

# Investigation on chemotactic drug targeting (chemotaxis and adhesion) inducer effect of GnRH-III derivatives in *Tetrahymena* and human leukemia cell line

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GnRH-III has been shown to exert a cytotoxic effect on the GnRH-R positive tumor cells. The chemotactic drug targeting (CDT) represents a new way for drug delivery approach based on selective chemoattractant guided targeting. The major goal of the present work was to develop and investigate various GnRH-III derivatives as potential targeting moieties for CDT. The cell physiological effects (chemotaxis, adhesion, and signaling) induced by three native GnRHs (hGnRH-I, cGnRH-II, and IGnRH-III) and nine GnRH-III derivatives were evaluated in two model cells (*Tetrahymena pyriformis* and Mono Mac 6 human monocytes). According to our results, the native GnRH-III elicited the highest chemoattractant and adhesion inducer activities of all synthesized peptides in micromolar concentrations in monocytes. With respect to chemoattraction, dimeric derivatives linked by a disulfide bridge ([GnRH-III(C)]<sub>2</sub>) proved to be efficient in both model cells; furthermore, acetylation of the linker region ([GnRH-III(Ac-C)]<sub>2</sub>) could slightly improve the chemotactic and adhesion effects in monocytes. The length of the peptide and the type of N-terminal amino acid could also determine the chemotactic and adhesion modulation potency of each fragment. The application of the chemoattractant GnRH-III derivatives was accompanied by a significant activation of phosphatidylinositol 3-kinase in both model cells. In summary, our work on low-level differentiated model cells of tumors has proved that GnRH-III and some of its synthetic derivatives are promising candidates to be applied in CDT: these compounds might act both as carrier, delivery unit, and antitumor agents. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

**Keywords:** gonadotropin-releasing hormone III; drug targeting; chemotaxis; adhesion; dimer derivative; *Tetrahymena*

## Introduction

The principle of CDT was developed in our laboratory in 2006 [1]. According to the theory, this special type of drug delivery is based on the chemotactic activity of a DDS, which is exploited to selectively target the attached drug to the target cells. The high-affinity binding of DDS to specific receptors could stimulate the positive chemotactic response of the target cells. This type of drug targeting could be utilized for the motile phenotype of cells, which is intrinsic or evoked in certain pathological stages (e.g. malignancy and metastasis of tumors) [2]. In our previous studies, tuftsin-based oligomeric carriers were investigated for CDT [1,3]. It was found that oligotuftsin preserved the chemoattractant behavior of the native terapeptide tuftsin on monocytes and macrophages. Furthermore, in DDSs, the chemoattractant potency of oligotuftsin and their branched derivatives with additional chemotactic peptides in branching compensate the chemorepellent effect of the attached drug; methotrexate [1,3]. In the last years, the GnRH derivatives were developed in our laboratory as targeting moieties for the preparation of DDSs [4]. Therefore, in the present study, the chemotactic activity of the GnRH derivatives as potential targeting moieties for CDT was investigated.

The decapeptide GnRH, also called luteinizing hormone-releasing hormone, plays a pivotal role in the regulation of the gonadal steroidogenesis and gametogenesis by inducing the synthesis and

release of the follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The mammalian GnRH-I (Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH<sub>2</sub>) was the first isoform discovered and found also in human (hGnRH-I = GnRH-I). The second variant of the hormone, chicken GnRH-II (cGnRH-II = GnRH-II; Glp-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH<sub>2</sub>) has been also found in the human body [5]. The GnRHs and their GnRH-R expressed by various cells and tissues inside and outside the central nervous system indicate numerous

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**Abbreviations:** CDT, chemotactic drug targeting; CI, cell index; DDS, drug delivery system; GnRH, gonadotropin-releasing hormone; GnRH-R, gonadotropin-releasing hormone receptor; *Inh*<sub>ind</sub>, inhibition index; LY, LY294002 PI3K inhibitor; MM6, Mono Mac 6 human monocytic cell line; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C.

and diverse functions. The GnRH–GnRH-R systems are potent regulators of the hypothalamic–pituitary–gonadal axis, and they act as autocrine/paracrine factors in several peripheral, hormone-related, and unrelated tissues (placenta, myometrium, and lymphoid cells) [6–8].

Several synthetic GnRH analogs, both agonists and antagonists, are used in the treatment of hormone-dependent tumors by suppressing the sex steroid production through downregulation and desensitization of the GnRH-Rs in pituitary [6,9]. However, it is widely accepted that the antitumor effect of the GnRH derivatives might be also mediated by a direct action, via the GnRH-Rs that are highly expressed in many cancer tissues either related (prostate, breast, ovarian, and endometrial) or unrelated (melanoma, colon, lung, pancreatic, etc.) to the reproductive system [6,10–12]. Therefore, the GnRH-Rs represent a good target for selective drug delivery. The targeted cancer chemotherapy may overcome the drawbacks of the traditional chemotherapy, such as toxicity on healthy cells, fast elimination from the circulation, and development of multidrug resistance of cancer cells. The full-length and the N-terminal truncated GnRH-I analogs were applied for selective drug targeting to tumor cells [13,14]. However, these compounds have significant endocrine effect that is not always favorable in tumor therapy, especially not in the case of the treatment of hormone-independent tumors. To avoid the endocrine side effects, the GnRH-III related peptides were developed [12].

The third isoform of GnRH (IGnRH-III = GnRH-III; Glp-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH<sub>2</sub>) was originally isolated from the sea lamprey (*Petromyzon marinus*) [15]. GnRH-III is not a natural ligand of the human GnRH-R, but it binds specifically to the GnRH-Rs on cancer cells. In this way, GnRH-III directly inhibits the growth of the GnRH-R positive tumors [12]. Besides its remarkable antiproliferative effect, GnRH-III has low endocrine potency in mammals [16], and it does not contain any D-amino acids or nonnatural amino acids, which are associated with numerous allergic side effects of synthetic derivatives with potent antitumor activity. All the facts mentioned earlier might indicate that GnRH-III is the most effective and selective native GnRH analog able of inhibiting the tumor growth [17]. Previous studies on structure-activity relationship indicated that the modification of the Lys<sup>8</sup> in GnRH-III did not result in the loss of receptor binding and antiproliferative effect [12,16,18,19]. Furthermore, the elimination of the free ε-amino group resulted in the decrease of endocrine effect of the parent hormone peptide [16,20]. In our previous experiments, a disulfide bridge linked symmetric dimer derivatives were developed to improve the therapeutic potency of GnRH-III. In the case of the symmetric dimers, elevated antiproliferative effect, enhanced stability to proteolysis, and decreased endocrine activity were determined compared with the native GnRH-III [4,20]. The dimers containing an enzyme labile spacer CGFLG sequence on the side chain of Lys<sup>8</sup> showed significant *in vivo* antitumor activity (44% tumor growth inhibition) in HT-29 bearing nude mice after continuous treatment for 40 days. The increased tumor cell-specific effect of the dimers was attributed to the protection of the peptides from proteolysis. The presence of dimers might result to microaggregation of receptors or an enhanced availability of receptor interactions as well as the internalization of the hormone dimers [4,19].

In spite of the importance of the conserved N-terminal sequence of the GnRHs for receptor binding [5], our preliminary data showed that the cellular uptake of the N-terminal truncated and the carboxyfluorescein-labeled GnRH-III derivatives proved to

be similar to the full-length GnRH-III peptide in case of HT-29 human colon and MCF-7 human breast cancer cells [21]. Therefore, both the dimer and the N-terminal truncated GnRH-III derivatives might be good candidates for selective drug targeting. For this purpose, the evaluation of their chemotactic properties may provide useful information for the development of DDSs.

Besides the well-established role of the GnRHs in the tumor progression, the widely expressed GnRH-Rs suggest numerous and diverse functions of the hormones, including modulation of neuronal migration during the development [8], adhesion, chemotaxis and homing in the T lymphocytes [7], cell invasion, and consequently the metastatic potential of, e.g. melanoma [22], ovarian, and prostatic carcinoma cells [23,24]. A GnRH agonist as a negative regulator of the melanoma proliferation exerts inhibitory effect on migration and invasiveness of the melanoma cells by interfering the expression of adhesion molecules (e.g. α3 integrin) and of matrix metalloproteinase-2 [22,25].

Many types of cancer cells exhibit defect in normal differentiation process, which was recognized as an important component of tumorigenesis. The degree of dedifferentiation (e.g. anchorage independent growth and resistance to apoptosis) basically determines the prognosis and treatment of a cancer [26]. This prompted us to investigate the therapeutic significance of GnRH-III and its derivatives (dimers and fragments) in monocytes derived from leukemia and in a eukaryote unicellular, the *Tetrahymena pyriformis*. Because the growth of *Tetrahymena* does not require a solid surface covered by substrates, and its proliferation rate is rather high similarly to the tumor cells; the *Tetrahymena* could be used as a model for preliminary studies of chemotaxis-based targeted tumor therapy [27,28]. The chemotactic responsiveness is a fundamental characteristic of monocytes and in the ciliate *Tetrahymena*. This unicellular model cell could recognize signal molecules derived from higher-ranked animals, has receptors for the mammalian hormones (e.g. insulin and histamine), and chemotactically responds to them in the same concentration range and direction as mammalian model cells. The presence of second messenger systems (e.g. cAMP, cGMP, Ca<sup>2+</sup>-calmodulin and inositol phospholipids) adequate for chemotactic signal transmission and immunologically identical substances to vertebrate signal molecules (e.g. insulin, human chorionic gonadotropin-like hormones: FSH and LH) was also described in *Tetrahymena* [29–32]. Moreover, its highly selective chemotactic responsiveness to structure-related ligands including hypothalamo–hypophyseal hormones (thyroid-stimulating hormone–FSH and oxytocin–vasopressin) [29,33] supports the selection of *Tetrahymena* for screening new, potentially chemotactic GnRH-III peptides.

In the present work, we report on the synthesis and cell biological characterization of different GnRH-III derivatives (symmetric dimers and fragments) to find suitable drug carrier for CDT.

The main objectives of our work were as follows:

- (i) To investigate the chemotactic properties of the GnRH-III derivatives in comparison with the native GnRH hormones in diverse model cells (*T. pyriformis* and the leukemic MM6).
- (ii) To examine the chemotactic signaling (activation of the PLC and PI3K enzymes) of different GnRH derivatives.
- (iii) To investigate the cell adhesion modulator effects of the GnRH ligands in monocytes.
- (iv) To evaluate the relationships between the structures of the ligands and the biological effects in order to establish the optimal structure for the CDT.

