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Identification of an aprotinin antiviral domain

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Abstract

Digestion of the proteinase inhibitor aprotinin, by clostripain, a cysteine proteinase, yielded five oligopeptide fragments. Two fragments exhibited both antiviral and antibacterial activities, two fragments only antiviral activity, and one fragment showed no antimicrobial activity. One of the former oligopeptides showed antiviral activity against human herpes simplex virus type 1 and bovine parainfluenza virus type 3. It consisted of the hexapeptide Y-F-Y-N-A-K corresponding to amino acids 21–26 of intact aprotinin. An identical synthetic peptide had the same antiviral spectrum as the natural hexapeptide, exhibited no antibacterial activity, and was also devoid of trypsin inhibiting activity. Intact aprotinin, in contrast, is ineffective against human herpes simplex virus 1 and bovine parainfluenza virus 3 but possesses antibacterial properties against several bacterial species [(1992) J. Appl. Bact. 72, 180–187].

Key words: Protease inhibitor; Aprotinin; Antiviral domain; Antimicrobial peptide

1. Introduction

Aprotinin, also known as bovine pancreatic trypsin inhibitor, is one of the most studied proteinase inhibitors. It has been isolated from bovine organs and tissues [1–3], and aprotinin-like inhibitors have been found in several organisms [4,5]. Although isolated in 1930 [6] and extensively studied since then, its precise biological function remains unknown [7].

One property of aprotinin is its antimicrobial activity. It inhibits the growth of several Gram-positive and -negative bacteria [8] and the replication of influenza viruses [9,10]. Both antibacterial and antiviral activities have been attributed to the proteinase inhibitory function of aprotinin.

We recently reported that the bactericidal and antiproteolytic activities of aprotinin are not related and suggested that the bactericidal potency of aprotinin is due instead to its cationic and hydrophobic properties [11].

The purpose of this study was to determine if the antimicrobial actions of aprotinin require the intact molecular conformation or whether discrete peptide sequences within the molecule can fulfil this function. Aprotinin was digested by the proteinase, clostripain, to yield 5 fragments, one of which had potent antiviral activity. We have completely characterized and synthetically produced this fragment. Its properties are discussed in this report.

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2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources: aprotinin (Contrykal 50000) was a gift from Prof. Oettel, VEB Pharmazeutisches Kombinat GERMED, Dresden (Germany); clostripain, methyl cellulose crystal violet and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Cbromide (MTT) were from Sigma; trypsin (from porcine pancreas) was from Serva; Fmoc-amino acid derivatives and Fmoc-Lys (t-Boc)-Resin were from Bachem (Switzerland); ProRPC (Hr 5/10 and HR 16/10) and PepRPC Hr 5/5 columns for reverse-phase chromatography were from Pharmacia; Bio Gel P-2 fine was from Bio-Rad; *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were from Roche; *Klebsiella pneumoniae* ATCC 13883, *Serratia marcescens* ATCC 8100, *Staphylococcus epidermitis* ATCC 12228 were from Eurodiagnostic, Shoreham-By Sea, Brighton; *Staphylococcus lentus* and *Bordetella bronchiseptica* wild-type strains were from the Institute of Bacteriology of the Veterinary Hospital Zürich; *Bacillus subtilis* BGA was from Merk; Eagle's minimum essential medium with L-glutamine was from Amimed; 24- and 96-well plates were from Nunc; Madin-Darby bovine kidney cells (MDBK) and pooled New Zealand white rabbit kidney cells (LLC-RK1) were from Flow laboratories; Rhabdomyosarkom cells (RD) and human herpes simplex virus type 1 (HSV-1) were a gift of Dr. Wunderli from the Institute for Immunology and Virology, University of Zürich; African green monkey kidney cells (VERO), swine testis cells (ST), equine herpes virus type 1 (EHV-1), bovine parainfluenza virus type 3 (PI-3) and porcine respiratory corona virus (PRCV) were from the Institute of Veterinary Virology, Zürich; embryonic horse lung cells (EHL) were prepared in our Institute from normal aborted fetuses at 3 months of gestation.

All chemicals for sequence analysis were purchased from Applied Biosystems Inc. Solvents and reagents used for peptide synthesis were purchased from Fluka.

