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Research Letter

New Alloferon Analogues: Synthesis and Antiviral Properties

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We have extended our study on structure/activity relationship studies of insect peptide alloferon (H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH) by evaluating the antiviral effects of new alloferon analogues. We synthesized 18 alloferon analogues: 12 peptides with sequences shortened from *N*- or *C*-terminus and 6 *N*-terminally modified analogues H-X^1-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH, where X^1 = Phe (13), Tyr (14), Trp (15), Phg (16), Phe(p-Cl) (17), and Phe(p-OMe) (18). We found that most of the evaluated peptides inhibit the replication of *Human Herpesviruses* or *Coxsackievirus* B2 in Vero, HEp-2 and LLC-MK$_2$ cells. Our results indicate that the compound [3-13]-alloferon (1) exhibits the strongest antiviral activity (IC$_{50}$ = 38 μM) among the analyzed compound. Moreover, no cytotoxic activity against the investigated cell lines was observed for all studied peptides at concentration 165 μM or higher.

Key words: alloferon, antiviral activity, insect peptides

Abbreviations: The symbols of amino acids, peptides and their derivatives are in accordance with the IUPAC-IUB Joint Commission on Biochemical Nomenclature Recommendation, 1983 (Eur. J. Biochem. 1984, 138, 9 and J. Pept. Sci. 2006, 12, 1).Boc, tert-butyloxycarbonyl; CPE, cytopathic effect; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; EDT, 1,2-ethanedithiol; FBS, foetal bovine serum; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, O-benzotriazole-*N,N,N′, N′*-tetramethyl-uronium-hexafluorophosphate; HOBt, *N*-hydroxybenxotriazole; HPLC, high-performance liquid chromatography; NEM, *N*-ethylmorpholine; MTT, 3-[4,5-dimethylthiazol -2-yl]-2,5-diphenyl tetrazolium bromide; TCID, tissue culture infected dose; TFA, trifluoroacetic acid; TLC, thin layer chromatography; TFMSA, trifluoromethanesulfonic acid; UV, ultraviolet.

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Viruses are pathogens that cause many serious diseases in humans such as flu, HIV/AIDS, smallpox and haemorrhagic fevers, liver cancer, fever blisters, genital herpes, etc. (1). In addition, viruses can also infect plants and animals, causing huge losses in agriculture (2).

Effective control of viral infections and diseases is limited, because of a relatively small number of efficient antiviral drugs (3,4). Furthermore, many of known antiviral drugs have a limited spectrum of activity; moreover, drugs that can eliminate viruses are dangerous to non-infected cells. The development of new antiviral drugs is difficult because the replication of viruses is very rapid and viruses mutate frequently, often rendering antiviral drugs ineffective. On the other hand in laboratory conditions, it is often problematic to cultivate viral cultures, thus establishing reliable tests for potential antiviral drugs is difficult (5,6).

To this day, some of the antiviral agents that are based on natural products, including a variety of polyphenols, flavonoids, polysaccharides, anthraquinones, terpenes and proteins (7–9). It seems that natural compounds from plants, animals and fungi present a promising strategy in the search for biological active compounds (1,7,10,11).

Recently, many antimicrobial peptides have been discovered in insects (1,12,13). However, relatively little data are available on insect peptides with the antiviral properties (13). Until now, only eight peptides with antiviral activity have been isolated from insects (13–19).

One of them is alloferon, a novel insect tridecapeptide (H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH) isolated in 2002 by Chernych from the blow fly *Calliphora vicina* (14). It is interesting that this short peptide contains four His residues in positions 1, 6, 9 and 12, and five Gly residues in position 2, 5, 7, 10 and 13. The primary structure of alloferon is also similar to some functionally relevant proteins such as precursors of influenza virus B haemagglutinin, bovine prion protein I and II and *Sarcophaga peregrina* antifungal protein (14).

The *in vitro* experiments show that alloferon stimulates natural killer lymphocytes. *In vivo*, alloferon induces the interferon (IFN) synthesis in mice (14), probably through activation of nuclear factor κB (20).

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Several other biological activities of alloferon have been discovered. The *in vivo* experiments in mice indicate that alloferon has antitumor properties (14,21), it also prevents mortality of most animals challenged by influenza virus A (14). Although rimantadine offers better protection, a much higher dose is required than in case of alloferon. An injection of alloferon also stimulates resistance to influenza virus B in dosage much smaller than ribavirin. Moreover, it was found that alloferon inhibits the replication of *Human Herpesvirus* 8 (*Kaposi's sarcoma*-associated *herpesvirus*) and *Human Papillomaviruses* (20,22).