## Materials and Methods

### Chemicals

All amino acid derivatives and 4-methylbenzhydrylamine (MBHA) resin were purchased from Reanal (Budapest, Hungary) or NovaBiochem (Läufelfingen, Switzerland). Chemicals for the syntheses of DCC, DIC, HOBt, piperidine, DBU, TFA, hydrogen fluoride, 1,4-DL-dithiothreitol (DTT), ethanedithiol, *p*-cresol, DIEA, and acetic anhydride (Ac<sub>2</sub>O) were obtained from Sigma-Aldrich Kft. (Budapest, Hungary), whereas the solvents (DCM, DMF, ethanol, and diethyl ether) were purchased from Reanal. Acetonitrile for HPLC was from Sigma-Aldrich Kft. All reagents and solvents were of analytical grade or highest available purity.

### Peptide Synthesis

#### *Synthesis of the linear native and truncated GnRH derivatives*

All the linear GnRH peptides were synthesized manually on MBHA resin (1.04 or 1.2 mmol/g capacity) using mixed Boc and Fmoc strategy. The peptides were built up by Boc chemistry up to the first Trp residue that was attached to the peptide chain as its Fmoc derivative. The rest of the peptide was built up by Fmoc chemistry. In the case of the full-length native GnRH isoforms, the pyroglutamic acid (Glp) was coupled to the N-terminus of the peptide without any protection. For couplings carbodiimide, (DCC or DIC)/HOBt procedure was applied. The N-termini of the truncated peptides were acetylated with Ac<sub>2</sub>O–DIEA–DMF (1:1:3, v/v/v) mixture for 30 min. Two-step cleavage method was used for the removal of side chain protecting groups and the peptide from the resin. In the first cleavage procedure, Trt and tBu groups were cleaved with a mixture of TFA–water–ethanedithiol (95:2.5:2.5, v/v/v) at room temperature for 1 h. After washing (DCM; 5 × 0.5 min), neutralization (10% DIEA/DCM; 3 × 1 min), and washing (DCM; 4 × 0.5 min and ethanol; 2 × 0.5 min) again, the peptide resin was dried. The remaining protecting groups were cleaved simultaneously with the removal of the peptide from the resin using anhydrous hydrogen fluoride in the presence of *p*-cresol and DTT (hydrogen fluoride–*p*-cresol–DTT = 10 ml:1 g:0.1 g). Crude peptides were purified by semi-preparative RP-HPLC, and the pure compounds were characterized by HPLC and ESI-MS (Table 1).

#### *Synthesis of the branched GnRH-III derivatives*

The synthesis of the Cys-containing branched GnRH-III derivatives without GFLG spacer was described previously [20]. The branched GnRH-III derivatives with GFLG spacer were prepared similarly to them. Briefly, the first two C-terminal amino acids (Gly and Pro) were coupled to the MBHA resin (1.04 mmol/g capacity) as their Boc derivatives. Then, Fmoc-Lys(Boc)-OH was attached to the peptide resin. The Boc group from the side chain of the Fmoc-Lys(Boc)-Pro-Gly-resin was removed, and Boc-Gly-OH, Boc-Leu-OH, Boc-Phe-OH, Boc-Gly-OH, and Boc-Cys (Meb)-OH were attached step by step in the branch. For the preparation of the acetylated version of the peptide, the Boc group was cleaved from the amino group of Cys, and it was acetylated using Ac<sub>2</sub>O–DIEA–DMF (1:1:3, v/v/v) mixture. The other part of the peptide chain was assembled by Fmoc chemistry. Two-step cleavage procedure was applied to obtain the free peptides as described previously. The crude products were purified by semi-preparative RP-HPLC, and the pure compounds were characterized by HPLC and ESI-MS (Table 1).

#### *Dimerization of the Cys-containing GnRH-III derivatives*

The purified cysteine-containing peptides were dissolved in 0.1-M Tris buffer (pH 8.1) at a peptide concentration of 10 mg/ml. The air oxidation at room temperature was performed for 2 h in the case of the peptides with free N<sup>α</sup>-amino group of Cys and overnight for the N<sup>α</sup>-acetylated compounds and monitored by analytical RP-HPLC. The reaction mixtures were acidified to pH 3 and purified on a semi-preparative HPLC column. Dimer peptides were characterized by RP-HPLC and ESI-MS mass spectrometry (Table 1).

### Purification and Analysis

#### *RP-HPLC*

Peptides were purified and analyzed by RP-HPLC on a Knauer (H. Knauer, Bad Homburg, Germany) HPLC system. The crude products were purified on a semi-preparative Phenomenex Jupiter C18 column (250 mm × 10 mm inside diameter) with 10-μm silica (300-Å pore size) (Torrance, CA, USA). The flow rate was 4 ml/min. Linear gradient of 10–60% B in 50 min was applied using eluent A (0.1% TFA in water) and eluent B [0.1% TFA in acetonitrile–water (80:20, v/v)]. Analytical HPLC was performed on a Phenomenex Synergy C12 column Max RP (250 × 4.6 mm inside diameter) with a 4-μm silica (80-Å pore size) as a stationary phase. Linear gradient elution (0 min 1% B; 5 min 1% B; 50 min 99% B) with the same eluents was used at a flow rate of 1 ml/min. Peaks were detected at λ = 220 nm.

#### *ESI-MS*

The identification of the products was achieved by MS. ESI-MS was performed with a Bruker Daltonics Esquire 3000 Plus (Bremen, Germany) mass spectrometer, operating in continuous sample injection at 4 μl/min flow rate. Samples were dissolved in 50% acetonitrile–water mixture. Mass spectra were recorded in positive mode in the *m/z* 50–2000 range.

### Cells and Culturing

Cultures of *Tetrahymena pyriformis* GL were grown in culture medium with 1% tryptone (Difco Laboratories, Detroit, MI, USA) and 0.1% yeast extract (Difco Laboratories) at 28 °C, in atmospheric CO<sub>2</sub>/O<sub>2</sub> ratio. *T. pyriformis* cultures were used in the logarithmic phase of growth, which was equivalent to a concentration of 10<sup>4</sup> cells/ml.

Cultures of MM6 [34] were maintained in RPMI 1640 (Sigma Ltd., St. Louis, MO, USA) containing 10% fetal calf serum, L-glutamine (2 mM/ml), gentamicin (16 μg/ml) at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere.

### Chemotaxis Assay

#### *Chemotaxis assay of T. pyriformis*

The chemotactic ability of *Tetrahymena* cells was determined by a modified version of Leick's two-chamber capillary chemotaxis assay [35,36]. In this assay, pipette tips of an eight-channel micropipette filled with the test substances were used as the upper chamber. Wells of a microtitration plate filled with cell cultures served as lower chambers. The incubation time was 15 min; previous experiments proved this time to be optimal as the concentration gradient is still present in the chamber [37]. After incubation, the samples were fixed with 4% formaldehyde solved in phosphate-buffered saline (pH = 7.2). The number of the cells was determined oculometrically by Neubauer hemocytometer

**Table 1.** Molecular structures and analytical parameters of the GnRH derivatives

Peptides	Structures	$t_R$ (min) <sup>a</sup>	$M_{mo}$ (calculated) <sup>b</sup>	M (measured) <sup>b</sup>
GnRH-I	<EHWSYGLRPG-NH <sub>2</sub>	18.0	1182.3	1182.0
GnRH-II	<EHWSHGWYPG-NH <sub>2</sub>	23.2	1236.6	1236.6
GnRH-III	<EHWSHDWKPG-NH <sub>2</sub>	21.5	1258.3	1258.6
Ac-HWSHDWKPG-NH <sub>2</sub>		21.9	1190.1	1189.8
Ac-WSHDWKPG-NH <sub>2</sub>		23.6	1053.0	1052.8
Ac-SHDWKPG-NH <sub>2</sub>		16.2	866.8	866.6
GnRH-III(C)	<EHWSHDWKPG-NH <sub>2</sub> H-C↓	22.1	1359.5	1359.0
GnRH-III(Ac-C)	<EHWSHDWKPG-NH <sub>2</sub> Ac-C↓	23.1	1404.5	1404.0
GnRH-III(CGFLG)	<EHWSHDWKPG-NH <sub>2</sub> H-CGFLG↓	22.9	1737.0	1737.5
GnRH-III(Ac-CGFLG)	<EHWSHDWKPG-NH <sub>2</sub> Ac-CGFLG↓	27.2	1779.0	1778.6
[GnRH-III(C)] <sub>2</sub>	<EHWSHDWKPG-NH <sub>2</sub> H-C↓ H-C↓ <EHWSHDWKPG-NH <sub>2</sub>	22.0	2723.0	2723.7
[GnRH-III(Ac-C)] <sub>2</sub>	<EHWSHDWKPG-NH <sub>2</sub> Ac-C↓ Ac-C↓ <EHWSHDWKPG-NH <sub>2</sub>	23.7	2807.0	2807.7
[GnRH-III(CGFLG)] <sub>2</sub>	<EHWSHDWKPG-NH <sub>2</sub> H-CGFLG↓ H-CGFLG↓ <EHWSHDWKPG-NH <sub>2</sub>	29.4	3471.4	3472.5
[GnRH-III(Ac-CGFLG)] <sub>2</sub>	<EHWSHDWKPG-NH <sub>2</sub> Ac-CGFLG↓ Ac-CGFLG↓ <EHWSHDWKPG-NH <sub>2</sub>	27.2	3555.9	3556.5

<sup>a</sup>Column: Phenomenex Synergy C12 column Max RP (250 × 4.6 mm inside diameter) with a 4-μm silica (80-Å pore size); eluents: eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile–water (80:20, v/v)); linear gradient elution: 0 min 1% B; 5 min 1% B; 50 min 99% B; flow rate: 1 ml/min; detection: λ = 220 nm.

<sup>b</sup>ESI-MS was carried out with Bruker Daltonics Esquire 3000 Plus mass spectrometer.

in light microscope Zeiss AXIO Observer A1 (Carl Zeiss Microscopy GmbH, München, Germany). All experiments were repeated five times. The evaluated value was normalized to the control and was given as 'chemotaxis index' in percents.

#### Chemotaxis assay of MM6 cells

Chemotactic responsiveness of the MM6 cell line was measured by a modified Boyden chamber technique in a NeuroProbe<sup>®</sup> MBB 96 chamber (NeuroProbe, Gaithersburg, MD, USA). This assay is also a two-chamber chemotactic assay; however, because of the diverse migratory behavior of monocytes to ciliates, the position of cells and test substances was changed. In this system, the examined substances were put into wells of a 96-well micro-titration plate as the lower chamber, while the cells were applied into the upper chambers separated from the lower one by polycarbonate filter (pore size 8 μm). The incubation time was

3 h at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. The number of the positive chemotactic responder cells was determined by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Sigma Ltd) assay. MTT solution was added to each sample at 0.5 mg/ml final concentration. After overnight incubation (16 h), the culture medium was removed and formazan crystals were dissolved in 100-μl DMSO. The absorbance was measured at 540 and 620 nm by ELISA reader (Labsystems Multiskan MS, Helsinki, Finland). Identical points of the concentration course study represent an average of 15 parallel measurements.

