THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Luteinizing hormon-releasing hormon (LHRH) receptor based new possible targeted therapy for human uveal melanoma

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The Examination takes place at Department of Biopharmacy Faculty of Pharmacy, University of Debrecen, 21st march 2019.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen , 13:00 21st march 2019.

2. Introduction

Uveal melanoma (UM) is the most common and aggressive primary intraocular malignancy in adults representing 85% of ocular melanomas, with a poor prognosis upon discovery. About half of the patients have already metastatic disease by the time of diagnosis. The prognosis of patients with metastases is generally considered to be poor, with a median survival of 4–15 months and a 1-year survival is only approximately 15%. No efficient systemic therapy has been established that could reduce the risk of metastases or prolong patient survival. Improvement in 5 year relative survival rate of UM patients has not been observed in the past three decades. Uveal and cutaneous melanomas are basically different diseases. However, our limited knowledge on the pathophysiology and treatment of metastatic UM has led to the widespread use of the same treatment for cutaneous and uveal melanomas inappropriate chemotherapy in many cases boosts cancer cells to become resistant to chemotherapy. Due to drug resistance, cancer therapy requires the use of elevated doses of antitumor drugs, which might lead to toxic side effects. Specific membrane receptors for LHRH (luteinizing hormone-releasing hormone) have been found in various animal and human cancers. High affinity binding sites for LHRH and the expression of LHRH receptors have been described in prostate breast, endometrial, ovarian and other cancers. Our research group has also demonstrated the presence of high-affinity membrane receptors for LHRH in 46% of human UM. Different transcript forms of mRNA for LHRH receptors have been reported in human benign prostate hyperplasia specimens, suggesting that the splicing mechanisms of LHRH receptors might be regulated in a tissue specific manner, or influenced by specific hormonal or metabolic effects. It is generally accepted, that drug resistance represents one of the major issues in the treatment of cancers. Targeted chemotherapy with cytotoxic LHRH analogs can alleviate side effects, compared to conventional chemotherapy. Thus, LHRH receptors expressed by UM could offer a novel, receptor targeted therapeutic approach. The cytotoxic analog AN-152 (AEZS-108, zoptarelin doxorubicin), consisting of [D-Lys6]-LHRH linked through a glutaric acid spacer to one molecule of doxorubicin (DOX) was specifically designed for receptor mediated chemotherapy aimed at the inhibition of the growth of tumors expressing LHRH receptors. However, according to our best knowledge, the efficacy of AN-152 in a DOX resistant human UM model has not been examined yet. In the current study, we have established a stable DOX resistant human UM cell line $OCM3_{DOX320}$, and confirmed the expression of LHRH receptor splice variants. Dose dependent toxicity of DOX and AN152 has been investigated in wild type (OCM3) and DOX resistant ($OCM3_{DOX320}$) UM cell lines by MTT (3-(4,5-dimethylthiazol-2-

yl)-2,5- diphenyltetrazolium bromide) assay. In addition, using confocal laser scanning microscopy, LHRH receptor-mediated internalization of AN152 has been shown in OCM3 and $OCM3_{DOX320}$ cells in a time dependent manner.

3. Aims

The aim of this study therefore was to evaluate as a potential therapeutic target the expression of luteinizing hormone-releasing hormone (LHRH) receptor in human uveal melanoma.

In the present study we investigated the cellular uptake of doxorubicin (DOX) and cytotoxic LHRH analog AN-152 (AEZS-108, zoptarelin doxorubicin) on human UM cell lines (OCM3) and its DOX resistant form $OCM3_{DOX320}$ by confocal laser scanning microscopy. The LHRH receptor expression was characterized by RT-PCR and immunocytochemistry.

