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Further studies on the antiviral activity of alloferon and its analogues

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The subject of our studies was the synthesis, biological evaluation, and conformational studies of insect tridecapeptide alloferon (H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH) and its analogues such as: [des-His[1]]-, [Lys[1]]-, [Arg[1]]-, and [Ala[1]]-alloferon. These peptides were synthesized to check the influence of the His residue at position 1 of the alloferon chain on its antiviral activity. Two aspects of the biological effects of these peptides were determined: (i) the cytotoxicity *in vitro* in the Vero, LLC-MK2, and HEp-2 cell lines, and (ii) the antiviral activity *in vitro* in respect to DNA and RNA viruses. We found that alloferon inhibited the herpes virus multiplication and failed to affect the coxsackie virus replication, whereas [Lys[1]]-alloferon exhibited a high inhibitory action towards both viruses. Moreover, the peptides did not show any cytotoxic activity against the Vero, LLC-MK2, and HEp-2 cells. The preliminary circular dichroism conformational studies showed that the peptides investigated seem to prefer an unordered conformation. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: alloferon; insect peptides; *Human Herpes Virus*; *Coxsackie* B2 virus; antiviral activity

Introduction

Alloferon is a tridecapeptide (H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH) isolated from the bacteria-challenged larvae of the blow fly *Calliphora vicina* [1]. It has a stimulatory effect on natural killer lymphocytes *in vitro,* and both antiviral and antitumour capabilities *in vivo* [1]. This peptide seems to induce interferon synthesis by activation of the nuclear factor-κB signalling pathway in Namalva cells [2]. In preliminary investigations, we found that alloferon inhibits the replication of *Human Herpes Virus* type 1 (HHV-1) *in vitro*. It inhibits the replication of virus HHV-1MC *in vitro* at the concentration of 90 μg/ml by 2log after 24-h incubation, but its inhibitory effect after 48-h incubation is weaker (1.5 log) [3].

The development of new antiviral products is very much required because viral diseases present a worldwide threat, moreover, modern medicine has relatively small number of efficient antiviral drugs. Many natural products contain biologically active substances which can inhibit viruses. They display antiviral action for different DNA- and RNA viruses, including, among others, enteroviruses, rotaviruses, herpes viruses, etc [4–13].

The antiviral activity of insect peptides against HHV-1 has been described in the literature for only a few substances, for example cecropin A, mellitin, *N*-myristoylated-peptide, and *Any*-GS [3,12–13]. It has been demonstrated that mellitin at the concentration of 3 μM reduces the HHV-1 yield by 80% in Vero cells; however, this compound is highly toxic for cells. Another peptide, cecropin A, is not toxic for Vero cells and exhibits a weak inhibitory action against HHV-1 [13]. Moreover, none of the known insect peptides or their analogues exhibits antiviral activity against *Coxsackie* B.

Continuing our studies on alloferon [3,14], we performed the synthesis of its analogues modified at position 1, such as:

H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (**I**)
H-Lys-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (**II**)
H-Arg-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (**III**)
H-Ala-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (**IV**)
H-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (**V**).

In the above compounds the His residue at position 1 was replaced by other basic amino acids, such as Lys and Arg (analogues **II** and **III**) and by the hydrophobic amino acid Ala (analogue **IV**). In addition, we synthesized [des-His[1]] -alloferon (**V**).

In this investigation, we would like to shed some light on the role of the His residue at position 1 of the alloferon peptide chain on the antiviral activity. These studies were inspired by the opinion presented in the literature that the presence of a His residue in the peptide chain of a suitable structure may endow such a peptide with potent antiviral capabilities [15]. At the same time, we performed these studies because the role of the basic amino acids at position 1 has not been sufficiently established in the previous investigations of the structure–biological function relationship [3].

To clarify the role of His-1 in alloferon and its analogues (**II**–**V**), we investigated their *in vitro* antiviral activity with respect to DNA viruses [*Human Herpes Virus* type 1 McIntire (HHV-1_{MC}) and the clinical strain of HHV-1 in a Vero or HEp-2 cells], and RNA viruses (971 PT *Coxsackie* type B2 and the clinical strain of *Coxsackie* type B2 using HEp-2 and LLC-MK2 cells). The antiviral activity of the peptides studied is presented in Tables 2 and 3. In the second part of the biological investigations, we examined the *in vitro* cytotoxic

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activity of those peptides against the Vero, LLC-MK2, and HEp-2 cell lines (Table 1). The conformational preferences of all the peptides were analysed by circular dichroism (CD) spectroscopy (Figure 1).

