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# Lactoferrin and lactoferricin inhibit Herpes simplex 1 and 2 infection and exhibit synergy when combined with acyclovir

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**Abstract**

Lactoferrin (LF) is a multifunctional glycoprotein, which plays an important role in immune regulation and defense mechanisms against bacteria, fungi, and viruses. Upon peptic digestion of LF, a peptide called lactoferricin (Lfcin) is generated. Lfcin corresponds to the N-terminal part of the protein. In this study we investigated the antiviral activity of bovine and human Lfcin against Herpes simplex virus (HSV)-1 and HSV-2. The 50% effective concentrations ($EC_{50}$) for LF and Lfcin against several clinical isolates of HSV-1 and HSV-2, including acyclovir (ACV)-resistant strains, were determined. We further evaluated the effect of the combination of either LF or Lfcin with ACV against HSV-1 and HSV-2. Synergy was observed between both LF or Lfcin in combination with ACV against the HSV laboratory strains.

The 50% effective concentration ($EC_{50}$) for ACV and LF or Lfcin, when combined with ACV, could be reduced by two- to sevenfold compared to the $EC_{50}$ when the drugs were used alone.


*Keywords:* Lactoferrin; Lactoferricin; Antiviral; Synergy; HSV

## 1. Introduction

Herpes simplex virus (HSV) is capable of causing a wide-spread spectrum of mild to severe disorders. These include acute primary and recurrent mucocutaneous disease in the otherwise healthy adult. For the immunocompromised and neonates this will often result in painful, disabilitating lesions and in worst case death (Whitley, 1995). Many therapeutic agents have been developed and used for HSV infections, most of these are nucleoside analogs with selective antiviral activity, acyclovir (ACV) being the most common. Resistance to nucleoside analogs has been reported (Coen, 1994). Therefore, the development of novel compounds with alternative mechanisms of antiviral action is important.

Some cationic peptides have been shown to exert antiviral activity against HSV and some other viruses. α-Helical peptides (magainins, cecropins, clavanins, and LL-37) have been shown to cause little HSV-1 or HSV-2 inactivation (Aboudy et al., 1994; Ourth et al., 1994; Yasin et al., 2000), while several β-sheet peptides (defensins, tachyplesin, and protegrins) inactivated one or both viruses (Yasin et al., 2000). Defensins directly inactivate certain enveloped viruses, such as HSV and vesicular stomatitis virus (Lehrer et al., 1985; Daher et al., 1986). Tachyplesin and protegrins, small β-sheet peptides found in horseshoe crabs and pigs, respectively, have been shown to inactivate HIV-1 (Tamamura et al., 1993).

Lactoferrin (LF) is a glycoprotein present in external secretions, especially milk. LF is also a major component of secondary granules of polymorphonuclear leukocytes (PMN) (Shau et al., 1992). LF possesses a variety of biological functions, such as immunomodulation and antimicrobial activity, towards bacteria, fungi, protozoa, and viruses (Vorland, 1999; Weinberg, 2001). LF has antiviral activity against several viruses, including rotavirus, respiratory syncytial virus, hepatitis C virus (HCV), herpes virus, and HIV (van Der Strate et al., 2001). LF has been found to prevent virus adsorption and/or penetration into host cell, indicating that LF has an effect on the early events of virus infection (Hasegawa et al., 1994; Harmsen et al., 1995). Lactoferricin (Lfcin) is a peptide generated by pepsin cleavage from the N-terminal part of LF (Tomita et al., 1991). In LF, the peptide structure forms a loop with a cationic charge. Bovine lactoferricin (Lfcin B) consists of 25 amino acid residues [17–41 in bovine LF (B-LF)] and human lactoferricin (Lfcin H) consists of 47 amino acids residues [1–47 in human LF (H-LF)], including a region homologous

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to Lfcin B (Bellamy et al., 1992). Lfcin B makes a cationic distorted β-sheet with a C–C bridge (Hwang et al., 1998). Lfcin B is active against a wide range of bacteria, fungi, protozoa, and tumors (Bellamy et al., 1992, 1993; Yoo et al., 1997). Lfcin B and H have also been shown to exert antiviral activity against human cytomegalovirus by inhibiting entry of the virus (Andersen et al., 2001).

