# Investigation of basic cell physiological activities induced by biactive molecules in mammalian and unicellular models

Ph.D. thesis

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## INTRODUCTION

The presence of bioactive compounds – secreted endogenous messengers (e.g. hormones) or synthetic drug molecules – with spatial and temporal information in the local environment of a cell could influence a whole range of cell physiological activities. The chemotaxis, the directed movement of the cells, is one of the most important cell physiological responses. The chemotaxis and its associated phenomena (adhesion, paracrine/autocrine communication, proliferation) participate in several regulatory and effector mechanisms of the cells representing different level of phylogeny. In the unicellular level the chemotaxis is important for the cells to survive, while in multicellular organisms, the co-operation of the adhesion, migration and proliferation is critical e.g. to the fertilization, to the development and to the normal immune response. The regulation or function of these cell biological activities can be subverted during neurodegenerative and autoimmune inflammatory processes as well as cancer metastasis. The altered adhesion, migratory potential of the cells appear to be promising therapeutic target, and drugs that specifically influence these processes could be effective in the prevention and treatment of the above-mentioned diseases.

The application of the more selective, targeted drugs could significantly improve the efficacy of the therapies. This targeted approach has became more important in the treatment of tumors, where the use of anticancer agents with low therapeutic index is restricted by the adverse events coming from the toxicity of the agents to the normal cells. The targeted tumor therapy is based on (i) the inhibition of an overactivated biochemical process, which has critical role in the malignant transformation, or on (ii) the hormone receptors that are overexpressed in tumor cells compared with their expression in normal tissues. The monoamine oxidase (MAO) is one of the identified molecular targets, which is overexpressed in many tumor types (renal, prostate, brain tumors), and significant in vitro and in vivo antitumor effects could be achieved by inhibition of this enzyme. *R*-deprenyl, an irreversible inhibitor of the MAO-B isotype has been used as an antiparkinsonian drug. Due to its complex pharmacological profile (e.g. antiapoptotic, anticancer, antioxidant effects) *R*-deprenyl could contribute to the prevention and treatment of different diseases outside the central nervous system, such as atherosclerosis and tumor progression. The well-established therapeutic effects of *R*-deprenyl proved to be stereoselective and are partly attributed to its metabolites formed by enzymes cytochrome P450, or flavin containing monooxygenase.

The application of drug delivery systems represents an other strategy for targeted cancer therapy. In this case, cytotoxic drugs are attached to hormone peptides, with the aim of delivering them selectively to the tumor’s overexpressed hormone receptors.

The gonadotropin-releasing hormone has receptors (GnRH-R) on different type of tumor cells, while limited number of GnRH-Rs is found on normal tissues. The GnRH itself is responsible for the regulation of gonadal steroidogenesis and gametogenesis in the pituitary, while it mediates direct antiproliferative effect via GnRH-Rs expressed on the cancer cells. The targeted chemotherapy favors GnRH derivatives (e.g. GnRH-III) which directly inhibit the growth of tumors without significant endocrine side effects. The GnRH-III in drug-containing conjugates intended for targeted tumortherapy serve as carrier and targeting unit for the specific delivery of a chemotherapeutic agent covalently linked to them. The dissemination of the tumor cells is an important aspect of the tumor progression and could significantly affect the success of the targeted tumor therapy. The breakdown of the cell adhesion contacts and the increased motility of the tumor cells play a key role in each step of the metastatic cascade (invasion, intra- and extravasation, travel to a distant site and colonization of a new environment). Based on the aforementioned facts, the manipulation of cancer cell adhesion and migration by different targeted approaches such as *R*-deprenyl and its derivatives that inhibit MAO-B, as well as GnRH-III based delivery units and their drug-containing conjugates that act through GnRH-R appears to be important to prevent the formation of metastasis.

Our aim was to investigate the cell physiological effects of bioactive compounds (serotonin, *R*-deprenyl and its derivatives, native GnRH analogues, synthetic variants and drug-containing conjugates of GnRH-III) that act on molecular targets (MAO or GnRH-R) allowing the possibility for development of targeted therapies in diverse model cells representing different level of phylogeny in the hope to find some candidate molecules that could inhibit both the growth and the dissemination of cancer cells.

**OBJECTIVES**

1. At the first period of our work the antimetastatic effects of the deprenyl derivatives, their role in the tumor progression and similarity of their cell physiological effects in model cells representing different level if phylogeny were examined. Our aims were as follows:

- to investigate the short term effects of the serotonin, as a MAO substrate, on endogenous hormone content of Tetrahymena, and the permanent character of pretreatment with serotonin (serotonin imprinting) by testing essential physiological indices;
- to study the serotonin content of the Tetrahymena treated by *R*-deprenyl, as MAO-B blocker, and the chemotactic responses of this model induced by deprenyl derivatives;

- to investigate the adhesion modulator, chemotactic and antiproliferative properties of *R*-deprenyl and its derivatives in tumor cell lines with different malignancy as well as to evaluate the relation of these cell functions and inhibitory action on tumor growth and metastatic process.