Materials and Methods

Peptides

Peptides were prepared by the manual solid-phase technique according to Ref. 3. The synthesis of peptides was performed using a standard Fmoc procedure on the Wang resin. Amino acids were assembled on an Fmoc-Gly-Wang resin. As a coupling reagent, HBTU in the presence of HOBT was used. The *N*-Fmoc group was removed with 20% Pip in DMF. The peptide-resin was cleaved with TFA in the presence of 1,2-ethanedithiol. Peptides were purified by the preparative HPLC. Finally, the peptides are re-dissolved in 50% acetic acid in water and then re-lyophilized. The peptide concentration was determined the nitrogen assay by elemental analysis using VarioEL III CHNS analyzer (Elementar Analysensysteme, Germany). The percentage peptide of each sample was determined by the following formula:

$$\%\text{peptide} = (\%\text{nitrogen found}/\%\ \text{theoretical nitrogen}) \times 100\%$$

The peptide content was >92%.

Stock solutions of alloferon (**I**), [Lys^1]-alloferon (**II**), [Arg^1]-alloferon (**III**), [Ala^1]-alloferon (**IV**), and [des-His^1]-alloferon (**V**) were prepared in sterile water and stored at 4 °C.

CD Spectroscopy

CD measurements were performed on a Jasco J-720 spectropolarimeter, at room temperature. A pathlength of 1 mm was used. Peptides were dissolved in water at the concentration of 0.07 mg/ml. Each spectrum represents the average of four scans. The data are presented as molar ellipticity (θ).

Cells

Three types of cell lines were used in this study: African green monkey kidney cells (Vero), Rhesus monkey kidney cells (LLC-MK2), and human larynx carcinoma cells (HEp-2). Cells were grown and maintained at 37 °C in the Eagle's medium 1959 (MEM) (Biomed Lublin) supplemented with 10% foetal bovine serum (FBS) from Gibco and 1% of antibiotic antimycotic solution (100×): penicillin, streptomycin, and amphotericin B (Sigma).

Viruses

Four viruses, representing the major families of DNA and RNA viruses pathogenic for humans, were used in the presented experiments. The viral strains used in this study were the standard strains of HHV-1_{MC} or 971 PT *Coxsackie* type B2 (971 PT CVB2), and the clinical strain of HHV-1 and *Coxsackie* type B2 (CVB2). The herpesviruses stock was propagated in Vero or HEp-2 cells. The coxsackie viruses stock was grown in LLC-MK2 or HEp-2 cells.

After the cytopathic effect (CPE) was evident, the cells were frozen-thawed several 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

The cytotoxic activity of the peptides was assessed by a light microscopy (Olympus CK2) and quantified by the MTT (ATCC bioproducts) assay *in vitro* using LLC-MK2, HEp-2, and Vero cell lines. The absorbance was determined with a reader (Reader 230, Organon Teknika) at 405 nm. Cells were inoculated in a 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 an additional 24 or 48 h. The control was prepared without any sample. All experiments were performed thrice. The toxicity was calculated as a percent of the control.

Antiviral Assay

The antiviral activity was assessed *in vitro* using Vero, LLC-MK2, or HEp-2 cell lines infected with 0.01TCID$_{50}$/cell (tissue culture infectious doses) of respective virus. After virus isolation, the cells were incubated for 2 days at 37 °C with the virus concentration of the respective compounds ranging from 1 : 2 to 1 : 64.

The antiviral activity of the tested peptides was determined using a CPE. The inhibition of the viral CPE was assessed by light microscopy. Virus titres were determined according to the Reed-Muench formula [16] and expressed in TCID$_{50}$/ ml at the particular stages of the experiments. Ribavirin (Sigma) and acyclovir (Sigma) were used as the control agents. The antiviral activity of the tested peptides was finally expressed as the compound concentration that reduces a virus yield by 50% (IC$_{50}$).

Results and Discussion

The microscopic observations showed that no changes occur in the Vero, LLC-MK2, and HEp-2 cells growth or morphology in the tested peptides presence. The MTT assay also proved that they have no effect on the cell proliferation (Table 1). The antiviral bioassay showed that most of the investigated peptides inhibit *in vitro* the replication of viruses in Vero, LLC-MK2, or HEp-2 cells (Tables 2 and 3).

