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A wide range of medium-sized, highly cationic, α-helical peptides show antiviral activity against herpes simplex virus

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Abstract

Ten highly cationic, α-helical peptides were synthesized and tested for antiviral activity against herpes simplex virus 1 and 2 (HSV-1 and HSV-2). Several of the peptides were found to exhibit antiviral activity. The peptides affinity for heparan sulfate (HS) increased with the number of cationic residues. Net charge could be decisive for the anti-HSV-1 activity, while secondary structure of the peptides seems more important for the anti-HSV-2 activity. The peptides were able to inhibit the entry of HSV-1 into the host cell, probably by blocking HS at the cell surface. HSV plaque formation was inhibited in a dose-dependent manner when cells were exposed to the peptides prior to the addition of virus. Lower inhibition activity was observed when the virus was allowed to attach to the cell surface before the addition of peptide. However, the plaque size was smaller compared to the untreated control, indicating that the peptides may also interfere with cell-to-cell spread of the virus. The two most potent antiviral peptides exhibited synergy with acyclovir against HSV.

Keywords: Lactoferricin; Heparan sulfate; Herpes simplex virus; Antiviral α-helical peptide

1. Introduction

Two human α-herpesvirus, herpes simplex virus 1 and 2 (HSV-1 and HSV-2) commonly cause recurrent facial and genital herpetic lesions, respectively (Mitchell et al., 2003; Esmann, 2001). Present antiviral treatments target specific viral replication processes that take place after the virus has entered the cell. Most of the treatment for HSV is based on acyclovir (ACV) and ACV-like nucleoside analogues. Some immunocompromised patients with recurrent HSV lesions develop resistance to ACV after repeated treatments (Reyes et al., 2003). A number of new antiviral drugs have been developed during the last decades. However, there is considerable room for improvement since many of the existing compounds are not well tolerated or particularly efficacious (Kleymann, 2003).

To establish an infection of HSV, the virus has to enter the host cell. The entry process is initiated after attachment of the viral glycoproteins to cellular glycosaminoglycan (GAG) chains (WuDunn and Spear, 1989). Heparan sulfate (HS) (Lindahl et al., 1994) is the main GAG chain for HSV attachment (Tal-Singer et al., 1995), although chondroitin sulfate (CS) may be used in the absence of HS (Stringer and Gallagher, 1997; Banfield et al., 1995; Mardberg et al., 2002). Viral gC is the major mediator of HS attachment, but gB is also able to bind GAG molecules (Herold et al., 1991). The functional site on HSV-1 gC involved in HS binding has been identified as a cluster of basic amino acids in addition to two hydrophobic residues contributing to the specific HS affinity (Trybala et al., 1994, 2004). However, viral attachment to HS does not enable viral entry. This process requires viral gD interaction with one or more cellular co-receptor molecules, divided into three structural families (Spear, 2004). One of these families is the 3-*O*-sulfated heparan sulfate (3-*O*-HS), which is broadly distributed on human

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cells and mediates efficient entry of HSV-1, but not HSV-2 (Shukla et al., 1999). 3-*O*-HS, identified as a heptasulfated octasaccharide, is able to specifically interact with HSV-1 gD molecule (Liu et al., 2002), and seems to play a crucial role in virus entry and cell fusion (Tiwari et al., 2004).

Anti-HSV peptides are often amphipathic α-helices or β-sheets. Several β-sheet peptides (defensins, tachyplesin and protegrins) inactivate HSV-1 and/or HSV-2 (Yasin et al., 2000; Daher et al., 1986). However, α-helical peptides like cecropins, clavanins and LL-37 show little anti-HSV activity (Aboudy et al., 1994; Yasin et al., 2000), while indolicidin, melittin and magainins show high antiviral activity towards both HSV-1 and HSV-2 (Albiol Matanic and Castilla, 2004).

