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

Investigation of luteinizing hormone-releasing hormone receptor based new targeted therapies and angiogenesis of tumors with high mortality

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1. Introduction

Numerous preclinical studies have demonstrated the efficacy of chemotherapy based on cytotoxic peptide conjugates targeted to receptors on different tumors in the last decades. A major challenge in the development of novel and highly effective anti-cancer drugs is the selective drug-delivery to the tumor site while healthy tissue is spared. Cell surface receptors are of high interest in the targeted cancer therapy approach as they provide the desired properties to allow selective tumor targeting. One of the most promising therapeutic strategies is based on the peptide receptors that are overexpressed in tumor cells, in comparison with their expression in normal tissues. Consequently, the ligand analogs of these receptors can be used as carriers for the preparation of diagnostic and therapeutic agents that have increased selectivity and decreased peripheral toxicity.

It was demonstraded by numerous studies, that luteinizing hormone-releasing hormone receptor (LHRH-R) is overexpressed in several types of cancers such as prostate, endometrial, epithelial ovarian, bladder, breast, lymphomas, and lung cancers and can also serve as targets for LHRH analogs that can be coupled to different cytotoxic agents. The cytotoxic agent attached to the peptide can enter the tumor cell by receptor mediated way and induce the death only of the tumor cell. For the generation of drug conjugates, the selective LHRH analog [D-Lys^6]-LHRH is frequently used and reached as a doxorubicin derivative (AEZS-108/AN-152). The receptor mediated antiproliferative effect of LHRH analog AEZS-108 has been considered on various tumors. This doxorubicin attached form of LHRH ligand showed exellent tissue permeabilty (except crossing the blood-brain barrier), high affinty to the LHRH-R-s, minimal side effects and rapid clearance from the body.

Tumor targeting with cytotoxic LHRH analogs would provide novel therapeutic strategies for LHRH positive tumors with high mortality and high recurrence rate such as uveal melanoma (UM) or baldder cancer. There are no running clinical studies investigating LHRH based therapeutic strategies for treatment of patients with UM or with bladder cancer.

Uveal melanoma is a rare but very agressive tumor. In our previous studies, we demonstrated that 46 % of UM specimens express the LHRH-R and AEZS-108 was found to be able to inhibit the proliferation of OCM3 UM cells even the doxorubicin resistant form of the cell line in a dose dependent manner.

Since it is unknown what type of signaling processes support the antiproliferative effects of AEZS-108 in addition of doxorubicin (DOX) induced reactive oxygene species (ROS) due to the receptor-ligand interaction, we aimed to study these signaling pathways. Angiogenesis has a pivotal role in the development of UM because lymphatic vessels are not present in the eyeball to promote distant metastasis. This special characteristics of UM also led us to investigate the effect of AEZS-108 on the expression of the genes involved in angiogenesis and metastasis in an *in vitro* model of UM.

In our further experiments we investigated the expression of LHRH-R-s as potential molecular targets for targeted cancer treatments in urological tissues. Bladder cancer is the 10th most common cancer worldwide and it is the second most common malignancy of the genitourinary tract after prostate cancer. The most common type of bladder cancer is the transitional cell carcinoma (TTC) (95.7%) and most tumors are of low malignant potential grade (39.7%). Despite its chemosensitivity, bladder cancer has high recurrence rate, nearly 50% and has the highest lifetime treatment cost per patient of all cancers. Systemic chemotherapy and immunotherapy result in partial response and only few cases show a complete response to this kind of combination therapy. These therapeutic approaches are accompanied by severe side effects and consequently by a low quality of life, therefore, new treatment strategies are urgently needed. Our aim was to investigate the expression pattern of LHRH ligand and LHRH-R in human bladder tumor specimens, in human bladder cancer cell lines and to study the potential correlation between the expression of receptors and clinicopathological characteristics of the specimens. Using radioligand competition assays, we investigate the bindig affinity of LHRH agonists or antagonists to the cell surface receptors.

