Lagunas F, et al. Oxyopinins, large amphipathic peptides isolated from the venom of the wolf spider Oxyopes kitabensis with cytolytic properties and positive insecticidal cooperativity with spider neurotoxins. J Biol Chem. 2002;277(26):23627-37.
12. Batista CV, da Silva LR, Sebben A, et al. Antimicrobial peptides from the Brazilian frog Phyllomedusa distincta. Peptides. 1999;20(6):679-86.
13. Castiglione-Morelli MA, Cristinziano P, Pepe A, et al. Conformation-activity relationship of a novel peptide antibiotic: structural characterization of dermaseptin DS 01 in media that mimic the membrane environment. Biopolymers. 2005;80(5):688-96.
14. Leite JR, Brand GD, Silva LP, et al. Dermaseptins from Phyllomedusa oreades and Phyllomedusa distincta: Secondary structure, antimicrobial activity, and mammalian cell toxicity. Comp Biochem Physiol A Mol Integr Physiol. 2008;151(3):336-43.
15. Moss, B. Poxviridae and their replication. In: Knipe, P.M, editor. Fields Virology. 5th ed. Lippincott-Williams and Wilkins: New York; 2007. p. 2079-81.
16. Pellet, P.E, Roizman, B. The Family Herpesviridae: a brief introduction. In: Knipe, P.M, editor. Fields Virology. 5th ed. Lippincott-Williams and Wilkins: New York; 2007. p. 2479-99.
17. Sampath A, Padmanabhan R. Molecular targets for flavivirus drug discovery. Antiviral Res. 2009;81(1):6-15.
18. Halstead SB. Dengue. Lancet. 2007;370(9599):1644-52.
19. Oishi K, Saito M, Mapua CA, et al. Dengue llness: clinical features and pathogenesis. J Infect Chemother. 2007;13(3):125-33.
20. Zhou Z, Khaliq M, Suk JE, et al. Antiviral compounds discovered by virtual screening of small-molecule libraries against dengue virus E protein. ACS Chem Biol. 2008;3(12):765-75.
21. Lee E, Pavy M, Young N, et al. Antiviral effect of the heparan sulfate mimetic, PI-88, against dengue and encephalitic flaviviruses. Antiviral Res. 2006;69(1):31-8.
22. Whitby K, Pierson TC, Geiss B, et al. Castanospermine, a potent inhibitor of dengue virus infection in vitro and in vivo. J Virol. 2005;79(14):8698-706.
23. Diamond MS, Zachariah M, Harris E. Mycophenolic acid inhibits dengue virus infection by preventing replication of viral RNA. Virology. 2002;304(2):211-21.