SMALL MOLECULE PEPTIDE-DRUG CONJUGATES FOR TARGETED DRUG DELIVERY IN CANCER THERAPY

PhD thesis

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Introduction

Cancer is a complex disease that can be caused by both environmental factors and genetic predisposition. Although chemotherapeutics still represent the most employed strategy for cancer treatment, the heterogeneity of cancer makes its treatment more challenging, thus chemotherapy has various drawbacks such as lack of selectivity for tumor cells and narrow therapeutic index, limiting its therapeutic benefits.

One of the approach to overcome limitation and to increase the selectivity of the cytotoxic agents is targeting therapy. Molecularly targeted therapy is represented by small molecule therapeutics designed to interact selectively with a specific target which is necessary for tumor progression. Monoclonal antibodies (mAbs) have ability to selectively detect and with high affinity bind to antigens of interest overexpressed in tumors, triggering cancer cell death through different cell killing mechanisms. A growing approach in targeted cancer therapy represent a strategy called targeted drug delivery system (DDS), where conjugation of chemotherapeutic to tumor-targeting devices leads to higher accumulation of the drug into the tumor, overcoming the systemic cytotoxicity. Besides antibody-drug conjugates (ADCs) technology, combining antibodies with cytotoxic drugs, which have been successfully approved for this objective in clinical use, small molecule-drug conjugates (SMDCs) have emerged in the last decades, combining small receptor binders with cytotoxic agents, that allow the selective delivery of the antitumor agent into receptor-positive malignant cells, and therefore overcome several drawbacks associated to ADCs approach. Among them peptide drug conjugates (PDC) and cell penetrating peptides (CPP) are the most employed.

PDCs contain ligands, such as vitamins or hormones, whose receptors are often overexpressed by tumors, conjugated to the drug via spacers and linkers, which prevent the premature release of the payload in the blood circulation, by cleaving through different modes of action, releasing the cytotoxic payload which reaches its target leading to cell death. Mechanism of action is that conjugate upon targeting ligand selectively recognized and bind for the receptor, internalize into the cancer cell via receptor-mediated endocytosis, where in compartments of endosomes and lysosomes dissociate and release of anti-tumor drugs at the tumor site, while receptor is recycled on the cell surface.

Among wide range of receptors which have been successfully targeted by this strategy, gonadotropin-releasing hormone receptor (GnRH-R) as highly expressed on the cell surface

of many different reproductive system related tumors such as breast (BC), ovarian, endometrial and prostate cancer, but also in non-reproductive cancers like melanoma, glioblastoma and colorectal cancer (CRC), while their presence on healthy tissues is limited, these receptors can serve as target for targeted anti-tumor therapy. Natural ligand for this receptor is gonadotropin-releasing hormone peptide (GnRH-I). Its natural isoform GnRH-III specifically binds to GnRH-Rs on cancer cells with higher affinity than GnRH-I, causing a direct anti-proliferative activity on many tumor types enabling its application as targeting moieties for anti-cancer drugs, while its endocrine side activity is strongly reduced compared to GnRH-I, both in vitro and in vivo.

In past years, Mező and colleagues developed and synthesized a various of GnRH-III-based DDSs, with a wide range of modifications on the primary GnRH-III sequence and different linkage systems for the drug, in order to increase the anti-tumor activity of the conjugates by eliciting a favorable influence on the GnRH-R binding affinity, stability, cellular uptake rates and drug releasing properties. Two GnRH-III conjugates (**1** and **2**) with the difference in ligand sequence carrying Daunorubicin (Dau) as drug were synthesized and investigated in my PhD study on 23 different cancer cell lines and human lung fibroblast. Based on these results, distinct cell lines were selected for cellular uptake studies of the compounds and for determination of the GnRH-R expression level, whereby not only the mRNA level, but also the absolute, as well as the cell surface receptor level was analyzed. Next to the in vitro analysis, the in vivo anti-tumor and anti-metastatic activity of 1 and 2 was investigated in orthotopic BC and CRC bearing mice to ensure the high potency of new GnRH-III based DDSs.

Besides peptide hormones used as ligands for their receptors, the search of new tumor homing peptides which could provide increased biological activity is a hot topic in targeted cancer therapy. One of the approach often used to explore new specific peptides which can be used efficiently as ligands in DDS, is a technique called the phage display, by which Zhang and his co-workers selected HT-29 human CRC specific heptapeptide VHLGYAT as the most selective peptide to HT-29 colon cancer cell line, while it is not able to recognize it in normal intestinal epithelial cells. Mező and co-workers developed conjugates with parent (**3**) and modified (**4**) heptapeptide targeting moiety coupled with Dau as cytotoxic agent, which anti-

tumor activity on 22 different origin cancer cell lines and one normal in vitro, and in vivo on HT-29 human colon tumor bearing mice were investigated as second topic of my study.