A need for developing novel antiviral agents and the use of combination of different antiviral drugs is becoming increasingly important for several reasons. The use of drug combinations reduces the possibility of selecting drug-resistant mutants, and the cytotoxicity may be lowered due to the need for lower concentrations of the different drugs. ACV is a potent inhibitor of HSV replication and is an apparent inactivator of the HSV DNA polymerase. The antiviral mechanism of action of LF and Lfcin is distinctly different from that of ACV, suggesting that combining these compounds could improve the efficacy against HSV infections.

In the present study, the antiviral activity of LF and Lfcin against clinical isolates of HSV, including ACV-resistant strains, was demonstrated. Combination of ACV with B-LF or Lfcin B against HSV-1 and HSV-2 was also investigated.

## 2. Materials and methods

### 2.1. Virus and cell cultures

The following strains of virus were obtained from the American Type Culture Collection (ATCC): HSV-1 MacIntyre, #VR-539 and HSV-2 strain G, #VR-734.

Clinical isolates of HSV-1 and HSV-2 were isolated in the Medical Microbiology Department at the University Hospital of North Norway. The ACV-resistant strains were a gift from Dr. K.D. Thompson, Clinical Virology Laboratory at the University of Chicago. MRC-5 and Vero cells were purchased from BioWhittaker, Walkersville, MD. Cell culture media consisted of minimum essential medium (MEM) buffered by HEPES buffer, containing non-essential amino acids, gentamicin (10 μg/ml) and Ultroser G or fetal bovine serum (FBS) (Gibco BRL, Life Technology Ltd., Paisley, Scotland).

### 2.2. Reagents

The following items were purchased from Sigma Co., St. Louis, MO: H-LF, human iron-saturated LF, and B-LF. Goat LF (G-LF) was purified from goat milk (gift from Tine Dairy, Tromsø, Norway) as earlier described (Gutteberg et al., 1989).

Lfcin B was purchased from the Center for Food Technology, Hamilton, Qld, Australia (Hoek et al., 1997). Collection of human milk was done at the University Hospital of North Norway, while the purification of H-LF and Lfcin H was performed at the Center for Food Technology, Hamilton, Qld, Australia. ACV was purchased from Alpharma Norge AS, Norway. Gamma-globulin (normal human immunoglobulin) was purchased from Pharmacia & Upjohn AS, Oslo, Norway.

The following items were purchased from DAKO, Glostrup, Denmark: rabbit immunoglobulins to HSV-1 or HSV-2, peroxidase conjugated goat anti-rabbit immunoglobulin, and *O*-phenylenediamine hydrochloride (OPD).

### 2.3. Plaque-reduction assay (PRA)

The 24-well plates were seeded with $1.0 \times 10^5$ Vero cells per well and incubated overnight. Two hundred microliters of diluted virus suspension containing 100–300 plaque-forming units (pfu) was inoculated in quadruplicates in each well of two plates, leaving four wells with only growth medium, as controls. The test substances LF or Lfcin were added to the cells simultaneously with the HSV dilution and incubated for 1 h at 37 °C in 5% $CO_2$. The virus-containing medium was removed after 1 h and replaced with MEM containing 3% gamma-globulin (ACV was included as a control in the assay and the growth medium added 1 h after the infection contained ACV).

The plates were incubated for 48 h at 37 °C in 5% $CO_2$, fixed and stained with 0.5% crystal violet in 50% methanol. Plaques were counted and were compared with plaque counts of controls not treated with drug.

### 2.4. Microplate susceptibility assay

MRC-5 ($1.5 \times 10^4$ cells per well) were grown in 96-well culture plates overnight at 37 °C to obtain a monolayer. The HSV-1 MacIntyre strain and HSV-2 G strain were titrated by plaque assay and pfu of several virus dilutions were plotted against optical density (OD) measured with the in situ ELISA. A total of 400–600 pfu of HSV-1 [multiplicity of infection (MOI): 0.03–0.04] and 150–300 pfu of HSV-2 (MOI: 0.01–0.02) were used in this assay. If the OD corresponded to an MOI outside this range for the clinical isolates, the data were not used to calculate drug susceptibility.

