DOI:10.14753/SE.2021.2555

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

PhD thesis

Ivan Ranđelović

Semmelweis University

Pathological Sciences Doctoral School

Experimental Oncology

Supervisor: József Tóvári, PhD

Official reviewers: Attila Marcell Szász, MD, PhD
Kata Horváti, PhD

Head of the Complex Examination Committee: Janina Kulka, MD, DSc

Members of the Complex Examination Committee:
Tamás Kardon, MD, PhD
Szilvia Bősze, PhD

Budapest
2021

DOI:10.14753/SE.2021.2555

Biti čovek, rođen bez svoga znanja i bez svoje volje, bačen u okean postojanja. Morati plivati. Postojati. Nositi identitet. Izdržati atmosferski pritisak svega oko sebe, sve sudare, nepredvidljive i nepredviđene postupke, svoje i tuđe, koji ponajčešće nisu po meri naših snaga. A povrh svega, treba još izdržati i svoju misao o svemu tome. Ukratko: biti čovek.

iz govora Ive Andrića na Nobelovom banketu u gradskoj kući u Stockholmu, 10. decembra 1961.

To be a human, to have been born without knowing it or wanting it, to be thrown into the ocean of existence, to be obliged to swim, to exist; to have an identity; to resist the pressure and shocks from the outside and the unforeseen and unforeseeable acts – one's own and those of others – which so often exceed one's capacities. And what is more, to endure one's own thoughts about all this: in a word, to be human.

from Ivo Andric's speech at the Nobel Banquet at the City Hall in Stockholm, December 10, 1961

Za sanjare…

For dreamers…

DOI:10.14753/SE.2021.2555

TABLE OF CONTENTS

DOI:10.14753/SE.2021.2555

DOI:10.14753/SE.2021.2555

DOI:10.14753/SE.2021.2555

THE LIST OF ABBREVIATIONS

All applied three-letter and one-letter codes for α-amino acids are in agreement with the recommendations from the IUPAC-IUB Commission on Biochemical Nomenclature

Ac: acetylation

ADC: antibody-drug conjugate

ADCC: antibody-dependent cellular cytotoxicity

ALL: acute lymphocytic leukemia

AML: acute myeloid leukemia

Aoa: aminooxyacetyl

BC: breast cancer

Bu: butyrylation

CLL: chronic lymphocytic leukemia

CML: chronic myeloid leukemia

CPP: cell penetrating peptide

CRC: colorectal cancer

Dau: Daunorubicin (Daunomycin)

DDS: drug delivery system

Dox: Doxorubicin

D-Tic: *D*-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid

EGFR: epidermal growth factor receptor

ER: estrogen receptor

FDA: Food and Drug Administration

FITC: fluorescein isothiocyanate

GnRH: Gonadotropin releasing hormone

GnRH-R: Gonadotropin-releasing hormone receptor

HER2: human epidermal growth factor receptor 2

Hsp: heat shock protein

ICI: immune checkpoint inhibitor

i.p.: intraperitoneal

KI: kinase inhibitor

mAbs: monoclonal antibodies

MDR: multi-drug resistance

MED: minimum effective dose

MFI: mean fluorescence intensity

MMAE: Monomethyl auristatin E

MMAF: Monomethyl auristatin F

MRP: MDR-associated protein

MTD: maximum tolerated dose

Mtx: Methotrexate

OEt: ethyl ester

OMe: methyl ester

OS: overall survival

PARP: poly ADP ribose polymerase

PD-L1: protein programmed cell death-ligand 1

PR: progesterone receptor

RP-HPLC: reversed phase high-pressure liquid chromatography

RT-qPCR: reverse transcription quantitative real time PCR

SAR: structure-activity relationship

SD: standard deviation

siRNA: small (short) interfering RNA

SMDC: small molecule drug conjugate

TFA: trifluoroacetic acid

TI: therapeutic index

TNBC: triple-negative breast cancer

VEGF: vascular endothelial growth factor

DOI:10.14753/SE.2021.2555

1 INTRODUCTION

1.1 Cancer

Cancer is a complex disease that can be caused by both environmental factors and genetic predisposition. According to Hanahan and Weinberg, there are hallmarks and necessary events along the carcinogenesis pathway which lead to cell transformation[1]. Carcinogenesis is a multi-step process that involves successive accumulation of genetic changes in cells as a consequence of random mutations that cause genomic instability, and lead to genetic diversity[2]. Uncontrolled and constant cell proliferation, with no entering a dormant or crisis state which should lead cell to death, is a common feature of every cancer cell and important for tumor development, caused by the abnormality in the cell cycle[3]. Moreover, in order to express a malignant phenotype, a cancer cell develops resistance to signals that inhibit the cell division and growth[4]. Like normal cells, tumor cells require nutrients for survival, as well as the ability to eject metabolic waste, thus the process of enhanced angiogenesis or neo vascularization through formation of blood vessels in the tumor occurs[5]. In highly malignant tumors cancer cells invade and colonize the local tissue, and metastasize to distant sites and organs, leading to incurability of the cancer[6]. Reprograming of tumor cell metabolism occurs to meet the need for rapid energy production imposed by constant growth and uncontrolled proliferation[7]. Although there are mechanisms by which the immune system responds to the presence of transformed cells, tumor cells developed the ability to evade the response[8].

Therefore, cancer is the second cause of death in the human population, behind heart disease, and especially malignant and metastatic tumors often lead to high mortality[9]. There are many types of cancers that differ in their properties such as the embryonic origin and molecular signature, thus cancer is a group of diseases rather than a specific disease, making their treatment more challeging[10]. Due to complexity and heterogeneity of tumors, and their resistance on therapy, the biology of a tumor can be understood only by studying the specific targets on tumor cells and within the tumor microenvironment which are establishing during the course of multistep tumorigenesis[11]. Hence, discovering and development of new therapeutics and approaches that target one or more cancer traits, or specifically deliver cytotoxic agents to the tumor (**Figure 1**), is of utmost importance.

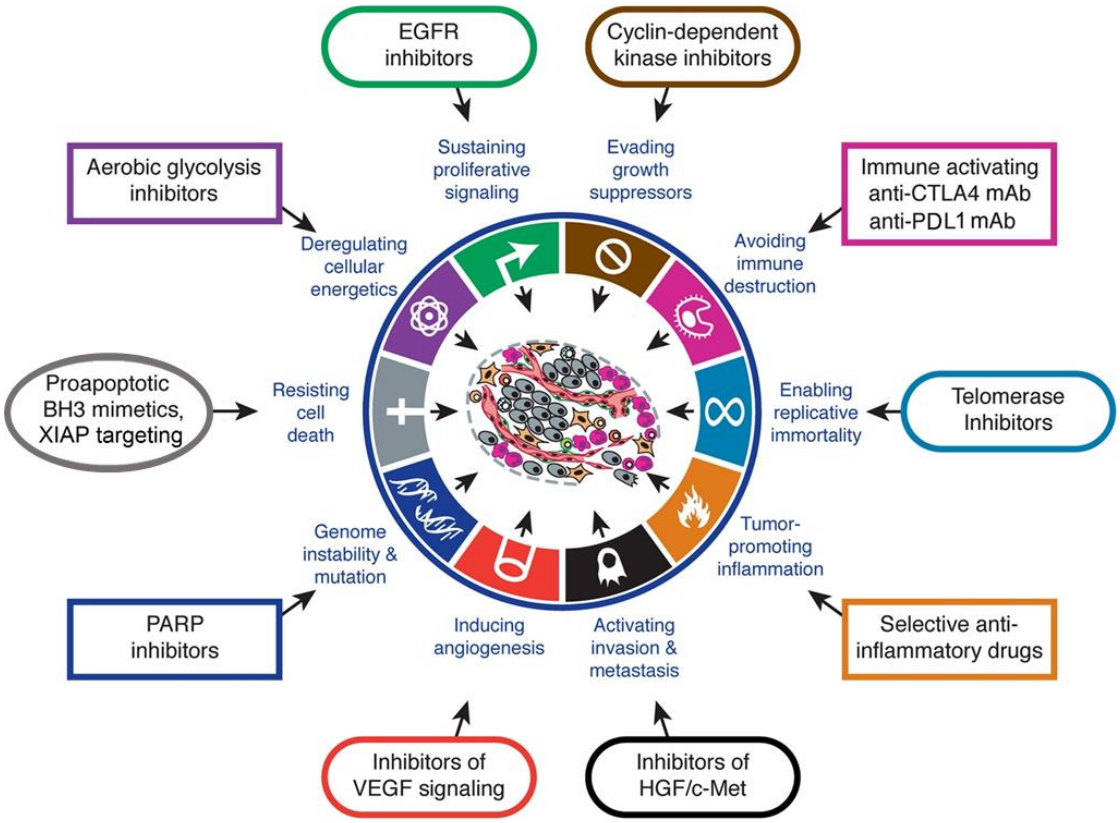

Figure 1. The hallmarks of cancer and some examples of their therapeutic targeting, adapted from D. Hanahan, R. A. Weinberg, Cell 2011, 144: 646–674[1].

1.1.1 Breast cancer

Histologically and genetically heterogeneous breast cancer (BC) is the most prevalent in women population with over 2 million new cases in 2018 (25.4 % of cancer in women) and over 0.6 million deaths worldwide (**Figure 2**)[12]. Population screening by mammography, magnetic resonance imaging, and pathology examinations aims to find early stage, non-metastatic BC which could be effectively treated with curability of 70–80%[13]. Unfortunately, advanced BC with distant organ metastases is considered incurable with currently available therapies[14]. Approximately 10% of BC are inherited and associated with a family history, while mutations in certain genes are associated with an average cumulative risk of developing BC[15]. By clinically used classification, based on histology and immunohistochemistry expression of key proteins such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and the proliferation marker KI-67, tumors expressing ER and/or PR are considered hormone receptor-positive BC, whereas tumors that do not express these markers are triple-negative BC (TNBC)[14]. Thus, drugs blocking the effects of estrogen on the mammary

gland, such as Tamoxifen, or drugs that block the production of estrogen, such as aromatase inhibitors (AIs), have major roles in the treatment of hormone-positive BC[16]. However, the highest death rates have HER2+ BC and TNBC[14]. HER2 is amplified in 13–15% of BC, causing an activation of the HER2 signaling pathway which activates proliferation and metastasis, and targeting HER2 has proven to be effective in HER2+ BC[17]. TNBCs are prone to early recurrences, within 2–3 years of first presentation, and tend to form lung and liver metastases, where the majority (~80%) of the driver alterations of the primary BC are conserved in the metastatic sites[14]. Moreover, TNBCs have high level of proliferation and show high grade aggressivity, while additionally do not express ER, PR or HER2, which makes treatment more difficult. Nevertheless, genetic expression studies have identified subtypes of TNBC with new molecular targets which may have prognostic and therapeutic implications, such as gonadotropin-releasing hormone receptor (GnRH-R)[18–20].

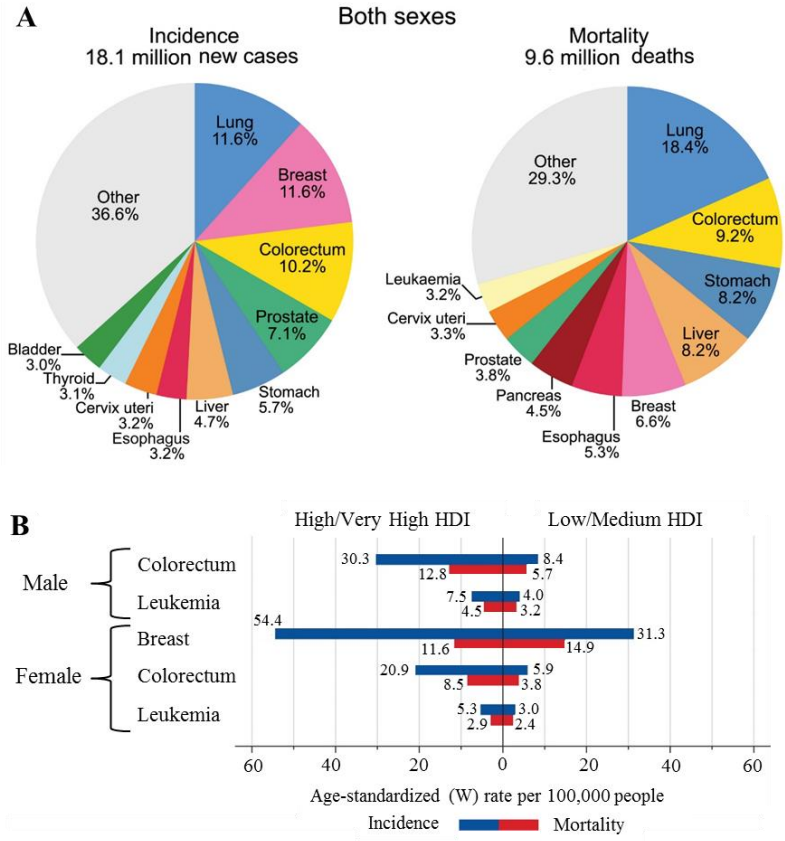

Figure 2. **A.** Estimated cancer incidences and mortality proportions. **B.** BC, CRC and leukemia age-standardized rates per 100000 people, in high/very-high human development index (HDI) regions versus low/medium HDI Regions; worldwide for both genders, in 2018. Adapted from World Cancer Report, F. Bray *et al*. CA Cancer J Clin. 2018, 68(6):394-424[12].

1.1.2 Colorectal cancer

According to the report from the International Agency for Research on Cancer in 2018, colorectal cancer (CRC) is the third most commonly occurring cancer in men and the second in women, with 1.8 million new cases diagnosed worldwide (10.6 % of all cancers), and 0.9 million deaths in 2018, placing it as the second leading cause of cancer deaths (**Figure 2**)[12]. It was reported that Hungary has the highest age-standardized incidence rate, with 51.2 cases on 100.000 persons per year[12]. The risk of CRC development and death increase rapidly with aging, as well as due to bad dietary patterns, lack of physical activity and obesity[21].

The majority of CRCs are adenocarcinomas (>90%), a malignant neoplasm that develops from glandular epithelial cells of the colon and rectum, while other rare types include squamous, adenosquamous and spindle cell carcinoma[22]. Around 25% of CRC cases have a family history without any obvious genetic cancer syndrome, while 5% are attributed to hereditary cancer syndromes[23]. Around 70% of CRC arise sporadically through acquired somatic genetic aberrations where mutations in APC, KRAS, TP53 and BRAF genes promote CRC development[24]. As in BC, CRC is a highly heterogeneous disease and also metastases to distal organs with different metastatic patterns making a big challenge for the treatment[21, 25]. The treatment repertoire for metastatic CRC, including targeted agents which target epidermal growth factor receptor (EGFR) such as monoclonal antibodies (mAbs), and anti-angiogenic agents targeting vascular endothelial growth factor (VEGF) signalling, as well as the broad spectrum kinase inhibitors (KI), improved the overall survival (OS) of patients to approximately 3 years, from 1 year in the era of 5-fluorouracil therapy alone[26]. The success of targeting therapeutics stimulates the discovery of another CRC targets such as GnRH-R[27, 28].

1.1.3 Leukemia

Leukemia is the general name for several malignant and progressive diseases of the blood-forming organs characterized by distorted proliferation and development of leukocytes and their precursors in the blood and bone marrow[29]. In 2018, number of new cases was close to a half million with double higher incidence in men, while number of deaths was around 0.3 million worldwide (**Figure 2**)[12].

According to the degree of cell differentiation, but not the duration of disease, leukemia can be classified as acute where are affected precursor cells of various lineage,

or chronic where are affected mature cells. Leukemia can be also classified according to the predominant type of cell involved on myelogenous or lymphocytic leukemia, and based on this several subtypes of leukemia have been identified with different characteristics such as acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML)[30–32]. Despite advances in management with mAbs and KI, the backbone of therapy remains multi-agent chemotherapy, as well as allogeneic stem cell transplantation for eligible candidates[33].

1.2 Cancer therapy

The heterogeneity of cancer makes its treatment more challenging, therefore specific treatment regimens and combined therapies are often applied according to the type of tumor, the stage of its development and specific genetic profile of the tumor[34]. Among a wide range of treatment options have been applied to treat cancer, classical options which include surgery, radiation therapy, hormone therapy and chemotherapy remain the backbone of cancer treatment[35].

1.2.1 Chemotherapy

Therapeutics which influence at least one of the cancer hallmark are considered as promising candidates for cancer therapy and numerous drugs with different molecular targets and mode of actions were developed. The traditional pharmaceutical approach using the administration of chemical substances, chemotherapeutics, which provide a pharmacologic effect to stop the rapid cell proliferation and prominently kill fast dividing cancer cells inducing apoptosis, is still widely applied and still represents the most employed strategy for cancer treatment, either alone or in combination with surgery and radiotherapy[36]. These agents affect fundamental cellular processes such as DNA replication and cell division, or interrupting the cell cycle at different stages[37], and based on their biological targets and mechanism of action are classified as in **Table 1**[38].

Table 1. Groups of chemotherapeutics based on their biological targets and mechanism of action approved by Food and Drug Administration (FDA) for BC, CRC and leukemias.

Category of chemo-therapeutic	**Biological targets and Mechanism of action**	**Chemotherapeutic name**	**Approval year**	**Therapy**
DNA damaging agents	form crosslinks or metal adducts or intercalate with DNA	Chlorambucil (Leukeran)	1957	CLL
		Bendamustine (Treanda)	2008	CLL
		Cyclophosphamide (Cytoxan)	1959	BC, CLL, ALL, CML
		Cisplatin	1978	BC
		Carboplatin (Paraplatin)	1989	BC
		Oxaliplatin (Eloxatin)	2002	CRC
Anti-metabolites	inhibit enzymes for synthesis of DNA	Methotrexate (Amethopterin)	1956	ALL, AML, BC
		Leucovorin (Fusilev)	2008	CRC
Nucleoside analogs	compete with natural nucleosides interfering DNA synthesis	5-FluoreUracil (Adrucil)	1962	CRC, BC
		Gemcitabine (Gemzar)	1995	BC
		Capecitabine (Xeloda)	1998	BC, CRC
Topoisomerase inhibitors	intercalate in DNA preventing its replication by interfering with the topoisomerases I and II	Irinotecan (Camptosar)	1996	CRC
		Doxorubicin (Adriamycin)	1974	BC, ALL
		Daunorubicin (Daunomycin)	1979	AML, ALL, CML
		Epirubicin (Ellence)	1999	BC
		Idarubicin (Idamycin)	2014	ALL
Anti-mitotic (anti-tubulin) agents	binding for tubulin and interfering microtubule polymerization inhibit mitosis	Vinblastine (Velban)	1965	ALL, CML
		Vincristine (Oncovin)	1963	ALL, CML
		Paclitaxel (Taxol)	1993	BC
		Docetaxel (Taxotere)	1995	BC

Among all of them, in my doctoral thesis study are used Daunorubicin (Dau) and Vinblastine (**Figure 3**). Dau is anthracycline antibiotic, isolated from Streptomyces peucetius, belonging to topoisomerase II inhibitors group. By intercalating between DNA bases and trapping the DNA-enzyme complex, Dau inhibits replication fork progression, prevent DNA replication and lead cell to death[39, 40]. Vinblastine, isolated from the *Catharanthus roseus*, is the most famous microtubule destabilizing agent from so called Vinca Alkaloids, which binds to monomeric tubulin and prevents its polymerization into microtubules and the mitotic spindle, thus blocking the mitosis of the cell[41, 42].

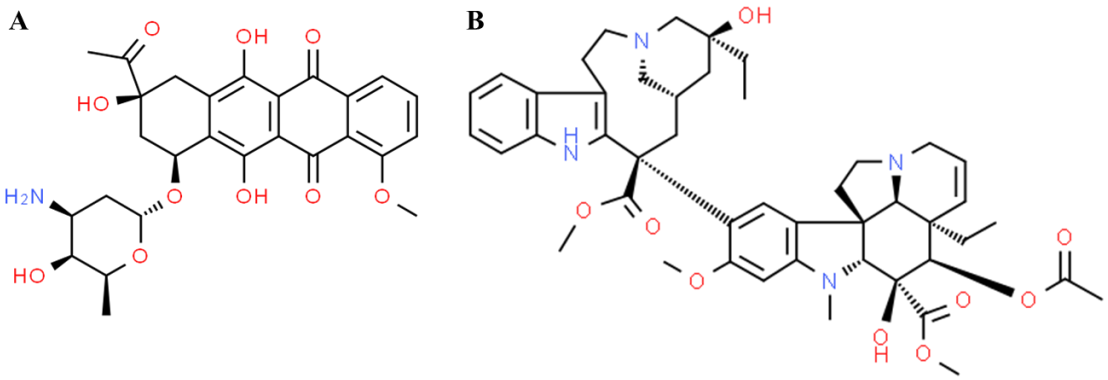

Figure 3. **A.** Daunorubicin structure. **B.** Vinblastin structure.

Although chemotherapy still plays a key role in the treatment of cancer, it has various drawbacks limiting its therapeutic benefits. The main limitation of traditional chemotherapeutics is the lack of selectivity because they do not accumulate and affect only in tumor cells, but also in the healthy tissues, displaying severe side-effects which cause systemic cytotoxicity[43]. Their low accumulation at the tumor site can be attributed to different factors such as the increased interstitial pressure in solid tumors and the rapid elimination from systemic circulation, which help accumulation of toxic agents in organs such as liver, kidneys and lung[44]. Another limitation is caused by high heterogeneity and mutation rates in tumor cells where particular population of cancer cells may be less affected by the treatment and it can make selection and then overgrow, leading to a drug-resistant tumor mass, which heavily affects the chemotherapy efficacy[45].

As consequence of drawbacks, traditional anti-cancer agents are characterized by a narrow therapeutic index (TI), which is commonly described as the difference between the lowest administered dose resulting to clinical benefits (minimum effective dose, MED) and the highest dose found to be free of undesired toxicities (maximum tolerated dose, MTD)[46]. Chemotherapeutics MED is not significantly lower than MTD associated to side-effects, and due to this the dosage range of a drug that can treat cancer effectively without displaying undesired effects is very narrow[47].

So far, two main approaches were used to make wider the TI and to improve the clinical effect of this class of compounds (**Figure 4**), mostly on the way to decrease the MED[48]. The first one is the combination of two or more chemotherapeutics without overlapping mechanism of action and different toxicity profiles[44, 49], and second, the discovery and the introduction of new more potent drugs that could be administrated at

lower dosage, but with improved anti-cancer efficacy[50]. In past decades a wide range of new anti-cancer drugs have been discovered from natural sources such as plants and other living organisms. Among them, the most famous are new DNA- and RNA- targeting agents such as derivatives of Calicheamicin and α-Amanitin, then tubulin-targeting molecules such as Cryptophycins, Maytansinoids, Dolastatins, and Monomethyl auristatin E and F (MMAE and MMAF), and a topoisomerase I inhibitor like Camptothecins and Exatecans, which inhibit cell proliferation at the picomolar range[51–54]. Despite the increased potency of these new cytotoxic agents compared to classic chemotherapeutics, the clinical performances were not improved, and they had to be discontinued from the clinic at early stages due to side-effects they caused even at low administration doses, unveiling the need for new pharmacologic approaches[55].

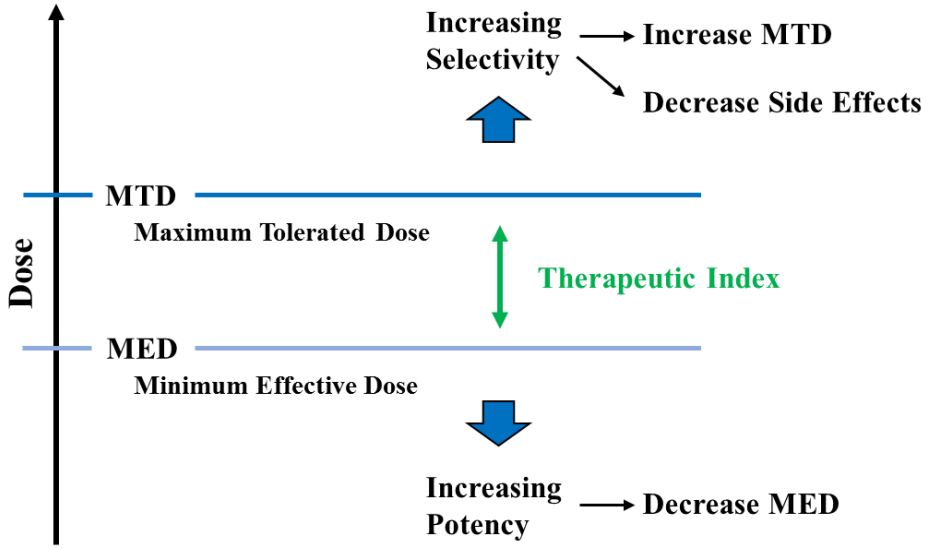

Figure 4. Strategy for the optimization of the therapeutic index. Increasing the anti-tumor potency of the chemotherapeutic through its modification, or development of more potent therapeutics (lower part). The improvement of the selectivity of anti-cancer drugs, through combining them with homing devices, and locally increasing their concentration without affecting the potency of these agents, could ideally lead to the increase of the therapeutic window and the decrease of the undesired side-effects generally observed in cancer patients (upper part). Adapted from S. Panowski *et al*. MAbs. 2014 6(1): 34–45[48].

Through years it was understood that the improvement of the selectivity of anti-cancer drugs could lead to the increased MTD without affecting the potency of these agents where MED stays same (**Figure 4**), by increasing the therapeutic window and avoiding

the systemic toxicity[55]. Thus, discovery of new targeted molecules or new homing devices which recognize and target a specific alteration or an antigen on tumor with enhanced selectivity, and deliver the cytotoxic payload, became a new approach in past years.

1.3 Targeted cancer therapy

With the purpose to improve the TI and to overcome the limitations of classic chemotherapy, modern strategies are oriented towards targeted therapies[56]. The aim of targeted cancer therapy is to affect cancer cells on selectively way by interfering directly with essential pathways involved in tumor growth such as signal-transduction pathways, or by efficiently delivering the cytotoxic agents to the tumor without compromising the healthy cells and reducing side-effects (**Figure 5**) [57–60]. Most common targets are molecular markers or antigens that play an important role in cell proliferation and that are overexpressed in tumor cells compared with normal tissues[34, 61]. Main approaches identified as targeting therapy are molecularly targeted therapy, monoclonal antibodies and targeted drug delivery systems (DDS)[62].

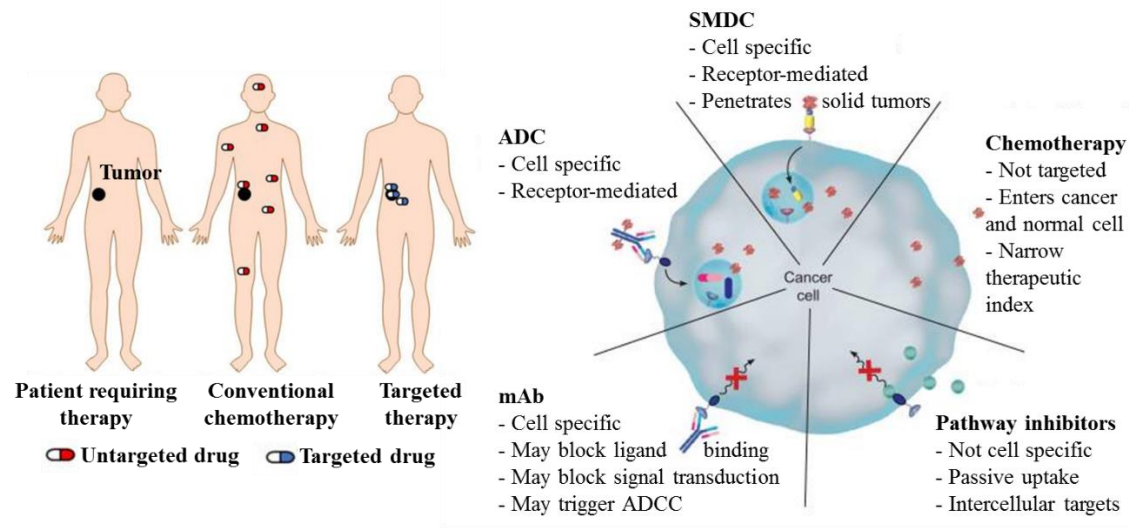

Figure 5. Targeted therapy *vs* traditional chemotherapy (left). Overview of the different classes of targeted therapy and chemotherapy (right). Adapted from E. Vrettos *et al*. Beilstein J Org Chem. 2018, 14: 930–954[63], and CP Leamon *et al*. Pharmgenomics Pers Med. 2013 6: 113–125[64].

1.3.1 Molecularly targeted therapy

This group consist of small molecule therapeutics designed to interact selectively with a specific target which is necessary for tumor progression, and to prevent the activation of signaling pathways deregulated in tumor cells[56, 65, 66]. Well known examples of

molecularly targeted drugs are small molecule KI aiming to bind for the catalytic site of protein kinases which are overexpressed or up-regulated in tumors, and block their activity[67, 68]. Among this class of therapeutics, Imatinib (Gleevec) has been first approved by FDA in 2001 for the treatment of CML opening the development and approval of another KI and inhibitors of enzymatic and cell signalling pathways[14, 69, 70]. Among them, the most famous are HER2 targeting Lapatinib (Tykerb, 2007) and Neratinib (Nerlynx, 2017) for the treatment of BC, PARP targeting Olaparib (Lynparza, 2014) and Talazoparib (Talzenna, 2018) for TNBC, Regorafenib (Stivarga, 2012) multiple KI used in CRC, and Dasatinib (Sprycel, 2006), Nilotinib (Tasigna, 2007) and Ibrutinib (Imbruvica, 2013) used for the treatment of leukemia.

Although KI and other small molecule inhibitors have often grouped as targeted therapeutics, they show the same pharmacokinetic limitations as traditional chemotherapeutics. Due to their ability to bind to multiple molecular targets including cell surface receptors and other intracellular proteins, they do not efficiently accumulate in the mass of solid tumors and therefore increase the risk of toxicity. Most of them have a short life span and fast clearance from systemic circulation due to their low molecular weight and the high lipophilicity thus requiring daily dosing treatments[71].

1.3.2 Monoclonal antibodies

The ability of mAbs to selectively detect and with high affinity bind to antigens of interest displayed on pathogens and abnormal cells with excellent specificity, promoted the development of mAbs targeting certain antigens which are overexpressed in tumors, triggering cancer cell death through different cell killing mechanisms[72, 73]. The anti-tumor mechanism can be direct by binding of mAbs for targeted proteins and receptors that may be fundamental for the disease progression and displaying an antagonist activity, blocking the dimerization, kinase activation and downstream signaling, thus inhibiting proliferation and inducing apoptosis (**Figure 6**)[74]. Another mechanisms are performed by immune-mediated cell killing such as antibody-dependent cellular cytotoxicity (ADCC), and complement dependent cytotoxicity (CDC) pathways[75].

The most famous mAbs used in the treatment of HER2+ BC are FDA approved Trastuzumab (Herceptin, 1998) and Pertuzumab (Perjeta, 2012), which significantly improved OS in patients[75]. Due to lack of classic BC targets in TNBC, immunotherapy has shown promise with immune checkpoint inhibitors (ICI) such as Atezolizumab

(Tecentriq, 2019) and Pembrolizumab (Keytruda, 2020), mAbs targeting protein programmed cell death-ligand 1 (PD-L1)[76, 77].

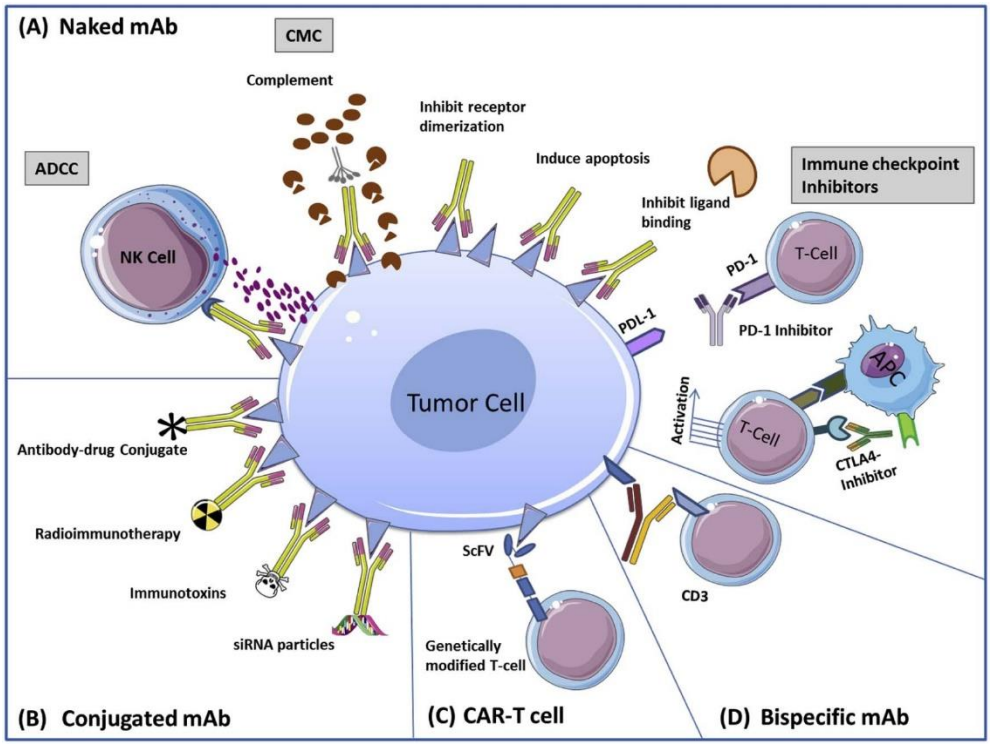

Figure 6. Mechanism of action of mAb therapy. Adapted from S. Charmsaz *et al*. Exp Hematol. 2017 54:31-39[78].

mAbs approved for CRC which showed a big benefit to patients were blood vessel formation targeting anti-VEGF agents Bevacizumab (Avastin, 2004) Ziv-aflibercept (Zaltrap, 2012) and Ramucirumab (Cyramza, 2015)[79–81], as well as anti-EGFR mAbs like Cetuximab (Erbitux, 2009) and Panitumumab (Vectibix, 2006)[82]. Moreover, for the treatment of CRC are also used ICIs such as PD-L1 inhibitors Pembrolizumab (Keytruda, 2017) and Nivolumab (Opdivo, 2017), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) inhibitor Ipilimumab (Yervoy, 2018)[83]. For the treatment of ALL and CLL different mAbs that target specific CD antigens are approved such as Blinatumomab (Blincyto, 2014), Obinutuzumab (Gazyva, 2013), Ofatumumab (Arzerra, 2014) and Alemtuzumab (Campath, 2001)[31, 84].

Although a wide range of mAbs therapeutics are approved and hundreds of them are in various pre-clinical or late stage clinical studies for cancer diseases, unfortunately, the development of antibodies for the treatment of solid tumors is generally difficult, and most of them often fail to cure patients when used as single agents, thus they are often

combined with chemotherapeutic drugs[85, 86]. However, the outstanding binding specificity of mAbs promote them to be considered as possible vehicles for selective tumor targeted release of anti-cancer drugs, and this strategy is used in the development of antibody-drug conjugates (ADC)[55].

1.3.3 Targeted drug delivery systems

A growing approach in targeted cancer therapy represent a strategy for increasing accumulation rate of the therapeutics into the tumor and overcoming the systemic cytotoxicity called targeted drug delivery system (DDS)[59]. This approach is inspired by the "magic-bullet" concept of Paul Ehrlich, awarded with the Nobel Prize in 1908 for his work, who coined the term referring to a therapeutic agent that could specifically attack the source responsible for disease without harming the rest of the host tissues[87]. This concept is applied to cancer therapy nowadays to propose the use of drug-delivery vehicles such as mAbs, small ligands, peptides, nanoparticles, polymers and micelles carrying powerful cytotoxic compounds after conjugation to them, and targeting a specific antigen or receptor to selectively deliver and liberate the payload at the tumor site without affecting the healthy tissue[60, 88–90].

The targeting by DDS can be mediated by two different mechanisms: passive or active[44, 91]. Passive targeting therapy represent group of molecules such as micelles, liposomes, polymers, nanoparticles and macromolecules which have the ability to passively accumulate into solid tumors, using so-called enhanced permeability and retention effect (EPR) described as the main mechanism of action of these large molecules which take advantage of poorly differentiated tumor vasculature that allows penetration of molecules with larger sizes, and the absence of lymphatic drainage which decrease the clearance of them[92, 93]. For example, different types of nanoparticles have been used to encapsulate cytotoxic agents with the aim to force drug accumulation within the tumor, following different mechanisms of action[94]. However, clinical success of nanomedicine has been limited so far to liposome-encapsulated Dau (DaunoXome, 1995) for the treatment of leukemia, and albumin-bound Paclitaxel (Abraxane, 2005) for the treatments of BC, while liposomal Vincristine (Marqibo, 2012) is approved for ALL[95].

Active targeting by DDS, also called ligand-receptor-mediated drug delivery, represent technologies which use ligands such as mAbs and small molecules as carriers for anti-cancer drugs, have been investigated to improve current chemotherapy outcome,

pointing out the selective receptor-dependent delivery and release of anti-tumor drugs at the tumor site preferentially *via* receptor-mediated endocytosis (**Figure 7**)[88, 96–99].

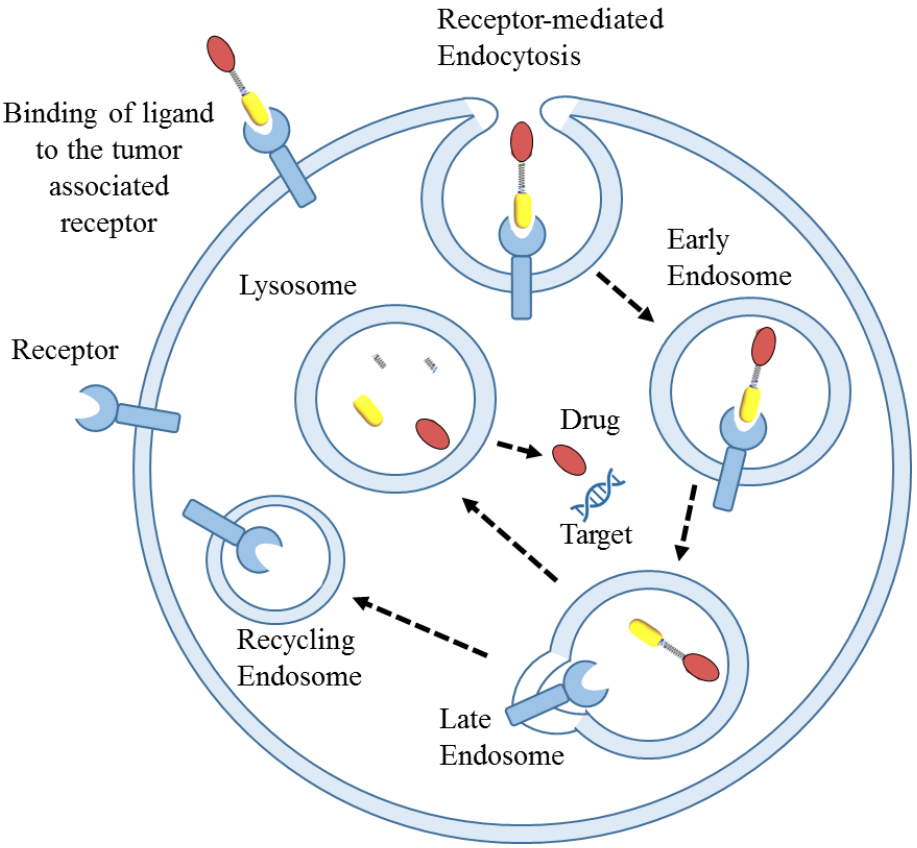

Figure 7. Receptor-mediated internalization of drug delivery systems.

