DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS

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Cell-penetrating peptides: cytotoxicity, immunogenicity and application for tumor targeting

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ABSTRACT

The delivery of therapeutic agents for the hard-to-treat tumors is restricted by inefficacy and non-selectivity of systemically administered drug delivery vectors. Therefore, novel approaches aimed at increasing the specificity and in turn the treatment efficacies of therapeutic drugs are required. During recent years, drug delivery systems, which comprise both targeting and anticancer chemotherapy strategies, have received much attention. In this regard, the application of cell-penetrating peptides appears to be a very promising strategy for targeted drug delivery. Cell-penetrating peptide-based vehicles have been developed for the delivery of different payloads into the cells in culture and in animals. However, similar issues as for any other drug delivery system: cytotoxicity and the tendency to induce innate immune response may limit their use. The current thesis is focused on the characterization of immunogenic and cytotoxic activities of cell-penetrating peptides PepFects, on the cytotoxic and apoptotic activities of newly designed cell-penetrating p53 analogues and on the development of a peptide-based glioma-targeted drug delivery vector.

In Paper I, the cytotoxic and immunogenic activities of PepFect peptides and PepFect/nucleic acid complexes were analyzed *in vitro* and *in vivo*. The current study confirmed that PepFect peptides possess promising potential in nucleic acid delivery without evidence of undesired cytotoxicity and inflammation at the concentrations of 10 μM and 5 μM, respectively *in vitro* and at a dose of 5 mg/kg *in vivo*.

In Paper II, we generated short p53-derived cell-penetrating protein analogues and analyzed their internalization efficacy, cytotoxic and apoptotic activities in the neuroblastoma and breast cancer cell lines. Even though the effect was observed under serum-free conditions, the novel protein analogues demonstrated selective apoptotic activity in the p53-non-active breast cancer cell line suggesting promising perspectives for a future anticancer strategy.

In Paper III, a novel tumor-selective peptide-based drug delivery platform gHoPe2 has been developed demonstrating potential application of CPP-s for tumor-targeted delivery.

In Paper IV, we showed that *N*-terminal modification of non-amphipathic $(RxR)_4$ with stearic acid moiety resulted in efficient peptide vector for nucleic acids delivery in non-toxic manner.

In conclusion, the current work provides evidence for the development of apoptotic protein-derived cell-penetrating peptides and tumor-targeted cell-penetrating peptide-based drug delivery vector with reduced toxicity and immunogenicity.

TABLE OF CONTENTS

LIST OF PUBLICATIONS

The current thesis is based on the following original publications, which will be referred to by the corresponding Roman numerals in the text:

I. **Suhorutšenko, J.,** Copolovici, D. M., Kurrikoff, K., Eriste, E., Oskolkov, N., Arukuusk, P., Langel, Ü (2012). Cell-penetrating peptides, PepFects show no evidence of toxicity and immunogenicity in vitro and in vivo. Bioconjug. Chem. 2255–62.

II. **Suhorutšenko, J.,** Eriste, E., Copolovici, D. M., Langel, Ü (2012). Human protein 53-derived cell-penetrating peptides. Int. J. Pept. Res.Ther. In press.

III. Eriste, E., Kurrikoff, K., **Suhorutšenko, J.,** Oskolkov, N., Copolovici, D. M., Jones, S., Laakkonen, P., Howl, J., Langel, Ü (2012). Peptide-based glioma-targeted drug delivery vector gHoPe2. Bioconjug. Chem. Submitted.

IV. Lehto, T., Abes, R., Oskolkov, N., **Suhorutšenko, J.,** Copolovici, D. M., Mäger, I., Viola, J.R., Simonsson, O., Guterstam, P., Eriste, E., Smith, C.I.E., Lebleu, B., EL-Andaloussi, S., and Langel, Ü (2010). Delivery of nucleic acids with a stearylated $(RxR)_4$ peptide using a non-covalent co-incubation strategy. J. Control.Release. 141, 42–51.

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My personal contribution to the articles referred to in this thesis is as follows:

Ref. I – planned and performed most of the experiments, wrote the Paper as corresponding author

Ref. II – performed the synthesis of the peptides, planned and carried out the experiments, wrote the Paper as corresponding author

Ref. III – planned and performed *in vitro* experiments, analyzed the data and participated in writing of the Paper

Ref. IV – planned and performed *in vitro* uptake and toxicity experiments, analyzed the data and participated in writing of the Paper

ADDITIONAL PUBLICATIONS

1. El-Andaloussi, S., Lehto, T., Mäger, I., Rosenthal-Aizman, K., Oprea, II., Simonson, O. E., Sork, H., Ezzat, K., Copolovici, D. M., Kurrikoff, K., Viola, J. R., Zaghloul, E. M., Sillard, R., Johansson, H. J., Said Hassane, F., Guterstam, P., **Suhorutšenko, J.,** Moreno, P. M., Oskolkov, N., Halldin, J., Tedebark, U., Metspalu, A., Lebleu, B., Lehtio, J., Smith, C. I. & Langel, Ü (2011). Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo. Nucleic Acids Res. 39, 3972–3987.

2. Lehto, T., Simonson, O. E., Mäger, I., Ezzat, K., Sork, H., Copolovici, D. M., Viola, J. R., Zaghloul, E. M., Lundin, P., Moreno, P. M., Mäe, M., Oskolkov, N., **Suhorutšenko, J.,** Smith, C. E. and El-Andaloussi, S (2011). A peptide-based vector for efficient gene transfer *in vitro* and *in vivo*. Molecular Therapy. vol. 19 no. 8, 1457–1467.

3. Kurrikoff, K., **Suhorutšenko, J.,** and Langel, Ü (2011) Cell-penetrating peptides in cancer targeting. In: Drug Delivery in Oncology. From Research Concepts to Cancer Therapy (vol 3). Wiley 1189–1210.

4. Patent application: Chimeric constructs between glioma-homing peptide and cell-penetrating peptides, gHoPe2; Owners: Cepep III AB, Tartu University, University of Wolverhampton; Inventors: Kurrikoff, K., Eriste, E., **Suhorutšenko, J.,** Oskolkov, N., Howl, J., Jones, S., Langel, Ü. Owners: Cepep III AB, Tartu University, University of Wolverhampton; EP application number: EP12171160; Filing date: 07.06.2012, Applicant: Cepep III AB3.