4. Materials and methots

Patients and Tissue Samples

Human uveal melanoma specimens were obtained from 39 patients, 30-84 years of age at the time of enucleation, at the Department of Ophthalmology, University of Debrecen, Hungary. Normal pituitary samples (anterior lobe) were collected at autopsy at the Department of Pathology, University of Debrecen and were used as positive controls. After surgical removal, selected portions of the melanoma tissues were flash frozen and stored at -70°C. Histopathological examination of each specimen was undertaken to confirm the diagnosis. The local Institutional Ethics Committee approved the collection and use of these specimens for the current study and informed consent was obtained from these patients.

RNA isolation, Reverse transcription and RT-PCR

Total RNA was isolated using AllPrep DNA/ RNA/Protein Mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Two hundred fifty nanograms of RNA from each sample were reverse transcribed into cDNA by QuantiTect Reverse Transcription kit (Qiagen) in a final volume of 20 µl. Two primer sets were designed to evaluate the expression of type I LHRH receptors (sense: 5'-GGTGGCATCAAGCATTTTAT-3', antisense: 5'–ACATAGTAGGGAGTCCAGCAGACA-3') and LHRH ligand (sense: 5'–GGCCTTATTCTACTGACTTGG-3', antisense: 5'-TCTTCTGCCCAGTTTCCTCT-3'). As internal control, β-actin housekeeping gene (sense: 5'-GGCATCCTCACCCTGAAGTA-3', antisense 5'-GGGGTGTTGAAGGTCTCAAA-3') was used. In all PCR reactions, 1 µl of cDNA was amplified in a 25 µl solution containing 1.5 mM MgCl2, 1x PCR buffer (Fermentas GmbH, St. Leon-Rot, Germany), 0.3 mM of each deoxynucleotide (Promega, Madison, WI), 1 unit of TrueStart HotStart DNA polymerase (Fermentas) and 0.25 µM of each primer. Samples were denatured for 3 min at 95°C, then subjected to 40 cycles at 95°C for 45 s, 59°C for 30 s, then 72°C for 1.5 min with a final extension at 72°C for 10 min. Ten µl of each amplification reaction was then electrophoretically separated on 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

Preparation of membranes and radioligand binding studies

Preparation of membranes for receptor studies was performed as described previously. Briefly, the samples were thawed, cleaned, and then homogenized in 50 mM Tris-HCl buffer (pH 7.4), supplemented with protease inhibitors (0.25mM Phenylmethylsulfonyl Fluoride, 0.4% (v/v) Aprotinin and 2 μg/ml Pepstatin A) using an Ultra-Turrax tissue homogenizer (IKA Works, Wilmington, NC) on ice. The homogenate was centrifuged at 500x g for 10 minutes at 4 ºC to remove nuclear debris and lipid layer. The supernatant containing the crude membrane fraction was ultracentrifuged (Beckman L8-80 M) twice at 70,000x g for 50 minutes at 4 ºC after resuspending in fresh buffer. The final pellet was resuspended in homogenization buffer and stored at -80 ºC until assayed. Protein concentration was determined by the method of Bradford using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Radio-iodinated derivatives of [D-Trp^6]LHRH were prepared by the chloramine-T method and purified by reverse-phase HPLC in our laboratory. LHRH receptor binding assays were carried out as reported [24] using in vitro ligand competition assays based on binding of [125I][D-Trp6]LHRH as radioligand to uveal melanoma membrane fractions. This radioligand has been wellcharacterized previously and shows high-affinity binding to human and rat pituitaries as well as human breast, prostate, and other cancers. In brief, membrane homogenates containing 50-160 μg protein were incubated in duplicate or triplicate with 60-80,000 cpm [^{125}I][D-Trp^6] LHRH and increasing concentrations (10^{-12} - 10^{-6} M) of nonradioactive peptides as competitors in a total volume of 150 μl of binding buffer. At the end of the incubation, 125 μl aliquots of suspension were transferred onto the top of 1 ml of ice-cold binding buffer containing 1.5% bovine serum albumin in siliconized polypropylene microcentrifuge tubes (Sigma-Aldrich GmbH, Munich, Germany). The tubes were then centrifuged at 12,000x g for 3 minutes at 4 ºC (Beckman J2-21M). Supernatants were aspirated and the bottoms of the tubes containing the pellet were cut off and counted in a gamma counter (Micromedic System, Huntsville, AL). Preliminary experiments were performed with membrane protein concentrations ranging from 20-250 μg/tube in order to determine the minimal amount of protein required to assess specific binding at a satisfactory level. Our work showed that accurate results can be obtained over a range of 40-180 μg of membrane protein in an incubation volume of 150 μl. The LIGAND-PC computerized curve-fitting program was used to determine the type of receptor binding, dissociation constant (K_d) and maximal