Earlier studies have indicated that the N-terminal α-helical region of the milk protein lactoferrin (LF) is important for its antiviral activity (Siciliano et al., 1999; Andersen et al., 2001). The cationic peptide, lactoferricin (Lfcin), generated by pepsin cleavage from this N-terminal region (Tomita et al., 1991), also possesses antiviral activity (Andersen et al., 2001, 2003; Jenssen et al., 2004). In the LF molecule, the Lfcin sequence makes an amphipathic α-helical structure (Baker et al., 1994; Haridas et al., 1994), while it makes a distorted β-sheet after pepsin cleavage (Hwang et al., 1998). To investigate the secondary structure requirements for antiviral activity, a group of small highly cationic α-helical peptides was synthesized. The peptides were tested for their anti-HSV activity. In addition, the combined drug effect of ACV and the peptides was investigated to evaluate synergistic properties.

2. Material and methods

2.1. Reagents

3,3′-Diaminobenzidine (DAB) tablets, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), IGEPAL CA-630 and *O*-nitrophenyl-β-D-galactopyranoside (ONPG) were all purchased from Sigma Chemical Co. (St. Louis, MO). PAL-PEG-PS resin and Fmoc-protected amino acids were purchased from Perseptive Biosystems GmbH (Hamburg, Germany). Heparan sulfate (HS) (bovine kidney) was from Seikagaku Corporation Inc. (Rockville, MD). CNBr-activated sepharose was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). HSV-1 (ATCC VR-539, MacIntyre), HSV-2 (ATCC VR-734, strain G), MRC-5 cells (human diploid lung fibroblasts, ATCC CLL 171) and Vero cells (African green monkey kidney, ATCC CCL-81) were purchased from Medprobe (Lund, Sweden). β-Galactosidase-expressing HSV-1 KOS-Rid-tk12 and HSV-1 KOS-tk12 (Dean et al., 1995) were kindly provided by Dr. Patricia Spear at Northwestern University in Chicago. Minimum essential medium (MEM) was purchased from Gibco BRL, Life Technology Ltd. (Paisley, Scotland). Primary antibody against HSV-1 or HSV-2, secondary antibody and 1,2-*O*-phenylenediamine dihydrochloride (OPD) were purchased from DAKO (Glostrup, Denmark).

2.2. Solid-phase peptide synthesis

Different cationic peptides were synthesized as described earlier (Jenssen et al., 2004) using Fmoc-amino acids and PAL-PEG-PS resin on a Milligen 9050 PepSynthesizer (Mildford, MA, USA). The peptides were purified to a purity of at least 97% on a preparative reverse-phase HPLC (Delta-Pak^{TM} C18, 100 Å, 15 μm, 25 mm × 100 mm, Waters Corporation, Milford, MA). The molecular mass was verified by electron-spray mass spectroscopy on a VG QUATTRO quadruple mass spectrometer VG Instruments Inc. (Altrincham, UK), before storage at −70 °C.

2.3. Affinity assay

Heparan sulfate affinity assays were performed on columns as described earlier (Jenssen et al., 2004). Peptide samples (4–6 parallels) were eluted with a gradient of NaCl (0–1.0 M) at a flow rate of 1.0 mL/min, and detected at 214 nm.

2.4. Cell cultures

MRC-5 and Vero cells were grown at 37 °C under an atmosphere of 5% CO_2 in minimum essential medium (MEM) buffered by HEPES buffer, containing non-essential amino acids, ultroser G from Gibco BRL, Life Technology Ltd. (Paisley, Scotland) and gentamicin (10 μg/mL). The cells were cultured as monolayers in 50 and 250 mL culture bottles from Nunc (Tamro MedLab, Skårer, Norway).

2.5. Infection assay

In situ ELISA were performed as described earlier on MRC-5 cells in 96-well microtiter plates (Jenssen et al., 2004). Different peptide concentrations (dose range 4–117 μM) were added before addition of the virus. HSV-1 or HSV-2 were inoculated to obtain an infectious dose (ID_{75}) equal to 75% infected cells. Inocula were removed after 2.5 h, and incubation terminated after an additional 18–20 h. The calculations of IC_{50} (inhibitory concentration giving 50% reduction of viral antigen) values were based on the median effect principle of Chou and Talalay (1984), from three to five independent experiments with two to eight replicates of each drug dilution.

The combined-drug effects were also analyzed with in situ ELISA, as described earlier by Andersen et al. (2003), using multiple drug effect equation (Chou and Talalay, 1984). Constant ratios of both drugs were used to set up the drug combinations and the combination index (CI) values were calculated using the mutually non-exclusive assumption. Concentrations of RJ8, RJ9 and ACV in the ranges of 2.0–39.5 μM, 2.2–43.3 μM and 4.9–970 nM, respectively, were used.

