In vitro antiviral activity of antimicrobial peptides against herpes simplex virus 1, adenovirus, and rotavirus

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Peptides with broad-spectrum antimicrobial activity, known as antimicrobial peptides, have been isolated from distinct organisms. This paper describes the in vitro evaluation of the cytotoxicity and antiviral activity of nine peptides with different structures and origins against herpes simplex virus type 1, human adenovirus respiratory strain, and rotavirus SA11. Most of the evaluated peptides presented antiviral activity but they were only active near cytotoxic concentrations. Nevertheless, these results seem promising, and further modifications on the peptide's structures may improve their selectivity and reduce their cytotoxicity.

Key words: antimicrobial peptides - antiviral activity - herpesvirus - adenovirus - rotavirus

Many peptides with broad-spectrum antimicrobial activity, typically known as antimicrobial peptides (AMPs), have been isolated from a wide panel of organisms, including mammals, amphibians, molluscs, tunicates, and arthropods (Bachère et al. 2000, Yasin et al. 2000, van der Strate et al. 2001, Zasloff 2002, Bulet et al. 2004, Chinchar et al. 2004, Park & Hahm 2005).

Most of them are cationic, amphipathic molecules that contain 15 to 40 amino acid residues, which can be loosely grouped into three major groups: α-helical peptides (e.g., magainins), cyclic and open-ended cyclic peptides with pairs of cysteine residues (e.g., defensins), and peptides with an over-representation of some amino acids (e.g., proline rich) (Yasin et al. 2000, Zasloff 2002, Bulet et al. 2004).

Although the antibacterial and antifungal activities of AMPs have been the main focus of the studies to date, some of these molecules have also been shown to be effective against viral pathogens (Yasin et al. 2000, van der Strate et al. 2001, Oevermann et al. 2003, Matanic & Castilla 2004, Sun et al. 2005). For instance, lactoferrin, a glycoprotein present in the milk, inhibited the human immunodeficiency virus (HIV-1), herpes simplex virus types 1 (HSV-1) and 2 (HSV-2), human cytomegalovirus, respiratory syncytial virus, hepatitis B and C virus, adenovirus, and rotavirus (van der Strate et al. 2001, Orsi 2004) in vitro, highlighting the importance of naturally occurring proteic molecules as antiviral agents.

This report describes the evaluation of the antiviral activity of nine antimicrobial peptides with different structures against HSV-1, human adenovirus respiratory strain (AdV-5), and rotavirus SA11 (RV-SA11), viruses that represent a challenge to the public health system due to their limiting symptomatic treatment.

MATERIALS AND METHODS

Antimicrobial peptides - The peptides used in this study are described in Table I. PW-2 and Gomesin were kindly donated by Dr Arnaldo Silva Junior (Unicamp, Campinas, SP) and by Dr Sirley Daffre (ICB-USP, SP, Brazil), respectively. The further evaluated peptides were extracted from the respective sources described in Table I. Stock solutions were prepared in sterile MilliQ® water at 1 μM and stored at – 20°C until use.

Cell culture and viruses - Vero (ATTC CCL-81), HEp-2 (ATCC CCL-23), and MA104 cells (ATTC CRL-2378.1) were grown and maintained in minimum essential medium (MEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and 1% of antibiotics PSA (100 IU/ml penicillin G, 100 μg/ml streptomycin and 0.025 mg/ml amphotericin B; Gibco BRL) at 37ºC in a humidified 5% CO_2 atmosphere. HSV-1, KOS strain (Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Rennes, France), AdV-5, and RV-SA11 (both from ICB-USP, SP, Brazil) were propagated and titrated in Vero, HEp-2, and MA104 cells, respectively.

Cytotoxicity evaluation - The cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (Mossmann 1983, Sieuwerts et al. 1995), with minor modifications. Vero, HEp-2, and MA104 cells were grown in 96-well plates for 24 h at 37ºC in a humidified 5% CO_2 atmosphere. Following incubation, media was replaced with fresh MEM containing two-fold serial dilutions of the pep-

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TABLE I
Characteristics of peptides used in this study