binding capacity of the receptors (B_{max}) againct Scatchard curve. This experiments was carried out the help of my co-authors, Gabor Halmos.

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded tissue samples from enucleation were immunostained as described earlier. [24] Briefly, following antigen-retrieving (pH: 6.0) and endogenous peroxidase-block, 3 μm thick sections were incubated with monoclonal antibody to LHRH-RI (NCL-GnRHR A9E4; Novocastra Laboratories Ltd., UK) at room temperature for 1 hour. After rinsing 3 times in phosphate-buffered saline (PBS; pH:7.4, 5 mins each), sections were treated with anti-mouse IgG (Fab)2- coupled to horse-radish-peroxidase (HRP) of EnVision+- HRP detection kit using amino-ethyl-carbasol (AEC; red; Vector Labs, UK) peroxidase substrate according to the manufacturer's instructions. The use of red chromogenic substrate avoided the color-interference between the brown melanin pigments of melanomas and the positive staining of IHC. Human pituitary glands (anterior lobe) obtained from autopsy were used as positive controls. Negative controls in which primary antibody was replaced by normal serum were also included for each IHC-run.
This experiments was carried out the help of my co-authors, Balázs Dezső.

Statistical analysis

Variables were described using standard statistics. Association between categorical variables were assessed using Fisher's exact tests, while those between continuous explanatory variables and binary outcomes were assessed using logistic regression, and expressed in terms of odds ratio (OR) and 95% confidence intervals (CI).

Cell lines and culturing conditions

The OCM3 cell line was kindly provided by the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen. Uveal melanoma cells were grown in monolayer and cultured in RPMI-1640 medium supplemented with L-glutamine (Biosera, USA), 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Biosera, USA) and 100 μg/ml penicillin-streptomycin (Biological Industries, USA) under standard conditions.

Development of doxorubicin resistant OCM3 sub-line

In order to induce resistance in OCM3 cells DOX (2 mg/ml injection solution, TEVA, Hungary, diluted in saline to 50 μM stock solution) was used. Initially, the cells were grown in a cell culture medium containing 10 nM DOX. Confluent cell cultures have been split and DOX concentration was doubled until final concentration of 320 nM was reached as described before (AbuHammad and Zihlif, 2013). This new sub-line became resistant to DOX and called $OCM3_{DOX320}$.

Total RNA isolation, reverse transcription and polymerase chain reaction

Total RNA was isolated using TRI reagent (Molecular Research Center, USA). Briefly, 30 mg of human pituitary (served as positive control) or 107 cells of OCM3 and $OCM3_{DOX320}$ were used for the standard isolation protocol. 500 ng of RNA from each sample was reverse transcribed into cDNA by Tetro cDNA Synthesis Kit (Bioline, Canada). Specific primer pairs were used to detect the expression of different exons of LHRH receptors as reported earlier (Grundker et al., 2001). All PCR (polymerase chain reaction) reactions were performed using MyTaq HS Mix (Bioline, Canada). Samples were denatured for 1 min at 95 °C, and then subjected to 40 cycles at 95 °C for 15 s, 60 °C for 30 s, then 72 °C for 10 s with a final extension at 72 °C for 2 min. Amplification reactions were then electrophoretically separated, stained with GelRed (Biotium, USA), and visualized under UV light.