We found that alloferon inhibits the replication of viruses HHV-1_{MC} and HHV-1 in Vero cells with IC$_{50}$ values of 305.50 and 479.00 µg/ml, respectively. However, this peptide did not inhibit the replication of virus HHV-1_{MC} in HEp-cells but it was slightly active against wild-type HHV-1 (IC$_{50}$ = 252.20 µg/ml). Moreover, alloferon exhibited a weak or no activity against 971 PT *Coxsackie* type B2 and the clinical strain of *Coxsackie* type B2.

The substitution of alloferon at position 1 of the peptide chain by other amino acids resulted in analogues with different activity. Among the examined analogues, only [Lys^1]-alloferon (**II**), [Arg^1]-alloferon (**III**), and [des-His^1]-alloferon (**V**) were active against herpesviruses and coxackieviruses. However, [Lys^1]-alloferon (**II**) was very active against HHV-1_{MC} (IC$_{50}$ = 147.09 µg/ml) and HHV-1 (IC$_{50}$ = 9.19 µg/ml) in Vero cells. This peptide strongly inhibited the replication of HHV-1 (IC$_{50}$ = 12.98 µg/ml) in HEp-2 cells, whereas it exhibited a weaker activity against HHV-1_{MC} with the IC$_{50}$ value of 241.3 µg/ml. However, the antiherpes effect of that peptide against Vero cells was very high as compared with the native peptide.

During the investigation of the influence of [Lys^1]-alloferon (**II**) on the replication of *Coxsackie* virus, we found that this analogue was very active against 971 PT *Coxsackie* B2 and *Coxsackie* B2 in both LLC-MK2 (IC$_{50}$ = 157.73 and 190.67 µg/ml, respectively) and HEp-2 cells (IC$_{50}$ = 107.04 and 74.00 µg/ml, respectively).

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Table 1. Cytotoxicity of alloferon and its analogues

Peptide	Concentration (μg/ml)	CPE inhibition (%) Vero cell	LLC-MK2 cell	HEp-2 cell
Alloferon **(I)**	300.00	100	100	100
	150.00	100	100	100
	75.00	100	100	100
	37.5	100	100	100
	18.75	100	100	100
	9.37	100	100	100
[Lys[1]]-alloferon **(II)**	218.75	100	100	100
	109.37	100	100	100
	54.68	100	100	100
	27.34	100	100	100
	13.67	100	100	100
	6.84	100	100	100
[Arg[1]]-alloferon **(III)**	143.75	100	100	100
	71.88	100	100	100
	35.94	100	100	100
	17.97	100	100	100
	8.98	100	100	100
	4.49	100	100	100
[Ala[1]]-alloferon **(IV)**	187.50	100	100	100
	93.75	100	100	100
	46.87	100	100	100
	23.44	100	100	100
	11.72	100	100	100
	5.86	100	100	100
[des-His[1]]-alloferon **(V)**	62.50	100	100	100
	31.25	100	100	100
	15.63	100	100	100
	7.81	100	100	100
	3.91	100	100	100
	1.95	100	100	100
Ribavirin	$>300^a$	–	–	–
	$>100^b$			
Acyclovir	$>250^a$	–	–	–
	$>250^b$			

[a] Vero cell; the 50% cytotoxic concentration for target cells in μg/ml.
[b] Hep-2 cell, the 50% cytotoxic concentration for target cells in μg/ml.

The substitution of the *N*-terminal residue by another basic amino acid (compound III) resulted in a peptide with a weak antiviral activity in Vero, LLC-MK2, and HEp-2 cells. [Arg[1]]-alloferon (**III**) shows the inhibitory effect on the replication of HHV-1 in Vero and HEp-2 cells with an IC_{50} values of 277.5 and 602.18 μg/ml, respectively. However, this peptide was inactive against $HHV\text{-}1_{MC}$ in Vero and HEp-2 cells. Moreover, [Arg[1]]-alloferon (**III**) was only slightly active against *Coxsackie* B2 and 971 PT *Coxsackie* B2 in LLC-MK2 cells (IC_{50} = 355.18 and 573.73 μg/ml, respectively). It is worth noting that this analogue inhibited the replication of viruses 971 PT CVB2 in HEp-2 cells with the value of 167.71 μg/ml but it exhibited no antiviral activity against the clinical strain CVB2.