2.6. Plaque reduction assay

The plaque reduction assay was performed as described earlier (Andersen et al., 2004), using Vero cells and 100–300 plaque-forming units (pfu) of HSV-1 or HSV-2. Virus was pre-absorbed for 1 h at 4 °C or added to the cells simultaneously with the peptide, and incubated for 1 h at 37 °C. Plaques were fixed and counted after 48 h. The experiments were always carried out with four parallel wells.

2.7. Entry assay

Vero cells were grown in 96-well cell culture plates overnight at 37 °C to obtain a monolayer. Various concentrations of the peptides and HSV-1 (KOS-Rid-tk12) were added to the cells. After 6 h of incubation, the cells were treated with 0.1 M citrate buffer pH 3.0 to inactivate extra-cellular virus (Warner et al., 1998). The cells were solublized with PBS containing 0.5% IGEPAL CA-630 and the β-galactosidase substrate ONPG (3 mg/mL). The entry of HSV-1 was measured as a function of the enzymatic activity. The results were plotted as percentages of controls in which no peptide was present. A similar assay was also performed with pre-absorbed virus at 4 °C for 1 h as described by Whitbeck et al. (1997). The experiments were carried out in at least two independent assays with at least four replicates of each drug dilution.

2.8. Toxicity assay

Sub-confluent (75% confluence) layers of MRC-5 cells were exposed to the peptides for 24 h, prior to MTT reduction assay (Mosmann, 1983), as described earlier (Jenssen et al., 2004). The experiments were carried out in at least two independent assays with at least two to four replicates of each drug dilution.

2.9. Calculations

Net charge at pH 7.0 for all peptides was calculated by Protean program (DNASTAR Inc., version 1.17). Grand average of hydropathicity (Kyte and Doolittle, 1982) and aliphatic index (Ikai, 1980) of the peptides was calculated using services at ExPASy ProtParam tool provided by the Swiss Institute of Bioinformatics. General two-tailed Spearman correlation with a confidence interval of 95% was used to correlate antiviral activity and other observed or calculated values, using PRISM program (GraphPad Software Inc., version 3.0, San Diego, CA).

2.10. Liposome preparation

Single-bilayer phospholipid vesicles were prepared essentially according to the procedure of Batzri and Korn (1973). Eight micromole of dioleoyl-L-α-phosphatidyl-DL-glycerol (DOPG, Sigma) or dioleoyl-L-α-phosphatidylcholine (DOPC, Sigma) dissolved in chloroform were dried in ultra pure nitrogen, re-dissolved in 1 volume of absolute ethanol and dried again. Subsequently, the lipids were re-dissolved in 200 μL of absolute ethanol and slowly (about 100 μL/min), injected into 4 mL of 10 mM potassium phosphate (pH 7.4) at 23 °C. The ethanol was removed by dialysis in 10 mM potassium phosphate (pH 7.4).

2.11. Circular dichroism

Circular dichroism (CD) spectra were recorded by using a Jasco J-810 spectropolarimeter (Jasco International Co.) calibrated with ammonium D-camphor-10-sulfonate (Icatayama Chemicals). Measurements were performed with 1.4 mM liposomes, 12 mM dodecyl-phosphocholine (DPC, Sigma) micelles and 50% (v/v) 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma) at 23 °C by using a quartz cuvette (Starna) with a path length of 0.1 cm. HFP was used instead of trifluoroethanol, giving the same results, but requiring threefold lower peptide concentration (Gast et al., 2001). All the measurements were performed, at least twice, with a protein concentration of 0.10 mg/mL in 10 mM potassium phosphate (KPI) buffer (pH 7.4). Samples were scanned five times at 20 nm/min with a bandwidth of 1 nm and a response time of 1 s, over a wavelength range 190–260 nm. The data were averaged and the spectrum of a sample-free control sample was subtracted. The α-helical content of the various peptides was calculated after smoothing (means-movement, convolution width 5) from mean residual ellipticity at 222 nm ($[\theta]_{222}$) using the formula $f_H = [\theta]_{222}/[-40{,}000(1 - 2.5/n)]$, where f_H and n represent the α-helical content and the number of peptide bonds, respectively (Scholtz et al., 1991).