2.2. Antibacterial assays

Single colonies of bacteria grown on trypticase soy agar plates were inoculated in 50 ml of trypticase soy broth (TSB) and grown overnight at 37°C. 1 ml of bacterial suspension was diluted 1:50 with TSB. Bacteria were grown at 37°C and logarithmic phase organisms were harvested at a density of $1–4 \times 10^8$ cfu per ml. The culture was then centrifuged at $2,000 \times g$ for 10 min. The sedimented bacteria were washed

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twice with 10 mM sodium phosphate buffer, pH 7.4, and adjusted to 10^6 cfu per ml. 50 μl of bacterial suspension was added to 50 μl of test solution. 100 μl of 2% TSB in 10 mM sodium phosphate buffer was then added. The mixture was incubated at 37°C for 2 h, serially ten-fold diluted from 1:10 to 1:1,000 in 10 mM sodium phosphate buffer, pH 7.4, and plated on trypticase soy agar. The plates were incubated for 24 48 h at 37°C until distinct colonies could be counted. All assays were carried out in duplicate.

2.3. Antiviral assays

The antiviral activity of aprotinin and its fragments was performed as follows: samples were dissolved in Eagle's minimum essential medium (serum free) containing 30 mM HEPES and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were grown to 90% confluency in 96-well plates and then infected with 10^4 tissue culture infective dose of 50% ($TCID_{50}$) of virus. After incubation for 60 min at 37°C, the non-adsorbed virus was discarded. The infected cells were washed with phosphate buffered saline (PBS) and then incubated with 50 μl of the sample prepared as described above. The cell cultures were incubated until a clearly detectable cytopathic effect (CPE) in unprotected infected cells was visible (about 24 h). Uninfected cells were incubated with the test substances without virus to evaluate their cytotoxicity. Antiviral assays were performed for each virus in a homologue system: HSV-1 on the human RD cell line, EHV-1 on the equine cell line EHL, PI-3 on the bovine cell line MDBK, and PRCV on the porcine cell line ST. Additionally HSV-1 and EHV-1 were tested in a heterologous system (VERO cells).

Quantification of HSV-1 inhibition was performed by plaque reduction assay as follows. Plates from the antiviral assay were frozen and thawed three times, supernatants were serially tenfold diluted in Eagle's minimal essential medium, and inoculated into the appriopriate cell monolayers grown in 24-well plates (VERO cells for HSV-1 and LLC-RK1 for EHV-1). Inoculations were performed with 250 μl supernatant for 60 min at 37°C. Cells were then washed and overlaid with Eagle's minimal essential medium containing 5% fetal calf serum and 0.8% (w/v) methyl cellulose and incubated at 37°C, 5% CO_2 in a humid atmosphere until plaques were visible (48–72 h). Methyl cellulose medium was then removed, the cultures were fixed with methanol and finally stained with 0.5% Crystal violet and the plaques counted.

Quantification of PI-3 inhibition were performed by MTT assay modified from [12,13] as follows. Plates from the antiviral assay were frozen and thawed three times, supernatants were serially tenfold diluted in Eagle's minimal essential medium, and inoculated to MDBK cell monolayers grown in 24-well plates. Inoculations were performed with 250 μl supernatant for 60 min at 37°C. Cells were then washed and overlaid with 1 ml Eagle's minimal essential medium containing 2% fetal calf serum and incubated at 37°C, 5% CO_2 in a humid atmosphere until clear damage of the monolayers was visible (3–4 days). Supernatant was then carefully removed and substituted with 0.5 ml fresh Eagle's minimal essential medium without fetal calf serum containing 1.25 mg/ml of MTT. After incubation for 1 h at 37°C, 0.5 ml of acidified isopropanol (2 ml of concentrated HCl per 500 ml of isopropanol) containing 10% (v/v) Triton X-100 was added to each well. After solubilisation of the cells and of the blue cristals (30 min), the A_{570}, which is inversely proportional to the extent of cell destruction, was read. Inhibition was calculated as the percent reduction in cell damage of Y-F-Y-N-A-K treated infected cultures compared to untreated infected cultures.

2.4. Assay for trypsin inhibitory capacity

The assay for the antitryptic activity was performed with *N*-benzoyl-L-arginine-*p*-nitroanilide as the substrate according to the method of Geiger and Fritz [14].

2.5. Peptide concentration

Peptide concentration was determined photometrically according to Gill et al. [15]. An extinction coefficient of 2,560 $M^{-1} \cdot cm^{-1}$ was assumed.