Interestingly, the elimination of His at position 1 of alloferon resulted only in compounds with a marked anti-HHV-1 activity in HEp-cells (IC_{50} = 104.44 μg/ml) and anti-971 PT CVB2 activity (IC_{50} = 89.56 μg/ml). Furthermore, the substitution of His by Ala

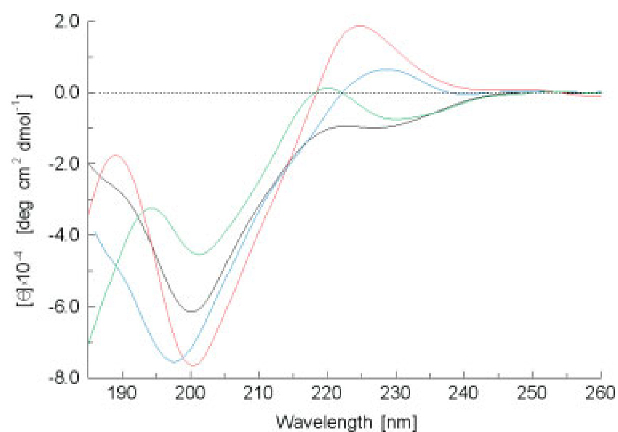

Figure 1. CD spectra of alloferon and its analogues. Alloferon (I) in black, [Lys[1]]-alloferon (II) in red, [Arg[1]]-alloferon (III) in blue, [Ala[1]]-alloferon in green (IV).

at position 1 resulted in a complete loss of activity against both viruses.

The biological effects of alloferon and its analogues presented here prompted us to perform the preliminary conformational studies by CD spectroscopy. The CD spectra of all investigated peptides dissolved in water (at neutral pH) contain a large negative band at ∼200 nm and much smaller positive or negative bands at 220–230 nm (Figure 1). The negative band at 200 nm indicates that the conformational equilibria of all the peptides are dominated by unordered structures [17]. In addition, the CD spectra show that the conformation of [Ala[1]]-alloferon (**IV**) seems to be the most similar to that of the native peptide.

The important problem is the search of effective drugs against the CVB2 and HHV-1 viruses. Many different compounds show antiviral activity against different viruses. Nowadays, the most commonly used drugs in the treatment of HHV-1 are synthetic nucleosides such as acyclovir, that selectively inhibit HHV DNA replication with low host-cell toxicity [18–20]. In addition, the emergence of resistance against antiviral drugs used in the treatment of HHV-1 in patients with immune deficiency, creates serious clinical and epidemiological problems. In recent years, resistance to acyclovir has been noted in patients receiving cancer chemotherapy due to the treatment. Moreover, nowadays, there are only few enterovirus-specific drugs available for clinical use but there are no effective drugs against Coxsackie B2 virus [21–23]. Therefore, a continued development of new antiviral agents directed at CVB and HHV is necessary.

The data presented here indicate that peptides isolated from insects can inhibit DNA and RNA viruses *in vitro*.

Our data demonstrate that the replacement of His by Lys at position 1 of the alloferon peptide chain ([Lys[1]]-alloferon) leads to a compound with strong antiviral activity. This analogue of alloferon is highly active against HSV-1 and CVB2 in all tested cells and does not show a cytotoxic activity. It is a very interesting result because many studies have demonstrated the importance of Lys in preventing and sometimes shortening outbreaks of herpes simplex infections [24]. In addition, poly-L-Lys also blocks the virus herpes infection. It was suggested that a high Lys to Arg ratio prevents herpes from replicating [25]. The proteins synthesized by HHV contain more Arg and less Lys than proteins synthesized by cells and Arg is required for HHV replication. The interesting result obtained for [Lys[1]]-alloferon may suggest that this peptide is involved in interactions with viral DNA or viral

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Table 2. Antiviral activity of alloferon and its analogues against HHV