In this process after recognition and binding of the ligand to the antigen or receptor on the cell surface, formed receptor-conjugate complex is internalized into the cancer cell, where in compartments of endosomes and lysosomes is dissociated. A chemically stable spacer which prevent the premature release of the payload in the blood circulation, is cleaved through different modes of action, releasing the cytotoxic payload which reaches its target leading to cell death, while the receptor is either degraded or recycled to the cell surface[100, 101]. This goal has been pursued by improving the tumor accumulation of anti-cancer drugs through their covalent conjugation to specific targeting ligands *via* a spacer that allows good plasma stability and efficient drug release at the tumor site due to the higher presence of certain enzymes or specific tumor physiological conditions, acidic pH in lysosomes, or degradation by lysosomal enzymes which ensure that the drug gets to its site of action[90].

ADCs and small molecule drug conjugates (SMDCs) represent two innovative classes of new generation biopharmaceutical therapeutics, designed to selectively bring cytotoxic agents to the tumor tissue as DDS[98]. In particular, mAbs and small ligands which display target specificity but limited anti-tumor activity are conjugated to cytotoxic agents, very potent but with poor safety and pharmaceutical profiles. These types of therapeutics will be described in the next sections.

1.3.3.1 Antibody-drug conjugates

The high specificity and affinity of mAbs for particular antigen, and the positive results obtained from the combination of mAbs with chemotherapy led to the idea to conjugate mAbs covalently *via* spacer and linker and to develop conjugated therapeutics called ADCs[55], which should ideally deliver and liberate the drug at the tumor site after mAb part selectively bind to its target expressed on tumor cells surface (**Figure 8**)[102, 103].

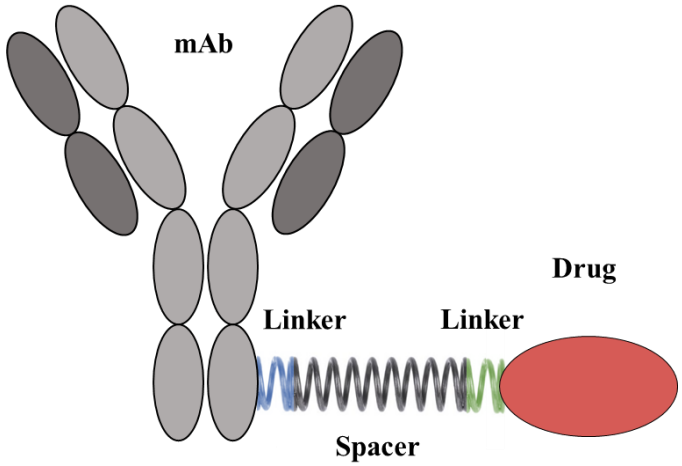

Figure 8. General structure of an antibody-drug conjugate.

Most ADCs, containing intracellular cleavable spacers, are designed to kill cancer cells in a target-dependent mechanism, which rely on the recognition and binding to the cell antigen, forming ADC-antigen complex, which internalize *via* a receptor-mediated endocytosis pathway explained above, and deliver the drug which reaches its target leading to cell death[55, 99, 104]. Another efficient pharmacological approach could be non-internalizing ADCs equipped with extracellular cleavable spacers which rely on extracellular drug release mechanism where upon binding of the antibody to the desired antigen, the drug is released in the tumor microenvironment under specific tumor acidic pH or enzymes[105]. Therefore, for the development of efficient ADCs, factors that should

be considered are the choice of the target, the binding affinity and immunogenicity of the antibody, the nature of the spacer and the potency of the cytotoxic drug[103].

The first generation ADCs such as BR96 and K1S/4, carried traditional chemotherapeutic drugs Doxorubicin (Dox) and Vinblastine, reached clinical trials, but the low anti-tumor activity in metastatic BC and CRC as well as observed toxicity halted their development[106]. The main reasons for their fail was the usage of a chimeric antibody which resulted in a considerable immunogenic response, fast clearance from the body, and not efficient release of the drug from the targeting vehicle, leading to their much less potency than the free drug[106]. These important aspects were improved in the next generation of ADC with adopting humanized or fully human antibodies as targeting devices[107]. Also, use of more potent anti-cancer drugs, mentioned before, and optimizing drug-to-antibody ratio (DAR) in the second and the third generation of ADCs leaded to eight ADCs have been approved by the FDA for cancer therapy so far (**Table 2**)[86, 108, 109].

Table 2. Antibody-drug conjugates approved by FDA, the drug they carry, and their targets.

Brand name	Name	Drug	Target	Company	Year	Therapy
Mylotarg	gemtuzumab-ozogamicin	NAGC	CD33	Pfizer/Wyeth	2000 2017	AML
Adcetris	brentuximab vedotin	MMAE	CD30	Seattle Genetics/Millennium/ Takeda	2011	Lymph oma
Kadcyla	ado-trastuzumab emtansine	DM1	HER2	Roche/Genentech	2013	BC
Besponsa	inotuzumab ozogamicin	NAGC	CD22	Pfizer/Wyeth	2017	ALL
Enhertu	fam-trastuzumab deruxtecan	Deruxtecan	HER2	AstraZeneca/Daiichi Sankyo	2019	BC
Padcev	Enfortumab vedotin	MMAE	Nectin-4	Astellas/Seattle Genetics	2019	Urotheli al
Polivy	Polatuzumab vedotin	MMAE	CD79b	Genentech and Roche	2019	NHL
Trodelvy	Sacituzumab govitecan	SN-38	Trop-2	Immunomedics	2020	TNBC

Mylotarg was re-approved. NAGC: drug N-acetyl-gamma-calicheamicin, DNA damaging agent. MMAE: Monomethyl auristatin E, microtubule polymerization blocking agent. DM1: emtansine, thiol-containing maytansinoid, microtubule polymerization blocking agent. Deruxtecan: a derivative of exatecan, topoisomerase I inhibitor. Nectin-4: cancer expressing cellular adhesion molecule. NHL: nonHodgkin lymphoma. SN-38: an active metabolite of irinotecan, an analog of camptothecin, topoisomerase I inhibitor. Trop-2: Tumor-associated calcium signal transducer 2.

Despite ADCs achieved a great success and represent the most promising DDSs, this strategy present drawbacks such as high manufacturing costs, unfavorable pharmacokinetics caused by low tissue penetration, low extravasation and low accumulation rate, the heterogeneity of the antigen expression in the tumor, and possible immune response which might cause some limitations for their use in the clinic[110]. However, exploring and developing alternative targeting devices, which may be easier to produce and which can reach tumor cells more efficiently, like SMDCs is gaining high interest among the pharmaceutical industry in recent years[98].

1.4 Small Molecule Drug Conjugates

With the aim to overcome the limitations associated with ADCs and to improve accumulation of anti-cancer drugs in the tumor, it was developed the approach where therapeutic agent is attached *via* covalent conjugation to specific ligand that with high affinity and selectivity target tumor associated receptor[111]. Ligands or targeting moieties in SMDC are represented by a low-molecular weight small molecules such as peptides, hormones, vitamins, and peptidomimetics, used as vehicle for targeted drug delivery, which should ideally accumulate in tumorous tissue avoiding healthy tissues and reducing harm side-effects. This valuable characteristics of particularly peptide-drug SMDCs emerged them as a promising alternative to antibodies as homing devices[63, 112–114]. The mechanism of drug release for SMDCs is same as for ADC through receptor-mediated endocytosis[51, 98]. Also, by SMDCs it is possible "non-internalizing" tumor targeting as with ADCs, using specific conditions of the tumor microenvironment for releasing of the drug extracellularly, which then can act against tumor cells by passive diffusion[90, 115].

While ADCs represent well known approach, which has already reached the market, increasing attention of researchers have been recently put into development of SMDCs because of their advantages. Small size of SMDC allows them to display a better pharmacokinetic properties such as high tissue permeability characterized by penetration into solid tumors with greater efficiency, and fast extravasation characterized by rapid excretion from circulation, in case of absence of good binding which lead to low immunogenicity[59, 116]. One more important advantage of SMDCs as DDS, especially peptide-drug conjugates is their structural simplicity and defined molecular structure that can be easily purified and characterized allowing fast a hit-to-lead optimization. SMDCs

can be easily developed in large scale by chemical synthesis, easily modified and produced with reduced cost compared to ADC[117, 118].

Despite all the advantages of small molecules as targeting moieties compared to antibodies, their clinical application has not been proven yet. Antibodies are more selective and they can be generated against any possible antigen, while targeting with SMDC is less selective and remains limited to a small number of targets[98, 119]. The biggest difference between ADCs and SMDCs is their pharmacokinetics. While ADCs remain in circulation up to one week, SMDCs peptides have short plasma half-life, shorter than one day, and poor stability against proteases which results in their fast clearance through the kidneys[120]. However, the fast pharmacokinetic profile of SMDCs allows more frequent dosing and higher payload concentration in the tumor tissue and reduce the side-effects to other organs[121]. Moreover, the progress in the field has minimized the limitations using different methods such as cyclization, N-methylation or sequence modifications with unnatural amino acids like introduction of D-amino acids[122].

Typical structure of SMDCs (**Figure 9**) consist in the connection of four core components: ligand - small targeting moiety connected with cytotoxic agent, *via* spacer and linker[90].

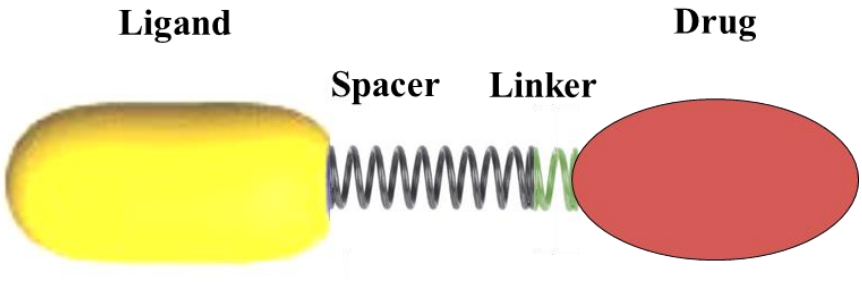

Figure 9. General structure of a small molecule drug conjugate.

Spacer is a longer chemical entity which connects the targeting ligand and the drug while keeping them at a distance, aiming to keep the integrity of the SMDC until the payload is delivered at its target in the tumor, achieving the selective drug release in the extracellular tumor environment, or intracellularly by receptor-mediated endocytosis.

A linker provides the functional groups to form a chemical bond for coupling, linking together the targeting ligand and the drug. It can also functionalize the spacer moiety between the ligand and the drug, and thus modify conjugate's physicochemical properties such as improving of solubility, or pharmacokinetic properties of drug release[123].

Often simple short linkers are also able to achieve the goal of selective drug release in the tumor environment. Several parameters, such as type of connection bond on the targeting ligand, polarity, hydrophobicity and drug release mechanisms, are of high relevance for optimization of the SMDCs and may contribute to the SMDC performance and modulate the safety and efficacy of it, because the nature of the spacer shows direct influence on the stability, efficacy and pharmacokinetic profile of the SMDC construct.

According to the physiological features of the targeted receptors, a variety of chemical structures with different chemical bond lability have been used as linkers or spacers to promote drug release with different mechanism from SMDCs under different conditions at the tumor site[51]. Reductively (metal complexes and disulfide bonds)[96, 124], hydrolytically (esters and hydrazones)[51], and enzymatically (peptides)[125, 126] cleavable linkers and spacers are completely stable at physiological conditions in circulation, but cleaved as a result of the highly reducing or acidic environment of the cancer intracellular compartments such as lysosomes and endosomes, or in the presence of certain enzymes inside cancer cells and within the tumor stroma[96]. To another group belong uncleavable linkers (triazoles, carbamates, and amides) which are in SMDCs generally stable both in circulation and inside the cell, and due to this properties have no significant anti-cancer activities, and they can be exploited for diagnostic imaging purposes by conjugation a fluorophore to the targeting ligand[127].

The most important prerequisite for a certain peptide to become a receptor-mediated carrier and its design are choice of the target receptor, which must be sufficiently overexpressed on the surface of tumor cells compared to healthy cells to avoid off-target cytotoxicity, and frequently recycled or resynthesized and ready for binding of SMDC[96]. Furthermore, high binding affinity and high specificity of ligand for the target are of high relevance, which allow better access to the targeted receptor and avoid other members of the receptor family, increasing the tumor:blood and tumor:organ accumulation ratio of the drug, and minimizing the fast clearance often associated to the use of small molecules[90]. Another important characteristics are efficient internalization of the ligand and its efficient synthesis and optimization through structure-activity relationship (SAR) studies to identify the sites where modification will not interfere with receptor binding[96].

Taking into account all this, the most used ligands are the natural-occurring ones, such as vitamins or hormones, whose receptors are often overexpressed by fast-growing

tumors. However, new technologies for hit identification such as high-throughput screening, phage display and DNA-encoded chemical libraries are increasingly exploited to discover new ligands for SMDC applications[52].

For SMDCs development, a variety of antigens have been investigated as targeted receptors and their targeted ligands have been synthesized, where the most common examples of targeted receptors-ligands are: integrin – RGD (arginine-glycine-aspartic acid peptides), CD13 receptor (aminopeptidase N) – NGR (asparagine-glycine-arginine) peptides, folate receptor – folate acid analogs.

In past decades specific hormone receptors are considered suitable targets for anti-cancer therapy, due to their high expression in different cancer cells[128, 129]. Therefore a wide range of agents have been developed and used to influence the endocrine system in hormone-dependent tumors and among them are the most famous are Somatostatin (SST) ligands such as Octreotide (Sandostatin, octapeptide) and Seglitide, and others like Lanreotide (Somatuline), Vapreotide, and Pasireotide which target Somatostatin receptors (SSTRs)[130], and GnRH ligands which will be described in further paragraphs.

1.4.1 Gonadotropin-releasing hormone-receptor targeting

GnRH-R belongs to group of G protein coupled receptors (GPCRs) with seven-transmembrane domain and normally they are expressed on anterior part of pituitary gland (**Figure 10**)[131].

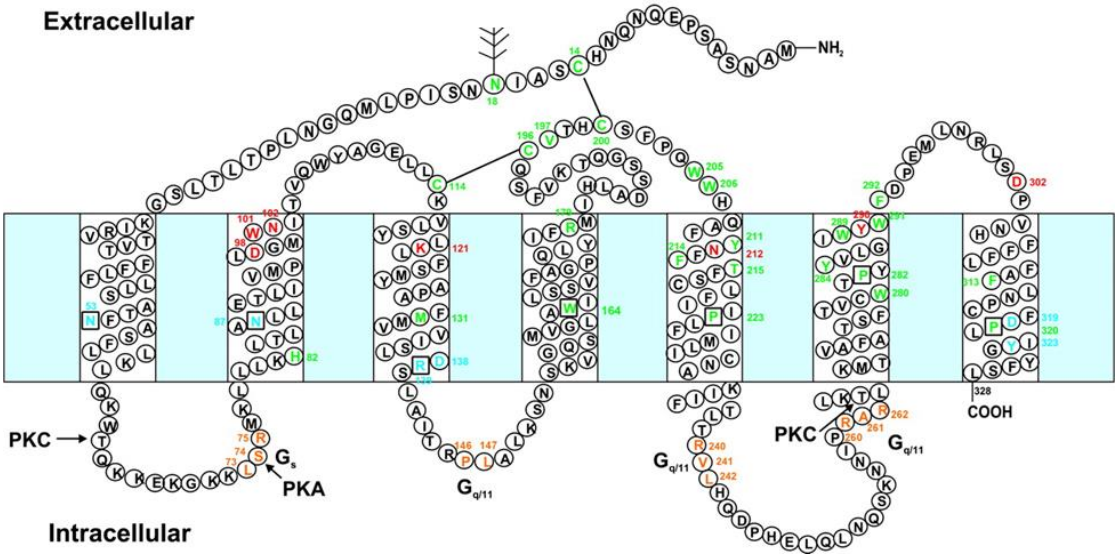

Figure 10. Two-dimensional representation of the human GnRH-R. Adapted from RP Millar *et al*. Endocr Rev. 2004 25(2): 235–275.

For GnRH-R binds gonadotropin-releasing hormone (GnRH), known also as GnRH-I or luteinizing hormone releasing hormone (LHRH), which is synthesized in hypothalamic neurons and released in bloodstream by pulsatile fashion (**Figure 11**), triggering the production and secretion of gonadotropic hormones such as luteinizing hormone (LH) and follicle stimulating hormone (FSH)[132].

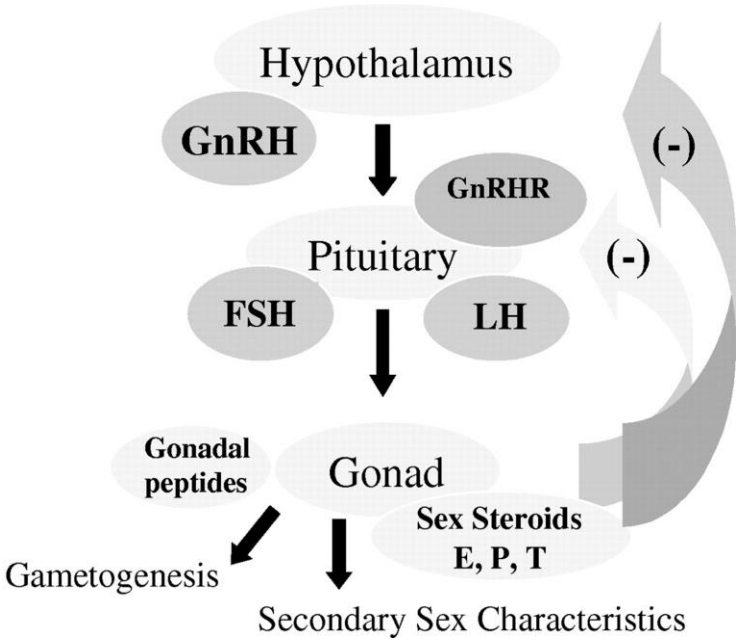

Figure 11. Physiological role of GnRH. The hypothalamic–pituitary–gonadal axis. E, estrogen; P, progesterone; T, testosterone. Adapted from GS Harrison *et al*. Endocr Relat Cancer. 2004 11(4):725-48[133].

Gonadotropins regulate gametogenesis and the production of androgen and estrogen, the sex steroids having important role in vertebrate reproduction[134]. GnRH-I is hormonal decapeptide (Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, where Glp is pyroglutamic acid) discovered by Andrew V. Schally and colleagues in 197[135].

Due to the fact that GnRH-R are not only present in pituitary gland, but also highly expressed on the cell surface of many different reproductive system related tumors such as BC, ovarian, endometrial and prostate cancer[19, 136, 137], but also in non-reproductive cancers like melanoma, glioblastoma and CRC[27, 138, 139], while their presence on healthy tissues is limited[28, 140], these receptor can serve as target for targeted anti-tumor therapy. Thus, a wide range of GnRH-I agonists and antagonists with different modification of the primary structure were developed and their *in vitro*/*in vivo* anti-tumor effect as well as good tolerance were demonstrated in hormone-dependent and independent tumors[141–148].

Anti-proliferative effect of GnRH-I derivatives can be exerted by an indirect way in reproductive system related tumors, where upon their binding for GnRH-R expressed on pituitary gland, cause the interrupting of signaling and suppression of gonadotropin and sex steroids secretion, which on indirect way suppress the development of hormone-dependent tumor[146]. GnRH analogs also affect on tumors by direct way, where their binding for GnRH-R expressed on the reproductive system related and also unrelated tumors, cause direct anti-tumor activity, while a reversible blockage of the pituitary GnRH-Rs occurs[149–152]. It was clearly shown, that the GnRH-R signal transduction mechanism activated by GnRH analogs in gonadotrophic cells of the pituitary, is different from that found in human malignant tumors, where anti-tumor effect is mediated by different pathway which prevents mitogenic signal transduction and reduces the expression of growth factor receptors[140, 147, 153–155]. This difference can be explained with presence of GnRH-I natural isoforms which are able to bind GnRH-Rs on cancer cells, and therefore activate distinct signaling complexes and inducing different effects[156–159].

One of GnRH isoforms, GnRH-II, was identified in chicken brain, but its expression was detected ubiquitously in all vertebrates including human[160]. Previously it has been shown that GnRH-II has an improved anti-tumor effect compared to GnRH-I, while its ability to induce the gonadotropin secretion pathway is less effective[157]. Although GnRH receptor type II (GnRH-IIR) is not present in human, its mRNA expression could be identified in human tumor cells[149]. However functional full length protein could not be determined[154, 161]. These data shows that GnRH-IR mediates the biological activity of GnRH-I, GnRH-II and their derivatives[157, 159, 160].

In addition to human GnRH-I, its natural isoform GnRH-III (Glp-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2) was identified and isolated from sea lamprey (*Petromyzon marinus*), and characterized by Sower and colleagues, has gained in importance and it was intensively studied within the past years[162]. It has been shown that 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[163], while its endocrine activity is strongly reduced compared to GnRH-I, both *in vitro* and *in vivo*[149, 163–165].

Various SAR studies have been performed in order to increase the anti-cancer potency of GnRH-III[165–168]. From these studies it was observed that the most significant difference

between GnRH-I and GnRH-III is their conformation, where GnRH-I has U-shape conformation, while GnRH-III has extended backbone structure[167, 168]. Also, it was showed that the positive impact on the tumor inhibitory effect could have amino acid substitutions in the peptide sequence[166, 167]. Therefore, GnRH-III and its derivatives can be considered as selective and efficacious targeting moieties which specifically bind to GnRH-Rs on cancer cells, but possess a much lower endocrine effects than GnRH-I and its analogs, and thus represent a promising approach especially for the treatment of hormone-independent tumors like CRC[169]. Because of that, different GnRH-III-based peptide carriers were developed and used as targeting moiety to deliver anti-cancer agents like Dau selectively to GnRH-R expressing cancer cells[170–172].

It has been demonstrated that GnRH targeting ligand internalize into the cell *via* receptor-mediated endocytosis by mechanism explained before. Internalization and recycling of the receptor is very fast, and already after 15 minutes, the ligand-receptor complex is transferred to the lysosomes, while after 30 minutes recycling is completed[173]. Also, apart from pituitary cells, reproductive organs and tumors, most of other tissues and hematopoietic stem cells do not express the GnRH-R, which makes it suitable target[174].

Thus, the development of the cytotoxic GnRH-I compounds started 40 years ago by Schally's group, and various GnRH DDSs have been synthesized[175–177]. Series of conjugates where various drugs such as Cisplatin, Melphalan, Methotrexate (Mtx) and Dox were inserted in position 6, either directly in the peptide sequence or conjugated to the side chain of Lysine (Lys), lead to development of zoptarelin-Dox (Zoptrex, AEZS-108, working name AN-152) reaching clinical trials[178]. It has been shown that Dox coupled GnRH analog, selectively accumulate in the nucleus of human GnRH-R positive BC cancer cell lines, while no intracellular AEZS-108 could be found in tumor cell lines which do not have membrane GnRH receptors[179]. The findings that this conjugate was more effective than Dox in inhibition growth of GnRH-R positive TNBC and CRC tumors, *in vitro* and *in vivo*, and less toxic[180–183], inspired clinical trials of it[184]. Unfortunately, although AEZS-108 reached phase clinical phase III studies proving to be effective and with low toxicity in patients with TNBC, it did not improve OS and progression free survival (PFS), or adverse events compared to Dox, and due to this it could not achieve its primary endpoint[185].

Parallel with the development of AEZS-108, another GnRH-I drug conjugates have been studied with the aim to generate compounds with higher potency[186, 187]. AEZS-108 modified analogs with more potent chemotherapeutics such as AN-207 coupled with 2-pyrrolino-Dox (pyDox) and AEZS-125 conjugated with Disorazol Z, which demonstrated 500-1000-times higher activity than Dox *in vitro* in TNBC MDA-MB-231 cells, and increased anti-cancer activity without enhancing organ toxicity, were not further investigated in clinical trials[20, 129, 136, 177, 188, 189].

Considering that the sea lamprey GnRH analog, GnRH-III, elicits a GnRH-R-mediated inhibitory effect on the growth of various human cancer cell types, while the hormone releasing effect is substantially reduced, GnRH-III represents a valuable targeting moiety for targeted tumor therapy. Taking into account that the side chain of Lys in position 8 (^{8}Lys) could be modified by conjugation without revealing the reduction of the receptor binding affinity and the anti-proliferative activity of GnRH-III, this Lys residue provides a valuable ligation site for cytotoxic payloads[165, 166, 190, 191]. This property of GnRH-III targeting ligand was used to couple a non-degradable poly-vinylpyrrolidone-co-maleic acid (P-VP-co-MA) moiety making the conjugate which showed a higher anti-proliferative activity on human ER positive BC and TNBC than the unconjugated GnRH-III, whereby also its *in vivo* anti-tumor activity on TNBC MDA-MB-231 xenograft in nude mice was improved[191, 192].

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 using the ^{8}Lys as ligation site 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[193–195].

Firstly, it was shown that even linkage systems such as ester or hydrazone bonds, used to couple Dox and Dau to the GnRH-III carrier, had a significantly higher anti-tumor activity *in vitro* on human BC and murine CRC compared to amide bond[171, 190], additional studies revealed that the relatively short half-life of the ester bond and pH sensitivity of hydrazone bond under physiological conditions might cause a partial release of the drug before it can reach the tumor tissue and enter tumor cells, limiting the efficacy of the conjugates and cause toxic side-effects[196].

Considering that structural properties of Dau don't allow it to be conjugated to the targeting moiety by ester bond like Dox, but allow the formation of the oxime bond, GnRH-III conjugates where Dox or Dau were linked to the targeting moiety by oxime bond were developed and displayed a substantial *in vitro* cytostatic effect[170]. These studies revealed that oxime linked Dau conjugates showed a slightly improved anti-tumor effect over the oxime linked Dox conjugates on BC and CRC cells indicating the advantage of Dau for this ligation system. Additionally, it was shown that the oxime bond is more stable under physiological conditions allowing the release of an active metabolite in lysosomes, thus representing an attractive linkage system, even if the lack of free drug release might limit the potency of these DDS[170, 193].

Furthermore, different strategies have been employed in order to improve the anti-cancer activity of the highly stabile oxime bond linked GnRH-III-Dau conjugates where Dau linked to the side chain of the ε-NH_2 group of ^{8}Lys by incorporation of an aminooxyacetyl (Aoa) moiety, demonstrated higher anti-tumor activity on MCF-7 human BC and HT-29 human CRC cells, than GFLG tetrapeptide spacer insertion in the structure of oxime bond, which should be cleved by enzyme Cathepsin B overexpressed in tumor cells[197, 198]. Importantly, in these studies was shown that the release of the free drug is not required for the anti-tumor activity of the conjugate since the amino acid-Dau metabolite is also able to intercalate with the DNA with sufficient efficiency[170]. Also, it was obtained similar anti-tumor efficacy of these conjugates *in vivo* on CRC-bearing mice[199]. Thus the attention was turned to modifications of the targeting sequence.

One of approach was the replacement of serine (Ser) in position 4 (^{4}Ser) by ^{4}Lys in GnRH-III sequence followed by acetylation (Ac) of ^{4}Lys which provides a more suitable structure for receptor binding, enhanced stability towards proteases, improved cellular uptake and increased *in vitro* anti-tumor activity on human BC and CRC cells, and this effect was further verified by *in vivo* studies on murine CRC-bearing mice[166, 193]. Further studies pointed out that the Ac of ^{4}Lys with short-chain fatty acids lead to an additional improvement of the biological activity of the GnRH-Dau conjugates, where the butyrylated (Bu) derivative GnRH-III-[^{4}Lys(Bu),^{8}Lys(Dau=Aoa)], (**compound 1**) (**Figure 12**) displayed not only increased cellular uptake and *in vitro* anti-tumor activity on human CRC and BC cells, but also *in vivo* an increased anti-tumor activity compared to its ^{4}Lys(Ac) counterpart on human CRC-bearing mice[194, 200].

DOI:10.14753/SE.2021.2555

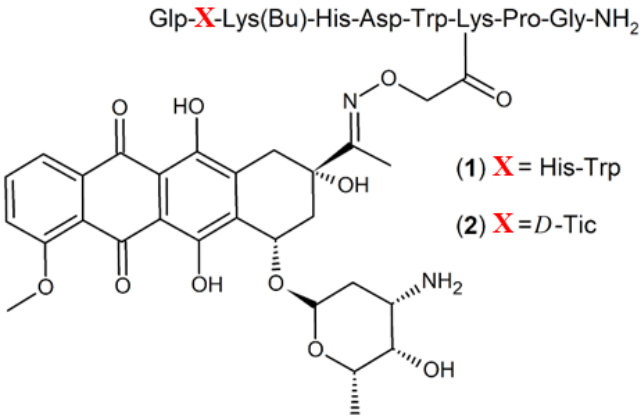

Figure 12. Structure of GnRH-III-Dau conjugates **1** and **2**.

One more benefit of ^{4}Ser to ^{4}Lys exchanging was the possibility for the development of multifunctional dual drug GnRH-III conjugates, but although developed di-Dau conjugates showed very encouraging anti-tumor activity, the mono-Dau conjugates represent the most promising candidates for GnRH-III-based targeted tumor therapy, since they possesses a comparable cytostatic effect and provides a better ratio between drug-content and achieved anti-cancer activity[194, 201, 202].

Further modifications through various amino acid substitutions selected conjugate GnRH-III-[^{2}ΔHis,^{3}*D*-Tic,^{4}Lys(Bu),^{8}Lys(Dau=Aoa)] (**compound 2**) (**Figure 12**), where ^{2}His was deleted and ^{3}Trp was exchanged by the unnatural amino acid *D*-Tic (*D*-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid), as the best candidate displaying an improved inhibitory effect on the growth of CRC and BC cell lines *in vitro*, as well as an improved cellular uptake and an accelerated delivery of the drug to its site of action[203]. In addition, it could be shown that compound **2** is highly stable in human plasma, while the high binding affinity to GnRH-Rs on cancer tissue and the release of the active drug metabolite in lysosomes were not affected.

For my doctoral thesis work, the *in vitro* anti-proliferative activity of **1** and **2** was investigated 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.

1.4.2 Heptapeptide selected by phage display as homing device for targeting CRC

The concept of selective drug targeting for cancer is based on the specificity towards cancer cells provided by the high expression of certain cell surface receptors or components on tumors, while sparing toxicity to off-target cells and avoiding multi-drug resistance (MDR) which are the major obstacles in cancer chemotherapy[56, 204]. As mentioned before the two main groups of targeting moieties are antibodies and small homing molecules such as peptides[118, 129]. In the last decades, many developments have been done in the field of peptide based SMDCs, particularly for cancer therapy[114]. Peptides having high binding affinities to tumor selective overexpressed receptors in cancer cells are useful because of their simple structure, low immunogenicity and easy, cost-effective chemical synthesis compared to ADCs. Nevertheless, the number of receptors in cancer cells is limited, and therefore, the elevation of the drug conjugate concentration alone might not enhance the anti-tumor effect.

To overcome this drawback, the search of new tumor homing peptides which could provide increased biological activity is a hot topic in targeted cancer therapy[205]. One of the approach often used to explore new peptide ligands is a technique called the phage display which represents a powerful tool to identify tumor specific peptides which can be used efficiently as DDS in anti-cancer drug targeting[206, 207]. Wide range cancer targeting peptides were selected by phage display panning whole cells, by *ex vivo* and *in vivo* panning on various cancers such as BC, CRC and leukemia[208–210]. The most famous example was identification of the tripeptide motif RGD which was able to recognize specific integrins present at the surface of cancer cells[211].

Several peptide drugs, developed using phage display, have been approved for use in the clinic or are in clinical trials[206]. The most promising in oncology field is Trebananib (AMG386) discovered in 2004, angiopoietin antagonist peptide that selectively inhibits the interaction of Ang-1 and Ang-2 with Tie2, which in preclinical experiments inhibited tumor endothelial cell proliferation and tumor growth[212]. Wide range of clinical trials with combinations of another therapeutics in BC[213] and CRC[214] did not lead to approvement of this therapeutic for clinical use.

CRC targeting peptides have been found using *in vitro* phage panning on CRC cells were CPIEDRPMC on HT29 cells[215], HEWSYLAPYPWF on WiDr cells[216], DWSSWVYRDPQT on COLO320HSR cells[217], CQARGDLGKIRC on T84[218],

CPKSNNGVC on RKO cells[219], by *ex vivo* panning on human colonic tissue were selected SPTKSNS[220] and VRPMPLQ[221], while QPIHPNNM[222] and CTPSPFSHC[223] peptides were identified by *in vivo* phage panning.

Zhang and his co-workers selected HT-29 human CRC specific heptapeptides by phage display technology in the *in vitro* panning experiment[224]. After three rounds of panning using colon cancer cell lines and two rounds of subtractive screening, the heptapeptide VHLGYAT was found as the most selective peptide to HT-29 CRC cell line, while it is not able to recognize it in normal intestinal epithelial cells. It has been demonstrated by the results of specific binding assays, that the targeting of this heptapeptide may have the potential for the diagnosis and anti-cancer therapy of CRC, although the receptor recognized by this peptide was not identified[224].

In the study of Malhotra and colleagues from 2013, this CRC targeting heptapeptide was conjugated to the nanoparticle carrying small interfering RNA (siRNA) which target Ser/threonine-protein kinase PLK1, a key regulator of mitosis in mammalian cells[225]. Silencing of the targeted PLK1 mRNA and protein in human colon tumor xenograft model with the heptapeptide coupled nanoparticles was approximately 50% of that in the control siRNA group. Although silencing differences between the targeted and untargeted nanoparticles groups were not compared, accumulation in the colon tumor was modestly greater for about 20% with targeted nanoparticles compared to untargeted nanoparticles.

Encouraged with these results, Mező and co-workers developed conjugates with heptapeptide targeting moiety that can be used for targeted therapy in CRC. For this purpose, the VHLGYAT heptapeptide, selected for HT-29 cells[224], was chosen as homing device which was coupled with Dau as cytotoxic agent leading to conjugate **3** (**Figure 13**). Ala scan and positional scan procedures were used to find more active conjugate **4** (**Figure 13**), with modified parent sequence in position 4, where Gly is exchanged for Phe (G/F). Stability of the conjugates under circumstances that will be used for *in vitro* and *in vivo* experiments, and in lysosomal homogenate revealed that the most potent and stable strategy would be to couple Dau for heptapeptide targeting moiety *via* oxime bond using LRRY spacer. In my doctoral study the anti-tumor activity of conjugate **3** with the parent homing peptide, and conjugate **4** (G/F) was investigated on 22 cancer cell lines of different origin and one normal *in vitro*, and *in vivo* on HT-29 human CRC bearing mice.

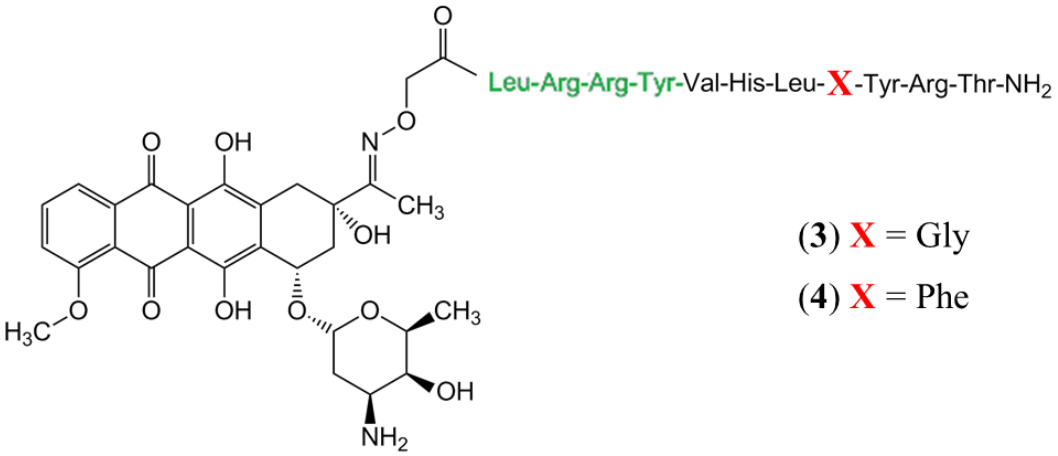

Figure 13. Structure of heptapeptide-Dau conjugates **3** and **4**.

1.4.3 Cell penetrating peptides

For survival and normal physiological function, cells are requiring biological membrane which works as natural permeable barrier[226]. However, presence of biological membrane could be a major obstacle for the efficient intracellular delivery of therapeutic agents. Therefore, the drug have to be or highly lipophilic or very small in order to transport through biomembranes, which is often not a case[227].

Hence, in recent decades, studies have been focused on the development of alternative DDSs such as liposomes and nanoparticles that enhance cell internalization[228]. More attention has been taken by using peptides as novel carriers for intracellular cargo delivery introducing the cell penetrating peptides (CPPs), mainly positively charged short peptide sequence with 5–30 amino acids and with low cytotoxicity that can penetrate through the cell membrane, and deliver a wide range of cargos into cells without destroying membrane integrity[229–233].

Classification of CPPs can be based on the origin of peptides which could be from the natural source protein such as transactivating transcriptional activator (TAT) and Penetratin which were first CPPs derived from a transactivating regulatory protein in human immunodeficiency virus (HIV)[234], and Drosophila antennapedia homeodomain[235], respectively. Among others natural CPPs very famous are VP22 from virus Herpes simplex, and pVEC a peptide of 18 amino acids derived from the cadherin of murine vascular endothelium[236]. Group of chimeric CPPs are composed of two or more motifs from dissimilar peptides such as Transportan[236]. Studies of the structure modifications in the frame of the function revealed that the amino acid arginine plays a fundamental role

in the uptake, and due to this various synthetic CPPs have been also developed, whereby oligopeptides R8/9 consisting in poly-arginine sequences display optimal length for efficient internalization. Based on these observations octaarginine was used frequently to transport different molecular cargos into cells[237–239].

Based on the physicochemical properties CPPs are categorized into three classes[240]: Cationic CPPs such as poly-arginine with positively charged residues. Amphipathic CPPs such as Transportan, have both hydrophilic and lipophilic sequences because of Lys residues in their structures. CPPs like Pept 1/2 contain only hydrophobic motif with non-polar sequences and belong to hydrophobic group[241].

There are three internalization pathways of CPPs into the cell which depend of the peptide sequence, the type of cargo molecule, and lipid components of the cell membrane[242, 243]. The first one is an energy-independent pathway *via* direct penetration, which involves destabilization and disruption of the cellular membrane accompanied by folding of the CPP and its translocation into the cell. Contrary to the first one, endocytosis pathway, including cellular digestion and receptor-mediated endocytosis, is energy-dependent approach of CPP transduction[244]. In the third uptake pathway, the electrostatic interaction of CPP positive residues, especially arginine, and the negative phospholipid layers within cellular membrane could cause the disruption of the lipid bilayer, followed with the formation of inverted transitory structure called micelle, which is responsible for the translocation of the CPP[241].

The favorable ability of CPPs to transport across cellular membrane, making them as a promising candidate for intracellular delivery without cellular injury[242, 245]. Thus, CPPs are employed as an appropriate carriers for delivery of various cargos including nucleic acid, proteins, plasmid, nanoparticles, siRNA and therapeutic agents, increasing their uptake in tumor cells as well as improving the treatment efficacy[231, 246, 247].

