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Studies of insect peptides alloferon, *Any*-GS and their analogues. Synthesis and antiherpes activity

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The subject of these studies was synthesis and determination of biological properties of a series of insect peptides, such as alloferon, *Any*-GS and their analogues. The synthesis of 14 peptides was performed by the solid-phase method. Biological effect of these peptides was evaluated by the antiviral test against *Human Herpes Virus* type 1 (HHV-1) *in vitro* using a Vero cell line. It was found that the investigated peptides inhibit the replication of HHV-1 in Vero cells. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

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

Introduction

The aim of the present study was to search for new antiviral activity among selected insect peptides such as alloferon, *Any*-GS and a series of their analogs.

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 is interesting that its primary structure is similar to some functionally relevant proteins, such as influenza virus B hemagglutinin, bovine prion protein I and II, and *Sarcophaga peregrina* antifungal protein. The *in vitro* experiments reveal that alloferon has a stimulatory activity toward natural killer lymphocytes [1]. *In vivo,* alloferon induces the IFN synthesis in mice [1]. A further study has shown that alloferon stimulates the synthesis of IFN through NF-κB activation [2]. Additional *in vivo* experiments in mice indicate that alloferon has both antiviral and antitumor capabilities [1]. Due to a unique immune modulating mechanism, alloferon may be used in conjunction with other antiviral and anticancer drugs to generate synergistic effects in combating disease.

The oligopeptide *Any*-GS (H-Asp-Ile-Leu-Arg-Gly-NH_2) was isolated from the wild silkmoth *Antheraea yamamai* [3]. This peptide suppresses the proliferation of rat hepatoma cells (dRLh84) *in vitro* [4]. Furthermore, it provokes the inhibition of growth and viability of human hepatoma cells HepG2 and decreases the activity of mitochondrial enzymes [5]. The mode of antitumor action of this molecule is still unknown; however, the biological study has shown that *Any*-GS causes the cell cycle arrest rather than apoptosis/necrosis [4]. Such evidence puts this peptide as a potentially interesting chemipharmaceutical. In addition, *Any*-GS shows several other biological activities. For example, *Any*-GS inhibits *in vitro* the growth of plant pathogens *Phoma narcissi* and *Botrytis tulipae* [6]. Moreover, *Any*-GS shows a strong cardioinhibitory effect in the myotropic test on the semi-isolated heart of insect *Tenebrio molitor* [7].

One of the most common viral infections in humans is caused by HHV. The *Herpesviridae* are a large family of DNA viruses that can cause skin and mucous membranes infections, tumor, and other serious diseases in animals and humans. A major problem of the modern medicine is a relatively small number of efficient antiviral drugs. Therefore, the identification of new biologically active substances which may be used as antiviral agents is very important [8]. Natural products isolated from arthropods are a significant source of bioactive compounds. In the last decade, several peptides with a large variety of biological activities were isolated from insects [1,4,9–12]. These peptides show, among others, myotropic, oostatic, antinociceptive, antimicrobial, and antitumor activity [9–12]. However, relatively little data are available on molecules from insects with the antiviral properties. Recently, several new peptides with antiviral or antitumor activity, such as: alloferon, *Any*-GS, *N*-myristoylated peptide and red fluorescent protein have been isolated from insects [11], opening new possibilities in the development of the much requested antiviral substances. Because both alloferon and *Any*-GS show a broad-spectrum activity [1,4], it inspired us to investigate their influence on the growth of the herpesviruses. Moreover, the insight into the relation between

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Abbreviations used: *CPE, cytopathic effect; DMF, dimethylformamide; DNA, deoxyribonucleic acid; FBS, fetal bovine serum; HBTU, O-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluorophosphate; HHV, Human Herpes Virus; HHV-1*$_{MC}$*, Human Herpes Virus type 1 McIntrie; HOBt, N-hydroxybenxotriazole; HPLC, high performance liquid chromatography; INF, interferon; NEM, N-ethylmorpholine; NF, nuclear factor; MTT, 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide; TCID, tissue culture infected dose; TFA, trifluoroacetic acid; TLC, thin layer chromatography; UV, ultraviolet.*

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Table 1. Physicochemical data of alloferon and its analogues modified at position 1