CPP is mainly positively charged short peptide sequence with 5–30 amino acids and with low cytotoxicity that can penetrate through the cell membrane, used as carriers for intracellular cargo delivery without destroying membrane integrity. Among various CPPs peptide consisting octa-arginine sequences display optimal length and properties for efficient internalization, and therefore it was used frequently to transport different molecular cargos into cells.

Knowing the fact that Vindoline is an antitumor inactive, even it is precursor of famous chemotherapeutic Vinblastine, and based on previous studies where conjugation of Vindoline with different chemical moieties promoted its anti-tumor activity against human leukemia cells, and based on studies where conjugation of chemotherapeutics to octa-arginine as CPP increased chemotherapeutic anti-tumor activity, Bánóczi and colleagues conjugated Vindoline with octa-arginine via Tryptophan with different configuration (**5** and **6**). In my doctoral thesis study it was evaluated whether this conjugation promote anti-tumor activity of Vindoline in vitro on P388 mouse leukemia and C26 murine colon cell lines. In addition in vivo anti-tumor effect of conjugates was studied in these tumor bearing mice.

OBJECTIVES

GnRH-III-Dau conjugates

Investigate the *in vitro* anti-proliferative activity of GnRH-III-Dau conjugates **1** and **2** on 23 different cancer cell lines and human lung fibroblast, and determine their relative potencies compared to anti-proliferative activity of free Dau.

Evaluate the cellular uptake of **1** and **2** on particular cancer and normal cell lines.

Determine mRNA, protein and the cell surface GnRH-R expression level on particular cancer and normal cell lines.

Evaluate toxicity of GnRH-III-Dau conjugates **1** and **2** in acute and chronic *in vivo* toxicity studies.

Investigate the *in vivo* anti-tumor, anti-proliferative and anti-metastatic activity of **1** and **2** in orthotopic 4T1 murine BC and MDA-MB-231 human BC, as well as in HT-29 human CRC bearing mice, and assess conjugates toxic effect.

Homing heptapeptide-Dau conjugates

The *in vitro* anti-proliferative activity of conjugates **3** and **4** on 22 different origin cancer cell lines and one normal, and determination of their relative potencies compared to anti-proliferative activity of free Dau.

Investigate the *in vivo* anti-tumor and anti-proliferative activity of **3** and **4** in orthotopic HT-29 human CRC bearing mice, and evaluate conjugates toxic effect.

Vindoline CPP conjugates

Determine *in vitro* anti-proliferative activity of Vindoline derivatives and CPP-conjugates, as well as free Vindoline on P388 mouse leukemia and C26 mouse colon cancer cell lines.

Investigate the *in vivo* anti-tumor effect of conjugates **5** and **6** on two subcutaneous mouse tumor models of P388 mouse leukemia and C26 mouse colon.

MATERIALS AND METHODS

Cell lines and culture conditions

In experimental procedures following cell lines were used, MDA-MB-231 and MCF-7 (human breast cancer), 4T1 (murine breast cancer), DU145 and PC-3 (human prostate cancer), A2780, OVCAR-3 and OVCAR-8 (human ovarian cancer), HepG2 (human liver cancer), A2058, WM983b, HT168-M1/9 and M24 (human melanoma), B16 (murine melanoma), H1975, H1650 and A549 (human lung cancer), HT-29, HCT116 and WiDr (human colorectal adenocarcinoma), C26 (murine colon cancer), P388 (murine leukemia) and PANC-1 (human pancreatic cancer) were cultured in RPMI 1640 (Lonza). Moreover, U87MG (human malignant glioma) and MRC-5 (human fibroblast) were cultured in DMEM (Lonza), while PE/CA-PJ15 and PE/CA-PJ41 (human head and neck cancer) were cultured in IMDM (Sigma Aldrich). All mediums were supplemented with 10% heat-inactivated FBS (Euroclone) and 1% Penicillin/Streptomycin (Sigma Aldrich).