2. The main objectives of the second part of our work were the complex analysis of the effects of GnRH peptides on the essential cell biological functions of Tetrahymena model cells, as well as the detailed in vitro study of GnRH-III peptide variants and their drug-containing conjugates in higher ranked, leukemic model cells.

- to characterize the chemotactic effects of GnRH peptides in Tetrahymena and to learn the possible structure – functional correlation of this ligands;
- to investigate the GnRH isoforms and synthetic derivatives on the hormone production and chemokinetic activity of ciliated model cells in comparison with some other signal hormones derived from higher ranked animals;

- to study the adhesion and chemotaxis of leukemic model cells induced by GnRH-III compounds and their conjugates containing different cytotoxic drugs
- to prove the antiproliferative/cytotoxic effects of the GnRH-III based conjugates and to compare these activities to the free drugs
- to exhibit the suitability of the GnRH-III based targeting moieties and conjugates for application in the targeted cancer therapy as antitumor and antimetastatic agents by reading their complex cell physiological activities.

## METHODS

### Chemicals

The assayed 10 deprenyl derivatives were provided to us as a generous gift of Chinoin Pharmaceutical and Chemical Works/Sanofi-Aventis (Budapest, Hungary).

The hormones tested in Tetrahymena model system were obtained from Sigma-Aldrich Ltd. (St. Louis, MO, USA) and TEVA Hungary Zrt.

All the GnRH peptides and conjugates assayed were synthesized, purified and analytically characterized by Research Group of Peptide Chemistry, Hungarian Academy of Science, Eötvös Loránd University and Laboratory of Analytical Chemistry and Biopolymer Structure Analysis, University of Konstanz.

**Model cells**

The preliminary studies of the native and synthetic compounds acting on MAO and GnRH-R on the basic cell physiological processes were done in *Tetrahymena pyriformis* model. Its practical advantages – simple culturing and due to its 150 min long generation time umpteenth progeny generation (e.g. $1000^{th}$) can be examined – its signal transduction system (receptors, paracrine/autocrine signaling) that shows similarities to the mechanism of mammalians and its highly selective chemotactic/chemocinetic responsiveness support the selection of Tetrahymena as model cells.

To evaluate the antimetastatic potency of deprenyl derivatives, analogues and conjugates of GnRH-III their cell physiological characters were also screened in higher ranked cell lines: in Mono Mac 6 (MM6) human monocytes derived from acute myeloid leukemia and in LM2 murine adenocarcinoma.

**Chemotaxis assay**

The chemotactic ability of Tetrahymena cells was determined by a modified version of Leick's two chamber capillary chemotaxis assay.

Chemotactic responsiveness of the MM6 cell line was measured by a modified Boyden chamber technique in a NeuroProbe® MBB 96 chamber. The number of the positive chemotactic responder cells was determined by MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay.

**Study of swimming behavior (tracking analysis)**

The swimming behavior of Tetrahymena was observed in an Axio-Observer invert microscope using AxioVision Rel 4.7.1. software. The swimming tracks of cells were registered with the Time-lapse module. The movement analysis was done by the Cell Tracker module of the software. For characterizing the swimming behavior the mean velocity of cells and the tortuosity of the swimming tracks were used.

**Signaling measurements**

In order to determine the role of the phospholipase C-γ and phosphatidylinositol 3-kinase pathways in the chemotactic response induced by GnRH peptides the cells treated with chemoattractant GnRHs were tested in inhibition assays (treatments with wortmannin and LY294002, blockers of PI3K) as well as in flow cytometry assays with antibody recognized phosphorylated (enzymatically active) form of PLC-γ1 were also carried out.

**Impedance-based cell adhesion assays**

The ECIS (Electrical Cell-substrate Impedance Sensing) and xCELLigence systems could monitor the cellular events by measuring electrical impedance across gold microelectrodes

integrated on the bottom of different arrays in real time manner. During the attachment of the cells, due to their insulating plasma membrane, an increase in the impedance could be registered. The detected impedance depends on the number and spreading of cells adhered to the surface of the electrodes. The adhesion properties of MM6 cells and the effects of various ligands on it were assessed using xCELLigence System. In the case of LM2 cells the ECIS proved to be convenient to detect both the adhesion and migration of the treated cells.

**Cell proliferation/cytotoxicity assay**

The growth characteristics of Tetrahymena cultures imprinted with different concentration of serotonin were studied after 6, 18, 24 and 48 hours in the $500^{th}$ and $1000^{th}$ generations.

To prove the direct anticancer activities of the deprenyl derivatives and drug-GnRH-III conjugates the antiproliferative/cytotoxic effects of the substances were analyzed in MM6 cells after 24, 48 and 72 hours of incubation.