In the last part of our study we focused on the angiogenesis of UM and the presence of cancer stem cell markers (CSCs) in UMs. The use of anti-angiogenic therapy as a primary treatment for patients with UM showed limited efficacy or even the acceleration of tumor growth in many clinical studies. Researchers drawn the conclusion, that targeting tumor neoangiogenesis may only be successful if the effect of the tumor microenvironment is considered. Recent studies have demonstrated that VEGF binds primarily to its receptors on endothelial cells but may also act on hematopoietic or neural stem cells. In addition, a primitive neural or ectodermal cell stem cell-like profile with significantly higher CSC marker gene expression was observed in densely vascularized UM specimens with a greater risk of metastasis. There is clear evidence for the complex process o neoangiogenic signals, the failures of anti-VEGFA treatments in UMs, the binding affinity of VEGF to hematopoietic or neural stem cells, and the presence of CSCs in circulating or metastatic UM tumors. One could still ask whether targeting the angiogenic factors is the real key to modifying the tumorigenesis of UM or are these processes only the consequences of other unknown key oncogenic factors like CSCs? Trying to answer this question, in the present study we aimed to evaluate the expression of cancer stem cell markers and angiogenic factors in UM and to understand the relationships between the expression of the detected stem cell markers and vasculogenic mimicry patterns in primary UM tumors.

2. Aims of the study

Since it is unknown what type of signaling processes support the antiprolifereative effects of AEZS-108 (AN-152) in addition of DOX induced ROS (reactive oxygene species) due to the receptor- ligand interaction, we aimed to study these signaling pathways in OCM3 uveal melanoma cell line. First, we demonstrated the expression of LHRH-R-s in OCM3 UM cells and human UM tissue specimens. The cytotocitcity of AEZS-108 was investigated by MTS assays in *in vitro* treatments with AEZS-108 and DOX in OCM3 cells. After the treatments with AEZS-108 and with DOX in OCM3 cells we investigated the altered gene expression profile of the genes involved in angiogenesis and metastasis by RT-qPCR array. Western blot analysis confirmed that the greatest changes of mRNA expression are at protein levels as well. Further qRT-PCR analysisis revealed that AEZS-108 is a more potent inducer of MASPIN tumor suppressor than free DOX in OCM3 cells. We investigated the role of [D-Lys^6]-LHRH ligand analog in the induction of MASPIN due to receptor- ligand interaction. We studied the expression of MASPIN gene in normal uvea and UM tumor specimens.

In the next part of our study we investigated the expression of LHRH-R-s as potential molecular targets for targeted cancer treatments in urological tissues. We investigated the expression of LHRH ligand and LHRH-R in human bladder tumor specimens and in three human bladder cancer cell lines (RT-112, UMUC3, and TCCSUP). We studied the potential correlation between the expression of receptors and clinicopathological characteristics of the specimens. Using radioligand competition assays, we investigated the presence of specific, high affinity receptors for LHRH on TCC samples and their bindig affinity to [D-Lys^6]-LHRH ligand analog, to LHRH agonist AEZS-108 or to LHRH antagonist Cetrorelix.

In the last part of our study we investigated the expression of stem cell markers in human UM specimens and we compared the reults with the expression of these markers in normal uvea tissues. Since, angiogenesis has a pivotal role in the development of UM we evaluated the expression of cancer stem cell markers and angiogenic factors in UM specimens and we aimed to understand the relationships between the expression of the detected stem cell markers an vasculogenic mimicry patterns of primary UM tumors. We performed the analysis by staining of FZD6 stem cell marker, VEGFA and HIF-1A angiogenic factors in UM specimens by IHC tissue microarray method. Statistical associations among the expression of FZD6, VEGFA and HIF-1A were evaluated with Spearman correlation,

correlations between the detected gene expression pattern and the clinicopathological characteristics were illustrated by Kaplan-Meier analysis.

3. Materials and methods

3.1. Cell lines and culturing conditons

OCM3 human primary UM cell lines, RT-112, UMUC3, and TCCSUP human bladder cancer 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. Human tissue specimens

Specimens of human UM were obtained at the time of enucleation at the Department of Ophthalmology of the University of Debrecen, Hungary. The human bladder cancer specimens were obtained at the time of initial open surgical treatment at the Department of Urology, University of Debrecen, Hungary. Informed consent was obtained before enucleation and sugery, and the study was performed according to the tenets of the Declaration of Helsinki and the local Institutional Ethics Committee. The collection and the use of the specimens were approved by the local institutional ethics committee named Regional Institutional Ethics Committee, Clinical Center, University of Debrecen. Immediately after surgical removal of UM or bladder tissues they were collected, saved and snap frozen in liquid nitrogen, and stored at −80°C until molecular biology analyses. For histopathological analysis formalin fixed paraffin embedded tissues were used.