The success of CPPs comes not only from their high transduction efficiency, but also from their versatility. Although they are simple to synthesize, to modify, and to improve, up to date there are still no FDA approved CPP-conjugated drugs, while several clinical trials have been discontinued[248]. A major challenge for the clinical trials of CPPs are their weak *in vivo* stability, due to proteolytic degradation, presence of immunogenicity and lack of cell specificity[249].

In order to overcome these disadvantages[250] and develop highly efficient CPPs for anti-tumor treatment, wide range of methods and strategies have been employed to optimize them[251]. In the fact that conventional cancer chemotherapies lack of satisfactory specificity towards tumor cells CPPs or another targeting moieties are used to be conjugated to chemotherapeutic agents in order to enhance their treatment efficacy[252].

Tumor-homing CPPs is an approach combining CPPs with homing peptides targeting certain receptors overexpressed on the surface of cancer cells[253]. Zhou *et al*. found a novel CPP called MT23 with B16 mouse melanoma cell specificity, which can significantly inhibit tumor growth and induce the cell apoptosis in B16 tumor-bearing mice[254]. Combination of the power of a CPP with an integrin-targeting unit provided enhanced cellular uptake and, as well as cytotoxicity in αvβ3 integrin receptor expressing cells[255]. Moreover, CPP-antibody conjugates where the antibody specifically binds to antigens overexpressed in the cancer cells, followed by releasing of CPP which internalizes into tumor cells were validated in CRC *in vivo* model[256].

Activatable CPPs are systems in which the CPP's cell-penetrating function is masked with cleavable spacer, which proteolysis occurs once the CPP is in the tumor tissue under different acidity of microenvironment compared to the normal tissue. Various acid-labile spacers were used to enhance the cell-penetrating ability of the CPP, when after reaching tumors deliver the drug and exhibit significant tumor growth inhibition *in vivo*[257]. Passive targeting by so called „smart" DDSs with intracellular specificity to a phenomenon common to the majority of cancer cells such as hypoxia or presence of glutathione compared to normal cells, demonstrated an enhance anti-tumor effect of the conjugate towards cancer cells compared to the drug alone, with a limited toxicity[258, 259].

In the past few years, a plenty of studies suggested that linking chemotherapeutic agents to CPPs could be therapeutically valuable, increasing their efficiency by promoting intracellular delivery, while reducing their undesired side-effects[260, 261]. For instance, oligoarginines as CPPs can transport covalently attached compounds into different kind of cells and enhance the efficiency of those compounds as showed in Szabo *et al*. study where Mtx covalently attached to the N-terminal of CPPs (R8) *via* peptide bond more efficiently enter BC cells, especially Mtx-resistant cells[262]. Moreover, polyarginine CPPs was used previously also to deliver anti-tumor drugs such as Dau, Vinblastine[263, 264].

As mentioned before Vinca alkaloids were first isolated from the leaves of *Catharanthus roseus*. The major alkaloid in this plant is Vindoline[265]. Some of the minor alkaloids of this plant, such as Vincristine and Vinblastine, have been widely used as anti-tumor drugs for a long time. These bisindole alkaloids, which contain a rearranged Catharanthine and Vindoline moiety, disturb the dynamics of microtubular system causing microtubule instability[266]. Vindoline, the most complex part of bisindole alkaloids, is considered as their bio- and synthetic- precursor. Unfortunately, early studies showed that Vindoline does not exhibit any anti-tumor activity, but it might be functioning as the anchor in the binding of bisindole alkaloids to tubulin[267]. Because small structural changes may cause drastic differences in the anti-tumor efficacies, potencies and side-effects, there is large effort to modify the structure of Vinca-alkaloids to obtain efficient drug candidates.

One of the first such attempts was the synthesis of 17-desacetylvinblastine derivatives by the replacement of the methyl ester group (OMe), at position C-16 in the Vindoline part, with an amide group (Vindesine)[268] and then to modifying this group by incorporating different alkyl groups or amino acids[269]. Among the amino acid derivatives, the presence of hydrophobic amino acid such as Leu, Ile, Val, or Trp exhibited pronounced activity. The Trp-OEt (ethyl ester) derivative was found to be very active on P388 and L1210 leukemia cells implanted intravenously in DBA/2 mice[270]. For further improvement, by modification of the Vindoline ring system a new set of compounds were prepared[271] and attached to *L*- or *D*-Trp-OMe isomers[272]. These conjugates showed higher *in vitro* cytostatic activity on HL-60 human leukemia cells, while the unmodified Vindoline was inefficient even at 100 μM concentration. However, the *L*- and *D*-Trp containing Vindoline derivatives showed lower activity as compared with Vinblastine.

Previously, Bánóczi and colleagues developed and studied the *L*-Trp modified derivatives of 17-desacetylvinblastine with essentially the same activity on human leukemia cells (HL-60) as that of Vinblastine, a drug used in cancer treatment[264]. It was shown that conjugation with octaarginine preserved the activity of the free isomers on both sensitive (HL-60) and drug-resistant leukemia cells (HL-60-MRP1 and HL-60-MDR1). Based on the biological data obtained with Trp derivatives of Vinblastine[264], and the fact that conjugated Vindoline showed higher *in vitro* cytostatic activity[272], have been developed Br-Vindoline *L*- and *D*-Trp isomer methyl ester derivatives and conjugates

with oligoarginine as CPP in order to induce anti-tumor effect of Vindoline (**Figure 14**). The conjugates were prepared by solution phase conjugation and they had higher *in vitro* cytostatic effect than that of free Vindoline derivatives. In addition, the *in vivo* anti-tumor effect of conjugates was studied in two mouse tumor models of P388 mouse leukemia and C26 murine colon. The results show that the modification of Vindoline and its conjugation with CPP may result in an efficient conjugate.

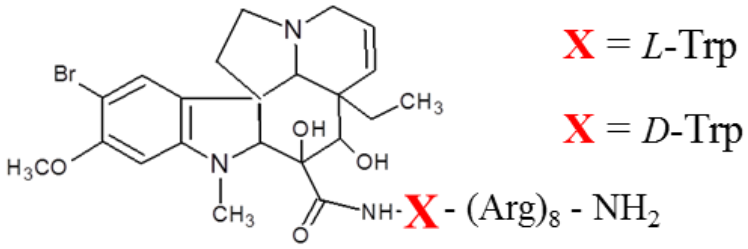

Figure 14. Structure of Br-Vind-*L*/*D*-Trp-Arg_8 cell penetrating peptide conjugates **5** and **6**.

DOI:10.14753/SE.2021.2555

2 OBJECTIVES

2.1 GnRH-III-Dau conjugates

Due to the promising findings that newly synthesized conjugate **2** (GnRH-III-[^{2}ΔHis,^{3}*D*-Tic,^{4}Lys(Bu),^{8}Lys(Dau=Aoa)]) is highly stable in human plasma, while the high binding affinity to GnRH-Rs on cancer cells and the release of the active drug metabolite in lysosomes are not compromised, accelerating delivery of the drug to its site of action, the aim of the present study was to gain information about the anti-tumor potential of this GnRH-III based DDS for targeted tumor therapy. Therefore, in the research for my doctoral thesis detailed *in vitro* and *in vivo* studies of the inhibitory effect on the cancer growth have been performed in direct comparison to the previous lead compound **1** (GnRH-III-[^{4}Lys(Bu),^{8}Lys(Dau=Aoa)]):

- 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.

2.2 Homing heptapeptide-Dau conjugates

Homing heptapeptide (VHLGYAT), selected that specifically binds for HT-29 CRC cells, was chosen as targeting moiety for which was coupled Dau as cytotoxic agent, *via* oxime bond using LRRY spacer, leading to conjugate **3** (Dau=Aoa-LRRY-VHLGYAT-NH_2) with parent homing peptide. Ala scan and positional scan procedures used to find more active conjugate revealed that conjugate **4** (Dau=Aoa-LRRY-VHLFYAT-NH_2), with modified parent sequence in position 4, where Gly is exchanged for Phe (G/F) is the most stable under circumstances that will be used for *in vitro* and *in vivo* experiments, and in lysosomal homogenate. In my doctoral thesis study it was investigated:

- 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.

2.3 Vindoline CPP conjugates

Based on the previous biological findings where it was found that Trp derivatives of Vinblastine have anti-tumor activity, and the fact that conjugated Vindoline showed higher *in vitro* anti-tumor effect compared to free one, in the study for my doctoral thesis newly developed Br-Vindoline *L*- and *D*-Trp isomer methyl ester derivatives as well as Br-Vind-*L*-Trp-Arg_8 (**5**) and Br-Vind-*D*-Trp-Arg_8 (**6**) conjugates with oligoarginine as CPP in order to induce anti-tumor effect of the Vindoline, were investigated:

- 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.

DOI:10.14753/SE.2021.2555

3 MATERIALS AND METHODS

3.1 Compounds for testing

3.1.1 GnRH-III-Dau peptide drug conjugates and Dau

The GnRH-III-Dau conjugates **1** and **2** were prepared with a combination of solid phase peptide synthesis of the peptide carrier and oxime bond formation in solution by Dr Sabine Schuster (Prof Dr Gábor Mező, Department of Organic Chemistry, MTA-ELTE Research Group of Peptide Chemistry, Eötvös Loránd University, Faculty of Science, Budapest, Hungary). The compounds were purified by reversed phase high-pressure liquid chromatography (RP-HPLC) using linear gradient elution with eluent A (0.1% trifluoroacetic acid (TFA)/water) and eluent B (0.1% TFA/acetonitrile (ACN)-water 80:20, *v*/*v*). The freeze-dried peptide drug conjugates were used, without changing the TFA counter ions, for the *in vitro* and *in vivo* studies in order to evaluate their tumor growth inhibitory effect. The free Dau (as HCl salt) and synthesized GnRH-III-Dau conjugates were dissolved in sterile water (Pharmamagist Kft., Budapest, Hungary) and used for the *in vitro* and *in vivo* studies.

3.1.2 Homing Heptapeptide-Dau conjugates that target colon cancer

Heptapeptide-Dau conjugates **3** and **4** were prepared by solid phase peptide synthesis using the standard Fmoc/tBu procedure on Rink-Amide MBHA resin by Krisztina Kiss (Prof Dr Gábor Mező, Department of Organic Chemistry, MTA-ELTE Research Group of Peptide Chemistry, Eötvös Loránd University, Faculty of Science, Budapest, Hungary). Dau was conjugated to the N-terminus of the peptide directly or through Cathepsin B cleavable spacers. The resulting conjugates were analyzed by analytical RP-HPLC and electrospray ionization mass spectroscopy (ESI-MS).

3.1.3 Vindoline derivatives, Vindoline and Vinblastine

The synthesis of the methyl esters derivatives Br-Vindoline-*L*-Trp-OMe and Br-Vindoline-*D*-Trp-OMe was described earlier, and it was performed by Dr Zoltán Bánóczi (Prof Dr Ferenc Hudecz, Department of Organic Chemistry, MTA-ELTE Research Group of Peptide Chemistry, Eötvös Loránd University, Faculty of Science, and Budapest, Hungary). Purification was done by RP-HPLC.

3.1.4 Vindoline-octaarginine conjugates

Vindoline-octaarginine conjugates Br-Vind-*L*-Trp-Arg_8 and Br-Vind-*D*-Trp-Arg_8 were synthesized on Rink Amide MBHA resin using standard Fmoc/tBu strategy as described before, by Dr Zoltán Bánóczi (Prof Dr Ferenc Hudecz, Department of Organic Chemistry, MTA-ELTE Research Group of Peptide Chemistry, Eötvös Loránd University, Faculty of Science, Budapest, Hungary). After cleavage from the resin, the crude product was purified by RP-HPLC and was allowed to react with Vindoline derivatives as reported earlier.

3.2 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 Medium with glutamine (Roswell Park Memorial Institute Medium, Lonza, Basel, Switzerland). Moreover, U87MG (human malignant glioma) and MRC-5 (human fibroblast) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Lonza), while PE/CA-PJ15 and PE/CA-PJ41 (human head and neck cancer) were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Sigma Aldrich, St. Louis, MO, USA). All mediums were supplemented with 10% heat-inactivated FBS (Fetal Bovine Serum; Euroclone, Pero, Milan, Italy) and 1% Penicillin/Streptomycin (Sigma Aldrich), while OVCAR-3 cells were cultured in 20% FBS containing RPMI medium. The cell lines were mainly obtained from the American Type Culture Collection (ATCC), except for A2780, PE/CA-PJ15 and PE/CA-PJ41 which were obtained from the European Collection of Authenticated Cell Cultures (ECACC), A2058 kindly provided by L.A. Liotta (NCI, Bethesda, MD, USA), HT168-M1/9 derived from A2058, M24 kind gift from B.M. Mueller (Scripps Research Institute, La Jolla, CA, USA) and WM983b kindly provided by Meenhard Herlyn (Wistar Institute, Philadelphia, PA, USA). Cells were cultured in sterile T25 or T75 flasks with ventilation cap (Sarstedt, Nümbrecht, Germany) at 37 °C in a humidified atmosphere with 5% CO_2.

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

For the evaluation of the *in vitro* anti-proliferative activity of the conjugates and free drugs, the cell viability was determined by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) that was obtained from Sigma Aldrich. MTT assay is based on mitochondrial enzymes dependent reduction of MTT to the colored formazan crystals. After standard harvesting of the cells by trypsin-EDTA (Lonza), 3×10^3 – 10×10^3 cells per well depending on cell line (2×10^4 cells per well for P388), 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** (32 nM – 100 µM) or free Dau (0.1 nM – 10 µM), dissolved in serum free medium and incubated for 24 h under standard conditions. The control wells were treated with serum free medium. 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 conjugates and free Vindoline at a 0.1, 1, 5, 10, 25, 50, 100 µM concentrations 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. Plates with not attaching P388 cells were centrifuged on plate rotor of the centrifuge (Labofuge 400R, Heraeus, Hanau, Germany) at 300 g for 3 min. to spin cells on the bottom, before each washing step.

Afterward, the MTT assay was performed, in order to determine cell viability, by adding 20 µL of MTT solution (5 mg/mL in PBS, 0.5 mg/mL final) to each well and after 2 h of incubation at 37 °C, the supernatant was removed. Plates containing P388 cells were centrifuged on the plate rotor of the centrifuge at 900 g for 5 min. to spin crystals on the bottom. The precipitated purple formazan crystals were dissolved in 100 µL of a 1:1 solution of dimethylsulfoxide (DMSO; Sigma Aldrich) – 96% Ethanol (Molar Chemicals Kft., Halásztelek, Hungary) and the absorbance was measured after 15 min. at λ = 570 nm by using Bio-Rad microplate reader model 550 (Hercules, CA, USA). Average background absorbance (DMSO-Ethanol) was subtracted from absorbance values of control and treated wells, and cell viability was determined relative to untreated (control) wells where cell viability was arbitrarily set to 100%. Absorbance values of

treated samples were normalized versus untreated control samples and interpolated by nonlinear regression analysis with GraphPad Prism 6 software (GraphPad, La Jolla, San Diego, CA, USA) to generate sigmoidal dose-response curves from which the 50% inhibitory concentration (IC_{50}) values of the conjugates and free drugs were calculated, and presented as micromolar (μM) units. The experiments were done in triplicate and each experiment was repeated twice.

3.4 RT-qPCR for GnRH-R mRNA Level of Expression

The GnRH-R mRNA expression of chosen cell lines was quantified *via* reverse transcription quantitative real time PCR (RT-qPCR). The total RNA was isolated while using Trizol® reagent (Ambion, by Life Technologies, Carlsbad, CA, USA), followed by chloroform (Carlo Erba Reagents S.A.S., Vel-de-Reuil, France) extraction and isopropanol (Carlo Erba Reagents S.A.S.) precipitation. The purity and concentration of RNA were determined by measuring the absorbance at 260 nm and 280 nm (NanoDrop ND-1000, Wilmington, DE, USA). For cDNA synthesis a GeneAmp PCR System 9700 thermo cycler (Applied Biosystems, Waltham, MA, USA) was used. 250 ng total RNA from cell lines and human colon RNA sample (Ambion) were reverse transcribed while using Moloney murine leukemia virus reverse transcriptase (MMLV), oligo (dT)18 primer (Takara, Mountain View, CA, USA), reaction mix, dNTPs mix and recombinant RNase inhibitor (Clontech, San Francisco, CA, USA). RNA-solutions were pre-incubated with oligo-primers for 2 min. at 70 °C. After cooling down and addition of dNTPs and reverse transcriptase, the incubation was continued for 1 h at 42 °C. To inactivate the reaction, the mixture was heated to 95 °C for 5 min. After reverse transcription, the cDNA samples were stored at −20 °C until further processing. Human total cDNA obtained from Clontech was used as control. In order to amplify the cDNA, qPCR was run on the StepOnePlus™ real-time PCR system (Applied Biosystems) in a final volume of 10 μL, containing 1.5 μL cDNA template equivalent to 3.75 ng of total RNA, 0.5 μL appropriate primer assay, 3 μL of water, and 5 μL 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 (IDT), Skokie, IL, USA); human β-actin gene with RefSeq NM_001101 (Qiagen, Hilden, Germany); and, human GAPDH gene with RefSeq NM_002046(1) (Qiagen, Hilden, Germany). No template control (containing only water) was used as

negative control. The cycling parameters were 40 cycles of 95 °C (10 sec) and 60 °C (30 sec). Each experiment was done in duplicates. Quantification was carried out using the comparative threshold cycle (Ct) method. An arbitrary threshold was chosen on the basis of the variability of the baseline. Ct values were calculated by determining the point at which the fluorescence exceeded the threshold limit. Ct was reported as the cycle number at this point. After cycling, the relative quantification (RQ) 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 (presented as RQ value = 1 on the graph), MRC-5 cells (normal human lung fibroblasts) was chosen. Data are representative of two independent experiments.

3.5 Quantitative Western Blot Studies

The cells were lysed in lysis buffer (cOmplete™ Lysis-M; Roche, Mannheim, Germany) in volume 3.5-fold higher than pellet of cells. The protein concentration was determined by Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) using 2 mg/ml bovine serum albumin (BSA, Sigma Aldrich) as protein standard and an EnSight™ multimode plate reader (Perkin Elmer, Waltham, MA, USA). Samples were denatured in Novex™ LDS sample buffer and NuPage™ sample reducing agent (Invitrogen, Carlsbad, CA, USA) at 70 °C for 2 min. in Eppendorf® Thermo-mixer Compact (Hamburg, Germany). 30 µL protein sample (30 µg) 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 washed in 0.1 % Tween-PBS (Sigma Aldrich), blocked for 1 h at room temperature (r.t.) with 5% nonfat dry milk (Bio-Rad), and incubated overnight at 4 °C with GnRH-R antibody (Proteintech, Rosemant, IL, USA, rabbit Polyclonal 19950-1-AP; 1:500 dilution). The secondary antibody (Cell Signaling Technology, Danvers, MA, USA, goat Anti-Rabbit-horseradish peroxidase (HRP) conjugated, 7074; 1:1000) was incubated 1 h at r.t. Amersham™ ECL™ prime western blotting detection reagent (GE Healthcare, Buckingamshire, UK) 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; 1:1000). 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. Data are representative of two independent experiments.

3.6 GnRH-R cell surface expression level determination by flow cytometry

Cells were harvested and one million cells from each cell line were used in the experiments. The cells were fixed with 4% Paraformaldehyde (PFA) for 10 min. at r.t., washed with phosphate buffered saline (PBS) and exposed to 3% BSA in PBS for 20 min. at r.t. Afterwards, GnRH-R antibody (Proteintech, rabbit polyclonal, 19950-1-AP) was used in a concentration of 0.2 µg/million cells (1:135 dilution), 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, 1:1000) 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, Franklin Lakes, NJ, USA). 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 cells. The results of two experiments are presented.

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

The cells were seeded to 24-well plates in a cell density of 1.5×10^5 cells per well, 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. Data are representative of two independent experiments.

3.8 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. The mice were kept in a sterile environment in Makrolon® cages at 22–24 °C (40–50% humidity), with a lighting regulation of 12/12 h light/dark. The animals had free access to tap water and were fed with a sterilized standard diet (SDS VRF1, autoclavable, Akronom Kft., Budapest, Hungary) ad libitum.

First generation of hybrid BDF1 (a cross between C57BL/6 female and DBA/2 male) adult female mice, from SPF animal colonies from Department of Experimental Pharmacology (National Institute of Oncology, Budapest, Hungary) were used in subcutaneous P388 murine leukemia tumor model experiment, and kept as described above.

The adult female severe combined immunodeficiency (SCID) mice on a C.B.-17 background were bred in specific opportunistic and pathogen free isolator breeding rooms. The breeding isolator was supplied with corn-cob bedding and standard VRF1 rodent chow and with acidified (pH 3) sterilized distilled water. The mice from the breeding rooms were used for the orthotopic model of human BC and human CRC. They were held in filter-top boxes in the experimental barrier rooms and every box-opening was performed under a Class 100 laminar-flow hood by an operator that was dressed in sterilized surgical attire. The cage components, corn-cob bedding and food (VRF1 from Special Diet Services) were steam-sterilized in an autoclave (121 °C, 20 min.). 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.

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

In order to determine the toxicity of the conjugates, *in vivo* acute and chronic toxicity studies were performed on healthy animals. In acute toxicity study, adult BALB/c female mice (20–23 g) were treated by a single intraperitoneal (i.p.) injection of conjugate **2**, whereby different doses (3.125, 6.25, 12.5, 25 and 50 mg/kg Dau content) were administrated to each groups (three mice per group). In chronic toxicity studies, adult female BALB/c mice (23–26 g) were treated with both GnRH-III-Dau conjugates by i.p. administration with a dose of 15 mg/kg Dau content on days 1, 3, 6, 8 and 10, or with free Dau (1 mg/kg) on days 1 and 8. In case of the control group, sterile water for injection as solvent was administered. Each group consisted of three mice. The toxicity was evaluated on the basis of life span, behavior and appearance of the mice, as well as the body weight. Parameters were followed for 14 days.

3.10 Mouse model of orthotopic 4T1 murine breast carcinoma, doses of treatments and measurements

Adult BALB/c female mice (20–25 g) were used in this experiment and kept under the same conditions, as described above. 4T1 murine BC cells, maintained as described above, were orthotopically injected into the lower quarter of the right mammary fat pad line of mice, whereby 1×10^6 cells were used per animal, suspended in 50 µL M199 medium (Sigma Aldrich). The treatment started seven days after cells inoculation, when the average tumor volume was 38 mm^3, by i.p. administration of the conjugates or free Dau. Four groups with seven animals per group were established and treated with certain doses and schedules. The mice in the control group were treated with sterile water for injection, while mice in free Dau group were treated with a dose of 1 mg/kg, once per week, on days 7, 14 and 21 after cells inoculation. The groups of GnRH-III-Dau conjugates **1** and **2** were treated on days 7, 10, 14, 17, 21 and 24 with a dose of 10 mg/kg Dau content. Animal weight and tumor volumes were measured initially when the treatment started and at periodic intervals according to the treatment schedule. A digital caliper was used to measure the length (a) and the width (b) of a given tumor, and the tumor volume was calculated while using the formula $V = ab^2 \times \pi/6$. The termination of the experiment was initiated 28 days after cell inoculation, respectively 22 days after treatment start, since the average volume of the tumors in the control group reached 1800 mm^3. The mice from all groups were sacrificed by cervical dislocation. 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.

3.11 Mouse model of orthotopic MDA-MB-231 human breast carcinoma, doses of treatments and measurements

Adult SCID female mice (22–28 g) were used in this experiment and kept under the same conditions, as described above. MDA-MB-231 human BC cells were injected orthotopically into the lower quarter of the right mammary fat pad line of mice, whereby 1.5×10^6 cells were used per animal, suspended in 50 µL of M199 medium. The treatments started 21 days after cells inoculation, when average tumor volume was 40 mm^3. Free Dau and conjugates were administrated by i.p. injection. Four groups with seven animals per group were established and treated with certain doses and schedules. The mice in the control group were treated with sterile water for injection, while the mice in the free Dau group were treated with a dose of 1 mg/kg, once per week, on days 21, 28, 35 and 42 after cell inoculation. The groups treated with the GnRH-III-Dau conjugates **1** and **2** were treated on days 21, 24, 28, 31, 35 and 38 with a dose of 15 mg/kg Dau content. The last treatment was performed on day 42 after cell inoculation, whereby a dose of 7.5 mg/kg Dau content was applied. The animal weights and tumor volumes were measured when the treatment was initiated and at periodic intervals according to the treatment schedule. The tumor volume was calculated in the same manner as described for murine BC. The experiment was terminated 45 days after cell inoculation (25 days after treatment initiation), due to bad conditions of animals in the control group. The mice from all groups were sacrificed by cervical dislocation and primary tumors and livers were harvested and weighed, while number of animals with metastases were counted. 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.

3.12 Mouse model of orthotopic HT-29 human colon cancer, doses of treatments and measurements

3.12.1 Development of the primary tumor for transplantation

Immunodeficient, 6–8 weeks old SCID female mice (21–27 g) were used in this experiment. HT-29 colon carcinoma cells were subcutaneously injected into one side of the intrascapular region, whereby 2×10^6 cells were used per animal, suspended in 200 µL of M199 medium, in order to establish the xenografts with primary tumor. After two weeks, the mice with palpable tumors were sacrificed by cervical dislocation, disinfected with iodine and the subcutaneous tumor was aseptically dissected out. Tumor pieces of 2 mm^3 were transplanted orthotopically under aseptic conditions into anesthetized (narcotic mixture: tiletamine (Virbac, Carros, France; 20.3 mg/kg), zolazepam (Virbac; 20.3 mg/kg), xylazine (Produlab Pharma B.V., SJ Raamsdonksveer, The Netherlands; 12.4 mg/kg) and butorphanol (Alvetra u. WERFFT GmbH, Vienna, Austria; 2.95 mg/kg) in saline solution; given i.p. 0.1 ml/10 g of animal body weight) SCID female mice.

3.12.2 Tumor transplantation

The abdomen of mice was disinfected with iodine and alcohol, a small midline incision (0.5 cm) was then made and the colorectal part of the intestine was exteriorized. Serosa of the site where the tumor pieces should be implanted were removed. Tumor tissue fragments of HT-29 human colon tumor were implanted on the top of the animal intestine, whereby an 8/0 surgical (polypropylene) suture was used to suture it on the wall of the intestine. The intestine was returned to the abdominal cavity and the abdominal wall was closed with 4/0 surgical (polyglycolic acid) sutures. The wound was disinfected with iodine and alcohol again and the animals were kept in a sterile environment. On the next day, no sign of pain and/or stress of the mice could be observed.

3.12.3 Doses and treatments

3.12.3.1 GnRH-III-Dau Conjugates and free Dau

The treatments started seven days after tumor transplantation by i.p. administration of the free Dau and conjugates which were dissolved in sterile water for injection. Six mice per group were used. One group of mice was treated with free Dau at a dose of 1 mg/kg body weight on days 7, 13 and 20 after tumor transplantation. The groups of GnRH-III-

Dau compounds **1** and **2** were treated with a dose of 10 mg/kg Dau content on days 7, 10, 13, 16, 20, 23 and 27 after tumor transplantation. The control group was treated with sterile water for injection. The experiment was terminated on day 30 after tumor transplantation (day 24 of treatment). The Dau group was terminated already on day 23 after tumor transplantation (day 17 of treatment), since the animals revealed a significant loss of weight. The mice from all groups were sacrificed by cervical dislocation. Their tumors and livers were harvested and weighed for determination of compounds anti-tumor as well as toxic activity.

3.12.3.2 Homing Heptapeptide-Dau conjugates and free Dau

The treatments started 13 days after tumor transplantation by i.p. administration of the compounds dissolved in sterile water for injection. For the treatment, 8 mice per group were used. One group of mice were treated with free Dau (1 mg/kg body weight) on days 13 and 20 after tumor transplantation. Groups that received conjugates **3** and **4** were treated with a dose of 10 mg/kg Dau content on days 13, 16, 20, 23 and 27 after tumor transplantation. Control group was treated with solvent. The experiment was terminated on day 30 after tumor transplantation. Dau group was terminated on day 23 after tumor transplantation due to significant weight loss of the animals. The mice from all groups were sacrificed by cervical dislocation. Their tumors and livers were removed and weighed for determination of compounds anti-tumor as well as toxic activity.

3.13 Mouse model of subcutaneous P388 murine leukemia, doses of treatments and measurements

The anti-tumor activity of Vindoline octaarginine conjugates and vinblastine was studied on subcutaneous P388 mouse leukemia model in hybrid BDF1 (a cross between C57BL/6 female and DBA/2 male) adult female mice, weighing 18 to 23 g. P388 tumors were established injecting 2×10^6 cells in 200μL serum free M199 medium per mouse subcutaneously into BDF1 female mice (5-7 animals per group). The animals were treated at day 1 and day 6 after tumor cell inoculation with the tested compounds at doses of 10 mg/kg. Vinblastine as a clinically used drug was used as positive control (1 mg/kg). During the treatment the animal weight was determined three times per week. The tumor volume was calculated in the same manner as described for BC models above. The experiment was terminated on day 25 after cell inoculation.

3.14 Mouse model of subcutaneous C26 murine colon cancer, doses of treatments and measurements

The anti-tumor activity of Vindoline octaarginine conjugates and vinblastine was studied also on subcutaneous C26 mouse colon carcinoma model in BALB/c female mice, weighing 19 to 25 g. Tumor model of C26 was established injecting 2×10^6 cells in 200µL serum free M199 medium per mouse subcutaneously into BALB/c female mice (5-7 animals per group). The animals were treated at days 13, 17, 20, 24 and 29 after tumor cell inoculation at dose of 10 mg/kg and 20 mg/kg body weight, while Vinblastine was used as positive control (1 mg/kg). During the treatment the animal weight was determined three times per week. The tumor volume was calculated in the same manner as described for BC models above. The treatments were terminated on day 31.

3.15 Immunohistochemical staining of KI-67

The routinely formalin-fixed tumors were dehydrated in a graded series of ethanol, infiltrated with xylene and embedded into paraffin at a temperature not exceeding 60 °C. Two micron thick sections were mounted on Superfrost slides (Thermo Shandon, Runcorn, UK) and then manually deparaffinized. To block endogenous peroxidase activity, slides were treated for 20 min. at r.t. with 3% H_2O_2 in methanol. The slides were immersed in 6% citrate buffer (pH = 6) and exposed to 98 °C water bath for 40 min. Afterwards, the slides were primarily treated with monoclonal mouse antibody against human KI-67 (Dako, Glostrup, Denmark, 1:40) 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, Fremont, CA, USA) was used with 3-amino-9-ethylcarbazole (AEC) as chromogen. Staining without the primary antibody served as the negative control.

3.16 Scoring of proliferation index, micro and macro-metastases

Liver, spleen, lung and kidneys were harvested and fixed in formalin. All of the visible macro-metastatic lesions of the peripheral organs of seven animals from the control and treated groups on a stereo microscope Kruss MSZ5600 (Kruss Optronic, Hamburg, Germany) under 7–45-fold magnification were counted. The percentages of macro-metastatic lesions in the treated groups compared to the control group was calculated.

Proliferation marker-stained samples were evaluated on light microscope Olympus BH-2 microscope (Olympus, Tokyo, Japan). The proliferation index in primary tumors and in lung metastases were determined counting the KI-67-positive tumor cells manually per field of view under light microscope (400-fold magnification), whereby five fields of vision per sample were evaluated. The proliferation index was calculated as percentage of KI-67 positive cells from all cells in the field of view.

The number of micro-metastases, which were KI-67 positive-stained in the lung samples sections on microscopic slides were manually counted per field of view under light microscope (100-fold magnification), whereby five fields of vision per sample were evaluated.

3.17 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.

DOI:10.14753/SE.2021.2555

4 RESULTS

4.1 Anti-tumor and anti-metastatic activity of GnRH-III-Dau conjugates

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

The anti-proliferative effect of the GnRH-III-Dau conjugates **1** and **2**, as well as free Dau, was tested on wide range of cancer cell lines from different origin and also on MRC-5 (human fibroblast) as non-cancerous control cell line. The cells were treated with the compounds for 24 h in serum free medium, followed by incubation in fresh serum containing medium for further 48 h. Afterwards, the cell viability was determined, and the IC_{50} values of the conjugates and free drug were calculated. The data showed that both conjugates possess an anti-proliferative effect on all cell types (**Table 3**). Except for the ovarian cancer cell lines A2780 and OVCAR-8, 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. The obtained IC_{50} values of the conjugates vary mostly in the low micromolar range and were one to two order of magnitude higher when compared to free Dau that can enter cells non-specifically by passive diffusion. 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. 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. Besides, the conjugates showed high anti-proliferative activity on murine CRC cell line C26, while the conjugates showed a moderate anti-proliferative activity on HT-29 human colon adenocarcinoma, but the relative potency was in the same range as for the BC cells.

DOI:10.14753/SE.2021.2555

Table 3. Anti-proliferative effect of free drug Dau and GnRH-III-Dau conjugates **1** and **2** on various cell lines.

Tumor Type	**Cell Line**	**IC_{50} [1] 24 h + 48 h**			**Relative Potency [2]**	
		Dau (nM)	**1 (μM)**	**2 (μM)**	**Dau/1**	**Dau/2**
Breast	MDA-MB-231	54.6 ± 7.4	5.8 ± 0.8	1.9 ± 0.2	0.0094	0.0287
Breast	MCF-7	63.9 ± 21.0	16.5 ± 1.2	4.0 ± 0.8	0.0039	0.0160
Murine breast	4T1	56.0 ± 14.7	6.3 ± 0.9	1.8 ± 0.1	0.0089	0.0311
Colon	HT-29	202.9 ±1.0	15.5 ± 1.7	7.3 ± 0.3	0.0131	0.0278
Murine colon	C26	117.5 ± 8.6	10.6 ± 0.2	2.6 ± 0.7	0.0111	0.0452
Prostate	DU145	16.3 ± 4.6	5.3 ± 0.4	2.1 ± 0.2	0.0031	0.0078
Prostate	PC-3	32.7 ± 4.7	6.3 ± 0.3	2.4 ± 0.6	0.0052	0.0136
Glio-blastoma	U87MG	126.4 ± 53.7	9.0 ± 0.8	2.3 ± 0.1	0.0140	0.0550
Ovarian	A2780	10.4 ± 1.6	1.4 ± 1.1	2.1 ± 0.5	0.0074	0.0050
Ovarian	OVCAR-3	404.0 ± 9.4	46.0 ± 1.3	8.2 ± 0.5	0.0088	0.0493
Ovarian	OVCAR-8	185.6 ± 99.8	5.7 ± 0.8	9.5 ± 0.8	0.0326	0.0195
Liver	HepG2	22.9 ± 1.4	6.8 ± 0.3	2.2 ± 0.7	0.0034	0.0104
Melanoma	A2058	35.1 ± 14.9	8.4 ± 0.3	2.6 ± 0.5	0.0042	0.0135
Melanoma	WM983b	49.8 ± 22.9	12.7 ± 1.5	2.6 ± 0.6	0.0039	0.0192
Melanoma	HT168-M1/9	27.5 ± 9.1	13.5 ± 1.1	2.9 ± 0.6	0.0020	0.0095
Melanoma	M24	118.8 ± 25.0	16.2 ± 0.2	3.5 ± 0.6	0.0073	0.0339
Murine melanoma	B16	26.0 ± 8.0	3.2 ± 0.8	1.1 ± 0.2	0.0081	0.0236
Head and neck	PE/CA-PJ41	45.6 ± 33.5	4.7 ± 0.8	1.7 ± 0.5	0.0097	0.0268
Head and neck	PE/CA-PJ15	50.5 ± 38.7	7.4 ± 0.8	2.9 ± 0.6	0.0068	0.0174
Lung	H1975	20.9 ± 2.7	4.1 ± 0.1	2.3 ± 0.7	0.0051	0.0091
Lung	H1650	50.3 ± 13.4	10.5 ± 1.1	4.0 ± 0.8	0.0048	0.0126
Lung	A549	69.3 ± 23.5	9.7 ± 0.6	4.3 ± 0.4	0.0071	0.0161
Pancreas	PANC-1	525.9 ± 24.7	>100	56.4 ± 4.5	0.0053	0.0093
Normal fibroblast	MRC-5	287.6 ± 35.1	41.9 ± 3.8	19.7 ± 1.2	0.0069	0.0146

[1] IC_{50} values (average ± SD). [2] Relative potency = IC_{50} Dau / IC_{50} Conjugate.

4.1.2 mRNA expression level of GnRH-R

Based on the results of the *in vitro* anti-proliferative activity, different cell lines (MDA-MB-231, HT-29, A2780, PANC-1, U87MG and MRC-5) were chosen, where the peptide drug GnRH-III-Dau conjugates **1** and **2** had either a high activity, or the lowest activity, to determine the GnRH-R expression level. The GnRH-R mRNA expression of these cell lines was quantified *via* RT-qPCR. The amount of GnRH-R mRNA 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 (**Figure 15A**). In MDA-MB-231, HT-29 and A2780 cancer cell lines, the level of GnRH-R mRNA expression was 7.3, 4.6 and 3.4 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.

4.1.3 Absolute protein expression level of GnRH-R

The GnRH-R protein level of the same cell lines was quantified by western blot. The protein level of GnRH-R 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 (**Figure 15B**). The highest protein level was obtained for A2780, U87MG and 4T1 cells, with 3.8-, 3.6- and 3.5-fold higher protein levels 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.

4.1.4 Cell surface protein expression level of GnRH-R

The GnRH-R cell surface expression level of these cell lines was quantified *via* flow cytometry. The GnRH-R expression on the cell surface was higher for all cancer cell lines in comparison to MRC-5 cells (**Figure 15C**). The highest level of GnRH-R was obtained for MDA-MB-231 and A2780 cells, with 5.7- and 5.6-fold higher surface expression, followed by U87MG, 4T1, and PANC-1 cell lines with around four-fold higher expression than MRC-5 cells, while HT-29 cancer cell line showed two-fold higher level of GnRH-R expression.