Immunocytochemistry

OCM3 and $OCM3_{DOX320}$ cells were cultured on coverslips in 12-well plates (SPL Life Science Co, Korea). Following treatments, cells were fixed, permeabilized and blocked with 10% bovine serum albumin (BSA) (Sigma-Aldrich, USA) for 1 h at room temperature. Samples were then incubated overnight with anti-LHRH-R (GnRHR(FL-328)) (Santa Cruz, USA) antibody at 4 °C and followed by FITC (fluorescein isothiocyanate) conjugated goat anti-rabbit IgG (sc-2012, Santa Cruz, USA) for 1 h at room temperature. Samples were covered with ProLong® Diamond Antifade Mountant with DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride) (Molecular Probes, USA). Fluorescence images were acquired using an Olympus IX71 camera (Olympus Corporation; Japan). Autofluorescence

and specific fluorescent signal were distinguished using unlabeled control cells under the same experimental conditions as fluorescently labeled cells. In the negative control samples, only secondary antibody was used in order to identify its non-specific binding.

Cell proliferation assay

The antiproliferative effects of DOX and AN-152 on OCM3 and $OCM3_{DOX320}$ cells were evaluated using Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich, USA), according to standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) protocol. Briefly, cells were seeded onto 96-well plates (SPL Life Science Co, Korea) at a concentration of 104 cells/well and cultured for 24 h. 40 nM, 320 nM, 1 μM, 2.5 μM and 5 μM DOX or AN-152 were added (100 μl/well). Cells were incubated at 37 °C for 72 h. Experiments were repeated 4 times. Optical density was measured at 570 nm using FLUOstart optima plate reader (BGM Labtech, Germany). Cytotoxic LHRH-conjugate, AN-152 (AEZS-108, zoptarelin doxorubicin) was originally synthesized in the laboratory of Andrew V. Schally, who kindly provided the compound to this experimental study.

Confocal laser scanning microscopy

10^4 OCM3 and $OCM3_{DOX320}$ cells were seeded in a μ-Slide 8-well, ibiTreat microscopy chamber (Ibidi, Germany). 24 h later, cells were treated with either 5 μM DOX or AN-152 for 0.5, 1, 6 or 24 h. Images were taken using an Olympus FluoView 1000 confocal laser scanning microscope (Olympus, Japan). Autofluorescence of DOX and AN-152 was detected. 543 nm line of an Argon ion laser was applied to excite DOX. Fluorescence emission of DOX was detected at 603 nm. Image stacks of 1 μm thick optical sections each containing 512 × 512 pixels were obtained using a 60× UPLSAPO oil immersion objective (NA 1.35).

Statistical analysis

Correlation analysis was carried out with the use of GraphPad Prism 7. Results were tested by 1-way ANOVA, followed by a Tukey's test for comparison for individual groups.