2.6. Sequence analysis of antiviral peptide

Sequence analysis was performed on a Model 470 A/970 A gas-phase sequencer (Applied Biosystem Inc.) equipped with an on-line PTH-amino acid analyzer Model 120 A (Applied Biosystem Inc.) using standard chemicals and programs.

2.7. Preparation of the synthetic peptide

Synthesis of Y-F-Y-N-A-K was performed on an Applied Biosystems Inc. Model 430 A Peptide Synthesizer. The hexapeptide was synthesized according to the FastMoc strategy using 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate as the activating agent [16]. The peptide was deprotected and cleaved from the resin by incubation in a mixture of 0.75 g crystalline phenol, 0.25 ml ethanedithiol, 0.5 ml thioanisole, 0.5 ml water and 10 ml trifluoroacetic acid.

The cleaved peptide was collected by precipitation in *t*-butylmethylether and then centrifuged. The pellet was collected and washed several times with *t*-butylmethylether. Aliquots of the peptide were dissolved in 5% (v/v) acetic acid and purified (see below).

2.8. Purification of commercial aprotinin by FPLC reverse-phase chromatography

12 mg aprotinin was dissolved in 2 ml 0.3% trifluoroacetic acid and loaded onto a ProRPC (Hr 16/10) column equilibrated with 0.3% trifluoroacetic acid. The column was eluted at a flow rate of 3 ml/min and fractions of 2 ml were collected. After collecting 5 fractions, a linear gradient from 0 to 30% (v/v) acetonitrile (80 ml vol.) was applied. Aprotinin was eluted as a single peak. Fractions corresponding to the peak were pooled and then freeze-dried.

2.9. Digestion of aprotinin with clostripain

Clostripain was activated as follows: 100 U of clostripain were dissolved in 500 μl of 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $CaCl_2$, 160 mM NaCl, 2.5 mM DTT, and incubated for 3 h at room temperature. The enzyme solution was then stored at 4°C until used (not longer than 24 h).

10 mg of purified aprotinin was dissolved in 1.5 ml of Tris buffer, pH 7.5, and added to 500 μl of clostripain solution. The mixture was then incubated at 37°C for 24 h and then cleared by centrifugation at 20,000 × *g* for 15 min.

2.10 Separation of aprotinin fragments

2.10.1. FPLC reverse-phase chromatography on ProRPC (Hr 16/10).

To the clostripain-digested aprotinin was added trifluoroacetic acid to a final concentration of 0.3% (v/v). The solution (2.5 ml total) was applied to the ProRPC column which was previously equilibrated with 0.3% trifluoroacetic acid in 10% acetonitrile (v/v). The column was eluted at a flow rate of 2 ml/min and fractions of 2 ml were collected. After collecting 10 fractions a linear gradient from 10 to 60% acetonitrile (v/v) was applied. Respective peak fractions were pooled and freeze-dried. The dried samples were dissolved in 700 μl H_2O and assayed for antiviral activity against HSV-1.

2.10.2. FPLC reverse-phase chromatography on ProRPC (Hr 5/10).

Pool II derived from the separation of the aprotinin fragments was acidified with trifluoroacetic acid (final concentration 0.3% (v/v)) and loaded onto a ProRPC column which was equilibrated with 0.3% trifluoroacetic acid. The column was eluted at a flow rate of 0.5 ml/min and fractions of 0.5 ml were collected. After collecting four fractions, a linear gradient from 0 to 60% acetonitrile was applied. The peak fractions were pooled, freeze-dried and sequenced.

2.11. Purification of the synthetic antiviral peptide fragment

Aliquots of the synthetic peptide dissolved in 5% (v/v) acetic acid were first purified by gel-filtration through a Bio-Gel P-2 column (1.6 × 87 cm) followed by reverse-phase chromatography through a PepRPC (Hr 5/5) column using a linear gradient of acetonitrile from 0 to 60% (30 ml) in 0.3% trifluoroacetic acid.