Peptide	Yield (%)[a]	$[\alpha]^{20}_{D}$ c = 1, methanol	R_t (HPLC)	Molecular weight calculated	Molecular weight found	R_f (TLC)[b]		
						X	Y	Z
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (**1**)	78	−28.5	11.2	1264.5	1265.5	0.32	0.32	0.25
H-Lys-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (**2**)	76	−18.3	12.2	1255.7	1256.6	0.33	0.34	0.26
H-Arg-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (**3**)	81	−21.4	12.8	1283.7	1284.5	0.33	0.35	0.28
H-Ala-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (**4**)	85	−17.8	11.7	1198.6	1199.6	0.30	0.33	0.26
H-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (**5**)	80	−14.4	11.3	1127.5	1128.5	0.36	0.31	0.22

[a] Crude yield: yield after cleavage of the resin. The purity of crude product was analyzed according to HPLC peak integrals at λ 210 nm on analytical HPLC. The crude peptide had a purity of >80%.
[b] TLC 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).

the peptide structure and function was applied to investigate the mechanism of action of selected insect antiviral peptides as well as in the design of their analogues as potential novel therapeutic agents.

Taking into account the frequent occurrence of histidines and aspartates in the active or binding sites of proteins [13–15], in our preliminary studies we decided to obtain information about the role of the His[1] of alloferon and the Asp[1] of *Any*-GS.

We performed the synthesis of the following peptides:

1. alloferon (H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH) (**1**) and its analogues modified at position 1: H-Lys-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (**2**), H-Arg-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (**3**), H-Ala-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (**4**), H-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH (**5**),
2. *Any*-GS (H-Asp-Ile-Leu-Arg-Gly-NH_2) (**6**) and its analogues modified at position 1, such as: H-Arg-Ile-Leu-Arg-Gly-NH_2 (**7**), H-Gly-Ile-Leu-Arg-Gly-NH_2 (**8**), H-Ala-Ile-Leu-Arg-Gly-NH_2 (**9**), H-Asn-Ile-Leu-Arg-Gly-NH_2 (**10**), H-Gln-Ile-Leu-Arg-Gly-NH_2 (**11**) and its shortened derivatives: H-Asp-Ile-Leu-Arg-NH_2 (**12**), H-Ile-Leu-Arg-Gly-NH_2 (**13**), H-Leu-Arg-Gly-NH_2 (**14**).

In the first group of peptides, the His residue at position 1 of alloferon was replaced by: (i) basic aliphatic amino acid – Lys (**2**) or Arg (**3**), (ii) hydrophobic aliphatic amino acid – Ala (**4**). We also obtained the analog without the His residue at position 1 (**5**).

In the next group of investigated peptides the Asp residue at position 1 of *Any*-GS was exchanged by: basic amino acid – Arg (**7**), neutral amino acid – Gly (**8**) or Ala (**9**) and neutral hydrophilic amino acid – Asn (**10**) or Gln (**11**). Moreover, we synthesized truncated analogues of *Any*-GS in which one or two amino acids were eliminated at the *C*-(**12**) or the *N*-terminus (**13–14**).

Peptides **1–5** were synthesized by the classical solid-phase method according to the Fmoc procedure. Their physicochemical data are presented in Table 1. Other peptides (**6–14**) were synthesized by use of the Boc solid-phase chemistry, according to the previously described procedure [7].

During the biological investigations of those peptides, we examined their *in vitro* antiviral activity against HHV-1_{MC} and cytotoxic activity using a Vero cell line.

Material and Methods

Peptides Synthesis

Peptides were prepared by the manual solid-phase techniques. The synthesis of peptides **1–5** was performed using the standard Fmoc procedure on a Wang resin. Other peptides (**6–14**) were synthesized by the classical solid-phase method according to the Boc-procedure [7]. The Wang resin preloaded with Fmoc-Gly (capacity 0.84 mmol/g) was purchased from Novabiochem. The 9-fluorenyl-methoxy-carbonyl amino acids, the coupling reagent HBTU and HOBt were purchased from Novabiochem. The side chain protecting groups for Fmoc-amino acids were *t*-butyl for Ser, trityl for His and Gln, and *t*-butoxycarbonyl for Lys. NEM was purchased from Fluka. TFA and *N*,*N*-dimethylformamide were obtained from Iris Biotech. HPLC-grade solvents were purchased from Fisher Scientific. All other reagents were purchased from Sigma-Aldrich (Steinheim, Germany). All solvents and reagents used for solid-phase synthesis were of analytical quality and were used without further purification.