5. Results

Expression of human type-I LHRH receptors in

Our specimens of uveal melanoma tissue consisted of 11 epithelioid, 21 spindle and 7 mixed cell type tumors. The tumor thickness range was 6-12.9 mm according to ultrasonography. The tumor basal diameters as measured with ultrasonography, ranged from 9-19 mm. If the tumor thickness was more than 8 mm and/or the largest tumor diameter was more than 13 mm, we enucleated the eye without prior treatment. In those cases where the thickness was less than 8 mm or the basal diameter was less than 13 mm, but the tumor was growing in spite of the previous transpupillary thermotherapy and/or Ruthenium-106 plaque brachytherapy, enucleation was performed. Based on our recent knowledge, type I LHRH receptor has two splice variants, but only the full length receptor is functional. Our primer set for LHRH receptor was designed to specifically amplify the mRNA of the full length receptor, but to give no signals for the splice variants. We used LHRH receptor primers encompassing the open reading frame from exon 2 to exon 3, overlapping the missing part in the above mentioned two isoforms. The predicted size of the PCR amplified cDNA for type I LHRH receptor was 241 bp. Fourty six percent of our samples expressed receptors for type I LHRH receptor (Fig. 1., Table 1.). Among epithelioid type uveal melanomas, 6 of 11 (55 %) were found to be positive for the expression of LHRH receptor while spindle type melanomas included 10 of 21 (48%) positive samples. In the mixed cell type group (containing both epithelioid and spindle cells, with no dominant pattern), 2 of 7 (29%) of the tumors expressed type I LHRH receptor. The presence of full length LHRH-I receptor was confirmed by immunohistochemistry and correlated well with the findings by RT-PCR. Among the specimens of RT-PCR positive uveal melanoma, the majority stained positive for LHRH receptors which could be detected in the form of red granules. The presence of specific LHRH binding sites and characteristics of binding of [^{125}I][D-Trp6]LHRH to membrane receptors on human uveal melanoma tissue was determined using ligand competition assays. Of the 10 specimens examined, 7 showed LHRH receptor binding. Analyses of the typical displacement of radiolabeled [D-Trp^{6}]LHRH by the same unlabeled peptide revealed that the one-site model provided the best fit, indicating the presence of one class of high-affinity LHRH receptors in crude membranes derived from human uveal melanoma specimens. The computerized nonlinear curvefitting and the Scatchard plot analyses of the binding data in the 7 receptor-positive tumor specimens indicated that the single class of binding sites had a mean dissociation constant (K_d) of 3.69 nM (range, 1.35 to 6.36 nM) with a mean maximal binding capacity (B_{max}) of 384.5 fmol/ mg of membrane protein (range, 251.5

to 511.6 fmol/mg protein). Biochemical parameters essential to establish the identity of specific binding sites were also determined. Thus the binding of [^{125}I][D-Trp^{6}] LHRH was found to be reversible, time- and temperature-dependent, and linear with protein concentration in the human uveal melanoma specimens examined (data not shown). The specificity of LHRH binding was demonstrated by competitive binding experiments using several peptides structurally related or unrelated to LHRH. The expression of mRNA for LHRH receptors was accompanied by ligand binding in all samples examined. Three of 10 tumor specimens did not exhibit mRNA expression for type I LHRH receptors, or show ligand binding; conversely, all receptor-positive specimens expressed a detectable amount of the receptor gene. There was no evident correlation between receptor binding characteristics or mRNA expression and clinical and pathological findings.

Expression of mRNA for human type I LHRH in human uveal melanoma

In addition to the expression of type-I LHRH receptor, we also studied the presence of LHRH ligand in our uveal melanoma samples. The majority of uveal melanomas (27 of 39, 69%) showed marked expression of mRNA for LHRH-I ligand. The expected size of the PCR product of 245 bp could be detected in 8 of 11 (73%) of epithelioid and 15 of 21 (71%) of spindle type tumors. In the mixed tumor type, 4 of 7 (57%) expressed LHRH-I. In 12 tumors (31%), both LHRH-I ligand and receptor were expressed. In 6 samples, only type I LHRH receptors were present, while in 15 melanomas only LHRH-I ligand was present. Statistical analysis showed an association between age and LHRH receptor mRNA and ligand co-expression (p=0.0407): a 10-year increase of age meant an estimated 87% increase in the odds of co-expression (OR=1.867, 95%CI 1.027 to 3.393). Otherwise, there were no correlations between LHRH-I ligand or receptor mRNA expression and tumor subtype or clinical parameters. The clinicopathological characteristics of patients with uveal melanoma and the result of mRNA expression.

Development of DOX resistant human uveal melanoma cell line

The DOX resistant $OCM3_{DOX320}$ cell line was established by stepwise treatment with DOX at 320 nM final concentration. $OCM3_{DOX320}$ cells showed similar morphology and comparable cell division rate to the wild type OCM3 cells. Confirming DOX resistance, $OCM3_{DOX320}$ cells

were able to grow in the presence of 320 nM DOX. DOX resistance remained after several cryopreservation steps.