DOI:10.14753/SE.2021.2555

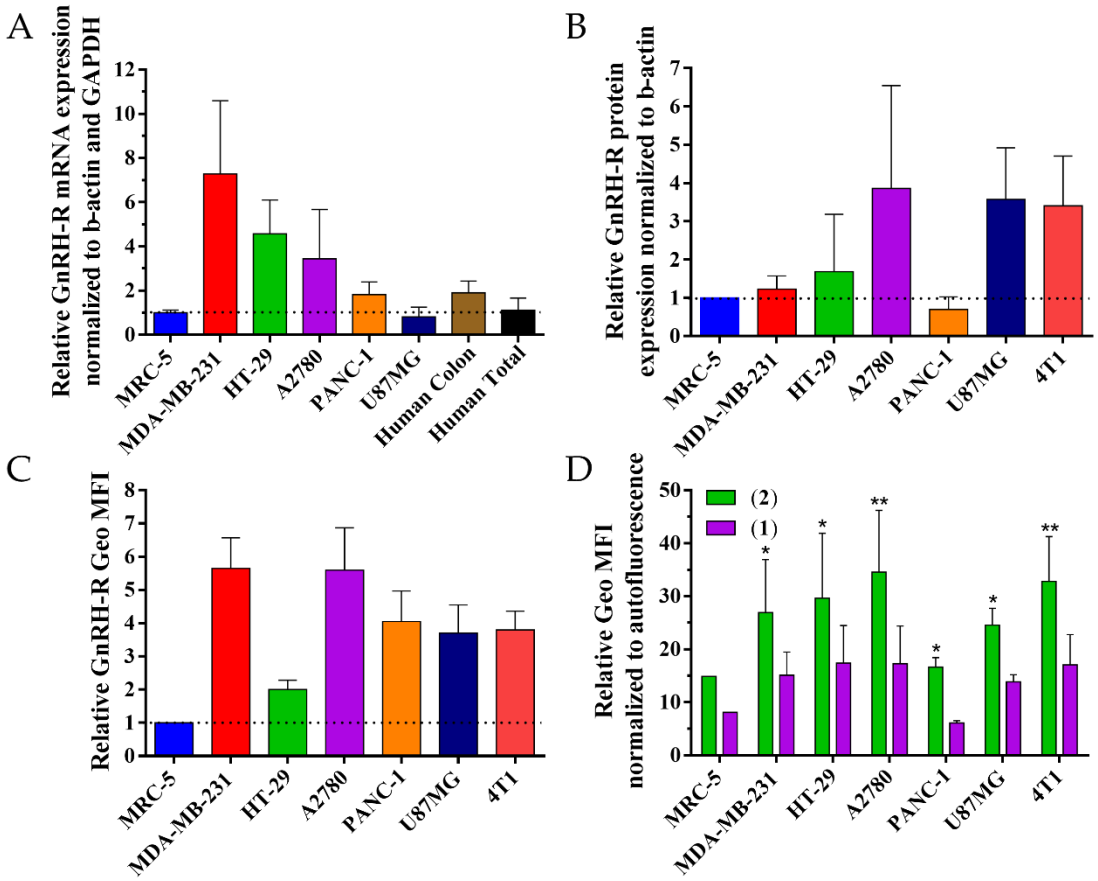

Figure 15. (**A**) GnRH-R mRNA expression level in cancer cell lines compared to MRC-5. (**B**) Absolute protein level of GnRH-R in cancer cell lines compared to MRC-5. (**C**) GnRH-R surface expression level in cancer cell lines compared to MRC-5. (**D**) Cellular uptake of GnRH-III-Dau conjugates **1** and **2** (40 µM), after 6 h treatment of the cell lines. Bar graphs represent average of data from two individual experiments and standard deviation (average ± SD). The dotted line represents relative value of normal cell line as reference sample (presented as 1 on the graph). By geo mean fluorescence intensity (geo MFI) are represented GnRH-R level and cellular uptake of conjugates (C, D). Statistical analysis for uptake studies of conjugates **1** and **2** was performed by two-way ANOVA test. * and ** mean significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

4.1.5 Determination of cellular uptake of GnRH-III-Dau conjugates

The cellular uptake of GnRH-III-Dau conjugates was measured by flow cytometry on the tested cell lines. The obtained results displayed 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 (**Figure 15D**). 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.

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

In vivo acute and chronic toxicity studies were performed on healthy animals in order to determine the toxicity of GnRH-III-Dau conjugates. An acute toxicity experiment with single injection of the treatments was performed for 14 days, whereby no significant change in body weight could be observed (**Figure 16A**) and also the general look and behavior of experimental animals was adequate, even with a dose of 50 mg/kg Dau content of conjugate **2**. Chronic toxicity experiments were also performed for 14 days and the animals were treated with both conjugates five times with a dose of 15 mg/kg Dau content and two times with free Dau (1 mg/kg). Also, in this experiment, it could not be observed a significant change in body weight (**Figure 16B**), general look and behavior of the mice.

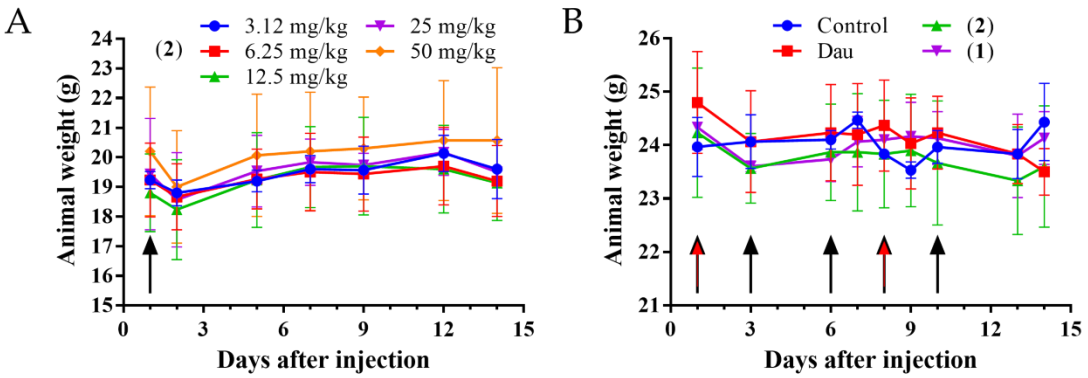

Figure 16. *In Vivo* toxicity study of conjugates and Dau on healthy BALB/c female mice. (**A**) Acute toxicity study of GnRH-III-Dau conjugate **2** with doses of 3.125, 6.25, 12.5, 25 and 50 mg/kg Dau content. (**B**) Chronic toxicity study of GnRH-III-Dau conjugates **1** and **2** with dose of 15 mg/kg Dau content, 5 treatments, black arrows; and free Dau 1 mg/kg, 2 treatments, red arrows. 3 mice per group. Animal body weight (grams, average ± SD).

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

The effect of GnRH-III-Dau conjugates **1** and **2**, as well as free Dau was evaluated on animal body weight in orthotopic 4T1 murine BC bearing mice. The body weight in orthotopic 4T1 murine BC bearing mice was partly changed during the treatment time. The animal body weights in the control and free Dau treated groups were slightly decreased for 3.3% and 3.8% respectively, while the animals treated with conjugates

showed an increase in body weight for 4.1% and 8% respectively, at the end of experiment, compared to the start (**Figure 17A**).

The anti-tumor effect of the GnRH-III conjugates **1**, **2** and free Dau was evaluated measuring the tumor volume in each group during the experiment. 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 on day 28. (**Figure 17B**). Moreover, significant inhibition of tumor volume was also obtained in all treated groups at day 24 after cells inoculation. The inhibition of tumor volume was on this day 20.6, 23.1 and 29.8% for **2**, free Dau and **1**, in comparison to the animals in control group.

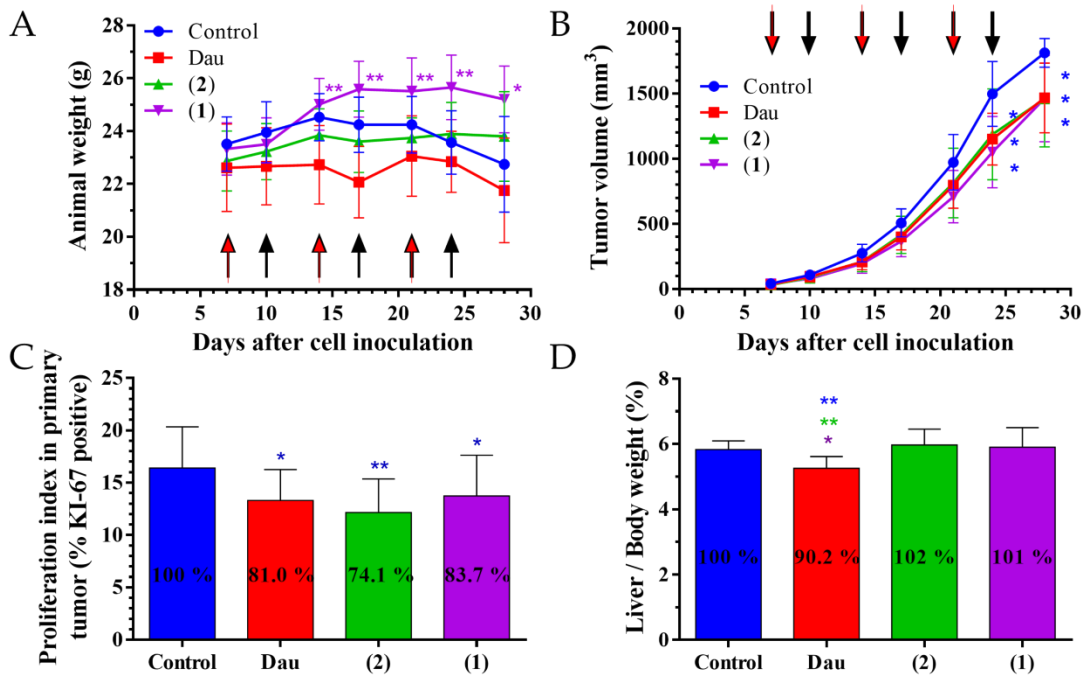

Figure 17. Effect of GnRH-III-Dau conjugates **1** and **2** (10 mg/kg Dau content, 6 treatments, black arrows) and free Dau (1 mg/kg, 3 treatments, red arrows) in orthotopic 4T1 murine BC bearing BALB/c female mice. (**A**) Animal body weight (grams, average ± SD). (**B**) Tumor volume (mm^3, average ± SD). (**C**) Proliferation in primary tumor (average proliferation index ± SD). (**D**) Liver weight/body weight ratio (percentage, average ± SD) after termination of experiment, 28 days subsequent to cells inoculation. 7 animals per group. Statistical analysis was performed by Mann–Whitney test. * and ** mean significant at $p \leq 0.05$ and $p \leq 0.01$, respectively. * blue, purple, and green mean significant difference in control, **1**, and **2** groups, respectively, at certain time point of experiment compared to the start (A), and significant difference compared to control, **1**, and **2** groups respectively (B, C, D).

The effect of free Dau and the GnRH-III-Dau conjugates on the proliferation of primary tumors in 4T1 orthotopic model was evaluated counting the percentage of KI-67 (proliferation marker) positive cells in comparison to all cells per field of view (magnification 400×) and calculating the proliferation index (**Figure 17C**). 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%.

The effect of the GnRH-III conjugates and free Dau on the liver toxicity was evaluated measuring the liver weight at the end of the experiment and calculating the liver weight/body weight ratio (**Figure 17D**). 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.

The number of macro-metastases in peripheral organs, such as spleen, lung, liver and kidneys, was counted, in order to determine the anti-metastatic effect of free Dau and the GnRH-III conjugates on aggressive 4T1 BC orthotopic model (**Figure 18A**). The number of macro-metastases in spleen was significantly decreased in all treated groups (Dau, **1** and **2**) by 64.3, 72.8 and 78.1%, in comparison to the control group. In the lung, the number of macro-metastases was also significantly reduced for all treated groups by 55.4, 55.2 and 64.4%, respectively. The numbers of macro-metastases in the liver and kidneys were decreased under treatments, whereby a significant decrease could be only obtained for conjugate **2**.

The effect of the GnRH-III-Dau conjugates and free Dau on the amount of micro-metastases and their proliferation in the lung (**Figure 18B,C**) was also evaluated, by counting the number of micro-metastases and proliferation marker KI-67 positive cells and the calculation of their ratio in comparison to all cells per field of view (magnification 100×). 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. The proliferation index of lung metastases was significantly inhibited by 27.8, 37.0 and 39.1% in groups that were treated with free Dau, **1** and **2**.

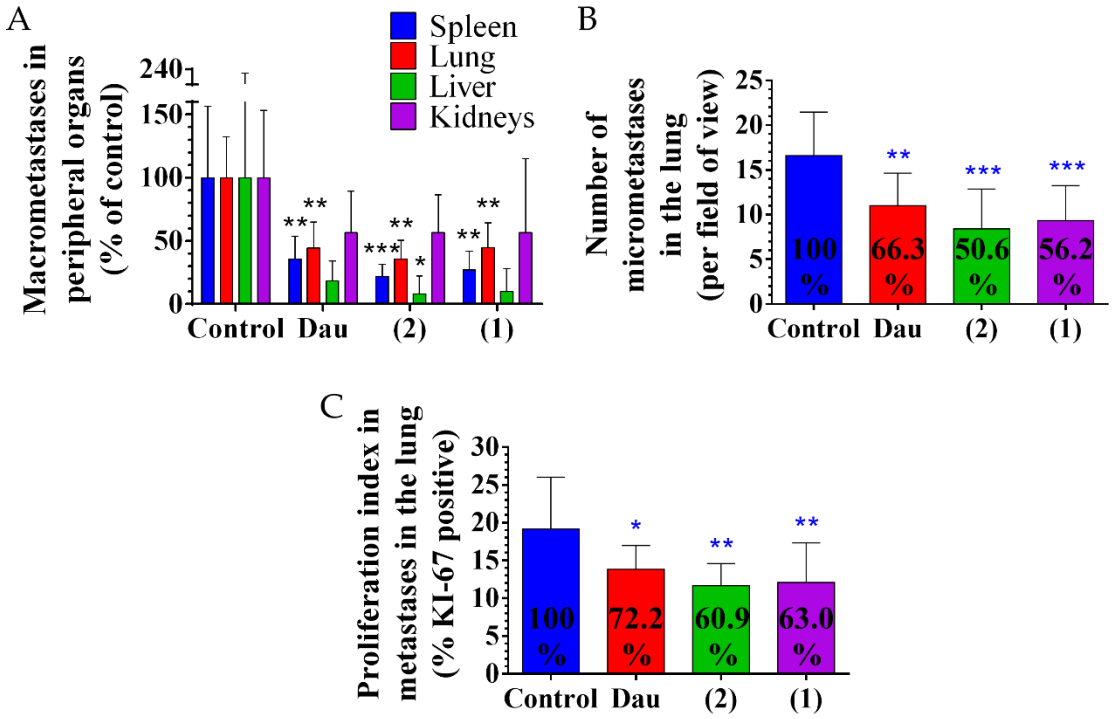

Figure 18. Effect of GnRH-III-Dau conjugates **1** and **2** (10 mg/kg Dau content, 6 treatments) and free Dau (1 mg/kg, 3 treatments) in orthotopic 4T1 murine BC bearing BALB/c female mice. (**A**) Number of macro-metastases in peripheral organs (average % of control ± SD). (**B**) Number of micro-metastases in lung (average number ± SD). (**C**) Proliferation of metastases in lung (average proliferation index ± SD). Statistical analysis was performed by Mann–Whitney test. *, ** and *** mean significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. * black and blue mean significant difference compared to the control group.

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

The effect of the GnRH-III conjugates **1** and **2**, as well as Dau on the animal body weight was evaluated in orthotopic MDA-MB-231 human BC bearing mice (**Figure 19A**). In all the groups, the body weight was decreased at the end of experiment in comparison to the beginning. The body weight of the mice in control group was decreased by 2.7%, while in the groups treated with **1** and **2**, it was decreased by 10.1 and 8.2%. In comparison, free Dau caused a significant decrease of mice body weight by 20% and considering that two animals of the control group were in bad condition, the experiment was terminated on day 45 after cells inoculation.

The anti-tumor effect of the GnRH-III conjugates **1**, **2** and Dau was evaluated by measuring the tumor volume in each group during the experiment. All treated groups

showed a significant inhibition of the tumor volume in comparison to the control group at the end of the experiment (**Figure 19B**). The treatment with Dau was most effectively, whereby the tumor volume was significantly inhibited by 46.3%. Apart from that, a significant inhibition of the tumor volume was also obtained in groups which were treated with conjugate **1** (34.1%) and **2** (23.1%). Moreover, from day 38 after cells inoculation, the inhibitory effect of free Dau was significant in comparison to control group.

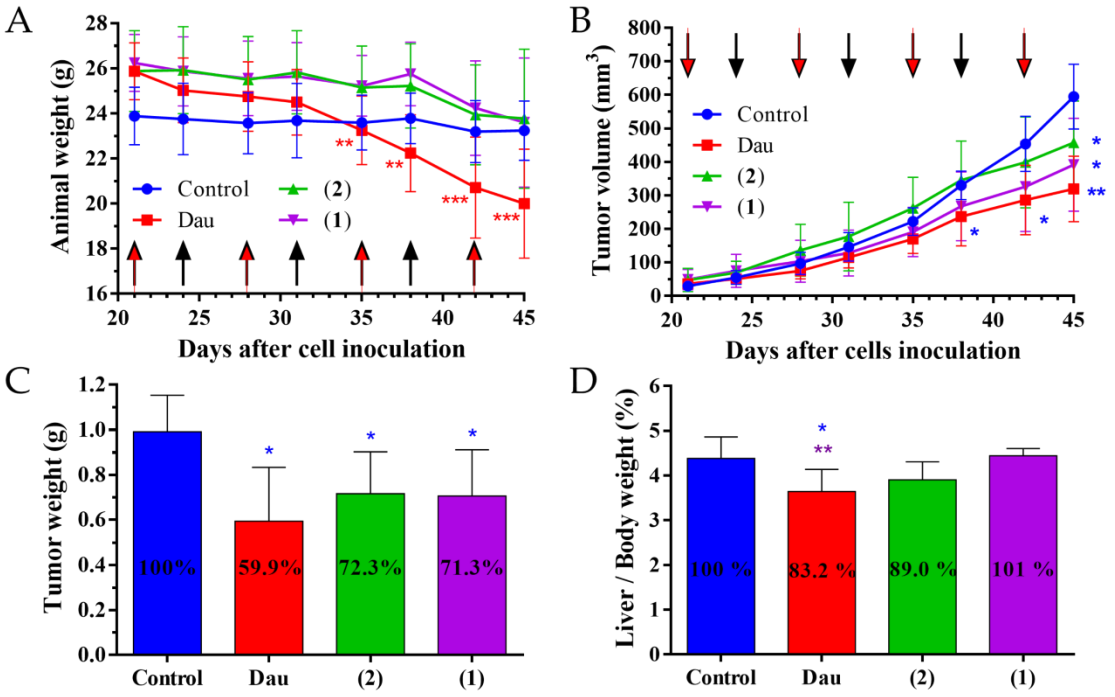

Figure 19. Effect of GnRH-III-Dau conjugates **1** and **2** (15 mg/kg Dau content, 6 treatments; 7.5 mg/kg Dau content of each conjugate, 1 treatment, black arrows) and free Dau (1 mg/kg, 4 treatments, red arrows) in orthotopic MDA-MB-231 human BC bearing SCID female mice. (**A**) Animal body weight (grams, average ± SD). (**B**) Tumor volume (mm^3, average ± SD). (**C**) Tumor weight (grams, average ± SD) after termination of the experiment, 45 days subsequent to cell inoculation. (**D**) Liver weight/body weight ratio (percentage, average ± SD) after termination of the experiment, 45 days after cell inoculation. 7 animals per group. Statistical analysis was performed by Mann–Whitney test. *, ** and *** mean significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. * blue, red, and purple mean significant difference in control, Dau, and **1** groups, respectively, at certain time point of experiment compared to the start (A), and significant difference compared to control, Dau, and **1** groups respectively (B, C, D).

The anti-tumor effect of the GnRH-III conjugates and Dau was evaluated also by measuring the tumor weight in each group after experiment termination (**Figure 19C**).

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 effect of the GnRH-III conjugates and Dau on the liver toxicity was evaluated by measuring the liver weights at the end of experiment and calculating the liver weight/body weight ratio (**Figure 19D**). 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. Conjugates treated groups showed non-significant changes in liver/body weight ratio.

The anti-metastatic effect of Dau and the GnRH-III conjugates in MDA-MB-231 orthotopic BC model was evaluated by counting animals containing metastases close to the primary tumor at the end of experiment (**Table 4**). It could be observed that all animals in the control group had metastases close to the primary tumor. In group treated with free Dau, four out of seven mice had metastases, while for the groups treated with conjugates **1** and **2** the best anti-metastatic effect could be obtained with three out of seven animals with metastases.

Table 4. Anti-metastatic effect of free Dau and GnRH-III-Dau conjugates **1** and **2** in orthotopic MDA-MB-231 human BC bearing SCID female mice on number of animals with metastases close to the primary tumor.

Treatment Groups of Mice	**Control**	**Dau**	**1**	**2**
Number of mice with metastases	5/5	4/7	3/7	3/7

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

The animal body weight in orthotopic HT-29 human colon carcinoma bearing mice decreased in all groups at the end of experiment when compared to the start (**Figure 20A**). The mice in free Dau treated group exhibit significantly decreased body weight, whereby the experiment was terminated on day 23 after tumor transplantation (day 17 of treatment). On the same day, the decrease of the body weights in the control and conjugates (**1** and **2**) treated groups were non-significantly lower. The body weight of the mice in the control group was significantly decreased on day 30 after tumor transplantation which was the reason for experiment termination. At the end of the

experiment, the body weight of the groups treated with GnRH-III conjugates **1** and **2** was also significantly reduced.

The anti-tumor effect of the GnRH-III conjugates and free Dau was evaluated by measuring the tumor weight in each group after the termination of the experiment (**Figure 20B**). 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.

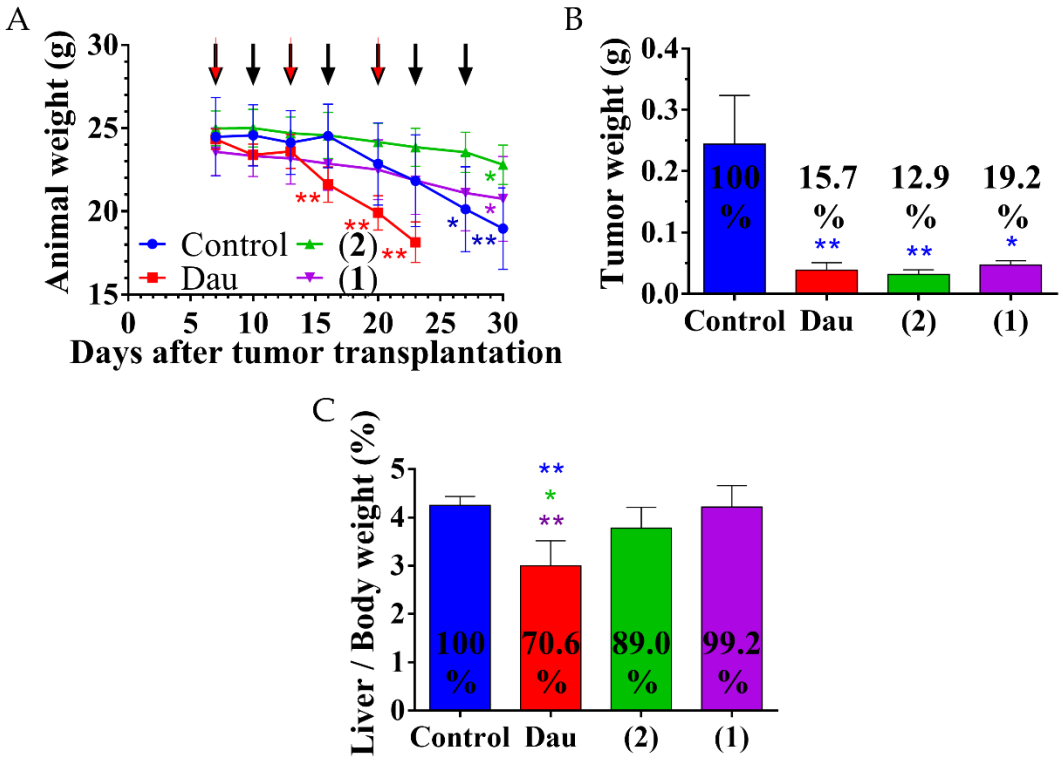

Figure 20. Effect of GnRH-III-Dau conjugates **1** and **2** (10 mg/kg Dau content, 7 treatments, black arrows) and free Dau (1 mg/kg, 3 treatments, red arrows) in orthotopic HT-29 human colon carcinoma bearing SCID female mice. (**A**) Animal body weight (grams, average ± SD). (**B**) Tumor weight (grams, average ± SEM) of mice from control, **1** and **2** groups after termination of experiment, 30 days after transplantation. (**C**) Liver weight/body weight ratio (percentage, average ± SD) of mice from control, **1** and **2** groups after termination of the experiment, 30 days after transplantation. Tumor and liver weight of mice from free Dau group on day 23 subsequent to transplantation. 6 animals per group. Statistical analysis was performed by Mann–Whitney test. * and ** mean significant at $p \leq 0.05$ and $p \leq 0.01$ respectively. * blue, red, purple, and green mean significant difference in control, Dau, **1**, and **2** groups, respectively, at certain time point of experiment compared to the start (A), and significant difference compared to control, Dau, **1**, and **2** groups respectively (B, C).

The average liver/body weight ratio of the mice in the group treated with free Dau was significantly decreased by 29.4% compared to the control group, as well as in comparison to the liver/body weights ratio of the mice in both conjugates treated groups (**Figure 20C**), showing the high toxicity of free Dau. The groups which were treated with the conjugates **1** and **2** showed non-significant changes in liver/body weight ratio.

All results and the corresponding specificity of each model is summarized in **Table 5** in order to provide a comprehensive overview.

DOI:10.14753/SE.2021.2555

Table 5. Summary of GnRH-R expression level, uptake level and effect of free drug Dau and GnRH-III-Dau conjugates **1** and **2** on 4T1, MDA-MB-231 and HT-29 in *in vitro*, *in vivo* and *ex vivo* models.

Model Type	**Experiment**	**4T1**			**MDA-MB-231**			**HT-29**		
in vitro	mRNA GnRH-R				+ + +			+ +		
	Protein GnRH-R	+ + +			+			+ +		
	Cell surface GnRH-R	+ +			+ + +			+		
	Uptake	+ +	+ + +		+ +	+ + +		+ +	+ + +	
	Cytotoxicity (24+48h)	6.3 µM	1.8 µM	56 nM	5.8 µM	1.9 µM	55 nM	15.5 µM	7.3 µM	203 nM
	Treatment	**1**	**2**	**Dau**	**1**	**2**	**Dau**	**1**	**2**	**Dau**
in vivo	Acute toxicity	NT up to 50 mg/kg Dau content								
	Chronic toxicity	NT on 15 mg/kg Dau content; NT 1 mg/kg free Dau								
	Tumor inhibition	S* 19%	S* 19%	S* 19%	S* 34%	S* 23%	S** 46%	S* 81%	S** 87%	S** 84%
	Animal weight	NT	NT	NT	NT	NT	T***	T* [1]	T* [1]	T*** [1]
	Liver toxicity	NT	NT	T**	NT	NT	T*	NT	NT	T**
	Macrometastases in peripheral organs	S**	S***	S**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Number of mice with metastases close to the primary tumor	n.a.	n.a.	n.a.	3/7[2]	3/7[2]	4/7[2]	n.a.	n.a.	n.a.
ex vivo	Proliferation index in primary tumor	S* 16%	S** 26%	S* 19%	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Number of micrometastases in the lung	S*** 44%	S*** 49%	S** 34%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Proliferation index in metastases in the lung	S** 37%	S** 39%	S* 28%	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

[1] Animal weight in control group was significantly reduced (T**). [2] All mice in control group had metastases close to the primary tumor. NT = non-toxic. T = toxic. S = significant inhibition. "+" represents level of expression or uptake. * represents level of significance. A higher level (double or triple + + +, ***). % represents percentage of inhibition compared to the control group. n.a.: not able to determine due to lack of metastases in particular model. n.d.: not determined due to the proliferation index inhibition by conjugates is already confirmed in aggressive 4T1 model.

DOI:10.14753/SE.2021.2555

4.2 Homing Heptapeptide-Dau conjugates

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

Tumor type specificity of two conjugates **3** and **4** was investigated on 22 different types of tumor cell line and on MCR-5 (human fibroblast) as non-cancerous control cell line. The cells were treated with the compounds for 24 h followed by incubation in fresh medium for further 48 h. The IC_{50} values of the conjugates were compared to each other and to the values obtained determining the effect of the free drug (**Table 6**). The data indicated that the conjugates had anti-proliferative effect on all cell types, but the lowest activity was measured on MRC-5 cells. However, the conjugates were not selective to the HT-29 human colon adenocarcinoma at all. Nevertheless, conjugate **4** showed higher anti-proliferative effect in all cases that was *ca.* 1.5-5 times higher activity depending on the type of cancer cells. The IC_{50} values were mainly at low micromolar range for conjugate **4** and they were 1-2 order of magnitude higher compared to the free Dau that can enter the cells by diffusion.

Table 6. Anti-proliferative effect of drug conjugates **3** and **4** compared to free Dau on various cell lines.

Tumor Type	**Cell Line**	**IC_{50} [1] 24 h + 48 h**			**Relative Potency [2]**	
		Dau (nM)	**3 (μM)**	**4 (μM)**	**Dau/3**	**Dau/4**
Glioblastoma	U87MG	27.9 ± 3.5	14.2 ± 3.5	6.6 ± 0.2	0.0020	0.0042
Breast	MCF-7	286.0 ± 24.7	22.2 ± 9.2	11.1 ± 3.8	0.0129	0.0258
Breast	MDA-MB-231	52.9 ± 10.3	6.3 ± 2.5	4.6 ± 0.8	0.0084	0.0115
Murine breast	4T1	40.8 ± 6.8	34.2 ± 0.4	11.6 ± 3.6	0.0012	0.0035
Colon	HCT116	127.1 ± 21.9	33.6 ± 4.4	7.2 ± 0.3	0.0038	0.0177
Colon	HT-29	202.9 ± 1.0	30.1 ± 0.3	11.6 ± 0.1	0.0067	0.0175
Colon	WiDr	240.1 ± 36.3	34.1 ± 3.2	15.1 ± 2.9	0.0070	0.0159
Murine colon	C26	126.0 ± 46.8	15.8 ± 2.5	8.3 ± 1.0	0.0080	0.0152
Liver	HepG2	21.3 ± 0.9	22.4 ± 4.4	4.7 ± 0.5	0.0010	0.0045
Lung	A549	68.2 ± 22.7	25.9 ± 2.3	5.9 ± 1.5	0.0026	0.0116
Lung	H1650	47.5 ± 1.6	4.4 ± 1.2	2.9 ± 0.6	0.0108	0.0164
Lung	H1975	13.3 ± 4.7	19.3 ± 0.1	3.7 ± 0.8	0.0007	0.0036
Melanoma	A2058	33.3 ± 0.4	10.5 ± 5.8	3.5 ± 1.3	0.0032	0.0095
Melanoma	M24	93.6 ± 25.8	15.4 ± 3.7	5.8 ± 0.9	0.0061	0.0161
Melanoma	WM983b	44.3 ± 19.2	7.1 ± 2.5	5.1 ± 0.4	0.0062	0.0087
Murine melanoma	B16	8.9 ± 5.4	15.2 ± 3.0	3.0 ± 0.5	0.0006	0.0030
Head and neck	PE/CA-PJ15	26.7 ± 5.0	20.2 ± 4.6	7.4 ± 3.4	0.0013	0.0036
Head and neck	PE/CA-PJ41	25.8 ± 5.4	9.4 ± 3.5	4.3 ± 0.1	0.0027	0.0060
Ovarian	OVCAR-3	472.9 ± 63.6	13.8 ± 0.5	11.3 ± 2.6	0.0343	0.0418
Pancreas	PANC-1	466.7 ± 36.6	31.7 ± 4.5	26.9 ± 8.1	0.0147	0.0173
Prostate	DU145	24.5 ± 5.4	6.1 ± 2.2	3.5 ± 0.5	0.0040	0.0070
Prostate	PC-3	26.0 ± 7.1	20.5 ± 0.3	5.9 ± 1.6	0.0013	0.0044
Normal fibroblast	MRC-5	254.7 ± 0.6	39.3 ± 24.6	52.1 ± 1.7	0.0065	0.0049

[1] IC_{50} values (average ± SD). [2] Relative potency = IC_{50} Dau / IC_{50} Conjugate.

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

Next to the parent conjugate **3**, conjugate **4** was selected for *in vivo* experiments and their anti-tumor activity was tested in orthotopic HT-29 human colon cancer model in SCID female mice. Conjugates **3** and **4** has a good water solubility and due to this they were dissolved in water for injection. The treatments were started on day 13 and the mice were terminated on day 30 after tumor transplantation, except for the free drug (Dau) group, which was terminated on day 23 after tumor transplantation due to the significant weight loss of the animals. The free drug was administered i.p. using 1 mg/kg dose once a week that is the maximal tolerated dose in this case. Conjugates were injected at a dose of 10 mg/kg Dau-content three times in the first and two times in the second week. During the experiment, one animal died from both the control and Dau treated groups, while two of them from the group treated with parent conjugate **3**. In contrast, no animals died from the group treated with modified conjugate **4**. After the termination, tumor growth inhibition was calculated by the measurement of tumor weight, while liver toxicity of the compounds was determined according to the liver/body weight ratio.

Animal weights did not differ from control mice in case of conjugates **3** and **4**, while Dau-treated mice showed an increased significant weight loss (**Figure 21A**). The results indicate that free Dau caused 83.7% tumor growth inhibition compared to the untreated control group (**Figure 21B**). However, significant liver toxicity according to the loss of liver compared to body weight for 22.7% was obtained (**Figure 21D**). Similarly to the *in vitro* data, conjugate **4** was also more active *in vivo* compared to conjugate **3** (**Figure 21B**). In comparison with the control group, tumor growth inhibition was significant for 89.1% in case of conjugate **4**, and not significant for 64.6% in case of conjugate **3**. 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 10% and 5%, respectively, that was not significant compared to the control group, and significantly different than it was observed in case of the free Dau treated group (**Figure 21D**).

To reveal the mechanism of the *in vivo* effect of conjugates and free drug (Dau), xenograft tumors were analyzed by immunohistochemistry. The proportion of proliferating tumor cells was determined by KI-67 labelling, which is a specific cellular marker for proliferation. Proliferation index was determined as percentage of KI-67-

positive-stained cells from viable cells in the tumor tissue. 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) (**Figure 21C**).

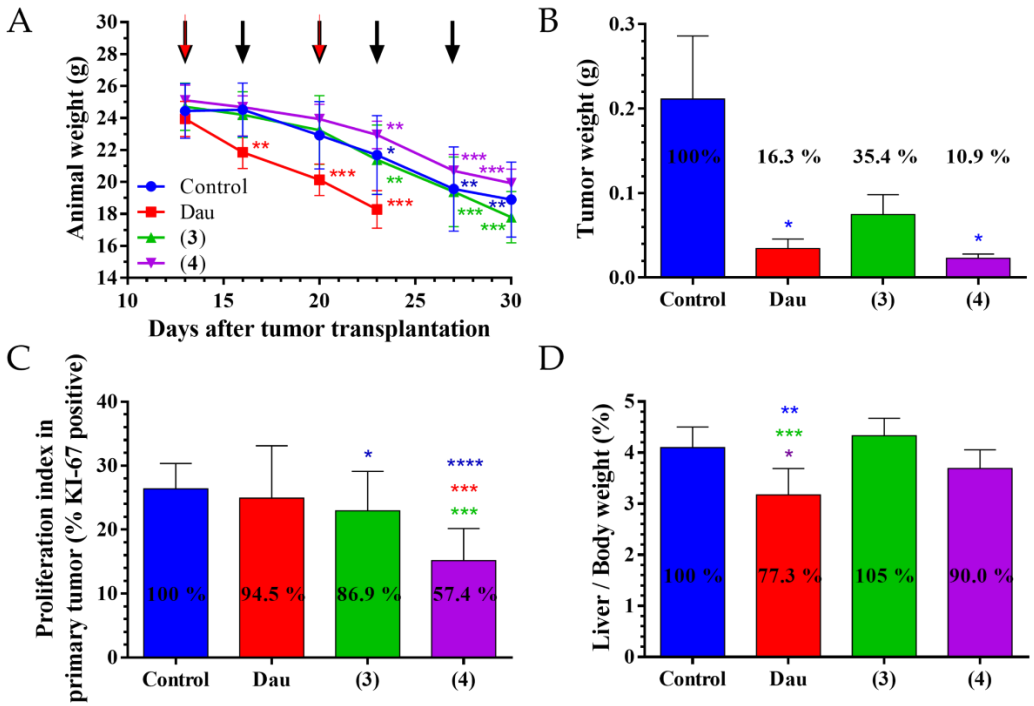

Figure 21. Effect of Heptapeptide-Dau conjugates **3** and **4** (10 mg/kg Dau content, 5 treatments, black arrows) and free Dau (1 mg/kg, 2 treatments, red arrows) in orthotopic HT-29 human colon carcinoma bearing SCID female mice. (**A**) Animal body weight (grams, average ± SD). (**B**) Tumor weight (grams, average ± SEM), after termination of the experiment, 30 days after transplantation. (**C**) Proliferation in primary tumor (average proliferation index ± SD). (**D**) Liver weight/body weight ratio (percentage, average ± SD), after termination of the experiment, 30 days after transplantation. Tumor and liver weight of mice from free Dau group on day 23 subsequent to transplantation. 8 animals per group. Statistical analysis was performed by Mann–Whitney test. *, **, *** and **** mean significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$, respectively. * blue, red, green, and purple mean significant difference in control, Dau, **3**, and **4** groups, respectively, at certain time point of experiment compared to the start (A), and significant difference compared to control, Dau, **3**, and **4** groups respectively (B, C, D).

DOI:10.14753/SE.2021.2555

4.3 Vindoline CPP conjugates

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

The analysis of *in vitro* anti-proliferative effect of Vindoline derivatives and conjugates was performed on C26 mouse colon carcinoma, and P388 mouse leukemia cell lines (**Table 7**; **Figure 22**).

As expected Vindoline and Br-Vindoline expressed no anti-tumor activity on studied cell lines. The presence of Trp-OMe moiety almost regardless to the configuration of the Trp residue increased the anti-tumor effect of Br-Vindoline, while the derivatives (Br-Vind-*L*-Trp-OMe, Br-Vind-*D*-Trp-OMe) had moderate biological activity in a range of IC_{50} = 21.6 to 5.3 μM. It is interesting to note that in case of P388 cells, Vindoline derivatives had not only the most marked effect, but also slightly different activity depending on the Trp configuration, IC_{50} = 5.3 μM for *L*-Trp, and IC_{50} = 15.9 μM for D-Trp, respectively.

The corresponding IC_{50} values obtained from the treatment of cells with octaarginine Vindoline conjugates indicated the same tendency against P388 murine leukemia cell line, whereby the *L*-Trp containing conjugate Br-Vind-*L*-Trp-Arg_8 (**5**) was more effective with IC_{50} = 3.1 μM, than the isomer conjugate Br-Vind-*D*-Trp-Arg_8 (**6**) with IC_{50} = 18.0 μM. The configuration of Trp notably determined the influence of the conjugates on the activity on C26 and P388 cells.

The anti-tumor effect of Vindoline-Trp-octaarginine conjugates was much higher in comparison with the free, unconjugated Vindoline derivatives, especially in C26 murine colon carcinoma cell line. It is also important to mention that conjugate of Br-Vindoline without the Trp moiety (Br-Vind-Arg_8) was less effective on both C26 and P388 cells in comparison to Vindoline derivatives and Vindoline octaarginine CPP conjugates.

Table 7. *In vitro* anti-proliferative activity of Vindoline derivatives and CPP conjugates on tumor cell lines.

Compound	**IC_{50} [1] 3 h + 69 h (μM)**	
	C26	**P388**
Vindoline	>100	>100
Br-Vindoline	>100	>100
Br-Vind-*L*-Trp-OMe	18.41 ± 2.06	5.27 ± 1.49
Br-Vind-*D*-Trp-OMe	21.62 ± 4.26	15.92 ± 2.80
Br-Vind-Arg_8	25.87 ± 2.70	26.55 ± 5.01
Br-Vind-*L*-Trp-Arg_8 (**5**)	4.29 ± 0.41	3.09 ± 0.45
Br-Vind-*D*-Trp-Arg_8 (**6**)	10.80 ± 0.72	17.98 ± 1.21

[1] IC_{50} values (average ± SD).