ABBREVIATIONS

CPP	cell-penetrating peptide
DCM	dichloromethane
DIEA	diisopropylethylamine
DMEM	Dulbecco's Modified Eagle Medium
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
HBTU	O-benzotriazole-N,N,N’,N’-tetramethyl-uronium-hexafluoro-phosphate
HOBt	hydroxybenzotriazole
IMDM	Iscove's Modified Dulbecco's Medium
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
NMP	N-methyl-2-pyrrolidone
OBzl	benzyl ester protecting group
PBS	phosphate buffered saline
PEG	polyethylene glycol
pDNA	plasmid DNA
PF	PepFect
PMO	phosphorodiamidate morpholino oligonucleotide
PNA	peptide nucleic acid
RNA	ribonucleic acid
siRNA	short interfering ribonucleic acid
SCO	splice-correcting oligonucleotides
SPPS	solid-phase peptide synthesis
TFA	trifluoroacetic acid
TIS	triisopropylsilane
TP10	transportan 10
TP	transportan

INTRODUCTION

Efficient intracellular internalization of therapeutic agents is often necessary to gain the desired therapeutic effect. The hydrophobic interior of the lipid bilayer makes the cellular membrane impermeable to molecules with high molecular weight such as nucleic acids (pDNA, siRNA, oligonucleotides), proteins, etc., serving as a protective wall between the cytoplasm and extracellular environment. Therefore, the use of these bioactive compounds for therapeutic applications has been restricted. The impermeability of the cellular membrane has become the major obstacle for current drug research and development.[1] Several drug delivery techniques have been proposed to promote efficient internalization of the therapeutics inside the cell. For instance, electroporation, encapsulation in polymers or liposomes, and viral delivery systems are widely utilized. However, several shortcomings including complex manipulation, low efficiency, cytotoxicity and undesired immunogenicity can limit their clinical application.[2, 3]

Cell-penetrating peptides (CPP-s) have proven their ability to deliver various drug molecules, DNA, RNA and proteins into cultured cells. CPP-s, also called PTD-s (protein transduction domains) are a class of peptides that has drawn much attention in the last few decades as non-toxic and non-immunogenic vehicles for the systematic delivery of different payloads. Being less than 30 amino acids in length with net positive charge and/or amphipathic nature these peptides are able to deliver a wide range of cargos through cell membrane both *in vitro* and *in vivo*. CPP-s can be utilized as delivery vectors for peptides, proteins, nucleic acids and chemotherapeutics.[4, 5] Most CPP-s are able to penetrate into the cell regardless to the cell type and proliferative state, which is a great advantage especially when delivery to such hard-to-reach tissues like brain or the parenchyma is preferred.[6] With regards to unspecific internalization and high membrane-penetrating activity, potential side effects such as cytotoxicity and immunogenicity of CPP-s should be considered. These undesired effects can be overcome by addition of the specificity to CPP-based drug delivery vectors using novel targeting strategies for instance homing peptide sequences determined by *in vitro* or *in vivo* phage display methodology.[7]

Prediction of short cell-penetrating peptide sequences using a quantitative structure-activity relationship algorithm gives an advantage for the generation of novel, more efficient therapeutic agents which comprise both cell-penetrating and biological activities within the primary sequence.[8]

The main aims of this thesis are:

1) to characterize the immunogenic and cytotoxic activities of the novel CPP-s PepFect-s as well as peptide/nucleic acid complexes *in vitro* and *in vivo*;
2) to analyze the cytotoxic and pro-apoptotic activities of novel cell-penetrating p53 analogues, whereof sequences were established using structure-activity prediction algorithm;
3) to develop a peptide-based glioma-targeted drug delivery vector;

4) to assess the effect of *N*-terminal stearyl modification of the CPP-s on the nucleic acids delivery *in vitro* in non-toxic manner.

The monitoring of newly designed CPP-s for cytotoxicity and immunogenicity is essential for the complete understanding of the effects of therapeutic agents *in vivo*.

The incorporation of a tumor-targeted sequence into the drug delivery vector enhances the efficacy of the anticancer drugs improving their biodistribution and reducing the toxicity in normal tissues.

Utilization of the mathematical prediction model allows the creation of peptides bearing both cell-penetrating and specific apoptogenic activity, demonstrating great possibilities for the identification of various proteomimetics, which would represent a new class of therapeutic agents.

I. LITERATURE OVERVIEW

I.1. Tumor Targeting

In the past few decades, significant progress has been achieved in understanding the molecular principles of malignant transformation and tumorigenesis. Insights into the genetic and physiological processes of tumorigenesis and tumor progression have resulted in the development of several novel classes of chemotherapeutic compounds. Different growth factor receptor and proteasome inhibitors and antiangiogenic agents have been shown to selectively interfere with tumor cell viability *in vitro* and *in vivo*, improving the balance between the efficacy and toxicity of systemic anticancer therapy.[9, 10] However, the non-specific biodistribution of intravenously administrated therapeutic agents often limit their application. For instance, the majority of routinely used, low molecular anticancer agents are cleared rapidly from systemic circulation by means of renal filtration, which prevents them from reaching tumor tissues. The small size and relatively high hydrophobicity of the most therapeutic molecules are also a reason for their non-specific distribution, resulting in toxicity in many different healthy tissues.[11] Additionally, several anatomical and physiological barriers such as vascular endothelium, the blood brain barrier, cellular and nuclear membranes, hepatic degradation, high interstitial fluid pressure and drug efflux pumps limit the delivery of intravenously-applied anticancer agents to the tumors.[12] The majority of tumor-targeted drug delivery systems have been developed to overcome these limitations. The principle of tumor-targeted delivery is to enhance the antitumor effect by targeting the drug to the tumor site, thereby increasing its specific delivery into the malignant tissues (site-specific delivery), and subsequently by directing the drug away from sites, which are particularly sensitive to the toxic effects (site-avoidance delivery), reducing the damage of normal tissues.[11, 13–17] Based on the mode of drug cargo transportation to the tumor site the targeting can be defined as passive targeting or active targeting.

I.1.1. Passive Targeting

Passive targeting was shown for the first time in 1986 by Hiroshi Maeda, who administered i.v. the albumin-binding Evans Blue dye into the mice bearing subcutaneously growing tumors and observed a surprisingly efficient accumulation of the Evans Blue-albumin complex within the tumors.[18] This phenomenon was further explained by the specific anomalies in the malignant tissues that result from the pathophysiology of the tumors. Unlike normal tissues, blood vessels of the malignants are characterized by irregular shape and poor alignment of the endothelial cells being disorganized with the relatively large fenestrations in the range around 100–500 nm in diameter.[19, 20] These anatomical features make the vasculature of tumor tissue permeable for different macromolecules including albumin and even larger nano-sized particles. In

contrast with smaller molecules that are rapidly cleared from the tumor intestitium, the larger molecules when reached the tumors start accumulating due to the absence of the lymphatic system.[21, 22] This so-called "enhanced permeability and retention" (EPR)[22, 23] effect has laid the foundation for the developing of different tumor-targeted drug delivery systems ranging from micro- and macro-particles, to liposomes, to various drug conjugates with synthetic polymers, to serum proteins.[21, 24]

1.1.2. Active Targeting

The study of active targeting received a great impulse in 1975, when Köhler and Milstein developed the strategy of monoclonal antibodies.[25] Utilization of this technique enabled derivation of pure antibodies that bound specifically to the targets overexpressed on tumor cells. In contrast to passive targeting that exploits the pathophysiologic anomalies of malignant tissues, active targeting is based on cellular differences between normal and cancer tissue, focusing on the specific molecular interactions with tumor-associated cell receptors or antigens.[24] Active targeting relies on the ability of various targeting ligands (antibodies, targeting peptides) to specifically bind to the receptors overexpressed in tumors.[11, 26, 27] Therefore, active targeting strategy is used to improve tumor cell recognition and cellular uptake of drug delivery agents, thereby diminishing their accumulation in normal tissues. Targeting ligands such as folate,[28] transferrin[29] and galactosamine[30] are routinely used for active targeting of nanomedicine formulations to tumor cells. Antibodies towards the surface proteins have also been used as targeting moieties for drug delivery systems. For instance, antibodies targeting CD20, CD25 and CD33 chemokine receptors, overexpressed in B-cell lymphoma, T-cell lymphoma and acute myeloid leukaemia, respectively, have been successfully used for delivering radionuclides (Zevalin), immunotoxins (Ontak) and antitumour antiobiotics (Mylotarg) selectively to tumour cells.[11, 12]