#### Cell Adhesion Assay of MM6 Cells

The effect of GnRH-III and its analogs on adhesion of the MM6 cells was assessed using the xCELLigence System (Roche Applied Science, Indianapolis, IN, USA), which monitor the cellular events by measuring electrical impedance across interdigitating gold



microelectrodes integrated on the bottom of tissue culture plate, so called E-plate. The xCELLigence SP System measures the change in impedance of gold microelectrodes to alternating current (AC) flow in real time manner. The detected impedance depends on the local ionic environment and whether the cells are attached to the surface of the electrodes. In the absence of the cells, the impedance is constant and determined as background. In the presence of the cells, their insulating plasma membranes constrain the electrical current and lead to an increase in the electrode impedance. The impedance depends on the number of the attached cells and on the dimensional change of the attached cells on the electrodes. More cells attached on the electrode, or spreading, cause larger increase in the impedance. The change in impedance is represented as CI. The CI is a relative and dimensionless value, and calculated by the following formula:

$$CI = \frac{(Z_i - Z_0)}{F_i}$$

where  $Z_i$  is the impedance at an individual point of time during the experiment,  $Z_0$  is the impedance at the start of the experiment, and  $F_i$  is a constant depending on the frequency ( $F_{10\text{kHz}} = 15$ ).

Briefly, the protocol is as follows: the electrodes were coated with human fibronectin (Chemicon® International Inc., Temecula, Canada). Mixture of  $1\ \mu\text{g}/\text{cm}^2$  human fibronectin in 0.1% gelatin (Sigma Ltd) was dropped to the bottom of each well. After 20-min incubation at  $4^\circ\text{C}$ , the protein solution was removed. To register the background value,  $100\ \mu\text{l}$  of culture medium was added to each well and impedance was recorded for 2 h. The given time interval was sufficient in each experiment to gain constant background curves of impedance. In the following two steps, the wells were filled with the solutions of the GnRH derivatives (peptide concentrations:  $10^{-11}$ – $10^{-6}\ \text{M}$ ), and finally, the wells were loaded with MM6 monocytes ( $10^4$  cells/ $100\ \mu\text{l}$ /well). Compound free wells served as a control. The adhesion of the MM6 cells was monitored in every 20 s for 24 h at 10 kHz.

The slope values calculated by the integrated software (RTCA 1.2 – Roche Applied Science, Indianapolis, IN, USA) were used for the statistical evaluation. The slope is used to describe the steepness, changing rate of an adhesion curve within a given time interval. Each data represents the mathematical average of three parallels.

### Inhibition of PI3K

Wortmannin, a metabolite of the fungus *Penicillium funiculosum*, is a specific natural inhibitor of PI3Ks whereas LY is a commonly used synthetic PI3K inhibitor acting on a different target [38,39]. The wortmannin and the LY were purchased from Sigma Ltd., and stock solutions were prepared in DMSO with a concentration of  $10^{-2}\ \text{M}$ . The role of PI3K in the chemotaxis of *T. pyriformis* cells elicited by the GnRH derivatives was studied by 5-min [40] pretreatments with  $10^{-5}\ \text{M}$  final concentration of wortmannin and LY [41,42]. The chemotactic ability of the pretreated cells was evaluated by the two-chamber capillary assay described earlier. The chemoattractant GnRH molecules in the optimal concentration were tested in this part of the study. To verify the effect of the solvent DMSO on ciliate migratory behaviors, the control experiments were performed with a culture medium containing the adequate volume of DMSO.

In case of MM6 cell line, the cells were pretreated for 25 min [41] with wortmannin and LY at the same concentration mentioned previously. After the treatment with the PI3K inhibitors, the cells were washed with fresh culture medium (RPMI 1640

containing 10% fetal calf serum, L-glutamine, and gentamicin). The chemotactic responsiveness of the pretreated MM6 cells was examined in NeuroProbe® chamber described earlier. DMSO, the solvent of the inhibitors was used as the control group, and the maximal chemoattractant concentrations of the identical GnRH derivatives were applied.

Chemotactic responsiveness of the pretreated cultures was determined in the following combinations: cells pretreated with DMSO were coursed towards control medium ( $C_c$ ) or a GnRH analog ( $G_c$ ); cells pretreated with wortmannin or LY were assayed for the control medium ( $C_{inh}$ ) or a GnRH derivative ( $G_{inh}$ ). The number of responding cells was used to determine  $Inh_{ind}$ , which was calculated by the following formula:

$$Inh_{ind}[\%] = \frac{G_{inh} \times C_c}{C_{inh} \times G_c} \times 100$$

### Statistical Evaluation of Data

The data shown in the figures represent averages expressed as the percentage of untreated control and  $\pm$ standard deviation (SD) values. Statistical analysis of data was carried out by the application of ANOVA (analysis of variance) of Origin Pro8.0 (OriginLab Corporation, Northampton, MA, USA). The level of significance is shown as follows:  $x - p < 0.05$ ;  $y - p < 0.01$ ;  $z - p < 0.001$ .

## Results

### Synthesis of the GnRH Derivatives

Three native forms of GnRH (GnRH-I, GnRH-II, and GnRH-III) and eight different GnRH-III derivatives, which might be potential targeting moiety for drug delivery, were synthesized by solid-phase peptide synthesis using mixed Boc/Fmoc strategy. Four GnRH symmetric dimers ([GnRH-III(C)]<sub>2</sub>, [GnRH-III(Ac-C)]<sub>2</sub>, [GnRH-III(CGFLG)]<sub>2</sub>, and [GnRH-III(Ac-CGFLG)]<sub>2</sub>) were formed using air oxidation to establish a disulfide bridge between the cysteine-modified branched GnRH-III monomers. The three N-terminal truncated GnRH-III derivatives were also prepared (Ac-HWSDWKPG-NH<sub>2</sub>, Ac-WSHDWKPG-NH<sub>2</sub>, and Ac-SHDWKPG-NH<sub>2</sub>). The peptides were characterized by RP-HPLC and ESI-MS. The molecular structures and analytical data are summarized in Table 1.

### Chemotactic Effects in *T. pyriformis*

#### Native hormones

In general, the three decapeptide hormones elicited rather neutral effects in wide concentration ranges. Nevertheless, slight chemotactic activities were also detected: GnRH-II proved to have a weak chemorepellent effect (76–77%) in the  $10^{-11}$ – $10^{-7}\ \text{M}$  concentration range; in contrast, GnRH-I had more – but not significant – chemoattractant effects at  $10^{-7}$ – $10^{-6}\ \text{M}$  concentrations (132–130%) (Table 2).

#### N-terminal truncated peptide fragments

The Ac-HWSDWKPG-NH<sub>2</sub> elicited chemoattractant effect in a wide concentration range ( $10^{-10}$ – $10^{-6}\ \text{M}$ ) with a maximal effect (192%) at  $10^{-9}\ \text{M}$  concentration. Further, truncation of the N-terminal part (Ac-WSHDWKPG-NH<sub>2</sub>) reversed the chemoattractant property to a chemorepellent effect at  $10^{-9}$ – $10^{-6}\ \text{M}$  concentrations (66–57%). Interestingly, the elimination of the next Trp from the sequence (Ac-SHDWKPG-NH<sub>2</sub>) resulted in a slightly chemoattractant (128–145%) compound at high concentrations ( $10^{-8}$ – $10^{-6}\ \text{M}$ ) (Table 2).

**Table 2.** Concentration course study of chemotaxis induced by the GnRH derivatives in *T. pyriformis*

Peptides	Chemotaxis index <sup>a</sup> (%) (control = 100%)						
	10 <sup>-12</sup> M	10 <sup>-11</sup> M	10 <sup>-10</sup> M	10 <sup>-9</sup> M	10 <sup>-8</sup> M	10 <sup>-7</sup> M	10 <sup>-6</sup> M
GnRH-I	110 ± 19.6	100 ± 11.6	116 ± 14.8	125 ± 14.0	103 ± 15.5	132 ± 21.6	130 ± 17.4
GnRH-II	92 ± 11.2	76 ± 11.6	71 <sup>x</sup> ± 10.6	77 ± 6.5	81 ± 6.9	77 ± 11.6	98 ± 9.8
GnRH-III	110 ± 9.3	91 ± 7.2	106 ± 9.2	113 ± 12.7	104 ± 9.5	86 ± 6.2	79 ± 7.7
Ac-HWSDWKPG-NH <sub>2</sub>	138 ± 26.3	135 ± 22.8	151 ± 27.7	192 <sup>y</sup> ± 26.9	165 ± 50.4	165 ± 26.9	159 ± 31.3
Ac-WSHDWKPG-NH <sub>2</sub>	75 ± 9.4	98 ± 17.4	92 ± 13.5	66 <sup>x</sup> ± 4.7	81 ± 10.6	69 <sup>x</sup> ± 6.1	57 <sup>y</sup> ± 7.6
Ac-SHDWKPG-NH <sub>2</sub>	83 ± 14.5	112 ± 10.4	84 ± 9.3	117 ± 11.9	128 ± 15.8	144 <sup>x</sup> ± 13.9	145 ± 22.8
GnRH-III(Ac-C)	81 ± 10.6	129 ± 17.8	103 ± 20.8	80 ± 6.5	49 <sup>y</sup> ± 8.1	97 ± 12.4	56 <sup>y</sup> ± 5.4
GnRH-III(Ac-CGFLG)	111 ± 20.1	88 ± 9.2	115 ± 10.2	149 <sup>x</sup> ± 19.5	71 ± 10.9	93 ± 13.5	96 ± 18.5
[GnRH-III(C)] <sub>2</sub>	189 <sup>x</sup> ± 32.5	237 <sup>y</sup> ± 48.2	226 <sup>x</sup> ± 23.7	161 ± 22.5	126 ± 24.6	176 ± 36.3	150 ± 30.0
[GnRH-III(Ac-C)] <sub>2</sub>	123 ± 16.5	117 ± 16.0	134 ± 14.4	76 ± 11.5	104 ± 12.1	110 ± 11.8	69 ± 9.3
[GnRH-III(CGFLG)] <sub>2</sub>	49 <sup>x</sup> ± 10.7	49 <sup>x</sup> ± 7.3	70 ± 18.8	57 ± 10.7	61 ± 12.2	49 <sup>x</sup> ± 12.5	38 <sup>x</sup> ± 11.5
[GnRH-III(Ac-CGFLG)] <sub>2</sub>	90 ± 14.0	105 ± 14.7	113 ± 19.6	92 ± 22.2	140 ± 28.2	112 ± 26.7	121 ± 25.2

<sup>a</sup>Chemotaxis index was expressed as a percentage of the control.