In addition, Lee *et al.* (23) suggest that alleferon has significant anti-inflammatory effects on the UVB-induced response on the cutaneous inflammation. In our preliminary investigation, we found that alloferon inhibits the replication of *Human Herpesvirus* 1 and fails to affect the *Coxsackievirus* replication *in vitro* (16,24). Furthermore, our results show that this peptide suppresses the development of plant pathogens (25). We also studied alloferon analogues modified in position 1 of the peptide chain such as: [des-His^1]-, [Lys^1]-, [Arg^1]- and [Ala^1]-alloferon (24). The most active compound in this series was peptide with lysine residue in position 1. This analogue was very active against reference and clinical strains of the *Human Herpesvirus* 1 (HHV-1) in Vero cells (IC_{50} = 147.09 and 9.19 μg/mL) and *Coxackievirus* B2 (CVB-2) in HEp-2 cells (IC_{50} = 107.04 and 74.0 μg/mL), respectively (24).

This result inspired us to perform further studies on alloferon analogues. In this study, on structure/activity relationship in alloferon, we present the synthesis and antiviral activity of two new series of analogues of alloferon (Table 1) such as: analogues with a shortened sequence and analogues modified in position 1.

In first group, to determinate the active core of alloferon, we synthesized 12 analogues partially truncated at *N*- or *C*-terminus. In the second series, histidine in position 1 was replaced: (i) by different proteinaceous aromatic amino acid residues as: phenylalanine (**13**), tyrosine (**14**), tryptophan (**15**); (ii) by non-proteinaceous aromatic amino acid residues as: phenylglycine (**16**) and *para*-substituted phenylalanine derivatives [-Cl (**17**) or -OMe (**18**)].

During the biological investigations of the peptides, we analyzed for their antiviral activity *in vitro* against *Herpesviruses* and *Coxsackieviruses* using Vero, HEp-2 and LLC-MK_2 cell lines.

Experimental

Materials

The Wang resins preloaded with Fmoc-Gly, Fmoc-Val and Fmoc-Gln(Trt), Fmoc-amino acids, HOBt, HBTU and TFA were purchased from IRIS Biotech (Marktredwitz, Germany). Boc-Gly-OH, Boc-Val-OH, Boc-His(π-Bom)-OH, Boc-Gln-OH, Merrifield resin and DCC were obtained from Bachem (Heidelberg, Germany). *N*-ethylmorpholine (NEM) was purchased from Fluka (as Sigma-Aldrich, St. Louis, MO, USA). HPLC-grade solvents were purchased from Fisher Scientific (Gliwice, Poland). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents and reagents used for solid-phase synthesis were of analytical quality and were used without further purification.

All the chemicals and reagents used for antimicrobial studies were of bacteriological grade. The viruses used for the antiviral studies came from the collection of the Department of Medical Microbiology, Medical University of Warsaw (Warsaw, Poland). The African green monkey kidney

Table 1: The primary structures of alloferon and its synthesized analogues

Peptide	Amino acid sequence
Alloferon	H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH
1	H-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH
2	H-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH
3	H-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH
4	H-His-Gly-Gln-His-Gly-Val-His-Gly-OH
5	H-Gly-Gln-His-Gly-Val-His-Gly-OH
6	H-Gln-His-Gly-Val-His-Gly-OH
7	H-His-Gly-Val-His-Gly-OH
8	H-Gly-Val-His-Gly-OH
9	H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-OH
10	H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-OH
11	H-His-Gly-Val-Ser-Gly-His-Gly-Gln-OH
12	H-His-Gly-Val-Ser-Gly-His-Gly-OH
13	H-Phe-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH
14	H-Tyr-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH
15	H-Trp-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH
16	H-Phg-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH
17	H-Phe(*p*-Cl)-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH
18	H-Phe(*p*-OMe)-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH

cells (Vero), rhesus monkey kidney cells (LLC-MK_2) and human larynx carcinoma cells (HEp-2) were obtained from the American Type Culture Collection (Manassas, VA, USA).