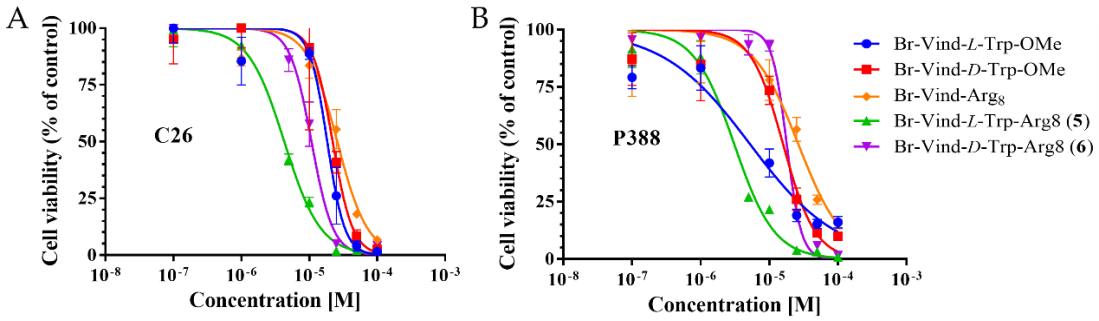

Figure 22. Anti-proliferative effect of the Vindoline derivatives and CPP conjugates. (**A**): C26 murine colon cancer cells, (**B**): P388 murine leukemia cancer cells, after 72 h (3 h treatment and an additional 69 h incubation). Dose-response curves obtained by non-linear regression. Error bars represent the SD of three parallels, the measurements were repeated twice.

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

The anti-tumor effect of Trp containing Vindoline-octaarginine CPP conjugates and Vinblastine on the tumor growth was studied *in vivo* by using tumor model of P388 mouse leukemia cells which were injected subcutaneously into mice. The effect of treatments was evaluated on animal body weight which slightly increased during the treatment time for 6-7% in the control and in all treated groups (**Figure 23A**).

The animals were treated with the conjugates on day 1 and 6 with dose of 10 mg/kg body weight. Vinblastine as a clinically applied drug was used as positive control in dose

of 1 mg/kg body weight. All treated groups showed inhibition of the tumor volume compared to the control group at the end of the experiment on day 25 after cell inoculation, but not significant (**Figure 23B**). 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. 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.

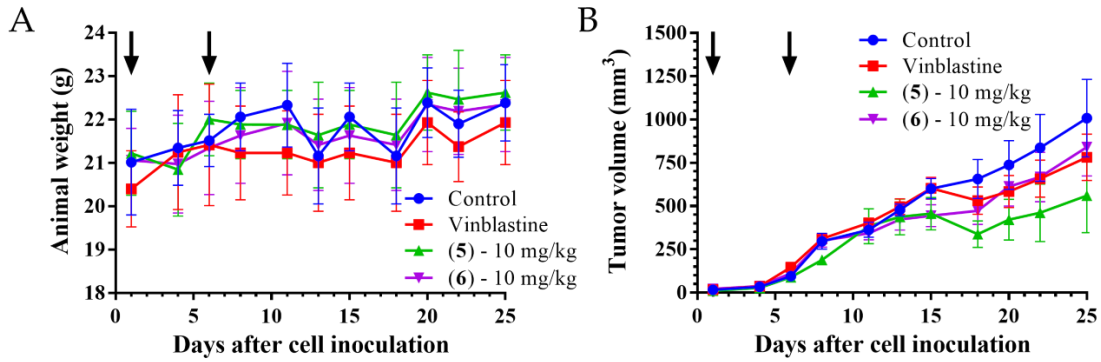

Figure 23. Effect of Br-vind-*L*-Trp-Arg_8 (**5**) and Br-Vind-*D*-Trp-Arg_8 (**6**) conjugates (10 mg/kg, 2 treatments, black arrows) and Vinblastine (1 mg/kg, 2 treatments, black arrows) in subcutaneous P388 murine leukemia bearing BDF1 female mice. (**A**) Animal body weight (grams, average ± SD). (**B**) Tumor volume (mm^3, average ± SEM). 5-7 animals per group.

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

The anti-tumor effect of Trp containing Vindoline-octaarginine CPP conjugates and Vinblastine on the tumor growth was studied *in vivo* also by using tumor model of subcutaneous C26 murine colon carcinoma bearing mice. The effect of treatments was evaluated on animal body weight which was not changed during the treatment time in the control and all treated groups (**Figure 24A**).

The animals were treated with the conjugates on days 13, 17, 20, 24 and 29 at dose of 10 mg/kg and 20 mg/kg body weight. Vinblastine as a clinically applied drug was used as positive control in dose of 1 mg/kg body weight. All treated groups showed inhibition of the tumor volume compared to the control group at the end of the experiment on day 31, but not significant (**Figure 24B**). The treatment of C26 colon carcinoma bearing mice

with *L*-isomer conjugate **5** showed higher anti-tumor activity compared to *D*-isomer conjugate **6** for same dose. Dose of 10 mg/kg of both conjugates showed 2-fold lower inhibition activity on tumor volume in colon tumor bearing mice compared to leukemia model, with inhibition of 19.9 and 7.3%, while the Vinblastine treatment had no marked influence on tumor growth, and inhibited tumor volume for 11.4% compared to the control. The inhibition of tumor volume was increased under double higher dose of conjugates where concentration of 20 mg/kg decreased tumor volume for 29.4 and 22.8% for *L*-Trp (**5**) and *D*-Trp (**6**) isomers, respectively, in comparison to the tumor volume in the control group.

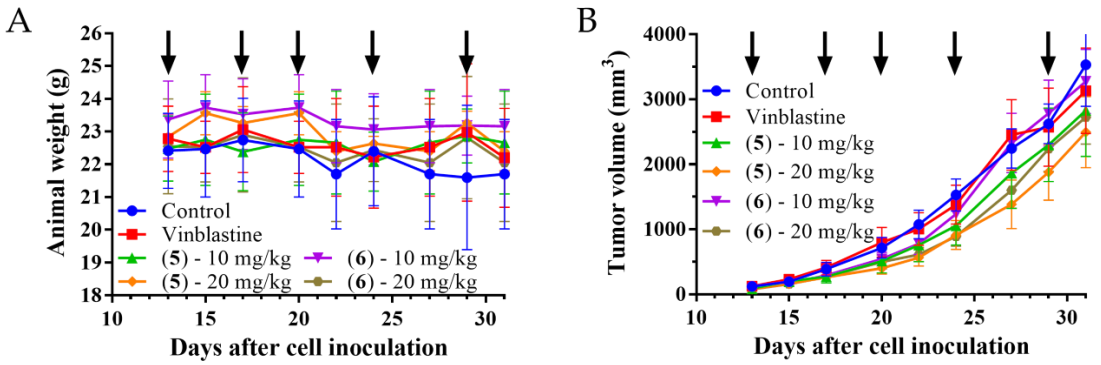

Figure 24. Effect of Br-vind-*L*-Trp-Arg_8 (**5**) and Br-Vind-*D*-Trp-Arg_8 (**6**) conjugates (10 mg/kg and 20 mg/kg 5 treatments, black arrows) and Vinblastine (1 mg/kg, 5 treatments, black arrows) in subcutaneous C26 murine colon carcinoma bearing BALB/c female mice. (**A**) Animal body weight (grams, average ± SD). (**B**) Tumor volume (mm^3, average ± SEM). 5-7 animals per group.

DOI:10.14753/SE.2021.2555

5 DISCUSSION

5.1 GnRH-III-Dau conjugates

The discovery and development of new drugs is a time-consuming, expensive and complex process which involves experts from a range of disciplines such as medicinal chemistry, biochemistry, molecular biology, medicine, and pharmacology. It is requiring a wide-ranging scan of thousands of potential candidates using reliable *in vitro* screening analysis. It has been estimated that from about 10 000 new chemical entities identified or synthesized as potential therapeutic agents, only one will reach the market in an average time of 16 years[273].

Moreover, high production costs and the need for several clinical trials to verify the tolerability, toxicity, and effectiveness of the candidate molecules delay production of marketable drugs, driving up their price and making them prohibitive to most patients. In this context, thanks to their biocompatibility, overall reproducibility of their synthetic processes, and lower production costs, peptides represent an attractive alternative and have received attention from the pharmaceutical industry in recent years[274].

The use of peptides in clinical trials has recently gained ground. They are now used widely as therapeutic drugs and diagnostics in clinical applications such as endocrinology, oncology, urology, and obstetrics[275]. Indeed, it is estimated that the global peptide therapeutics market, which was valued at 23 billion American dollars in 2017, will reach 43 billion American dollars by 2024, with annual growth rate of around 9%[276].

When considering that receptors for many regulatory peptides are overexpressed in various tumor cells, compared with their expression in normal tissues, peptide based drug tumor targeting is a promising therapeutic approach for cancer and enables the specific delivery of anti-cancer drugs to cancerous cells[130]. Among various homing devices, GnRH-III peptide represents a suitable targeting moiety and different GnRH-III conjugates have been developed and used as DDS[129]. Moreover, GnRH-Rs were not only detected in cancers related to the reproductive system, but also to unrelated ones, such as colon cancer[27, 28].

Recently, a novel promising GnRH-III-based DDS with an improved *in vitro* activity was developed in Mező group[203]. Its activity was analyzed on a wide set of cancer cell lines and also on MRC-5 normal cell line in comparison to previous lead conjugate **1** to further characterize the potency of the new conjugate **2**[200]. Furthermore, the *in vivo*

activity of both compounds in tumor bearing mice was determined to gain a deeper insight of their potency.

The *in vitro* anti-proliferative effect measurements, on a wide set of cancer cell lines, revealed that both conjugates elicited a substantial growth inhibitory effects with IC_{50} values in the low micromolar range, whereby conjugate **2** displayed mostly a higher cytostatic effect than **1**. These results are in line with previously reported results and confirm the high potential of the novel lead compound **2**[203]. In this study, higher IC_{50} values were obtained in CRC and BC cell lines than in the previous one. This might be related to the difference in the assays which have been used for cell viability determination[277]. In the present study a MTT assay was used and in previous studies a resazurin based assay. Furthermore, beneficial relative potencies were obtained of compounds **1** and **2**, on breast, colon, malignant glioma, melanoma and ovarian cancer cell lines. On the contrary, relatively high IC_{50} and low relative potency values were obtained in case of non-cancerous MRC-5 cells, suggested that the conjugates are highly tumor selective, except for PANC-1 cells, where the IC_{50} value of the free Dau was also substantially higher than for the other cancer cell lines. Lower values of relative potency indicate a loss of targeting capacity of the conjugates with respect to free Dau[278].

It is well known that pancreatic cancers reveal very often MDR towards a variety of classical anti-cancer drugs[279]. Miller *et al*. has determined that the MDR of PANC-1 cells is mainly related to the presence of the MDR-associated protein (MRP)[280]. Moreover, it has been shown that MRP mediates the adenosine triphosphate (ATP)-dependent efflux of anthracyclines, like Dau and other anti-cancer agents[281]. Based on these findings, it can be assumed that the low activity of the free Dau is mainly related to the efflux of the drug from cytosol directly after passive diffusion.

The endocytic uptake of Dau *via* conjugates might overcome the MRP-derived MDR in PANC-1 cells, as shown by Zheng *et al*.[282]. Therefore, it can be assumed that the Dau resistance of PANC-1, can be reduced when the drug enters the cells by an endocytic route, which could not be obtained with tested conjugates. Thus, the low activity of the conjugates on PANC-1 cells might be related with a lower receptor level even if other reports verified GnRH-R expression on PANC-1 cells[283]. The GnRH-R expression was determined on a selection of cell lines, where **1** and **2** had a high activity and also on the two cell lines with the lowest activity, to better interpret the results. Thus, the GnRH-R

mRNA, absolute protein level and cell surface receptor level of MDA-MB-231, 4T1, HT-29 and A2780, as well as PANC-1 and MRC-5 cells was determined.

The GnRH-R mRNA expression level was analyzed using RT-qPCR. The amount of GnRH-R mRNA in MRC-5 was almost the same as the level in human total cDNA sample, which indicates that MRC-5 normal cell line provides a good reference sample, thus the relative quantification of each sample was normalized to MRC-5. All cancer cell lines showed a higher level of GnRH-R mRNA expression than the normal cell line which is in accordance with previous studies[28]. The detected GnRH-R mRNA level in PANC-1 cells was substantially reduced compared to MDA-MB-231, HT-29 and A2780 cancer cells which might be an explanation for the high IC_{50} values on PANC-1 cells. Interestingly, human colon sample showed almost two-fold higher level of GnRH-R mRNA in comparison to normal cell line and human total cDNA sample, which is in accordance with reported data, illustrating that GnRH-R is present on healthy intestine and colon[284, 285]. On the contrary, no correlation between the GnRH-R mRNA level and the IC_{50} value could be obtained in the case of U87MG cancer cells, which exhibit just a low mRNA level of GnRH-R expression, but low IC_{50} values. This observation might be explained by post-transcriptional processes that are involved in the final synthesis of the protein leading to different levels of its expression[286, 287]. It is well known that mRNA and protein expression do not always correlate, thus the absolute GnRH-R protein level was also determined[288].

Whole cell lysates of the appropriate cell lines were analyzed using western blot studies in order to determine the GnRH-R protein expression level. All cancer cell lines showed higher protein level of GnRH-R in comparison to normal cells, except for PANC-1. Moreover, these results are in line with the determined IC_{50} with exception of MDA-MB-231. For the biological activity not only the absolute GnRH-R level is of high relevance, but also the location of the receptor on the cell surface[289].

Thus, the GnRH-R expression level on the cell surface was analyzed using flow cytometry. All of the cancer cell lines showed higher GnRH-R receptor surface level in comparison to the normal cell line. The obtained results are in accordance with previously published data for breast[137, 290], ovarian[291, 292], colon[27], brain[139] and pancreatic cancer[283]. Moreover, these results correlate very well with the determined IC_{50} values. However,

both conjugates showed low cytotoxic effect on PANC-1 cells, although a high surface expression level of GnRH-R was obtained. This effect could depend on a number of factors next to the binding to receptors, including the rate of internalization, efficiency of the drug release, interaction of drug with its targets and the suitability of the drug for a certain tumor[63, 293]. The reason for this effect could be very low basal protein level. Thus it might be possible that the amount of receptors which are recycled to the cell surface, after the initial internalization of the surface receptors together with the conjugate, is quite low. Therefore, it could be the case that only in the beginning, a smaller amount of conjugates enter the cell by receptor mediated endocytosis, and then the internalization is slowed down which might cause the reduced activity of the conjugates on PANC-1 cells. In comparison, the cell lines with a high and moderate cell surface and basal protein level display enhanced biological activities. Thus, it can be assumed that the surface receptor level is more constant over the treatment time of 24 hours which might explain better *in vitro* activity.

Apart from that just a relative low basal and surface protein GnRH-R expression level was determined in case of MDA-MB-231 cells, while both compounds reveal a high biological activity on these cells. This effect might be explained by the high mRNA level which could be detected for MDA-MB-231 cells. It could be possible that the translation of the GnRH-R mRNA might be initiated and accelerated when GnRH-III conjugates are bound to the receptors, which would explain high *in vitro* activity of the conjugates on MDA-MB-231. However, cellular uptake studies were performed to further evaluate this assumption.

The cellular uptake of conjugates was analyzed using flow cytometry after six hours of treatment, whereby non treated samples were used as controls. All of the cancer cell lines showed higher uptake rates for the conjugates in comparison to normal cells. These results are in line with the GnRH-R expression level and the IC_{50} values. In accordance with previous results, the obtained cellular uptake rates of the new lead compound **2** were for each cancer cell line significantly higher than that of **1**, but not for the normal cell line MRC-5, where could not be obtained a significant difference between the conjugates. This indicates that the uptake capacity on cancer cells is higher for conjugate **2** which is in line with previous data[203]. Apart from that, the cellular uptake rates on PANC-1 cells were for both conjugates lower than for the other cancer cell lines and in the same range

as the rates for MRC-5. These results are in line with the low anti-tumor effect of the conjugates on these two cell lines and the GnRH-R expression level.

Taking all of the results into account, it can be proposed that the biological activity of the conjugates strongly depend on the expression of the GnRH-Rs which ensures the selectivity of the GnRH-III based DDSs, and on cellular uptake capacity of the conjugates.

Furthermore, the *in vivo* anti-tumor activity of the conjugates was analyzed on tumor-bearing mice due to the promising *in vitro* results of **1** and **2** and the fact that the stability of the conjugates in murine plasma revealed that both compounds were stable for at least 24 h. Although both conjugates have been already proven to be stable in human blood plasma[203], this experiment provides valuable information and avoids misinterpretation of preclinical results that might be caused by differences between the enzymatic activity of laboratory animals and humans[294].

The analysis of the toxicity of new compounds on healthy mice is essential for the drug development process[295]. Animal weight and behavioral changes are the critical characteristics in toxicity testing as animals should be protected from stress and pain[296]. Thus, an acute toxicity study experiment was performed, which revealed that neither the body weights of the mice were significantly changed after administration of the new conjugate **2**, nor the general look and behavior of experimental animals, even at a dose of 50 mg/kg Dau content. Chronic toxicity experiment with 5 treatments of conjugates with a dose of 15 mg/kg Dau content, revealed no significant change in the body weights, general look and behavior of mice. Based on these results, it is concluded that both conjugates can be used at this concentration for the treatment of tumor bearing mice, since this dose was not toxic for the animals.

Initially, the *in vivo* anti-tumor activity in an orthotopic 4T1 murine BC model was determined. Moreover, the anti-metastatic effect of the conjugates and free Dau was analyzed, since the majority of deaths of BC patients are related to tumor cell metastasis. Unfortunately, most BC are invasive and frequently metastasize to distant organs, including lung and liver[297, 298]. There is currently no effective therapy for metastatic BC. Therefore, the development of therapeutic agents to suppress metastatic BC is of great significance. To determine the efficacy of anti-tumor drugs, the choice of a suitable *in vivo* model that mimics the initiation and progression of BC is highly important[299, 300]. A

number of murine models have become available during the last two decades and syngeneic BC murine models, including the 4T1 murine mammary cancer models, originally isolated as subpopulation derived from a spontaneously arising mammary tumor in BALB/c mice[301, 302], are widely used to study the mechanisms of tumor growth and metastasis[303–306]. Orthotopic BC models mimic the tumor microenvironment in an adequate way and they provide more tumorigenic and metastatic cancer cell population[307]. Moreover, these models represent beneficial tools to investigate the anti-tumor and anti-metastatic effects of various drugs according to their aggressive growth and high invasive nature[308, 309]. In could be shown that 4T1 tumor metastasizes *via* a hematogenous route to liver and lungs, making it a beneficial model of human metastatic BC[310], which grows progressively and causes a uniformly lethal disease[311]. Therefore, the orthotopic implantation of 4T1 cells by injection in the mammary fat pad was performed, which leads to the formation of primary tumors and subsequent metastatic growth, establishing a fast and quantitative method[312] to determine the *in vivo* anti-tumor activity of conjugates **1** and **2**, and Dau. Moreover, the anti-metastatic effect of the conjugates and free Dau was analyzed, since the majority of deaths of BC patients are related to tumor cell metastasis.

The change of the animal body weight during the *in vivo* experiment is an important parameter that shows the condition of drug treated and non-treated tumor bearing animal[313]. In 4T1 tumor bearing mice, decreasing body weights were obtained for the control and Dau group, while an increase in animal body weight could be observed in both conjugate treated groups, indicating that the conjugates did not cause toxicity and side-effects on the animals during the treatment.

The GnRH-III conjugates **1** and **2**, as well as free Dau showed a significant tumor volume inhibition activity against a very aggressive syngeneic orthotopic 4T1 mouse breast tumor. Apart from that, the expression of the KI-67 protein is strictly associated with cell proliferation and tumor progression, therefore we determined the proliferation index of KI-67 positively-stained cells[314]. The results display, for both conjugates, as well as free Dau a significantly lower proliferation index in primary tumor when compared to the control group, which supports their significant inhibitory effect on the tumor growth.

Furthermore, not only the elicited anti-tumor activity is of high relevance for the success of an anti-cancer drug, but also the selectivity to cancerous cells and reduction of

side-effects. Dau is known to be rapidly and widely distributed in tissues, whereby the highest levels were found in the liver, spleen, kidneys, lungs and heart[315]. Since the liver is the vital organ in metabolism of Dau, production of toxic intermediates that may trigger liver injury and impair with the liver function can increase the risk of toxicity[316]. Analysis of organ weight in toxicology studies is an important factor for the identification of potentially harmful effects of drugs, thus the liver weight/body weight ratio analysis provides better understanding of drug toxicity[306, 317]. The free Dau caused a significant decrease of liver/body weight ratio in comparison to control and conjugates treated groups, revealing that the treatment with Dau caused toxic side-effect in mice. In comparison, non-significant liver/body weight ratio could be detected in GnRH-III conjugates treated groups proving evidences for their selectivity and non-toxicity to healthy tissue.

The number of metastases close to the primary tumor and on distant organs are often clinical picture in breast tumors[318]. Conjugates and the free Dau both exhibited a decreased number of macro-metastases in peripheral organs, especially in the lung and the spleen, while the new conjugate **2** revealed also significantly decreased metastases in the liver. Further was evaluated the effect of GnRH-III-Dau conjugates and Dau on the number of micro-metastases and on the proliferation of metastases in the lung in order to confirm the significant decrease of the number of macro-metastases. The number of micro-metastases was significantly reduced for all treated groups in comparison to the control group, especially in conjugates treated groups. Both conjugates and free Dau showed a significant inhibition of the proliferation index in lung metastases, which allows for drawing the conclusion that both conjugates possess an anti-metastatic activity.

The significant *in vivo* tumor growth inhibition in 4T1 BC bearing mice, together with the significant inhibition of the proliferation in primary tumor and the significant anti-metastatic activity, supports the assumption that both conjugates are promising therapeutics for BC without causing significant side-effects and toxicity.

Although subcutaneous inoculations of the cells are easier to perform, orthotopic models give rise to a more tumorigenic and more metastatic cancer cell population. Thus, results obtained by means of the subcutaneous inoculations might be either false-negative or false-positive, encouraging the use of orthotopic models to study the tumor growth[307].

The use of an orthotopic xenograft model[319] in SCID mice[320] provide a powerful tool for therapeutic investigation of anti-cancer agents which should act on BC.

Further studies have been performed in orthotopic MDA-MB-231 human BC bearing mice, because the use of human BC cell lines in orthotopic xenograft mouse model provide a powerful tool for therapeutic investigation of anti-cancer agents and give valuable information about specific drug targeting in human[321]. In addition, this TNBC cell line is extremely aggressive, expresses a high level of GnRH-R and it has been shown to be a suitable model to evaluate the efficiency of GnRH peptide drug conjugates[19, 20, 181, 322]. This prompted us to determine the *in vivo* anti-tumor activity of both GnRH-III compounds in orthotopic MDA-MB-231 human breast tumor bearing mice.

In this model, the animal body weight of the mice was decreased in all groups at the end of experiment when compared to the beginning. But, only in the group treated with free Dau, the body weight of the mice was significantly decreased. Taking into account that two animals in the control group were in bad condition, but not the mice in the groups treated with the conjugates, it can be concluded that the conjugates did not cause a substantial toxicity, even if the administrated Dau content was much higher than the maximum tolerated dose of the free drug[200].

If we consider the tumor volume inhibition, the strongest effect was obtained for the treatment with free Dau, but also both conjugates significantly inhibited the tumor volume. The anti-tumor effect of the GnRH-III conjugates and free Dau was evaluated also measuring the tumor weight in each group after termination of the experiment. Also, these data demonstrate that the conjugates **1** and **2**, as well as free Dau possess a significant anti-tumor activity in orthotopic human MDA-MB-231 TNBC bearing mice.

In accordance with the results that were obtained in the 4T1 mouse breast model, the treatment with free Dau caused a significant decrease of the liver/body weight ratio compared to the liver/body weight ratios of the control and conjugates treated groups. When considering that was not detected a significant change in liver/body weight ratio, it can be suggested that the treatment with the GnRH-III conjugates do not cause harmful side-effects.

It has been reported that, in SCID mice, the remaining innate immune cells reduce the metastasis formation in distal organs[323]. Due to this, the anti-metastatic effect of the conjugates and free drug was evaluated based on the number of animals containing

metastases close to the primary tumor. The obtained results provide evidence that both conjugates cause a significant reduction in the number of animals with metastases. Moreover, the anti-metastatic effect of the conjugates was higher in comparison with the Dau treated group, suggesting that these two conjugates are potential anti-metastatic therapeutics for BC.

Based on these results, it can be concluded that both GnRH-III conjugates, as well as Dau inhibit the tumor growth significantly in MDA-MB-231 BC bearing mice. Furthermore, the anti-metastatic activity of the conjugates was significant and higher than that of Dau. Moreover, the results of the animal body weight, as well as the liver/body weight ratio, indicate that no toxicity side-effects are caused by the treatment with the conjugates, even if the Dau content of the injected dose was much higher than the maximum tolerant of free Dau.

Next to the *in vivo* studies on BC bearing mice, we were interested to analyze the anti-cancer activity of the two lead compounds on tumors that are not related to the reproductive system. Due to *in vitro* results and previous *in vivo* studies of **1**, CRC might represent an adequate model for these studies[200]. Although orthotopic colon cancer xenograft models are technically challenging and labor-intensive, orthotopic transplants are able to more accurately mimic human tumors. This approach simulates better the natural microenvironment for tumor development, providing an effective approach to investigate tumor pathophysiology and to develop therapeutic strategies which allow a better prediction of patient's response to chemotherapy in comparison with heterotopic transplants[324–326]. Apart from that, different studies pointed out that many colon tumors possess an increased expression level of GnRH-R[27], therefore a variety of synthetic therapeutics have been used which target this receptor and hence revealed significant tumor growth inhibition *in vitro* and *in vivo*[190, 193, 195, 199, 200, 327]. Thus, HT-29 human colon tumors were implanted to the intestine of immunodeficient SCID mice[200, 328, 329].

We observed that the free Dau cause a significant decrease in mice body weights, which compelled us to terminate the experiment for this group already on day 23 after tumor transplantation. Apart from that, the significant loss of the body weight in the control group also prompted us to terminate the experiment on day 30 after tumor transplantation. A reduction of the animal weight was also obtained for mice which were treated with the GnRH-III conjugates, but here the effect was not that serious, especially

on the day when the Dau group was terminated. This indicates that both conjugates cause less harmful side-effects than Dau. Moreover, it might be possible that a distinct decrease of the body weight was caused by a higher susceptibility of the animals after surgery procedures, which was necessary for establishing the orthotopic CRC model[200, 328, 329].

The anti-tumor effect of the conjugates and Dau was evaluated after isolation of the tumors at the end of experiment. A significant inhibition of the tumor weight was obtained in all treated groups, whereby the highest inhibition was obtained for the treatment with the novel lead compound **2** even the experiment took longer in comparison with the free drug. Furthermore, this effect could be achieved without substantial toxic effects and by a lower dose than in the previous studies with **1** and free Dau[200].

In addition, no significant changes were detected in the liver/body weight ratios of the groups treated with the conjugates, while a significant decrease of the liver/body weight ratios was observed for the group that was treated with the free drug. This indicates that the conjugates did not cause toxicity to the mice, unlike free Dau.

Based on these results, we can conclude that both GnRH-III conjugates, as well as free Dau inhibited the tumor growth efficiently in HT-29 CRC bearing mice. However, the new conjugate **2** showed the highest anti-tumor effect against CRC, while its impact on the animal body weight and liver/body weight ratio was the lowest demonstrating that compound **2** is a promising candidate for the targeted chemotherapy of CRC.

5.2 Homing Heptapeptide-Dau conjugates

As it can be seen from previous paragraphs, targeted tumor chemotherapy might be an efficient tool for cancer treatment, and development of peptide based SMDCs is a current topic in this field. In spite of the higher tissue penetration of small conjugates over ADCs, the uptake of appropriate amount of drug molecules is still problematic. Therefore, a combination of peptide based SMDCs where conjugates could contain different homing peptides recognizing different cell surface compartments attached to different drug molecules with various site of action, and discovering of new homing peptides might be necessary for effective tumor regression *in vivo*. One of the approaches to find tumor selective peptides is phage display technique[330, 331]. In this study, homing peptides with selectivity to HT-29 human CRC were searched in the literature. According to the finding by Zhang *et al*.[224], it was selected VHLGYAT heptapeptide as homing moiety which was identified from phage library. This sequence was optimized for better tumor recognition.

After series of experiments such as Ala-scan and positional scanning it was identified that the substitution of Gly with a bulky nonpolar amino acid Phe in position 4 of the homing motif, is the best choice to increase the anti-tumor activity of the conjugates where Dau is coupled through Cathepsin B cleavable spacer (LRRY) *via* oxime linkage. As one of the key point of the biological effect of the conjugates is their efficient entry into the cells, the cellular uptake experiments indicated that the cytostatic effect and the uptake of the conjugates correlate[332].

The stability of conjugates under the conditions which will be used for the biological tests, as well as their metabolism in lysosomal homogenates are very important due to difference in the stability of the conjugates might have some influence both on the *in vitro* and *in vivo* studies. It was shown that, replacing the Gly for Phe in conjugate **4**, did not only improved the anti-tumor effect, compared to conjugate **3** that contains parent homing peptide, but also the stability of the conjugates[332].

In my doctoral thesis study was measured the selectivity of the conjugates for HT-29 colon cancer cells. Also, the anti-tumor activity of conjugate **3** with the parent homing peptide and conjugate **4** (G/F) were measured on 22 different cancer cell lines and on MRC-5 human fibroblast cells. Surprisingly, both conjugates showed one of the highest IC_{50} values on HT-29 cells. Therefore, we can draw a conclusion, that the targeting moiety is not selective to HT-29 colon cancer cells. Nevertheless, conjugate **4** was more effective

than conjugate **3** on all cancer cell lines. The lowest effect was observed on fibroblast cells, suggesting selectivity of the conjugates to tumor cells.

The selectivity of the VHLGYAT heptapeptide was not checked earlier on a broad spectrum of tumor types. Interestingly, the measurements on different types of cancer cell lines did not show selectivity to HT-29 cells. Therefore, sequence homology was searched in the literature. In the manuscript published by Fourie *et al.*, two peptides A6R (AS**HL**GLAR) and HbS (**VHL**TPVEK) were found that have overlapping sequence with the parent peptide (see in bold)[333]. Both of them bind to heat shock protein 70 (Hsp70). According to the literature, Hsp70 is overexpressed in a large variety of different tumor types and it is localized not only intracellularly, but also tumor selective Hsp70 expression in the plasma membrane was determined[334]. A membrane Hsp70 positive tumor phenotype is associated with aggressiveness and therapy resistance of cancer and the membrane-bound Hsp70 plays a pivotal role in eliciting anti-tumor immune response. Furthermore, it can be a good target for targeted tumor therapy. All of these make a strong suggestion that VHLGYAT based homing peptides might recognize membrane-bound Hsp70.

The *in vivo* tumor growth inhibition effect of two conjugates was measured on orthotopically developed HT-29 colon cancer bearing mice. The results indicate that modified conjugate **4** has better anti-tumor effect than conjugate **3** with the parent homing peptide. This effect was similar to the activity of the free Dau used in maximal tolerated dose, however, much lower liver toxicity was observed in the groups treated with the conjugates, even content of Dau was 10 times higher. In addition to the other positive quality of conjugate **4**, much lower proliferation index was obtained in tumors in the group treated with conjugate **4** than in groups treated with conjugate **3** which resulted in significantly higher tumor growth inhibition.

Based on this study, it is worth to modify tumor homing peptides selected by phage display technique for the development of small molecule drug conjugates with increased bioactivity and stability that can be applied efficiently for targeted tumor therapy. In addition, the selectivity of the conjugates has to be determined. In this case, it seems that the homing peptide selected by phage display has affinity to a broad spectrum of different tumor cells that might be related to the cell surface protein Hsp70 as possible target, which can be evaluated in further studies.

5.3 Vindoline CPP conjugates

Vinca alkaloids such as Vinblastine and Vincristine are effective and clinically used anti-tumor drugs. Contrary, one of the major alkaloid, Vindoline, has no anti-tumor activity[269, 270].

Previously wide range of Vindoline derivatives were synthesized and their *in vitro* cytostatic activity was characterized[272]. While Vindoline has no anti-tumor activity[335], some of its derivatives, such as methyl esters, Br-Vindoline-*L*/*D*-Trp-OMe, showed modest activity. Thus, these compounds might provide new avenues for tumor treatment with a new group of Vinca alkaloid derivatives. The mechanism of action of this novel Vindoline derivatives could be different from the clinically used compounds (Vinblastine, Vincristine) and could result in more efficient treatment.

It was observed earlier that conjugation of Vinblastine to octaarginine retains the drug anti-tumor activity, and the conjugates showed selectivity for inhibiting the mitotic spindle formation[264]. Thus, Br-Vindoline-*L*/*D*-Trp-OMe derivatives were selected for studying the effect of conjugation with oligoarginine as a CPP.

Considering that Vinca alkaloids are typically used in the treatment of cancerous diseases of the blood (leukemias, non-Hodgkin's lymphoma, Hodgkin's disease)[336], the effect of conjugation with tetra-, hexa-, and octaarginine was previously studied on HL-60 cells[337]. It was obtained that cytostatic effect of oligoarginine bearing compounds was drastically increased in comparison to the effect of methyl esters or a carboxylic acid derivative. Although all conjugates had a cytostatic effect, the conjugate with octaarginine showed the highest activity.

For analyzing the anti-tumor activity, 10-Br-Vindoline, 10-Br-Vindoline-*L*-Trp-OH and 10-Br-Vindoline-*D*-Trp-OH were conjugated with octaarginine. The effect of the chirality of Trp on the biological activity was investigated. For conjugation, Br-Vindoline derivatives were coupled to the N-terminal amino group of octaarginine in solution. In this research also were tested octaarginine free compounds such as Vindoline, 10-Br-Vindoline and Br-Vindoline-*L*/*D*-Trp-OMe derivatives, in order to compare with them the anti-tumor activity of CPP coupled compounds.

Previously, octaarginine conjugates, Br-Vindoline derivatives with/without Trp residue, and free Vindoline was studied *in vitro*. These conjugates showed an anti-tumor effect *in vitro* on human leukemia (HL-60) and BC (MDA-MB-231 and MCF-7) cells,

DOI:10.14753/SE.2021.2555

where although the Trp-OMe residue increased the efficacy of Br-Vindoline, it had no influence in the conjugates on HL-60 and MDA-MB-231 cells, and all conjugates had essentially the same activity. In contrast, conjugates with Trp were more active on the MCF-7 cells, but the cytostatic activity was independent of the configuration of Trp moiety[337].

In my doctoral thesis study, the anti-tumor effect of octaarginine conjugates and Br-Vindoline derivatives with/without Trp residue, as well as free Vindoline was studied on mouse leukemia (P388) and mouse colon carcinoma cells (C26). On these cells, free Vindoline and 10-Br-Vindoline did not show anti-tumor activity as expected, while all conjugates have cytostatic effect. Presence of Trp increased anti-tumor activity as previously, but in these two cell lines the configuration has influenced the activity. Conjugate with *L*-Trp was twice or six times more potent on P388 and C26 cells, respectively, than its *D*-Trp counterpart, with IC_{50} in low micromolar range. These promising data showed that the conjugation with octaarginine, the well-known CPP, increased the activity of the moderately active Vindoline derivatives. Both, the presence of Trp and octaarginine could increase the cellular uptake and thus the activity of Br-Vindoline derivatives[338].

Based on encouraging *in vitro* results, the Br-Vind-*L*-Trp-Arg_8 (**5**) and Br-Vind-*D*-Trp-Arg_8 (**6**) conjugates were selected for *in vivo* studies using P388 and C26 tumor bearing mouse models, where Vinblastine as a clinically used drug was used as positive control.

It was observed that the configuration of Trp had the impact on the activity, where the conjugate **5** containing *L*-isomer was more active than the conjugate **6** containing *D*-isomer. This phenomenon could be interpreted by an interaction between the Br-Vindoline and octaarginine part, which is influenced by the configuration of the Trp.

The treatment with conjugate containing *L*-Trp showed higher inhibition of the P388 leukemia tumor growth than conjugate with *D*-Trp, and than Vinblastine, a known cytostatic agent, suggesting potent anti-tumor activity of *L*-Trp containing Vindoline-octaarginine CPP conjugate. In comparison, the tumor volume inhibitory effect of both conjugates was lower in C26 murine colon carcinoma bearing mice than in leukemia, although were more treatments, where also *L*-Trp containing conjugate showed higher anti-tumor activity than *D*-Trp containing counterpart, in a dose dependent manner.

Considering that *L*-Trp containing CPP conjugate showed higher anti-tumor effect than free Vinblastine in both models, it can be suggested that this strategy promotes Vindoline anti-tumor activity, although native Vindoline is not anti-tumorigenic.

It is also worth to mention that the effectiveness of L-isomer conjugate **5** over the D-isomer conjugate **6** in *in vivo* studies was correlated with obtained *in vitro* results. Based on the number of administrations, doses and times of administration, we can conclude that the isomer related effect is also dependent on the properties of the tumor investigated.

Although both conjugates were significantly potent *in vitro* on cells, especially conjugate **5**, even high tumor volume inhibition was not statistically significant *in vivo*. This non-significance can be attributed to properties of tumors investigated, where even free Vinblastine inhibited tumor volume with much lower potency than both conjugates. *In vivo* results presented here, might also indicate the anti-tumor potency of conjugate, especially against leukemia. It is important to emphasize that the role of CPP part of conjugates, octaarginine, might increase the tumor accumulation of conjugates[339].

In both *in vivo* tumor models conjugates did not show adverse effect on animal body weight, general looking and behavior of experimental animals, even at the dose of 20 mg/kg, revealing that conjugates are not toxic and that do not cause side-effects to the animal.

This study demonstrated that Vindoline, the main alkaloid component of *Catharanthus roseus*, and precursor of Vinblastine, has a potency to be chemically transformed to an active anti-cancer agent. To the best of our knowledge, the conjugates studied here are the first Vindoline derivatives with significant *in vitro* anti-tumor activity. This results indicate that conjugation with octaarginine as CPP could increases the *in vitro* anti-tumor effect of the free Vindoline derivative. It is attractive to hypothesize that the Vidoline-based derivatives/conjugates could be considered as a potential new member of the Vinca alkaloid family with different tumor cell targeting mechanism.

DOI:10.14753/SE.2021.2555

6 CONCLUSIONS

6.1 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.

6.2 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.

6.3 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.

DOI:10.14753/SE.2021.2555

7 SUMMARY

Among various homing devices, gonadotropin-releasing hormone-III (GnRH-III) peptide represents a suitable targeting moiety for drug delivery systems in cancer therapy. The study of the previously developed GnRH-III-[^{4}Lys(Bu),^{8}Lys(Dau=Aoa)] conjugate and the novel synthesized GnRH-III-[$^{2}\Delta$His,^{3}*D*-Tic,^{4}Lys(Bu),^{8}Lys(Dau=Aoa)] conjugate, containing the anti-cancer drug Daunorubicin (Dau) demonstrated that both GnRH-III-Dau conjugates possess an efficient anti-tumor effect on more than 20 cancer cell lines, whereby the biological activity is strongly connected to the expression of gonadotropin-releasing hormone receptors (GnRH-R), where the novel conjugate showed a higher *in vitro* anti-proliferative activity and a higher uptake capacity. Moreover, the treatment with GnRH-III-Dau conjugates cause a significant *in vivo* tumor growth and metastases inhibitory effect in three different orthotopic models, including 4T1 murine and MDA-MB-231 human breast carcinoma, as well as HT-29 human colorectal cancer bearing BALB/c and SCID mice, while toxic side-effects were substantially reduced in comparison to the treatment with free drug, suggesting conjugates as a highly promising candidates for targeted tumor therapy in both colon cancer and metastatic breast cancer.