1.2. Drug delivery systems

The concept of cancer drug delivery implies transporting the anticancer drug to the tumor tissue and cells with subsequent release, either intra- or extracellularly. Drug delivery systems can be generally classified as micro- and macro-particulate drug delivery systems, high-molecular-, and low-molecular-weight drug conjugates.[21, 24] The drugs can be covalently bound to various drug carriers for development of low- and high-molecular weight drug conjugates or encapsulated in liposomes, nanoparticles, hydrogels or micelles forming micro- and macro-molecular drug delivery systems (**Table 1**).[24]

Table 1. Classification of drug delivery systems[24]

Micro- and macro-molecular drug delivery systems	High-molecular weight drug conjugates	Low-molecular weight drug conjugates
• Liposomes • Nanoparticles • Hydrogels • Micelles	Drug carriers: • Antibodies • Synthetic polymers • Natural polymers • Serum proteins	Drug carriers: • Vitamins • Homing peptides • CPP-s • Aptamers • Fatty acids • Pro-drugs

Drug conjugates developed for active targeting include both high- and low-molecular weight carriers, although high-molecular delivery systems with diameters exceeding 50 nm are preferred.[24, 31] These drug delivery systems comprise the encapsulation of a drug inside a suitable carrier as well as conjugation of the drugs with vitamins,[32] lipids,[33] peptides,[34, 35] aptamers,[36] antibodies,[37, 38] synthetic or natural polymers,[39–48] liposomes,[49, 50] or protein[51–53]- or polymer-based nano- or microparticles.[54–56] Similar approaches can also be used for the delivery of DNA and RNA molecules.[57–63] The covalent coupling or the physical encapsulation of the drug inside the carrier enables active or passive targeting drug delivery strategies. Low- and high-molecular weight drug delivery systems interacting with tumor-associated receptors are internalized into the tumor cell via receptor-mediated endocytosis. The drug delivery systems using passive targeting strategy are internalized by adsorptive or fluid-phase endocytosis.[64] Endosomes, which form during endocytosis, are either transported to certain cell organelles (e.g., the Golgi apparatus), return to the cell surface, or form primary and secondary lysosomes. These sorting procedures depend on the drug carrier and the kind of endocytosis this drug carrier utilizes for internalization.[65]

Macromolecular drug delivery systems are more heterogeneous than low-molecular weight ones, being dispersed in molecular weight, charge distributions, and a range of drug loading ratios. These drug delivery systems comprise drug conjugates with antibodies, synthetic polymers or liposomes, and nanoparticles and microparticles.[24, 66, 67]

An ideal drug carrier should combine optimal loading and release properties (effective release at the tumor site via enzymatic cleavage or in a pH-dependent manner), long-term circulation (sufficient stability in the bloodstream), low toxicity, and high affinity for the receptor or antigen without increasing drug levels in healthy tissue.[68] Although the majority of drug delivery approaches have obtained convincing results in tumor-bearing animal models and even passed the phase I–III trials, only a few of them have reached market approval, for instance liposomes[69] (Doxil®, Daunosome®, Myocet®), the albumin taxol nanoparticle[70] Abraxane®, and the drug–polymer conjugate SMANCS[71] (a

conjugate of poly (styrene-co-maleic acid/anhydride) and the antitumor agent neocarzinostatin).[24]

1.2.1. Cell-penetrating peptides

Cell-penetrating peptides (CPP-s) are relatively short, up to 30 amino acid-long peptides that are capable of crossing the plasma membrane and transport various cargo molecules into the cell (**Table 2**). These properties make them attractive drug delivery vectors, especially considering their high cell internalization efficacy and low cytotoxicity.[6] In 1988, the cellular uptake of HIV-1 transcriptional trans-activator protein TAT was demonstrated.[72, 73] Later, the cell-penetrating activity of penetratin,[74] a peptide derived from the antennapedia homeodomain protein of *Drosophila* and TAT[75] were described. The greatest breakthrough in the CPP field occurred when it was found that the cell-penetrating properties of some proteins depend on the short protein domains and that these protein domains are able not only to translocate through the cellular membrane themselves but also are capable of taking cargo with them.

Another breakthrough occurred when the application of CPP-s *in vivo* for the delivery of PNA, small peptides and large proteins was demonstrated.[76, 77] Thereafter numerous applications of the CPP-s *in vivo* have been reported suggesting that they are one of the most promising approaches for the development of non-invasive and non-toxic drug delivery systems.

It was first suggested that the CPP-s internalize inside the cells using an energy independent mechanism. However, in last decade it has become more evident that endocytosis is the preferred mechanism for internalization of cargo-attached CPP-s.[78] Most probably different CPP-s and CPP-cargo conjugates use different internalization mechanisms or even multiple combinations of these mechanisms.[79]

During the last decade, the number of known CPP sequences has increased rapidly, and it still continues to grow. The novel CPP-s derived from well established proteins are often developed to have the shortest possible peptide sequences capable of translocation through the plasma membrane.[8]

Table 2. Examples of CPP-s and their classification

Protein derived CPP-s

CPP	Sequence	Origin	References
Penetratin	RQIKIWFQNRRMKWKK[a]	*Drosophila* homeoprotein Antennapedia	[80]
TAT (48–60)	GRKKRRQRRRPPQ	Human immunodeficiency virus type 1 (HIV-1) TAT	[81]
pVEC	LLIILRRRIRKQAHAHSK[a]	Mouse protein-VE-cadherin	[82]
VP22	NAKTRRHERRRKLAIER	Human herpes simplex virus protein	[83]

Chimeric CPP-s

CPP	Sequence	Origin	References
MPG	[c]GALFLGFLGAAGSTMGAWSQPKKKRKV[b]	HIV gp41 and SV40 T-antigen	[84]
Pep-1	KETWWETWWTEWSQPKKKRKV[b]	NLS from SV40 large T-antigen and reverse transcriptase of HIV	[85]
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL[a]	Galanin and mastoparan	[86]
TP10	AGYLLGKINLKALAALAKKIL[a]	Galanin and mastoparan	[87]
M918	[c]MVTVLFRRLRIRRACGPPRVRV	The tumor suppressor protein p14ARF	[88]

Synthetic CPP-s

CPP	Sequence	Origin	References
Oligoarginine	$(R)_n$[d]	Chemically synthesized positively charged sequence	[89]
MAP	KLALKLALKALKAALKLA[a]	Chemically synthesized amphipatic peptide	[90]
CADY	GLWRALWRLLRSLWRLLWRA[b]	PPTG1-derived, tryptophan-rich peptide	[91]

[a]amide; [b]cysteamide modification; [c]acetyl modification; [d]n=6–12[d]

The utilization of CPP-s as therapeutic drug delivery vectors comprises both active and passive targeting mechanisms.[6] Passive targeting can be characterized as conditional mechanism that exploits extracellular properties of the tumor tissues including high levels of matrix metalloproteases and the pH differences between the tumor and normal tissues (**Figure 1. A, B**). Following this strategy CPP and cargo internalize into the cell only after tumor-specific extracellular enzymes cleave the CPP-shielding domain, revealing the CPP.[92] Alternatively the CPP can be revealed upon the lower pH environment, typical for the tumors.[93] This pH-dependent CPP activation leads to the cargo specific internalization into the tumor cells.