3.3. Methods used in our studies focusing on uveal melanoma cancers

3.3.1. *In vitro* treatments with [D- Lys^6]LHRH analog, with cytotoxic AEZS-108 and with doxorubicin in OCM3 cell lines

The AEZS-108 (formerly known as AN-152/ INN: Zoptarelin Doxorubicin Acetate) and [D-Lys^6]LHRH used in our experiments was kindly provided by Æterna Zentaris (Frankfurt am Main, Germany). Doxorubicin HCl (2 mg/ml) (DOX) was purchased from Teva Pharmaceutical Works Ltd. (Hungary). The compounds were dissolved in NaCl and used in 5 μM concentration in complete RPMI 1640 medium. Cells were incubated with the media containing compounds for 24 or 48 hours long.

3.3.2. Determination of cell viability by MTS assay

Cells were seeded into a 96-well plate at the density of 10.000 cells/ well in complete growth medium and incubated for 24 hours. Afterwards, medium was completely replaced with 5 μM cytotoxic compound containing medium and cells were incubated for additional

24 hours. Subsequently, MTS assay was performed in order to quantify viable cells. All treatments were performed in hexaplicates (n= 6). Absorbance was measured at 490 nm.

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

After *in vitro* treatments we used TRIzol reagent (MRC, USA) to isolate total RNA from OCM3 cells. RNA from each sample (500 ng) was reverse transcribed to cDNA using IScript Reverse Transcriptase Kit (Bio-Rad Laboratories, USA). In order to investigate the expression of genes involved in angiogenesis and metastasis regulatory factors Human Angiogenesis 96 StellArray™ qRT-PCR arrays (Lonza Ltd., USA) plates and SYBR Green Supermix (Bio-Rad Laboratories, USA) were used. In order to evaluate the expression of MASPIN in furter qRT-PCR analysises specific primer set was designed. All real-time amplifications were measured in triplicates. Altered mRNA expression levels were calculated using the $2^{40\text{-}Ct}$ method results were normalized to expression of housekeeping genes.

In every other experiments, in which we worked with uveal melanoma cells (OCM3) or with UM tissues total RNA was isolated using AllPrep DNA/RNA/Protein Mini kit (Qiagen, Hilden, Germany). RNA from each sample (250- 500 ng) was reverse transcribed to cDNA using QuantiTect Reverse Transcription kittel (Qiagen, Germany). In order to evaluate the expression of genes in our interest primer sets were designed. 1 µl of the cDNA was amplified using PCR amplification kit (Fermentas, Germany). β-actin was used as an internal control. PCR products were separated electrophoretically in 1.5% agarose gel and stained with GelRed® Nucleic Acid Gel Stain (Biotium, UK).

3.3.4. Protein analyisis of UM cells and samples

Immuncytochemistry

To detect LHRH-R-s OCM-3 cells were fixed in ice-cold methanol. After permeabilisation of the cells and blocking the aspecific binding sites we incubated the cells overnight with primary antibody (anti-LHRHR (Santa Cruz, USA, dilution:1:100). After washing steps EnVision Flex, HRP (Agilent Technologies, USA) secondary antibody was used. Signals were detected using ready-to-use DAB substrate kit (Agilent Technologies, USA).