In this study also VHLGYAT heptapeptide selected by phage display technique for HT-29 human colon cancer was investigated as homing peptide for drug delivery. Modified conjugate in which Gly was replaced by Phe amino acid, Dau=Aoa-LRRY-VHLFYAT-NH_2, provided much higher anti-tumor efficacy than conjugate with parent sequence, in various cancer cell lines, and showed significant tumor growth inhibition of orthotopically developed HT-29 colon cancer in mice, with much less toxic side-effects compared to the free drug, illustrating that it is worth to modify tumor homing peptides selected by phage display technique for development of small molecule drug conjugates with increased bioactivity which can be applied efficiently for targeted tumor therapy.

In vitro and *in vivo* studies on murine leukemia P388 and murine colon C26 tumor models indicate that the modification of inactive Vindoline and conjugation to cell penetrating peptide (CPP) octa-arginine could result in an effective conjugate which promotes Vindoline anti-tumor activity, which is dependent and notably influenced by the presence and configuration of Trp in the conjugate, where conjugate with *L*-Trp configuration is more active than conjugate with the *D*-isomer, providing a new strategy for improving the drugs efficacy in tumor targeting treatments.

DOI:10.14753/SE.2021.2555

8 ÖSSZEFOGLALÁS

A különféle irányító molekulák közül a gonadotropin-releasing hormon-III (GnRH)-III (GnRH-III) peptid egy ígéretes hodozópeptid az irányított tumor terápiában. A Daunorubicin (Dau) gyógyszerhatóanyagót tartalmazó, korábban kifejlesztett GnRH-III-[^{4}Lys(Bu), ^{8}Lys(Dau=Aoa)] és az újonnan szintetizált GnRH-III-[$^{2}\Delta$His,^{3}*D*-Tic,^{4}Lys(Bu),^{8}Lys(Dau=Aoa)] konjugátumok esetében kimutatható, hogy mindkét GnRH-III-Dau konjugátum hatékony tumorellenes hatást fejt ki több mint 20 rákos sejtvonalon. A biológiai aktivitás szorosan kapcsolódik a gonadotropin-felszabadító hormon receptorok (GnRH-R) expressziójához. Az új konjugátum magasabb *in vitro* antiproliferatív aktivitást és nagyobb sejtfelvételt mutatott. Továbbá, a GnRH-III-Dau konjugátumokkal végzett kezelés szignifikáns *in vivo* tumor növekedés- és metasztázist gátló hatást vált ki három különböző BALB/c és SCID egerekbe táplált ortotróp tumormodellben: a 4T1 egér és az MDA-MB-231 humán emlő karcinóma, valamint a HT-29 humán vastagbélrák. A toxikus mellékhatások lényegesen csökkentek a szabad hatóanyaggal végzett kezeléshez képest, ami arra utal, hogy a konjugátumok ígéretes jelöltek a célzott tumor terápiában mind a vastagbélrák, mind az áttétes emlőrák esetében.

A HT-29 humán vastagbélrákra specifikus fágkönyvtárból kiválasztott VHLGYAT heptapeptidet is vizsgálták, mint irányító peptidet. Az a konjugátum analóg, amelyben a Gly-t Phe aminosavra cserélték, Dau = Aoa-LRRY-VHLFYAT-NH_2, sokkal nagyobb tumorellenes hatást mutatott a különböző rákos sejtvonalakban, mint a natív szekvenciát tartalmazó konjugátum. Továbbá, ez utóbbi, jóval kevesebb toxikus mellékhatást okozott, mint a hatóanyag az ortotróp HT-29 vastagbélrák tumorban, szemléltetve, hogy érdemes módosítani a fág technikával kiválasztott irányító peptideket, mert ezek a módosítások hatékonyabb bioaktivitást és célzottabb terápiás lehetőségeket biztosíthatnak a kismolekulás gyógyszerkonjugátumok fejlesztésében.

Az egér leukémia P388 és az egér vastagbél C26 tumor modelljein végzett *in vitro* és *in vivo* vizsgálatok azt mutatják, hogy az inaktív Vindoline módosítása és az okta-arginin sejtpenetráló peptidhez (CPP) való konjugálása hatékony konjugátumot eredményezhet. Ez a Trp jelenlététől és konfigurációjától függ, az *L*-Trp konfigurációval rendelkező konjugátum aktívabb, mint a *D*-izomert tartalmazó, ezzel új stratégiát kínálva a gyógyszerek hatékonyságának javítására különböző tumorellenes kezelésekben.

9 BIBLIOGRAPHY

1. Hanahan D, & Weinberg RA (2011) Hallmarks of cancer: the next generation *Cell* 144(5) 646–674, https://doi.org/10.1016/j.cell.2011.02.013.

2. Negrini S, Gorgoulis VG, & Halazonetis TD (2010) Genomic instability--an evolving hallmark of cancer *Nat Rev Mol Cell Biol* 11(3) 220–228, https://doi.org/10.1038/nrm2858.

3. Evan G, & Littlewood T (1998) A matter of life and cell death *Science* 281(5381) 1317–1322, https://doi.org/10.1126/science.281.5381.1317.

4. Sherr CJ, & McCormick F (2002) The RB and p53 pathways in cancer *Cancer Cell* 2(2) 103–112, https://doi.org/10.1016/s1535-6108(02)00102-2.

5. Carmeliet P (2005) VEGF as a key mediator of angiogenesis in cancer *Oncology* 69 Suppl 3 4–10, https://doi.org/10.1159/000088478.

6. Fidler IJ (2003) The pathogenesis of cancer metastasis: the “seed and soil” hypothesis revisited *Nat Rev Cancer* 3(6) 453–458, https://doi.org/10.1038/nrc1098.

7. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, & Thompson CB (2008) The biology of cancer: metabolic reprogramming fuels cell growth and proliferation *Cell Metab* 7(1) 11–20, https://doi.org/10.1016/j.cmet.2007.10.002.

8. Kim R, Emi M, & Tanabe K (2007) Cancer immunoediting from immune surveillance to immune escape *Immunology* 121(1) 1–14, https://doi.org/10.1111/j.1365-2567.2007.02587.x.

9. Seyfried TN, & Huysentruyt LC (2013) On the origin of cancer metastasis *Crit Rev Oncog* 18(1–2) 43–73, https://doi.org/10.1615/critrevoncog.v18.i1-2.40.

10. Dagogo-Jack I, & Shaw AT (2018) Tumour heterogeneity and resistance to cancer therapies *Nature Reviews Clinical Oncology* 15(2) 81–94, https://doi.org/10.1038/nrclinonc.2017.166.

11. Balkwill FR, Capasso M, & Hagemann T (2012) The tumor microenvironment at a glance *J Cell Sci* 125(Pt 23) 5591–5596, https://doi.org/10.1242/jcs.116392.

12. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, & Jemal A (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries *CA Cancer J Clin* 68(6) 394–424, https://doi.org/10.3322/caac.21492.

13. Lauby-Secretan B, Scoccianti C, Loomis D, Benbrahim-Tallaa L, Bouvard V, Bianchini F, Straif K, & International Agency for Research on Cancer Handbook Working Group (2015) Breast-cancer screening--viewpoint of the IARC Working Group *N Engl J Med* 372(24) 2353–2358, https://doi.org/10.1056/NEJMsr1504363.

14. Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, Ruddy K, Tsang J, & Cardoso F (2019) Breast cancer *Nat Rev Dis Primers* 5(1) 66, https://doi.org/10.1038/s41572-019-0111-2.

15. Shiovitz S, & Korde LA (2015) Genetics of breast cancer: a topic in evolution *Ann Oncol* 26(7) 1291–1299, https://doi.org/10.1093/annonc/mdv022.

16. Early Breast Cancer Trialists' Collaborative Group (EBCTCG) (2015) Aromatase inhibitors versus tamoxifen in early breast cancer: patient-level meta-analysis of the randomised trials *The Lancet* 386(10001) 1341–1352, https://doi.org/10.1016/S0140-6736(15)61074-1.

17. Gianni L, Pienkowski T, Im Y-H, Tseng L-M, Liu M-C, Lluch A, Starosławska E, Haba-Rodriguez J de la, Im S-A, Pedrini JL, Poirier B, Morandi P, Semiglazov V, Srimuninnimit V, Bianchi GV, Magazzù D, McNally V, Douthwaite H, Ross G, & Valagussa P (2016) 5-year analysis of neoadjuvant pertuzumab and trastuzumab in patients with locally advanced, inflammatory, or early-stage HER2-positive breast cancer (NeoSphere): a multicentre, open-label, phase 2 randomised trial *The Lancet Oncology* 17(6) 791–800, https://doi.org/10.1016/S1470-2045(16)00163-7.

18. Burstein MD, Tsimelzon A, Poage GM, Covington KR, Contreras A, Fuqua SAW, Savage MI, Osborne CK, Hilsenbeck SG, Chang JC, Mills GB, Lau CC, & Brown

PH (2015) Comprehensive genomic analysis identifies novel subtypes and targets of triple-negative breast cancer *Clin Cancer Res* 21(7) 1688–1698, https://doi.org/10.1158/1078-0432.CCR-14-0432.

19. Kwok CW, Treeck O, Buchholz S, Seitz S, Ortmann O, & Engel JB (2015) Receptors for luteinizing hormone-releasing hormone (GnRH) as therapeutic targets in triple negative breast cancers (TNBC) *Targ Oncol* 10(3) 365–373, https://doi.org/10.1007/s11523-014-0340-y.

20. Seitz S, Buchholz S, Schally AV, Weber F, Klinkhammer-Schalke M, Inwald EC, Perez R, Rick FG, Szalontay L, Hohla F, Segerer S, Kwok CW, Ortmann O, & Engel JB (2014) Triple negative breast cancers express receptors for LHRH and are potential therapeutic targets for cytotoxic LHRH-analogs, AEZS 108 and AEZS 125 *BMC Cancer* 14(1) 847, https://doi.org/10.1186/1471-2407-14-847.

21. Keum N, & Giovannucci E (2019) Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies *Nat Rev Gastroenterol Hepatol* 16(12) 713–732, https://doi.org/10.1038/s41575-019-0189-8.

22. Fleming M, Ravula S, Tatishchev SF, & Wang HL (2012) Colorectal carcinoma: Pathologic aspects *J Gastrointest Oncol* 3(3) 153–173, https://doi.org/10.3978/j.issn.2078-6891.2012.030.

23. Henrikson NB, Webber EM, Goddard KA, Scrol A, Piper M, Williams MS, Zallen DT, Calonge N, Ganiats TG, Janssens ACJW, Zauber A, Lansdorp-Vogelaar I, van Ballegooijen M, & Whitlock EP (2015) Family history and the natural history of colorectal cancer: systematic review *Genet Med* 17(9) 702–712, https://doi.org/10.1038/gim.2014.188.

24. Armaghany T, Wilson JD, Chu Q, & Mills G (2012) Genetic alterations in colorectal cancer *Gastrointest Cancer Res* 5(1) 19–27.

25. Riihimäki M, Hemminki A, Sundquist J, & Hemminki K (2016) Patterns of metastasis in colon and rectal cancer *Sci Rep* 6 29765, https://doi.org/10.1038/srep29765.

26. Sveen A, Kopetz S, & Lothe RA (2020) Biomarker-guided therapy for colorectal cancer: strength in complexity *Nat Rev Clin Oncol* 17(1) 11–32, https://doi.org/10.1038/s41571-019-0241-1.

27. Szepeshazi K, Schally AV, & Halmos G (2007) LH-RH receptors in human colorectal cancers: unexpected molecular targets for experimental therapy *Int J Oncol* 30(6) 1485–1492, https://doi.org/10.3892/ijo.30.6.1485.

28. Aguilar-Rojas A, & Huerta-Reyes M (2009) Human gonadotropin-releasing hormone receptor-activated cellular functions and signaling pathways in extra-pituitary tissues and cancer cells (Review) *Oncol Rep* 22(5) 981–990, https://doi.org/10.3892/or_00000525.

29. Juliusson G, & Hough R (2016) Leukemia *Prog Tumor Res* 43 87–100, https://doi.org/10.1159/000447076.

30. Terwilliger T, & Abdul-Hay M (2017) Acute lymphoblastic leukemia: a comprehensive review and 2017 update *Blood Cancer J* 7(6) e577, https://doi.org/10.1038/bcj.2017.53.

31. Hallek M (2019) Chronic lymphocytic leukemia: 2020 update on diagnosis, risk stratification and treatment *Am J Hematol* 94(11) 1266–1287, https://doi.org/10.1002/ajh.25595.

32. De Kouchkovsky I, & Abdul-Hay M (2016) "Acute myeloid leukemia: a comprehensive review and 2016 update" *Blood Cancer J* 6(7) e441, https://doi.org/10.1038/bcj.2016.50.

33. Estey EH (2014) Acute myeloid leukemia: 2014 update on risk-stratification and management *Am J Hematol* 89(11) 1063–1081, https://doi.org/10.1002/ajh.23834.

34. Verma M (2012) Personalized Medicine and Cancer *J Pers Med* 2(1) 1–14, https://doi.org/10.3390/jpm2010001.

35. Urruticoechea A, Alemany R, Balart J, Villanueva A, Viñals F, & Capellá G (2010) Recent advances in cancer therapy: an overview *Curr Pharm Des* 16(1) 3–10, https://doi.org/10.2174/138161210789941847.

36. DeVita VT, & Chu E (2008) A history of cancer chemotherapy *Cancer Res* 68(21) 8643–8653, https://doi.org/10.1158/0008-5472.CAN-07-6611.

37. Chabner BA, & Roberts TG (2005) Timeline: Chemotherapy and the war on cancer *Nat Rev Cancer* 5(1) 65–72, https://doi.org/10.1038/nrc1529.

38. Espinosa E, Zamora P, Feliu J, & González Barón M (2003) Classification of anticancer drugs--a new system based on therapeutic targets *Cancer Treat Rev* 29(6) 515–523, https://doi.org/10.1016/s0305-7372(03)00116-6.

39. Binaschi M, Zunino F, & Capranico G (1995) Mechanism of action of DNA topoisomerase inhibitors *Stem Cells* 13(4) 369–379, https://doi.org/10.1002/stem.5530130408.

40. Dezhenkova LG, Tsvetkov VB, & Shtil AA (2014) Topoisomerase I and II inhibitors: chemical structure, mechanisms of action and role in cancer chemotherapy *Russ Chem Rev* 83(1) 82, https://doi.org/10.1070/RC2014v083n01ABEH004363.

41. Hait WN, Rubin E, Alli E, & Goodin S (2007) Tubulin Targeting Agents *Update on Cancer Therapeutics* 2(1) 1–18, https://doi.org/10.1016/j.uct.2006.10.001.

42. Martino E, Casamassima G, Castiglione S, Cellupica E, Pantalone S, Papagni F, Rui M, Siciliano AM, & Collina S (2018) Vinca alkaloids and analogues as anti-cancer agents: Looking back, peering ahead *Bioorg Med Chem Lett* 28(17) 2816–2826, https://doi.org/10.1016/j.bmcl.2018.06.044.

43. Carelle N, Piotto E, Bellanger A, Germanaud J, Thuillier A, & Khayat D (2002) Changing patient perceptions of the side effects of cancer chemotherapy *Cancer* 95(1) 155–163, https://doi.org/10.1002/cncr.10630.

44. Lammers T, Kiessling F, Hennink WE, & Storm G (2012) Drug targeting to tumors: principles, pitfalls and (pre-) clinical progress *J Control Release* 161(2) 175–187, https://doi.org/10.1016/j.jconrel.2011.09.063.

45. Kerru N, Singh P, Koorbanally N, Raj R, & Kumar V (2017) Recent advances (2015-2016) in anticancer hybrids *Eur J Med Chem* 142 179–212, https://doi.org/10.1016/j.ejmech.2017.07.033.

46. Muller PY, & Milton MN (2012) The determination and interpretation of the therapeutic index in drug development *Nature Reviews Drug Discovery* 11(10) 751–761, https://doi.org/10.1038/nrd3801.

47. Schirrmacher V (2019) From chemotherapy to biological therapy: A review of novel concepts to reduce the side effects of systemic cancer treatment (Review) *Int J Oncol* 54(2) 407–419, https://doi.org/10.3892/ijo.2018.4661.

48. Panowski S, Bhakta S, Raab H, Polakis P, & Junutula JR (2014) Site-specific antibody drug conjugates for cancer therapy *MAbs* 6(1) 34–45, https://doi.org/10.4161/mabs.27022.

49. Bayat Mokhtari R, Homayouni TS, Baluch N, Morgatskaya E, Kumar S, Das B, & Yeger H (2017) Combination therapy in combating cancer *Oncotarget* 8(23) 38022–38043, https://doi.org/10.18632/oncotarget.16723.

50. Xie X, Wu Y, Luo S, Yang H, Li L, Zhou S, Shen R, & Lin H (2017) Efficacy and Toxicity of Low-Dose versus Conventional-Dose Chemotherapy for Malignant Tumors: a Meta-Analysis of 6 Randomized Controlled Trials *Asian Pac J Cancer Prev* 18(2) 479–484, https://doi.org/10.22034/APJCP.2017.18.2.479.

51. Kratz F, Müller IA, Ryppa C, & Warnecke A (2008) Prodrug strategies in anticancer chemotherapy *ChemMedChem* 3(1) 20–53, https://doi.org/10.1002/cmdc.200700159.

52. Krall N, Scheuermann J, & Neri D (2013) Small targeted cytotoxics: current state and promises from DNA-encoded chemical libraries *Angew Chem Int Ed Engl* 52(5) 1384–1402, https://doi.org/10.1002/anie.201204631.

53. Miller ML, Shizuka M, Wilhelm A, Salomon P, Reid EE, Lanieri L, Sikka S, Maloney EK, Harvey L, Qiu Q, Archer KE, Bai C, Vitharana D, Harris L, Singh R, Ponte JF, Yoder NC, Kovtun Y, Lai KC, Ab O, Pinkas J, Keating TA, & Chari RVJ (2018) A DNA-Interacting Payload Designed to Eliminate Cross-Linking Improves the Therapeutic Index of Antibody-Drug Conjugates (ADCs) *Mol Cancer Ther* 17(3) 650–660, https://doi.org/10.1158/1535-7163.MCT-17-0940.

54. Akaiwa M, Dugal-Tessier J, & Mendelsohn BA (2020) Antibody-Drug Conjugate Payloads; Study of Auristatin Derivatives *Chem Pharm Bull* 68(3) 201–211, https://doi.org/10.1248/cpb.c19-00853.

55. Chari RVJ, Miller ML, & Widdison WC (2014) Antibody-drug conjugates: an emerging concept in cancer therapy *Angew Chem Int Ed Engl* 53(15) 3796–3827, https://doi.org/10.1002/anie.201307628.

56. Baudino TA (2015) Targeted Cancer Therapy: The Next Generation of Cancer Treatment *Curr Drug Discov Technol* 12(1) 3–20, https://doi.org/10.2174/1570163812666150602144310.

57. Dai L, Liu J, Luo Z, Li M, & Cai K (2016) Tumor therapy: targeted drug delivery systems *J Mater Chem B* 4(42) 6758–6772, https://doi.org/10.1039/c6tb01743f.

58. Syn NL-X, Yong W-P, Goh B-C, & Lee S-C (2016) Evolving landscape of tumor molecular profiling for personalized cancer therapy: a comprehensive review *Expert Opin Drug Metab Toxicol* 12(8) 911–922, https://doi.org/10.1080/17425255.2016.1196187.

59. Cazzamalli S, Corso AD, & Neri D (2017) Targeted Delivery of Cytotoxic Drugs: Challenges, Opportunities and New Developments *Chimia (Aarau)* 71(10) 712–715, https://doi.org/10.2533/chimia.2017.712.

60. Richter M, & Zhang H (2005) Receptor-targeted cancer therapy *DNA Cell Biol* 24(5) 271–282, https://doi.org/10.1089/dna.2005.24.271.

61. Tu S-M, Bilen MA, & Tannir NM (2016) Personalised cancer care: promises and challenges of targeted therapy *J R Soc Med* 109(3) 98–105, https://doi.org/10.1177/0141076816631154.

62. Gharwan H, & Groninger H (2016) Kinase inhibitors and monoclonal antibodies in oncology: clinical implications *Nat Rev Clin Oncol* 13(4) 209–227, https://doi.org/10.1038/nrclinonc.2015.213.

63. Vrettos EI, Mező G, & Tzakos AG (2018) On the design principles of peptide–drug conjugates for targeted drug delivery to the malignant tumor site *Beilstein J Org Chem* 14 930–954, https://doi.org/10.3762/bjoc.14.80.

64. Leamon CP, Lovejoy CD, & Nguyen B (2013) Patient selection and targeted treatment in the management of platinum-resistant ovarian cancer *Pharmgenomics Pers Med* 6 113–125, https://doi.org/10.2147/PGPM.S24943.

65. Huang M, Shen A, Ding J, & Geng M (2014) Molecularly targeted cancer therapy: some lessons from the past decade *Trends Pharmacol Sci* 35(1) 41–50, https://doi.org/10.1016/j.tips.2013.11.004.

66. Sun G-C, Yang X, Yu Y, & Zhao D-W (2015) The Applications of Targeting Anti-Cancer Agents in Cancer Therapeutics *Anticancer Agents Med Chem* 15(7) 869–880, https://doi.org/10.2174/1871520615666150318101845.

67. Zhang J, Yang PL, & Gray NS (2009) Targeting cancer with small molecule kinase inhibitors *Nat Rev Cancer* 9(1) 28–39, https://doi.org/10.1038/nrc2559.

68. Ferguson FM, & Gray NS (2018) Kinase inhibitors: the road ahead *Nat Rev Drug Discov* 17(5) 353–377, https://doi.org/10.1038/nrd.2018.21.

69. Bhullar KS, Lagarón NO, McGowan EM, Parmar I, Jha A, Hubbard BP, & Rupasinghe HPV (2018) Kinase-targeted cancer therapies: progress, challenges and future directions *Mol Cancer* 17(1) 48, https://doi.org/10.1186/s12943-018-0804-2.

70. Sun J, Wei Q, Zhou Y, Wang J, Liu Q, & Xu H (2017) A systematic analysis of FDA-approved anticancer drugs *BMC Syst Biol* 11(Suppl 5) 87, https://doi.org/10.1186/s12918-017-0464-7.

71. Fabbro D (2015) 25 years of small molecular weight kinase inhibitors: potentials and limitations *Mol Pharmacol* 87(5) 766–775, https://doi.org/10.1124/mol.114.095489.

72. Dillman RO (1999) Infusion reactions associated with the therapeutic use of monoclonal antibodies in the treatment of malignancy *Cancer Metastasis Rev* 18(4) 465–471, https://doi.org/10.1023/a:1006341717398.

73. Nelson PN, Reynolds GM, Waldron EE, Ward E, Giannopoulos K, & Murray PG (2000) Monoclonal antibodies *MP, Mol Pathol* 53(3) 111–117, https://doi.org/10.1136/mp.53.3.111.

74. Scott AM, Wolchok JD, & Old LJ (2012) Antibody therapy of cancer *Nat Rev Cancer* 12(4) 278–287, https://doi.org/10.1038/nrc3236.

75. Escrivá-de-Romaní S, Arumí M, Bellet M, & Saura C (2018) HER2-positive breast cancer: Current and new therapeutic strategies *The Breast* 39 80–88, https://doi.org/10.1016/j.breast.2018.03.006.

76. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, Diéras V, Hegg R, Im S-A, Shaw Wright G, Henschel V, Molinero L, Chui SY, Funke R, Husain A, Winer EP, Loi S, Emens LA, & IMpassion130 Trial Investigators (2018) Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer *N Engl J Med* 379(22) 2108–2121, https://doi.org/10.1056/NEJMoa1809615.

77. Schmid P, Cortés J, Dent R, Pusztai L, McArthur HL, Kuemmel S, Bergh J, Denkert C, Park YH, Hui R, Harbeck N, Takahashi M, Foukakis T, Fasching PA, Cardoso F, Jia L, Karantza V, Zhao J, Aktan G, & O'Shaughnessy J (2019) LBA8_PR - KEYNOTE-522: Phase III study of pembrolizumab (pembro) + chemotherapy (chemo) vs placebo (pbo) + chemo as neoadjuvant treatment, followed by pembro

vs pbo as adjuvant treatment for early triple-negative breast cancer (TNBC) *Annals of Oncology* 30 v853–v854, https://doi.org/10.1093/annonc/mdz394.003.

78. Charmsaz S, Scott AM, & Boyd AW (2017) Targeted therapies in hematological malignancies using therapeutic monoclonal antibodies against Eph family receptors *Experimental Hematology* 54 31–39, https://doi.org/10.1016/j.exphem.2017.07.003.

79. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, & Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer *N Engl J Med* 350(23) 2335–2342, https://doi.org/10.1056/NEJMoa032691.

80. Van Cutsem E, Tabernero J, Lakomy R, Prenen H, Prausová J, Macarulla T, Ruff P, van Hazel GA, Moiseyenko V, Ferry D, McKendrick J, Polikoff J, Tellier A, Castan R, & Allegra C (2012) Addition of aflibercept to fluorouracil, leucovorin, and irinotecan improves survival in a phase III randomized trial in patients with metastatic colorectal cancer previously treated with an oxaliplatin-based regimen *J Clin Oncol* 30(28) 3499–3506, https://doi.org/10.1200/JCO.2012.42.8201.

81. Tabernero J, Yoshino T, Cohn AL, Obermannova R, Bodoky G, Garcia-Carbonero R, Ciuleanu T-E, Portnoy DC, Van Cutsem E, Grothey A, Prausová J, Garcia-Alfonso P, Yamazaki K, Clingan PR, Lonardi S, Kim TW, Simms L, Chang S-C, Nasroulah F, & RAISE Study Investigators (2015) Ramucirumab versus placebo in combination with second-line FOLFIRI in patients with metastatic colorectal carcinoma that progressed during or after first-line therapy with bevacizumab, oxaliplatin, and a fluoropyrimidine (RAISE): a randomised, double-blind, multicentre, phase 3 study *Lancet Oncol* 16(5) 499–508, https://doi.org/10.1016/S1470-2045(15)70127-0.

82. Pietrantonio F, Cremolini C, Petrelli F, Di Bartolomeo M, Loupakis F, Maggi C, Antoniotti C, de Braud F, Falcone A, & Iacovelli R (2015) First-line anti-EGFR monoclonal antibodies in panRAS wild-type metastatic colorectal cancer: A

systematic review and meta-analysis *Crit Rev Oncol Hematol* 96(1) 156–166, https://doi.org/10.1016/j.critrevonc.2015.05.016.

83. Overman MJ, Lonardi S, Wong KYM, Lenz H-J, Gelsomino F, Aglietta M, Morse MA, Van Cutsem E, McDermott R, Hill A, Sawyer MB, Hendlisz A, Neyns B, Svrcek M, Moss RA, Ledeine J-M, Cao ZA, Kamble S, Kopetz S, & André T (2018) Durable Clinical Benefit With Nivolumab Plus Ipilimumab in DNA Mismatch Repair-Deficient/Microsatellite Instability-High Metastatic Colorectal Cancer *J Clin Oncol* 36(8) 773–779, https://doi.org/10.1200/JCO.2017.76.9901.

84. Jabbour E, O'Brien S, Ravandi F, & Kantarjian H (2015) Monoclonal antibodies in acute lymphoblastic leukemia *Blood* 125(26) 4010–4016, https://doi.org/10.1182/blood-2014-08-596403.

85. Li F, Zhao C, & Wang L (2014) Molecular-targeted agents combination therapy for cancer: developments and potentials *Int J Cancer* 134(6) 1257–1269, https://doi.org/10.1002/ijc.28261.

86. Kaplon H, & Reichert JM (2019) Antibodies to watch in 2019 *MAbs* 11(2) 219–238, https://doi.org/10.1080/19420862.2018.1556465.

87. Strebhardt K, & Ullrich A (2008) Paul Ehrlich's magic bullet concept: 100 years of progress *Nat Rev Cancer* 8(6) 473–480, https://doi.org/10.1038/nrc2394.

88. Allen TM (2002) Ligand-targeted therapeutics in anticancer therapy *Nat Rev Cancer* 2(10) 750–763, https://doi.org/10.1038/nrc903.

89. Wong PT, & Choi SK (2015) Mechanisms of drug release in nanotherapeutic delivery systems *Chem Rev* 115(9) 3388–3432, https://doi.org/10.1021/cr5004634.

90. Srinivasarao M, Galliford CV, & Low PS (2015) Principles in the design of ligand-targeted cancer therapeutics and imaging agents *Nat Rev Drug Discov* 14(3) 203–219, https://doi.org/10.1038/nrd4519.

91. Attia MF, Anton N, Wallyn J, Omran Z, & Vandamme TF (2019) An overview of active and passive targeting strategies to improve the nanocarriers efficiency to

tumour sites *J Pharm Pharmacol* 71(8) 1185–1198, https://doi.org/10.1111/jphp.13098.

92. Iyer AK, Khaled G, Fang J, & Maeda H (2006) Exploiting the enhanced permeability and retention effect for tumor targeting *Drug Discov Today* 11(17–18) 812–818, https://doi.org/10.1016/j.drudis.2006.07.005.

93. Maeda H, Wu J, Sawa T, Matsumura Y, & Hori K (2000) Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review *J Control Release* 65(1–2) 271–284, https://doi.org/10.1016/s0168-3659(99)00248-5.

94. Sun T, Zhang YS, Pang B, Hyun DC, Yang M, & Xia Y (2014) Engineered nanoparticles for drug delivery in cancer therapy *Angew Chem Int Ed Engl* 53(46) 12320–12364, https://doi.org/10.1002/anie.201403036.

95. Jabir NR, Anwar K, Firoz CK, Oves M, Kamal MA, & Tabrez S (2018) An overview on the current status of cancer nanomedicines *Curr Med Res Opin* 34(5) 911–921, https://doi.org/10.1080/03007995.2017.1421528.

96. Srinivasarao M, & Low PS (2017) Ligand-Targeted Drug Delivery *Chem Rev* 117(19) 12133–12164, https://doi.org/10.1021/acs.chemrev.7b00013.

97. Kiseleva RY, Glassman PM, Greineder CF, Hood ED, Shuvaev VV, & Muzykantov VR (2018) Targeting therapeutics to endothelium: are we there yet? *Drug Deliv Transl Res* 8(4) 883–902, https://doi.org/10.1007/s13346-017-0464-6.

98. Casi G, & Neri D (2015) Antibody-Drug Conjugates and Small Molecule-Drug Conjugates: Opportunities and Challenges for the Development of Selective Anticancer Cytotoxic Agents *J Med Chem* 58(22) 8751–8761, https://doi.org/10.1021/acs.jmedchem.5b00457.

99. Chalouni C, & Doll S (2018) Fate of Antibody-Drug Conjugates in Cancer Cells *J Exp Clin Cancer Res* 37(1) 20, https://doi.org/10.1186/s13046-017-0667-1.

100. Chari RVJ (1998) Targeted delivery of chemotherapeutics: tumor-activated prodrug therapy *Adv Drug Deliv Rev* 31(1–2) 89–104, https://doi.org/10.1016/s0169-409x(97)00095-1.

101. Ríhová B (1998) Receptor-mediated targeted drug or toxin delivery *Adv Drug Deliv Rev* 29(3) 273–289, https://doi.org/10.1016/s0169-409x(97)00084-7.

102. Teicher BA, & Chari RVJ (2011) Antibody conjugate therapeutics: challenges and potential *Clin Cancer Res* 17(20) 6389–6397, https://doi.org/10.1158/1078-0432.CCR-11-1417.

103. Lin JH, Guo Y, & Wang W (2018) Challenges of Antibody Drug Conjugates in Cancer Therapy: Current Understanding of Mechanisms and Future Strategies *Curr Pharmacol Rep* 4(1) 10–26, https://doi.org/10.1007/s40495-018-0122-9.

104. Ritchie M, Tchistiakova L, & Scott N (2013) Implications of receptor-mediated endocytosis and intracellular trafficking dynamics in the development of antibody drug conjugates *MAbs* 5(1) 13–21, https://doi.org/10.4161/mabs.22854.

105. Gébleux R, Stringhini M, Casanova R, Soltermann A, & Neri D (2017) Non-internalizing antibody-drug conjugates display potent anti-cancer activity upon proteolytic release of monomethyl auristatin E in the subendothelial extracellular matrix *Int J Cancer* 140(7) 1670–1679, https://doi.org/10.1002/ijc.30569.

106. Chari RVJ (2008) Targeted cancer therapy: conferring specificity to cytotoxic drugs *Acc Chem Res* 41(1) 98–107, https://doi.org/10.1021/ar700108g.

107. Beck A, Goetsch L, Dumontet C, & Corvaïa N (2017) Strategies and challenges for the next generation of antibody-drug conjugates *Nat Rev Drug Discov* 16(5) 315–337, https://doi.org/10.1038/nrd.2016.268.

108. Sammet B, Steinkühler C, & Sewald N (2012) Antibody-drug conjugates in tumor therapy *Pharm Pat Anal* 1(1) 65–73, https://doi.org/10.4155/ppa.12.4.

109. Chari RVJ (2016) Expanding the Reach of Antibody-Drug Conjugates *ACS Med Chem Lett* 7(11) 974–976, https://doi.org/10.1021/acsmedchemlett.6b00312.

110. Diamantis N, & Banerji U (2016) Antibody-drug conjugates--an emerging class of cancer treatment *Br J Cancer* 114(4) 362–367, https://doi.org/10.1038/bjc.2015.435.

111. Kue CS, Kamkaew A, Burgess K, Kiew LV, Chung LY, & Lee HB (2016) Small Molecules for Active Targeting in Cancer *Med Res Rev* 36(3) 494–575, https://doi.org/10.1002/med.21387.

112. Le Joncour V, & Laakkonen P (2018) Seek & Destroy, use of targeting peptides for cancer detection and drug delivery *Bioorg Med Chem* 26(10) 2797–2806, https://doi.org/10.1016/j.bmc.2017.08.052.

113. Chatzisideri T, Leonidis G, & Sarli V (2018) Cancer-targeted delivery systems based on peptides *Future Med Chem* 10(18) 2201–2226, https://doi.org/10.4155/fmc-2018-0174.

114. Gilad Y, Firer M, & Gellerman G (2016) Recent Innovations in Peptide Based Targeted Drug Delivery to Cancer Cells *Biomedicines* 4(2) 11, https://doi.org/10.3390/biomedicines4020011.

115. Casi G, & Neri D (2015) Noninternalizing targeted cytotoxics for cancer therapy *Mol Pharm* 12(6) 1880–1884, https://doi.org/10.1021/mp500798y.

116. Ma L, Wang C, He Z, Cheng B, Zheng L, & Huang K (2017) Peptide-Drug Conjugate: A Novel Drug Design Approach *Curr Med Chem* 24(31) 3373–3396, https://doi.org/10.2174/0929867324666170404142840.

117. Fosgerau K, & Hoffmann T (2015) Peptide therapeutics: current status and future directions *Drug Discov Today* 20(1) 122–128, https://doi.org/10.1016/j.drudis.2014.10.003.

118. Hagimori M, Fuchigami Y, & Kawakami S (2017) Peptide-Based Cancer-Targeted DDS and Molecular Imaging *Chem Pharm Bull* 65(7) 618–624, https://doi.org/10.1248/cpb.c17-00098.

119. Cazzamalli S, Dal Corso A, Widmayer F, & Neri D (2018) Chemically Defined Antibody- and Small Molecule-Drug Conjugates for in Vivo Tumor Targeting Applications: A Comparative Analysis *J Am Chem Soc* 140(5) 1617–1621, https://doi.org/10.1021/jacs.7b13361.

120. Di L (2015) Strategic approaches to optimizing peptide ADME properties *AAPS J* 17(1) 134–143, https://doi.org/10.1208/s12248-014-9687-3.

121. Deonarain MP, Yahioglu G, Stamati I, Pomowski A, Clarke J, Edwards BM, Diez-Posada S, & Stewart AC (2018) Small-Format Drug Conjugates: A Viable Alternative to ADCs for Solid Tumours? *Antibodies (Basel)* 7(2) 16, https://doi.org/10.3390/antib7020016.

122. Werle M, & Bernkop-Schnürch A (2006) Strategies to improve plasma half life time of peptide and protein drugs *Amino Acids* 30(4) 351–367, https://doi.org/10.1007/s00726-005-0289-3.

123. Alouane A, Labruère R, Le Saux T, Schmidt F, & Jullien L (2015) Self-immolative spacers: kinetic aspects, structure-property relationships, and applications *Angew Chem Int Ed Engl* 54(26) 7492–7509, https://doi.org/10.1002/anie.201500088.

124. Yang J, Chen H, Vlahov IR, Cheng J-X, & Low PS (2006) Evaluation of disulfide reduction during receptor-mediated endocytosis by using FRET imaging *Proc Natl Acad Sci USA* 103(37) 13872–13877, https://doi.org/10.1073/pnas.0601455103.

125. Chen K-C, Schmuck K, Tietze LF, & Roffler SR (2013) Selective cancer therapy by extracellular activation of a highly potent glycosidic duocarmycin analogue *Mol Pharm* 10(5) 1773–1782, https://doi.org/10.1021/mp300581u.

126. Zhang P, Cheetham AG, Lock LL, & Cui H (2013) Cellular uptake and cytotoxicity of drug-peptide conjugates regulated by conjugation site *Bioconjug Chem* 24(4) 604–613, https://doi.org/10.1021/bc300585h.

127. Maecke HR, & Reubi JC (2011) Somatostatin receptors as targets for nuclear medicine imaging and radionuclide treatment *J Nucl Med* 52(6) 841–844, https://doi.org/10.2967/jnumed.110.084236.

128. Schally AV, & Nagy A (2004) Chemotherapy targeted to cancers through tumoral hormone receptors *Trends Endocrinol Metab* 15(7) 300–310, https://doi.org/10.1016/j.tem.2004.07.002.

129. Mezo G, & Manea M (2010) Receptor-mediated tumor targeting based on peptide hormones *Expert Opin Drug Deliv* 7(1) 79–96, https://doi.org/10.1517/17425240903418410.

130. Reubi JC (2003) Peptide receptors as molecular targets for cancer diagnosis and therapy *Endocr Rev* 24(4) 389–427, https://doi.org/10.1210/er.2002-0007.

131. Millar RP, Lu Z-L, Pawson AJ, Flanagan CA, Morgan K, & Maudsley SR (2004) Gonadotropin-releasing hormone receptors *Endocr Rev* 25(2) 235–275, https://doi.org/10.1210/er.2003-0002.

132. Nair RM, & Schally AV (1972) Structure of a hypothalamic peptide possessing gonadotropin-releasing activity *Int J Pept Protein Res* 4(6) 421–430, https://doi.org/10.1111/j.1399-3011.1972.tb03449.x.

133. Harrison GS, Wierman ME, Nett TM, & Glode LM (2004) Gonadotropin-releasing hormone and its receptor in normal and malignant cells *Endocr Relat Cancer* 11(4) 725–748, https://doi.org/10.1677/erc.1.00777.

134. Schally AV, Arimura A, Kastin AJ, Matsuo H, Baba Y, Redding TW, Nair RM, Debeljuk L, & White WF (1971) Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle-stimulating hormones *Science* 173(4001) 1036–1038, https://doi.org/10.1126/science.173.4001.1036.

135. Schally AV, Arimura A, Baba Y, Nair RM, Matsuo H, Redding TW, & Debeljuk L (1971) Isolation and properties of the FSH and LH-releasing hormone *Biochem Biophys Res Commun* 43(2) 393–399, https://doi.org/10.1016/0006-291x(71)90766-2.

136. Nagy A, & Schally AV (2005) Targeting of cytotoxic luteinizing hormone-releasing hormone analogs to breast, ovarian, endometrial, and prostate cancers *Biol Reprod* 73(5) 851–859, https://doi.org/10.1095/biolreprod.105.043489.