***In vitro* anti-proliferative activity of the conjugates and free drug**

After standard harvesting, the cells were seeded in serum containing growth medium to 96-well plates with flat bottom (Sarstedt), in a 200 μL final volume per well, and incubated at 37 °C. After 24 h, the growth medium was removed and cells were treated with various

concentrations of conjugates **1** – **4** or free Dau, dissolved in serum free medium and incubated for 24 h under standard conditions. After 24 h of treatment, the cells were washed twice with serum free medium and then cultured in serum containing medium for an additional 48 h. C26 and P388 cells were treated with various concentrations of the Vindoline derivatives, conjugates (**5** and **6**) and free Vindoline for 3 h in a 200 μL final volume per well. After incubation, the cells were washed twice with serum free medium and further cultured up to 72 h in serum containing medium.

Afterward, the MTT assay was performed, in order to determine cell viability. Absorbance values of treated samples, after subtraction with blank values, were normalized versus untreated control samples and interpolated by nonlinear regression analysis with GraphPad Prism 6 software (GraphPad) to generate sigmoidal dose-response curves from which the 50% inhibitory concentration (IC_{50}) values of the conjugates and free drugs were calculated. The experiments were done in triplicate and each experiment was repeated twice.

RT-qPCR for GnRH-R mRNA Level of Expression

The total RNA was isolated while using Trizol® reagent (Ambion, by Life Technologies), followed by chloroform (Carlo Erba Reagents) extraction and isopropanol (Carlo Erba Reagents) precipitation. The purity and concentration of RNA were determined NanoDrop ND-1000 (Thermo Scientific). For cDNA synthesis a GeneAmp PCR System 9700 thermo cycler (Applied Biosystems) was used. RNA were reverse transcribed to cDNA. In order to amplify the cDNA, qPCR was run on the StepOnePlus™ real-time PCR system (Applied Biosystems) using PowerUp™ SYBR® green master mix (Applied Biosytems). SYBR green primer assays were obtained for: Human GnRH-R gene with reference sequence (RefSeq) NM_000406(2) and exon location 1-1 (Integrated DNA Technologies); human β-actin gene with RefSeq NM_001101 (Qiagen); and, human GAPDH gene with RefSeq NM_002046(1) (Qiagen). The relative quantification of GnRH-R mRNA expression was calculated and normalized in comparison to the both internal controls, endogenous housekeeping genes β-actin and GAPDH, conducting *via* the ΔCt method and analyzed by the $RQ = 2^{-\Delta\Delta Ct}$ method. As reference sample, MRC-5 cells was chosen. Two experiments with two biological replicates were performed.

Quantitative Western Blot Studies

The cells were lysed in lysis buffer (cOmplete™ Lysis-M; Roche). The protein concentration was determined by Pierce™ BCA protein assay kit (Thermo Fisher Scientific). Samples were denatured in Novex™ LDS sample buffer and NuPage™ sample reducing agent (Invitrogen) in Eppendorf® Thermo-mixer Compact. Protein samples were loaded to a 1 mm thick 12% Bis-Tris plus gel (Invitrogen) and run with Novex™ NuPage™ MES SDS buffer (Invitrogen) while using a Bio-Rad 1000/500 Constant Voltage Power Supply, on voltage 200 V. Blotting was performed by iBlot Gel Transfer Stacks Kit (Invitrogen), whereby proteins were transferred to a nitrocellulose membrane while using a Invitrogen iBlot™ dry blotting system. The membrane was incubated overnight at 4 °C with GnRH-R antibody (Proteintech, rabbit Polyclonal 19950-1-AP). The secondary antibody (Cell Signaling Technology, goat Anti-Rabbit-horseradish peroxidase (HRP) conjugated, 7074) was incubated 1 h at r.t. Amersham™ ECL™ prime western blotting detection reagent (GE Healthcare) was used to visualize bands on a Bio-Rad ChemiDoc™ MP Imaging System. The software Image Lab (Bio-Rad) was used to evaluate the signal level of the bands, followed by normalization of GnRH-R expression signal to the reference housekeeping β-actin signal (Cell Signaling Technology, rabbit mAb; HRP conjugated, D6A8). Normalized values from each sample were compared to value that was obtained from MRC-5 cells which arbitrary set as 1, by relative quantification to enable a better comparison of the obtained results.

GnRH-R cell surface expression level determination by flow cytometry

The cells were fixed with 4% Paraformaldehyde, washed with PBS and exposed to 3% BSA in PBS. Afterwards, GnRH-R antibody (Proteintech, rabbit polyclonal, 19950-1-AP) was used, diluted in PBS and 3% BSA solution and incubated for 2 h at r.t. A fluorescent secondary antibody was used for detection (Cell Signaling Technology, Alexa 488-conjugated anti-rabbit IgG Fab fragment, CST 4412) and incubated at r.t. for 30 min. As control, samples only exposed to secondary antibody were used. The fluorescence was detected using the FITC-A channel of FACSVerse™ Flow Cytometer (BD Biosciences). The BD FACSuit™ software was applied to evaluate geo mean fluorescence intensity (geo MFI). For each cell line, the ratio between GnRH-R geo MFI and secondary antibody control geo MFI was calculated. The ratio values from all cell lines were normalized to the results obtained from MRC-5.