In both experimental setup for the determination of cell numbers an other impedance based technique the CASY TT® cell counter and analyzer was used, which could also provide the size distribution (differentiation of the viable and dead cells) and the viability of the samples.

**Flow cytometric analysis of the intracellular hormone content**

The effects of various mammalian signal molecules on the intracellular hormonal content of Tetrahymena were measured by flow cytometric method following indirect immunostaning (anti-serotonin, anti-histamine, anti-T3, anti-ACTH primary antibody + FITC-labeled anti-rabbit secondary antibody).

**Experiments of hormonal imprinting**

The duration of the single imprinter effect of serotonin was investigated in two concentrations ($10^{-6}$ M and $10^{-15}$ M) by testing essential physiological indices, such as (i) serotonin content (ii) cell growth as well as (iii) chemotactic and (iv) swimming behaviors in $500^{th}$ and $1000^{th}$ offspring generations. (In case of migratory experiments retreatments with $10^{-15}$ M or $10^{-6}$ M concentration of hormone were also applied.). The control and pretreated cells were maintained in tryptone/yeast medium and transferred twice a week for 120 days.

**Statistical analysis**

Data obtained from each experiment represent averages and ±SD values. Statistical analysis was performed by using the ANOVA algorithm (OriginPro 8.0). Histograms provided by CASY TT and flow cytometry were further analyzed by the Kolmogorov-Smirnov test (XLSTAT module of MS Excel). The chemotactic values were given as chemotactic index. The slope analysis of the adhesion assay was performed by RTCA 1.2. program. Significance levels correspond to x: $p < 0.05$; y: $p < 0.01$; z: $p < 0.001$.

## RESULTS

### *R*-deprenyl and its derivatives

Short and long term effects of serotonin in Tetrahymena model cells

In addition to studying the cell physiological functions (chemotaxis, hormone content) induced by MAO inhibitor *R*-deprenyl and its derivatives the short and long term effects of serotonin as a native substrate of MAO enzyme were also investigated in Tetrahymena model.

At the low level of phylogeny the components of a hormonal system (receptors, signal transduction pathways) typical for the higher ranked animals could be observed. In the watery condition of Tetrahymena the cell physiological effects of the hormonal interactions could be influenced by the extreme dilution of secreted hormones, the amount of the dissolved nutrients in the environment of the cells and the time of the hormonal exposure. The effects of serotonin on the hormone content of Tetrahymena were different depending on the milieu in which the treatments took place. The significant stimulating character of serotonin on the intracellular concentration of histamine and ACTH could be observed only in Losina-Losinsky solution containing inorganic salts. The femtomolar or even lower concentrations of serotonin were sufficient for provoking these responses. However the length of exposure could markedly determine the activity of serotonin. Considering the natural watery conditions, where the dilution of materials produced and secreted by Tetrahymena is very high a nutrient free salt milieu is more physiological for the cells than a medium containing bacto tryptone and yeast extract (normal culturing medium). It became clear that due to its sensitive receptors the Tetrahymena sensed signals even in such high dilutions of hormones.

The hormonal imprinting is a special type of treatment, which is developed at the first encounter with a signal molecule resulting in the altered responsiveness of the cells, and the memory transferred to the daughter cells. At the second exposure with the imprinter chemical the imprinted cells or its offsprings express a changed, often enhanced response compared to the reaction at the first encounter. In our experiments the durability of the imprinting induced by $10^{-6}$ M or $10^{-15}$ M serotonin – instead of the usual time period: 24h (~9 generations) or one week (~70 generations) – was measured up to generations 500 and 1000 by testing essential physiological indices: *(1)* serotonin content, *(2)* cell growth, *(3)* chemotaxis, *(4)* swimming behavior of the imprinted and control cells.

In all of the indices tested in generations 500 and 1000, the results of the imprinted cells were quantitatively different from those of the control cells. *(1)* In case of the serotonin content the strength and the direction of the imprinting were dependent on the concentration of the imprinter. Serotonin imprinting with both concentrations caused similar effect

(decrease) on serotonin production up to the generation 500; however, the negative character of $10^{-6}$ M has disappeared by generation 1000, while $10^{-15}$ M imprinting elevated the intracellular serotonin level. *(2)* The significant elevation of the growth rate in the progeny generation 500 and 1000 showed that the serotonin pretreatment durably modified the cellular function, and the memory formed by imprinting persisted over as many as 1000 subsequent generations. *(3)* The single pretreatment with both concentrations of serotonin in generations 500 and 1000 decreased the chemotactic response of the cells; the repeated treatment with serotonin could further improve this chemorepellence. The robustness of the chemorepellent character indicated the ability of serotonin pretreatment to form hormonal imprinting and thereby long lasting memory. *(4)* The effect of serotonin imprinting is complex: the negative migratory character – initiation of slow, winding swimming – occurred at re-exposure to the hormone. The long lasting effects of the serotonin pretreatment even 1000 generation after the imprinting lead to the assumption that memory of serotonin imprinting is a heritable epigenetic change which is passed from generation to generation.