One hundred microliters of diluted virus suspension was inoculated in quadruplicates in each well of two plates, leaving eight uninfected cell control wells. There were four virus-infected control wells without drug, and four replicates of four dilutions of the drug.

Dilutions of the virus were prepared in MEM with Ultroser G. The test substances (LF, Lfcin, or ACV either alone or in combination) were added to cell cultures simultaneously with the HSV dilution. LF and Lfcin were tested within a concentration range of 25–500 μg/ml. This corresponds to 0.3–6.3 μM LF, 7.8–156.5 μM Lfcin B, and 4.5–90.0 μM Lfcin H. The ACV-resistant isolates were tested against ACV concentrations of 0.1–100 μM.

After a virus adsorption period of 2 h, the inocula were removed. Maintenance medium was then added to the cells and the plates were incubated at 37 °C. Since LF is known

to exert its antiviral activity in the early phase of infection, it was only present during the adsorption period. For the assay testing with ACV, the drug was also included in the maintenance medium added after the inocula had been removed. After 18 h the expression of viral antigen was measured by in situ ELISA.

### 2.5. In situ ELISA

The medium was removed and the plates were washed in phosphate-buffered saline (PBS), and then fixed with 100% methanol at room temperature for 10 min. To block unspecific binding, 100 μl of PBS with 4% bovine serum albumin was added to each well and incubated at 37 °C for 30 min. Then, 100 μl of rabbit immunoglobulin to HSV-1 or HSV-2 was added to each well following producers recommended working dilution (1:1000). The plates were incubated at 37 °C for 30 min and washed three times in PBS. Then, 100 μl peroxidase conjugated goat anti-rabbit immunoglobulin (working dilutions 1:1000) was added to each well and incubated at 37 °C for 30 min. Further, the plates were washed three times in PBS. Freshly prepared OPD was added at 100 μl to each well and incubated in the dark at room temperature. The reaction was stopped after 10 min by the addition of 100 μl of 0.5 M $H_2SO_4$. OD was read at 492 nm using a Mikrowell Reader 510 (Organon Teknika, Durham, SC).

HSV-1 MacIntyre strain and HSV-2 G strain were tested with B-LF, Lfcin B, and Lfcin H in both the PRA and microplate susceptibility assay. The clinical isolates, including the ACV-resistant strains of HSV-1 and HSV-2, were tested with B-LF, Lfcin B, and Lfcin H with the microplate susceptibility assay to determine the 50% effective concentration ($EC_{50}$) values. The combination studies were also performed with microplate susceptibility assay. Ultroser G was used instead of serum (FBS) in the antiviral testing of LF and Lfcin. To investigate whether the addition of serum could have an inhibitory effect on LF and Lfcin, PRA was performed with medium containing 10% FBS instead of Ultroser G for B-LF and Lfcin B against HSV-1 and HSV-2.

### 2.6. Cytotoxicity assay

To determine the cytotoxic effect on MRC-5 cells and/or Vero cells, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) reduction assay was employed (Sigma) (Mosmann, 1983). The assays were performed in 96-well microtiter plates, the cells were seeded (to obtain confluence) at 24 h prior to the addition of peptides in serum-free medium. The cells and the peptides were incubated for 24 or 48 h at 37 °C in humidified atmosphere of 5% $CO_2$. Cells in serum-free medium were used as a negative control, whereas cells treated with 1% Triton X-100 served as a positive control. After the incubation period, 10 μl MTT solution (5 mg MTT per ml PBS per 100 μl medium) was added to each well and incubated for 2 h. A volume of 130 μl was removed from the cells. In order to dissolve the formazan crystals, 100 μl of 0.04 M HCl in isopropanol was added and the plates were shaken for 1 h. A microtiter plate reader (Thermomax Molecular Devices, Sunnyvale, CA) finally measured the absorbance at 590 nm. The 50% cytotoxic concentration ($CC_{50}$) or concentration of compound required to reduce the viability of the cells was determined from the dose–response curves.