Cellular uptake of GnRH-III-Dau conjugates by flow cytometry

The cells were seeded to 24-well plates, incubated 24 h at 37 °C and then treated with GnRH-III-Dau conjugates **1** and **2** (40 μM) for 6 h. Control wells remained untreated. After harvesting, cells were washed in PBS and quenched with 0.04% Trypan-Blue (Invitrogen). The fluorescence intensity of Dau was detected while using the PE-A channel of FACSVerse™ Flow Cytometer (BD Biosciences) and geo MFI was evaluated using BD FACSuite™ software. The geo MFI ratio between control and samples treated with conjugates was calculated in order to compare the uptake on different cell lines.

Experimental animals

Adult female BALB/c mice from a specified pathogen free (SPF) breeding of the Department of Experimental Pharmacology (National Institute of Oncology, Budapest, Hungary) were used in acute and chronic toxicity studies, in orthotopic 4T1 murine BC, and in subcutaneous C26 murine colon tumor model *in vivo* experiments. First generation of hybrid BDF1 (a cross between C57BL/6 female and DBA/2 male) adult female mice, from SPF animal colonies from same animal house were used in subcutaneous P388 murine leukemia tumor model experiment. The adult female severe combined immunodeficiency (SCID) mice were used for the orthotopic model of human BC and human CRC. All animals used in experiments were cared according to the "Guiding Principles for the Care and Use of Animals" based upon the Helsinki declaration and they were approved by the ethical committee of National Institute of Oncology. The animal housing density was according the regulations and recommendations from directive 2010/63/EU of the European Parliament and of the Council of the European Union on the protection of animals used for scientific purposes. Permission license for breeding and performing experiments with laboratory animals: PEI/001/1738-3/2015 and PEI/001/2574-6/2015.

Acute and chronic toxicity studies of GnRH-III-Dau conjugates

In order to determine the toxicity of the conjugates, *in vivo* acute (single intraperitoneal (i.p.) administration of conjugate **2**, with different doses 3.125, 6.25, 12.5, 25 and 50 mg/kg Dau content) and chronic (five treatments, 15 mg/kg Dau content) or with free Dau (two treatments, 1 mg/kg) toxicity studies were performed on healthy female BALB/c mice. The

toxicity was evaluated on the basis of life span, behavior and appearance of mice, as well as the body weight. Parameters were followed for 14 days.

Mouse model of orthotopic 4T1 murine breast carcinoma

4T1 murine BC cells were orthotopically injected into the lower quarter of the right mammary fat pad line of BALB/c female mice. The control group were treated with solvent, free Dau group were treated with a dose of 1 mg/kg, once per week, while GnRH-III-Dau conjugates **1** and **2** groups were treated twice per week with a dose of 10 mg/kg Dau content by i.p. administration. Animal weight and tumor volumes were measured initially when the treatment started and at periodic intervals according to the treatment schedule. The anti-tumor effects and the liver toxicity of the conjugates and free Dau were evaluated based on the tumor volume and the liver weight/body weight ratio in each group. Moreover, the anti-proliferative and anti-metastatic activity of the conjugates and free Dau was evaluated in primary tumor and in metastases on the peripheral organs.

Mouse model of orthotopic MDA-MB-231 human breast carcinoma

MDA-MB-231 human BC cells were injected orthotopically into the lower quarter of the right mammary fat pad line of SCID female mice. The mice in the control group were treated with solvent, while the free Dau group were treated with a dose of 1 mg/kg, once per week. The groups treated with the GnRH-III-Dau conjugates **1** and **2** were treated on with a dose of 15 mg/kg Dau content, twice per week, while the last treatment was with a dose of 7.5 mg/kg Dau content. The animal weights and tumor volumes were measured when the treatment was initiated and at periodic intervals according to the treatment schedule. The anti-tumor effects of the conjugates were evaluated measuring the tumor volume and the tumor weights, while the toxicity effects of conjugates were evaluated measuring liver weights and calculating the liver weight/body weight ratio. Anti-metastatic effect was evaluated counting animals containing metastases close to the primary tumor.