Peptides were purified by the preparative HPLC using a Varian ProStar system with a column: Tosoh Bioscience ODS-120T C18 (ODS 300 × 21.5 mm) in a linear gradient of 15–30% B in A (A – 0.1% aqueous TFA, B – 80% acetonitrile in water, containing 0.1% TFA) for 40 min at a flow rate of 7 ml/min with the 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) with a linear gradient 0–100% B in A for 60 min at a flow rate of 1 ml/min with the UV absorption determined at 210 nm.

The molecular weight of the peptides was determined with a Bruker Daltonics micrOTOF-Q mass spectrometer.

The optical activity of the chiral compounds was measured with a Jasco DIP-1000 polarimeter (Jasco).

TLC was performed on aluminum sheets precoated with silica gel 60 from Merck.

H-His-Gly-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 above. 0.5 g of the Fmoc-Gly-resin (capacity 0.84 mmol/g) was suspended in 20% solution of

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piperidine in 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, 3 equiv), was dissolved in DMF and coupled to the resin in the presence of HBTU (1.352 g, 3 equiv), HOBt (0.180 g, 3 equiv) and NEM (293 µl, 6 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, 3 equiv), Fmoc-Gly-OH (0.397, 3 equiv), Fmoc-His(Trt)-OH (0.826 g, 3 equiv), Fmoc-Gln(Trt)-OH (0.815 g, 3 equiv), Fmoc-Gly-OH (0.397, 3 equiv), Fmoc-His(Trt)-OH (0.826 g, 3 equiv), Fmoc-Gly-OH (0.397, 3 equiv), Fmoc-Ser(Bu^t)-OH (0.512 g, 3 equiv), Fmoc-Val-OH (0.453 g, 3 equiv), Fmoc-Gly-OH (0.397, 3 equiv) and Fmoc-His(Trt)-OH (0.826 g, 3 equiv) were connected to the 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 ethanedithiol and 0.125 ml of water at room temperature according to the standard procedure. Then the peptide was purified by the preparative HPLC. The main fractions were combined and lyophilized. The purity and homogeneity of all final products were checked by HPLC, TLC, optical activity and molecular weight determinations. Peptides **2–5** were obtained and purified in the same manner as peptide **1**. Their analytical data are presented in Table 1.

Biological Investigation

Cell culture and viruses

Vero cells were grown and maintained in the Eagle's minimum essential medium 1959 (Biomed Lublin) supplemented with 10% FBS from Gibco and 1% of antibiotic antimycotic solution (100×): penicilin, streptomycin, amfotericin B (Sigma-Aldrich) at 37 °C.

The viral strain used in this study was HHV-1_{MC}.

The virus stock was grown on Vero cells. After a 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

The cytotoxic activity of peptides was assessed by a light microscopy (Olympus CK2) and quantified by the MTT (ATCC bioproducts) assay *in vitro* using a Vero cell line. The absorbance was read at 405 nm (Reader 230, Organon Teknika Turnhout, Belgium) [16]. The stock solutions of peptides were prepared in sterile water and stored at 4 °C. Their concentrations are presented in Tables 2 and 3. Vero cells were inoculated in 96-microwell plate. After incubation for 24 h, the peptides in the serial twofold dilutions from 1 : 4 to 1 : 128 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 calculated as a per cent of the control.

Antiviral assay

The antiviral activity was assessed against HHV-1_{MC} *in vitro* using a Vero cell line. In these studies the cell cultures were infected with HHV-1_{MC} 1$TCID_{50}$/cell. The tested compounds were added at their maximum non-toxic concentrations (∼10^{-5} M) to the cell culture with the virus. The antiviral activity of the peptides was determined initially using a CPE. The inhibition of the viral CPE was assessed by a light microscopy. Virus titers were determined according to the Reed–Muench formula [16] and expressed in $TCID_{50}$/ ml at the particular stages of the experiments. The antiviral activity of the investigated peptides was finally expressed as a reduction ratio of the virus titers by comparison with the virus control.

Results and Discussion

The antiviral bioassay shows that all the investigated peptides evidently inhibit the replication of HHV-1_{MC} virus *in vitro* at their maximum non-toxic concentrations after 24 and 48 h (Tables 2 and 3).

We found that alloferon at the concentration of 90 µg/ml inhibits *in vitro* the replication of virus HHV-1_{MC} by 2 log after 24 h of incubation but its inhibitory effect after 48 h of incubation is weaker (1.5 log). A similar activity was observed after 24 h for analogs of alloferon modified at position 1 (peptides **2**–**5**). However, these peptides after 48 h of incubation demonstrate a weak inhibitory effect on the replication of virus HHV-1.