3. Results

A set of highly cationic hydrophilic peptides with either arginine or lysine was designed (Table 1). Five of the ten peptides exhibited antiviral activity towards both HSV-1 and HSV-2, while other peptides exhibited more strain-specific activity. The results showed that more than 43% cationic amino acids in the peptide were required to give antiviral activity (Tables 1 and 2). Arginine in RJ8 resulted in higher antiviral activity than lysine in RJ5. The peptides with 14 and 15 lysines (RJ9 and RJ10) and 12 arginines (RJ8) exhibited the best antiviral activity towards HSV-1, while HSV-2 was most susceptible towards RJ5 and RJ8. HS affinity of the peptides (Table 2) was evaluated in vitro using a column coated with HS. The affinity correlated with the increasing number of cationic amino acids. Arginine in the peptide resulted in a significantly higher HS affinity than lysine in RJ8 and RJ5, respectively (Table 2). RJ4 was the only peptide with some toxicity against human fibroblast cells (MRC-5) (Table 2).

To evaluate the peptides' ability to block viral entry of HSV-1, β-galactosidase activity was measured 6 h post-infection. The seven most antiviral peptides were analyzed

Table 1
Synthesized peptide sequences

Name	Amino acid sequence (single letter code)																				Charge[a]	Molecular weight[b]
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
RJ1	K	A	K	A	K	A	K	A	K	A	K	A	K	A	K	A	K	A	K	A	K	11	2138.7
RJ2	K	A	A	K	K	A	A	K	A	A	K	K	A	A	K	A	A	K	K	A	A	9	2024.5
RJ3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	W	–	–	–	–	–	9	2139.6
RJ4	–	–	–	–	–	–	W	–	–	–	–	–	–	–	–	W	–	–	–	–	–	9	2254.8
RJ5	A	K	K	A	A	K	K	A	K	K	A	A	K	K	A	K	K	A	A	K	K	12	2195.8
RJ6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	W	–	–	–	12	2310.9
RJ7	–	–	–	–	W	–	–	–	–	–	–	–	–	–	–	–	–	W	–	–	–	12	2426.0
RJ8	–	R	R	–	–	R	R	–	R	R	–	–	R	R	–	R	R	–	–	R	R	12	2531.9
RJ9	–	–	–	–	K	–	–	–	–	–	–	–	–	–	–	–	–	K	–	–	–	14	2310.0
RJ10	–	–	–	–	K	–	–	–	–	–	–	K	–	–	–	–	–	–	K	–	–	15	2367.1

Note: The peptides are divided into three structural groups. The small horizontal lines indicate that the amino acid in this position is identical as in the first peptide in the respective group.

[a] Number of charged amino acids.

[b] Calculated molecular weight, also verified by ES-MS.

Table 2
Peptide affinity, toxicity and antiviral activity

Peptide	IC_{50} [μM][a] HSV-1	IC_{50} [μM][a] HSV-2	Toxicity CC_{50} [μM][b]	HS [mM] NaCl[c]	GRAVY[d]	Aliphatic index[e]	Net charge[f]
RJ1	ND	53.2	>455	200	−1.186	47.62	9.90
RJ2	ND	ND	>494	143	−0.643	57.14	8.90
RJ3	117.0	ND	>467	147	−0.771	52.38	8.90
RJ4	ND	ND	268	190	−0.900	47.62	8.90
RJ5	41.0	14.3	>468	233	−1.457	42.86	11.90
RJ6	47.3	ND	>433	250	−1.586	38.10	10.90
RJ7	40.8	44.9	>412	257	−1.714	33.33	10.90
RJ8	18.2	22.2	>395	357	−1.800	42.86	11.91
RJ9	13.0	46.0	>433	277	−2.000	33.33	13.90
RJ10	24.6	54.7	>422	345	−2.271	28.57	14.89

ND: no detectable activities within test range (4–117 μM).

[a] Concentration required for 50% reduction in virus amplification detected with in situ ELISA.

[b] CC_{50}: 50% cytotoxic concentration against human fibroblasts.

[c] Concentration of NaCl required to elute the peptide from the HS column.

[d] Grand average of hydropathicity combine the hydrophobicity and hydrophilicity of the amino acids to predict if the peptide is hydrophilic (negative values) or hydrophobic (positive values).