SDS-PAGE-Western blot

In case of *in vitro* treated OCM3 samples total protein was isolated using TRIzol reagent (MRC, USA). Total protein from tissue samples was isolated using AllPrep DNA/RNA/Protein Mini kit (Qiagen, Hilden, Germany). 40 μgs of proteins were separated in 12 % SDS-polyacrylamide gels and then transferred to PVDF membrane using standard procedures. After blocking of aspecific binding sites of the membrane we incubated the samples overnight with primary antibodies to MASPIN, VEGFA, VEGFB, HIF1A, GAPDH, β-actin or FZD6. After washing the membranes with TRIS-buffer or PBS-buffer primary antibodies labeled with horseradish peroxidase or alkaline phosphatase were conjugated to secondary antibodies. Protein signals were detected using WesternBright™ ECL Substrate Kit (Advanstra Corporation, USA) or AP Conjugate Substrate Kit (Bio-Rad Laboratories, Hercules, CA, USA). Results were acquired and analyzed with the Molecular Image Chemidoc XRS+ System using Image Lab Software 5.2 (Bio-Rad Laboratories, USA).

Immunhistochemistry

TMA blocks were created by a computer-controlled TMA Master instrument (3DHISTECH, Budapest, Hungary). Formalin-fixed parafin- embedded, 3–4 μm thick tissue samples from enucleation were deparafinized, rehydrated using ethanol, and treated with EnVision™ FLEX Peroxidase-Blocking Reagent (Dako, Denmark) to inhibit endogenous peroxidase activity. After heat- induced epitope retrieval and blocking sections were incubated with antibody specific for VEGF in 1:100 (Santa Cruz Biotechnology, USA); for FZD6 in 1:100 (Santa Cruz Biotechnology, USA) and for HIF-1A (Covalab, France) in 1:3000 at room temperature in wet chamber at 4°C overnight. After washing steps primary antibodies were labeled with secondary antibodies linked to horseradish-peroxidase (HRP). Signals were developed using VectorVIP DAB+ Chromogen Substrate Kit (Vector® Labs, USA). According to the intensity, the proportion of positive cells was ranked in five groups: 0 (negative), 1 (1–25%), 2 (26–50%), 3 (51–75%) and 4 (76–100%).

3.4. Methods used in our studies focusing on bladder cancer

3.4.1. RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using Nucleospin Total RNA and Protein Isolation Kit (Macherey- Nagel, Germany) according to the manufacturer's instructions. For reverse transcription, 150–250 ng of total RNA, oligo (dT) 15 primer, and MMLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA) were used. cDNA was amplified using gene- specific primers for LHRH-R-I: sense 5′-GAC CTT GTC TGG AAA GAT CC-3′, antisense 5′-CAG GCT GAT CAC CAC CAT CA-3′, for LHRH-I: sense: 5′-CTA CTG ACT TGG TGC GTG GA-3′ and antisense: 5′-CTG CCC AGT TTC CTC TTC AA-3′. For β-actin houskeeping gene, sense 5′- GGC ATC CTC ACC CTG AAG TA-3′, and antisense 5′-GGG GTG TTG AAG GTC TCA AA-3′ were used. 1 μl of the cDNA was amplified using PCR amplification kit (Invitrogen, UK). PCR products were separated electrophoretically in 1.5% agarose gel and stained with GelRed® Nucleic Acid Gel Stain (Biotium, UK).

3.4.2. Immunhistochemistry

For immunohistochemistry (IHC), serial sections of paraffin- embedded tissues (12 bladder cancer, 3 normal bladder samples and human pituitary glands) were used. After dewaxing in xylene and rehydrating in graded alcohol, antigen retrieval was performed (Target Retrieval Solution, Dako, Denmark). For biotin-free immune staining, a 'Bond-TM Automated Immune-Stainer' (Leica Microsystems, UK) was used according to the manufacturer's instruction. As a primary antibody, GnRH-R (equivalent to LHRH-R) antibody (Novocastra, UK) was applied in 1:20 dilution. Slides were incubated for 30 min with NCL-GnRHR mAB targeting the human GnRH terminal region. The immunoreaction was visualized by using diamino-benzidine (DAB). The slides were then counterstained with Mayer's hematoxylin. Immunoreactions were evaluated by a pathologist with respect to the intensity of the immunoreaction using the following scoring system: 0, no staining; 1+, weak; 2+, moderate; 3+, strong staining.

