SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Investigation of Luteinizing hormone-releasing hormone (LH-RH-I) receptor type I and copy number of chromosome 3 and 4 in human uveal melanoma

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES DEBRECEN
DEBRECEN, 2018
Investigation of Luteinizing hormone-releasing hormone (LH-RH-I) receptor type I and copy number of chromosome 3 and 4 in human uveal melanoma

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1 Introduction

Uveal melanoma (UM) is a rare but very aggressive malignancy: independently of the currently available therapies, 50% of the patients develop metastasis and the median survival time of these patients is only about 2-8 months. Fluorescein angiography and ocular echography are the most effective diagnostic tools available for clinicians. The most important clinical prognostic factor is the size of the tumor, moreover, the therapy is often chosen based on tumorsize. Enucleation (removal of the globe) remains a reasonable option for very large tumors and in patients for whom radiotherapy is likely to be problematic. Tumor cell type is an important prognostic factor. Three histopathological uveal melanoma categories are being recognised: spindle, epithelioid and mixed cells. Monosomy 3 strongly predicts metastatic risk and other chromosomal abnormalities also correlate with metastatic diseases. About half of the patients develop metastases, most frequently in the liver. Gain of 8q (+8q) is found in around 40% of UM cases and proved to be an independent significant prognostic marker for decreased survival. In uveal melanoma, other recurrent chromosome alteration, such as lack of 1p and 16q, have been described. The discovery of specific receptors for hypothalamic hormones on cancer cells has led to the development of radiolabeled and cytotoxic hormone analogs. These analogs are more selective in wiping out cancer cells and less toxic than conventional chemotherapeutic agents. Hypothalamic luteinizing hormone-releasing hormone type I
(LH-RH-I) is the primary link between the hypothalamus and the pituitary gland in the regulation of gonadal functions and it has a pivotal role in vertebrate reproduction. The actions of LH-RH-I are mediated by specific G protein-coupled receptors for LH-RH-I present on the plasma membranes of the pituitary gonadotrophs. LH-RH-R-I is mainly localized in the hypothalamus, however, it has also been detected in the endometrium, placenta, breasts, ovary, testis, prostate, thymus, kidneys and in lower levels in several other organs. Tumoral receptors for LH-RH have been detected on human breast, prostate, ovarian, endometrial, colorectal, pancreatic cancers and in human melanomas, uveal melanomas, non-Hodgkin’s lymphomas, and renal cell carcinomas. The receptors for LH-RH on human tumors might also serve as targets for LH-RH analogs linked to cytotoxic agents.

However, chromosome aberrations in UM are obvious, the relationship between their occurrence and prognosis is quite complex and it remains unsolved. The gene encoding LH-RH-R is harbored by chromosome 4q21.2, however the numerical aberrations of chromosome 4 have never been studied by fluorescence in situ hybridization (FISH) in UM.
2 Aims

In this study, our aim was to investigate the mRNA expression of LH-RH-I receptor and LH-RH ligand in OCM-1 and OCM-3 (Ocular Choroidal Melanoma-1 and 3) human uveal melanoma cell lines as useful models for further in vitro and in vivo studies. The presence and binding characteristics of LH-RH-I receptor protein have also been examined by Western blotting, immunocytochemistry and ligand competition assays. In addition, we have studied the expression of mRNA and protein of LH-RH-I receptors in tumor tissue samples from nude mice xenografted with OCM-1 and OCM-3 cell lines.

The gene encoding LH-RH-R-I is harbored by chromosome 4q21.2, however the numerical aberrations of chromosome 4 have never been studied by fluorescence in situ hybridization (FISH) in UM. Therefore our aim was also to investigate the copy number of chromosome 3, particularly the monosomy of chromosome 3 which has been extensively described in the aggressive behavior of UM, and chromosome 4 in 46 human UM specimens using FISH. Furthermore, chromosome index (CI) and „dominant” cell population values for chromosome 3 and 4 have been determined. Additionally, we analyzed the survival rate of the UM patients according to their CI. The correlation between LH-RH and LH-RH-R-I expression and the copy number of chromosome 3 and 4 has also been investigated.
3 Materials and methods

3.1 Cell lines and culturing

OCM-1 and OCM-3 human primary uveal melanoma cell lines were cultured in RPMI 1640 medium supplemented with L-glutamine, 10 % FBS, and 1 % penicillin/streptomycin in a humidified chamber in 5 % CO2 at 37°C.