Basic cell physiological effects of *R*-deprenyl and its derivatives in Tetrahymena

The MAO enzyme, responsible for the metabolism of serotonin is supposed to regulate the widespread cell physiological effects of serotonin as an intracellular messenger by decomposing the hormone. According to our results about the effects of the MAO-A and MAO-B selective inhibitors it is possible that in spite of the case of mammalian cells the B isoform is responsible for the breakdown of serotonin. The *R*-deprenyl as a MAO-B blocker seemed to enhance the endogenous serotonin content by inhibition of the serotonin metabolism. Concerning the direct chemotactic effect of *R*-deprenyl it proved to be independent of MAO-B inhibition and regulation of the serotonin metabolism since its stereoselective chemoattractant effect was demonstrated in a much lower concentration ($10^{-11}$ M) than that required to inhibit MAO-B. The different modifications of *R*-deprenyl including its metabolic conversions such as desalkylation and N-oxidation resulted in mostly repellent or neutral chemotactic effects. Solely in case of the metabolites containing propargyl moiety (N-desmethyl-*R*-deprenyl, *R*-deprenyl-N-oxyde) a positive chemotactic tendency was shown in low concentration range. Our result in Tetrahymena model corresponding closely to the structure function relations (the presence of the propargyl moiety is required for *R*-deprenyl to exert its effects; the modification of the side chain in the C1 position of *R*-deprenyl diminish its activities) defined already in the literature confirm the previous data of our research group about the discriminative chemotactic potency of the unicellular model and also its suitability to examine the cell physiological effects of deprenyl derivatives.

Cell biological effects induced by *R*-deprenyl and its derivatives in higher ranked model cells

To evaluate the possible utilization of the deprenyl derivatives in targeted chemotherapy the investigations of the cell biological effects of *R*-deprenyl and its derivatives were continued in MM6 human leukemic and LM2 mammary adenocarcinoma cell lines since these cells seemed promising targets regarding the application of the aforementioned ligands.

In the two different, above-mentioned MM6 and LM2 tumor models a high percentage of concordant responses were elicited, nevertheless, in some cases the two types of tumor cells exerted different responses. The basic molecules (*R*- and *S*-deprenyls) and the metabolites were working in a similar way in the two model cells, while in the case of synthetic derivatives significant diversities were detected in the sign and amplitude of their effects.

The first crucial steps in metastasis cascade are the impairment of the adhesion contacts of the tumor cells and in parallel their increasing motility. These two events provide substantially for the tumor cells to detach from the primary tumor and migrate to the surrounding tissue. The adhesion and chemotactic phenotypes of the tumor cells have also key roles in the subsequent stages of the metastatic dissemination such as intra- and extravasation as well as migration to a distant site.

The interpretation of the antimetastatic potency of the deprenyl derivatives is based on the relation of their simultaneous effects on the cell adhesion and migration. The so called chemotaxis-adhesion ratio (CAR = chemotaxis [Chtx. ind.] / adhesion [Slope]) defined by our research group serves to characterize the relation of the above-mentioned activities. In case of an ideal antimetastatic agent increasing the cell adhesion and in parallel having chemorepellent or negative migratory properties at the primary tumor this CAR index is much smaller than one (CAR < 0.8). The CAR value of *R*-deprenyl in a concentration required to inhibit MAO-B was found far less than one in both model cells. Considering also its antiproliferative effect, the *R*-deprenyl is supposed to prevent the tumor cell proliferation and dissemination in a clinically relevant concentration. In the lower concentrations of R deprenyl its chemotactic effect in leukemic cell line was the dominant character accompanied by an antiproliferative effect ($10^{-9}$ M) which might compensate for its unfavorable activities in respect of metastasis formation (high CAR value). Although the *S*-deprenyl failed to exert any antiproliferative effect, low CAR values of *S*-deprenyl observed in the entire concentration range in both model cells suggest that regarding the antimestatic activity this derivative might be more efficient than the *R*-isomer. The metabolic conversion of *R*-deprenyl is probably not necessary for its cell physiological activity, but all in all the metabolites could contribute to the hypothetical antitumoral and antimestastatic effects of the parent compound: (i) the

adhesion inducer effect of *R*-amphetamine accompanied by a chemorepellent character, (ii) the *R*-methamphetamine had an antiproliferative effect in leukemia model, (iii) if the dissemination of the tumor cells has occurred the *R*-desmethyl-deprenyl and *R*-methamphetamine might interfere with the extravasation of the circulating tumor cells due to their adhesion reducer and negative migratory effects. The effects of p-fluoro-deprenyls are contradictory; their antimetastatic activity was below the effectiveness of reference compounds (*R*- and *S*-deprenyl). Although, in respect of antiproliferation this alteration proved to be highly efficient in both enantiomer, especially in casy of p-fluoro-*S*-deprenyl which had a long lasting antiproliferative effect, while cells could recover from the cytotoxic effect elicited by other derivatives (*R*-deprenyl, *R*-methamphetamine, etc.).