[e] Aliphatic index.

[f] Net charge at pH 7.0.

and all but RJ1 were found to block viral entry. RJ8 and RJ10 were the most potent peptides blocking the entry of HSV-1 (Fig. 1).

To further evaluate the antiviral mode of action, a plaque reduction assay was performed with the two most potent antiviral peptides, RJ8 and RJ9. Both peptides inhibited plaque formation of HSV-1 (Fig. 2). Decreasing number of plaques was also observed when the virus was allowed to attach to the cell surface at 4 °C for 1 h, prior to peptide exposure (Fig. 2). The plaque size was also significantly reduced in this study (data not shown).

The two most potent antiviral peptides, RJ8 and RJ9, were also tested in combination with acyclovir (ACV) against HSV-1 and HSV-2. Previous results with Lfcin:ACV showed synergy in a ratio from 100,000:3 to 1:3 (unpublished data). To reduce the amount of ACV, it was decided to evaluate whether RJ8 and RJ9 exhibited synergy with ACV in the concentration ratio 100,000:3, equal to 2.0–39.5 μM

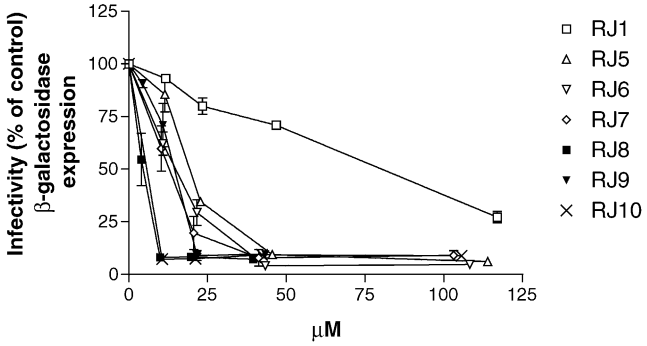

Fig. 1. The peptides' effect on infection of Vero cells with an HSV-1 mutant (KOS-Rid1-tk12). Vero cells were incubated with an HSV-1 mutant, which expresses β-galactosidase in the host cell. The peptides were tested at a concentration of 4–117 μM, and all but RJ1 showed relatively high ability to block HSV-1 entry.

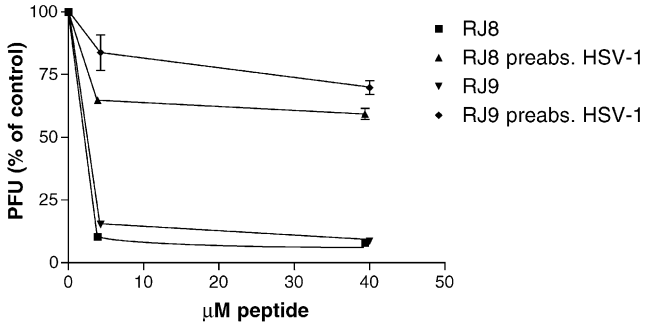

Fig. 2. The peptides' antiviral effect before and after viral attachment to the host cell. HSV-1 was pre-incubated with the cells before the addition of RJ8 (▲) or RJ9 (♦). HSV-1 and RJ8 (■) or RJ9 (▼) were added simultaneously to the Vero cells.

RJ8, 2.2–43.3 μM RJ9 and 48.8–968 pM of ACV. A dose-dependent inhibition was observed for the combination of both peptides (RJ8 and RJ9) and ACV, with a CI values <1, with a range of 0.219–0.899 and 0.058–0.213, respectively (Table 3). These results show synergism against both HSV-1 and HSV-2, when ACV is combined with either peptide.

The secondary structures of the different peptides were analyzed by CD spectrometry under various conditions, in an attempt to verify structural requirements for anti-HSV activity. The CD spectra of all the peptides in pure water and in 10 mM potassium phosphate (pH 7.4) were characteristic of a non-structured conformation, with α-helical contents of not more than 0–4% (Table 4). HFP induces and stabilize α-helical structure in peptides that have an intrinsic tendency to adopt this type of secondary structure (Lehrman et al., 1990; Sonnichsen et al., 1992; Jasanoff and Fersht, 1994). In the presence of HFP, the calculated α-helical contents for the various peptides were in the range of 34–64% (Table 4). DPC micelles and negatively charged liposomes (DOPG), but not uncharged liposomes (DOPC), also induced helical structuring in the different peptides (Table 4).