## 3. Calculations

The calculations of $EC_{50}$ values were based on the median-effect principle of Chou and Talalay (1984). This method calculates the $D_m$ value, which is analogous to the $EC_{50}$.

The combined-drug effects were analyzed using multiple-drug effect equation, based on the median-effect principle. This method involves the plotting of dose–effect curves for each compound and for multiply diluted fixed-ratio combinations of the compounds, i.e. plotting the dose (in μM) versus HSV antigen fraction affected ($f_a$). Fraction affected is the percent reduction of HSV antigen expression with a given drug concentration expressed in decimals; for example a dose of 0.6 μM B-LF results in an $f_a$ of 0.5 (50% HSV-1 antigen expression reduction). The slope of the linearised median-effect equation plot (log fraction affected/log fraction unaffected against the log of the dose of the two separate compounds and the dose of the combination), which signifies the shape of the dose–effect curve, and the *X*-intercept of the plot which signifies the potency of each compound, and each combination were then used to calculate the combination index (CI) (Chou and Talalay, 1984).

The CI value compares the amount of drug A which gives 90% effect when used in combination with another drug B, with the amount of drug A, which gives a 90% effect when the drug is used alone. A combination is either synergistic, additive, or antagonistic if CI $<$ 1, =1, >1, respectively. Constant ratios of both drugs were used to set up the drug combinations and the CI values were calculated using the mutually non-exclusive assumption.

With the combination index method used in this study one examines effects along diagonal lines across the dose–response surface. This will reveal synergy, antagonism, or additive effects for a fixed drug ratio. One should, therefore, describe dose–response surfaces with several drug ratios.

## 4. Results

Table 1 summarizes the results of the antiviral effect of LFs from different species, Lfcin B and Lfcin H against HSV-1 and HSV-2. For B-LF, Lfcin B, and Lfcin H, the $EC_{50}$ values were also determined with PRA. The results showed that the $EC_{50}$ measured with the microplate

Table 1
Antiviral activity of lactoferrins and lactoferricin

| | $EC_{50}$ (μM)[a] | | $CC_{50}$ (μM) |
|---|---|---|---|
| | HSV-1 | HSV-2 | |
| B-LF | 0.6 (0.2) | 0.2 (0.7) | >13 |
| H-LF | 1.2 | 0.7 | >13 |
| G-LF | 2.0 | 35.4 | – |
| Lfcin B | 14.6 (5.2) | 12.3 (12.2) | 101 |
| Lfcin H | 42.6 (19.2) | 31.0 (38.3) | 155 |
| ACV | 2.8 (0.9) | 0.4 (1.1) | – |

The 50% effective inhibitory concentration ($EC_{50}$) values (in μM) for bovine LF, H-LF, G-LF, Lfcin B, Lfcin H, and ACV for HSV-1 and HSV-2 performed with the microplate susceptibility assay (HSV-1: 400–600 pfu, HSV-2: 150–300 pfu). Numbers in parentheses are $EC_{50}$ values from plaque-reduction assay (100–300 pfu for both HSV-1 and HSV-2). The 50% cytotoxic concentration ($CC_{50}$) values represent concentrations of the compounds required to reduce the viability of the cells after 48 h of exposure.

[a] The values were derived from two independent assay of either microplate susceptibility assay or plaque-reduction assay.

susceptibility assay corresponded well with the $EC_{50}$ from the PRA for HSV-2. While the $EC_{50}$ values for HSV-1 measured with the microplate susceptibility assay were higher than the values measured with PRA, this is due to the fact that the number of pfu used in the microplate susceptibility assay (400–600 pfu) is higher than in the PRA (100–300 pfu).

The B-LF was the most effective against both viruses. The human was slightly, and the goat version markedly, less inhibitory than the bovine. All LFs were more effective than Lfcin B and Lfcin H, with the exception of G-LF against HSV-2. Both peptides had modest antiviral activity, Lfcin B was significantly more effective than the human peptide. The peptides were more cytotoxic than the LFs, but the cytotoxic concentrations of Lfcin B and Lfcin H were well below those that inhibited HSV-1 and HSV-2 (Table 1).