**GnRH-III peptide-variants and different hormones of higher ranked animal**

Effects on Tetrahymena model cells

As it has been seen in the case of serotonin, the hormones of the higher ranked animals could influence many cell physiological reactions of Tetrahymena including swimming behavior – vectorial movement, namely chemotaxis, or chemokinesis, which is a random response – production and secretion of endogenous substances.

Based on the chemotactic response of Tetrahymena varied with the sequence of the peptides, the chemotactic effects of the native GnRHs (GnRH-I, GnRH-II, GnRH-III) and the fragments of GnRH-III (Ac-HWSHDWKPG-$NH_2$, Ac-WSHDWKPG-$NH_2$, Ac-SHDWKPG-$NH_2$) appeared to be basically determined by the expressed amino acids in 5.-8. position of the GnRHs, and the physico-chemical character of the N-terminal amino acid. The results of the monomers and dimers synthesized with different composition suggested that the impact of different modifications of $Lys^8$ on the chemotactic activity of the native GnRH-III proved to be dependent on the length of the branch linked to the hormone on this position (GnRH-III(Ac-C), GnRH-III(Ac-CGFLG)). In case of the dimers formed via this $Lys^8$ of GnRH-III the length and chemical modification (acetylation) of the linker sequences appeared to define the chemotactic effect.

The specific responses of Tetrahymena induced by GnRH peptides also indicated the sensitivity of chemoreception and the induction of adequate signal transduction mechanisms. The phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3K) identified also in Tetrahymena are included in the intracellular signaling elements activated by GnRH peptides. The tested chemoattractant GnRH-III derivatives were failed to activate the PLC, while the PI3K inhibitors appeared to inhibit significantly the chemoattractant reactions. These results

showed that the GnRHs besides their chemotactic behaviors in Tetrahymena could be inducers of the classic intracellular signaling pathways known in the higher ranked (tumor) cells.

The GnRH peptides acting along a concentration gradient could induce chemotaxis of Tetrahymena and when presented in uniform concentration could influence the chemokinetic activity – speed and turning behavior – of the cells. The swimming velocity and the tortuosity of the path were changed mostly in the opposite way; the movement of the cells became more rectilinear (lower tourtosity) along with the increasing speed of the cells. The slow, serpentine-like movements of Tetrahymena uniformly provoked by the chemorepellent compounds were in good agreement with the literature. The chemokinetic activity of the attractants was not so consequent, these kinds of substances resulted in rather straight path of the unicellular model. The more or less identical effects of the chemorepellent compounds – GnRH peptides and glycoprotein type hormones – on the chemokinetic activity of Tetrahymena even though their molecular weight distributed in wide range verified that the correlation observed between the chemotactic and chemokinetic effects of a given molecule is a general character.

The GnRH peptides not only had direct effect on cell migration, but also would affect the migratory response of Tetrahymena by modulating the production and secretion of hormones that have chemotactic and chemokinetic activity in Tetrahymena after their release. An unambiguous correlation between the effects of the tested GnRH ligands on chemotaxis and hormone content of Tetrahymena was failed to observe. The positive effects of GnRH-III dimers ($[GnRH\text{-}III(C)]_2$, $GnRH\text{-}III(CGFLG)]_2$) and fragment (Ac-SHDWKPG-$NH_2$) derivatives on the histamine content and their negative effects on the adrenalin content of Tetrahymena proved to be independent of their chemotactic character. Some differences between the repellent and attractant GnRHs were manifested in their effects on intracellular level of serotonin and endorphin. In general, in case of the chemoattractant GnRH peptides the differences observed in the intensity of their chemotactic activity and in their effects on the swimming behavior of Tetrahymena could be partly arise from their indirect effect on the migratory responses of Tetrahymena by increasing the content of chemoattractant endorphin and in parallel decreasing the level of repellent serotonin in Tetrahymena.

Effects of GnRH-III derivatives on higher ranked model cells (MM6)

In order to evaluate the possible use of the GnRH peptides in the targeted tumor therapy as drug delivery systems with both antimetastatic and anticancer activity, the effects of the GnRH peptides serving as targeting moieties and