Eagle's medium was purchased from Biomed (Lublin, Poland). FBS was obtained from Gibco (Paisley, UK). Peptides were purified by preparative high-performance liquid chromatography on a Varian ProStar HPLC system, column: Tosoh Biosciences ODS-120T C18 (ODS 300 × 21.5 mm) (Tokyo, Japan) with UV detection at 210 nm.

Analytical HPLC was performed on a Thermo Separation Products HPLC system with a Vydac C18 column (ODS 250 × 4.6 mm) (Grace, Deerfield, IL, USA) with UV absorption determined at 210 nm. The molecular weight of the peptides was confirmed with a Bruker Daltonics micrOTOF-Q mass spectrometer (Bremen, Germany). The optical activity of the chiral compounds was measured with a Jasco DIP-1000 polarimeter (Jasco, Japan). TLC was performed on aluminium sheets precoated with silica gel 60 from Merck (Darmstadt, Germany).

Cytotoxic activity of peptides was assessed by light microscopy Olympus CK2 (Olympus Corp., Hamburg, Germany). The plate readings were recorded spectrophotometrically on a reader (Reader 230, Organon Teknika Turnhout, Belgium).

Synthesis

Synthesis of H-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (1)

The peptide was obtained by a stepwise elongation of the peptide chain by the method outlined below.

0.5 g of the Fmoc-Gly-resin (capacity 0.84 mmol/g) was suspended in 20% solution of piperidine in dimethylformamide (DMF). The mixture was stirred for 20 min at room temperature. Then, it was filtered and washed with DMF. The next amino acid, Fmoc-His(Trt)-OH (0.826 g, three equiv), was dissolved in DMF and coupled to the resin in the presence of HBTU (1.352 g, three equiv), HOBt (0.180 g, three equiv) and NEM (293 μL, six equiv) for 2 h. The end of the reaction was determined by the Kaiser test. Other Fmoc-amino acid derivatives: Fmoc-Val-OH (0.453 g, three equiv), Fmoc-Gly-OH (0.397 g, three equiv), Fmoc-His(Trt)-OH (0.826 g, three equiv), Fmoc-Gln(Trt)-OH (0.815 g, three equiv), Fmoc-Gly-OH (0.397 g, three equiv), Fmoc-His(Trt)-OH (0.826 g, three equiv), Fmoc-Gly-OH (0.397, three equiv), Fmoc-Ser(Bu^t)-OH (0.512 g, three equiv), Fmoc-Val-OH (0.453 g, three equiv) were connected to the peptide-resin in the same way. After the final removal of the N^{α}-Fmoc group, the peptide-resin was washed with DMF, MeOH:DMF (1:1, v/v), MeOH and then dried overnight over KOH under reduced pressure. The free peptide was obtained by deprotection with 4.75 mL of TFA in the presence of 0.125 mL of EDT and 0.125 mL of water for 2 h at room temperature according to the standard procedure. The peptide was purified by preparative HPLC. The main fractions were combined and lyophilized. Finally, the peptide was redissolved in 50% acetic acid in water and then relyophilized. The purity of all final products was checked by HPLC, TLC, optical activity and molecular weight determinations.

Peptides **2**–**3** and **9**–**18** were obtained and purified in the same manner as peptide **1**. The purity of free peptides was checked by HPLC and found to exceed 95% in each case. Their analytical data are presented in Table 2.

Synthesis of H-His-Gly-Gln-His-Gly-Val-His-Gly-OH (4)

The peptide was obtained by a stepwise elongation of the peptide chain by the method outlined below. The *C*-terminal amino acids were bound to the Merrifield resin by the caesium salt procedure to the substitution level of 0.68 mmol glycine per gram.

One gram of Boc-Gly-resin was suspended in solution of 30% TFA in CH_2Cl_2. The mixture was stirred for 30 min at room temperature. Then, it was filtered and washed for 10 min with CH_2Cl_2 and EtOH, three times each. The resin was neutralized with 10% TEA in CH_2Cl_2 for 10 min and washed with EtOH and CH_2Cl_2, three times each. The next amino acid, Boc-His(π-Bom)-OH (0.50 g, three equiv), was dissolved in CH_2Cl_2 and coupled to the resin in the presence of three equivalents of DCC/HOBt for 2 h. The end of the reaction was determined by the Kaiser test. The following amino acids (three equivalents) were coupled to the resin by the DCC method: Boc-Gly-OH, Boc-His(π-Bom)-OH. Boc-Val-OH (0.80 g, six equiv) was introduced to the peptide chain by the symmetrical anhydride method. Boc-Gln-OH (1.0 g, three equiv) was coupled to the resin in the presence of HBTU (0.75 g, three equiv), HOBt (0.30 g, three equiv), NEM (0.9 mL, six equiv). The peptide-resin was washed with CH_2Cl_2, MeOH:CH_2Cl_2 (1:1, v/v), MeOH and then dried overnight over KOH under reduced pressure.