3.2 Animal studies

Athymic (nude) mice (Ncr nu/nu) were obtained from Charles River Laboratories (Germany). Mice were housed in sterile, individually ventilated cages in an air-conditioned (21±2 ºC), humidity-controlled room (≈50 %) with a 12/12 hour light/dark cycle. Animals were fed with autoclaved chow and water ad libitum. All experiments were in accordance with the institutional guidelines for the welfare of experimental animals and regulations of the European Union. The experimental protocol was approved by the Laboratory Animal Care and Use Committee of the University of Debrecen. Six million tumor cells were subcutaneously injected into the femoral region of the mice. Four weeks after the initiation of donor animals, when tumors had developed in donor animals, tumors were aseptically dissected and mechanically minced. Approximately 3 mm³ tumor tissue was transplanted subcutaneously into nude mice by a trocar needle. At the end of each experiment, mice were sacrificed under 3 % isoflurane anaesthesia using a small animal anaesthetic device. Tumors were excised, weighed and necropsy was
done. Tumor specimens were snap-frozen and stored at -80°C until further experiments.

3.3 Human uveal melanoma tissues

Specimens of human uveal melanoma were obtained from 46 patients 30-84 years of age at the time of enucleation at the Department of Ophthalmology of the University of Debrecen in Debrecen, Hungary. Normal lymphocyte samples, used as positive controls were collected at the Department of Pathology of the University of Debrecen. Informed consent was obtained before enucleation, and the study was performed according to the tenets of the Declaration of Helsinki and the local Institutional Ethics Committee. Fresh tumor tissue was obtained less than one hour after enucleation, according to a standardized protocol. Conventional histopathological examination was performed on all tumors and the origin of the tumor was confirmed.

3.4 RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated using Nucleospin RNA and Protein purification kit (Macherey-Nagel, Germany) according to the manufacturer’s instructions. RNA from each sample (2000 ng) was reverse transcribed to cDNA using Tetra cDNA synthesis kit (Bioline, UK) in a final volume of 20 µl. In order to evaluate the expression of type I LH-RH receptors and LH-RH ligand, primer sets were designed. Hypoxanthine phosphoribosyltransferase 1
(HPRT1) was used as an internal reference gene. mRNA levels of LH-RH-R-I, LH-RH and HPRT1 have been assessed using iQ™ SYBR® Green Supermix (BIO-RAD, USA). Reactions were conducted according to the manufacturer's protocol using MyiQ2 two color real time PCR detection system (BIO-RAD, USA). All real-time amplifications were measured in triplicates. Results were evaluated with BIO-RAD iQ5 (BIO-RAD, USA) software and changes in mRNA levels were calculated using the $2^{-\Delta\Delta C_{t}}$ method.

3.5 Immunocytochemistry

3.5.1 Immunoperoxidase staining

To detect LH-RH-I receptors, OCM-1 and OCM-3 cells were fixed in ice-cold methanol (10 minutes). Samples were incubated with primary anti-LH-RH-R antibody (sc-13944 rabbit polyclonal Santa Cruz, USA, 1:50) (overnight, 4°C) and EnVision Flex, HRP (Agilent Technologies, USA) (room temperature, 1 hour). Signals were detected using ready-to-use DAB substrate kit (Agilent Technologies, USA).

3.5.2 Immunofluorescent labeling

To investigate LH-RH-I receptors, OCM-1 and OCM-3 cells were fixed in 4 % paraformaldehyde at room temperature for 10 min. Samples were incubated with primary anti-LH-RH-R antibody (sc-13944 rabbit polyclonal Santa Cruz, USA, 1:50) (overnight, 4°C) and anti-rabbit FITC secondary antibody (ThermoFisher Scientific,
USA, 1:1000). Samples were rinsed and mounted with ProLong® Diamond Antifade Mountant with DAPI (Molecular probes, USA). Staining was evaluated using the Olympus FV-1000 confocal microscope (Olympus Corporation, Japan).