137. Fekete M, Wittliff JL, & Schally AV (1989) Characteristics and distribution of receptors for [D-TRP6]-luteinizing hormone-releasing hormone, somatostatin, epidermal growth factor, and sex steroids in 500 biopsy samples of human breast cancer *J Clin Lab Anal* 3(3) 137–147, https://doi.org/10.1002/jcla.1860030302.

138. Keller G, Schally AV, Gaiser T, Nagy A, Baker B, Westphal G, Halmos G, & Engel JB (2005) Human malignant melanomas express receptors for luteinizing hormone releasing hormone allowing targeted therapy with cytotoxic luteinizing hormone releasing hormone analogue *Cancer Res* 65(13) 5857–5863, https://doi.org/10.1158/0008-5472.CAN-04-3816.

139. Montagnani Marelli M, Moretti RM, Mai S, Müller O, Van Groeninghen JC, & Limonta P (2009) Novel insights into GnRH receptor activity: role in the control of human glioblastoma cell proliferation *Oncol Rep* 21(5) 1277–1282, https://doi.org/10.3892/or_00000351.

140. Gründker C, & Emons G (2017) The Role of Gonadotropin-Releasing Hormone in Cancer Cell Proliferation and Metastasis *Front Endocrinol (Lausanne)* 8 187, https://doi.org/10.3389/fendo.2017.00187.

141. Engel JB, & Schally AV (2007) Drug Insight: clinical use of agonists and antagonists of luteinizing-hormone-releasing hormone *Nat Clin Pract Endocrinol Metab* 3(2) 157–167, https://doi.org/10.1038/ncpendmet0399.

142. Huerta-Reyes M, Maya-Núñez G, Pérez-Solis MA, López-Muñoz E, Guillén N, Olivo-Marin J-C, & Aguilar-Rojas A (2019) Treatment of Breast Cancer With Gonadotropin-Releasing Hormone Analogs *Front Oncol* 9 943, https://doi.org/10.3389/fonc.2019.00943.

143. Schultze-Mosgau A, Griesinger G, Altgassen C, von Otte S, Hornung D, & Diedrich K (2005) New developments in the use of peptide gonadotropin-releasing hormone antagonists versus agonists *Expert Opin Investig Drugs* 14(9) 1085–1097, https://doi.org/10.1517/13543784.14.9.1085.

144. Chengalvala MV, Pelletier JC, & Kopf GS (2003) GnRH agonists and antagonists in cancer therapy *Curr Med Chem Anticancer Agents* 3(6) 399–410, https://doi.org/10.2174/1568011033482251.

145. Padula AM (2005) GnRH analogues--agonists and antagonists *Anim Reprod Sci* 88(1–2) 115–126, https://doi.org/10.1016/j.anireprosci.2005.05.005.

146. Mezo G, & Manea M (2009) Luteinizing hormone-releasing hormone antagonists *Expert Opin Ther Pat* 19(12) 1771–1785, https://doi.org/10.1517/13543770903410237.

147. Gründker C, Günthert AR, Westphalen S, & Emons G (2002) Biology of the gonadotropin-releasing hormone system in gynecological cancers *Eur J Endocrinol* 146(1) 1–14, https://doi.org/10.1530/eje.0.1460001.

148. Montagnani Marelli M, Moretti RM, Januszkiewicz-Caulier J, Motta M, & Limonta P (2006) Gonadotropin-releasing hormone (GnRH) receptors in tumors: a new rationale for the therapeutical application of GnRH analogs in cancer patients? *Curr Cancer Drug Targets* 6(3) 257–269, https://doi.org/10.2174/156800906776842966.

149. Millar RP (2005) GnRHs and GnRH receptors *Anim Reprod Sci* 88(1–2) 5–28, https://doi.org/10.1016/j.anireprosci.2005.05.032.

150. Sealfon SC, Weinstein H, & Millar RP (1997) Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor *Endocr Rev* 18(2) 180–205, https://doi.org/10.1210/edrv.18.2.0295.

151. Kovacs M, Schally AV, Nagy A, Koppan M, & Groot K (1997) Recovery of pituitary function after treatment with a targeted cytotoxic analog of luteinizing hormone-releasing hormone *Proc Natl Acad Sci USA* 94(4) 1420–1425, https://doi.org/10.1073/pnas.94.4.1420.

152. Limonta P, Moretti RM, Montagnani Marelli M, & Motta M (2003) The biology of gonadotropin hormone-releasing hormone: role in the control of tumor growth and progression in humans *Front Neuroendocrinol* 24(4) 279–295, https://doi.org/10.1016/j.yfrne.2003.10.003.

153. Gründker C, Völker P, & Emons G (2001) Antiproliferative signaling of luteinizing hormone-releasing hormone in human endometrial and ovarian cancer cells through G protein alpha(I)-mediated activation of phosphotyrosine phosphatase *Endocrinology* 142(6) 2369–2380, https://doi.org/10.1210/endo.142.6.8190.

154. Limonta P, Montagnani Marelli M, Mai S, Motta M, Martini L, & Moretti RM (2012) GnRH receptors in cancer: from cell biology to novel targeted therapeutic strategies *Endocr Rev* 33(5) 784–811, https://doi.org/10.1210/er.2012-1014.

155. Imai A, Takagi H, Horibe S, Fuseya T, & Tamaya T (1996) Coupling of gonadotropin-releasing hormone receptor to Gi protein in human reproductive tract tumors *J Clin Endocrinol Metab* 81(9) 3249–3253, https://doi.org/10.1210/jcem.81.9.8784077.

156. Cheung LWT, & Wong AST (2008) Gonadotropin-releasing hormone: GnRH receptor signaling in extrapituitary tissues *FEBS J* 275(22) 5479–5495, https://doi.org/10.1111/j.1742-4658.2008.06677.x.

157. Millar RP, Pawson AJ, Morgan K, Rissman EF, & Lu Z-L (2008) Diversity of actions of GnRHs mediated by ligand-induced selective signaling *Front Neuroendocrinol* 29(1) 17–35, https://doi.org/10.1016/j.yfrne.2007.06.002.

158. Kakar SS, Grizzle WE, & Neill JD (1994) The nucleotide sequences of human GnRH receptors in breast and ovarian tumors are identical with that found in pituitary *Mol Cell Endocrinol* 106(1–2) 145–149, https://doi.org/10.1016/0303-7207(94)90196-1.

159. Millar RP, & Pawson AJ (2004) Outside-in and inside-out signaling: the new concept that selectivity of ligand binding at the gonadotropin-releasing hormone receptor is modulated by the intracellular environment *Endocrinology* 145(8) 3590–3593, https://doi.org/10.1210/en.2004-0461.

160. White RB, Eisen JA, Kasten TL, & Fernald RD (1998) Second gene for gonadotropin-releasing hormone in humans *Proc Natl Acad Sci USA* 95(1) 305–309, https://doi.org/10.1073/pnas.95.1.305.

161. Gründker C, Günthert AR, Millar RP, & Emons G (2002) Expression of gonadotropin-releasing hormone II (GnRH-II) receptor in human endometrial and ovarian cancer cells and effects of GnRH-II on tumor cell proliferation *J Clin Endocrinol Metab* 87(3) 1427–1430, https://doi.org/10.1210/jcem.87.3.8437.

162. Sower SA, Chiang YC, Lovas S, & Conlon JM (1993) Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain *Endocrinology* 132(3) 1125–1131, https://doi.org/10.1210/endo.132.3.8440174.

163. Lovas S, Pályi I, Vincze B, Horváth J, Kovács M, Mezö I, Tóth G, Teplán I, & Murphy RF (1998) Direct anticancer activity of gonadotropin-releasing hormone-III *J Pept Res* 52(5) 384–389, https://doi.org/10.1111/j.1399-3011.1998.tb00662.x.

164. Kovacs M, Seprodi J, Koppan M, Horvath JE, Vincze B, Teplan I, & Flerko B (2002) Lamprey gonadotropin hormone-releasing hormone-III has no selective follicle-stimulating hormone-releasing effect in rats *J Neuroendocrinol* 14(8) 647–655, https://doi.org/10.1046/j.1365-2826.2002.00828.x.

165. Kovács M, Vincze B, Horváth JE, & Seprodi J (2007) Structure-activity study on the LH- and FSH-releasing and anticancer effects of gonadotropin-releasing hormone (GnRH)-III analogs *Peptides* 28(4) 821–829, https://doi.org/10.1016/j.peptides.2007.01.003.

166. Mezö I, Lovas S, Pályi I, Vincze B, Kálnay A, Turi G, Vadász Z, Seprödi J, Idei M, Tóth G, Gulyás E, Otvös F, Mák M, Horváth JE, Teplán I, & Murphy RF (1997) Synthesis of gonadotropin-releasing hormone III analogs. Structure-antitumor activity relationships *J Med Chem* 40(21) 3353–3358, https://doi.org/10.1021/jm9700981.

167. Pappa EV, Zompra AA, Diamantopoulou Z, Spyranti Z, Pairas G, Lamari FN, Katsoris P, Spyroulias GA, & Cordopatis P (2012) Structure-activity studies of lGnRH-III through rational amino acid substitution and NMR conformational studies *Biopolymers* 98(6) 525–534, https://doi.org/10.1002/bip.22123.

168. Mezo G, Czajlik A, Manea M, Jakab A, Farkas V, Majer Z, Vass E, Bodor A, Kapuvári B, Boldizsár M, Vincze B, Csuka O, Kovács M, Przybylski M, Perczel A, & Hudecz F (2007) Structure, enzymatic stability and antitumor activity of sea lamprey GnRH-III and its dimer derivatives *Peptides* 28(4) 806–820, https://doi.org/10.1016/j.peptides.2006.12.014.

169. Manea M, & Mező G (2013) lGnRH-III -- a promising candidate for anticancer drug development *Protein Pept Lett* 20(4) 439–449, https://doi.org/10.2174/0929866511320040008.

170. Orbán E, Mezo G, Schlage P, Csík G, Kulić Z, Ansorge P, Fellinger E, Möller HM, & Manea M (2011) In vitro degradation and antitumor activity of oxime bond-linked daunorubicin-GnRH-III bioconjugates and DNA-binding properties of daunorubicin-amino acid metabolites *Amino Acids* 41(2) 469–483, https://doi.org/10.1007/s00726-010-0766-1.

171. Schlage P, Mezo G, Orbán E, Bosze S, & Manea M (2011) Anthracycline-GnRH derivative bioconjugates with different linkages: synthesis, in vitro drug release and cytostatic effect. *J Control Release* 156(2) 170–178, https://doi.org/10.1016/j.jconrel.2011.08.005.

172. Szabó I, Bősze S, Orbán E, Sipos É, Halmos G, Kovács M, & Mező G (2015) Comparative in vitro biological evaluation of daunorubicin containing GnRH-I and GnRH-II conjugates developed for tumor targeting *J Pept Sci* 21(5) 426–435, https://doi.org/10.1002/psc.2775.

173. Suarez-Quian CA, Wynn PC, & Catt KJ (1986) Receptor-mediated endocytosis of GnRH analogs: differential processing of gold-labeled agonist and antagonist derivatives *J Steroid Biochem* 24(1) 183–192, https://doi.org/10.1016/0022-4731(86)90049-x.

174. Schally AV, & Nagy A (1999) Cancer chemotherapy based on targeting of cytotoxic peptide conjugates to their receptors on tumors *Eur J Endocrinol* 141(1) 1–14, https://doi.org/10.1530/eje.0.1410001.

175. Bajusz S, Janaky T, Csernus VJ, Bokser L, Fekete M, Srkalovic G, Redding TW, & Schally AV (1989) Highly potent analogues of luteinizing hormone-releasing hormone containing D-phenylalanine nitrogen mustard in position 6 *Proc Natl Acad Sci USA* 86(16) 6318–6322, https://doi.org/10.1073/pnas.86.16.6318.

176. Milovanovic SR, Monje E, Szepeshazi K, Radulovic S, & Schally A (1993) Effect of treatment with LHRH analogs containing cytotoxic radicals on the binding characteristics of receptors for luteinizing-hormone-releasing hormone in MXT mouse mammary carcinoma *J Cancer Res Clin Oncol* 119(5) 273–278, https://doi.org/10.1007/BF01212725.

177. Nagy A, Schally AV, Armatis P, Szepeshazi K, Halmos G, Kovacs M, Zarandi M, Groot K, Miyazaki M, Jungwirth A, & Horvath J (1996) Cytotoxic analogs of luteinizing hormone-releasing hormone containing doxorubicin or 2-pyrrolinodoxorubicin, a derivative 500-1000 times more potent *Proc Natl Acad Sci USA* 93(14) 7269–7273, https://doi.org/10.1073/pnas.93.14.7269.

178. Nagy A, Plonowski A, & Schally AV (2000) Stability of cytotoxic luteinizing hormone-releasing hormone conjugate (AN-152) containing doxorubicin 14-O-hemiglutarate in mouse and human serum in vitro: implications for the design of preclinical studies *Proc Natl Acad Sci USA* 97(2) 829–834, https://doi.org/10.1073/pnas.97.2.829.

179. Westphalen S, Kotulla G, Kaiser F, Krauss W, Werning G, Elsasser HP, Nagy A, Schulz KD, Grundker C, Schally AV, & Emons G (2000) Receptor mediated antiproliferative effects of the cytotoxic LHRH agonist AN-152 in human ovarian and endometrial cancer cell lines *Int J Oncol* 17(5) 1063–1069, https://doi.org/10.3892/ijo.17.5.1063.

180. Engel JB, Schally AV, Dietl J, Rieger L, & Hönig A (2007) Targeted therapy of breast and gynecological cancers with cytotoxic analogues of peptide hormones *Mol Pharm* 4(5) 652–658, https://doi.org/10.1021/mp0700514.

181. Föst C, Duwe F, Hellriegel M, Schweyer S, Emons G, & Gründker C (2011) Targeted chemotherapy for triple-negative breast cancers via LHRH receptor *Oncol Rep* 25(5) 1481–1487, https://doi.org/10.3892/or.2011.1188.

182. Schally AV (2008) New approaches to the therapy of various tumors based on peptide analogues *Horm Metab Res* 40(5) 315–322, https://doi.org/10.1055/s-2008-1073142.

183. Engel J, Emons G, Pinski J, & Schally AV (2012) AEZS-108 : a targeted cytotoxic analog of LHRH for the treatment of cancers positive for LHRH receptors *Expert Opin Investig Drugs* 21(6) 891–899, https://doi.org/10.1517/13543784.2012.685128.

184. Emons G, Kaufmann M, Gorchev G, Tsekova V, Gründker C, Günthert AR, Hanker LC, Velikova M, Sindermann H, Engel J, & Schally AV (2010) Dose escalation and pharmacokinetic study of AEZS-108 (AN-152), an LHRH agonist linked to doxorubicin, in women with LHRH receptor-positive tumors *Gynecol Oncol* 119(3) 457–461, https://doi.org/10.1016/j.ygyno.2010.08.003.

185. Engel JB, Schally AV, Buchholz S, Seitz S, Emons G, & Ortmann O (2012) Targeted chemotherapy of endometrial, ovarian and breast cancers with cytotoxic analogs of luteinizing hormone-releasing hormone (LHRH) *Arch Gynecol Obstet* 286(2) 437–442, https://doi.org/10.1007/s00404-012-2335-1.

186. Nagy A, Armatis P, & Schally AV (1996) High yield conversion of doxorubicin to 2-pyrrolinodoxorubicin, an analog 500-1000 times more potent: structure-activity relationship of daunosamine-modified derivatives of doxorubicin *Proc Natl Acad Sci USA* 93(6) 2464–2469, https://doi.org/10.1073/pnas.93.6.2464.

187. Engel JB, Tinneberg H-R, Rick FG, Berkes E, & Schally AV (2016) Targeting of Peptide Cytotoxins to LHRH Receptors For Treatment of Cancer *Curr Drug Targets* 17(5) 488–494, https://doi.org/10.2174/1389450117051603031547 17.

188. Stepankova J, Studenovsky M, Malina J, Kasparkova J, Liskova B, Novakova O, Ulbrich K, & Brabec V (2011) DNA interactions of 2-pyrrolinodoxorubicin, a

distinctively more potent daunosamine-modified analogue of doxorubicin *Biochem Pharmacol* 82(3) 227–235, https://doi.org/10.1016/j.bcp.2011.04.010.

189. Stangelberger A, Schally AV, Nagy A, Szepeshazi K, Kanashiro CA, & Halmos G (2006) Inhibition of human experimental prostate cancers by a targeted cytotoxic luteinizing hormone-releasing hormone analog AN-207 *Prostate* 66(2) 200–210, https://doi.org/10.1002/pros.20335.

190. Szabó I, Manea M, Orbán E, Csámpai A, Bosze S, Szabó R, Tejeda M, Gaál D, Kapuvári B, Przybylski M, Hudecz F, & Mezo G (2009) Development of an oxime bond containing daunorubicin-gonadotropin-releasing hormone-III conjugate as a potential anticancer drug *Bioconjug Chem* 20(4) 656–665, https://doi.org/10.1021/bc800542u.

191. Pályi I, Vincze B, Lovas S, Mező I, Pató J, Kálnay A, Turi G, Gaál D, Mihalik R, Péter I, Teplán I, & Murphy RF (1999) Gonadotropin-releasing hormone analogue conjugates with strong selective antitumor activity *Proc Natl Acad Sci U S A* 96(5) 2361–2366, https://doi.org/10.1073/pnas.96.5.2361.

192. Mezo G, Manea M, Szabí I, Vincze B, & Kovács M (2008) New derivatives of GnRH as potential anticancer therapeutic agents. *Curr Med Chem* 15(23) 2366–2379, https://doi.org/10.2174/092986708785909157.

193. Manea M, Leurs U, Orbán E, Baranyai Z, Öhlschläger P, Marquardt A, Schulcz Á, Tejeda M, Kapuvári B, Tóvári J, & Mezo G (2011) Enhanced enzymatic stability and antitumor activity of daunorubicin-GnRH-III bioconjugates modified in position 4 *Bioconjug Chem* 22(7) 1320–1329, https://doi.org/10.1021/bc100547p.

194. Hegedüs R, Manea M, Orbán E, Szabó I, Kiss E, Sipos E, Halmos G, & Mező G (2012) Enhanced cellular uptake and in vitro antitumor activity of short-chain fatty acid acylated daunorubicin-GnRH-III bioconjugates *Eur J Med Chem* 56 155–165, https://doi.org/10.1016/j.ejmech.2012.08.014.

195. Schuster S, Biri-Kovács B, Szeder B, Farkas V, Buday L, Szabó Z, Halmos G, & Mező G (2018) Synthesis and in vitro biochemical evaluation of oxime bond-linked

daunorubicin-GnRH-III conjugates developed for targeted drug delivery *Beilstein J Org Chem* 14 756–771, https://doi.org/10.3762/bjoc.14.64.

196. Yang F, Teves SS, Kemp CJ, & Henikoff S (2014) Doxorubicin, DNA torsion, and chromatin dynamics *Biochim Biophys Acta* 1845(1) 84–89, https://doi.org/10.1016/j.bbcan.2013.12.002.

197. Thomssen C, Schmitt M, Goretzki L, Oppelt P, Pache L, Dettmar P, Jänicke F, & Graeff H (1995) Prognostic value of the cysteine proteases cathepsins B and cathepsin L in human breast cancer *Clin Cancer Res* 1(7) 741–746.

198. Mai J, Waisman DM, & Sloane BF (2000) Cell surface complex of cathepsin B/annexin II tetramer in malignant progression *Biochim Biophys Acta* 1477(1–2) 215–230, https://doi.org/10.1016/s0167-4838(99)00274-5.

199. Manea M, Tóvári J, Tejeda M, Schulcz A, Kapuvári B, Vincze B, & Mezo G (2012) In-vivo antitumour effect of daunorubicin-GnRH-III derivative conjugates on colon carcinoma-bearing mice *Anticancer Drugs* 23(1) 90–97, https://doi.org/10.1097/CAD.0b013e32834bb6b4.

200. Kapuvári B, Hegedüs R, Schulcz Á, Manea M, Tóvári J, Gacs A, Vincze B, & Mező G (2016) Improved in vivo antitumor effect of a daunorubicin - GnRH-III bioconjugate modified by apoptosis inducing agent butyric acid on colorectal carcinoma bearing mice *Invest New Drugs* 34 416–423, https://doi.org/10.1007/s10637-016-0354-7.

201. Leurs U, Mező G, Orbán E, Öhlschläger P, Marquardt A, & Manea M (2012) Design, synthesis, in vitro stability and cytostatic effect of multifunctional anticancer drug-bioconjugates containing GnRH-III as a targeting moiety *Biopolymers* 98(1) 1–10, https://doi.org/10.1002/bip.21640.

202. Hegedüs R, Pauschert A, Orbán E, Szabó I, Andreu D, Marquardt A, Mező G, & Manea M (2015) Modification of daunorubicin-GnRH-III bioconjugates with oligoethylene glycol derivatives to improve solubility and bioavailability for

targeted cancer chemotherapy *Biopolymers* 104(3) 167–177, https://doi.org/10.1002/bip.22629.

203. Schuster S, Biri-Kovács B, Szeder B, Buday L, Gardi J, Szabó Z, Halmos G, & Mező G (2018) Enhanced In Vitro Antitumor Activity of GnRH-III-Daunorubicin Bioconjugates Influenced by Sequence Modification *Pharmaceutics* 10(4) 223, https://doi.org/10.3390/pharmaceutics10040223.

204. Padma VV (2015) An overview of targeted cancer therapy *BioMed* 5(4) 19, https://doi.org/10.7603/s40681-015-0019-4.

205. Kapoor P, Singh H, Gautam A, Chaudhary K, Kumar R, & Raghava GPS (2012) TumorHoPe: a database of tumor homing peptides *PLoS ONE* 7(4) e35187, https://doi.org/10.1371/journal.pone.0035187.

206. Hamzeh-Mivehroud M, Alizadeh AA, Morris MB, Church WB, & Dastmalchi S (2013) Phage display as a technology delivering on the promise of peptide drug discovery *Drug Discov Today* 18(23–24) 1144–1157, https://doi.org/10.1016/j.drudis.2013.09.001.

207. Molek P, Strukelj B, & Bratkovic T (2011) Peptide phage display as a tool for drug discovery: targeting membrane receptors *Molecules* 16(1) 857–887, https://doi.org/10.3390/molecules16010857.

208. Gray BP, & Brown KC (2014) Combinatorial Peptide Libraries: Mining for Cell-Binding Peptides *Chem Rev* 114(2) 1020–1081, https://doi.org/10.1021/cr400166n.

209. Saw PE, & Song E-W (2019) Phage display screening of therapeutic peptide for cancer targeting and therapy *Protein Cell* 10(11) 787–807, https://doi.org/10.1007/s13238-019-0639-7.

210. Joyce JA, Laakkonen P, Bernasconi M, Bergers G, Ruoslahti E, & Hanahan D (2003) Stage-specific vascular markers revealed by phage display in a mouse model of pancreatic islet tumorigenesis *Cancer Cell* 4(5) 393–403, https://doi.org/10.1016/s1535-6108(03)00271-x.

211. Pasqualini R, Koivunen E, & Ruoslahti E (1995) A peptide isolated from phage display libraries is a structural and functional mimic of an RGD-binding site on integrins *J Cell Biol* 130(5) 1189–1196, https://doi.org/10.1083/jcb.130.5.1189.

212. Oliner J, Min H, Leal J, Yu D, Rao S, You E, Tang X, Kim H, Meyer S, Han SJ, Hawkins N, Rosenfeld R, Davy E, Graham K, Jacobsen F, Stevenson S, Ho J, Chen Q, Hartmann T, Michaels M, Kelley M, Li L, Sitney K, Martin F, Sun J-R, Zhang N, Lu J, Estrada J, Kumar R, Coxon A, Kaufman S, Pretorius J, Scully S, Cattley R, Payton M, Coats S, Nguyen L, Desilva B, Ndifor A, Hayward I, Radinsky R, Boone T, & Kendall R (2004) Suppression of angiogenesis and tumor growth by selective inhibition of angiopoietin-2 *Cancer Cell* 6(5) 507–516, https://doi.org/10.1016/j.ccr.2004.09.030.

213. Diéras V, Wildiers H, Jassem J, Dirix LY, Guastalla J-P, Bono P, Hurvitz SA, Gonçalves A, Romieu G, Limentani SA, Jerusalem G, Lakshmaiah KC, Roché H, Sánchez-Rovira P, Pienkowski T, Seguí Palmer MÁ, Li A, Sun Y-N, Pickett CA, & Slamon DJ (2015) Trebananib (AMG 386) plus weekly paclitaxel with or without bevacizumab as first-line therapy for HER2-negative locally recurrent or metastatic breast cancer: A phase 2 randomized study *Breast* 24(3) 182–190, https://doi.org/10.1016/j.breast.2014.11.003.

214. Peeters M, Strickland AH, Lichinitser M, Suresh AVS, Manikhas G, Shapiro J, Rogowski W, Huang X, Wu B, Warner D, Jain R, & Tebbutt NC (2013) A randomised, double-blind, placebo-controlled phase 2 study of trebananib (AMG 386) in combination with FOLFIRI in patients with previously treated metastatic colorectal carcinoma *Br J Cancer* 108(3) 503–511, https://doi.org/10.1038/bjc.2012.594.

215. Kelly KA, & Jones DA (2003) Isolation of a colon tumor specific binding peptide using phage display selection *Neoplasia* 5(5) 437–444, https://doi.org/10.1016/s1476-5586(03)80046-5.

216. Rasmussen UB, Schreiber V, Schultz H, Mischler F, & Schughart K (2002) Tumor cell-targeting by phage-displayed peptides *Cancer Gene Ther* 9(7) 606–612, https://doi.org/10.1038/sj.cgt.7700476.

217. Hou L, Zhu D, Liang Y, Tian X, Li L, Wang P, Zhu L, Weng X, Wang Y, Li Y, Wu T, Wang J, & Meng X (2018) Identification of a specific peptide binding to colon cancer cells from a phage-displayed peptide library *British Journal of Cancer* 118(1) 79–87, https://doi.org/10.1038/bjc.2017.366.

218. Odermatt A, Audigé A, Frick C, Vogt B, Frey BM, Frey FJ, & Mazzucchelli L (2001) Identification of receptor ligands by screening phage-display peptide libraries ex vivo on microdissected kidney tubules *J Am Soc Nephrol* 12(2) 308–316, https://doi.org/10.1681/ASN.V122308.

219. Ferreira D, Silva AP, Nobrega FL, Martins IM, Barbosa-Matos C, Granja S, Martins SF, Baltazar F, & Rodrigues LR (2019) Rational Identification of a Colorectal Cancer Targeting Peptide through Phage Display *Sci Rep* 9 3958, https://doi.org/10.1038/s41598-019-40562-1.

220. Kubo N, Akita N, Shimizu A, Kitahara H, Parker AL, & Miyagawa S (2008) Identification of oligopeptide binding to colon cancer cells separated from patients using laser capture microdissection *J Drug Target* 16(5) 396–404, https://doi.org/10.1080/10611860802088796.

221. Hsiung P-L, Hsiung P-L, Hardy J, Friedland S, Soetikno R, Du CB, Wu AP, Sahbaie P, Crawford JM, Lowe AW, Contag CH, & Wang TD (2008) Detection of colonic dysplasia in vivo using a targeted heptapeptide and confocal microendoscopy *Nat Med* 14(4) 454–458, https://doi.org/10.1038/nm1692.

222. Miller SJ, Joshi BP, Feng Y, Gaustad A, Fearon ER, & Wang TD (2011) In Vivo Fluorescence-Based Endoscopic Detection of Colon Dysplasia in the Mouse Using a Novel Peptide Probe *PLoS One* 6(3) e17384, https://doi.org/10.1371/journal.pone.0017384.

223. Li ZJ, Wu WKK, Ng SSM, Yu L, Li HT, Wong CCM, Wu YC, Zhang L, Ren SX, Sun XG, Chan KM, & Cho CH (2010) A novel peptide specifically targeting the vasculature of orthotopic colorectal cancer for imaging detection and drug delivery *Journal of Controlled Release* 148(3) 292–302, https://doi.org/10.1016/j.jconrel.2010.09.015.

224. Zhang Y, Chen J, Zhang Y, Hu Z, Hu D, Pan Y, Ou S, Liu G, Yin X, Zhao J, Ren L, & Wang J (2007) Panning and identification of a colon tumor binding peptide from a phage display peptide library *J Biomol Screen* 12(3) 429–435, https://doi.org/10.1177/1087057106299164.

225. Malhotra M, Tomaro-Duchesneau C, Saha S, & Prakash S (2013) Systemic siRNA Delivery via Peptide-Tagged Polymeric Nanoparticles, Targeting PLK1 Gene in a Mouse Xenograft Model of Colorectal Cancer *Int J Biomater* 2013 252531, https://doi.org/10.1155/2013/252531.

226. Groves JT (2019) Membrane Mechanics in Living Cells *Dev Cell* 48(1) 15–16, https://doi.org/10.1016/j.devcel.2018.12.011.

227. Schaffhausen J (2015) Remaining hurdles to effective cancer therapy *Trends Pharmacol Sci* 36(6) v, https://doi.org/10.1016/j.tips.2015.04.008.

228. Swain S, Sahu PK, Beg S, & Babu SM (2016) Nanoparticles for Cancer Targeting: Current and Future Directions *Curr Drug Deliv* 13(8) 1290–1302, https://doi.org/10.2174/1567201813666160713121122.

229. Jafari S, Maleki Dizaj S, & Adibkia K (2015) Cell-penetrating peptides and their analogues as novel nanocarriers for drug delivery *Bioimpacts* 5(2) 103–111, https://doi.org/10.15171/bi.2015.10.

230. Kristensen M, Birch D, & Mørck Nielsen H (2016) Applications and Challenges for Use of Cell-Penetrating Peptides as Delivery Vectors for Peptide and Protein Cargos *Int J Mol Sci* 17(2) 185, https://doi.org/10.3390/ijms17020185.

231. Bolhassani A, Jafarzade BS, & Mardani G (2017) In vitro and in vivo delivery of therapeutic proteins using cell penetrating peptides *Peptides* 87 50–63, https://doi.org/10.1016/j.peptides.2016.11.011.

232. Ye J, Liu E, Yu Z, Pei X, Chen S, Zhang P, Shin M-C, Gong J, He H, & Yang VC (2016) CPP-Assisted Intracellular Drug Delivery, What Is Next? *Int J Mol Sci* 17(11) 1892, https://doi.org/10.3390/ijms17111892.

233. Hudecz F, Bánóczi Z, & Csík G (2005) Medium-sized peptides as built in carriers for biologically active compounds *Med Res Rev* 25(6) 679–736, https://doi.org/10.1002/med.20034.

234. Frankel AD, & Pabo CO (1988) Cellular uptake of the tat protein from human immunodeficiency virus *Cell* 55(6) 1189–1193, https://doi.org/10.1016/0092-8674(88)90263-2.

235. Derossi D, Joliot AH, Chassaing G, & Prochiantz A (1994) The third helix of the Antennapedia homeodomain translocates through biological membranes *J Biol Chem* 269(14) 10444–10450, https://doi.org/10.1016/S0021-9258(17)34080-2.

236. Milletti F (2012) Cell-penetrating peptides: classes, origin, and current landscape *Drug Discov Today* 17(15–16) 850–860, https://doi.org/10.1016/j.drudis.2012.03.002.

237. Mitchell DJ, Kim DT, Steinman L, Fathman CG, & Rothbard JB (2000) Polyarginine enters cells more efficiently than other polycationic homopolymers *J Pept Res* 56(5) 318–325, https://doi.org/10.1034/j.1399-3011.2000.00723.x.

238. Futaki S, Suzuki T, Ohashi W, Yagami T, Tanaka S, Ueda K, & Sugiura Y (2001) Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery *J Biol Chem* 276(8) 5836–5840, https://doi.org/10.1074/jbc.M007540200.

239. Suzuki T, Futaki S, Niwa M, Tanaka S, Ueda K, & Sugiura Y (2002) Possible existence of common internalization mechanisms among arginine-rich peptides *J Biol Chem* 277(4) 2437–2443, https://doi.org/10.1074/jbc.M110017200.

240. Ziegler A (2008) Thermodynamic studies and binding mechanisms of cell-penetrating peptides with lipids and glycosaminoglycans *Adv Drug Deliv Rev* 60(4–5) 580–597, https://doi.org/10.1016/j.addr.2007.10.005.

241. Huang Y-W, & Lee H-J. Cell-Penetrating Peptides for Medical Theranostics and Targeted Drug Delivery. In: Koutsopoulos S (editor), Peptide Applications in Biomedicine, Biotechnology and Bioengineering. Woodhead Publishing (Elsevier), Cambridge, MA, USA, 1998: 359–370, https://doi.org/10.1016/B978-0-08-100736-5.00013-2.

242. Guidotti G, Brambilla L, & Rossi D (2017) Cell-Penetrating Peptides: From Basic Research to Clinics *Trends in Pharmacological Sciences* 38(4) 406–424, https://doi.org/10.1016/j.tips.2017.01.003.

243. Ruseska I, & Zimmer A (2020) Internalization mechanisms of cell-penetrating peptides *Beilstein J Nanotechnol* 11(1) 101–123, https://doi.org/10.3762/bjnano.11.10.

244. Kauffman WB, Fuselier T, He J, & Wimley WC (2015) Mechanism Matters: A Taxonomy of Cell Penetrating Peptides *Trends Biochem Sci* 40(12) 749–764, https://doi.org/10.1016/j.tibs.2015.10.004.

245. Borrelli A, Tornesello AL, Tornesello ML, & Buonaguro FM (2018) Cell Penetrating Peptides as Molecular Carriers for Anti-Cancer Agents *Molecules* 23(2) 295, https://doi.org/10.3390/molecules23020295.

246. Derakhshankhah H, & Jafari S (2018) Cell penetrating peptides: A concise review with emphasis on biomedical applications *Biomedicine & Pharmacotherapy* 108 1090–1096, https://doi.org/10.1016/j.biopha.2018.09.097.

247. Kurrikoff K, Gestin M, & Langel Ü (2016) Recent in vivo advances in cell-penetrating peptide-assisted drug delivery *Expert Opin Drug Deliv* 13(3) 373–387, https://doi.org/10.1517/17425247.2016.1125879.

248. Habault J, & Poyet J-L (2019) Recent Advances in Cell Penetrating Peptide-Based Anticancer Therapies *Molecules* 24(5) 927, https://doi.org/10.3390/molecules24050927.

249. Regberg J, Srimanee A, & Langel U (2012) Applications of cell-penetrating peptides for tumor targeting and future cancer therapies *Pharmaceuticals (Basel)* 5(9) 991–1007, https://doi.org/10.3390/ph5090991.

250. Reissmann S (2014) Cell penetration: scope and limitations by the application of cell-penetrating peptides *J Pept Sci* 20(10) 760–784, https://doi.org/10.1002/psc.2672.

251. Raucher D, & Ryu JS (2015) Cell-penetrating peptides: strategies for anticancer treatment *Trends Mol Med* 21(9) 560–570, https://doi.org/10.1016/j.molmed.2015.06.005.

252. Derakhshankhah H, Izadi Z, Alaei L, Lotfabadi A, Saboury AA, Dinarvand R, Divsalar A, Seyedarabi A, Barzegari E, & Evini M (2017) Colon Cancer and Specific Ways to Deliver Drugs to the Large Intestine *Anticancer Agents Med Chem* 17(10) 1317–1327, https://doi.org/10.2174/1871520617666170213142030.

253. Gao H, Zhang Q, Yang Y, Jiang X, & He Q (2015) Tumor homing cell penetrating peptide decorated nanoparticles used for enhancing tumor targeting delivery and therapy *International Journal of Pharmaceutics* 478(1) 240–250, https://doi.org/10.1016/j.ijpharm.2014.11.029.

254. Zhou N, Wu J, Qin Y-Y, Zhao X-L, Ding Y, Sun L-S, He T, Huang X-W, Liu C-B, & Wang H (2017) Novel peptide MT23 for potent penetrating and selective targeting in mouse melanoma cancer cells *Eur J Pharm Biopharm* 120 80–88, https://doi.org/10.1016/j.ejpb.2017.08.011.

255. Feni L, Parente S, Robert C, Gazzola S, Arosio D, Piarulli U, & Neundorf I (2019) Kiss and Run: Promoting Effective and Targeted Cellular Uptake of a Drug Delivery Vehicle Composed of an Integrin-Targeting Diketopiperazine Peptidomimetic and

a Cell-Penetrating Peptide *Bioconjug Chem* 30(7) 2011–2022, https://doi.org/10.1021/acs.bioconjchem.9b00292.

256. Shin MC, Zhang J, Ah Min K, Lee K, Moon C, Balthasar JP, & Yang VC (2014) Combination of antibody targeting and PTD-mediated intracellular toxin delivery for colorectal cancer therapy *J Control Release* 194 197–210, https://doi.org/10.1016/j.jconrel.2014.08.030.

257. Cheng H, Zhu J-Y, Xu X-D, Qiu W-X, Lei Q, Han K, Cheng Y-J, & Zhang X-Z (2015) Activable Cell-Penetrating Peptide Conjugated Prodrug for Tumor Targeted Drug Delivery *ACS Appl Mater Interfaces* 7(29) 16061–16069, https://doi.org/10.1021/acsami.5b04517.

258. Xiang B, Jia X-L, Qi J-L, Yang L-P, Sun W-H, Yan X, Yang S-K, Cao D-Y, Du Q, & Qi X-R (2017) Enhancing siRNA-based cancer therapy using a new pH-responsive activatable cell-penetrating peptide-modified liposomal system *Int J Nanomedicine* 12 2385–2405, https://doi.org/10.2147/IJN.S129574.

259. Karagiota A, Kourti M, Simos G, & Mylonis I (2019) HIF-1α-derived cell-penetrating peptides inhibit ERK-dependent activation of HIF-1 and trigger apoptosis of cancer cells under hypoxia *Cell Mol Life Sci* 76(4) 809–825, https://doi.org/10.1007/s00018-018-2985-7.

260. Bechara C, & Sagan S (2013) Cell-penetrating peptides: 20 years later, where do we stand? *FEBS Lett* 587(12) 1693–1702, https://doi.org/10.1016/j.febslet.2013.04.031.

261. Aroui S, & Kenani A. Cell-Penetrating Peptides: A Challenge for Drug Delivery. In: Stefaniu A, Rasul A and Hussain G (editors), Cheminformatics and its Applications. IntechOpen, London, UK, 2020: 1-11. https://doi.org/10.5772/intechopen.91684.

262. Szabó I, Orbán E, Schlosser G, Hudecz F, & Bánóczi Z (2016) Cell-penetrating conjugates of pentaglutamylated methotrexate as potential anticancer drugs against

resistant tumor cells *Eur J Med Chem* 115 361–368, https://doi.org/10.1016/j.ejmech.2016.03.034.

263. Bánóczi Z, Peregi B, Orbán E, Szabó R, & Hudecz F (2008) Synthesis of daunomycin-oligoarginine conjugates and their effect on human leukemia cells (HL-60) *Arkivoc* 2008(3) 140–153, https://doi.org/10.3998/ark.5550190.0009.313.

264. Bánóczi Z, Gorka-Kereskényi Á, Reményi J, Orbán E, Hazai L, Tökési N, Oláh J, Ovádi J, Béni Z, Háda V, Szántay C, Hudecz F, Kalaus G, & Szántay C (2010) Synthesis and in vitro antitumor effect of vinblastine derivative-oligoarginine conjugates *Bioconjug Chem* 21(11) 1948–1955, https://doi.org/10.1021/bc100028z.