Data represent the mean of five parallels ± SD.

The level of significance is shown as follows: x -  $p < 0.05$ ; y -  $p < 0.01$ ; z -  $p < 0.001$ .

### Cysteine-containing monomers and dimers

The chemotactic effects of the GnRH-III monomers were dependent on the structure of the branch on Lys<sup>8</sup>. GnRH-III(Ac-C) proved to acquire a chemorepellent character at high concentrations (10<sup>-8</sup> M: 49%, 10<sup>-6</sup> M: 56%), whereas elongation of the side chain with a GFLG sequence (GnRH-III(Ac-CGFLG)) resulted in a chemoattractant peak (149%) at 10<sup>-9</sup> M (Table 2). The nonacetylated versions of the monomers ([GnRH-III(C)] and GnRH-III(CGFLG)) dimerize very fast; therefore, they were not studied in this experiment [43]. The [GnRH-III(C)]<sub>2</sub> and [GnRH-III(Ac-C)]<sub>2</sub> dimers were built up from two GnRH-III molecules modified by the cysteine residue via a disulfide bridge. Formation of the nonacetylated dimer ([GnRH-III(C)]<sub>2</sub>) resulted in a compound that was chemoattractant at low (10<sup>-12</sup>–10<sup>-10</sup> M: 189–237%) and high concentrations (10<sup>-7</sup>–10<sup>-6</sup> M: 176–150%) as well. Acetylation of the cysteines ([GnRH-III(Ac-C)]<sub>2</sub>) depressed the chemotactic profile of [GnRH-III(C)]<sub>2</sub> to neutral level, whereas incorporation of a GFLG sequence between the cysteine residue and GnRH-III ([GnRH-III(CGFLG)]<sub>2</sub>) resulted to a wide range (10<sup>-12</sup>–10<sup>-6</sup> M) chemorepellent (38–49%) compound. The acetyl group in GnRH-III(Ac-CGFLG)]<sub>2</sub> could not elicit significant effects, and neutral character was detected at the concentrations studied (Table 2).

### Chemotactic Effects in MM6 Cell Line

#### Native hormones

GnRH-II and GnRH-III showed similar chemotactic characteristics. They were chemoattractant at the 10<sup>-7</sup> and 10<sup>-6</sup> M concentrations, and their effects were repellent in the 10<sup>-11</sup>–10<sup>-9</sup> M concentration range. Nevertheless, GnRH-III proved to be more chemoattractant (190–177%) at higher concentration and more repellent (58%) at the lower concentration than GnRH-II (range of attractance: 138–149%, range of repellence: 80–82%). Contrary to the aforementioned two molecules, GnRH-I exhibited its chemoattractant effect only at 10<sup>-9</sup> M (142%) (Table 3).

### N-terminal truncated peptide fragments

As it was detected in MM6 cell line, the chemotactic effect of the peptide fragments depended on the length of the GnRH-III sequence and/or on the type of amino acid at the N-terminus. The truncation of the first amino acid (Glp) resulted in Ac-HWSDWKPG-NH<sub>2</sub> possessing a chemorepellent effect (79–49%) in a wide concentration range (10<sup>-11</sup>–10<sup>-6</sup> M) (Table 3). In the case of Ac-WSHDWKPG-NH<sub>2</sub> fragment, an aromatic amino acid (Trp) is expressed at the N-terminus and a chemoattractant effect (126–148%) was detected at low concentrations (10<sup>-11</sup>–10<sup>-9</sup> M). The further truncation step (Ac-SHDWKPG-NH<sub>2</sub>) resulted in a moderate but a wide range chemorepellent character (Table 3).

### Cysteine-containing monomers and dimers

GnRH-III(Ac-C) monomer elicited a remarkable chemorepellent response both at low (10<sup>-11</sup>–10<sup>-10</sup> M: 46–26%) and high concentrations (10<sup>-7</sup>–10<sup>-6</sup> M: 41–24%) (Table 3). The [GnRH-III(C)]<sub>2</sub> dimer had biphasic chemotactic character, similarly to the parent hormone (GnRH-III), and it was repellent (67–26%) at low concentrations (10<sup>-11</sup>–10<sup>-9</sup> M) and attractant (151–135%) at 10<sup>-7</sup>–10<sup>-6</sup> M range. The profile of the concentration dependence of the chemotactic responsiveness induced by [GnRH-III(Ac-C)]<sub>2</sub> resembles to the activity elicited by the nonacetylated derivative. However, the acetylation of the dimer ([GnRH-III(Ac-C)]<sub>2</sub>) diminished the strong repellent effect (from 26% to 64%) at lower concentration (10<sup>-9</sup> M) and slightly enhanced the chemoattractant (from 151% to 171%) activity at 10<sup>-7</sup> M of [GnRH-III(C)]<sub>2</sub> (Table 3).

The concentration course study of GnRH-III(Ac-CGFLG) monomer showed a mirror arrangement of effectiveness compared with GnRH-III: the ligand had chemoattractant activity (197%) at 10<sup>-10</sup> M and it was significantly repellent (43–65%) at high concentrations (10<sup>-8</sup>–10<sup>-6</sup> M) (Table 3). The dimerization ([GnRH-III(CGFLG)]<sub>2</sub>) sustained the chemoattractant character (182%) but attenuated the repellence of the monomer to neutral level (10<sup>-9</sup>–10<sup>-6</sup> M). In this case, the acetylation ([GnRH-III(Ac-CGFLG)]<sub>2</sub>) resulted in the loss of the chemoattractant activity and showed a chemorepellent property both at low (10<sup>-11</sup> M: 68%) and at high concentrations (10<sup>-8</sup>–10<sup>-6</sup> M: 55–72%) (Table 3).

**Table 3.** Concentration course study of chemotaxis induced by the GnRH derivatives in MM6

Peptides	Chemotaxis index <sup>a</sup> (%) (control = 100%)					
	10 <sup>-11</sup> M	10 <sup>-10</sup> M	10 <sup>-9</sup> M	10 <sup>-8</sup> M	10 <sup>-7</sup> M	10 <sup>-6</sup> M
GnRH-I	137 ± 11.7	116 ± 11.1	142 <sup>x</sup> ± 14.0	111 ± 12.3	96 ± 8.4	132 ± 7.1
GnRH-II	80 <sup>x</sup> ± 5.0	99 ± 8.1	82 <sup>x</sup> ± 5.9	103 ± 15.7	149 <sup>x</sup> ± 16.8	138 ± 15
GnRH-III	118 ± 8.5	78 ± 10.0	58 <sup>y</sup> ± 5.5	104 ± 15.9	190 <sup>z</sup> ± 14.7	177 <sup>z</sup> ± 9.9
Ac-HWSDWKPG-NH <sub>2</sub>	79 ± 12.4	74 ± 8.7	85 ± 14.0	74 ± 18.4	73 ± 21.7	49 <sup>x</sup> ± 19.2
Ac-WSHDWKPG-NH <sub>2</sub>	126 ± 13.9	123 ± 18.6	148 <sup>x</sup> ± 16.4	100 ± 11.4	119 ± 12.0	83 ± 8.9
Ac-SHDWKPG-NH <sub>2</sub>	96 ± 9.1	81 ± 8.9	80 ± 10.9	82 ± 9.6	87 ± 10.4	76 ± 14.2
GnRH-III(Ac-C)	46 <sup>z</sup> ± 8.8	26 <sup>z</sup> ± 6.7	72 <sup>x</sup> ± 10.4	79 ± 17.1	41 <sup>z</sup> ± 12.0	24 <sup>z</sup> ± 8.8
GnRH-III(Ac-CFLG)	116 ± 30.7	197 <sup>x</sup> ± 27.5	73 ± 21.9	43 <sup>z</sup> ± 7.8	65 <sup>z</sup> ± 10.3	120 ± 8.9
[GnRH-III(C)] <sub>2</sub>	67 <sup>z</sup> ± 8.1	71 <sup>z</sup> ± 4.4	26 <sup>z</sup> ± 4.3	75 <sup>x</sup> ± 8.6	151 <sup>y</sup> ± 15.9	135 <sup>y</sup> ± 7.8
[GnRH-III(Ac-C)] <sub>2</sub>	77 ± 10.1	98 ± 7.7	64 <sup>y</sup> ± 4.3	107 ± 11.0	171 <sup>y</sup> ± 19.3	81 ± 9.7
[GnRH-III(CFLG)] <sub>2</sub>	39 <sup>z</sup> ± 10.1	182 <sup>z</sup> ± 14.7	96 ± 11.7	94 ± 10.7	99 ± 9.5	106 ± 8.9
[GnRH-III(Ac-CFLG)] <sub>2</sub>	68 <sup>y</sup> ± 5.6	93 ± 9.0	114 ± 6.8	55 <sup>z</sup> ± 6.5	63 <sup>y</sup> ± 7.2	72 <sup>x</sup> ± 6.8

<sup>a</sup>Chemotaxis index was expressed as a percentage of the control.

Data represent the mean of 15 parallels ± SD.

The level of significance is shown as follows: x – *p* < 0.05; y – *p* < 0.01; z – *p* < 0.001.

### Cell Adhesion in MM6 Cell Line

#### Native hormones

The three isoforms of natural GnRH could increase the adhesion of the monocyte model cells with the following order of activity: GnRH-III > GnRH-II > GnRH-I (Figure 1). GnRH-II (123–136%) and GnRH-I (128–119%) showed this adhesion inducer effect in wider concentration range (10<sup>-10</sup>–10<sup>-7</sup> M), whereas GnRH-III had a positive effect on adhesion (125–169%) only at high concentrations (10<sup>-7</sup>–10<sup>-6</sup> M). To compare the effect of GnRH-III to the human hormones, this analog was more potent, however, at different concentration (Figure 1).