Peptides	Reference	Source	Amino acid sequence
PW-2	da Silva Jr et al. 2001	Recombinant	HPLKQYWWRPSI
Tachyplesin-1	Murakami et al. 1991	Limulid	KWCFRVCYRGICYRRCR [a]
Gomesin	Mandard et al. 2002	Spider	ZCRRLCYKQRCVTYCRGR [a]
Clavanin A	Lee et al. 1997	Tunicate	VFQFLGKIIHHVGNFVHGFSHVF [a]
Magainin [b]	Zasloff 1987	Frog	GIGKFLKKAKKFGKAFVKIMKK [a]
HCTF [c]	Destoumieux-Garzon et al. 2001	Synthetic	FEDLPNFGHHIQLKVFNHGEHIHH
Penaeidin-3 [d]	Yang et al. 2003	Shrimp	QGA: VYKGGYTRPIPRPPPPVRPL PGGPIGPYNGCFV SCRGISFSQAR SCCSRLGRCCHVGKGYSG pEGA: PEVYKGGYTRPIPRPPPPV RPLPLPGGPIGPYNGCFVSCRGIS FQARSCCSRLGRCCHVGKGYSG
ALF [e]	Somboonwiwat et al. 2005	Shrimp	QGWEAVAAAVASKIVGLWRNEK TELLGHECKFTVKPYLKRFQVY YKGRMWCPGWTAIRGEASTRSQ SGVAGKTAKDFVRKAFQKGLISQ QEANQWLSS
Mytilin A	Charlet et al. 1996	Mussel	GCASRCKAKCAGRRCKGWASAS FRGRCYCKCFRC

a: C-terminal amidation; *b*: the magainin used in this work is the synthetic analog MSI-94; *c*: hemocyanin C-terminal factor; *d*: the penaeidin-3 used in this work is a mixture (1:1) of two recombinant analogs QGA and pEGA; *e*: antilipolysaccharide factor.

TABLE II
Cytotoxicity of peptides obtained by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Peptides	CC_{50} [a] Vero	CC_{50} HEp-2	CC_{50} MA104
PW-2	> 125	84.90 ± 5.83	> 125
Tachyplesin-1	Nt	Nt	23.70 ± 3.96
Gomesin	78.30 ± 11.31	50.63 ± 1.55	24.30 ± 2.65
Clavanin A	Nt	25.50 ± 5.57	50.23 ± 7.62
Magainin	39.72 ± 0.85	37.15 ± 5.28	33.52 ± 2.38
HCTF	> 80	Nt	Nt
Penaeidin-3	> 100	42.85 ± 4.85	21.03 ± 4.27
ALF	47.20 ± 11.00	17.13 ± 1.23	35.91 ± 6.15
Mytilin A	49.84 ± 3.34	Nt	> 80

CC_{50}: concentration (μM) that reduced the absorbance of treated cells by 50% when compared to cell control. Results are expressed as mean ± SDM of three separate experiments; Nt: not tested; HCTF: hemocyanin C-terminal factor; ALF: antilipolysaccharide factor.

tides. After 72, 96, and 120 h of incubation, respectively, for MA104, Vero, and HEp-2 cells at the same conditions mentioned above, the cytotoxicity was assessed and expressed as CC_{50} (concentration that reduced the absorbance of treated cells by 50% when compared to control – untreated cells). All assays were performed in triplicate.

Antiviral assays - The antiviral assays were based upon cell viability also using the MTT method as reported by Takeuchi et al. (1991) in their studies with HSV. The technical details are described below, depending on the used strategy. For the simultaneous assay, Vero, HEp-2, and MA104 cells were grown in 96-well plates for 24 h at 37ºC in a humidified 5% CO_2 atmosphere. Following incubation, media was replaced with fresh MEM containing two-fold serial dilutions of non-cytotoxic concentrations (below CC_{50} values) of the peptides and each one of the evaluated viruses (MOI = 0.5). Plates were incubated for different periods of time according to each virus: 72 h for RV-SA11, 96 h for HSV-1, and 120 h for AdV-5 at the same conditions mentioned above. The percentages of protection were calculated as [(A-B)/(C-B) × 100], where A, B, and C indicate the absorbance of the peptides, virus and control cells, respectively. The calculated EC_{50} value was defined as the concentration that reduced the absorbance of infected cells to 50% when compared to infected cells and control cells. To determine whether the compounds inhibited viruses replication by affecting their adsorption or penetration on the host cells another strategy was adopted, the pretreatment assay (Bettega et al. 2004). Vero, HEp-2, and MA104 cells were grown as described above and following incubation, media was replaced with fresh MEM containing two-fold serial dilutions of non-cytotoxic concentrations of the peptides. Plates were incubated for 3 h prior to virus infection and further incubation periods.