Mouse model of orthotopic HT-29 human colon cancer

HT-29 colon carcinoma cells were subcutaneously injected into one side of the intrascapular region of SCID female mice in order to establish the xenografts with primary tumor, which after two weeks was aseptically dissected out. Tumor pieces were transplanted

orthotopically under aseptic conditions on the top of the animal intestine by surgical procedure into anesthetized SCID female mice. The control group was treated with solvent, free Dau group with dose of 1 mg/kg body weight once per week, while the groups of GnRH-III-Dau compounds **1** and **2**, and homing heptapeptide-Dau conjugates **3** and **4**, were treated with a dose of 10 mg/kg Dau content, twice per week, by i.p administration. The control group was treated with sterile water for injection. The Dau group was terminated already earlier, since the animals revealed a significant loss of weight. Their tumors and livers were harvested and weighed for determination of compounds anti-tumor as well as toxic activity.

Mouse model of subcutaneous P388 murine leukemia

The anti-tumor activity of Vindoline octaarginine conjugates (10mg/kg) and vinblastine (1mg/kg), two treatments each, was studied on subcutaneous P388 mouse leukemia model in hybrid BDF1 female mice. During the treatment the animal weight and tumor volume were followed.

Mouse model of subcutaneous C26 murine colon cancer

The anti-tumor activity of Vindoline octaarginine conjugates (10mg/kg and 20mg/kg) and vinblastine (1mg/kg), five treatments each, was studied also on subcutaneous C26 mouse colon carcinoma model in BALB/c female mice. During the treatment the animal weight and tumor volume were followed.

Immunohistochemical staining of KI-67

The routinely formalin-fixed tumors were dehydrated. Two micron thick sections were mounted on Superfrost slides (Thermo Shandon), then manually deparaffinized, and protected from endogenous peroxidase activity. After the slides were immersed in 6% citrate buffer (pH = 6) and exposed to 98 °C water bath. the slides were primarily treated with monoclonal mouse antibody against human KI-67 (Dako,) and then incubated for 1 h at r.t. After washing with PBS, biotinylated secondary antibody (Dako) was applied and incubated for 10 min. at r.t. After washing periods, for visualization, supersensitive one step polymer HRP (Biogenex) was used with 3-amino-9-ethylcarbazole (AEC) as chromogen. Staining without the primary antibody served as the negative control.

Scoring of proliferation index, micro and macro-metastases

All of the visible macro-metastatic lesions of the peripheral organs such as liver, spleen, lungs and kidneys in 4T1 model, on a stereo microscope Kruss MSZ5600 (Kruss Optronic) were counted, and the percentages of them in the treated groups compared to the control group was calculated. Proliferation marker-stained samples on microscopic slides were evaluated on light microscope Olympus BH-2 microscope (Olympus). By counting the KI-67-positive tumor cells manually per field of view under light microscope, and calculating percentage of KI-67 positive cells from all cells in the field of view, the proliferation index in primary tumors and in lung metastases were determined. The number of micro-metastases, as KI-67 positive-stained in the lung samples sections were also manually counted per field of view under light microscope.

Statistical analysis

In case of *in vivo* studies, statistical analyses were performed by GraphPad Prism 6 (GraphPad Software) using the non-parametric Mann-Whitney test, while the statistical analysis for uptake studies of conjugates was performed using two-way ANOVA test. The experimental data where *p*-values equal or lower than 0.05 were considered statistically significant. *, **, *** and **** mean significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$, respectively.

RESULTS

***In vitro* anti-proliferative activity of GnRH-III-Dau conjugates and free Dau**

The data showed that both conjugates possess an anti-proliferative effect on all cancer cell types, where conjugate **2** displayed higher anti-proliferative activity than conjugate **1**, which were 1.8 – 5.6 times higher, depending on the type of cancer cells. The lowest activity was measured on PANC-1 pancreatic cancer cells, whereby a high IC_{50} value was also obtained on MRC-5 cells, showing selectivity of the conjugates for cancer cell lines.

Moreover, the relative potency was calculated as a ratio of free Dau's IC_{50} and conjugates' IC_{50} in order to show the potency of the conjugates independently from the cell line, due to different activity of free Dau, which can enter cells non-specifically by passive diffusion. A higher value of relative potency indicates that the conjugate's IC_{50} value is closer

to the free Dau's IC_{50} value, which implies that the targeting capacity of the conjugate as well as its anti-tumor effect is stronger on a particular cell line, as compared to a cell line with lower relative potency. The BC cell lines showed good response to the conjugates by IC_{50} values, as well as by relative potency. Although conjugates showed showed a moderate anti-proliferative activity on HT-29 human colon adenocarcinoma, the relative potency was in the same range as for the BC cells.

mRNA expression level of GnRH-R

The amount of GnRH-R mRNA on chosen cell lines, quantified via RT-qPCR, was higher in all cancer cell lines in comparison to the normal cell line MRC-5, except for U87MG cells, where it was slightly lower. In MDA-MB-231 and HT-29, the level of GnRH-R mRNA expression was 7.3 and 4.6 times higher in comparison to normal cell line, while only 1.8-fold higher expression was obtained for PANC-1 cell line. The human total cDNA sample showed almost same level of GnRHR mRNA expression as normal cell line, suggesting it as suitable reference sample to which all results will be compared.