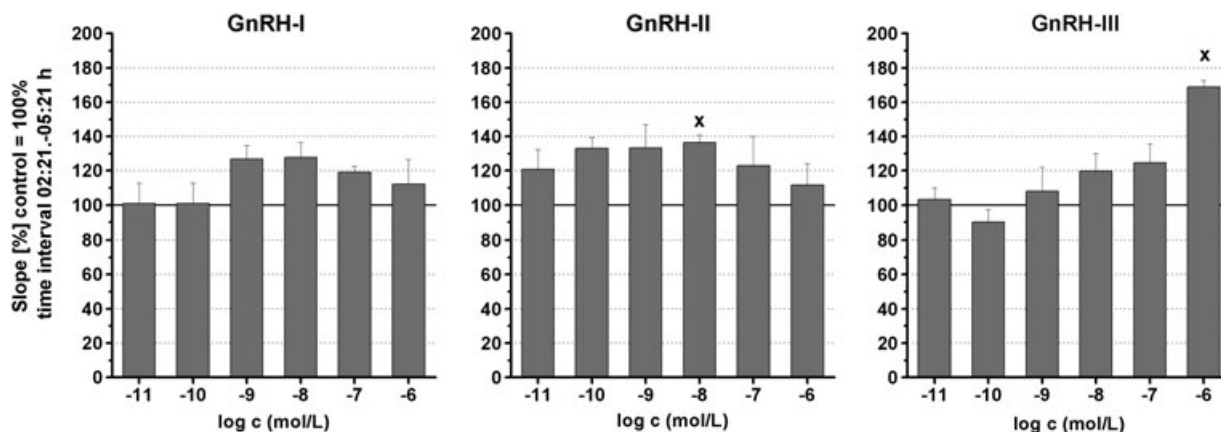
#### N-terminal truncated peptide fragments

The truncation of the first (Ac-HWSDWKPG-NH<sub>2</sub>) and the second amino acids (Ac-WSHDWKPG-NH<sub>2</sub>) from GnRH-III did not influence markedly the adhesion inducer character of the parent hormone (Figure 2). These fragments increased also the adhesion but in lower rate and in lower concentration range than GnRH-III. This

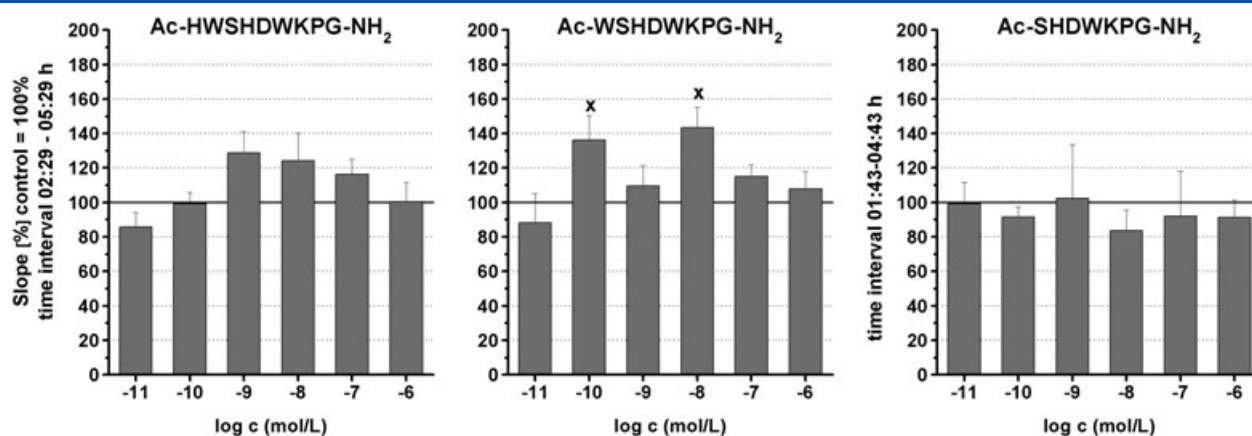
positive effect was more pronounced in case of Ac-WSHDWKPG-NH<sub>2</sub> fragment at 10<sup>-10</sup> and 10<sup>-8</sup> M concentration (136% and 143%, respectively). The further truncation of the sequence (Ac-SHDWKPG-NH<sub>2</sub>) resulted in a fragment, possessing neutral effect on adhesion in a short-term period (0–3 h) (Figure 2) and negative effect with long-term characteristic after 6–10 h (data not shown).

#### Cysteine-containing monomers and dimers

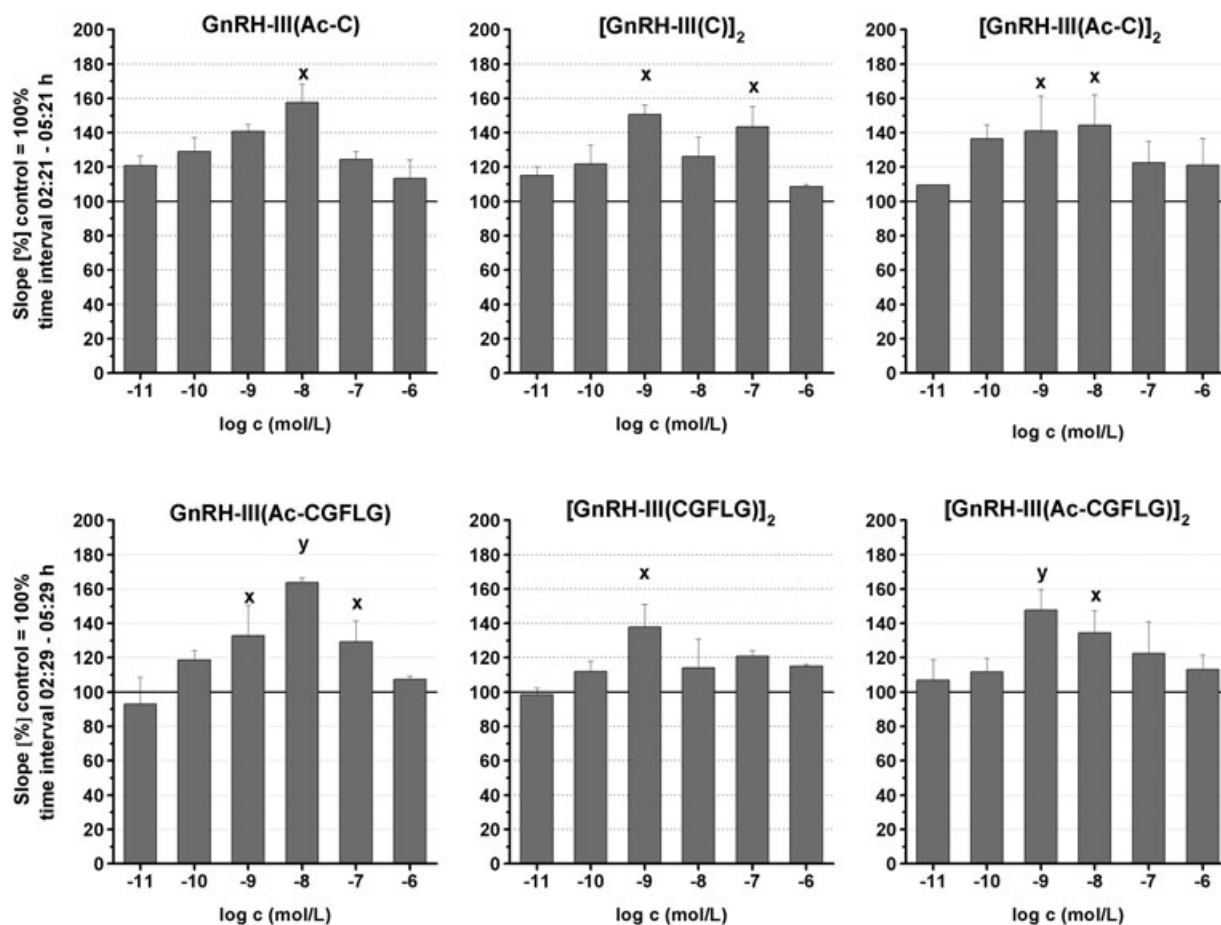
The monomers, independent of the length of the side chain, had uniform adhesion enhancer effects in 10<sup>-10</sup>–10<sup>-7</sup> M range (Figure 3). Their maximal effects were detected at 10<sup>-8</sup> M [GnRH-III (Ac-C): 158%, GnRH-III(Ac-GFLG): 163%], whereas GnRH-III elicited its effect only at higher concentration. The dimers possessed similar concentration dependence on the adhesion enhancement activity as the monomers (Figure 3). The dimerization of GnRH-III(Ac-C) did not alter significantly the adhesion of the monocytes; the effect was not influenced by the presence or absence of the acetyl group on Cys ([GnRH-III(C)]<sub>2</sub>: 151% and [GnRH-III(Ac-C)]<sub>2</sub>: 143%).



**Figure 1.** The concentration dependence of the effect on the adhesion of MM6 cells induced by the native GnRHs. The slope value computed in a chosen period (marked on the graphs) is expressed as a percentage of the untreated control. Data represent the mean of three parallels ± SD. The level of significance is shown as follows: x – *p* < 0.05.



**Figure 2.** The concentration dependence of the effect on the adhesion of MM6 cells induced by the GnRH-III fragments. The slope value computed in a chosen period (marked on the graphs) is expressed as a percentage of the untreated control. Data represent the mean of three parallels  $\pm$  SD. The level of significance is shown as follows: x –  $p < 0.05$ .



**Figure 3.** The concentration dependence of the effect on the adhesion of MM6 cells induced by the GnRH-III monomer and dimer derivatives. The slope value computed in a chosen period (marked on the graphs) is expressed as a percentage of the untreated control. Data represent the mean of three parallels  $\pm$  SD. The level of significance is shown as follows: x –  $p < 0.05$ ; y –  $p < 0.01$ .

[GnRH-III(CGFLG)]<sub>2</sub> was the least potent dimer derivatives with 137% efficiency only at  $10^{-9}$  M concentration. In GFLG containing dimers, the acetylation of the linker region ([GnRH-III (Ac-CGFLG)]<sub>2</sub>) improved the adhesion inducer activity (147%), and this effect was expressed in wider concentration range ( $10^{-9}$ – $10^{-7}$  M) (Figure 3).

### Effects of the GnRH Peptides on Intracellular Signaling

To determine the relationship between chemotactic activity induced by the GnRH peptides and activation PI3K, the model cells treated with the GnRH peptides were tested in inhibition assays (treatments with wortmannin and LY, blockers of PI3K).



Inhibition assay of PI3K in *T. pyriformis*

Chemotactic responses of *Tetrahymena* to [GnRH-III(C)]<sub>2</sub> and Ac-HWSDWKPG-NH<sub>2</sub> at the chemotactically effective concentrations (10<sup>-11</sup> and 10<sup>-9</sup> M) were inhibited (62.7%; 65.3%) by LY, whereas the wortmannin was ineffective (100.2%; 93.4%). The opposite result was observed in the case of Ac-SHDWKPG-NH<sub>2</sub> (10<sup>-7</sup> M); here, only the wortmannin was able to inhibit (34%) the chemotactic effect of this fragment. It is worth to be mentioned that the chemotactic behavior of the cells to GnRH-III(Ac-CGFLG) (10<sup>-9</sup> M) was increased by the inhibition of PI3K (wortmannin: 170%; LY: 130%) (Table 4).