The free peptide was obtained according to the following procedure: the peptide-resin was mixed with 0.9 mL of anisole, 0.45 mL of EDT, 7.5 mL of TFA and 1.2 mL of TFMSA. The mixture was kept at room temperature for 2 h. The resin was filtered off and the filtrate was triturated with diethyl ether (200 mL). The above reaction mixture gave a precipitate, which was separated, washed with diethyl ether, dried *in vacuo* over KOH and then dissolved in water and lyophilized. The peptide was purified by preparative HPLC. Finally, the peptide was redissolved in 50% acetic acid in water and then relyophilized. The purity of all final products was checked by HPLC, TLC, optical activity and molecular weight determinations.

Table 2: Physicochemical data of alloferon analogues

Peptide	Yield (%)[a]	$[\alpha]^{20}_D$ (c = 1.0, methanol)	R_t (HPLC)	MW calculated	$[M+H]^+$ *m/z* found	R_f (TLC)[b]		
						X	Y	Z
1	78	−23.5	14.2	1070.5	1071.5	0.35	0.27	0.17
2	74	−27.3	13.2	971.7	972.4	0.34	0.29	0.21
3	82	−31.4	12.8	884.4	886.4	0.35	0.28	0.19
4	74	−20.8	12.9	827.7	828.4	0.35	0.18	0.22
5	75	−30.4	12.5	690.6	691.3	0.33	0.30	0.19
6	78	−22.0	12.3	633.5	634.3	0.30	0.18	0.33
7	86	−30.0	12.6	505.4	506.2	0.32	0.29	0.22
8	83	−19.3	11.8	368.2	369.2	0.32	0.30	0.22
9	76	−25.6	15.6	1070.5	1071.5	0.41	0.22	0.18
10	84	−24.5	14.9	971.4	972.3	0.36	0.19	0.23
11	85	−18.4	14.1	777.4	778.4	0.34	0.19	0.28
12	78	−39.6	13.4	649.3	650.3	0.37	0.22	0.26
13	75	−47.6	15.0	1274.6	1275.3	0.28	0.30	0.15
14	79	−12.0	13.5	1290.6	1291.3	0.23	0.19	0.29
15	75	−17.5	16.2	1313.6	1314.3	0.22	0.30	0.26
16	84	−23.6	14.5	1260.6	1261.3	0.20	0.17	0.23
17	81	−18.5	15.1	1309.6	1310.3	0.32	0.22	0.20
18	79	−17.3	15.6	1305.6	1306.3	0.28	0.31	0.19

[a]Crude yield: yield after cleavage from the resin. The purity of crude product was analysed according to HPLC peak integrals at λ 210 nm on analytical HPLC. The crude peptide had a purity of >80%.
[b]T.L.C. on silica gel plates; eluents: X = n-butanol/acetic acid/methanol (4:1:1), Y = chloroform/methanol/acetic acid (5:3:1), Z = n-butanol/pyridine/acetic acid/water (30:20:6:24).

Peptides **5**–**8** were obtained and purified in the same manner as peptide **4**. The purity of free peptides were checked by HPLC and found to exceed 95% in each case. Their analytical data are presented in Table 2.

Molecular modelling study

Theoretical calculations were performed using the HyperChem version 7.01 (Hypercube Inc., Gainsville, FL, USA). The peptide structures were built using the Database Amino Acids option. First geometric parameters of the peptides in the zwitterionic form were optimized by molecular mechanic computations with Amber force field. Subsequently, the peptide was placed in a box containing 200 water molecules and its geometry was optimized again.

Biology

Cells

Three types of cell lines were used in this study: Vero, LLC-MK_2, HEp-2. Cells were grown and maintained at 37 °C in Eagle's medium 1959 supplemented with 10% FBS and 1% of antibiotic antimycotic solution (100 ×): penicillin, streptomycin, amphotericin B.