3.4.3. Preparation of membranes and radioligand binding studies

To investigate the ligand binding affinity of LHRH-R-s on membranes preparated from human TCC type of bladder cancer samples we used a radioactive LHRH analog ([^{125}I][D-Trp^{6}] LHRH). This radioligand was well- characterized previously and showed high affinity binding to LHRH receptors expressed in various human cancers. 60–150 μg protein were

incubated in duplicate with 70–90,000 cpm [^{125}I][D-Trp^{6}]LHRH in the presence of increasing concentrations of nonradioactive peptides as competitors ([D-Lys^{6}]LHRH, AN-152 ([D-Lys^{6}]LHRH- DOX) and Cetrorelix ([Ac-D-Nal(2)^{1},DPhe(4Cl)^{2},D-Pal(3)^{3},D-Cit^{6},D-Ala^{10}]LHRH), in a total volume of 150 μL of binding buffer. At the end of the incubation, 125 μL of suspension were transferred onto the top of 1 mL of ice- cold binding buffer containing 1.5% BSA in microcentrifuge tubes. The tubes were centrifuged at 12,000× g for 3 min at 4°C. Supernatants were aspirated and the pellet was counted in a gamma counter. Protein concentration was determined by Bio- Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The final binding affinities were expressed as IC_{50} values and were calculated by using the LIGAND-PC program.

3.4.4. Protein isolation Western blot with bladder cancer cell lines

We demonstrated the expression of LHRH-R-s in RT-112, UMUC3, and TCCSUP human bladder cancer cell lines by SDS-PAGE-Western blot. Afterwards, the cells were scraped from the flasks and lysed in ice-cold M-PER protein lysis buffer (Thermo Fisher Scientific, USA), supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, USA). Protein quantification was performed using BCA reagent (Thermo Fisher Scientific, USA). All the samples were diluted with 4× Laemmli buffer. 40 μg protein in equal volume of each sample was boiled at 95°C for 8 min. The lysates were separated on 10% SDS-polyacrylamide gels and then transferred to PVDF membrane using standard procedures. After blocking of aspecific binding sites of the membrane we incubated the samples overnight with primary antibodies to LHRH-R in 1:200 dilution (Santa Cruz Biotechnology, USA), to GAPDH in 1:1000 dilution (Cell Signaling Technology, USA). After washing steps primary antibodies were labeled with secondary antibodies linked HRP. Protein signals were detected using WesternBright™ ECL Substrate Kit (Advanstra Corporation, USA). Results were acquired and analyzed with the Molecular Image Chemidoc XRS+ System using Image Lab Software 5.2 (Bio-Rad Laboratories, USA).

3.5. Statistical analysis

Data of RT- PCR, RT- qPCR and Western blot experiments were analyzed by Students's t-test or one- way ANOVA followed by Tukey post hoc test to determine the differences between the selected groups using the program Prism 5 software (GraphPad Software, USA). Data are presented as a mean value ± standard error of the mean (SEM). A p value

* <0.05 was considered to be statistically significant (p value ** < 0.01: highly significant, p value *** < 0.005: extremely significant).

Correlations between FZD6, VEGFA and HIF-1A gene expression of the paraffin-embedded UM tissues were evaluated with Spearman correlation analysis. Survival of the patients depending of the variables was plotted against the post- operative days (elapsed until death or the follow-up period) according to the Kaplan–Meier method. Calculations were performed using IBM SPSS Statistics (IBM Corp. USA).

Correlations between expression of LHRH-R and clinicopathological status of TCC bladder cancer specimens Pearson analysis was performed (Microsoft Excel, USA).

4. Results

4.1. Results of our studies focusing on uveal melanoma

4.1.1. OCM3 human uveal melanoma cell line expresses the human LHRH receptor

Expression and cellular distribution of the full length LHRH-R in OCM3 cells was demonstrated by RT-PCR and by immunocytochemistry. We found that full length LHRH receptors are present in the cell membrane and therefore in the cytoplasm, so they might play a role in the facilitation of the selective uptake of AEZS-108 in OCM3 cells.

4.1.2. AEZS-108 and doxorubicin induces comparable cytotoxicity in OCM3 cells

Our result showed that 5 μM AEZS-108 reduced cell viability by 36.3% after 24 hours ($p < 0.001$) and by 84.7% after 48 hours ($p < 0.001$). In contrast, free DOX led to 62.9% ($p < 0.001$) and 89.7% ($p < 0.001$) cell death after 24 and 48 hours, respectively.