## Inhibition assay of PI3K in MM6 cell line

The chemoattractant profile of the two native isoforms, GnRH-II and GnRH-III, was sensitive to the inhibitors. The chemoattractant response to GnRH-II (10<sup>-7</sup> M) could be suppressed by both inhibitors with the same degree (wortmannin: 79%; LY: 77%), whereas in the case of GnRH-III (10<sup>-7</sup> M), only the wortmannin pretreatment showed this kind of inhibition (83%) (Table 5). Moreover, LY also inhibited the [GnRH-III(C)]<sub>2</sub> (10<sup>-7</sup> M) stimulated chemotactic effect (82%). The positive chemotactic effects elicited by [GnRH-III(Ac-C)]<sub>2</sub> (10<sup>-7</sup> M), GnRH-III(Ac-CGFLG) (10<sup>-10</sup> M), and its dimer derivative ([GnRH-III(Ac-CGFLG)]<sub>2</sub>) (10<sup>-10</sup> M) were not inhibited by neither wortmannin nor LY. Similar result was detected – none of the inhibitors had effect on the chemotactic responsiveness – in the case of Ac-WSHDWKPG-NH<sub>2</sub> (10<sup>-9</sup> M), too (Table 5).

## Discussion

In the present paper, we report on the development and cell biological (chemotaxis, adhesion, and signaling) characterization of GnRH-III and some of its novel derivatives (fragments and dimers) in two model cells of tumorigenesis representing different, lower levels of differentiation [27,28,34]. In comparison with normal tissues, the overexpression of the GnRH-R on tumors might represent the basis for targeted tumor therapy [14]. In accordance with data obtained, we evaluate the suitability of the GnRH-III-based ligands for CDT as carriers with targeting activity.

It is well known that the efficient chemotactic drug delivery requires a suitable conjugate of at least three components: a carrier, a chemotactic ligand, and a drug molecule. In respect of the basic concept of CDT design, the GnRH-III derivatives are rather special as they fulfill the function of both the carrier and the trigger of chemotactic response in the target cell [1,3,4]. Furthermore, GnRH-III and some of its derivatives have own anti-proliferative effect. The cell migration in case of most mammalian cells is governed by cell – extracellular matrix interactions. To mediate directed migration of the target cell, the optimal CDT ligands are required to associate with extracellular matrix, which results in an increased expression of their adhesion-dependent migration inducer activity [44]. In this purpose, the chemotactic actions and the evoked chemotactic signaling of these substances were examined on two model cells representing lower levels of differentiation (*T. pyriformis* ciliate protozoon and MM6

**Table 4.** The effect of the inhibition of PI3K on the chemotactic response of *T. pyriformis* cells induced by the GnRH derivatives

Compound	Concentration (M)	Chemotactic effect (%)	Inhibition index <sup>a</sup> (%)	
			Wortmannin	LY
Ac-HWSDWKPG-NH <sub>2</sub>	10 <sup>-9</sup>	192 <sup>x</sup>	93.4	65.3 <sup>y</sup>
Ac-SHDWKPG-NH <sub>2</sub>	10 <sup>-7</sup>	144 <sup>x</sup>	34 <sup>y</sup>	92
GnRH-III(Ac-CGFLG)	10 <sup>-9</sup>	149 <sup>x</sup>	170 <sup>y</sup>	130
[GnRH-III(C)] <sub>2</sub>	10 <sup>-11</sup>	237 <sup>y</sup>	100.2	62.7 <sup>x</sup>

<sup>a</sup>The chemotactic responsiveness of the inhibited cells was characterized by inhibition index ( $\text{Inh}_{\text{ind}} = (G_{\text{inh}} \times C_c) / (C_{\text{inh}} \times G_c) \times 100\%$ ).

Data shown in the table were calculated from the averages of 10 parallel measurements.

The level of significance is shown as follows: x –  $p < 0.05$ ; y –  $p < 0.01$ .

**Table 5.** The effect of the inhibition of PI3K on the chemotactic response of MM6 cells induced by the GnRH derivatives

Compound	Concentration (M)	Chemotactic effect (%)	Inhibition index <sup>a</sup> (%)	
			Wortmannin	LY
GnRH-II	10 <sup>-7</sup>	149 <sup>x</sup>	79	77 <sup>x</sup>
GnRH-III	10 <sup>-7</sup>	190 <sup>y</sup>	83	99
Ac-WSHDWKPG-NH <sub>2</sub>	10 <sup>-9</sup>	148 <sup>y</sup>	106	147 <sup>x</sup>
[GnRH-III(C)] <sub>2</sub>	10 <sup>-7</sup>	151 <sup>y</sup>	110	82
[GnRH-III(Ac-C)] <sub>2</sub>	10 <sup>-7</sup>	171 <sup>y</sup>	102.8	93.2
GnRH-III(Ac-CGFLG)	10 <sup>-10</sup>	197 <sup>y</sup>	95.8	99
[GnRH-III(CGFLG)] <sub>2</sub>	10 <sup>-10</sup>	182 <sup>z</sup>	96	116.7

<sup>a</sup>The chemotactic responsiveness of the inhibited cells was characterized by inhibition index ( $\text{Inh}_{\text{ind}} = (G_{\text{inh}} \times C_c) / (C_{\text{inh}} \times G_c) \times 100\%$ ).

Data shown in the table were calculated from the averages of 15 parallel measurements.

The level of significance is shown as follows: x –  $p < 0.05$ ; y –  $p < 0.01$ ; z –  $p < 0.001$ .

human monocytic cell line derived from acute myeloid leukemia). The adhesion of the monocytes as a preliminary step of the chemotaxis was also measured in the presence of different GnRH-III ligands.

The central part of the decapeptide native hormones exhibits considerable variations in 5–8 position, which defines the bioactive conformations, consequently the selectivity, and ligand-specific actions. Furthermore, the conservative N-terminal and C-terminal parts of the sequence (Glp-His-Trp-Ser and Pro-Gly-NH<sub>2</sub>) appear to be necessary for receptor binding and partly for receptor activation [5].

Comparing the chemotactic effects and the sequences of the three examined native hormones in *Tetrahymena*, we can conclude that the more expressed aromatic amino acids (Tyr<sup>6</sup>-Trp<sup>7</sup>) in the central part of GnRH-II are presumably responsible for the repellent character of the molecule. In our former experiments on the chemotactic potency of the L-amino acids in *Tetrahymena*, it was demonstrated that the aromatic side chain of amino acids was accompanied with uniform and wide range chemorepellent effect [45].

In monocytes, GnRH-III and the reference GnRH-II elicited similar biphasic chemotactic profiles: they were attractant at high concentrations (10<sup>-7</sup>–10<sup>-6</sup> M) and repellent in a lower range (10<sup>-11</sup>–10<sup>-9</sup> M). It has been shown by a radioreceptor assay with H<sup>3</sup>-labeled GnRH-III that the hormone could recognize both the high-affinity and low-affinity binding sites in a membrane suspension prepared from tumor cells [19]. This ability could be associated with the dichotomy of concentration dependence of chemotaxis elicited by GnRH-III and GnRH-II in monocytes.

In respect of the enhanced chemoattraction and adhesion, GnRH-III has proved to be more effective than the other native GnRHs at micromolar concentrations in the leukemia model. The preferable combination of the three effects (chemoattractant, adhesion inducing, and antiproliferative [17]) possessing also pharmacological significance suggests the applicability of GnRH-III in the CDT.

Truncated GnRH-I peptide variants were synthesized by Janáky and coworkers to evaluate the optimal size in respect of their suitability in drug targeting. These fragments conjugated with different cytotoxic drugs were capable to inhibit the growth of a breast cancer cell line (MCF-7) [13]. These results prompted us to investigate the chemotaxis and adhesion inducer activity of the GnRH-III fragments truncated on the N-terminus.

The chemotactic results gained on our model cells demonstrated the significance of the expressed N-terminal amino acid in the GnRH-III fragments. The presence of the aromatic amino acid Trp on the N-terminus (Ac-WSHDWKPG-NH<sub>2</sub>) was associated with the chemoattractant character in monocytes, whereas non-aromatic amino acid bearing fragments (Ac-HWSHDWKPG-NH<sub>2</sub> and Ac-SHDWKPG-NH<sub>2</sub>) were chemorepellent. In contrast, the opposite structure–function relation was provided in *Tetrahymena* model: the aromatic N-terminal moiety (Ac-WSHDWKPG-NH<sub>2</sub>) induced negative chemosensory response. This latter observation corresponds closely with our previous study on the chemotactic effect of peptides containing SXWS motif in *Tetrahymena*. Our data confirmed that addition of Trp residue to the N-terminus of peptides (WSXWS) resulted in a reduction of the chemotactic behavior elicited by SXWS peptides [46]. Our present results supply further evidence to the dominant role of the aromatic moiety at the N-terminus in avoidance response of *Tetrahymena*. It is important to note that the effect of a chemotactic peptide on different model cells could be determined by not only the physicochemical

character of the N-terminal part but also by other factors, e.g. the length and conformation of the whole peptide are significant in this respect [47].

The effect of the fragments on adhesion was rather influenced by the length of the peptide than the physicochemical character of the N-terminus. The absence of the first and second amino acids (Glp and His) was permissible; in the truncated forms, the adhesion inducer effects of GnRH-III were still retained, whereas further truncation of the molecule was accompanied with neutral or negative effect on adhesion. The structure–antitumor relationships of the GnRH-III analogs synthesized by Mező and coworkers suggest the contribution of the indol rings of Trp<sup>3,7</sup> to the interaction of GnRH-III with the receptor. The cleavage of Trp<sup>3</sup> might interfere with the receptor binding of the GnRH-III fragment [12]. It is also conceivable that the deletion of the Glp-His-Trp tripeptide from the N-terminal part changes drastically the structure and consequently the behavior of the molecule. According to our aforementioned data, the advantaged size regarding CDT is the octamer Ac-WSHDWKPG-NH<sub>2</sub>. This fragment still retains the chemoattractant and adhesion inducer characters of GnRH-III in monocytes.

The results of the Ala scan at positions 5–8 of GnRH-III suggest [18] that dimer structures formed via Lys<sup>8</sup> of GnRH-III could be promising to improve the selective antiproliferative character of the ligand [20] and dimers might have enhanced modulator effect on chemotaxis and adhesion.