TABLE III

Percentages of inhibition of the different tested viruses by peptides, obtained by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, using different strategies of evaluation

	Herpes simplex virus type 1		Human adenovirus respiratory strain		Rotavirus SA11	
Peptides	Conc	%	Conc.	%	Conc.	%
PW-2	125		80		125	
Sim		70.91 ± 2.46		36.67 ± 6.61		18.66 ± 4.67
Pre		25.08 ± 9.90		24.60 ± 5.70		31.69 ± 14.09
Tachyplesin-1					20	
Sim		At		At		51.41 ± 20.92
Pre		At		At		32.14 ± 9.36
Gomesin	75		50		20	
Sim		59.14 ± 2.46		31.73 ± 2.46		18.32 ± 9.16
Pre		19.31 ± 9.64		52.25 ± 4.27		Nt
Clavanin A			25		50	
Sim		At		94.72 ± 5.59		69.40 ± 13.13
Pre		At		61.54 ± 13.60		95.46 ± 34.42
Magainin	35		35		30	
Sim		24.06 ± 13.19		29.35 ± 2.74		29.90 ± 11.18
Pre		23.77 ± 5.70		20.36 ± 3.88		13.84 ± 3.18
HCTF	80					
Sim		30.27 ± 2.03		Nt		Nt
Pre		Nt		Nt		Nt
Penaeidin-3	100		40		20	
Sim		85.17 ± 7.11		28.00 ± 7.51		12.75 ± 9.08
Pre		34.26 ± 9.10		32.37 ± 11.79		56.65 ± 2.58
ALF	45		15		35	
Sim		72.07 ± 17.02		98.17 ± 4.24		9.58 ± 3.39
Pre		58.50 ± 8.20		33.85 ± 7.50		27.07 ± 22.51
Mytilin A	40				80	
Sim		34.28 ± 7.40		Nt		33.12 ± 2.56
Pre		96.11 ± 4.33		Nt		17.64 ± 5.83

Conc: concentrations are expressed in μM; %: results are expressed as mean ± SDM of three separate experiments; Sim: simultaneous assay; Pre: pretreatment assay; HCTF: hemocyanin C-terminal factor; ALF: antilipolysaccharide factor; At: already tested by other authors (see Murakami et al. 1991 and Yasin et al. 2000); Nt: not tested.

The percentages of protection as well as the EC_{50} values were calculated as described above. All assays were performed in triplicate.

RESULTS AND DISCUSSION

All evaluated peptides were cytotoxic, in different degrees, for at least one of the cell lines after the respective period of incubation, which were equivalent to the length of time the monolayers would be exposed to the peptides during the antiviral assays (Table II). The compounds were therefore assayed for antiviral activity at concentrations below or equal to the CC_{50} values.

Anti-HSV-1 effects - As shown in Table III, PW-2, ALF, Gomesin, and Penaeidin-3 exhibited significant activity against HSV-1 in the simultaneous treatment. Penaeidin-3 inhibited over 85% of the viral replication at 100 μM, with an EC_{50} value of 1.56 ± 0.18, resulting in a selectivity index ($SI = CC_{50}/EC_{50}$) of 64 (data not shown). Its unique mixed structure among the evaluated peptides (a linear proline-rich N-terminal fragment with a cyclic C-terminal fragment with three disulfide bonds) may have contributed to the detected activity, since another study has already described this feature (Yasin et al. 2000, Yang et al. 2003).

In the pretreatment strategy only ALF and Mytilin A inhibited viral replication notably (Table III). Although ALF exhibited similar percentage of inhibition in both strategies, Mytilin A presented a low percentage of inhibition in the simultaneous assay (34.28 ± 7.40) and a complete inhibition of viral replication in the pretreatment. Therefore, we speculate that this peptide, with a highly compact cysteine-rich structure, may exert its antiviral activity through a competition with the viral attachment/entry sites for binding to cell surface (van der Strate et al. 2001).

Anti-AdV-5 effects - Even though significant percentage of inhibition were obtained with ALF (98.17 ± 4.24) and Clavanin A (94.72 ± 5.59) in the simultaneous treatment, and with Gomesin (52.25 ± 4.27) and Clavanin A (61.54 ± 13.60) in the pretreatment, the active concen-

trations were too close to the CC_{50} values of these peptides, resulting in low selectivity indices (data not shown). Since most AMPs exert their antiviral activity by interfering with membranes, the activity against non-enveloped viruses is of particular interest, justifying further modifications of peptide structures to increase their selectivity (Yasin et al. 2000, Orsi 2004).

Anti-RV-SA11 effects - The majority of the evaluated peptides presented low percentage of inhibition (Table III), except by clavanin A, which inhibited 69.40% ± 13.13 and 95.46% ± 34.42 of viral replication in the simultaneous and pretreatment assays, respectively. Although a study has described the role of clavanin A glycine residues in its interaction with lipid bilayers (van Kan et al. 2001), the RV-SA11 does not possess a lipid envelope in its structure, thus we speculate that this peptide may exert its antiviral activity through an additional mechanism that interferes in the early steps of viral infection, since a higher percentage of viral replication inhibition was detected in the pre-treatment assay.

Despite the fact that most of the evaluated peptides that presented antiviral activity in this study were only active at concentrations too close to their CC_{50} values, the results obtained here are promising, and further modifications on the peptide structures may increase selectivity, allowing upcoming investigations about their mode of action.

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