4.1.3. AEZS-108 alters the expression of angiogenesis and metastatis regulatory factors in OCM3 cells

We have investigated the role of AEZS-108 in the expression of 94 key regulatory genes involved in angiogenesis and development of metastasis in OCM3 cells. MASPIN (SERPINB5), SERPINE1 (PAI-1), CXCR4, VEGFA and MAPK7 genes have been found to be significantly upregulated, while ANGPT1, HIF1-A, ANGPTL3, ETS1, VEGFB, CEACAM and SURVIVIN genes were significantly downregulated ($p < 0.05$) as compared to control (untreated) cells. The tumor suppressor gene MASPIN showed the highest overexpression (203.19× upregulation), while the most significantly downregulated gene was HIF-1A (8.67× downregulation).

4.1.4. AEZS-108 provokes higher expression of MASPIN than free DOX

The expression of MASPIN tumor suppressor gene was further investigated by qRT-PCR. Our results clearly showed that [D-Lys^6]LHRH treated OCM3 cells do not express MASPIN, while free DOX and AEZS-108 induces MASPIN expression. However, equal dose of AEZS-108 and DOX showed significantly different effect on MASPIN expression, namely, that AEZS-108 treatment results in significantly higher MASPIN expression than free DOX treatment.

4.1.5. The tumor suppressor MASPIN is not expressed in uveal melanoma or in normal uvea tissue specimens

The mRNA expression of MASPIN has been investigated in 3 healthy and 18 human uveal melanoma tissue specimens by RT-PCR. Our results did not show mRNA expression of MASPIN in healthy or in uveal melanoma tissues.

4.1.6. AEZS-108 alters MASPIN, HIF-1A, VEGFA and VEGFB protein expression in OCM3 cells

SDS-PAGE-Western blots confirmed the qRT-PCR results, namely, that AEZS-108 is a more potent inducer of MASPIN than free DOX. Our data showed that treatment with AEZS-108 and DOX significantly decreased HIF-1A, VEGFA and VEGFB expression (HIF-1A, $p < 0.05$.)

4.1.7. Investigation of the gene expression of nestin, NGFR, SOX10, FZD6 and PROM1 cancer stem cell markers (CSCs) in 18 human UM specimens and in 3 normal uvea samples

The expression of cancer stem cell markers was demonstrated by RT-PCR and by densitometric analysis of the agarose gel electrophoresis of the PCR products. Snap frozen uveal melanoma tissue specimens consisted of seven epitheloid, eight spindle and three mixed cell type tumors from 6 female and 12 male patients with an average age of 63.1 years (range between 30–84 years). Normal uvea samples showed considerably lower positivity compared to tumor samples (nestin: $p = 0.007$; SOX10: $p = 0.004$). The 18 UM specimens showed stronger CSC expression with the exception of NGFR. mRNA levels of nestin, FZD6 and SOX10 could be detected in all the samples. PROM1 was expressed in 82% and NGFR was expressed in 94% of the specimens. We found no statistical correlation between expression of CSCs and previous treatment of patients with ruthenium applicator.

4.1.8. Protein expression of FZD6, HIF-1A and VEGFA in 18 human UM specimens by SDS-PAGE-Western blot

To confirm the results of RT-PCR, protein levels of FZD6, HIF-1A and VEGFA have also been examined with SDS-PAGE-Western blot analysis. Our results showed that only a low number of samples (11.11%) were positive for FZD6; 38.88% and 33.33% of the samples were positive for the expression of HIF-1A and VEGFA, respectively.

4.1.9. Overall survival could depend on the tumor cell type constituting the UM tumor

Considering the low number of samples, we decided to modify our method in order to be able to investigate a great number of UM specimens with a more sensitive and cost-effective tissue microarray (TMA) system. Our study included 22 women and 30 men, with an average age of 59.55 years (range between 30- 83 years). The parafin-embedded primary uveal melanoma specimens consisted of 16 epitheloid, 24 spindles and 12 mixed cell type tumors. The results of Kaplan–Meier curves confirmed that the epithelioid subtype is associated with a worse prognosis than the other two tumor subtypes (Mantel–Cox test, n =49, p =0.02). The best survival rate was associated with the proportion of patients with mixed cell type.

