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Novel analogs of alloferon: Synthesis, conformational studies, pro-apoptotic and antiviral activity

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ABSTRACT

In this study, we report the structure-activity relationships of novel derivatives of the insect peptide alloferon (H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH). The peptide structure was modified by exchanging His at position 9 or 12 for natural or non-natural amino acids. Biological properties of these peptides were determined in antiviral *in vitro* test against *Human Herpes Virus* 1 McIntrie strain (HHV-1_{MC}) using a Vero cell line. The peptides were also evaluated for the pro-apoptotic action *in vivo* on hemocytes of the *Tenebrio molitor* beetle. Additionally, the structural properties of alloferon analogs were examined by the circular dichroism in water and methanol. It was found that most of the evaluated peptides can reduce the HHV-1 titer in Vero cells. [Ala^9]-alloferon exhibits the strongest antiviral activity among the analyzed compounds. However, no cytotoxic activity against Vero cell line was observed for all the studied peptides. *In vivo* assays with hemocytes of *T. molitor* showed that [Lys^9]-, [Phg^9]-, [Lys^{12}]-, and [Phe^{12}]-alloferon exhibit a twofold increase in caspases activity in comparison with the native peptide. The CD conformational studies indicate that the investigated peptides seem to prefer the unordered conformation.

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1. Introduction

Alloferon is a tridecapeptide (H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH) isolated from blood of an experimentally infected larvae of the blow fly *Calliphora vicina*. Larvae were experimentally infected by pricking cuticle with a needle soaked in a suspension of heat-killed *Escherichia coli* and *Micrococcus luteus* cells [1].

It has been reported that this peptide displays the antitumor [1–4] and antiviral activities toward influenza virus, herpes viruses, and coxsackievirus [1,5,6]. Alloferon induces also the interferon (IFN) synthesis *in vivo* which was demonstrated using animal and human models. This compound also stimulates the cytotoxic activity of natural killer (NK) cells [2,3]. Thus, alloferon is interesting as a potential anticancer or antiviral drug.

Additionally, several new biological properties of alloferon have been found [7–12]. It was observed that alloferon at a dose of 10 nM strongly induces *Tenebrio molitor* hemocytes to undergo apoptosis [9] and *in vitro* studies revealed a weak cardiostimulatory activity of alloferon in *Zophobas atratus* [9]. Recently, our works also demonstrated that alloferon exhibits antinociceptive activity in rats and this effect is mediated by opioid receptors [7]. Moreover, it significantly decreased the level of tumor necrosis factor alpha (TNF-α) and vascular endothelial growth factor (VEGF), insignificantly decreased the IFN γ level, and increased the production of IL-2 (interleukin 2) in rats' plasma [10].

Recently, it has been reported that a structural analog of alloferon, referred to as allostatine (H-His-Gly-Val-Ser-Gly-Trp-Gly-Gln-His-Gly-Thr-His-Gly-OH), has antitumor properties and it is interesting as a potential anticancer drug [4].

However, in the literature not much attention has been paid to the influence of individual amino acids in the alloferon peptide chain on its structure and biological activity [5,9,13–17].

Abbreviations: CD, circular dichroism; CPE, cytopathic effect; DMF, dimethylformamide; FBS, fetal bovine serum; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluorophosphate; HOBt, N-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NMM, N-methylmorpholine; TCID, tissue culture infected dose; TFA, trifluoroacetic acid; TIS, triisopropylsilane; UV, ultraviolet.

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The preliminary structure-activity studies show that the presence of the aromatic ring at position 1 of the alloferon peptide chain can play a role in the expression of antiviral properties *in vitro*. In addition, [Lys[1]]-alloferon exhibits a strong antiviral activity against reference and clinical strains of the *Human Herpesvirus* type 1 (HHV-1) in Vero cells and Coxsackievirus B-2 (CVB-2) in HEp-2 cells [5]. It was also observed that removal of two *N*-terminal amino acids ([3-13]-alloferon) caused a greater reduction of the titer of the standard strain of 971 PT Coxsackievirus B-2 in HEp-2 cells [17]. Furthermore, the biological studies show that the *N*- and *C*-terminally truncated alloferon analogs containing the *C*-terminal sequence His-Gly strongly induce *T. molitor* hemocytes to undergo apoptosis. Moreover, [Phe(p-NH_2)[1]]- and [Tyr[6]]-alloferon exhibit a twofold increase in caspases activity in comparison with the native peptide [9]. On the basis of our results, we concluded that not only basic or aromatic character but also the volume of the side chain of amino acid at position 1 or 6 is responsible for the biologically active alloferon conformation.