3.6 Western blot

Total protein was isolated using Nucleospin RNA and Protein purification kit (Macherey-Nagel, Germany) according to the manufacturer’s instructions. Total protein amount of the supernatant was determined by Nanodrop ND-1000 UV-Vis Spectrophotometer (USA). Equal amount of proteins (20 µg) were separated in 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred to PVDF membrane using standard procedures (89). Upon blocking with 5 % BSA, membranes were incubated with primary antibodies (overnight, 4°C): anti-LH-RH-R (sc-13944 rabbit polyclonal Santa Cruz, USA 1:200) and anti-GAPDH (D16H11 rabbit monoclonal Cell Signaling 1:1000). Proteins were detected with anti-rabbit horseradish peroxidase conjugated antibody (mouse sc-2357 Santa Cruz, USA) and Luminata Forte Western HRP substrate (Merck Millipore, Germany). The protein bands were quantified using Image Lab software (Bio-Rad Laboratories, USA).

3.7 Preparation of membranes and radioligand binding studies

Preparation of membranes for receptor studies was performed as described previously. Receptor binding was characterized using
sensitive *in vitro* ligand competition assay based on the binding of 
$[^{125}\text{I}][\text{D-Trp}^6]^{-}\text{LH-RH}$ as radioligand to membrane homogenates. The binding characteristics of receptors for LH-RH-I were determined in membrane fraction of OCM-1 and OCM-3 human uveal melanoma cell lines (1.8-2.4 x 108 cells each) and in OCM-1 and OCM-3 tumors grew in nude mice. Radioiodinated derivatives of $[\text{D-Trp}^6]^{-}\text{LH-RH}$ were prepared by the chloramine-T method and purified by reverse-phase HPLC as described previously. Briefly, membrane homogenates containing 50-160 µg protein were incubated in duplicate or triplicate with 60-80,000 cpm $[^{125}\text{I}][\text{D-Trp}^6]^{-}\text{LH-RH}$ and increasing concentrations (10-12 - 10-6 M) of nonradioactive peptides as competitors in a total volume of 150 µl binding buffer. At the end of incubation time, 125 µl aliquots of suspension were transferred onto the top of 1 ml of ice-cold binding buffer containing 1.5 % BSA in siliconized polypropylene microcentrifuge tubes (Sigma-Aldrich, USA). The tubes were centrifuged at 12,000x g, 4 °C for 3 minutes. Supernatants were aspirated and the bottom of the tube containing the pellet was cut off and counted in a gamma counter. Protein concentration was determined by Bradford method using a Bio-Rad protein assay kit (Bio-Rad Laboratories, USA). The LIGAND-PC computerized curve-fitting program of Munson and Rodbard was used to determine the type of receptor binding, dissociation constant ($K_d$) and maximal binding capacity of the receptors ($B_{\text{max}}$)
3.8  Fluorescence in situ hybridization

3.8.1  Touch preparations

The tumor tissues were transferred from -80°C to -20°C. The tissue samples were used for touch preparations, which were obtained by pressing frozen tissue samples several times on the surface of a silanized slide. The slides were fixed in methanol-acetic acid (3:1), air dried, washed with 70 % acetic acid solution and distilled water, dehydrated with 70 %, 80 % and 90 % ethanol and air dried. The slides were stored at -20°C until further use.

3.8.2  DNA FISH probes

Numerical aberrations of chromosome 3 and 4 were studied by FISH with centromere specific probes (CEP; Chromosome Enumeration DNA FISH Probes, Vysis, Germany). The probes consist of chromosome 3 or 4-specific tandem-repeat DNA sequences. The CEP probes are directly labeled with SpectrumOrange (chromosome 3) and SpectrumGreen (chromosome 4) fluorophores. The centromeric probes contain 7 μl CEP Hybridization Buffer, 1 μl probe and 1 μl distilled water.

3.8.3  FISH hybridization

Fluorescence in situ hybridization was done according to a general protocol with some modifications. The slides containing the touch
preparations were fixed in methanol-acetic acid (3:1) at -20°C, then incubated in 15 μl 10 % pepsin in 100 μl 1 M HCl. The slides were washed with 1x PBS buffer and then dehydrated in 70 %, 85 %, 100 % alcohol series and air dried. DNA FISH probe was added, coverslips were applied and sealed to the slide with rubber cement. The slides were denatured at 75°C for 5 minutes and hybridized at 42°C overnight. After hybridization, the slides were washed with 50 % formamide / 2x standard saline citrate (SSC) solution at 42°C for 7 minutes then with 2x SSC solution at 42°C for 7 minutes. Slides were then counterstained with ProLong® Diamond Antifade Mountant with DAPI (Molecular probes, USA).