The active or affinity based targeting mechanism relies on the binding activity of tumor-specific homing peptides[94, 95] and antibodies.[96] Selective CPP-cargo internalization takes place after the specific recognition of tumor cells by the antibodies or after the tumor-specific binding of the homing peptides (**Figure 1. C, D**).

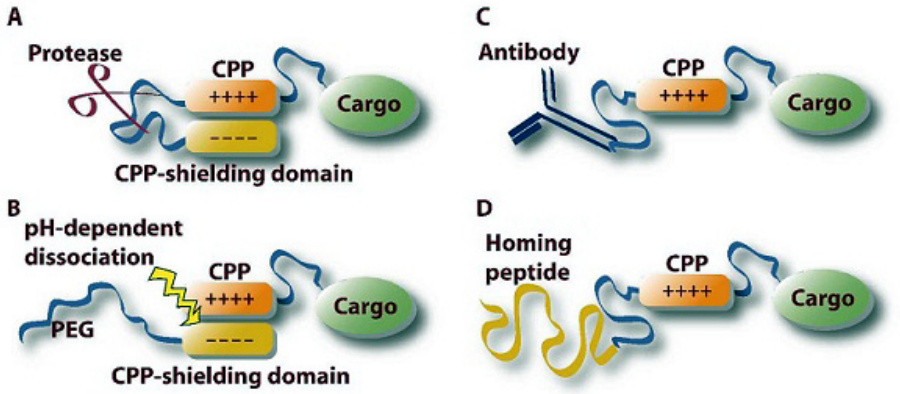

Figure 1. Tumor-targeting strategies of CPP-based drug delivery vectors. **A.** protease-dependent CPP activation. CPP and cargo intracellular internalization occurs when tumor-specific extracellular enzymes cleave the CPP-shielding domain; **B.** pH-dependent CPP activation. Under the lower pH conditions the CPP can be revealed from the CPP-shielding domain leading to the rapid internalization of CPP-cargo vector; **C.** antibody-dependent tumor targeting is based on the specific recognition and binding of the antibodies to the tumor cells; **D.** tumor targeting by homing peptides relies on the affinity of the homing sequences to the specific receptors of the tumor cells.[6]

Most CPP-s are able to penetrate the cells regardless of the cell type and proliferation stage, which is a great advantage especially when the delivery to hard-to-reach tissues (like the brain or into the parenchyma tissue, beyond blood vessels) is considered. The specificity of CPP-based drug delivery vectors can be improved by incorporating novel targeting strategies, for instance, homing peptide sequences determined by *in vitro*, *in vivo* or *ex vivo* phage display methodology.[97] The studies of Ruoslahti and Laakkonen have described the

development of homing peptides, which recognize the molecular alterations in the vasculature of different organs and the tumors.[98–101] The tumor-targeted peptide sequences were identified by using *in vivo* phage displayed peptide libraries and applied further for tumor-targeting therapies, significantly enhancing the efficacy of anticancer drugs.[102] The detected sequences, capable of specific penetration activity in a certain cell types, are attractive vehicles for targeted drug delivery.[103–107]

1.3. Identification of tumor targeting sequences using phage display technology

The majority of pathological conditions, including tumors, diabetes, atherosclerosis, and inflammatory diseases are accompanied by the upregulation of disease-specific molecules on the endothelium of the affected tissues. For instance, during tumor progression, many vascular changes take place contributing to the process of angiogenesis, important for the enhanced blood supply of the growing tumors.[108] Molecular changes that occur during the process of vascular rearrangement can be profiled by using phage display technology. Phage-displayed random peptide libraries enable to map regional and disease-specific differences in the vasculature.[109]

Initial studies utilizing phage display libraries or epitope libraries were performed *in vitro*, where the peptide ligands were searched against the immobilized antigen or cell-specific targets.[110] Thereafter phage display was applied *in vivo* in order to identify the tumor vasculature homing peptides for anticancer therapy.[103] Further *in vivo* studies have proven the fact that not only tumor vasculature, but also normal organs and cells can be targeted using the peptide sequences identified by *in vivo* phage display technology.[101, 111] It was found that each normal tissue expresses its own specific set of cell-surface proteins and carbohydrates on vascular endothelial cells, that can be recognized by phage display screening and that constitute a vascular "zip code" system. These molecules act as novel biomarkers of vascular heterogeneity, being expressed by the normal vasculature and up- or downregulated in tumors.[109]

The identification of tumor-targeting peptides has employed the advanced phage display technology, also known as biopanning. The biopanning method utilizes phage display approach, where the cells or the tissues of interest are exposed to a large, randomized library expressed on the surface of T7 or filamentous phage (M13) particles.[112] The phage display library usually consists of billions of peptide sequences, representing a pool sufficient in size and diversity for the identification of the peptide sequences specific for a variety of target molecules.[113] After exposure, the phage binds to the target cell and the non-relevant phage are removed. The remaining phage are released from the cells and expanded in bacteria, and selected again reexposing to the target of interest. The whole process can be repeated several (6–10) times in order to achieve the adequate enrichment with the relevant clone phage. The peptide

sequence carried as a fusion protein by this phage can be further identified by the peptide sequencing.[112]

In the *in vivo* phage display the peptide library expressing phage are injected into the tail vein of a mouse and allowed to circulate for 5–15 min. Thereafter the organ of interest is removed and the bound phage is rescued and amplified. The whole process is repeated several times (biopanning).[109, 113] Biopanning using phage display can be performed in cell culture, in animals or using the combination of *in vitro* and *in vivo* approaches.[112, 113]

The combination of *ex vivo* and *in vivo* phage display screening methods can be applied for the detection of novel tumor-targeting sequences.[114] According to this strategy the phage library is exposed to the cells prior isolated from the target tissue in order to enrich for peptides binding all cells in the tissue. Thereafter the phage library is introduced into the mouse blood circulation in order to screen for the peptide sequences, capable of specifically targeting the organs of interest. The advanced *ex vivo*/*in vivo* phage display approach allows detection of the homing peptide sequences for more effectively targeting the tissue of interest.[115–117]

1.4. Cytotoxicity of CPP-s

CPP-s play an important role in eukaryotic, prokaryotic and viral biology, being evolutionarily optimized on the one hand as killing agents and on the other as non-toxic cytosolic delivery agents. These biological properties of CPP-s encouraged the rapid development of CPP-based therapeutics either for antimicrobial, anticancer or for cytoprotective, anti-inflammatory or diagnostic purposes. Most CPP-s are cationic peptides, capable not only of translocation themselves but also of the intracellular delivery of various bioactive cargos. This implies an interaction with the plasma membrane, which can result in membrane disturbance. The combination of cationic and membrane-active properties leading to membrane perturbation is also the characteristic of antimicrobial peptides, which also belong to the group of cationic peptides.[118] Moreover, the derivation of CPP-s is also essential (**Table 2**). For example, some CPP-s such as pVEC,[82] are derived directly from membrane proteins and are less toxic, whereas others such as transportans,[86] originate from membrane lytic proteins and are designed to have high amphipathic properties can disturb the plasma membrane.[90] The monitoring of CPP-s regarding their possible cytotoxic side effects on non-targeted membranes is of central importance, especially when the peptides are under consideration for therapeutic applications: gene therapy, cancer treatment.