Absolute protein expression level of GnRH-R

The GnRH-R protein level of the same cell lines, quantified by western blot, was higher in all cancer cell lines as compared to the normal cell line MRC-5, except for PANC-1 cells, where it was 0.3-fold lower. Protein level in 4T1 cells, was 3.5-fold higher than in MRC-5 cells, while MDA-MB-231 and HT-29 cancer cell lines showed 1.2- and 1.7-fold higher level of GnRH-R protein expression.

Cell surface protein expression level of GnRH-R

The GnRH-R cell surface expression level, quantified *via* flow cytometry, was higher for all cancer cell lines in comparison to MRC-5 cells. The highest level of GnRH-R was obtained for MDA-MB-231 with 5.6-fold higher surface expression. 4T1 and HT-29 showed four- and two- fold higher expression level of GnRH-R than MRC-5 cells.

Determination of cellular uptake of GnRH-III-Dau conjugates

The cellular uptake determination by flow cytometry showed that the new conjugate **2** was taken up significantly more efficiently than conjugate **1**, with 1.7–2.7 times higher uptake

rates, depending on the cell line. The normal cell line MRC-5, as well as PANC-1 cancer cell line showed two-fold lower uptake capacity in comparison to the other cancer cell lines, confirming data from anti-proliferative experiment, and promoting BC and CRC as suitable models for testing *in vivo* anti-tumor activity of these conjugates.

Acute and chronic toxicity studies of GnRH-III-Dau conjugates

An acute toxicity experiment with single injection revealed that conjugate **2** is not toxic for using in animals even with a dose of 50 mg/kg Dau content. Chronic toxicity experiment with five treatments of both conjugates (15 mg/kg Dau content) and two times with free Dau (1 mg/kg) also showed non-toxicity for regimes which will be used further.

Effect of GnRH-III-Dau conjugates and free Dau in orthotopic 4T1 murine breast tumor model *in vivo*

The animal body weight was non-significantly changed during the treatment time in all groups. All treated groups showed significant inhibition of the tumor volume by approximately 19% as compared to the control group at the end of the experiment. It was observed that both GnRH-III conjugates **1** and **2** caused a significant decrease of the proliferation index by 16.3 and 25.9%, as compared to the control, while free Dau decreased the proliferation index also significantly by 19%, confirming tumor volume inhibition. The average liver/body weight ratio of the mice in the group that was treated with free Dau was significantly decreased by 9.8% compared to the control group, as well as in comparison to the liver/body weight ratio of mice treated with the conjugates which showed no significant changes in liver/body weights ratio, showing high toxicity of Dau in comparison with conjugates. The number of macro-metastases in spleen and lungs was significantly decreased in all treated groups (Dau, **1** and **2**) by 64.3, 72.8 and 78.1%, and by 55.4, 55.2 and 64.4%, respectively, in comparison to the control group, while in the liver and kidneys were decreased under treatments, whereby a significant decrease could be only obtained only for conjugate **2**. The obtained data revealed that free Dau and both conjugates (**1**, **2**) significantly inhibited the number of micro-metastases in the lung by 33.7, 43.8 and 49.4%, as compared to the control group, which was confirmed with significant inhibition of the proliferation index of lung metastases by 27.8, 37.0 and 39.1% in groups that were treated with free Dau, **1** and **2**.

Effect of GnRH-III-Dau conjugates and free Dau in orthotopic MDA-MB-231 human breast tumor model *in vivo*

Free Dau treatment caused a significant decrease of mice body weight by 20%, while conjugates treatments did not change it significatly. All treated groups showed a significant inhibition of the tumor volume in comparison to the control group at the end of the experiment where inhibition by Dau was the most effective (46.3%), followed with with conjugate **1** (34.1%) and **2** (23.1%). Based on these tumor weights, it was determined that free Dau, **1** and **2** inhibited tumor weight significantly by 40.1, 28.7 and 27.7% in the case of orthotopic human MDA-MB-231 breast tumor model. The average liver/body weight ratio in the group treated with free Dau was significantly decreased by 16.8% as compared to the control group, while conjugates treated groups showed non-significant changes. The highest anti-metastatic effect showed GnRH-III conjugates **1** and **2** where only three out of seven animals contain metastases close to the primary tumor. Four out of seven mice treated with free Dau contain metastases, it could be observed that all animals in the control group had metastases close to the primary tumor.