Activities of GnRH peptides serving as targeting moieties on MM6 model cells

The effect of the GnRH-III peptides with targeting potency on cell adhesion is a rather general character; with exception of the Ac-SHDWKPG-$NH_2$ fragment all derivatives could increase the cell adhesion in MM6 cells, although there were some differences in the degree and the concentration dependence of their effects. In contrast, the formation of N-terminal truncated GnRH-III fragments and dimer derivatives from two GnRH-III molecules containing branch on $Lys^8$ changed the chemotactic response of MM6 cells as it was detected in Tetrahymena model. The monomer with short branch (GnRH-III(Ac-C)) elicited strong chemorepellent effects in both model cells, while in case of the leukemia model the chemoattractant activity of $[GnRH\text{-}III(C)]_2$ built up from two monomers modified by cysteine residue *via* disulfide bridge was less effective than in Tetrahymena and appeared only at high concentrations. The incorporation of a GFLG sequence between the cysteine residue and GnRH-III could reverse the chemotactic effects compared to the reference molecules containing only cysteine or acetyl-cysteine in both model cells. The biphasic (repellent – low conc.; attractant – high conc.) chemotactic profile of the GnRH-III in monocytes was retained in the similar chemotactic effects of $[GnRH\text{-}III(C)]_2$, $[GnRH\text{-}III(Ac\text{-}C)]_2$, which raises the possibility that the GnRH-III itself determines the receptor binding and the chemotactic activity of these dimers.

The association of the PI3K and PLC enzymes with the GnRHs mediated cellular events and the induction of chemotactic response of monocytes prompted us to investigate the involvement of these enzymes in the chemotactic signaling induced by GnRH-III derivatives in MM6 cells. In case of the natural variants (GnRH-II and GnRH-III) and some dimers (e.g. $[GnRH\text{-}III(C)]_2$) the role of the PI3K in the transduction of their chemotactic effects could be demonstrated, while the tested chemoattractant GnRH-III derivatives could not activate the PLC in monocytes. The presence of acetyl moiety or the GFLG spacer might induce distinct – PI3K and PLC independent – signal pathways as the activation of PI3K and PLC was failed to be detected in the chemotactic signaling mediated by these peptides in either of the model cells.

The antimetastatic effect of the GnRH-III based targeting units was evaluated by calculating their CAR indices. In some cases the considerably low CAR values suggested that the GnRH-III derivatives themselves or as a part of drug-containing conjugates might help to keep the cancer cells at the site of the primary tumor. In respect of antimetastatic activity the monomers and dimers of GnRH-III modified on the $Lys^8$ proved to be more effective then the native GnRH-III. Due to the complex cell physiological activity of both monomers ((GnRH-III(Ac-C); GnRH-III(Ac-CGFLG)) and some dimers ($[GnRH\text{-}III(C)]_2$, $[GnRH\text{-}III(Ac\text{-}CGFLG)]_2$) in the leukemia model these peptides could be promising candidates to be applied in the targeted cancer therapy as targeting moieties of drug delivery systems.

Effects of drug-containing GnRH-III conjugates on MM6 model cells

Beside the targeting moiety the GnRH-III based conjugates contain a drug molecule as well. In most of the tested conjugates the anthracycline derivatives doxorubicin or daunorubicin were incorporated as anticancer agents. One of the important features of GnRH-III based drug delivery systems is the ability to display an antiproliferative/cytotoxic effect of the drug, and to interfere with the dissemination of the cancer cells by modulating their adhesion and migration. Since the attached drug and the chemical bond between the drug and the targeting moiety itself were able to significantly influence the biological effects of the GnRH-III peptides it can be concluded that the targeting units, the drug and the mode of conjugation together configured the cells physiological effects of the conjugates.

The combination of the adhesion inducer, chemorepellent and cytotoxic effects occurred in the case of GnRH-III(Dox-ester), in which the doxorubicin was coupled to the GnRH-III via ester bond. This conjugate is an analogue of the AN-152 which is built up from a superactive GnRH agonist ([D-Lys$^6$]-GnRH-I) and doxorubicin via ester bond. Since the An-152 is currently in the phase III clinical trial it served as a reference compound in our experiments. The cytotoxic effect of GnRH-III(Dox-ester) did not differ significantly from the activity of the AN-152 and the free doxorubicin, while its antimetastatic potency exceeded the efficiency of AN-152. The relatively high sensitivity of conjugates containing ester bond against proteolytic enzyme resulting in toxic side effects, prompted us to investigate conjugates having a more stable chemical bond, in which a peptide spacer (e.g. GFLG, YRRL) cleavable by a lysosomal enzyme was applied between the drug and the GnRH-III to provide appropriate intracellular drug release. The effects of the GnRH-III(Dox-amide-GFLG) expressed more stability due to the amide bond, could be beneficial regarding the antitumoral and antimetastatic activity.

In contrast, the GnRH-III(Dau) and GnRH-III(Dau-GFLG) possessing oxime bond did not have such above-mentioned effects. Since the daunorubicin has less cardiotoxic side effects typical for anthracyclines than doxorubicin it proved to be necessary to study different designs of daunorubicin containing conjugates. The modification of daunorubicin-GnRH-III conjugates in position 4 by acetyl-lysine not only improved the cytotoxic activity but the (Lys(Ac))$^4$GnRH-III(Dau) beside its chemorepellent character proved to have the most significant adhesion inducer effect; these activities predicted the antimetastatic potency of (Lys(Ac))$^4$GnRH-III(Dau).