Toxicity analysis of the peptide-based delivery agents is performed in most of the studies, although only a few studies comparing the toxicity of different classes of CPP-s under similar conditions are available.[119–122] The presence and the origin of cargo may influence translocation mechanisms and the efficiency of the peptide-cargo conjugate, and also the toxicity may differ from that

exerted by the peptide alone. Several independent studies demonstrate that the conjugation of different cargos to the CPP reduces the toxicity produced by CPP alone. Maiolo *et al.,* showed that the incorparation of a tri- or tetra- peptide cargo to Arg_7 and Arg_7Trp diminished the intrinsic toxicities of Arg_7 and Arg_7Trp alone.[123] Jones *et al.,* compared the toxicities of rhodamine-labelelled and unlabelled pAntp (43–58), pTAT (48–60), TP10, and Arg_{11}, and their conjugates with NBD peptide cargo in A549 cells.[120] The CPP-NBD or its scrambled sequence increased the toxicity of TP10 and pTAT (48–60), decreased the toxicity of Arg_{11} and had no effect on pAntp (43–58) response. One of the examples of negative influence on the cellular viability produced by cargo can be the conjugation of fluorophores such as carboxyfluorescein or tetramethylrhodamine to the peptides commonly utilized in order to monitor the internalization of the CPP-s. In their study El-Andaloussi *et al.,* recently showed that the attachment of the carboxyfluorescein to CPP increases the cytotoxicity of the peptide.[122] Similarly, Cardozo *et al.,* showed that the cytotoxicity of TAT (48–57) and penetratin was higher for CPP conjugates with larger cargo peptides then for those unlabelled or with smaller cargo.[119] Increased cytotoxicity of fluorescently labelled CPP-s was also reported by Dupont *et al.,* whereby the conjugation of carboxyfluorescein to penetratin remarkably enhanced plasma membrane leakage in MDCK cells, while the biotin-labelled or unlabelled peptides showed no influence on cellular viability.[124]

1.5. Innate immune response and immunogenicity of drug delivery agents

The administration of almost any foreign macromolecule to an animal can elicit the immune response. Innate immunity comprises a variety of induced mechanisms to clear the organism from foreign, potentially dangerous molecules. These mechanisms are well regulated by pathogen-recognition receptors of macrophages, neutrophils and dendritic cells that are able to discriminate between the molecules present on the normal cells and infectious foreign ligands such as mannose-rich oligosaccharides, peptidoglycans and lipopolysaccharides from the bacterial cells as well as unmethylated DNA, common for many pathogens. Once activated, the immune cells secrete pro-inflammatory chemokines and cytokines in turn activating naive T-lymphocytes and inducing the adaptive immune response.[125, 126]

Although the majority of therapeutic drug delivery agents themselves are generally considered to be non-toxic and non-immunogenic, the size and the surface characteristics of cargo-assembled complexes or nanoparticles can affect the protein adsorption on the surface of these macromolecules in the blood stream.[127, 128] The proteins that can specifically adsorb on the surface of the nanoparticles may address their uptake to the certain cell types including the immune cells.[127, 129, 130] As a result, a cascade of immune responses can be initiated, attracting increased numbers of macrophages and triggering phago-

cytosis and releasing cytokine signalling molecules, which further stimulate a systemic immune response.[130–133]

Various *in vitro* assays have evaluated the effect that nanomaterials can produce on immune cell uptake, viability and cytokine expression. For instance, Shukla *et al.,* examined the effects that 3–8 nm gold nanoparticles (AuNP) produced on the RAW264.7 macrophage cell line.[134] They demonstrated that AuNP-s at the concentration of 100 μM and of the certain size do not reduce viability and do not stimulate inflammatory cytokines such as TNF-α and IL-1 β. Yen *et al.,* evaluated AuNP effect on J774 A1 macrophages testing different size ranges: 2–4 nm, 5–7 nm and 20–40 nm.[135] They showed that AuNP triggered the inflammatory response, producing higher levels of IL-1 β, IL-6 and TNF-α cytokines within 6 h compared to controls. TNF-α and IL-6 expression was higher than in controls after 48 h of incubation with the particles with smaller size range, demonstrating the importance of the particle size for the immunological response. One of the explanations can be the difference in the adsorption of serum proteins to the particles of different sizes, although the mechanism of this effect remains unclear.[136] Additionally, undesired toxicity can be mediated through interactions of the nucleic acids with the proteins. These effects can be sequence-dependent, as for interaction of nucleic acids with Toll-like receptors (TLR-s), or sequence-independent.[137] For example, RNA molecules can also affect TLR-s 3, 7, and 8, eliciting undesired immune stimulation that manifests as hyperplasia in lymphoid organs and mononuclear cell infiltrates in non-lymphoid organs.[138, 139]

Application of CPP-s for drug delivery has shown to be of great advantage as synthetic peptides contain less than 30 amino acids and show no evidence of cytotoxicity, immunogenicity or oncogene activation.[140] However, the research into biological side effects of cationic and amphipathic peptides has still received great attention.[141] Several independent studies on biological side effects of cationic and amphipathic peptides suggest that amphipathic peptides such as TP10 affect cellular metabolism already at a concentration of 5 μM,[142] whereas the cationic peptides such as TAT are not cytotoxic *in vitro* even at concentrations up to 400 μM.[143] However, *in vivo* studies by the same group indicated some mild cytotoxic effect on a rabbit's eyelid after 7 days of application of TAT peptide. Similarly, poly-Arg peptides also did not induce any cellular toxicity at siRNA/peptide ratio of negative/positive charges of 40.[143–145] The studies indicate applicability of CPP-s for anticancer therapy, without the non-specific activation of the immune system.[3] However, there are still a number of studies describing the activation of macrophages, and recruitment of leukocytes, which leads to the systemic induction of the immune response by the therapeutic drug delivery agents *in vivo*.[136, 146–149] Therefore, the *in vivo* studies that characterize the immunological response to the drug delivery agents are very essential.

1.6. Prediction of CPP-s

The opportunity to predict the cell-penetrating functions of novel peptide sequences provides new possibilities for the development of novel peptide-based delivery systems. The predictions, based on such important CPP sequence characteristics as abundance of basic amino acids, length and ability to form secondary structures have helped to determine several effective CPP-s. However, those predictions were often quite imprecise. For instance, uptake efficiency can vary widely between the different laboratories: from 100 fold enrichment of the peptide in the cytoplasm to equilibrium concentrations, comparable to the extracellular concentrations.[150, 151] The prediction of CPP functionality requires the independent and precise calculations of several peptide structure-dependent parameters that contribute to the CPP characteristics. Such analyses would not be an easy task, especially when taking into the account that various parameters can differ between the cell lines or due to different methods of measurement. In order to simplify the analysis it would be reasonable to reduce the number of predicted variables assuming that the CPP properties are bulky and does not depend on specific motifs, but rather on the net properties of the amino acid residues in the peptide.[151] Thus the properties important for the membrane penetration have been established.[152, 153] As the membrane surface contains charged and neutral groups of the lipid layer it would be reasonable to assume that the electrostatic and hydrophobic interactions should be essential for the initial process of cellular penetration.[154, 155] According to their structures the CPP-s can be classified in three different groups: the peptides with low amphipaticity like penetratin[80] and TAT,[75] the peptides with high amphipaticity like MAP[90] and transportan,[86] and the peptides in which the charged and hydrophobic residues are separated longitudinally in the chain, such as pVEC[82] and Pep-1.[85] Most probably the peptides, which belong to different groups internalize inside the cells following different endocytotic pathways. The quantitative structure-activity relationship analysis (QSAR) for the peptides is generally performed on the amino acid residue basis, interpreting the physico-chemical characteristics of the compounds into sets of descriptor variables. Three descriptive scales for the twenty natural amino acids z1, z2 and z3[156] were first introduced by Hellberg *et al.* The scales were settled down using such physicochemical variables as molecular weight, pKa and partition coefficients. Z-scales quantify the structural variation within a group of related peptides organizing the z-scales according to the particular amino acid sequence.