Effect of GnRH-III-Dau conjugates and free Dau in orthotopic HT-29 human colon tumor model *in vivo*

The animal body weight in mice decreased in all groups at the end of experiment when compared to the start, but only significanly in free Dau treated group triggering earlier termination of this group. The obtained data reveal that Dau, **1** and **2** significantly inhibited the tumor growth, whereby the tumor weights were reduced by 84.3, 80.8 and 87.1%, as compared to the control group. The average liver/body weight ratio was significantly decreased by 29.4% in Dau treated group showing the high toxicity of free Dau in comparison to conjugates where this ratio was not significantly changed compared to the control.

***In vitro* anti-proliferative activity of Homing Heptapeptide-Dau conjugates and Dau**

Tumor type specificity of two conjugates **3** and **4** investigated on 22 different types of tumor cell line and on MCR-5 (human fibroblast) as non-cancerous control cell line, showed that the conjugates had anti-proliferative effect on all cell types, but the lowest activity was measured on MRC-5 cells, telling us about selectivity of the conjugates for cancer cell lines, which was confirmed calculating the relative potency. Modified conjugate **4** showed higher

anti-proliferative effect than parent conjugate **3** in all cases, which was *ca.* 1.5-5 times higher activity depending on the type of cancer cells.

Effect of Homing Heptapeptide-Dau conjugates and free Dau in orthotopic HT-29 human colon tumor model *in vivo*

Animal weights did not differ from control mice in case of conjugates **3** and **4**, while Dau-treated mice showed significant weight loss, triggering earlier termination of this group. Moreover, significant liver toxicity according to the loss of liver compared to body weight for 22.7% was obtained, supporting results that free Dau treatment is significantly toxic, although reach tumor weight inhibition by 83.7% compared to the control.

Modified conjugate **4** significantly inhibited tumor weight for 89.1%, and similarly to the *in vitro* data, it was also more active *in vivo* compared to parent conjugate **3** which non-significantly inhibited tumor weight for 64.6%, in comparison with the control group. Inhibition effect on tumor growth of conjugate **4**, in comparison with conjugate **3**, was on the border of significance ($p = 0.0593$). The liver/body mass changes were not significant compared to the control group, and significantly different than it was observed in case of the free Dau treated group showing non toxicity of conjugates although administered Dau content was much higher than of free Dau. Moreover, both conjugates significantly inhibited the number of KI-67-positive cells in the xenograft tumor, in comparison with control tumors, where conjugate **4** significantly inhibited the number of KI-67-positive cells in comparison with conjugate **3** and free drug (Dau) confirming *in vivo* data.

***In vitro* anti-proliferative activity of Vindoline CPP conjugates**

As expected Vindoline and Br-Vindoline expressed no anti-tumor activity on C26 mouse colon carcinoma, and P388 mouse leukemia cell lines. The presence of Trp-OMe moiety in derivatives increased the anti-tumor effect of Br-Vindoline, with biological activity in a range of IC_{50} = 21.6 to 5.3 μM. This activity was influenced by configuration of Trp, where in case of P388 cells, *L*-Trp containing derivative (IC_{50} = 5.3 μM) was more effective than *D*-Trp containing (IC_{50} = 15.9 μM). Same Trp configuration dependent anti-tumor activity was obtained with Vindoline-Trp-octaarginine conjugates, which were more effective than the free, unconjugated Vindoline derivatives, especially in C26 murine colon carcinoma cell line.

The conjugate of Br-Vindoline without the Trp moiety was less effective on both cells in comparison to Vindoline derivatives and Vindoline octaarginine CPP conjugates.

Effect of Vindoline-octaarginine CPP conjugates and free Vinblastine in subcutaneous P388 murine leukemia tumor model *in vivo*

The effect of all treatments slightly increased animal body weight during the treatment for 6-7% in the control and in all treated groups. The inhibition of tumor volume was 22.5, 44.5 and 16.7% for Vinblastine, conjugate **5** and conjugate **6**, respectively, in comparison to the tumor volume in control group, at the end of the experiment, but not significant. The treatment with conjugate **5** containing *L*-Trp could inhibit the tumor growth almost three times more than conjugate **6** with *D*-Trp, after two administrations at 10 mg/kg concentration. Moreover, conjugate **5** inhibited double more tumor volume than Vinblastine, a known cytostatic agent.