Viruses

Two viruses, representing DNA and RNA viruses pathogenic for humans, were used in these experiments. The viral strains used in this study were the standard strains of *Human Herpesvirus 1* McIntire (HHV-1_{MC}) or 971 PT *Coxsackievirus* B2 (971 PT, CVB-2), and the clinical strain of *Human Herpesvirus 1* (HHV-1) and *Coxsackievirus* B2 (CVB-2). The herpesviruses stock was propagated in Vero or HEp-2 cells. The CVB-2 strains were grown in LLC-MK_2 or HEp-2 cells.

After the cytopathic effect was evident, the cells were frozen-thawed three times. The cell debris was removed by centrifugation. The supernatant was aliquoted, titrated and kept at −70 °C. In the antiviral assay, the medium was supplemented with 2% FBS and the above-mentioned antibiotics.

Cytotoxicity assay

Cytotoxic activity of peptides was assessed by a light microscopy and quantified by the MTT assay *in vitro* using LLC-MK_2, HEp-2 and Vero cell lines. The absorbance was read in a reader at 405 nm. Cells were inoculated in 96-microwell plate. After incubation for 24 h, the peptides in the serial twofold dilutions from 1:2 to 1:64 were added to the culture medium and cultured for further 24 or 48 h. The control was prepared without any sample. All experiments were performed in triplicate. The toxicity was expressed as maximum cytotoxic concentration to cause a microscopically detectable alteration of normal cell morphology.

Antiviral assay

Antiviral activity was assessed *in vitro* using Vero, LLC-MK_2 or HEp-2 cell lines infected with 0.01 $TCID_{50}$/cell

(Tissue Culture Infectious Doses) of respective virus. The cells after virus isolation were incubated for 2 days at 37 °C with various concentrations of the respective compounds, ranging from 1:2 to 1:64.

The antiviral activity of the tested peptides was determined using a cytopathic effect. The inhibition of the viral CPE was assessed by a light microscopy. Virus titres were determined according to the Reed–Muench formula (26) and expressed in $TCID_{50}$/mL at particular stages of the experiments. The antiviral activity of the tested peptides was finally expressed as the compound concentration that reduces virus yield by 50% (IC_{50}).

Results and Discussion

The 18 new analogues of alloferon were prepared by the manual solid-phase techniques. The synthesis of peptides **1**–**3** and **9**–**18** were performed using the standard Fmoc procedure on Wang resin. As a coupling reagent, HBTU in the presence of HOBT was used. The *N*-Fmoc group was removed with 20% piperidine in DMF. The peptide-resin was cleaved with TFA in the presence of EDT and water. Other peptides (**4**–**8**) were synthesized by the classical solid-phase method according to the Boc-procedure. DCC in the presence of HOBt was used as a coupling reagent. The Boc-protecting group was removed with 30% TFA in CH_2Cl_2. Peptides were released from the resin using TFA, TFMSA and EDT. All peptides were purified by preparative HPLC. In each case, the free peptides had a purity of >95%. Their analytical data are presented in Table 2.

Cytotoxic activity of new analogues of alloferon was examined against the Vero, LLC-MK_2 and HEp-2 cell lines. The cells were incubated in the presence of various doses of tested peptides. As shown in Table 3, the investigated peptides did not show any cytotoxic activity against examined cell lines and did not affect the growth or morphology of the tested cells. The MTT assay also proved that they have no effect on cell proliferation.

The antiviral activity of investigated peptides was tested *in vitro* with respect to: DNA viruses (HHV-1_{MC} and the clinical strain of HHV-1 in a Vero or HEp-2 cells), and RNA viruses (971 PT *Coxsackievirus* B2 and the clinical strain of *Coxsackievirus* B2 using HEp-2 and LLC-MK_2 cells).