Values for grand average of hydropathicity (GRAVY), aliphatic index and net charge at pH 7.0 (Table 2) showed all good correlation with HS-affinity (Table 2). A good correlation was also observed between HS affinity, GRAVY, aliphatic index, net charge and the antiviral activity for HSV-1. However, neither HS affinity, nor GRAVY or aliphatic index gave a good correlation with anti-HSV-2 activity, the only correlating parameter with anti-HSV-2 activity being net charge.

Table 3
Combination index (CI_{75} and CI_{90}) for RJ8:ACV and RJ9:ACV

	HSV-1		HSV-2	
	CI_{75}	CI_{90}	CI_{75}	CI_{90}
RJ8:ACV (100,000:3)	0.820	0.899	0.219	0.233
RJ9:ACV(100,000:3)	0.123	0.213	0.064	0.058

Note: Combination index (CI) of <1, 1, >1 indicate synergism, additive effects and antagonism, respectively. CI_{90} and CI_{75} are the CI at 90% and 75% inhibition of HSV replication, detected with in situ ELISA. Drug concentration: 2.0–39.5 μM RJ8, 2.2–43.3 μM RJ9 and 48.8–968 pM ACV (six drug combinations diluted 1.33-, 2-, 4-, 10- and 20-fold from the highest concentration). ACV IC_{50} values as obtained earlier (Andersen et al., 2003).

4. Discussion

Based on the knowledge of the anti-HSV activity and the structure of LF and Lfcin (Baker et al., 1994; Hwang et al., 1998; Andersen et al., 2003), 10 short, highly cationic hydrophilic peptides were synthesized (Table 1). The spatial positioning of charged amino acids in a stabilized secondary structure were evaluated as important factors (Jenssen et al., 2004), and for that reason, the peptides were made as α-helical as possible by introducing alanines (Chou and Fasman, 1978; O'Neil and DeGrado, 1990; Bessalle et al., 1993).

The peptides affinity for HS increased as expected with the number of charged amino acids. Specific distribution of charged residues in the cationic sector and the size of the sector were of minor importance for the affinity (Table 2). Arginine gave higher affinity than lysine in RJ8 versus RJ5, which is in accordance with earlier substitution assays in several different types of peptides (Fromm et al., 1995; Hileman et al., 1998; Stenlund et al., 2002). The introduction of hydrophobic residues in RJ6 and RJ7, resulted in an increase in HS affinity. This is in accordance with earlier findings by Trybala et al. (2004), illustrating that two hydrophobic residues are crucial for the HSV-1 gC interaction with HS.

RJ4 is the only peptide proven toxic within the concentrations tested. RJ3 and RJ4 are almost identical except that RJ4 has a tryptophan in position 7, while RJ3 has alanine. The toxic effect is most likely related to the positioning of this tryptophan or the ratio between tryptophans and charged amino acids, since RJ7 also contains two tryptophans with no toxicity. The tryptophans side chains may also be buried in the water–phospholipid interface (Yau et al., 1998), or be

Table 4
Estimated α-helical content (%) of the peptides in different membrane simulation solutions

Peptide	10 mM Kpi[a]	50% HFP[b]	12 mM DPC[c]	1.4 mM DOPC[d]	1.4 mM DOPG[e]
RJ1	0	41	8	0	41
RJ2	1	36	26	1	31
RJ3	1	34	34	2	11
RJ4	0	39	4	0	18
RJ5	0	64	8	0	10
RJ6	0	36	22	0	12
RJ7	0	40	37	0	10
RJ8	4	51	45	5	19
RJ9	0	38	4	0	9
RJ10	0	41	0	0	8

[a] Potassium phosphate (pH 7.4).
[b] 1,1,1,3,3,3-Hexafluoro-2-propanol.
[c] Dodecyl-phosphocholine.
[d] Dioleoyl-L-α-phoshatidylcholine.
[e] Dioleoyl-L-α-phoshatidyl-DL-glycerol. All assays were performed at 23 °C.

used as a needle to get the peptide into the cell (Wimley and White, 2000), thereby causing toxicity.