	Vero cells				HEp-2 cells			
	$HHV\text{-}1_{MC}$		HHV-1		$HHV\text{-}1_{MC}$		HHV-1	
Peptides	IC_{50} (μg/ml)	IC_{50} range (μg/ml)	IC_{50} (μg/ml)	IC_{50} range (μg/ml)	IC_{50} (μg/ml)	IC_{50} range (μg/ml)	IC_{50} (μg/ml)	IC_{50} range (μg/ml)
Alloferon **(I)**	305.00	280.00–333.4	479.00	388.50–605.20	Without effect	–	252.66	133.65–566.26
[Lys^1]-alloferon **(II)**	147.09	124.30–175.18	9.19	−27.80–136.80	241.90	186.19–325.09	12.98	−24.16–130.90
[Arg^1]-alloferon **(III)**	321.10	163.90–773.30	277.50	226.50–351.00	Without effect	–	602.18	284.36–782.76
[Ala^1]-alloferon **(IV)**	Without effect	–	Without effect	–	Without effect	–	Without effect	–
[des-His^1]-alloferon **(V)**	307.74	121.67–1370.73	Without effect	–	Without effect	–	104.44	79.26–145.21
Ribavirin	162.0	–	–	–	–	–	–	–
Acyclovir	1.0	–	1.5	–	2.0	–	2.5	–

Table 3. Antiviral activity of alloferon and its analogues against CVB2

	LLC-MK2 cells				HEp-2 cells			
	971 PT CVB2		CVB2		971 PT CVB2		CVB2	
Peptides	IC_{50} (μg/ml)	IC_{50} range (μg/ml)	IC_{50} (μg/ml)	IC_{50} range (μg/ml)	IC_{50} (μg/ml)	IC_{50} range (μg/ml)	IC_{50} (μg/ml)	IC_{50} range (μg/ml)
Alloferon **(I)**	Without effect		Without effect	–	Without effect	–	256.96	231.34–286.48
[Lys^1]-alloferon **(II)**	157.73	113.96–209.10	190.67	119.90–330.68	107.04	47.69–250.43	74.00	43.85–120.98
[Arg^1]-alloferon **(III)**	577.73	341.57–1477.35	355.18	263.18–519.22	167.71	149.44–189.43	Without effect	–
[Ala^1]-alloferon **(IV)**	358.00	167.20–1078.70	Without effect	–	Without effect	–	Without effect	–
[des-His^1]-alloferon **(V)**	89.56	38.20–190.45	Without effect	–	299.35	217.73–447.96	Without effect	–
Ribavirin	–	–	–	–	200.0	–	–	–

proteins. The mechanism of action of alloferon can be similar to the previously described action of poly-*L*-Lys and other positively charged proteins [25]. However, our results indicate that alloferon is a less potent antiviral agent than [Lys^1]-alloferon. This is probably due to the fact that the imidazole NH group of His is only weakly ionized at neutral pH. Moreover, the exchange of His with Arg or Ala at position 1 of the alloferon chain leads to compounds with a reduced or none antiviral activity. However, the most active compound (analogue **V**) against 971 PT CVB2 was obtained by elimination of His at position 1. This means that not only the electric charge but also the structure of the *N*-terminal amino acid residue side chain is important for the antiviral activity. In addition, it is interesting that a substitution of His with a hydrophobic amino acid Ala at position 1 caused a complete loss of activity against HHV. This result can suggest that the hydrophilicity at position 1 of the peptide is related to antiherpes activity.

The data also show that the antiviral activity of alloferon and its analogues against HHV in Vero cells requires higher concentration of peptides comparable to the reference drug, acyclovir, which has a IC_{50} value 1.5 μg/ml and it shows a cytotoxicity (CC_{50} = 250 μg/ml), but on the other hand [Lys^1]-alloferon was more active than ribavirin. Moreover, the interesting result was that [Lys^1]-alloferon and [Arg^1]-alloferon showed higher inhibitory activity against CVB2 in HEp-2 cells than ribavirin.

In this study, we noticed the difference between inhibition of HHV and CVB2 *in vitro*. Some peptides were active only against the herpes viruses. This effect can be due to the dissimilarity of the viral structure and the replication cycles between HHV-1 and CVB2.

Our results indicate that the activity of the tested peptides depends on the virus and the cell line used. Further studies are planned to elucidate the antiviral mechanism of action of the tested peptides in different cell-virus models.

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Conclusions

From the analysis of the biological effect of alloferon analogues presented here, the following conclusions can be reached: Most of the evaluated peptides inhibit the replication of DNA and RNA viruses in Vero and HEp-2 cells; the most active compound was [Lys^1]-alloferon; all the peptides did not show a cytotoxic activity against Vero, LLC-MK2, and HEp-2 cells; the hydrophilicity at position 1 of alloferon is important for the inhibition activity HHV.

The results obtained indicate that the insect peptides may be useful antiviral agents against HHV-1 and *Coxsackie* B2.

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