In 1998, Sandberg *et al.,* in search for the parameters that would allow predicting the biological activity of peptides presented five-descriptor sets z1-z5, collecting a vast amount of variables for total 87 coded and non-coded amino acids.[157] The bulk of the amino acids corresponding to the number of heavy atoms (C, N, S and O) in the amino acids side-chains was also utilized. Additionally, the net donated hydrogen bonds of the amino acid, which were obtained by the calculation of accepted hydrogen bonds of the side-chains

subtracted from the donated hydrogen bonds was also shown to be very important in order to predict the cell-penetrating activity of the newly designed peptides.[158]

Recently Dobchev *et al.,* developed the Artificial Neural Network-based computational model (ANN) using principle component analysis (PCA) for the prediction of CPP-s, which internalize by similar mechanisms.[159] Application of ANN and PCA allowed predicting the penetrating capability of 101 studied peptides with 83 % accuracy. Moreover further optimization of the analyzed data sets improved the prediction up to 100 %.

The ANN model comprises the input layer consisting of six molecular descriptors D1-D6, derived from 3D peptide structures: topographic electronic index for all bonds, difference in charged partial surface areas, Schultz average vertex, molecular weight, hydrogen acceptor dependent charged surface area, difference (positive-negative) in charged partial surface area. These descriptors were determined by performing PCA analysis using STATISTICA[160] software (StatSoft Inc., USA). Additionally the ANN model also contains hidden layers and an output layer where a single neuron related to the cell penetration.

1.6.1. CPP prediction algorithm

The mathematical model to predict CPP sequences was introduced by Hällbrink *et al.,* in 2005. This model was assigned to establish the CPP sequences using either already existing proteins or random peptide sequences.[158] The principle of this prediction model is also based on z-descriptors reflecting certain chemical and physical properties of each amino acid[157] and providing a well-documented and accurate platform for CPP prediction. Utilizing the Sandberg's z-scales, Hällbrink *et al.,* developed a CPP prediction algorithm, analyzing the bulk property values ($z\Sigma/n$), for the set of 24 cell-penetrating and 17 non-penetrating sequences. The possibility to perform *in silico* predictions using a CPP prediction algorithm would be of great advantage,[161] however, the main drawback would be its inability to consider the order of the amino acid residues in the peptide backbone calculating only the overall sum of the residues in the sequence. The algorithm allows obtaining equal predictions for CPP-s as well as for their inversed and scrambled analogues, which might not internalize into the cells that effectively.[158]

Nevertheless several reports were published recently describing the successful utilization of the prediction algorithm for the detection of the biologically active CPP-s. The study of Howl *et al*., confirmed the applicability of a CPP prediction algorithm by generating cytochrome C (Cyt C) protein-derived analogue, capable of cellular internalization and the reduction of the viability of cancer cells.[162] Using a CPP prediction algorithm Johansson *et al*., designed p14ARF protein-derived 22 amino-acids-long peptide, which was prone to translocation inside the breast cancer cells inducing apoptosis.[163]

I.7. Apoptosis

Apoptosis is an evolutionarily conserved energy-dependant process of programmed cell death that is important for normal development of eukaryotic organisms, the maintenance of tissue homeostasis and an effective functioning of the immune system.[164] In contrast to necrosis, apoptosis does not result in inflammation because the damaged cells do not disintegrate and expose their interior to the neighbouring cells, but form the apoptotic bodies, which are further phagocytosed by the surrounding cells.[165] During the last few decades, the process of apoptosis has received significant attention because of it involvement in such widespread diseases as cancer and cardiovascular disorders. Therefore, the identification of the mechanisms standing behind the apoptosis is of particular interest. Many different pathways are involved in the induction of apoptosis however two major pathways are commonly defined: death receptor and mitochondrial pathways[166] (**Figure 2**). Both pathways converge to induce the activation of caspases, the final executioners of cell death, although, it should be noted that caspase-independent forms of apoptosis have also been reported.[167]

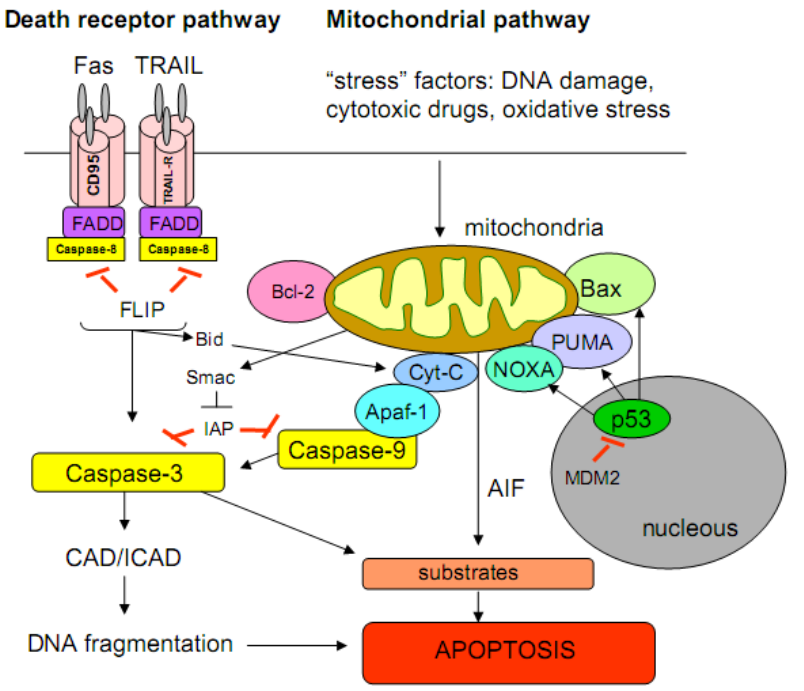

Figure 2. Death receptor and mitochondrial apoptotic pathways.[168, 169] In response to different extra- or intracellular stress signals, p53 transactivates pro-apoptotic genes activating the death receptor pathway (CD95, TRAIL, Bid), or the mitochondrial pathway (Bax, Noxa, Puma, Cyt-C, APAF-1). The pathways induce the activation of caspase-9 and caspase-3 leading to apoptosis.