Bifunctional conjugates containing two drugs were tested to increase the efficacy of GnRH-III conjugates and to avoid the loss of effectiveness due to the low GnRH-R

expression and its desensitization. However, these bifunctional conjugates could not result an unequivocal improvement in cytotoxicity due to some hypothetic conformational reasons or the hampered accessibility. Better effect profile was gained by application of $(Lys)^4$GnRH-III carrier bearing drugs in position 4. and 8., $(Lys)^4$GnRH-III($Dau^4$-$Dau^8$), $(Lys)^4$GnRH-III($Mtx^4$-$Dau^8$). The antitumor or antimetastatic property of these compounds was better compared to those ones where the side-chain of $Lys^8$ was used to link the drug to the conjugate (GnRH-III($Dau^4$-$Dau^8$), GnRH-III($Mtx^4$-$Dau^8$)).

One drug per monomer unit was present in the dimer-based conjugates. The effect of these dimeric conjugates was proper in ratio to the monomers, their cytotoxic activity was at least the twice of the monomers. The CAR values (CAR<<1) of dimeric conjugates suggest that they could significantly inhibit detachment and spreading of tumor cells due to their chemorepellent and adhesion inducer combined effects.

## CONCLUSIONS

1. It was described that the exogenously applied serotonin increases the endogenous hormone content of Tetrahymena in a concentration- and time dependent manner, especially when the cells were obtained in a nutrient-poor medium which models better the natural conditions of the cells. The above-mentioned effects were elicited by femtomolar or even lower concentrations of serotonin, which demonstrates the high receptor level sensibility of the ciliate model cell.

   The serotonin imprinting has resulted in increased (hormone content, proliferation, chemokinetic activity) or decreased (chemotactic responsiveness) reactions in the 500th and 1000th generations which points to the long term effectiveness of hormonal imprinting (induction of memory) and raises the possibility of development an epigenetic inheritance.

2. According to our results it is probable that Tetrahymena possesses B-isotype of the MAO enzyme, which has been previously demonstrated. This MAO-B might be responsible for the metabolism of serotonin and the inhibition of the enzyme by *R*-deprenyl might influence a wide range of cell physiological activities of cells.

3. *R*-deprenyl and its 9 derivatives were demonstrated as potent chemotaxis inducers in Tetrahymena. Chemoattractant behavior of *R*-deprenyl has proved to be stereoselective as the modifications of the molecular structure resulted in mainly repellent or neutral ligands and the concentration dependence of these activities was independent of MAO-B inhibition.

4. In higher ranked models (MM6 and LM2 cell lines) the adhesion and chemotaxis inducer effects of *R*-deprenyl and its derivatives proved to have a good correlation. *R*-deprenyl was

detected as antiproliferative to tumor cells in concentrations required to block MAO-B while inhibiting the spreading of tumor cells due to its parallel cell adhesion inducer and chemorepellent/migration blocker effects. *S*-deprenyl was found to be the most effective antimetastatic derivative considering its influences on cell adhesion and chemotaxis. The antimetastatic effects of *R*-deprenyl could be contributed by its metabolites with their cell adhesion decreasing and chemorepellent effects elicited on the extravasation of tumor cells in the circulatory system.

5. Chemotactic activity of GnRH peptide derivatives and activation of PI3K in the chemo-attractant signaling of GnRHs was demonstrated in the ciliated model cell. The sequence and structure dependent chemotactic reactions of Tetrahymena refer to the decisive role of the chemical character of the N-terminal amino acid and the $Lys^8$ side chain of GnRH-III.

6. The characterization of GnRH-III derivatives and glycoprotein hormones (different molecular weight and structure) showed that repellent compounds uniformly provoked slow, serpentine-like movement, while chemoattractants mainly resulted in linear swimming path of Tetrahymena. It was also documented that their effects on hormone content can also influence (auto- and paracrine manner) the migratory character of these ligands.

7. In leukemia model (monocyte) it was proved that the GnRH-III derivatives designed for drug delivery and their drug-containing conjugates have significant effects on cell adhesion and chemotaxis as well. The structure-function relationships of GnRH-III detected originally in Tetrahymena were verified in monocyte cell lines, too. Effects of the conjugates on chemotaxis and cell adhesion were determined not only by the carrier GnRH-III or its derivatives, but the drug itself or the way of coupling (type of the bonds, presence and type of the linker sequence) could also influence the biological activity of the conjugates. Adhesion inducer and chemorepellent character of the doxorubicin containing conjugates or molecules modified in position 4 were more underlined compared to the daunorubicin containing conjugates or the GnRH-III parent ligands.

8. Assays of cytotoxicity (antiproliferative activity) documented that majority of the GnRH-III based conjugates possess effects characteristic to the free drug. The doxorubicin containing conjugates proved to be the most effective; their cytotoxicity was comparable to the effects of reference molecule AN-152 and the activity of the free cytostatic drug. In the case of daunorubicin containing conjugates the dimerization and application of a $(Lys(Ac))^4$GnRH-III carrier could improve significantly their cytostatic potential.