Interesting results in the antiviral test were also observed in the case of the second group of investigated peptides.

Any-GS (**6**) at the concentration of 52.5 µg/ml inhibits *in vitro* the replication of HHV-1_{MC} after 24 and 48 h by 1.5 and 1 log, respectively (Table 3). The analogues of *Any*-GS modified at position 1 of the peptide chain (peptides **7**–**11**), in which the *N*-terminal Asp residue was exchanged by Arg, Gly, Ala, Asn or Gln, reduce the virus titer by 2 to 1 log after 24 h. After 48 h of incubation, this effect is becoming weaker and the peptides reduce the virus titer by 0.5 to

Table 2. Influence of alloferon and its analogues on the replication of the HHV-1_{MC} virus *in vitro*

	Concentration (µg/ml)		Effect after 24-h incubation			Effect after 48-h incubation		
			Virus titer ($TCID_{50}$/ ml)			Virus titer ($TCID_{50}$/ ml)		
Peptide	Stock solution	MNC[a]	Control	Sample	Inhibition ($logTCID_{50}$/ ml)	Control	Sample	Inhibition ($logTCID_{50}$/ ml)
1	360	90	8×10^3	1.1×10^2	2	1.8×10^4	8×10^2	1.5
2	155	38.7	1.7×10^3	7×10	1.5	3×10^3	3×10^2	1
3	160	40	1.7×10^3	1.3×10	2	3×10^3	3×10^2	1
4	150	37.5	1.7×10^3	3×10	2	3×10^3	3×10^2	1
5	150	37.5	1.7×10^3	0.7×10	2.5	3×10^3	3×10^2	1

[a] Maximal non-toxic concentration.

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Table 3. Influence of *Any*-GS and its analogues on the replication of the HHV-1_{MC} virus *in vitro*

Peptide	Concentration (μg/ml)		Effect after 24-h incubation			Effect after 48-h incubation		
			Virus titer ($TCID_{50}$/ ml)		Inhibition ($logTCID_{50}$/ ml)	Virus titer ($TCID_{50}$/ ml)		Inhibition ($logTCID_{50}$/ ml)
	Stock solution	MNC[a]	Control	Sample		Control	Sample	
6	210	52.5	8×10^3	3×10^2	1.5	1.8×10^4	1×10^3	1
7	250	62.5	1.7×10^3	1.6×10	2	3×10^3	2.1×10^2	1
8	135	33.7	1.7×10^3	3×10	2	3×10^3	1.1×10^2	1
9	160	40	1.7×10^3	2.8×10	2	3×10^3	8×10^2	1.5
10	195	48.8	1.7×10^3	7×10	1.5	3×10^3	8.3×10^2	0.5
11	105	26.3	1.7×10^3	3×10	2	3×10^3	3×10^2	1
12	230	57.5	8×10^3	1.8×10^2	1.5	1.8×10^4	3×10^2	2
13	206	51.5	8×10^3	1.3×10^2	2	1.8×10^4	5×10^2	1.5
14	230	57.5	8×10^3	1.2×10^3	1	1.8×10^4	1.1×10^3	1.5

[a] Maximal non-toxic concentration.

1 log. It is interesting that the truncated analogues of *Any*-GS without the *C*-terminal Gly (**12**), *N*-terminal Asp (**13**) or the fragment Asp-Ile (**14**) preserved the antiviral properties of native *Any*-GS.

These results can indicate that the *C*-terminal amino acid residues of *Any*-GS are important for the antiviral activity. However, the deletion of the first two residues of *Any*-GS resulted in a loss of the cell growth suppression activity on dRLh84 cells [4]. A more detailed study to clarify this effect is in progress.

Moreover, alloferon, *Any*-GS, and their investigated analogs did not show any cytotoxic activity against the Vero cells at tested concentrations. Microscopic observations showed that no changes occurred in the Vero cell growth or morphology in the presence of the tested peptides. The MTT assay also proved that they had no effect on the cell proliferation.

Conclusions

Based on the biological results presented here we can conclude that:

- The investigated peptides strongly inhibit the replication of HHV-1 in Vero cells.
- The presence of hydrophilic residues at position 1 of alloferon is not important for inhibition of the virus replication.
- The basic or acidic character of residues at position 1 of *Any*-GS does not affect the antiviral activity.
- No cytotoxic activity was observed for all tested compounds against the Vero cells.
  These effects constitute a basis for future studies on the antiviral activity of insect peptides and may lead to development of novel antiviral drug.

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