3.8.4 Fluorescence microscopy

Slides were evaluated using a fluorescence microscope (Axio Imager Z2, Zeiss, Germany). Image capture was performed by a monochrome charge-coupled device camera attached to the fluorescence microscope and ISIS software (Metasystems, Germany).

3.8.5 FISH analysis

Numerical aberrations of chromosome 3 and 4 were assessed by analysing chromosome copy number on the basis of 100 relevant tumor cell nuclei. Chromosome index (CI) values for chromosome 3 and 4 were determined for the ratio of the whole FISH signal in the
sample and the number of nuclei. „Dominant” cell population value was determined.

3.9 Statistical analysis

Correlation analysis was carried out between the expression of mRNA for LH-RH-I receptor and LH-RH ligand with the use of GraphPad Prism 7. The two data sets were evaluated using Kolmogorov–Smirnov normality test (KS test), and then Pearson correlation analysis was performed. Indices for chromosome 3 and 4 were analysed from the UM samples. The two data sets were evaluated using D'Agostino & Pearson omnibus normality test, and then Spearman correlation analysis was performed. Chromosome results, receptor findings and clinicopathological data were also analysed. Statistical analysis was carried out with the use of GraphPad Prism 7 (USA). Survival data was plotted against the postoperative days (elapsed until death or the end of the follow-up period), according to the Kaplan-Meier method. Statistical analysis was carried out with GraphPad Prism 7 software (USA).
4 Results


mRNA expression of LH-RH-I receptors has been analyzed in OCM-1 and OCM-3 cell lines by RT-qPCR. LH-RH-I receptors were detected in both these human uveal melanoma cell lines, with slightly higher expression of LH-RH-I receptor observed in OCM-3 cells. Western blot analysis confirmed the presence of LH-RH-I receptors in OCM-1 and OCM-3 cells. In accordance with the receptor mRNA data, a slightly higher protein expression of LH-RH-I receptors was observed in OCM-3 cells by Western blot and immunocytochemical analysis.

4.2 Expression of type I LH-RH receptors in vivo in tumor xenograft models

The mRNA expression of LH-RH-I receptors in OCM-1 and OCM-3 tumors grown in nude mice was analyzed by RT-qPCR. mRNA expression of LH-RH-I receptors could be detected in all tumor xenograft. In accordance with our in vitro results, the level of LH-RH-I receptor transcript in our OCM-3 model was considerably higher than in the OCM-1 tumor samples. Western blot analysis also confirmed the presence of LH-RH-I receptor protein in OCM-1 and OCM-3 tumor tissues. Similarly to our in vitro findings, Western
blot analysis confirmed higher protein expression levels of LH-RH-I receptor in OCM-3 tumor xenografts.

4.3 Expression of mRNA for LH-RH in human uveal melanoma cell lines and tumor xenografts

In addition to LH-RH receptor studies, the expression of LH-RH ligand in OCM-1 and OCM-3 cellular models was also investigated by RT-qPCR. The presence of mRNA of LH-RH ligand was detected in both cell lines and tumors grown in nude mice. Although the expression of LH-RH-R was considerably higher in OCM-3 cells, the mRNA expression of LH-RH ligand was slightly higher in OCM-1 cells.