The results obtained in our study have inspired us to further studies on the structure/function relationship of alloferon.

In this work, we synthesized the following peptides:

1. Alloferon and its analogs modified at position 9 of the peptide chain

H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**His**-Gly-Val-His-Gly-OH (I)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Ala**-Gly-Val-His-Gly-OH (II)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Arg**-Gly-Val-His-Gly-OH (III)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Lys**-Gly-Val-His-Gly-OH (IV)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Phe**-Gly-Val-His-Gly-OH (V)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Phg**-Gly-Val-His-Gly-OH (VI)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Tyr**-Gly-Val-His-Gly-OH (VII)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Trp**-Gly-Val-His-Gly-OH (VIII)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Phe(*p*-Cl)**-Gly-Val-His-Gly-OH (IX)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-**Phe(*p*-OMe)**-Gly-Val-His-Gly-OH (X)

2. Alloferon analogs modified at position 12 of the peptide chain

H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Ala**-Gly-OH (XI)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Lys**-Gly-OH (XII)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Arg**-Gly-OH (XIII)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Phe**-Gly-OH (XIV)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Phg**-Gly-OH (XV)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Tyr**-Gly-OH (XVI)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Trp**-Gly-OH (XVII)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Phe(*p*-Cl)**-Gly-OH (XVIII)
H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-**Phe(*p*-OMe)**-Gly-OH (XIX)

Biological effects of these peptides were evaluated *in vitro* against *Human Herpes Virus* 1 McIntyre strain (HHV-1_{MC}) using a Vero cell line and *in vivo* in relation to hemocytes of *T. molitor*.

Furthermore, CD studies were carried out to obtain information about the role of a peptide conformation in the biological activity of alloferon.

2. Material and methods

2.1. Materials

The Wang resins preloaded with Fmoc-Gly and Fmoc-amino acids, HOBt, HBTU, and TFA were purchased from IRIS Biotech. HPLC-grade solvents were purchased from Fisher Scientific. All other reagents were purchased from Sigma-Aldrich. All solvents and reagents used for the 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.

2.2. Synthesis

Peptides were obtained by a stepwise elongation of the peptide chain according to procedures described previously by Kuczer et al. [9]. In brief, the analogs were synthesized by the classical solid phase method using the Fmoc procedure starting from an Fmoc-Gly-Wang resin. Synthesis was performed in disposable plastic reactors (Intavis AG). Fmoc protecting groups were removed using 20% piperidine in DMF. Subsequently, Fmoc-protected amino acids (3 equiv) were attached by using 3 equiv of HBTU as the coupling agent in the presence of HOBt (3 equiv) and NMM (6 equiv) for 2 h at room temperature.

The completeness of each coupling reaction was monitored by the Kaiser test [18].

Final cleavage of the peptides was achieved with TFA, TIS, and water (95:2.5:2.5 v/v) for 2 h at room temperature. The crude peptides were precipitated from cold diethyl ether, washed with diethyl ether, dissolved in water, and lyophilized. The peptides were purified by semipreparative HPLC using a Varian ProStar chromatograph equipped with a TOSOH Bioscience C18 column (21.5 mm × 300 mm) (Tosoh, Tokyo, Japan) and a 210/254 nm dual-wavelength UV detector. Water–acetonitrile gradients containing 0.1% TFA at a flow rate of 7 ml/min were used for purification. The final purity of the lyophilized peptides was >95% according to analytical HPLC (Thermo Separation Product; column: Vydac Protein RP C18 (4.6 mm × 250 mm) (Grace, Deerfield, IL, USA); linear gradient 0–100% B in 60 min, solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in 80% acetonitrile/water, UV detection at 210 nm). Additional HPLC analyses were performed, using a Varian Microsorb-MV 100-5 CN column (4.6 mm × 250 mm) (Varian, Palo Alto, CA, USA) with a linear gradient from 0% to 100% B for 40 min, flow rate 1.0 ml/min, solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in 80% acetonitrile/water.

Finally, the peptides were re-dissolved in 50% acetic acid in water and then re-lyophilized. The chemical identities of the peptides were confirmed by ESI-MS using micrOTOF-Q or Apex-Qe Ultra 7T FT-ICR instruments (Bruker Daltonic, Bremen, Germany).

2.3. 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 or methanol at the concentration

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