3. Results

3.1. Characterization of peak II antiviral peptide

Digestion of aprotinin by clostripain over a period of 24 h at 37°C resulted in the appearance of fragments which were resolved by reverse-phase chromatography

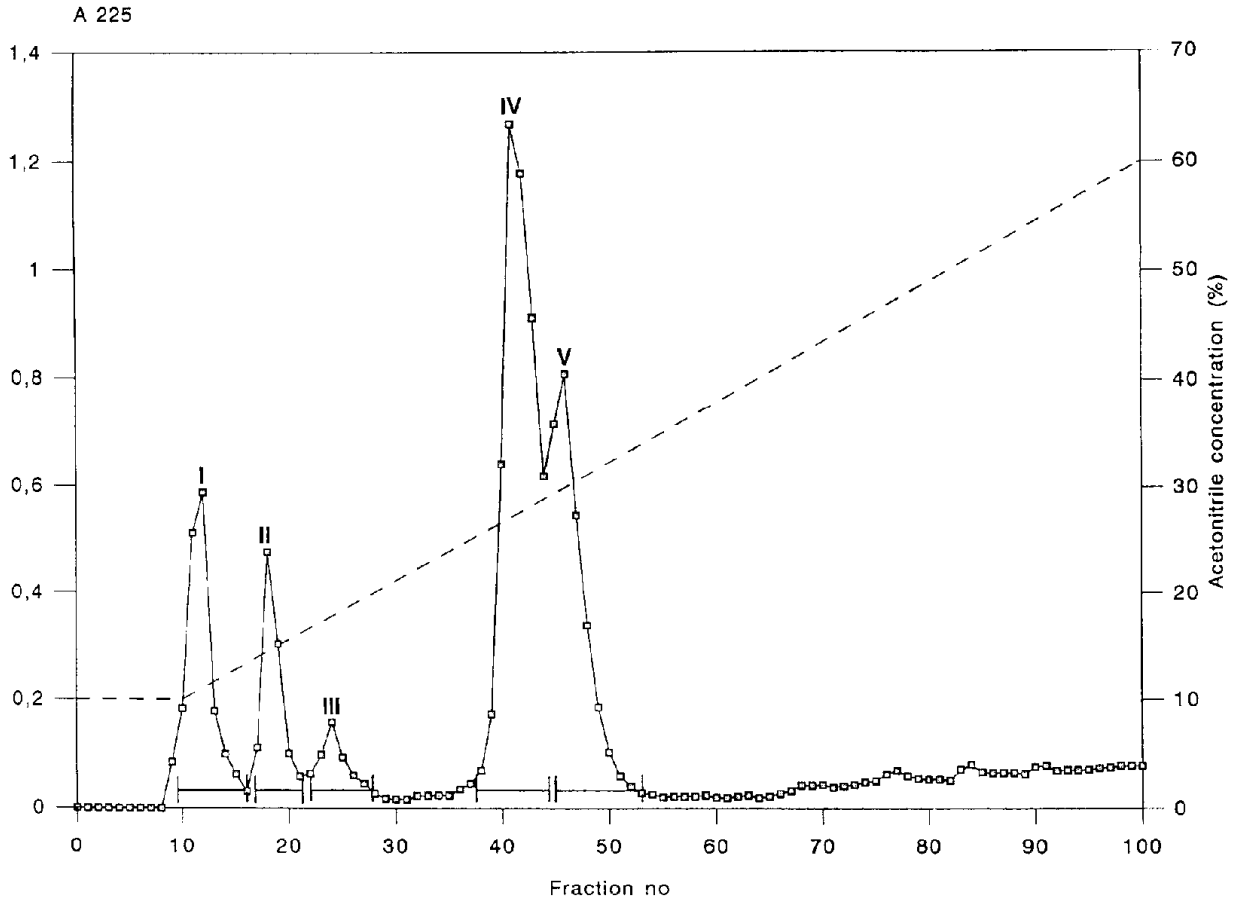

Fig. 1. Reverse-phase chromatography on ProRPC (Hr 16/10) of the fragments derived from aprotinin digestion by clostripain. (——) Absorbance at 225 nm. (– – –) Gradient elution.

on a ProRPC Hr 16/10 column. A typical elution diagram is shown in Fig. 1 and consisted of five peaks. The fractions corresponding to each of the five peaks were pooled and assayed for antiviral activity. Pool I contained neither antiviral nor antibacterial activity but was cytotoxic for the cells used in the antiviral assay. Pools II and III were antiviral for HSV-1 and PI-3 but devoid of antibacterial activity. Pools IV and V contained antiviral and antibacterial activities. Pool II was re-chromatographed on ProRPC (Hr 5/10). The elution diagram from this step consisted of one major peak (Fig. 2) in which the antiviral activity toward HSV-1 was detected. This oligopeptide was sequenced and found to be Y-F-Y-N-A-K, corresponding to amino acids 21–26 of aprotinin [7]. The oligopeptide was then artificially synthesized and purified, and the sequence and antiviral activity against HSV-1 were confirmed. All further characterizing assays were performed with the synthetic oligopeptide.