The addition of FBS to the antiviral assay (PRA) showed that B-LF and Lfcin B was slightly inhibited by the presence of serum, with the $EC_{50}$ for B-LF being 0.5 and 0.9 μM for HSV-1 and HSV-2, respectively. The $EC_{50}$ value for Lfcin B was 11.8 and 17.4 μM against HSV-1 and HSV-2, respectively. The $EC_{50}$ increased by 2.5-fold for B-LF against HSV-1, while for the other combinations tested only minor increases were found (1.3–1.5-fold).

Experiments with clinical isolates of HSV confirmed the antiviral activity of B-LF and Lfcin B against HSV-1 and HSV-2, while Lfcin H exerted the same modest activity as against the laboratory strains. Table 2 shows the range of the $EC_{50}$ values (in μM) for B-LF, Lfcin B, and Lfcin H. The ACV-resistant strains were also susceptible to B-LF, Lfcin B, and Lfcin H (Table 3).

A combination between B-LF and ACV (ratio 1:4.25) against HSV-1 showed synergy at a given inhibition (75 and 90%) (Table 4). This combination had an $EC_{50}$ of 1.79 μM, whereas 0.34 μM of B-LF and 1.45 μM of ACV were needed to achieve 50% inhibition, compared with 0.60 μM of B-LF and 2.8 μM of ACV when the two drugs were used alone. This results in an almost twofold drop in the $EC_{50}$ for both B-LF and ACV. Another drug ratio (1:42.5) tested against HSV-1 showed synergy at both 75 and 90% inhibition. Synergy was also observed for Lfcin B and ACV (ratio 5.7: 1) against HSV-1 at 75 and 90% inhibition. The $EC_{50}$ for this combination was 3.84 μM, whereas 3.27 μM of Lfcin B and

Table 2
Antiviral activity against HSV clinical isolates

| | HSV-1 | | | | HSV-2 | | | |
|---|---|---|---|---|---|---|---|---|
| | *n* | Minimum (μM) | Maximum (μM) | Mean (μM) | *n* | Minimum (μM) | Maximum (μM) | Mean (μM) |
| B-LF | 15 | 0.3 | 1.7 | 0.8 | 20 | 0.1 | 2.3 | 0.7 |
| Lfcin B | 16 | 4.2 | 34.9 | 17.9 | 11 | 1.9 | 24.0 | 11.0 |
| Lfcin H | 15 | 11.0 | 63.0 | 22.1 | 3 | 26 | 69 | 53 |

The 50% effective inhibitory concentration ($EC_{50}$) values of B-LF, Lfcin B, and Lfcin H against clinical isolates of HSV-1 and HSV-2 measured with microplate susceptibility assay. The $EC_{50}$ is calculated from one microplate assay with at least four replicates of each drug dilution. The table shows the minimum, maximum, and mean $EC_{50}$ values for the tested clinical isolates ($n$ = number of clinical isolates tested).

Table 3
Antiviral activity against acyclovir-resistant HSV isolates

| | HSV-1 | | | | HSV-2 | | | |
|---|---|---|---|---|---|---|---|---|
| | *n* | Minimum (μM) | Maximum (μM) | Mean (μM) | *n* | Minimum (μM) | Maximum (μM) | Mean (μM) |
| B-LF | 2 | 0.37 | 0.65 | 0.51 | 6 | 0.10 | 0.75 | 0.40 |
| Lfcin B | 2 | 10.50 | 13.00 | 11.75 | 8 | 10.5 | 25.00 | 15.00 |
| Lfcin H | 2 | 23.00 | 48.00 | 35.50 | 5 | 30.00 | 61.00 | 43.4 |

The 50% effective inhibitory concentration ($EC_{50}$) values of B-LF, Lfcin B, and Lfcin H against HSV-1 and HSV-2 acyclovir-resistant strains measured with microplate susceptibility assay. The values were derived from two independent microplate susceptibility assays for each drug. The table shows the minimum, maximum, and mean $EC_{50}$ values for the tested clinical isolates ($n$ = number of clinical isolates tested).