4.1.10. FZD6 expression is related to VEGFA expression

We detected in 28 cases (58.8%) 1+ intensity, in 7 cases (13.7%) 2+ intensity of FZD6 staining and 17 cases (29%) no expression of FZD6 was detected. Normal nevi samples were negative to FZD6 expression. According to the Spearman analysis, there is a statistically significant strong correlation (Spearman r =0.411, n =48, p =0.004) between FZD6 and VEGFA expression, but not between FZD6 and HIF-1A expression (Spearman r =0.061, n =50, p =0.672). Kaplan–Meier curves showed no significant correlation (Mantel–Cox test, n =51, p =0.867) between the FZD6 expression and the survival of the patients. Comparing the survival rate of the three tumor subtypes separately and considering their FZD6 expression levels, an obvious but not significant difference was revealed. Based on the Kaplan–Meier curves (plotted against the number of postoperative days depending on FZD6 expression) there was a detectable trend (Mantel–Cox test, n =16, p =0.541) for a poor survival rate in the epithelioid subtype compared to the other two subtypes of UM.

4.1.11. No significant correlation was detected between the expression of angiogenic factors and survival rates in primary UM specimens

We detected in 5 cases (10.4%) 4+ intensity, in 12 cases (25%) 3+ intensity, in 12 cases (25%) 2+ intensity, in 12 cases (25%) 1+ intensity of VEGFA staining and in 7 cases (14.6%) no VEGFA expression was detected. Normal nevi samples were negative to VEGFA expression. Survival analysis showed no significant correlation between the VEGFA expression and the survival rate.

We detected in 10 cases (20%) 4+ intensity, in 26 cases (52%) 3+ intensity, in 10 cases (20%) 2+ intensity, in 2 cases (4%) 1+ intensity of HIF-1A staining and in 2 cases (4%) no expression was detected. Normal nevi samples showed high positivity (3+) to HIF-1A expression. No correlation was found between the HIF-1A expression and the survival of the patients.

4.1.12. The expression of melanin pigment negatively correlates with the overall survival rate in patients with primary UM

We detected in 10 cases (20.4%) 3+ intensity, in 13 cases (26.53%) 2+ intensity, in 20 cases (40.81%) 1+ intensity of melanin expression and in 6 cases (12.24%) no expression of melanin. Kaplan–Meier curves showed a significant difference in the overall survival depending on the melanin content of the samples (Mantel–Cox test, n=49, p=0.033). The highest survival rate was associated with the samples in which the expression of melanin was only weak.

4.2. Results of our studies focusing on TCC type of bladder cancer

4.2.1. Expression of mRNA for LHRH ligand and LHRH-R-I in human bladder cancer tissue samples and in human bladder cancer cell lines

We detected in 20 (83%) of 24 specimens the expression of LHRH receptor and in all cell lines (RT-112, UMUC3 and TCCSUP) examined. Ligand of LHRH was detected in 19 of 24 specimens examined (79%) and also in all three cell lines.

4.2.2. Immunohistochemistry of LHRH-R in human bladder cancer and in healthy bladder tissues

Positive staining for LHRH-R was found in all of the 12 samples examined. The majority of TCCs expressed the LHRH-R at a moderate level and the majority of cells showed cytoplasmic or membranous LHRH-R expression. The less differentiated TCC cases (Grade 3–4) showed no or weak staining (0, 1+), but the well or moderately differentiated tumors (Grade 1–2) appeared to exhibit moderate to strong LHRH-R expression. Result of Pearson analysis confirmed our visual observation: the expression of LHRH-R-s is negatively correlated ($r = -0.91$) with the pathological grade of the cases examined. No correlation among LHRH-R expression and patients' age and gender was observed. No LHRH-R expression was detected in 3 healthy bladder tissue samples.

4.2.3. Expression of LHRH receptors in human bladder cancer cell lines

The presence of LHRH-R-s was investigated in RT-112, UMUC3, and TCCSUP cell lines by Western blot analysis. Our results confirmed the presence of LHRH-R-s in all three bladder cancer cell lines examined.