4.4 Radioligand Binding Studies

The presence of specific LH-RH binding sites and binding characteristics of $[^{125}\text{I}][\text{D-Trp}^6]$-LH-RH to membrane receptors in OCM-1 and OCM-3 human uveal melanoma models were determined using ligand competition assays. Analysis of the typical displacement of radiolabeled $[^{125}\text{I}][\text{D-Trp}^6]$-LH-RH by the same unlabeled peptide revealed that the one-site model provided the best fit, indicating the presence of one class of high-affinity LH-RH-I receptors in crude membranes derived from human uveal melanoma cells. In cell membranes of OCM-1 and OCM-3 cells, ligand competition studies revealed a single class of high affinity binding...
sites for LH-RH-I with a mean dissociation constants ($K_d$) of 4.11±0.3 nM and 4.26±0.6 nM, respectively. The concentration of LH-RH-I receptors was 233.6±21.7 fmol/mg membrane protein in OCM-1 cells while OCM-3 cells showed remarkably higher receptor level (1029.1±68.5 fmol/mg membrane protein). Receptors for LH-RH-I have also been found in the membranes of OCM-1 and OCM-3 tumor tissue samples. Radiolabeled [$^{125}$I][D-Trp$^6$]-LH-RH was found to be bound to a single class of specific, high affinity binding sites in both human uveal melanoma models investigated. Mean $K_d$ values were 5.85±0.7 nM in OCM-1 tumors and 6.18±0.8 nM for OCM-3 tumors. Mean $B_{max}$ values were as the followings; 267.3±38.5 fmol/mg membrane protein in OCM-1 tumors and about 2.7 times higher (713.0±29.4 fmol/mg membrane protein) in OCM-3 xenografts. Biochemical parameters, which are essential to establish the identity of specific binding sites, were also determined. The binding of [$^{125}$I][D-Trp$^6$]-LH-RH was found to be reversible, time- and temperature-dependent, and linear with protein concentration in human uveal melanoma samples. The specificity of LH-RH binding was demonstrated by competitive binding experiments using several peptides structurally related or unrelated to LH-RH. The binding of radiolabeled [$^{125}$I][D-Trp$^6$]-LH-RH was completely displaced by increasing concentrations (10$^{-12}$ - 10$^{-6}$ M) of LH-RH agonist buserelin and LH-RH antagonist cetrorelix.
4.5 Correlation between type I LH-RH receptor and LH-RH ligand expressions

According to our statistical analysis, there is a significant correlation between the expression of mRNA for LH-RH-I receptor and LH-RH ligand in OCM-1 cell line and in OCM-1 tumor xenografts (Pearson r=0.8380; p=0.0373, CI=0.95 %). Moreover, significant correlation was also observed between the expressions of LH-RH-R-I and LH-RH ligand in OCM-3 cells and OCM-3 tumors grown in nude mice (Pearson r=0.9878; p=0.0002, CI=0.95 %)

4.6 Distribution of chromosome 3

Based on CI values, monosomy of chromosome 3 could be found in 16 (35 %) samples. In 6 specimens (13 %), more than 2 copies of chromosome 3 have been found. Normal biparental disomy was observed in 24 samples (52 %). In 26 samples one signal per cell per „dominant” cell population could be detected, whereas in 9 cases, clones containing 3 or more chromosomes per nucleus have been found. In two specimens, either loss of chromosome 3 or polysomy have been observed. Normal distribution of chromosome 3 was detected in 13 cases. In addition, normal tissue samples contained negligible abnormal cell population (<15 %)
4.7 Distribution of chromosome 4

Based on CI values, chromosome 4 could be detected in normal biparental disomy in 14 samples (30 %), while 32 cases (70 %) showed more than 2 signals per nucleus. In 8 samples one signal per cell per „dominant” cell population has been observed, whereas in 41 cases, clones containing 3 or more chromosomes per nucleus have been found. In 6 specimens either loss of chromosome 4 or polysomy has been observed. Normal distribution of chromosome 4 was detected only in 3 cases.

4.8 Statistical results

According to the statistical analysis, there is a statistically significant (p<0.05) correlation between the copy number of chromosome 3 and 4 (Spearman r=0.42; 0.139-0.639; CI=0.95%).

Chromosome index values (CI) of chromosomes 3 and 4 were determined for the samples and were considered to be normal (N: 1.75-2.25) or pathological (P: <1.75 or >2.25). Comparing the survival rate of the four groups (NN, NP, PN, PP), obvious difference has been revealed, however, statistically significant differences could not be shown (p=0.38 for the Mantel-Cox test and p=0.43 for the Gehan-Breslow-Wilcoxon test). Even the two major groups (N, P) have not been found to be significantly different (p=0.12 by both the Mantel-Cox and Gehan-Breslow-Wilcoxon tests), in spite of the considerable difference between their survival
curves. The correlation between chromosome 3 and 4 aberrations and LH-RH-R was also investigated in 17 UM samples where receptor data were available. No significant correlation was found between the chromosome copy number and the expression and binding characteristics of LH-RH-R. Furthermore, based on our findings and the clinicopathological data, there is no correlation between the clinical outcome and chromosome 3 and 4 status.
5 Summary

Previously, we have demonstrated that approximately 50 % of UMs express LH-RH-R-I. The gene encoding LH-RH-R-I is located in chromosome 4, however the occurring numerical aberrations of chromosome 4 have never been studied in UM.