The ligation of two GnRH-III peptides directly through a disulfide bridge proved to be important in respect of the chemoattractant and adhesion inducer characters. According to the concentration course study, [GnRH-III(C)]<sub>2</sub> elicited significant chemoattractant responses in both model cells, however, in different concentration ranges. In the case of the monocyte model, the acetylation of the linker cysteine amino acids ([GnRH-III(Ac-C)]<sub>2</sub>) has slightly improved the two cell physiological responses tested. Results gained in the acetylated dimer were in good agreement with the reported stronger anticancer activity [20]. The biphasic (repellent – low concentration; attractant – high concentration) chemotactic profile of GnRH-III in monocytes was retained in the similar chemotactic effects of [GnRH-III(C)]<sub>2</sub> and [GnRH-III(Ac-C)]<sub>2</sub>, which raises the possibility that GnRH-III determines the receptor bindings and the chemotactic activities of these dimers. Our former report about the structural analysis supports this theory; the ECD (electron-capture dissociation) spectroscopy measurements suggest that the aforementioned dimers and GnRH-III adopt mainly the same conformation [20].

On the basis of the described chemoattractant characters and the abilities of [GnRH-III(C)]<sub>2</sub> and [GnRH-III(Ac-C)]<sub>2</sub> to influence positively the cell adhesion, we can conclude that besides GnRH-III, its dimeric derivatives are also promising candidates for CDT.

The other group of dimers was formed by 'GFLG' spacer, which provides a lysosomal enzyme labile moiety. The presence of the spacer has more advantages, e.g. facilitates the optimal ligand–receptor interactions in extracellular space or promotes transition of dimers into monomers by enzymatic cleavages after internalization [4]. Collating of the chemotactic abilities of molecules containing GFLG spacer sequence showed that the presence of the tetrapeptide could reverse the chemotactic effects compared with the referent molecules containing only cysteine or acetylcysteine in both model cells. In the case of *Tetrahymena*, this kind of modification ([GnRH-III(CGFLG)]<sub>2</sub>) turned the wide range chemoattractant effect of [GnRH-III(C)]<sub>2</sub> into a chemorepellent

effect. In monocytes, the concentration dependence of [GnRH-III (CGFLG)]<sub>2</sub> showed a reciprocal relation to the reference [GnRH-III (C)]<sub>2</sub> – in low concentrations, [GnRH-III(CGFLG)]<sub>2</sub> was chemoattractant, whereas in higher concentrations, it turned to neutral. Besides the presence of the GFLG sequence, the acetylation ([GnRH-III(Ac-CGFLG)]<sub>2</sub>) could enhance only the adhesion inducer activity of the referent dimer ([GnRH-III(CGFLG)]<sub>2</sub>). These reversal effects of the spacer sequence containing dimers suggest that this new, large, hydrophobic tetrapeptide chain provides a new component to the GnRH-III peptide available for interactions with GnRH-Rs [48,49].

A wide range of studies have certified that the binding affinity and the signaling of type-I GnRH-R (GnRH-IR) can be different depending on the cell type and location; however, the genes and the GnRH-IR peptides are the same in distinct cells [10,24]. Induction of the GnRH-IR of the pituitary activates the PLC via G $\alpha_{q/11}$  protein. The GnRH-IR in different tumor cells mediates antiproliferative and apoptotic effects through G $\alpha_i$  protein, which interferes with the activation of several downstream signaling pathways such as mitogen-activated protein kinases and PI3K signaling [11,24]. It has been recently shown that the second messenger pathways (e.g. G $\alpha_i$ -cAMP and G $\alpha_{q/11}$ -PLC) responsible for antiproliferative action of GnRH-III vary in tumor cells of diverse origin [50].

On the basis of several experiments, the presence of membrane receptors for vertebrate hormones, adequate functioning of signaling mediators (cAMP, calmodulin-Ca<sup>2+</sup> and inositol phospholipids), and activity of enzymes such as PLA<sub>2</sub>, PLC, and PI3K systems [31,51] were proved also in *Tetrahymena*. The association of the PI3K and PLC enzymes with the GnRHs-mediated cellular events prompted us to investigate the involvement of the PLC and PI3K enzymes in the chemotactic signaling induced by the GnRH-III derivatives in *T. pyriformis* and MM6 cells.

Our results showed that the tested chemoattractant GnRH-III derivatives failed to activate the PLC on either of the model cells (our unpublished data, not shown in Results). It seems that the putative GnRH-R expressed in both the tumorous and unicellular model cells might partly and functionally differ from the receptor in the pituitary. The natural variants (GnRH-II and GnRH-III) appear to activate the PI3K in monocytes, as the chemotactic action of the hormones was wortmannin sensitive. The mechanisms mentioned earlier were found in *Tetrahymena* treated with fragments Ac-HWSDWKPG-NH<sub>2</sub> and Ac-SHDWKPG-NH<sub>2</sub>. Formation of dimer via a disulfide bridge did not alter notably the chemotactic signaling of the investigated components: LY appeared to inhibit chemotactic effect stimulated by [GnRH-III(C)]<sub>2</sub> in both model cells. According to our data, the presence of acetyl moiety or the GFLG enzyme labile spacer might induce distinct – PI3K independent – signal pathways as none of the inhibitors applied could prevent the events mediated by the derivatives. It is assumed that the receptors in both model cells behave more like the receptors in some cancer cells [24,50]. The divergences in the blocking profile of inhibitors might be explained by the different properties (structure and target on PI3K) of the inhibitors [38,39].

The results of our experiment in *T. pyriformis* have confirmed further the high sensitivity of its chemosensory behavior and showed that the GnRHs – over their chemotactic behaviors in *Tetrahymena* – could be inducers of the classic intracellular signaling pathways known in the higher-ranked (tumor) cells. However, it is worth mentioning that in spite of the typical chemotactic behavior of monocytes and *Tetrahymena*, they use completely different mode of migration. So while the GnRH-based

reception could be detected in both model cells, the sensitivity of their chemotactic responses was different because of difference in, e.g. downstream regulation of migration mechanism and cellular milieu.

A wide range of studies have been shown that besides the chemotactic reaction, many other cell physiological activities of *Tetrahymena* – including swimming behavior, binding and production of endogenous substances – could be influenced by hormones of the higher-ranked animals [31,52]. Unlike several hormones (e.g. insulin, biogenic amines, and hypothalamo-hypophyseal hormones) [31,53], there is no available data about the chemotactic unrelated effects of GnRHs in the literature. According to our results under publication, the GnRH-III derivatives could also modulate the swimming behavior – velocity and tortuosity of the swimming tracks – and the intracellular hormone content of the cells. In general, the chemorepellent GnRH-III related peptides uniformly provoked slow, serpentin-like movements, whereas the chemoattractants resulted in a rather straight path of *Tetrahymena*. The significant elevation in the intracellular histamine level and the reduction in the epinephrine content of *Tetrahymena* induced by the GnRH-III variants proved to be independent of their chemotactic effects.

The concentration-dependent dual effects of GnRH-III and its dimer derivatives indicate that they might act on different types of GnRH-Rs (GnRH-IR and GnRH-IIR) in monocytes when they are applied on different concentrations [16,20]. It cannot be excluded that the same GnRH-III derivative could stimulate different signal transduction pathways depending on time, dose of exposure, and cellular milieu, which might cause different cellular outcomes [10,24].

In summary, the investigated GnRH-III derivatives could evoke significant chemotactic responses of both model cells that proved to be sensitive to even slight structural modifications. Furthermore, the adhesion increasing activity of these peptides is a rather general character.

In monocytes, the parent peptide GnRH-III (10<sup>-7</sup>–10<sup>-6</sup> M) was the most potent in respect of the chemoattraction and adhesion enhancement of all the peptides synthesized and tested in this study. The detected cell physiological activities in both models indicate their sensitivity to the length and the physicochemical character of the peptide. In monocytes, the optimal size regarding adhesion inducing and chemoattractant effect is the octamer Ac-WSHDWKPG-NH<sub>2</sub>. The dimer formation, especially in the case of [GnRH-III(C)]<sub>2</sub> and [GnRH-III(Ac-C)]<sub>2</sub>, could preserve the chemotactic profile and the adhesion inducer effect of the native GnRH-III. Data obtained from the investigations of chemotactic signaling suggest that PI3K is predominantly involved in the pathway of the intracellular signaling of chemoattraction exerted by the GnRH-III derivatives in both model cells.

The results of the cell biological tests bring into attention that not only GnRH-III but also some of their dimer and fragment derivatives themselves could act as a part of CDT conjugates and make the possibility of the further development to deliver cytotoxic agents directly to the tumor cells by CDT.