4.2.4. Radioligand binding studies

Using ligand competition assays high affinity binding sites for LHRH-R were found in 10 of 12 human specimens. The mean concentration of LHRH-R-s (maximal binding capacity, B_{max}) was 473.09 fmol/mg membrane protein in crude membranes derived from human bladder cancers. The binding of [^{125}I][D-Trp^{6}]LHRH was found to be specific, reversible, time- and temperature-dependent, and linear with protein concentration in human bladder cancer samples. The binding affinity of LHRH analogs and cytotoxic LHRH analog AN-152 (AEZS-108) to membrane receptors of human bladder cancer cells expressing LHRH receptors was also investigated by ligand competition assay. Displacement of [^{125}I][D-Trp^{6}]LHRH as a radioligand by the unlabeled LHRH analogs as competitors was determined. Our results showed that LHRH agonist analog [D-Lys^{6}]LHRH, or is DOX conjugated form (AN-152) and LHRH antagonist Cetrorelix could effectively bind to LHRH-R-s even at low nanomolar concentration.

5. Summary

In our study we aimed to support the development of targeted therapeutic strategies focusing on LHRH-R-s for tumors with high mortality and recurrence rate, such as uveal melanoma (UM) of TCC type of bladder cancer.

In our first study, our aim was to demonstrate the expression of LHRH-R-s in OCM3 UM cells and to compare the antiproliferative effects of AEZS-108 and DOX in an *in vitro* model of UM, and to elucidate the signaling processes support the antitumor effects of AEZS-108. First, we found that full length LHRH-R-s are present in the cell membrane and therefore in the cytoplasm, so they might play a role in the facilitation of the selective uptake of AEZS-108 in OCM3 cells. The *in vitro* treatments with equal dose of AEZS-108 an DOX showed that AEZS-108 and DOX induces comparable cytotoxicity in OCM3 cells. Our analysis revealed that AEZS-108 is a more potent inducer of MASPIN tumor suppressor than free DOX in OCM3 cells and free LHRH ligand is not able to induce its overexpression. Based on our RT-PCR analysis MASPIN tumor suppressor is not expressed in in healthy uvea or in uveal melanoma tissues. Furthermore, the treatment with AEZS-108 altered the expression of many angiogenic factors e. g. VEGFA, VEGFB, HIF-1A ANGPT1, ANGPTL3 and of key regulators of migration e. g. CXCR4, ETS1 and SERPINE1.

In further studies we demonstrated high rate of expression of LHRH-R and LHRH ligand in human specimens with TCC type of bladder cancers. We demonstrated the mRNA expression of LHRH-R in 83% of 24 specimens and in all cell lines (RT-112, UMUC3 and TCCSUP) examined. Ligand of LHRH was detected in 79% of 24 specimens examined and also in all three cell lines.

Positive staining for LHRH-R proteins was found in all of the samples examined by IHC. The expression of LHRH-R showed negative correlation with TCC grade. Radioligand binding studies also showed the presence of specific LHRH-R on cell membrane of tissue specimens and high binding affinity of LHRH analogs like AN-152 or Cetrorelix.

In the last part of our studies, we investigated the regulation of angiogenesis in uveal melanoma in correlation with the presence of cancer stem cells (CSCs). RT-PCR analysis showed high expression of CSC markers, particularly nestin, FZD6 and SOX10 and somewhat lower expression of NGFR and PROM1 in UM tissues. The protein expression

of FZD6, HIF-1A and VEGFA was further evaluated in 52 uveal melanoma specimens by IHC-TMA. We report for the first time that Spearman analysis showed a significant correlation between FZD6 and VEGFA expression in uveal melanoma. The observed correlation between FZD6 and VEGFA suggests the presence of CSCs in UM that are associated with the vascularization process. Surprisingly, statistical analysises showed no correlations between the overall survival and expression of HIF-1A or VEGFA, despite targeting angiogenesis, often be assumed to be a promising way to treat a densely vascularized UM tumors.

Our results support the merit of additional investigation of the role of LHRH and its receptors in human uveal melanoma and in bladder cancer and open up a new avenue in the further developmen of LHRH analogs for therapeutic and imaging purposes in thes type of cancers.

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List of publications related to the dissertation

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