The antiviral bioassay showed that most of investigated peptides inhibit *in vitro* the replication of viruses in Vero, LLC-MK_2 or HEp-2 cells (Figures 1–5). We found that among the truncated analogues the most active against HHV-1_{MC} in Vero cells were these without three (compound **2,** [4-13]-alloferon, IC_{50} = 186 μM), five (compound **4**, [6-13]-alloferon, IC_{50} = 179 μM) and six *N*-terminal amino acids (compound **5,** [7-13]-alloferon, IC_{50} = 215 μM), whereas the deletion of four residues causes

Table 3: Cytotoxicity of alloferon analogues

Peptide	CPE[a] (μM)
1	165
2	>420
3	>448
4	468
5	>775
6	380
7	>826
8	>1174
9	260[b]
10	350[b]
11	447[b]
12	504[b]
13	290[b]
14	>315[b]
15	282[b]
16	350[b]
17	255[b]
18	312[b]
Ribavirin	>300[c], >100[d]
Acyclovir	>250[c,d]

[a]Maximal non-cytotoxic concentration for target cells (Vero, HEp-2, LLC-MK_2).
[b]Maximal non-cytotoxic concentration for Vero cells.
[c]Vero cells; the 50% cytotoxic concentration for target cells in μg/mL.
[d]HEp-2 cells, the 50% cytotoxic concentration for target cells in μg/mL.

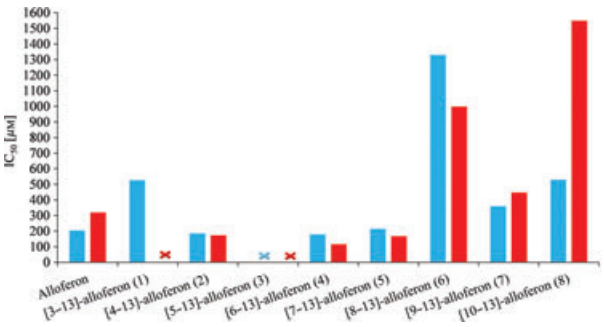

Figure 1: Antiviral activity of alloferon and its analogues against HHV-1_{MC} and HHV-1 in Vero cells. ■: standard strain of HHV-1_{MC}; ■: clinical strain of HHV-1, X without effect (concentration of tested peptide >> 500 μM), acyclovir IC_{50} = 4 μM.

complete loss of antiviral activity (analogue **3**, [5-13]-alloferon) (Figure 1). A similar activity was observed for these peptides against the clinical strain of HHV-1 in Vero cells with IC_{50} values 173, 117 and 168 μM, respectively (Figure 1).

As shown in Figure 2, only the *C*-terminal truncated analogue of alloferon without the fragment His-Gly ([1-11]-alloferon, analogue **9**) displays the antiviral activity against HHV-1_{MC} at lower concentrations (IC_{50} = 178 μM) than alloferon.

During the investigation of the influence of alloferon derivatives on the replication of the standard strain HHV-1_{MC} in

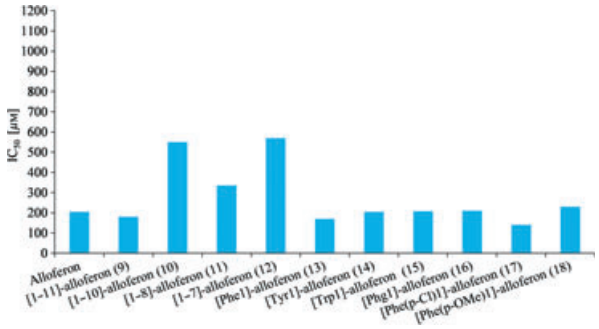

Figure 2: Antiviral activity of alloferon and its analogues against HHV-1_{MC} in Vero cells. Acyclovir IC_{50} = 4 μM.

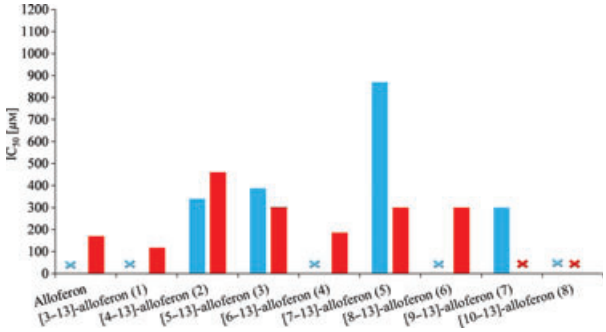

Figure 3: Antiviral activity of alloferon and its analogues against HHV-1_{MC} and HHV-1 in HEp-2 cells. ■: standard strain of HHV-1_{MC}; ■: clinical strain of HHV-1, X without effect (concentration of tested peptide >> 500 μM), acyclovir IC_{50} = 8 μM.