Table 4
Antiviral activity of acyclovir in combination with lactoferrin or lactoferricin against HSV-1

| Combination | $EC_{50}$ (μM)[a] | CI at the following percent inhibition | |
|---|---|---|---|
| | | 75 | 90 |
| B-LF + ACV (1:4.25) | 1.79 | 0.28 | 0.18 |
| B-LF + ACV (1:42.5) | 1.20 | 0.51 | 0.53 |
| Lfcin B + ACV (5.7:1) | 3.84 | 0.27 | 0.17 |

The table shows the CIs for 75 and 90% inhibition of HSV-1 and the $EC_{50}$ for the combinations. The CI value compares the amount of drug A which gives 75 or 90% effect when used in combination with another drug B, with the amount of drug A which gives 75 or 90% effect when the drug is used alone. A combination is either synergistic, additive, or antagonistic if CI < 1, =1, >1, respectively. The values were determined from two independent microplate susceptibility assays. Fifty percent cytotoxic concentration ($CC_{50}$) of B-LF + ACV (ratio 1:4.25) was 22.4 μM.

[a] The $EC_{50}$ for the combination is the concentration of ACV added to the concentration of LF or Lfcin at which 50% inhibition was achieved.

0.57 μM of ACV were needed to achieve 50% inhibition. This results in almost a fivefold drop in the $EC_{50}$ for both compounds.

B-LF and ACV in combination also showed synergy against HSV-2 (Table 5). The combination B-LF and ACV (ratio 1:1.87) against HSV-2 showed an $EC_{50}$ of 0.29 μM whereas 0.10 μM of B-LF and 0.19 μM of ACV were needed to achieve 50% inhibition, and hence resulted in a twofold drop in the $EC_{50}$ for both compounds. Another drug ratio (5:1) tested against HSV-2 showed additive effect at 75% inhibition and synergy at 90% inhibition.

For Lfcin B and ACV against HSV-2 several different combination ratios were tested, as shown in Table 5. All of these combinations showed synergy at a given inhibition (75 and 90%). The combination of ACV:Lfcin B at a drug ratio of 1:33 had an $EC_{50}$ value of 1.87 μM (Table 5). In combination, 0.05 μM ACV and 1.82 μM Lfcin B were needed to achieve 50% inhibition compared with 0.37 μM for ACV and 12.30 μM for Lfcin B when the two drugs were used alone. This represents a sevenfold drop in $EC_{50}$ for both ACV and Lfcin B. The drug ratio of 1:330 for ACV:Lfcin B had an $EC_{50}$ value of 6.30 μM; this resulted in a 19-fold drop in $EC_{50}$ for ACV and a twofold drop in the $EC_{50}$ for Lfcin B.

Table 5
Antiviral activity of acyclovir in combination with lactoferrin or lactoferricin against HSV-2

| Combination | $EC_{50}$ (μM)[a] | CI at the following percent inhibition | |
|---|---|---|---|
| | | 75 | 90 |
| B-LF + ACV (1:1.87) | 0.29 | 0.66 | 0.53 |
| B-LF + ACV (5:1) | 0.62 | 1.00 | 0.50 |
| ACV + Lfcin B (1:33) | 1.87 | 0.33 | 0.35 |
| ACV + Lfcin B (1:330) | 6.30 | 0.38 | 0.25 |
| ACV + Lfcin B (1:3300) | 7.40 | 0.39 | 0.25 |
| ACV + Lfcin B (1:33000) | 6.36 | 0.80 | 1.25 |

The table shows the CIs for 75 and 90% inhibition of HSV-2 and the $EC_{50}$ for the different combinations. The CI value compares the amount of drug A which gives 75 or 90% effect when used in combination with another drug B, with the amount of drug A which gives 75 or 90% effect when the drug is used alone. A combination is either synergistic, additive, or antagonistic if CI < 1, =1, >1, respectively. The values were determined from two independent microplate susceptibility assays. Fifty percent cytotoxic concentration ($CC_{50}$) of ACV + Lfcin B (ratio 1:33) was 131 μM.