9. On the basis of the antiproliferative and cytotoxic effects of GnRH-III drug conjugates it is assumed that the peptide derivatives of GnRH-III used as targeting components are able to guard drugs to the target cells and to promote their release and antitumor activity. However, the combined adhesion inducer and chemorepellent effect which is characteristic to both carriers and their conjugates suggests that they might inhibit invasion of tumor cells and decrease the chance of development metastasis in the level of the primary tumor. In summary, all these data predict the choice of application of some doxorubicin conjugates as well as the daunorubicin containing dimer and the $(Lys(Ac))^4$GnRH-III based conjugates in the targeted tumor therapy as novel complex antitumor and antimetastatic therapeutics.

**SUMMARY**

The cell physiological activities (adhesion, chemotaxis, proliferation) induced by endogenous or synthetic bioactive compounds fundamentally determine the cell fate in unicellular level and have pivotal roles in many clinically relevant processes (e.g., formation of tumor and metastasis) in mammalians. Furthermore targeted therapy for the influence of these activities represents a new way for example to increase the selectivity of antitumoral effect, and to prevent the formation of metastasis.

The cell physiological effects of *R*-deprenyl and its derivatives that target the MAO-B, as well as GnRH-III, an agonist of GnRH receptor, and related peptides designed for drug delivery were first investigated on Tetrahymena and elucidated in respect of effects induced by native ligands (serotonin, hypothalamo-hypophyseal hormones). Then, in higher ranked cells in point of antitumoral and antimetastatic effects important structure-function relations were described to find small drugs and peptide based drug delivery systems for targeted tumor therapy.

In Tetrahymena the acute effects of serotonin (substrate of MAO) on the intracellular hormone contents proved to depend on concentration, time of treatment and nutritive conditions, while the enhanced or decreased reactions of the cells in progeny generations 500. and 1000. after serotonin imprinting indicated the development of a durable and heritable imprinting. The elevation in the serotonin level caused by *R*-deprenyl appeared to be due to MAO-B inhibition, while chemotactic effects of deprenyl derivatives were rather MAO-B independent actions. By screening different types of GnRH-III derivatives and trophormones good correlation was found between their chemotactic and chemokinetic reactions regulated by endogenous autocrine/paracrine signal molecules.

In different types of cancer cells, the adhesion inducer, chemorepellent and antiproliferative effects of *R*-deprenyl in the clinically relevant concentration and for some derivatives (e.g. *S*-deprenyl, p-fluoro-*S*-deprenyl) indicate that these molecules might have targeted inhibitory effects in tumor growth and in metastasis formation at primary tumors. The drug-targeting conjugates (e.g. GnRH-III(Dox-ester), Lys(Ac)$^4$GnRH-III(Dau)) containing chemotherapeutic agents coupled to GnRH-III targeting moiety or its analogues with chemotactic and adhesion modulator potency could trigger efficient toxic effects and their adhesion enhancer and chemorepellent effects confirmed the feasibility of the GnRH-III-based conjugates as antimetastatic drug delivery systems for targeted tumor therapy.

## LIST OF OWN PUBLICATIONS

### Articles related to the PhD thesis

1. Csaba G, **Lajkó E**, Pállinger É. (2010) Serotonin in Tetrahymena. How does it work? Acta Protozool 49: 133-138. *IF: 0,881*
2. Csaba G, **Lajkó E**, Pállinger É. (2010) Comparison of effect of hormones (histamine, serotonin, insulin) on the hormone (serotonin, histamine, triiodothyronine, ACTH) synthesis of Tetrahymeny in medium or salt solution. Cell Biol Int. 34: 1095-1098. *IF: 1,747*
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6. Leurs U*, **Lajkó E***, Mező G, Orbán E, Öhlschläger P, Marquardt A, Kőhidai L, Manea M. (2012) GnRH-III based multifunctional drug delivery systems containing both daunorubicin and methotrexate. Eur J Med Chem. 52:173-183. *IF: 3,346 (*Contributed equally)*
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### Other publications

9. Csaba G, **Lajkó E**, Pállinger É. (2010) Hormonal effects on Tetrahymena: change in case of combined treatment. Acta Microbiol Immunol Hung. 57: 393-3999. *IF: 0,625*
10. **Lajkó E**, Csaba G, Pállinger É. (2012) Durable effect of heat-stress on the hormone production of Tetrahymena. Effect of insulin on the consequences of stress. Acta Microbiol Immunol Hung. 59: 249-256. *IF: 0,787*
11. **Lajkó E**, Pállinger É, Csaba G (2013) Effect of glucose on the insulin production and insulin binding of Tetrahymena. Acta Microbiol Immunol Hung. 59: 461-468. *IF: 0.787*