LHRH receptor expression in OCM3 and OCM3$_{DOX320}$ cells

Primers for the LHRH receptors were designed to amplify the different exons of mRNA of the LHRH receptors. The predicted sizes of the PCR products were the followings: exon I: 436 bp, exon II: 411 bp and exon III: 473 bp. Using RT-PCR, alternatively spliced LHRH receptors could be detected. Both cell lines expressed dominantly exon I, but exon II and III were also detected with a very low intensity in OCM3$_{DOX320}$ cell line. The presence of LHRH receptor protein isoforms was confirmed by immunocytochemistry in OCM3 and OCM3$_{DOX320}$ cells. The LHRH receptor was mainly localized in the cytoplasm and in the cell membrane

Cell proliferation assay

OCM3 and OCM3$_{DOX320}$ cell viabilities were measured by MTT assay after 72 h of incubation with different concentrations (40 nM, 320 nM, 1 μM, 2.5 μM, 5 μM) of DOX or AN-152. OCM3$_{DOX320}$ cells did not show significant difference in cell viability in the presence of 1 μM DOX when compared to untreated control cells. 1 μM DOX induced significant cell death of OCM3 cells but did not cause significant cell death in OCM3$_{DOX320}$ cells, confirming DOX resistance. AN152 at lower concentration (40 nM, 320 nM) increased cell proliferation significantly compared to equimolar dose of DOX which does not have any effect on cell viability at this concentration in OCM3 cells. However, higher concentrations of AN-152 can effectively inhibit cell proliferation in both cell lines. Higher concentrations (1–5 μM) of DOX and AN-152 showed no significantly different effect either on OCM3 or OCM3$_{DOX320}$ cells.

Visualization of cellular uptake and distribution of DOX and AN-152 in OCM3 and OCM3$_{DOX320}$ cells by confocal laser scanning microscopy

Exposure of OCM3 human UM cells to DOX resulted in a homogenous distribution of DOX related fluorescence mainly in the nucleus in all recorded time segments. In comparison, OCM3 cells exposed to AN-152 showed higher fluorescence intensity in the cytoplasm, the nucleus and the plasma membrane suggesting membranebound internalization. In contrast, in OCM3$_{DOX320}$ cells exposed to DOX the fluorescence signal slowly accumulated in the nucleus at the 0.5–1 h timeframe. The distribution of the fluorescence signal in the 6 and 24 h time

period showed that the fluorescent signal of DOX is mainly localized in the cytoplasm with lower intensity in the nucleus. In comparison, in $OCM3_{DOX320}$ cells treated with AN-152 no accumulated fluorescence was found in the nucleus at any time point. A stronger fluorescence signal was found in the cytoplasm and in the cytoplasmatic granules, indicating the gradual membrane bound uptake of AN-152 in the 0.5 and 1 h time segments. Our results indicate that both DOX sensitive and resistant cells can successfully accumulate AN-152, but in DOX resistant $OCM3_{DOX320}$ cells the signal of DOX was vanished especially from the nucleus as seen on the 24 h images.