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## References

- 1 Mező G, Láng O, Jakab A, Bai KB, Szabó I, Schlosser G, Láng J, Kóhidai L, Hudecz F. Synthesis of oligo(tufts)-based branched oligopeptide conjugates for chemotactic drug targeting. *J. Pept. Sci.* 2006; **12**: 328–336.
- 2 Kay RR, Langridge P, Traynor D, Hoeller O. Changing directions in the study of chemotaxis. *Nat. Rev. Mol. Cell Biol.* 2008; **9**: 455–463.
- 3 Bai KB, Láng O, Orbán E, Szabó R, Kóhidai L, Hudecz F, Mező G. Design, synthesis, and in vitro activity of novel drug delivery systems containing tufts derivatives and methotrexate. *Bioconjug. Chem.* 2008; **19**: 2260–2269.
- 4 Mező G, Manea M, Szabó I, Vincze B, Kovács M. New derivatives of GnRH as potential anticancer therapeutic agents. *Curr. Med. Chem.* 2008; **15**: 2366–2379.
- 5 Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR. Gonadotropin-releasing hormone receptors. *Endocr. Rev.* 2004; **25**: 235–275.
- 6 Limonta P, Moretti RM, Marelli MM, Motta M. The biology of gonadotropin hormone-releasing hormone: role in the control of tumor growth and progression in humans. *Front. Neuroendocrinol.* 2003; **24**: 279–295.
- 7 Chen A, Ganor Y, Rahimpour S, Ben-Aroya N, Koch Y, Levite M. The neuropeptides GnRH-II and GnRH-I are produced by human T cells and trigger laminin receptor gene expression, adhesion, chemotaxis and homing to specific organs. *Nat. Med.* 2002; **8**: 1421–1426.
- 8 Hapgood JP, Sadie H, van Biljon W, Ronacher K. Regulation of expression of mammalian gonadotropin-releasing hormone receptor genes. *J. Neuroendocrinol.* 2005; **17**: 619–638.
- 9 Mezo G, Manea M. Luteinizing hormone-releasing hormone antagonists. *Expert Opin. Ther. Pat.* 2009; **19**: 1771–1785.
- 10 Grundker C, Gunthert AR, Westphalen S, Emons G. Biology of the gonadotropin-releasing hormone system in gynecological cancers. *Eur. J. Endocrinol.* 2002; **146**: 1–14.
- 11 Aguilar-Rojas A, Huerta-Reyes M. Human gonadotropin-releasing hormone receptor-activated cellular functions and signaling pathways in extra-pituitary tissues and cancer cells (Review). *Oncol. Rep.* 2009; **22**: 981–990.
- 12 Mező I, Lovas S, Pályi I, Vincze B, Kálnay A, Turi G, Vadász Z, Seprődi J, Idei M, Tóth G, Gulyás E, Ötvös F, Mák M, Horváth JE, Teplán I, Murphy RF. Synthesis of gonadotropin-releasing hormone III analogs. Structure-antitumor activity relationships. *J. Med. Chem.* 1997; **40**: 3353–3358.
- 13 Janáky T, Juhász A, Rékási Z, Serfőző P, Pinski J, Bokser L, Srkalovic G, Milovanovic S, Redding TW, Halmos G, Nagy A, Schally AV. Short-chain analogs of luteinizing hormone-releasing hormone containing cytotoxic moieties. *Proc. Natl. Acad. Sci. USA* 1992; **89**: 10203–10207.
- 14 Nagy A, Schally AV. Targeting cytotoxic conjugates of somatostatin, luteinizing hormone-releasing hormone and bombesin to cancers expressing their receptors: a “smarter” chemotherapy. *Curr. Pharm. Des.* 2005; **11**: 1167–1180.
- 15 Sower SA, Chiang YC, Lovas S, Conlon JM. Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. *Endocrinology* 1993; **132**: 1125–1131.
- 16 Kovács M, Vincze B, Horváth JE, Seprődi J. Structure-activity study on the LH- and FSH-releasing and anticancer effects of gonadotropin-releasing hormone (GnRH)-III analogs. *Peptides* 2007; **28**: 821–829.
- 17 Herédi-Szabó K, Murphy RF, Lovas S. Is IgNHR-III the most potent GnRH analog containing only natural amino acids that specifically inhibits the growth of human breast cancer cells? *J. Pept. Sci.* 2006; **12**: 714–720.
- 18 Herédi-Szabó K, Lubke J, Tóth G, Murphy RF, Lovas S. Importance of the central region of lamprey gonadotropin-releasing hormone III in the inhibition of breast cancer cell growth. *Peptides* 2005; **26**: 419–422.
- 19 Pályi I, Vincze B, Lovas S, Mező I, Pató J, Kálnay A, Turi G, Gaál D, Mihalik R, Péter I, Teplán I, Murphy RF. Gonadotropin-releasing hormone analogue conjugates with strong selective antitumor activity. *Proc. Natl. Acad. Sci. USA* 1999; **96**: 2361–2366.
- 20 Mező G, Czajlik A, Manea M, Jakab A, Farkas V, Majer Z, Vass E, Bodor A, Kapuvári B, Boldizsár M, Vincze B, Csuka O, Kovács M, Przybylski M, Perczel A, Hudecz F. Structure, enzymatic stability and antitumor activity of sea lamprey GnRH-III and its dimer derivatives. *Peptides* 2007; **28**: 806–820.
- 21 Mező G, Szabó I, Bősze S, Szabó R, Jakab A, Bai KB, Kapuvári B, Vincze B, Boldizsár M, Csuka O, Kovács M, Hudecz F. in *Peptides 2006*, Rolka K, Rekowski P, Stibberring J (eds.). Kenes International: Geneva, Switzerland, 2006; 558–559.
- 22 Moretti RM, Montagnani Marelli M, Van Groeninghen JC, Limonta P. Locally expressed LHRH receptors mediate the oncostatic and antimetastatic activity of LHRH agonists on melanoma cells. *J. Clin. Endocrinol. Metab.* 2002; **87**: 3791–3797.
- 23 Dondi D, Festuccia C, Piccolella M, Bologna M, Motta M. GnRH agonists and antagonists decrease the metastatic progression of human prostate cancer cell lines by inhibiting the plasminogen activator system. *Oncol. Rep.* 2006; **15**: 393–400.
- 24 Cheung LW, Wong AS. Gonadotropin-releasing hormone: GnRH receptor signaling in extrapituitary tissues. *FEBS J.* 2008; **275**: 5479–5495.
- 25 Moretti RM, Montagnani Marelli M, Mai S, Limonta P. Gonadotropin-releasing hormone agonists suppress melanoma cell motility and invasiveness through the inhibition of alpha3 integrin and MMP-2 expression and activity. *Int. J. Oncol.* 2008; **33**: 405–413.
- 26 Savagner P. The epithelial-mesenchymal transition (EMT) phenomenon. *Ann. Oncol.* 2010; **21**: 89–92.
- 27 Csaba G. The biological basis and clinical significance of hormonal imprinting, an epigenetic process. *Clin. Epigenetics.* 2011; **2**: 187–196.
- 28 Kovacs P, Csaba G, Torok O. Interrelationships between the imprinting potential and biological activity of insulin derivatives: studies on *Tetrahymena* and Chang liver cells. *Cytobios* 1989; **60**: 97–101.
- 29 Kóhidai L. Chemotaxis: the proper physiological response to evaluate phylogeny of signal molecules. *Acta Biol. Hung.* 1999; **50**: 375–394.
- 30 Kóhidai L, Csaba G. Chemotaxis and chemotactic selection induced with cytokines (IL-8, RANTES and TNF-alpha) in the unicellular *Tetrahymena pyriformis*. *Cytokine* 1998; **10**: 481–486.
- 31 Csaba G. The hormonal system of the unicellular *Tetrahymena*: a review with evolutionary aspects. *Acta Microbiol. Immunol. Hung.* 2012; **59**: 131–156.
- 32 Csaba G, Kovács P. Human chorionic gonadotropin (HCG)-like hormones (FSH, LH, TSH) in *Tetrahymena*. A confocal microscopic analysis. *Acta Protozool.* 2000; **39**: 191–198.
- 33 Kóhidai L, Csaba G. Different and selective chemotactic responses of *Tetrahymena pyriformis* to two families of signal molecules: lectins and peptide hormones. *Acta Microbiol. Immunol. Hung.* 1996; **43**: 83–91.
- 34 Ziegler-Heitbrock HW, Thiel E, Futterer A, Herzog V, Wirtz A, Riethmüller G. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. *Int. J. Cancer* 1988; **41**: 456–461.
- 35 Kóhidai L, Lemberkovic E, Csaba G. Molecule dependent chemotactic responses of *Tetrahymena pyriformis* elicited by volatile oils. *Acta Protozool.* 1995; **34**: 181–185.
- 36 Leick V, Helle J. A quantitative assay for ciliate chemotaxis. *Anal. Biochem.* 1983; **135**: 466–469.
- 37 Sáfár O, Kóhidai L, Hegedűs A. Time-delayed model of the unbiased movement of *Tetrahymena pyriformis*. *Period. Math. Hung.* 2011; **63**: 215–225.
- 38 Arcaro A, Wymann MP. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem. J.* 1993; **296**: 297–301.
- 39 Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* 1994; **269**: 5241–5248.
- 40 Nam SW, Kim ST, Lee KM, Kim SH, Kou S, Lim J, Hwang H, Joo MK, Jeong B, Yoo SH, Park S. N-Methyl-D-aspartate receptor-mediated chemotaxis and Ca(2+) signaling in *Tetrahymena pyriformis*. *Protist* 2009; **160**: 331–342.
- 41 Harakawa N, Sasada M, Maeda A, Asagoe K, Nohgawa M, Takano K, Matsuda Y, Yamamoto K, Okuma M. Random migration of polymorphonuclear leukocytes induced by GM-CSF involving a signal transduction pathway different from that of fMLP. *J. Leukoc. Biol.* 1997; **61**: 500–506.
- 42 Thelen M, Uguccioni M, Bosiger J. PI 3-Kinase-dependent and independent chemotaxis of human neutrophil leukocytes. *Biochem. Biophys. Res. Commun.* 1995; **217**: 1255–1262.
- 43 Mező G, Manea M, Jakab A, Kapuvári B, Bősze S, Schlosser G, Przybylski M, Hudecz F. Synthesis and structural characterization of bioactive peptide conjugates using thioether linkage approaches. *J. Pept. Sci.* 2004; **10**: 701–713.
- 44 Schmidt S, Friedl P. Interstitial cell migration: integrin-dependent and alternative adhesion mechanisms. *Cell Tissue Res.* 2010; **339**: 83–92.
- 45 Kóhidai L, Láng O, Csaba G. Chemotactic-range-fitting of amino acids and its correlations to physicochemical parameters in *Tetrahymena pyriformis* – evolutionary consequences. *Cell. Mol. Biol. (Noisy-le-Grand)* 2003; **49**: 487–495.



- 46 Illyés E, Bösze S, Láng O, Sebestyén F, Kóhidai L, Hudecz F. A new class of chemotactic peptides containing EWS motif: a mini-review. *Chim. Oggi*. 2002; **20**: 55–61.
- 47 Kóhidai L, Kovacs K, Csaba G. Direct chemotactic effect of bradykinin and related peptides-significance of amino- and carboxyterminal character of oligopeptides in chemotaxis of *Tetrahymena pyriformis*. *Cell Biol. Int.* 2002; **26**: 55–62.
- 48 Flanagan CA, Millar RP, Illing N. Advances in understanding gonadotrophin-releasing hormone receptor structure and ligand interactions. *Rev. Reprod.* 1997; **2**: 113–120.
- 49 Padula AM. GnRH analogues – agonists and antagonists. *Anim. Reprod. Sci.* 2005; **88**: 115–126.
- 50 Herédi-Szabó K, Murphy RF, Lovas S. Different signal responses to lamprey GnRH-III in human cancer cells. *Int. J. Pept. Res. Ther.* 2006; **12**: 359–364.
- 51 Kovács P, Pállinger E. Phosphatidylinositol 3-kinase-like activity in *Tetrahymena*. Effects of wortmannin and LY294002. *Acta Protozool.* 2003; **42**: 277–285.
- 52 Kovács P, Lovas G, Csaba G. Influence of insulin on the movement of *Tetrahymena pyriformis*. Hormonal imprinting alters the velocity. *Comp. Biochem. Physiol. Comp. Physiol.* 1994; **107**: 375–379.
- 53 Lajko E, Pallinger E, Csaba G. Investigations on the triiodothyronine (T3)-specificity of thyrotropic (TSH) and gonadotropic (HCG) hormone in the unicellular *Tetrahymena*. *Acta Microbiol. Immunol. Hung.* 2011; **58**: 85–91.