In the present study, our aim was to investigate the expression of mRNA for receptors of LH-RH-I and LH-RH ligand in OCM-1 and OCM-3 human UM cell lines. The presence and binding characteristics of LH-RH-I receptor protein was further studied by Western blot, immunocytochemistry and ligand competition assay. The mRNA and protein expression of LH-RH-I receptors have also been studied using tumor samples originating from nude mice xenografted with OCM-1 or OCM-3 cells. Moreover, we investigated the abnormalities of chromosome 3 and 4 and the possible correlation between them and with the expression of LH-RH-R. 46 UM specimens were obtained after enucleation. Numerical aberrations of chromosome 3 and 4 were studied by FISH.

mRNA expression of LH-RH-I receptor has been detected in OCM-1 and OCM-3 cell lines and was found higher in OCM-3 cells. LH-RH-I receptor mRNA was also observed in both UM xenograft models with higher levels in OCM-3 xenografted mice. The presence of LH-RH-I receptor protein was found in both cell lines, and also in tumor tissue samples grown in nude mice. Both human UM models investigated showed specific high affinity receptors for LH-RH-I using ligand competition assay. Expression of mRNA for LH-RH
ligand has also been detected in both cell lines and cancer tissues. Chromosome 4 could be detected in normal two copies only in 14 samples (30 %), however, 32 cases (70 %) showed more than 2 signals per nucleus. Monosomy of chromosome 3 could be found in 16 samples (35 %). In 6 specimens (13 %), more than 2 copies of chromosome 3 were found, while normal biparental disomy could be detected in 24 samples (52 %). Statistical analysis indicates significant correlation (p<0.05) between the copy number of chromosome 3 and 4. Moreover, moderate difference has been revealed in the survival rate of the UM patients with various pathological profiles. No correlation was found between chromosome aberrations and LH-RH-R expression. In summary, the expression of LH-RH-I receptors in OCM-1 and OCM-3 human UM cell lines suggests that these receptors could serve as potential molecular target for novel therapies.

In conclusion, the results presented in the current dissertation could contribute to a more precise determination of the prognosis of human UM and to the development of new therapeutic approaches to this malignancy.
6 Acknowledgements

I would like to express my special thanks to my supervisor, Gábor Halmos PharmD PhD the head of the Department of Biopharmacy, for his guidance throughout my PhD period, his support and his useful advice in my experimental work. This work is dedicated to the late Andrea Treszl PhD, who died of metastatic breast cancer. She introduced me to the world of research and inspired me to start my scientific work. Her intellectual, spiritual and personal contributions provided a great inspiration to our work in UM.

I express my gratitude to all my colleagues in the Department of Biopharmacy for creating a supportive and pleasant atmosphere.

I express my gratitude to our collaborating partners. It was a great privilege to perform scientific work with Nobel laureate Andrew V. Schally.

I am deeply grateful to my husband and my little son, Botond, for their patience and support throughout my studies.

This work was supported by Hungarian Scientific Research Fund (OTKA) K 81596 (G.H.), TAMOP 4.2.2.A-11/1/KONV-2012-0025 project (G.H.), GINOP-2.3.2-15-2016-00043 (G.H.), TAMOP-4.2.2/B-10/1-2010-0024 (E.S), the Gedeon Richter's Talentum Foundation (E.S.), EFOP-3.6.1-16-2016-00022 (E.S) and the ÚNKP-17-3 New National Excellence Program of the Ministry of Human Capacities (E.S.). The project is co-financed by the European Union and the European Regional Development Fund. The research was also financed by the Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary, within the framework of the Biotechnology thematic programme of the University of Debrecen.
Candidate: Eva Sipos
Neptun ID: V1LB4Q
Doctoral School: Doctoral School of Pharmacy

List of publications related to the dissertation


List of other publications


Total IF of journals (all publications): 22,646
Total IF of journals (publications related to the dissertation): 5,274

The Candidate’s publication data submitted to the IDEa Tudostér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

07 March, 2018
Published abstracts:

Sipos Éva, Fodor Klára, Rózsa Dávid, Treszl Andrea, Schally Andrew, Halmos Gábor: I-es típusú LH-RH receptorok (LH-RH-R) karakterizálása OCM-1 és OCM-3 humán uvealis melanoma sejtvonalakon
Magyar Onkológia 61: (1)p. 72. Magyar Onkológusok Társasága XXXII. Kongresszusa 2017.11. 16-18. Debrecen

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