[a] The $EC_{50}$ for the combination is the concentration of ACV added to the concentration of LF or Lfcin at which 50% inhibition was achieved.

The cytotoxic effects of the combinations B-LF + ACV [$CC_{50}$ = 22.4 μM (ratio 1:4.25)] and ACV + Lfcin B [$CC_{50}$ = 131 μM (ratio 1:33)] were well below those that inhibited HSV-1 and HSV-2.

## 5. Discussion

The antiviral effect of LF has previously been described for several viruses, including HSV. This study shows that both the human and the bovine Lfcin have antiviral activity against HSV-1 and HSV-2, the bovine peptide being more potent. The native protein, LF is more effective than the peptide Lfcin, indicating that LF may have other regions contributing to the antiviral activity or that the size of the molecule is important (Hammer et al., 2000). This is in accordance with the study of Siciliano et al. (1999) that showed that a tryptic digest of LF exerted antiviral activity against HSV, although the native protein was more potent. Ikeda et al. (2000) have shown that Lfcin was ineffective against HCV, while LF exerted antiviral activity.

The screening of clinical isolates of HSV-1 and HSV-2 showed that the antiviral activity of LF and Lfcin corresponded to the results obtained with the laboratory strains. Evaluating new antiviral molecules against several clinical isolates in addition to laboratory-adapted strains is necessary to draw conclusion about the true antiviral effect. As expected the ACV-resistant isolates were also susceptible to LF and Lfcin, due to the difference in mode of action of the drugs.

The literature mentions the antiviral effects of several cationic peptides, in common for several of these peptides is the β-sheet conformation. (Lehrer et al., 1985; Yasin et al., 2000) The structure of the molecule may be of importance for the antiviral activity. Lfcin B has been described as a distorted β-sheet, while the exact structure for Lfcin H has not yet been described. Lfcin B is more active against HSV-1 and HSV-2 than Lfcin H. Lfcin H and B have a homologous region, but Lfcin B is structurally simpler than Lfcin H. Lfcin B consists of 25 residues in a single chain, with a somewhat different sequence than Lfcin H (Bellamy et al., 1992). Lfcin H has a lower net positive charge than Lfcin B, i.e. 5.85 and 7.84 at pH 7.0, respectively. This again may be responsible for the overall higher antiviral activity of Lfcin B. The significance of the structural diversity of the peptides has yet to be clarified.

The antiviral mechanism for these peptides has not been described, but for LF it has been suggested that it blocks the initial binding of HSV to the host cell by binding to heparan sulfate at the cell surface (Hasegawa et al., 1994; Marchetti et al., 1996). LF has a relative high net positive charge and its N-terminal region is extremely basic (Baker et al., 2002). LF and Lfcin B block the entry of HCMV into human fibroblasts (Harmsen et al., 1995; Andersen et al., 2001).

Combined antiviral therapy with two or more drugs has now been shown to be beneficial in the treatment for several infections. In vitro studies have shown that LF exhibit synergy in combination with antifungal agents against *Candida* isolates and with zidovudine against HIV-1 (Kuipers et al., 1999; Viani et al., 1999). In the present study, we showed a synergistic activity between ACV and B-LF or Lfcin B.

A synergistic antiviral activity of these combinations can be anticipated, since the compounds have a different mode of action. ACV inhibits the HSV replication, while LF and Lfcin B inhibit the first steps in the HSV infection.

The best drug combination tested here resulted in a sevenfold drop in $EC_{50}$ for both ACV and Lfcin B. The moderately active peptide is becoming quite more potent when used in combination with ACV, as compared to when used alone.

Importantly, reduced amounts of ACV could be used when combined with Lfcin B or B-LF. Combination therapy may also reduce the possibility for the incidence of drug-resistant strains.

An in vivo model has shown that both systemically and orally administered LF could protect mice from bacterial infection (Bhimani et al., 1999). LF and Lfcin in combination with ACV may find its utility as preventive therapy in individuals at high risk of viral infections, especially in immunocompromised patients.

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