Effect of Vindoline-octaarginine CPP conjugates and free vinblastine in subcutaneous C26 murine colon tumor model *in vivo*

Animal body weight was not changed during the treatment time in the control and all treated groups. Conjugates **5** and **6** inhibited tumor volume on dose dependent manner where dose of 10 mg/kg inhibited for 19.9 and 7.3%, respectively, while dose of 20 mg/kg decreased tumor volume for 29.4 and 22.8%, in comparison to the tumor volume in the control group, at the end of the experiment, but not significant. Here also *L*-isomer conjugate **5** showed higher anti-tumor activity compared to *D*-isomer conjugate **6**. The Vinblastine treatment had no marked influence on tumor growth, and inhibited tumor volume for 11.4% compared to the control.

CONCLUSIONS

GnRH-III-Dau conjugates

It was demonstrated that both GnRH-III-Dau conjugates **1** and **2** possess efficient growth inhibitory effect on various cancer cells, whereby the biological activity is strongly connected to the expression of GnRH-Rs.

GnRH-R mRNA, protein and cell surface level studies pointed out that cells with higher receptor expression level remain higher affected by the conjugates than cells with lower GnRH-R expression level.

The selectivity of the compounds to GnRH-R positive cancer cells was ensured by uptake studies, which is of high relevance for the therapeutic success of targeted chemotherapy.

GnRH-III-Dau conjugates **1** and **2** did not trigger toxic side-effect in acute and chronic toxicity *in vivo* studies on healthy mice, promoting them as good candidates for testing their anti-tumor activity *in vivo* on tumor bearing mice.

It was clearly showed that the treatment with the GnRH-III-Dau conjugates **1** and **2** elicit a significant *in vivo* tumor growth inhibitory effect in orthotopic 4T1 murine and MDA-MB-231 human BC tumor bearing models.

The anti-metastatic effect of the conjugates on human and murine BC bearing mice was significantly improved in comparison to the free drug, whereby especially the novel conjugate (GnRH-III-[^{2}ΔHis,^{3}*D*-Tic,^{4}Lys(Bu),^{8}Lys(Dau=Aoa)]; **2**) exhibited a reduced metastasis development in the spleen, lung and liver in the 4T1 murine BC model.

Compound **2** revealed a significant higher anti-tumor activity on orthotopically developed HT-29 human CRC bearing mice than the free Dau and compound **1**.

Toxic side-effects were substantially reduced in comparison to the treatment with free Dau. This indicates clearly that the administration of GnRH-III based DDSs provide valuable benefits over the application of the free drug.

All of these findings confirm that novel compound **2** is a promising candidate for targeted tumor therapy in both CRC and metastatic BC.

Homing Heptapeptide-Dau conjugates

Conjugate **4** with modified targeting sequence is more effective than conjugate **3** with the parent homing peptide on all investigated cancer cell lines.

The conjugates have lowest effect on normal cells, suggesting selectivity of the conjugates to tumor cells.

Conjugate **4** has significantly higher anti-tumor effect than conjugate **3** on orthotopic HT-29 colon cancer bearing mice, supported by much lower proliferation index in the tumor.

Free Dau revealed significant liver toxicity compared to both conjugates although they have 10 times higher Dau content.

Based on all, it is worth to modify tumor homing peptides selected by phage display technique for the development of SMDC with increased bioactivity and stability that can be applied efficiently for targeted tumor therapy.

Vindoline CPP conjugates

Octaarginine CPP Br-Vindoline conjugates and derivatives showed anti-tumor effect on mouse leukemia and colon carcinoma cells compared to free Vindoline and Br-Vindoline which did not.

Conjugation with octaarginine, the well-known CPP, increased the anti-tumor activity of the moderately active Vindoline derivatives.

The configuration of Trp influence the anti-tumor activity, where conjugate with *L*-Trp is much more potent than its *D*-Trp counterpart *in vitro* and *in vivo*.

L-Trp containing CPP conjugate inhibits more tumor volume in tumor bearing mice than free Vinblastine in both murine leukemia and colon carcinoma models.

In both *in vivo* tumor models conjugates did not show adverse effect on animal body weight, general looking and behavior of experimental animals, revealing that conjugates are not toxic and that do not cause side-effects to the animals.

This study demonstrated that Vindoline has a potency to be chemically transformed to an active anti-cancer agent, where especially the conjugation with octaarginine as CPP could increase anti-tumor effect.

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