1.7.1. Death receptor pathway

The death receptor or extrinsic pathway involves death receptors that are expressed on the surface of the cells and induce apoptosis upon ligand binding. Death receptors belong to the tumor necrosis factor (TNF) super family the best characterized of which are Fas[170] (CD95/Apo1), TNF receptor 1[171] (p55), TRAMP[172, 173] (WSL-1/Apo3/DR3/LARD), TRAIL-R1[174] (DR4) and TRAIL-R2[175] (DR5/Apo2/KILLER). Fas Ligand (CD95 ligand) binds Fas, TNF and lymphotoxin A bind to TNFR1[176], TWEAK (Apo3 ligand) binds to TRAMP[177] and TRAIL (Apo2 ligand) is the ligand for both TRAIL-R1[174] and TRAIL-R2.[178] Activated by ligand these receptors formed in an intracellular death domain DD, associate directly with Fas-associated death domain (FADD) or indirectly via TNFR-associated death domain[176, 179] (TRADD). FADD further interacts with pro-caspase-8 forming a complex at the death inducing signalling complex (DISC), which induces the activation of caspase-8 and the activation of other downstream effector caspases. Caspase-8 in turn also triggers the intrinsic apoptotic pathway by cleaving the BH3 interacting domain agonist Bid and translocating to the mitochondria.[180] In addition to the TNF death receptor family there are the decoy receptors DcR1, DcR2 and osteoprotegerin (OPG), which inhibit death signalling through the sequestration of ligand.[176]

1.7.2. Mitochondrial pathway

The mitochondrial or intrinsic pathway is activated in response to a variety of extra- and intracellular stress factors such as DNA damage, ischaemia and oxidative stress resulting in the activation of numerous apoptotic signals, which lead to cell death. The majority of anticancer drugs and irradiation also contribute to the activation of mitochondrial apoptotic pathway, which commonly starts with the permeabilization of the mitochondrial outer membrane. Although the mechanisms of this process remain unclear, it is suggested that the permeabilization can be either permeability transition (PT) pore dependent or independent.[181] The opening of the PT pore triggers the dissipation of the proton gradient created by electron transport, causing the water entrance into the mitochondrial matrix, which results in swelling of the intermembranal space and rupturing of the outer membrane. These induces the release of several apoptogenic proteins[181, 182] such as Cyt C,[183] apoptosis inducing factor[184] (AIF) and endonuclease G.[185, 186] Cyt C together with apoptosis protease activating factor (APAF-1) and caspase-9 forms an "apoptosome"[187] that in turn activates the effector caspases collectively mediating the apoptosis. AIF[184] and endonuclease G[186] mediate DNA fragmentation and subsequent chromosomal condensation. PT pore independent mitochondrial membrane permeabilisation is regulated by Bcl-2 family members, which can be subdivided into anti-apoptotic members such as Bcl-2 and Bcl-xL and pro-apoptotic members such as Bax, Bak, and Bok, Bad, Bik, Bid, Puma, Bim, Bmf and Noxa.[188–192]

1.7.3. Tumor suppressor protein p53

Elucidation of the function of many mutated gene products supports the importance of the proper regulation of cellular pathways. Cells continuously respond to different external and internal signals through oncogenes and tumor suppressor genes, which both control the crucial steps in the cell cycle: transitions from G_0/G_1 (resting) stage to S (replicating) stage, inhibit cell growth and induce apoptosis in response to cellular stress or other stimuli.[193] Both oncogene and tumor suppessor gene products are in most cases involved in the signalling cascades and are integrated in protein interaction networks.[194] The heterogeneity of tumor cells that comes from the variety of mutations makes it difficult to define the most promising drug targets. However, the proteins that are functionally participating in tumor formation and progression can provide a source of potential therapeutic targets as they are functionally crucial for cancer cells and are at least temporarily not vital for normal cells.[195] One such potential anticancer therapeutic target is p53, which in normal cells is present at low levels being inhibited by murine double minute-2 (MDM2) or the human homologue[196] (HDM2) protein. Following apoptotic signals, p53 is normally activated during the mitochondrial apoptotic pathway. Activation of p53 involves stabilization of the protein by post-translational modifications disrupting the interaction between p53 and MDM2. p53 mediates the expression of APAF-1 and certain pro-apoptotic Bcl-2 family members[197] as well as eliciting transcriptional independent cell death pathways.[198, 199] Being a transcription factor p53 suppresses the tumorigenesis, and is mutated in over 50 % of tumors.[166] The pro-apoptotic activity of p53 can be characterized as transcriptional dependent and independent, because it induces transcription of the genes involved in both extrinsic (CD95 and DR5) and intrinsic (PUMA, Bax, Bid) pathways. Moreover, the cytoplasmic p53 inhibits the pro-survival activity of Bcl-2 and Bcl-XL and/or activates the pro-apoptotic Bax, independently of its transcriptional activity.[199] As the p53 is pleiotropic, being involved in the regulation of cellular development,[200, 201] metabolism,[202] mitosis[203, 204] and reproduction[205] many anticancer strategies aim to restore the p53 activity in tumors in order to induce apoptosis in cancer cells.[206]

1.8. Mimicking CPP-s

Modulation of the protein-protein interactions using mimicking proteins would be beneficial for the regulation of intracellular procesess. The main drawback for the application of such proteins is their complex production and inability to penetrate the cell, where the most protein interactions occur. Therefore, the conjugation of biologically active proteins and peptides to the CPP-s would facilitate their entry.[207] The outcomes of protein-protein interactions are not always easy to predict as the examined protein can either bind to the target protein producing an effect or it can block protein-protein interactions by inducing conformational changes, which can either activate or inhibit the target

protein functions. Similarly, a peptide designed to mimic a protein can also activate or inhibit the interactions, depending on the function of the protein from which it is derived. Regarding the activating versus inhibiting mimicking peptides, the activating mimicking peptides would be more pharmacologically advantageous as lower concentrations are needed to produce the effect. Inhibitory mimicking peptides compete with the native ligands therefore higher doses are needed to produce the effect, which can also induce the non-specific side effects, namely immunogenicity. Additionally, since the mimicking peptides represent short fragments from the original protein, to acquire all the interacting interfaces and regulatory sites of the full-length protein would be quite complicated.[207]

An example of protein-protein interaction inhibiting peptide is VIVIT peptide, which inhibits the calcineurin-NFAT interaction,[208, 209] and shepherdin, which in turn blocks the surviving-Hsp90 interaction.[210] Generally the therapeutic targets of mimicking peptides that inhibit protein-protein interactions are focused on the suppression of cancer cell progression.[207]

Fulda *et al*., described the conjugation of the mitochondrial caspase activator SMAC-derived, 7 amino acid-long peptide with TAT. This conjugate allowed sensitizing the tumor cells to the anticancer treatment in the intracranial xenograft model.[211] The conjugation of TAT to the cytotoxic cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ -derived mimicking peptide resulted in the translocation of the peptide inside the U251 human glioblastoma cells inhibiting their proliferation.[212]

Restoration of p53 functions would also lead to the induction of apoptosis in tumor cells. Selivanova *et al.,* conjugated penetratin to the C-terminus of p53 peptide and demonstrated the activation of specific DNA binding induced by fusion, which restored the transcriptional trans-activating function of the mutant p53 protein in the living cells.[213] Penetratin-p53 fusion peptide induced apoptosis both in mutant and in wild-type p53-bearing cancer cell lines, whereas normal cells remained non-affected.[214] Furthermore Araki *et al*., generated peptides using TAT, oligoarginine and FHV conjugating them to the C-terminal domain of p53. As a result the application of oligoarginine-p53 and FHV-p53 significantly inhibited bladder cancer cells prolifiration, decreased tumor burden and prolonged survival in bladder cancer xenografts.[215]

Several studies have reported the successful application of CPP-s in MDM-2 protein suppression in cancer cells. Kanovsky *et al*., designed p53 MDM-2-binding region-derived peptides linked to penetratin in order to conduct cellular internalization.[216] The competitive binding to MDM-2 by a p53 mimicking peptide subsequently preserved the intracellular p53 activity, prolonging its half-life in pancreatic cells. The treatment did not influence the growth of non-cancer cells. These observations were further confirmed *in vivo* by Harbour *et al*., who showed that TAT-MDM-2-inhibiting fusion peptide induced the accumulation of p53, and preferential apoptosis in tumour cells, but not in normal cells, inducing no toxic side effects *in vivo*.[217] Similar results have been obtained using penetratin fusion with MDM-2-binding residues.[218]

For therapeutic effect, the combination of the effector and the CPP in the same peptide sequence is preferred. This strategy would eliminate the requirement of the additional conjugation to the CPP making the production easier. Cyt C is an important protein in the electron transport chain of the mitochondria and is released in response to the apoptotic stimulus. Howl *et al*., discovered that the Cyt C protein also contains sequences possessing the CPP properties.[219] The mimicking peptides Cyt $C^{86–101}$ and Cyt $C^{77–101}$ were found to reduce the cancer cell viability, inducing DNA fragmentation and caspase-3 activation, which are the hallmarks of apoptosis.