6. Discussion

Targeted chemotherapy represents a modern approach to cancer therapy. More specific delivery of chemotherapeutic agents to malignant tissues is more effective and less toxic than conventional systemic chemotherapies. As a molecular target, LHRH receptor has been selected for the development of cytotoxic LHRH analogs, based on the presence of specific receptor for LHRH in various types of cancers including endometrial, breast, ovarian, prostate, colorectal, pancreatic, bladder cancers, glioblastoma, cutaneous and uveal melanomas. Cancer resistance to chemotherapy poses an enormous challenge to oncologists, and new therapeutic approaches are to be explored. DOX resistance has not been deeply studied in the therapy of metastases of UM due to the short estimated survival, but the administration of DOX in combination with other therapies has been reported. Conventional cytotoxic treatment prior to targeted cytotoxic therapeutic approaches can significantly influence the outcome of the therapy inducing overcoming resistance. In order to support a better understanding of the molecular mechanisms and sequencing of patients' therapy we investigated the cellular uptake mechanisms and effectiveness of DOX and AN-152 treatment in DOX sensitive and resistant forms of UMs. Cytotoxic LHRH analogs enter cancer cells with LHRH receptor mediated internalization. 46% of human UM expresses full length (type I) receptors for LHRH but the potential role of receptor isoforms in targeted therapy has not been investigated so far. The present study describes mRNA expression of LHRH receptor splice variants in OCM3 and $OCM3_{DOX320}$ human UM cell lines. The presence of LHRH receptor isoforms was also demonstrated by immunocytochemistry. To elucidate the effects of AN-152 treatment in OCM3 and $OCM3_{DOX320}$ cell lines, we investigated cell viability and intracellular distribution of the chemotherapeutic agent DOX and targeted cytotoxic LHRH analog AN-152. Treatment with DOX and AN152 significantly decreased OCM3 and $OCM3_{DOX320}$ cell viability in micromolar doses, demonstrated by MTT assay. In our in vitro study, AN-152 and DOX showed similar results on cell viability in $OCM3_{DOX320}$ cells. However, the in vivo effect particularly of AN-152 might be somewhat different from the in vitro results. Based on the present study, AN-152, at 40 nM concentration increased cell proliferation compared to equimolar dose of DOX. Acquired resistance of $OCM3_{DOX320}$ cell line inhibited the effectiveness of DOX and AN-152. In low doses, AN-152 most likely exhibited hormonal properties since it enhanced cell proliferation in both cell lines. On the other hand, DOX was not effective as a cytotoxic agent in equimolar concentration. This phenomenon suggests a possible signal transduction mechanism through LHRH receptor isoforms. The strong binding characteristics of AN-152 to

LHRH receptors might be sufficient to activate signaling mechanisms but in nanomolar concentration DOX does not effectively decrease cell viability. It highlights the importance of superactive cytotoxic LHRH analog such as AN-207 that shows significant cytotoxicity in nanomolar concentrations. AN-207 contains 2-pyrrolino-DOX that can be more beneficial for patients with DOX resistant cancer. AN-207 is 500–1000× more effective than DOX and there is no cross resistance between 2-pyrrolino-DOX and DOX. The present data suggest that resistance to DOX can reduce the efficiency of AN-152. The confocal laser scanning microscopy results demonstrated slower receptor mediated uptake of AN-152 compared to the passive diffusion of DOX. Acquired resistance of $OCM3_{DOX320}$ might be a result of active efflux pump mechanisms, which can successfully eliminate free DOX from the cell nucleus, but does not influence receptor mediated uptake of AN-152. Interestingly, $OCM3_{DOX320}$ cells treated for 24 h were able to reduce DOX concentration in the cell nucleus. In our present study, in $OCM3_{DOX320}$ cells treated with AN-152 no accumulated fluorescence was found in the nucleus at any time period. However, a stronger fluorescent signal was found in the cytoplasm and in the cytoplasmatic granules, indicating the gradual membrane bound uptake of AN-152 in 0.5 and 1 h time segments. The half-life (t1/2) of AN-152 is about 2 h in human serum in vitro. In our modeling system the expected half-life of AN-152 could be similar, since the 6 and 24 h fluorescent images were similar irrespectively of DOX or AN-152 treatment. Our results indicate that both DOX sensitive and resistant cells can successfully accumulate AN-152, but in DOX resistant $OCM3_{DOX320}$ cells the signal of DOX was vanished especially from the nucleus as seen on the 24 h images. The importance of the rapidly developing chemoresistant cells should not be underestimated in cancer therapy. The development of resistant cells and metastases with high frequency results in an enormous challenge in clinical practice. Fifty per cent of the patients with primary uveal melanoma develop metastases independently from surgical or radiation therapy. Currently, there is no standard systemic therapy that could increase the overall survival. The role of LHRH receptor splice variants and protein isoforms in oncogenesis and therapy are most likely underestimated. Limited information exists about the potential role of the LHRH receptor isoforms in targeted therapy. Our results indicate that LHRH receptor isoforms could be suitable for targeted therapies based on LHRH analogs such as AN-152. Cytotoxic analog AN-152 was tested in Phase I/II clinical trials in castration-resistant prostate cancer and in Phase II and III trials in endometrial and ovarian. Clinical trials with zoptarelin doxorubicin in various human cancers were either completed or terminated. Regrettably, some of these studies did not achieve their primary endpoint. Our findings support a growing body of evidence that the use of DOX for treatment of patients with UM can