3.2. *Specificity of the antiviral activity of the oligopeptide, Y-F-Y-N-A-K*

The oligopeptide inhibited replication of HSV-1 in RD cells in a dose-dependent manner (Fig. 3). The 50% effective dose (ED_{50}) was 38 μM. A concentration of 600 μM Y-F-Y-N-A-K reduced PI-3 replication by 25%. No inhibitory effects were observed against EHV-1 or PRCV. In order to investigate whether cell type is important for the inhibitory effect of the oligopeptide, the antiviral assays for HIV-1 and EHV-1 were also performed on VERO cells. At a concentration of 600 μM the oligopeptide did not inhibit the replication of HSV-1 if VERO cells were infected with the same amount of virus used for RD cells. Because VERO cells are 100-times more sensitive to HSV-1 the compound was also tested for inhibitory activity using 100-times less virus. Under this condition the oligopeptide inhibited HSV-1 replication by 70%. No inhibition of EHV-1 replication in VERO cells was observed. Aprotinin assayed up to a concentration of 1.5 mM had no effect on the four viruses tested. Both compounds, aprotinin and the oligopeptide, were not cytotoxic for the cells used in the assays.

Preincubation of the cells with oligopeptide for 24 h before inoculation of the virus resulted in no antiviral effect. Preincubation of the virus with peptide for 1 h at 37°C before inoculation of the cells with the mixture was also ineffective. Immediate addition of the virus–peptide

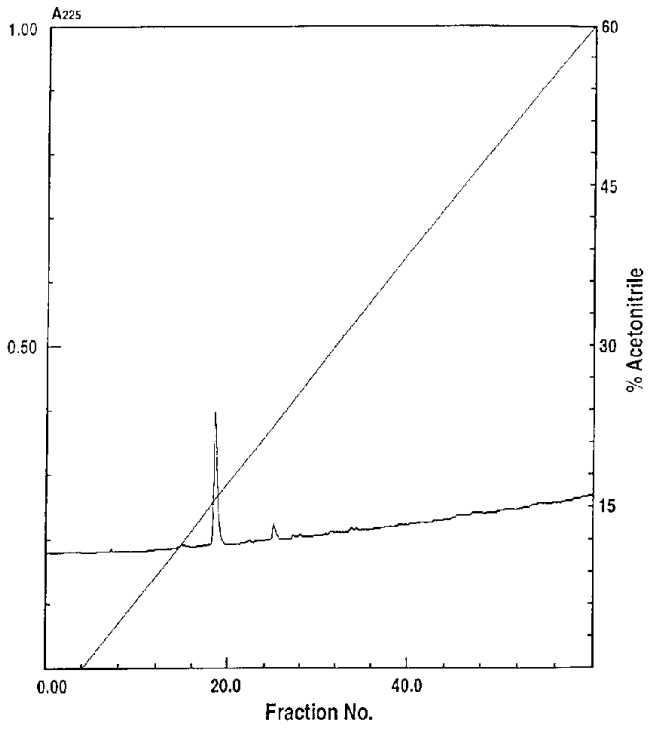

Fig. 2. Reverse-phase chromatography on ProRPC (Hr 5/10) of pool II from ProRPC (Hr 16/10).

mixture to the cells without preincubation was likewise ineffective.

3.3. Antibacterial activity

The antibacterial activity of the hexapeptide was determined at the same protein concentration used for the antiviral assays. No antibacterial activity was detected against the following Gram-positive and -negative bacteria: *E. coli, K. pneumoniae, B. subtilis, Bord. bronchiseptica, S. marcescens, Staph. lentus, Staph. epidermitis, Staph. aureus.*

3.4. Trypsin inhibiting activity

Antitryptic activity of the oligopeptide was assayed at a concentration of 1 mM. No inhibition of trypsin occurred although intact aprotinin completely inhibits trypsin at the same equimolar concentration.

4. Discussion

Digestion of aprotinin with clostripain yielded five oligopeptide fragments, four of which had antiviral activity against HSV-1, although the native molecule exhibits no antiviral activity. Clostripain was previously used for the fragmentation of human cathepsin G, a basic enzyme which has antibacterial activity not related to its catalytic properties [17]. Here, only fragment II has been completely analysed since difficulties in sequencing and peptide synthesis occurred with pools III, IV and V which would have unduely delayed this report. Fragment II (Fig. 1) consisted of the hexapeptide Y-F-Y-N-A-K, which corresponds to amino acids 21–26 of the aprotinin sequence.