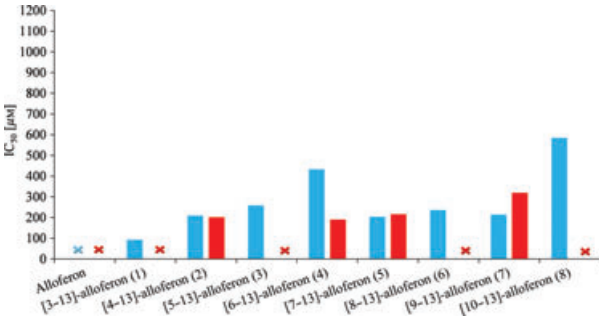

Figure 4: Antiviral activity of alloferon and its analogues against 971 PT CVB-2 and CVB-2 in LLC-MK_2 cells. ■: standard strain of 971PT CVB-2; ■: clinical strain of CVB-2, X without effect (concentration of tested peptide >> 500 μM), ribavirin IC_{50} = 820 μM.

HEp-2 cells, we found that most of the *N*-truncated analogues did not inhibit the replication of this virus (Figure 3). However, alloferon and most of truncated analogues of alloferon inhibited the replication of the clinical strain of HHV-1 in HEp-2 cells. The highest antiviral effect was observed for compounds **1** ([3-13]-alloferon, IC_{50} = 117 μM) and **4** ([6-13]-alloferon, IC_{50} = 186 μM). Other analogues such as [4-13]- (**2**) and [5-13]- alloferon (**3**) show weak inhibitory activity, but the analogues without eight

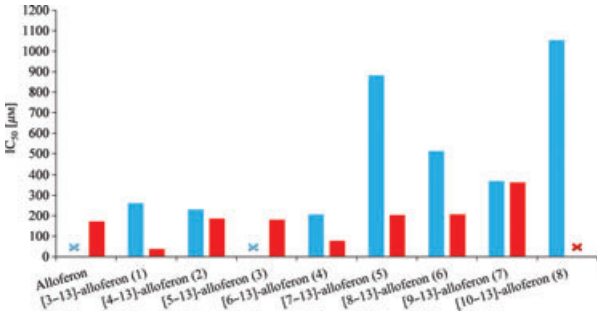

Figure 5: Antiviral activity of alloferon and its analogues against 971 PT CVB-2 and CVB-2 in HEp-2 cells. ■: standard strain of 971PT CVB-2; ■: clinical strain of CVB-2, X without effect (concentration of tested peptide >> 500 μM), ribavirin IC_{50} = 820 μM.

(peptide **7**) and nine amino acid residues (peptide **8**) at the *N*-terminal region were completely inactive.

The antiviral bioassay against RNA viruses demonstrated that alloferon did not show inhibitory effect on the replication of examined CVB-2 in LLC-MK_2 cells, whereas all truncated analogues were active against the reference strain of CVB-2 (Figure 4). The most active analogue was peptide **1** ([3-13]-alloferon), without the *N*-terminal dipeptide His-Gly (IC_{50} = 93 μM). Moreover, only four *N*-truncated analogues of alloferon showed an inhibitory effect against the clinical strain of CVB -2 in LLC-MK_2 (Figure 4).

Additionally, as shown in Figure 5, the *N*-truncated analogues weakly inhibited the replication of the standard strain of 971 PT *Coxsackievirus* B 2 in HEp-2 cells. The antiviral test against the clinical strain of CVB-2 in HEp-2 showed that the majority of the peptides have the antiviral activity. The most active compounds were peptides without *N*-terminal fragments: His-Gly (analogue **1**, [3-13]-alloferon, IC_{50} = 38 μM) or His-Gly-Val-Ser-Gly (analogue **4**, [6-13]-alloferon, (IC_{50} = 78 μM) (Figure 5).

These results indicated that the *C*-terminal amino acid residues of alloferon are more important for antiviral activity than the *N*-terminal ones. From the analysis of analogues modified in position 1, we found that the substitution of the *N*-terminal histidine by aromatic amino acids such as Phe, Trp, Phg, Tyr, Phe(*p*-OMe) or Phe(*p*-Cl) gives analogues with antiviral activity. All analogues modified in position 1 inhibit the replication of the virus HHV-1_{MC} in Vero cells as it was also observed by us (24) for the native peptide (Figure 2). Moreover, we discovered that the alloferon analogue modified in position 1 by Ala is inactive (24). These results suggest that for optimal biological function an aromatic structure is required at *N*-terminal position of alloferon.