potentially reduce the effectiveness of later initiated AN-152 therapy. AN-152 may overwhelm chemoresistance due to receptor-mediated accumulation and slow gradual LHRH receptor mediated uptake. LHRH receptor based uptake and prolonged cleavage of AN-152 predicts a less overcoming of chemoresistance of cancer cells. Chemoresistance is one of the major drawbacks of anthracyclines systemic therapies in malignancies. In conclusion, our findings provide new insights into novel therapeutic approaches to human uveal melanoma and could help to establish an effective systemic treatment for UM.

7. Abstract

In the past decades, the average survival of patients has not changed in the case of uveal melanoma (UM). The average survival of metastatic patients with UM is less than one year, and despite the treatment of the primary tumor, metastases develop in more than half of the patients. The drawbacks of bad statistics are the rapid dissemination of the tumor cells but resistance to chemotherapy also plays a major role. Cytotoxic LHRH (luteinizing hormone-releasing hormone) analogs can be effectively used for targeting sexual hormone-dependent malignancies like endometrial, ovarian and prostatic cancers but the application of these analogs in hormone-independent cancers such as human uveal melanoma (UM) is not examined jet.

The aims of the current study were to investigate the expression of LHRH receptor and ligand in human UM tissues, as a potential novel therapeutic target. We also established a new in vitro model to study the cellular uptake and efficacy of cytotoxic LHRH analog AN-152 (AEZS-108, zoptarelin doxorubicin) in a doxorubicin-resistant UM cell line.

The expression of LHRH receptor and LHRH ligand was tested in 39 human UM specimens by RT-PCR with specific primer set for full length LHRH receptor. Radio ligand binding characteristics of LHRH receptors were studied in tumor membranes of ten UM specimen. The presence of LHRH receptor protein has been confirmed by immunohistochemistry. We established a new doxorubicin (DOX) resistant UM cell line by stepwise administration of DOX to OCM3 human UM cells. The LHRH receptor expression of DOX sensitive (OCM3) and DOX resistant ($OCM3_{DOX320}$) UM cell lines was determined by exon specific RT-PCR and the immunocytochemistry. Cellular uptake and intracellular distribution of DOX and AN-152 were imaged with confocal laser scanning microscopy. Comparative cytotoxic activity of DOX and AN-152 were tested on both cell line by MTT assay

High percentage (46%) of UM specimens expressed the mRNS of full length type 1 LHRH receptor and 69% of samples were positive to LHRH ligand expression. 70% of the tissue samples showed high ligand binding affinity to LHRH receptors. Immunohistochemistry also confirmed the presence of LHRH receptors. We were able to establish a new DOX resistant UM cell line. Our study demonstrated the expression of LHRH receptor splice variants and protein isoforms in OCM3 and $OCM3_{DOX320}$ cell lines. The cellular uptake of AN-152 was confirmed by fluorescent microscopy. Based on the MTT assay AN-152 effectively inhibited cell proliferatin in both cell line in dose dependent manner.

Our results demonstrated that LHRH receptors and its isoforms can be potential molecular targets for an effective targeted therapy of UM and its metastases.

8. Aknowledgement

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List of publications related to the dissertation

1. **Oláh, G.**, Dobos, N., Vámosi, G., Szabó, Z., Sipos, É., Fodor, K., Harda, K. M., Schally, A. V., Halmos, G.: Experimental therapy of doxorubicin resistant human uveal melanoma with targeted cytotoxic luteinizing hormone-releasing hormone analog (AN-152).
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