The degree of secondary structure in our peptides, measured by CD spectroscopy in the HFP buffer might be a bit overestimated because of the nature of the buffer, however, no CD buffer will provide more correct estimated values. Tryptophan may also cause overestimation because of its absorbance. Highest percentage of helicity is estimated in RJ5 and RJ8. An ideal relation between helix-stabilizing alanines and the positioning of the different amino acids might explain this. The lysine containing RJ5 gave significantly higher helicity than the arginine containing RJ8. This is in accordance with CD-results on other peptides done by Javadpour et al. (1996).

Several cationic peptides have shown antiviral activity against HSV (Egal et al., 1999; Belaid et al., 2002; Sinha et al., 2003). In this study, the peptides required more than 50% charged amino acids to exert antiviral activity, and the activity was favored by an amphipathic distribution of the amino acids (Table 2). By introducing one tryptophan in RJ6, compared to RJ5, the anti-HSV-1 activity decreased and the anti-HSV-2 activity was lost. Introduction of two tryptophans in RJ7 appeared to increase the anti-HSV-1 activity compared to RJ6 and RJ5, but the anti-HSV-2 activity was still three-fold lower than for RJ5. This might be a result of increased specific binding of HS as a result of two hydrophobic residues (Trybala et al., 2004). Since both alanine and tryptophan stabilizes α-helical structure (Chou and Fasman, 1978), the substitution should not theoretically influence the secondary structure.

When two additional lysines are incorporated, in RJ9 compared to RJ5, the antiviral specificity switches from HSV-2 to HSV-1. It appears as if the anti-HSV-1 activity requires a larger cationic angle than anti-HSV-2 activity. If the cationic angle is too large, the antiviral activity against both viruses decreases. This can also explain the decreasing antiviral activity when one additional lysine is incorporated into RJ10. The high amount of charged residues in RJ9 and RJ10 results in fewer alanines, which apparently can alter the secondary structure (Table 4). The lack of a conserved secondary structure may explain the decrease in anti-HSV-2 activity for RJ9 and RJ10. At the same time, it appears that the anti-HSV-1 activity can be more influenced by charge and not by the secondary structure. Arginine gives higher HS affinity than lysine (R8 and R5), as a result of the chemistry of the side chains, but their structure are quite similar, indicating that HS affinity is more mandatory for high HSV-1 activity than the secondary structure. The high HS affinity and anti-HSV-1 activity of RJ9–10, compared with their less conserved secondary structure, supports this hypothesis.

All the peptides, except for RJ1, showed a high ability to block viral entry in a entry assay, as we have reported earlier for lactoferricin (Andersen et al., 2004). Adding peptides and virus simultaneously to the cell culture, the peptides inhibited plaque formation. When allowing virus to bind to the cells at 4 °C for 1 h prior to peptide exposure, fewer and smaller plaques were observed. Mikloska and Cunningham have shown a similar reduction in plaque size by interferon α and γ (Mikloska and Cunningham, 2001). The plaque size reduction illustrates the peptides' ability to interfere with viral spread, even after viral attachment to cellular receptor molecules.

The combined drug effect was investigated for the two most antiviral peptides, RJ8 and RJ9, in combination with ACV. A synergistic effect between both peptides and ACV was observed against HSV-1 and HSV-2, in accordance with results reported earlier for the cationic lactoferricin peptide (Andersen et al., 2003).

Anti-HSV-1 activity is well correlated with HS affinity, GRAVY and aliphatic index. We have also indications that the secondary structure is less important than HS affinity for anti-HSV-1 activity. The anti-HSV-2 activity is only correlated with net charge, thus pointing to the importance of negatively charged amino acid residues in the peptides. The positioning of the charged residues is of greater influence on the antiviral activity, rather than the total charge. This indicates that the peptide structure is more important for antiviral activity towards HSV-2 than HSV-1, which is in accordance with our earlier findings (Jenssen et al., 2004).

5. Conclusion

We have designed highly cationic α-helical peptides with anti-HSV activity compared to other antiviral peptides. The peptides exhibit antiviral activity also when added after the virus was allowed to bind to the cellular receptor molecules. The two most potent antiviral peptides showed synergy when combined with acyclovir against HSV.

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