The biological effects of alloferon and its analogues modified in position 1 presented here and in our earlier report (24), prompted us to perform the preliminary conformational studies by molecular modelling. Theoretical calculations

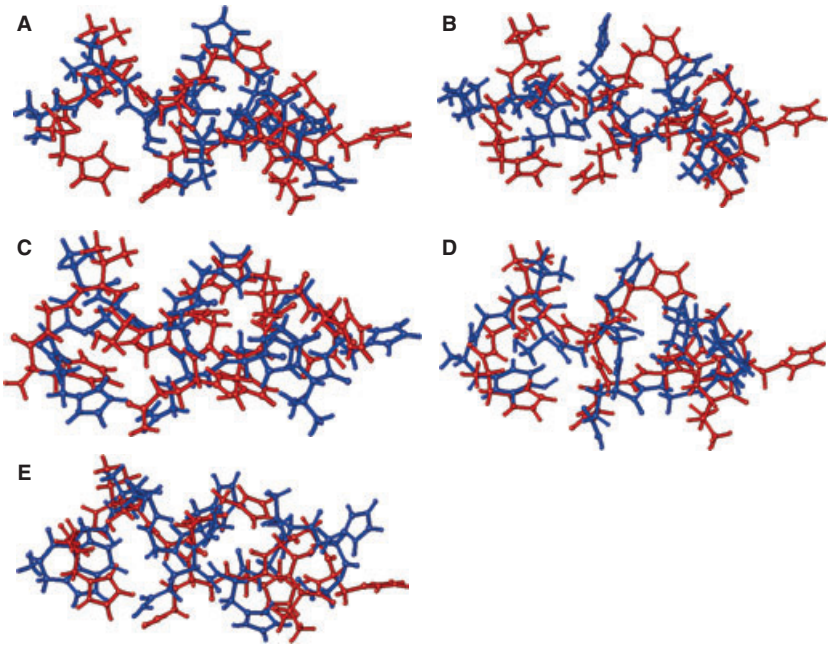

Figure 6: Superposition of minimum energy conformations of [Ala[1]]-alloferon (A), [Lys[1]]-alloferon (B), [Phe[1]]-alloferon (C), [Tyr[1]]-alloferon (D) and [Trp[1]]-alloferon (E) (blue) upon alloferon (red).

were performed for alloferon and its selected analogues modified in position 1 such as [Phe[1]]-, [Tyr[1]]-, [Trp[1]]-, [Lys[1]]- and [Ala[1]]-alloferon (Figure 6).

The conformations of the peptides were optimized using Amber force field (HyperChem 7.01) in a two-step procedure. First, the conformation of molecules was optimized. Subsequently, the peptide was placed in a box containing 200 water molecules and its geometry was optimized again.

The obtained structures of analogues of alloferon were superimposed to compare their conformation with conformation of alloferon. The similarities between the conformations of alloferon with peptides **13–15** were found (Figure 6). The differences between alloferon and [Lys[1]]- or [Ala[1]]-alloferon were observed for the relative spatial arrangement of the side chains of lysine, alanine and histidine in position 1 (Figure 6). These results are in good agreement with the biological data presented above and our earlier investigations (24) and suggest that the positively charged side chain of Lys, which is conformationally flexible, can create the optimal conditions for biological activity of such analogue.

However, the mechanism of antiviral activity of alloferon is still unknown; therefore, only the hypotheses resulting from structure/activity studies of alloferon analogues could be used in design of potential new antiviral compounds.

Conclusion

We found that in a series of new analogues of alloferon most of the evaluated peptides inhibit the replication of DNA and RNA viruses *in vitro* at lower or equal dose as compared with the native peptide. However, the activity of the tested peptides depends on the virus and the cell line used. The analysis of biological effects shows that the reference CVB-2 is less sensitive to investigated peptides.

Furthermore, both our previous (24) and the present work with the analogues of alloferon suggest that the presence of the aromatic ring in position 1 of the peptide chain can play a role in the expression of antiviral properties.

It is worth noting that the tested peptides show no cytotoxic activity against Vero, LLC-MK_2 and HEp-2 cells. Our results indicate that among the analogues of alloferon could be new non-toxic antiviral agents.

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

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