The oligopeptide did not inhibit the replication of EHV-1 in EHL and VERO cells and of PRCV but inhibited replication of HSV-1 in RD and VERO cells and PI-3 on MDBK cells. This differentiated effect indicates that this compound may interact with specific and, for some viruses, essential viral replication mechanisms. HSV-1 replication is inhibited by the peptide in both RD and VERO cells if the same multiplicity of infection, specific for the cell, was used. In addition EHV-1 replication was not inhibited either on EHL or on VERO cells at different multiplicities of infection. Thus we conclude that the effect of Y-F-Y-N-A-K is not dependent on the cell type infected.

The peptide was devoid of antiproteinase activity, and, in intact aprotinin, it is separated from the active site (amino acid 13–18) by 2 amino acids (19 and 20) [7]. Thus no relationship between antiviral activity and proteinase inhibition is indicated.

Intact aprotinin had no effect on any of the four viruses tested although it was previously demonstrated to inhibit influenza virus propagation in chicken embryos [10]. This observation was attributed to the ability of aprotinin to block proteolytic activation of the virus spikes. However, no control experiments were performed using inactivated aprotinin. While aprotinin also has antibacterial properties [8,11], these are most probably unrelated to its antiproteinase activity [11].

The new antiviral activity of the aprotinin-derived oligopeptide, not detectable in the original compound, con-

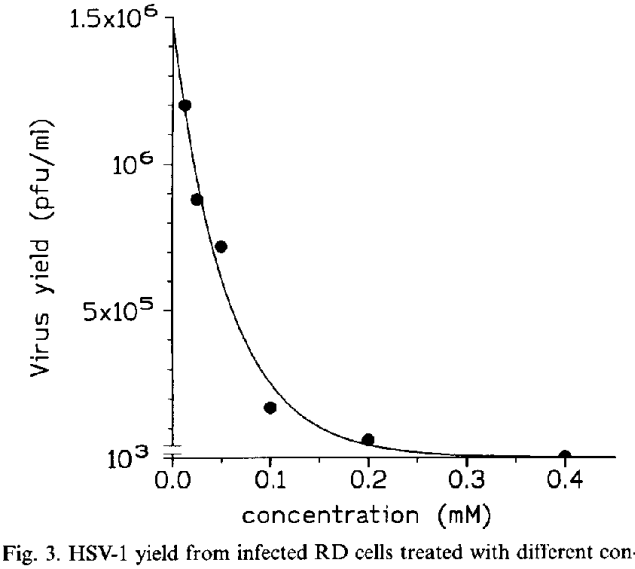

Fig. 3. HSV-1 yield from infected RD cells treated with different concentrations of the oligopeptide, Y-F-Y-N-A-K.

trasts with the situation of cystatin, an inhibitor of cysteine proteinases and a tripeptide derived therefrom [18]. The short oligopeptide, which corresponds to its active site and inhibits proteinases, also inhibits viral multiplication of HSV-1 as does the parent molecule. This suggests that proteinase inhibition could be the underlying mechanism in that system.

The cystatin- and aprotinin-derived peptides may have similarities concerning their site and chronology of action in the virus replication cycles. This is suggested by the dependence of observed antiviral activity on the order of addition of virus vs. oligopeptide in both cases. As a prerequisite for antiviral activity, the virus had to be applied to the cells 1 h before the respective peptides were added. Since preincubation of viruses with oligopeptides did not affect viral infectivity, we can also conclude that the aprotinin- and cystatin-peptides do not function as defensins, i.e. by directly neutralizing viruses [19]. Another feature common to both the cystatin and aprotinin oligopeptides is their requirement for prolonged incubation (> 1 h) in the virus–cell system before suppressing viral activity. This, plus the fact that simultaneous addition of the virus and oligopeptide to the cells did not suppress infectivity, indicate that the oligopeptide does not compete with the virus for receptor sites on the cell surface. Successful inhibition of viral activity occurred only when the respective oligopeptide was added after virus adsorption and internalization and was continually present in the system. How the oligopeptide functions to inhibit viral replication remains unknown. It is possible that viruses alter the cell surface thereby providing new binding sites, or that they stimulate uptake of exogenous material by the cells. Such speculations have yet to be tested as possible interaction mechanisms.

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