265. Gorman M, Neuss N, & Biemann K (1962) Vinca Alkaloids. X1. The Structure of Vindoline *J Am Chem Soc* 84(6) 1058–1059, https://doi.org/10.1021/ja00865a042.

266. Owellen RJ, Hartke CA, Dickerson RM, & Hains FO (1976) Inhibition of tubulin-microtubule polymerization by drugs of the Vinca alkaloid class *Cancer Res* 36(4) 1499–1502.

267. Prakash V, & Timasheff SN (1991) Mechanism of interaction of vinca alkaloids with tubulin: catharanthine and vindoline *Biochemistry* 30(3) 873–880, https://doi.org/10.1021/bi00217a042.

268. Barnett CJ, Cullinan GJ, Gerzon K, Hoying RC, Jones WE, Newlon WM, Poore GA, Robison RL, Sweeney MJ, Todd GC, Dyke RW, & Nelson RL (1978) Structure-activity relationships of dimeric Catharanthus alkaloids. 1. Deacetylvinblastine amide (vindesine) sulfate *J Med Chem* 21(1) 88–96, https://doi.org/10.1021/jm00199a016.

269. Conrad RA, Cullinan GJ, Gerzon K, & Poore GA (1979) Structure-activity relationships of dimeric Catharanthus alkaloids. 2. Experimental antitumor activities of N-substituted deacetylvinblastine amide (vindesine) sulfates *J Med Chem* 22(4) 391–400, https://doi.org/10.1021/jm00190a008.

270. Bhushana Rao KS, Collard MP, Dejonghe JP, Atassi G, Hannart JA, & Trouet A (1985) Vinblastin-23-oyl amino acid derivatives: chemistry, physicochemical data,

toxicity, and antitumor activities against P388 and L1210 leukemias *J Med Chem* 28(8) 1079–1088, https://doi.org/10.1021/jm00146a017.

271. Gorka-Kereskényi Á, Szabó L, Hazai L, Lengyel M, Szántay, Jr. C, Sánta Z, Kalaus G, & Szántay C (2007) Aromatic Electrophilic Substitutions on Vindoline *Heterocycles* 71(7) 1553–1563, https://doi.org/10.3987/COM-07-11049.

272. Keglevich P, Hazai L, Gorka-Kereskényi Á, Péter L, Gyenese J, Lengyel Z, Kalaus G, Dubrovay Z, Dékány M, Orbán E, Szabó I, Bánóczi Z, Szántay, Jr. C, & Szántay C (2013) Synthesis and in vitro Antitumor Effect of New Vindoline Derivatives Coupled with Amino Acid Esters *Heterocycles* 87(11) 2299–2317, https://doi.org/10.3987/COM-13-12827.

273. Gad SC. Introduction: Drug Discovery in the 21st Century. In: Gad SC, Cavagnaro JA, Nassar AF, Hollenberg PF, Scatina J, Bleavins MR, Carini C, Jurima-Romet M, Rahbari R (editors), Pharmaceutical Sciences Encyclopedia: Drug Discovery, Development, and Manufacturing, American Cancer Society. John Wiley & Sons, New York, 2010: 1–10, https://doi.org/10.1002/9780470571224.pse001.

274. Obexer R, Walport LJ, & Suga H (2017) Exploring sequence space: harnessing chemical and biological diversity towards new peptide leads *Current Opinion in Chemical Biology* 38 52–61, https://doi.org/10.1016/j.cbpa.2017.02.020.

275. Vergote V, Burvenich C, Van de Wiele C, & De Spiegeleer B (2009) Quality specifications for peptide drugs: a regulatory-pharmaceutical approach *J Pept Sci* 15(11) 697–710, https://doi.org/10.1002/psc.1167.

276. Global Peptide Therapeutics Market Size 2017: By Type, Industry Share, Trends, Growth Analysis and Forecast to 2024 Retrieved online May 11, 2020 from: https://www.zionmarketresearch.com/report/peptide-therapeutics-market.

277. Sumantran VN. Cellular Chemosensitivity Assays: An Overview. In: Cree IA (editor), Cancer Cell Culture: Methods in Molecular Biology (Methods and Protocols), vol 731. Humana Press, Totowa, NJ, USA, 2011: 219–236, https://doi.org/10.1007/978-1-61779-080-5_19.

278. López Rivas P, Ranđelović I, Raposo Moreira Dias A, Pina A, Arosio D, Tóvári J, Mező G, Dal Corso A, Pignataro L, & Gennari C (2018) Synthesis and Biological Evaluation of Paclitaxel Conjugates Involving Linkers Cleavable by Lysosomal Enzymes and αVβ3-Integrin Ligands for Tumor Targeting *European Journal of Organic Chemistry* 2018(23) 2902–2909, https://doi.org/10.1002/ejoc.201800447.

279. Chand S, O'Hayer K, Blanco FF, Winter JM, & Brody JR (2016) The Landscape of Pancreatic Cancer Therapeutic Resistance Mechanisms *Int J Biol Sci* 12(3) 273–282, https://doi.org/10.7150/ijbs.14951.

280. Miller DW, Fontain M, Kolar C, & Lawson T (1996) The expression of multidrug resistance-associated protein (MRP) in pancreatic adenocarcinoma cell lines *Cancer Lett* 107(2) 301–306, https://doi.org/10.1016/0304-3835(96)04384-4.

281. Zaman GJ, Flens MJ, van Leusden MR, de Haas M, Mülder HS, Lankelma J, Pinedo HM, Scheper RJ, Baas F, & Broxterman HJ (1994) The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump *Proc Natl Acad Sci USA* 91(19) 8822–8826, https://doi.org/10.1073/pnas.91.19.8822.

282. Zheng Z, Aojula H, & Clarke D (2010) Reduction of doxorubicin resistance in P-glycoprotein overexpressing cells by hybrid cell-penetrating and drug-binding peptide *J Drug Target* 18(6) 477–487, https://doi.org/10.3109/10611860903548347.

283. Friess H, Büchler M, Kiesel L, Krüger M, & Beger HG (1991) LH-RH receptors in the human pancreas. Basis for antihormonal treatment in ductal carcinoma of the pancreas *Int J Pancreatol* 10(2) 151–159, https://doi.org/10.1007/BF02924118.

284. Sand E, Bergvall M, Ekblad E, D'Amato M, & Ohlsson B (2013) Expression and distribution of GnRH, LH, and FSH and their receptors in gastrointestinal tract of man and rat *Regul Pept* 187 24–28, https://doi.org/10.1016/j.regpep.2013.09.002.

285. Ohlsson B (2016) Gonadotropin-Releasing Hormone and Its Physiological and Pathophysiological Roles in Relation to the Structure and Function of the

Gastrointestinal Tract *Eur Surg Res* 57(1–2) 22–33, https://doi.org/10.1159/000445717.

286. Greenbaum D, Colangelo C, Williams K, & Gerstein M (2003) Comparing protein abundance and mRNA expression levels on a genomic scale *Genome Biol* 4(9) 117, https://doi.org/10.1186/gb-2003-4-9-117.

287. Nagaraj N, Wisniewski JR, Geiger T, Cox J, Kircher M, Kelso J, Pääbo S, & Mann M (2011) Deep proteome and transcriptome mapping of a human cancer cell line *Mol Syst Biol* 7 548, https://doi.org/10.1038/msb.2011.81.

288. Maier T, Güell M, & Serrano L (2009) Correlation of mRNA and protein in complex biological samples *FEBS Letters* 583(24) 3966–3973, https://doi.org/10.1016/j.febslet.2009.10.036.

289. Hapgood JP, Sadie H, van Biljon W, & Ronacher K (2005) Regulation of expression of mammalian gonadotrophin-releasing hormone receptor genes *J Neuroendocrinol* 17(10) 619–638, https://doi.org/10.1111/j.1365-2826.2005.01353.x.

290. Eidne KA, Flanagan CA, & Millar RP (1985) Gonadotropin-releasing hormone binding sites in human breast carcinoma *Science* 229(4717) 989–991, https://doi.org/10.1126/science.2992093.

291. Emons G, Pahwa GS, Brack C, Sturm R, Oberheuser F, & Knuppen R (1989) Gonadotropin releasing hormone binding sites in human epithelial ovarian carcinomata *Eur J Cancer Clin Oncol* 25(2) 215–221, https://doi.org/10.1016/0277-5379(89)90011-4.

292. Irmer G, Bürger C, Müller R, Ortmann O, Peter U, Kakar SS, Neill JD, Schulz KD, & Emons G (1995) Expression of the messenger RNAs for luteinizing hormone-releasing hormone (LHRH) and its receptor in human ovarian epithelial carcinoma *Cancer Res* 55(4) 817–822.

293. Wang Y, Cheetham AG, Angacian G, Su H, Xie L, & Cui H (2017) Peptide-drug conjugates as effective prodrug strategies for targeted delivery *Adv Drug Deliv Rev* 110–111 112–126, https://doi.org/10.1016/j.addr.2016.06.015.

294. Mak IW, Evaniew N, & Ghert M (2014) Lost in translation: animal models and clinical trials in cancer treatment *Am J Transl Res* 6(2) 114–118.

295. Parasuraman S (2011) Toxicological screening *J Pharmacol Pharmacother* 2(2) 74–79, https://doi.org/10.4103/0976-500X.81895.

296. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the Care and Use of Laboratory Animals, 8th ed. National Academies Press (US), Washington (DC), 2011: 11-35, https://doi.org/10.17226/12910.

297. Gerratana L, Fanotto V, Bonotto M, Bolzonello S, Minisini AM, Fasola G, & Puglisi F (2015) Pattern of metastasis and outcome in patients with breast cancer *Clin Exp Metastasis* 32(2) 125–133, https://doi.org/10.1007/s10585-015-9697-2.

298. Jin L, Han B, Siegel E, Cui Y, Giuliano A, & Cui X (2018) Breast cancer lung metastasis: Molecular biology and therapeutic implications *Cancer Biol Ther* 19(10) 858–868, https://doi.org/10.1080/15384047.2018.1456599.

299. Ottewell PD, Coleman RE, & Holen I (2006) From genetic abnormality to metastases: murine models of breast cancer and their use in the development of anticancer therapies *Breast Cancer Res Treat* 96(2) 101–113, https://doi.org/10.1007/s10549-005-9067-x.

300. Jordan VC (2015) Proven value of translational research with appropriate animal models to advance breast cancer treatment and save lives: the tamoxifen tale *Br J Clin Pharmacol* 79(2) 254–267, https://doi.org/10.1111/bcp.12440.

301. Dexter DL, Kowalski HM, Blazar BA, Fligiel Z, Vogel R, & Heppner GH (1978) Heterogeneity of tumor cells from a single mouse mammary tumor *Cancer Res* 38(10) 3174–3181.

302. Heppner GH, Dexter DL, DeNucci T, Miller FR, & Calabresi P (1978) Heterogeneity in drug sensitivity among tumor cell subpopulations of a single mammary tumor *Cancer Res* 38(11 Pt 1) 3758–3763.

303. Rashid OM, & Takabe K (2015) Animal models for exploring the pharmacokinetics of breast cancer therapies *Expert Opin Drug Metab Toxicol* 11(2) 221–230, https://doi.org/10.1517/17425255.2015.983073.

304. Singh M, Ramos I, Asafu-Adjei D, Quispe-Tintaya W, Chandra D, Jahangir A, Zang X, Aggarwal BB, & Gravekamp C (2013) Curcumin improves the therapeutic efficacy of Listeria(at)-Mage-b vaccine in correlation with improved T-cell responses in blood of a triple-negative breast cancer model 4T1 *Cancer Med* 2(4) 571–582, https://doi.org/10.1002/cam4.94.

305. Tao K, Fang M, Alroy J, & Sahagian GG (2008) Imagable 4T1 model for the study of late stage breast cancer *BMC Cancer* 8(1) 228, https://doi.org/10.1186/1471-2407-8-228.

306. Clarke R (1996) Animal models of breast cancer: Their diversity and role in biomedical research *Breast Cancer Res Tr* 39(1) 1–6, https://doi.org/10.1007/BF01806073.

307. Bibby MC (2004) Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages *Eur J Cancer* 40(6) 852–857, https://doi.org/10.1016/j.ejca.2003.11.021.

308. Fantozzi A, & Christofori G (2006) Mouse models of breast cancer metastasis *Breast Cancer Res* 8(4) 212, https://doi.org/10.1186/bcr1530.

309. Weigelt B, Peterse JL, & van't Veer LJ (2005) Breast cancer metastasis: markers and models *Nat Rev Cancer* 5(8) 591–602, https://doi.org/10.1038/nrc1670.

310. Heppner GH, Miller FR, & Shekhar PM (2000) Nontransgenic models of breast cancer *Breast Cancer Res* 2(5) 331–334, https://doi.org/10.1186/bcr77.

311. Morecki S, Yacovlev L, & Slavin S (1998) Effect of indomethacin on tumorigenicity and immunity induction in a murine model of mammary carcinoma *Int J Cancer* 75(6) 894–899, https://doi.org/10.1002/(sici)1097-0215(19980316)75:6<894::aid-ijc12>3.0.co;2-3.

312. Kocatürk B, & Versteeg HH (2015) Orthotopic injection of breast cancer cells into the mammary fat pad of mice to study tumor growth *J Vis Exp* (96) e51967, https://doi.org/10.3791/51967.

313. Denayer T, Stöhr T, & Roy MV (2014) Animal models in translational medicine: Validation and prediction *European Journal of Molecular & Clinical Medicine* 2(1) 5–11, https://doi.org/10.1016/j.nhtm.2014.08.001.

314. Scholzen T, & Gerdes J (2000) The Ki-67 protein: from the known and the unknown *J Cell Physiol* 182(3) 311–322, https://doi.org/10.1002/(SICI)1097-4652(200003)182:3<311::AID-JCP1>3.0.CO;2-9.

315. Danesi R, Paparelli A, Bernardini N, & Del Tacca M (1988) Cytofluorescence localization and disposition of doxorubicin and doxorubicinol in rat cardiac tissue *Eur J Cancer Clin Oncol* 24(7) 1123–1131, https://doi.org/10.1016/0277-5379(88)90118-6.

316. Paul C, Baurain R, Gahrton G, & Peterson C (1980) Determination of daunorubicin and its main metabolites in plasma, urine and leukaemic cells in patients with acute myeloblastic leukaemia *Cancer Lett* 9(4) 263–269, https://doi.org/10.1016/0304-3835(80)90016-6.

317. Michael B, Yano B, Sellers RS, Perry R, Morton D, Roome N, Johnson JK, Schafer K, & Pitsch S (2007) Evaluation of organ weights for rodent and non-rodent toxicity studies: a review of regulatory guidelines and a survey of current practices *Toxicol Pathol* 35(5) 742–750, https://doi.org/10.1080/01926230701595292.

318. Paget S (1989) The distribution of secondary growths in cancer of the breast. 1889 *Cancer Metastasis Rev* 8(2) 98–101, https://doi.org/10.1016/S0140-6736(00)49915-0.

319. Miller FR, Medina D, & Heppner GH (1981) Preferential growth of mammary tumors in intact mammary fatpads *Cancer Res* 41(10) 3863–3867.

320. Bosma GC, Custer RP, & Bosma MJ (1983) A severe combined immunodeficiency mutation in the mouse *Nature* 301(5900) 527–530, https://doi.org/10.1038/301527a0.

321. Chavez KJ, Garimella SV, & Lipkowitz S (2010) Triple negative breast cancer cell lines: one tool in the search for better treatment of triple negative breast cancer *Breast Dis* 32(1–2) 35–48, https://doi.org/10.3233/BD-2010-0307.

322. Herédi-Szabó K, Murphy RF, & Lovas S (2006) Is lGnRH-III the most potent GnRH analog containing only natural amino acids that specifically inhibits the growth of human breast cancer cells? *J Pept Sci* 12(11) 714–720, https://doi.org/10.1002/psc.783.

323. Dewan MZ, Terunuma H, Ahmed S, Ohba K, Takada M, Tanaka Y, Toi M, & Yamamoto N (2005) Natural killer cells in breast cancer cell growth and metastasis in SCID mice *Biomed Pharmacother* 59 Suppl 2 S375-379, https://doi.org/10.1016/s0753-3322(05)80082-4.

324. Talmadge JE, Singh RK, Fidler IJ, & Raz A (2007) Murine models to evaluate novel and conventional therapeutic strategies for cancer *Am J Pathol* 170(3) 793–804, https://doi.org/10.2353/ajpath.2007.060929.

325. Mittal VK, Bhullar JS, & Jayant K (2015) Animal models of human colorectal cancer: Current status, uses and limitations *World J Gastroenterol* 21(41) 11854–11861, https://doi.org/10.3748/wjg.v21.i41.11854.

326. McIntyre RE, Buczacki SJA, Arends MJ, & Adams DJ (2015) Mouse models of colorectal cancer as preclinical models *Bioessays* 37(8) 909–920, https://doi.org/10.1002/bies.201500032.

327. Schreier VN, Mező G, Orbán E, Dürr C, Marquardt A, & Manea M (2013) Synthesis, enzymatic stability and in vitro cytostatic effect of Daunorubicin-GnRH-III derivative dimers *Bioorg Med Chem Lett* 23(7) 2145–2150, https://doi.org/10.1016/j.bmcl.2013.01.114.

328. Tseng W, Leong X, & Engleman E (2007) Orthotopic mouse model of colorectal cancer *J Vis Exp* (10) e484, https://doi.org/10.3791/484.

329. Liao H-W, & Hung M-C (2017) Intracaecal Orthotopic Colorectal Cancer Xenograft Mouse Model *Bio Protoc* 7(11) e2311, https://doi.org/10.21769/BioProtoc.2311.

330. Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface *Science* 228(4705) 1315–1317, https://doi.org/10.1126/science.4001944.

331. Smith GP, & Petrenko VA (1997) Phage Display *Chem Rev* 97(2) 391–410, https://doi.org/10.1021/cr960065d.

332. Kiss K, Biri-Kovács B, Szabó R, Ranđelović I, Enyedi KN, Schlosser G, Orosz Á, Kapuvári B, Tóvári J, & Mező G (2019) Sequence modification of heptapeptide selected by phage display as homing device for HT-29 colon cancer cells to improve the anti-tumour activity of drug delivery systems *Eur J Med Chem* 176 105–116, https://doi.org/10.1016/j.ejmech.2019.05.016.

333. Fourie AM, Sambrook JF, & Gething MJ (1994) Common and divergent peptide binding specificities of hsp70 molecular chaperones *J Biol Chem* 269(48) 30470–30478, https://doi.org/10.1016/S0021-9258(18)43837-9.

334. Shevtsov M, Huile G, & Multhoff G (2018) Membrane heat shock protein 70: a theranostic target for cancer therapy *Philos Trans R Soc Lond, B, Biol Sci* 373(1738) 20160526, https://doi.org/10.1098/rstb.2016.0526.

335. Sertel S, Fu Y, Zu Y, Rebacz B, Konkimalla B, Plinkert PK, Krämer A, Gertsch J, & Efferth T (2011) Molecular docking and pharmacogenomics of vinca alkaloids and their monomeric precursors, vindoline and catharanthine *Biochem Pharmacol* 81(6) 723–735, https://doi.org/10.1016/j.bcp.2010.12.026.

336. Almagro L, Fernández-Pérez F, & Pedreño MA (2015) Indole alkaloids from Catharanthus roseus: bioproduction and their effect on human health *Molecules* 20(2) 2973–3000, https://doi.org/10.3390/molecules20022973.

337. Bánóczi Z, Keglevich A, Szabó I, Ranđelović I, Hegedüs Z, Regenbach FL, Keglevich P, Lengyel Z, Gorka-Kereskényi Á, Dubrovay Z, Háda V, Szigetvári Á, Szántay C, Hazai L, Tóvári J, & Hudecz F (2018) The effect of conjugation on antitumor activity of vindoline derivatives with octaarginine, a cell-penetrating peptide *J Pept Sci* 24(10) e3118, https://doi.org/10.1002/psc.3118.

338. Maiolo JR, Ferrer M, & Ottinger EA (2005) Effects of cargo molecules on the cellular uptake of arginine-rich cell-penetrating peptides *Biochim Biophys Acta* 1712(2) 161–172, https://doi.org/10.1016/j.bbamem.2005.04.010.

339. Nakase I, Konishi Y, Ueda M, Saji H, & Futaki S (2012) Accumulation of arginine-rich cell-penetrating peptides in tumors and the potential for anticancer drug delivery in vivo *J Control Release* 159(2) 181–188, https://doi.org/10.1016/j.jconrel.2012.01.016.

10 BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

10.1 Publications included into the PhD thesis

1. Ranđelović I, Schuster S, Kapuvári B, Fossati G, Steinkühler C, Mező G, & Tóvári J (2019) Improved In Vivo Anti-Tumor and Anti-Metastatic Effect of GnRH-III-Daunorubicin Analogs on Colorectal and Breast Carcinoma Bearing Mice *Int J Mol Sci* 20(19) 4763, https://doi.org/10.3390/ijms20194763. IF: 4.556
2. Kiss K, Biri-Kovács B, Szabó R, Ranđelović I, Enyedi KN, Schlosser G, Orosz Á, Kapuvári B, Tóvári J, & Mező G (2019) Sequence modification of heptapeptide selected by phage display as homing device for HT-29 colon cancer cells to improve the anti-tumour activity of drug delivery systems *Eur J Med Chem* 176 105-116, https://doi.org/10.1016/j.ejmech.2019.05.016. IF: 5.573
3. Bánóczi Z, Keglevich A, Szabó I, Ranđelović I, Hegedüs Z, Regenbach FL, Keglevich P, Lengyel Z, Gorka-Kereskényi Á, Dubrovay Z, Háda V, Szigetvári Á, Szántay C Jr, Hazai L, Tóvári J, & Hudecz F (2018) The effect of conjugation on antitumor activity of vindoline derivatives with octaarginine, a cell-penetrating peptide *J Pept Sci* 24(10) e3118, https://doi.org/10.1002/psc.3118. IF: 2.081

10.2 Publications related to the PhD thesis

1. Dókus LE, Lajkó E, Ranđelović I, Mező D, Schlosser G, Kőhidai L, Tóvári J, & Mező G (2020) Phage Display-Based Homing Peptide-Daunomycin Conjugates for Selective Drug Targeting to PANC-1 Pancreatic Cancer *Pharmaceutics* 12(6) 576, https://doi.org/10.3390/pharmaceutics12060576. IF: 4.421
2. Tripodi AAP, Ranđelović I, Biri-Kovács B, Szeder B, Mező G, & Tóvári J (2020) In Vivo Tumor Growth Inhibition and Antiangiogenic Effect of Cyclic NGR Peptide-Daunorubicin Conjugates Developed for Targeted Drug Delivery *Pathol Oncol Res* 26(3) 1879-1892, https://doi.org/10.1007/s12253-019-00773-3. IF: 2.826
3. López Rivas P, Ranđelović I, Raposo Moreira Dias A, Pina A, Arosio D, Tóvári J, Mező G, Dal Corso A, Pignataro L, & Gennari C (2018) Synthesis and Biological Evaluation of Paclitaxel Conjugates Involving Linkers Cleavable by Lysosomal Enzymes and αVβ3-Integrin Ligands for Tumor Targeting *European*

Journal of Organic Chemistry 2018(23) 2902-2909, https://doi.org/10.1002/ejoc.201800447. IF: 3.029

4. Gronewold A, Horn M, Ranđelović I, Tóvári J, Muñoz Vázquez S, Schomäcker K, & Neundorf I (2017) Characterization of a Cell-Penetrating Peptide with Potential Anticancer Activity *ChemMedChem* 12(1) 42-49, https://doi.org/10.1002/cmdc.201600498. IF: 3.009
5. Mezõ G, Dókus L, Schlosser G, Lajkó E, Szász Z, Ranđelović I, Biri-Kovács B, Tóvári J, & Kõhidai L (2019) Hasnyálmirigytumort célzó terápiás irányítópeptidek összehasonlítása [Comparison of therapeutic peptides targeting pancreatic cancer] *Magy Onkol* 63(4) 301-308.
6. Mezõ G, Biri-Kovács B, Pethõ L, Sabine S, Kiss K, Oláhné Szabó R, Ranđelović I, & Tóvári J (2019) Célzott tumorterápiára alkalmas peptidhatóanyag konjugátumok szerkezetének optimalizálása [Optimization of peptide-drug conjugates for targeted tumor therapy] *Magy Onkol* 63(4) 290-300.

10.3 Publications not related to the PhD thesis

1. Rádai Z, Windt T, Nagy V, Füredi A, Kiss NZ, Ranđelović I, Tóvári J, Keglevich G, Szakács G, & Tóth S (2019) Synthesis and anticancer cytotoxicity with structural context of an α-hydroxyphosphonate based compound library derived from substituted benzaldehydes *New J Chem* 43 14028-14035, https://doi.org/10.1039/C9NJ02144B. IF: 3.288
2. Madera-Sandoval RL, Tóvári J, Lövey J, Ranđelović I, Jiménez-Orozco A, Hernández-Chávez VG, Reyes-Maldonado E, & Vega-López A (2019) Combination of pentoxifylline and α-galactosylceramide with radiotherapy promotes necro-apoptosis and leukocyte infiltration and reduces the mitosis rate in murine melanoma *Acta Histochem* 121(6) 680-689, https://doi.org/10.1016/j.acthis.2019.06.003. IF: 2.107
3. Nyíri K, Koppány G, Pálfy G, Vida I, Tóth S, Orgován Z, Ranđelović I, Baranyi M, Molnár E, Keserû MG, Tóvári J, Perczel A, Vértessy BG, & Tímár J (2019) Allélspecifikus inhibitorok nyomában: a RASopátia konzorcium célpontjában a KRAS fehérje onkogén mutációi [Allele-specific inhibitors of mutant KRAS are in the focus of RASopathy consortium] *Magy Onkol* 63(4) 310-323.

4. Ludwig G, Ranđelović I, Maksimović-Ivanić D, Mijatović S, Bulatović MZ, Miljković D, Korb M, Lang H, Steinborn D, & Kaluđerović GN (2014) Anticancer potential of (pentamethylcyclopentadienyl)chloridoiridium(III) complexes bearing κP and κP,κS-coordinated Ph2 PCH2 CH2 CH2 S(O)x Ph (x=0-2) ligands *ChemMedChem* 9(7) 1586-93, https://doi.org/10.1002/cmdc.201300479. IF: 2.968
5. Ludwig G, Mijatović S, Ranđelović I, Bulatović M, Miljković D, Maksimović-Ivanić D, Korb M, Lang H, Steinborn D, & Kaluđerović GN (2013) Biological activity of neutral and cationic iridium(III) complexes with κP and κP,κS coordinated $Ph_2PCH_2S(O)xPh$ (x = 0-2) ligands *Eur J Med Chem* 69 216-22, https://doi.org/10.1016/j.ejmech.2013.08.025. IF: 3.432

DOI:10.14753/SE.2021.2555

11 ACKNOWLEDGMENTS

I have the greatest gratitude to my wife Anna, my love, my inspiration and motivation, my strength, my will, my happiness, my life, for magical energy, for magical connection, for immense support and help, for sincere understanding, for tremendous patience, for heavenly encouragement, for her believing in me, for her always being with me, in my heart, whose pure love I feel and which gives me meaning in life.

Posebnu zahvalnost dugujem mojoj porodici, mojoj mami Višnji, tati Radovanu, sestri Ivani i sestričinama Minji i Vanji, na beskonačnom verovanju, razumevanju i strpljenju, na bezuslovnoj ljubavi, na bezgraničnoj podršci i bodrenju, na svemu dobrom i kvalitetnom što su ugradili u mene. Ponosim se vama i time što sam deo vas, i vi deo mene. POBEDILI SMO I POBEDIĆEMO.

DOI:10.14753/SE.2021.2555

I am immensely grateful to my supervisor Dr. József Tóvári (National Institute of Oncology) for giving me a chance to be part of his lab, for believing in me every second, for letting me to grow and develop independently, but always near in crucial moments to hold me, support me, help me, and with surgically precise advices improve my science. Thank you for making it possible for me to come to the lab with a smile every day, to enjoy my work, to go home satisfied, and thank you for every help I asked you for regarding private life, you answered with the speed of light. It is my biggest happiness to have you in my life as a great supervisor and a great human.

I am deeply grateful to Dr. Mihály Cserepes (National Institute of Oncology), my direct colleague, for all helpful and golden advice regarding experiments from the planning to the evaluation of results, for the time and dedication with which he reviewed and evaluated my presentation for doctoral thesis defense, for everything he selflessly shared with me, and for great attitude and atmosphere he always provides on our friendly meetings.

Special thanks to Zita Hegedüs (National Institute of Oncology), my direct colleague, for always fast respond to help me with experiments and everyday lab activities, for a lot of translation on and from Hungarian language, and for great years together.

Huge thanks to Anita Hidvégi, Irén Bodrogi-Mayer, and Anna Mária Tóth for introducing me to the *in vivo* experiments, for your willing to help me always in working with animals, for your precise work, and for your big contribution in my every day research.

Nagy köszönet illeti az egész Kísérletes Farmakológiai Osztályt: Mónikát, Editet, Lizát, Izoldát, Karolát, Marikát, Vikit, Zolit, Janit a rendkívül fontos munkájukért, amivel lehetővé tették az enyémet.

Huge thanks to Dr. Andrea Ladányi, head of Tumor Biology Lab at National Institute of Oncology for big support and encouragement during all these years, especially in my early days at the Institute.

Hálás köszönet Parragné Derecskei Katalinnak, hogy mindig segített az *ex vivo* kísérletekben, illetve köszönet a barátságáért, az összes mosolyért és közös boldog pillanatért, amit átéltünk, annak ellenére is, hogy nem egy nyelvet beszélünk.

DOI:10.14753/SE.2021.2555

I want to thank also my freshly joined colleagues Diána Mező, Sára Surguta, Violetta Léner, Eszter Bajtai and Balázs Vári for encouraging, supporting and for providing me maximum peaceful atmosphere and conditions while writing the last pages of this thesis. It is my richness to have you as colleagues.

Special thanks to Attila Kigyós, CEO of KinetoLab, for supporting and believing in me all these years, and for giving me a chance to work in an industrial environment, making my dream true. Yes, that is it!

Thanks to Dr. Enikő Tátrai for nice welcoming at my starts.

Big thanks to the Department of Biochemistry, head Dr. Krisztina Kőhalmy, and Bence Kapuvári for help and advice regarding experiments, for providing the instruments and reagents, for all support and encouragement during these years.

I am very thankful to Edit Marosi and Zsuzsanna Lénárt for help regarding administration during visa application, and for friendship with such nice persons.

Special thanks to Edit Sánta, Molecular Pathology Laboratory, for nice friendship, enjoyable talks, for care, support and encouraging.

Thanks to Prof. Dr. Péter Nagy, Dr. Katalin Erdélyi and Dr. Éva Dóka from the Department of Molecular Immunology and Toxicology at National Institute of Oncology for all support and help.

Big thanks to Dr. Eleonóra Imrédi (National Institute of Oncology Budapest) for always willing to find time to help me whenever I asked for.

Big thanks to Dr. Beatrix Kotlán (passed away) for kind care if I eat or drink something during my long days in the lab, for advice about beautiful places in Hungary, and for introducing me to healthy life actions and sport activities in Budapest, such as the famous Budapest Marathon.

I am greatly acknowledged to Prof. Dr. Miklós Kásler and Prof. Dr. Csaba Polgár, previous and nowadays director of National Institute of Oncology for their guaranties and supports giving me always during my visa application, and for providing me conditions for work.

DOI:10.14753/SE.2021.2555

I am especially grateful to Prof. Dr. Gábor Mező (Eötvös Loránd University), member of MAGICBULLET network, for immensely big support and help he provides me during all these years. Thank you for always quick and clear explanations of chemistry, thank you for your trust to work with me in wide range of projects with together aim to find magic compound, and thank you for giving me a project which results I published in my first author paper, and which I used as main results in my PhD thesis.

It was my great privilege to have for the closest collaborators and friends Dr. Sabine Schuster and Dr. Andrea Angelo Pierluigi Tripodi, from Eötvös Loránd University, members of MAGICBULLET network, with whom I had great scientific results, unforgettable time together in Budapest, and made friendship for whole life. Thank you for trust to analyze biological activity of your conjugates, thank you for your sincerity, for your warm support in heavy moments, and for a lot of happy moments which will stay in my hear forever.

Great thanks to Krisztina Kiss (Eötvös Loránd University) for synthesis of conjugates which I tested as part of my PhD study.

Special thanks to Dr. Beáta Biri-Kovács (Eötvös Loránd University) for all help regarding experiments, for huge support, and for nice friendship.

Big thanks to Prof. Dr. Ferenc Hudecz (Eötvös Loránd University) for introducing me to the world of bioconjugates, for all pleasant and useful discussions, and for a trust to be part of his research.

Great thanks to Dr. Zoltán Bánóczi (Eötvös Loránd University) for giving me opportunity to be a part of the research to test the compounds he synthesized, which results I used in my PhD thesis.

On my journey it was a pleasure to have Dr. Christian Steinkühler to whom I am thankful for providing great conditions for research and development while my secondment in Preclinical R&D of Italfarmaco, for his big support, advice, encouragement and believing in me.

Big thanks to Dr. Gianluca Fossati, head of the Department for Biochemistry and Molecular Biology at Preclinical R&D, Italfarmaco for giving me a chance to be part of his lab and for implanting in me a critical point of view regarding science.

Special huge thanks to members of the Department for Biochemistry and Molecular Biology at Preclinical R&D, Italfarmaco, Dr. Monica Forino, Dr. Chiara Ripamonti, Dr. Valeria Spadotto, Dr. Gianluca Caprini and Edoardo Cellupica for their great welcoming and warm acceptance in their team where I felt like at home, for English speaking lab meetings, for always their availability when I need advice or help, and for their time they gave to me to improve my knowledge and skills in molecular biology from such a great professionals as they are.

I am very thankful to Dr. Daniela Modena for help and advice regarding *in vitro* assays, and for always pleasant talks while my being in Italfarmaco.

I would like to express my big gratitude to members of the European Training Network MAGICBULLET: Prof. Dr. Norbert Sewald (Bielefeld University) for the excellent organization of the network, Prof. Dr. Cesare Gennari (Milan University), Prof. Dr. Ines Neundorf (Cologne University) and Prof. Dr. Umberto Piarulli (Insubria University) for successful collaboration, Dr. Katherina Sewald (Fraunhofer ITEM), Dr. Torsten Hechler (Heidelberg Pharma), Dr. Ralf Palmisano (Optical Imaging Center Erlangen) and Dr. Hans-Georg Lerchen (Bayer Pharma) for all support and advice.

Special thanks to Prof. Dr. Pirjo Laakkonen (Helsinki University) for drafting me for MAGICBULLET project, and for all constructive critiques from which I learn to be stronger scientist with high level of results expectations.

Many thanks to my colleagues and friends from the MAGICBULLET network Ana Martins, Dr. Clémence Robert, Dr. André Raposo Moreira Dias, Dr. Eduard Figueras, Abiodun Ayo, Jayendrakishore Tanjore Ramanathan, Dr. Lucia Feni, Dr. Barbara Korsak

and Dr. Francesca Gallo for an amazing three years together, through the storms and through the flower fields, through unforgettable moments from which we learned, which improved us and made us family.

Great thanks to Dr. Lizeth Bodero for nice moments, pleasant talks and support during all these years, and for the warm welcoming in Como and unforgettable time there from which grew our lovely friendship.

Big thanks to Dr. Silvia Gazzola for the friendship and unique connections we establish from first view, which grows through years by supporting, encouraging, and believing in each other.

Thanks to Dr. Adina Borbély for useful advice to settle and accommodate in Budapest, and for always pleasant relationship, earlier mostly on the network meetings, and later in Budapest on interesting and enjoyable excursions she organized.

Thanks to Dr. Paula López Rivas for nice friendship established in Budapest, Milan and during network meetings, for huge support and help in professional and private life.

Magnificent thanks to Dr. Marcel Frese, MAGICBULLET network manager, for great organization of every network meeting, for always precise information, for fast answers and help, and for all huge support during these years.

Special thanks to Dr. Katalin Uray (Eötvös Loránd University) for pre-reviewing my PhD thesis for the home defense. I greatly appreciate your comments, suggestions, notifications, questions and precise explanations of very important concepts and points, which was very useful for the improvement of my doctoral thesis, as well as for my knowledge in the field of peptide chemistry and drug conjugates.

I am very grateful to Prof. Dr. József Tímár, Prof. Dr. Sándor Paku, Dr. Katalin Dezső, Dr. Edina Bugyik, Dr. Vanessza Szabó, Dr. István Kenessey, Dr. Gábor Barna and Marcell Baranyi from Semmelweis University for all professional help, for useful advice in experiments, and for improving my knowledge and quality of experimental work.

Big thank to Krisztina Tölgyesi-Lovász, Head of the Semmelweis University Doctoral School Secretariat for huge support, fast answers and help during my studies.

DOI:10.14753/SE.2021.2555

Thank very much to Dr. Gretchen Repasky for big professionalism in her work, who evaluate dedicatedly and studiously my application, and for recommended me a Magicbullet project as the best to my research interest and research background. You were one of the most important sign on the road in my career.

I am grateful to Dr. Danijela Maksimović-Ivanić, Dr. Sanja Mijatović, Dr. Mirna Bulatović, Dr. Marija Mojić, Dr. Stanislava Stošic-Grujičić from the Department of Immunology, Institute for Biological Research "Siniša Stanković", Belgrade University my first mentors, my first guides in the cancer drug development field, who introduced me to the world of science and taught me that it is my world which I should enjoy. Thank you for the energy and time you have dedicated to me, for the tremendous help and support you have given me in my beginnings and all these years.

Special thanks to Dr. Marija Kojić, from The University of Queensland Diamantina Institute, a great scientist, and even greater person, for unreservedly spreading her rich knowledge, love and a positive spirit, for dedicated her time to me, and always available to help me.

Prof. Dr. Zoran Rašić, Director of Veterinary Specialist Institute Jagodina, big thank you for your huge help and support all these years, for always right words and useful advice, for encouraging me and giving me safe wind, and for providing me to walk bravely, persistently and with dignity.

Huge thanks to Prof. Dr. Bogomir Dimitrijević (passed away), Belgrade University, for introducing me and interested me in the world of cancer biology with his interesting, original and always overcrowded lectures.

Big thanks to my dear friends Giga, Jela, Filip, Dijana, Andrija, Neda, Violeta, Dr. Mihailo Jelić, Dr. Ivan Radin, Dr. Ruth Madera Sandoval, Dora, Sebastian, Marko, Irina, Viktor, Ksenija, Elena, Aleksandar, Anna, Vera, Veronika, Zhenechka, Liza, Dima, Mia, Natalia, Maksim, Lada, Borislav and Dr. Mónika Gyugos for the immense support and encouragement they have given me all these years.

Большое спасибо семье Одинцовых (Людмиле и Артему) и Боровиных (Светлане, Владиславу, Екатерине, Соне, Вере и Андрею) за их большую поддержку, веру в меня и безграничную любовь.

DOI:10.14753/SE.2021.2555

The experimental part of my PhD Thesis was done at the Department of Experimental Pharmacology, National Institute of Oncology, Budapest, Hungary, and at the Department for Biochemistry and Molecular Biology, Preclinical R&D, Italfarmaco S.p.A., Cinisello, Milan, Italy.

This research was funded by the European Union's Horizon 2020 research and innovation program Magicbullet under the Marie Sklodowska-Curie Grant No. 642004 and by the Hungarian National Research, Development and Innovation Office (K116295 and KFI_16-1-2017-0439). Financial support from the 2019 and 2020 Thematic Excellence Programs (TUDFO/51757/2019-ITM and TKP2020-NKA-26) is greatly acknowledged.