Cell cycle control mechanism disorders are common for all types of cancers. p14ARF, MDM2 and p53 belong to the same regulatory pathway and are altered in all the tumors. p14ARF suppresses tumor progression by inhibiting the MDM2 protein leading to the activation of p53 and the induction of apoptosis.[220, 221] Johansson *et al*., generated four p14ARF protein-derived 13–37 amino-acid-long peptides, all designed from the N-terminal region.[163] The 22 amino-acid-long N-terminal peptide denoted as ARF(1–22) was found to be a CPP, capable of inducing apoptosis in breast cancer cell lines MCF7 and MDA-231 MB by mimicking the functions of the full-length p14ARF protein.

2. AIMS OF THE STUDY

The thesis is focused on the characterization of immunogenic and cytotoxic activities of PepFect (PF) peptides, on cytotoxic and apoptotic activities of newly designed cell-penetrating p53 analogues, on the development of peptide-based tumor-targeted drug delivery vector gHoPe2 and on the evaluation of nucleic acid delivery efficiency of the *N*-terminally stearylated CPP-s.

Paper I
The aim of the study was to analyze the cytotoxic and immunogenic activity of PF peptides and PF/nucleic acid complexes *in vitro* and *in vivo* and to compare it with the cytotoxic and immunogenic activities of unmodified TP10, TAT (48–60) and stearyl-$(RxR)_4$ and the widely used lipofectamine transfection reagents.

Paper II
The aim of the study was to design and synthesize short p53-derived cell-penetrating protein analogues and to analyze their cytotoxic and apoptotic activity in breast cancer cell lines.

Paper III
The aim of the study was to develop a novel glioma-targeted drug delivery vehicle based on a new glioma-specific homing peptide sequence conjugated to a CPP.

Paper IV
The aim of the study was to evaluate the effect of *N*-terminal stearylation of the $(RxR)_4$ peptide on plasmid and oligonucleotide delivery and to estimate the possible cytotoxic effect of peptides/nucleic acid complexes *in vitro* comparing it with the cytotoxic activity of the lipofectamine transfection reagent.

3. METHODOLOGICAL CONSIDERATIONS

Methods and materials used in the thesis are described extensively in respective papers, therefore only theoretical and practical aspects of the methods will be discussed in this section.

3.1. Choice and design of CPP-s

The sequences of all CPP-s studied in this thesis are presented in **Table 3**.

In Paper I, cytotoxicity and immunogenicity of PF3, PF4 and PF6 peptides as well as peptide/nucleic acid complexes were analyzed *in vitro* and *in vivo*. The capability to induce immunogenicity and *in vivo* toxicity can limit the application of CPP-s as drug delivery vectors in clinical settings. We estimated the influence of the PF delivery agents on the immune response by measuring caspase activity, cytotoxicity and cytokine release *in vitro* and *in vivo*. Several well-established peptides such as TAT (48–60), stearyl-$(RxR)_4$, transfection reagents Lipofectamine™ 2000 and Lipofectamine™ RNAiMAX as well as LPS were used in parallel in this experiments in order to provide a comparison with the immunogenic profile of PF.

Previous studies have shown that the rational design by using QSAR-based algorithm allows creating protein analogues in which cell-penetrating and biological activities are organized within the primary sequence. In Paper II we designed and synthesized short p53 analogues Peptide4 and Peptide5, which were capable of penetrating breast cancer cells and induce apoptosis. By using the primary amino acid sequence of p53 available at NCBI Human Protein Database and a CPP prediction algorithm we generated potential CPP sequences thereafter utilized for apoptotic analyses.

In order to improve chemotherapeutic activity of antineoplastic agents the incorporation of the tumor-specific targeting sequences into a CPP-based drug delivery vectors has been studied. In Paper III, novel tumor-selective CPP-based drug delivery platform gHoPe2 was developed. The novel drug delivery vector comprises a glioma-specific peptide sequence that has been covalently conjugated to the CPP pVEC and the commonly used anticancer drug doxorubicin. The gHoPe2 drug delivery vehicle and unmodified doxorubicin were utilized for the treatment of glioblastoma *in vitro* and in mouse xenografts.

In Paper IV, $(RxR)_4$ and oligoarginine (Arg9) CPP-s were used for the delivery of plasmid DNA and splice-correcting oligonucleotides (SCO-s) *in vitro*. The main problem for CPP-s taken up by endocytic pathways is the escape from endolysosomal compartments. The introduction of a stearyl group in the structure of CPP-s was utilized to overcome the problem of endosomal entrapment.

Table 3. Sequences of CPP-s used in this thesis

Names	Sequence	Reference
Transportan 10, TP10	AGYLLGKINLKALAALAKKIL-NH_2	87
TAT (48–60)	GRKKRRQRRRPPQ-NH_2	81
Stearyl-$(RxR)_4$	[a]RxRRxRRxRRxR-NH_2	59
Stearyl-Arg9	[a]RRRRRRRRR-NH_2	222
PepFect3, stearyl-TP10, PF3	[a]AGYLLGKINLKALAALAKKIL-NH_2	58
PepFect4, stearyl-TP10, PF4	AGYLLGK[b]INLKALAALAKKIL-NH_2	223
PepFect6, PF6	[a]AGYLLGK[c]INLKALAALAKKIL-NH_2	57
Peptide1, p53 (369–382)	GKKHRSTSQGKKSKL-NH_2	224
Peptide2, FAM-p53(369–382)[d]	[d]GKKHRSTSQGKKSKL-NH_2	224
Peptide3, Stearyl-(Lys^3-εNH-Stearyl)-p53(369–382)[b,d]	[d]GKK[a]HRSTSQGKKSKL-NH_2	224
Peptide4, Stearyl-p53(369–382)[d]	[d]GKKHRSTSQGKKSKL-NH_2	224
Peptide5,(Lys^3-εNH-Stearyl)-p53(369–382)[b]	GKK[b]HRSTSQGKKSKL-NH_2	224
pVEC	LLIILRRRIRKQAHAHSK-NH_2	82
gHo	NHQQQNPHQPPM-NH_2	95
FAM-pVEC	[d]LLIILRRRIRKQAHAHSK-NH_2	95
FAM-gHo	[d]NHQQQNPHQPPM-NH_2	95
FAM-pVEC-gHo (FAM-gHoPe2)	[d]LLIILRRRIRKQAHAHSKNHQQQNPHQPPM-NH_2	95
FAM-gHo-pVEC (FAM-gHoPe3)	[d]NHQQQNPHQPPMLLIILRRRIRKQAHAHSK-NH_2	95
Dox-pVEC-gHo (Dox-gHoPe2)	[e]XCLLIILRRRIRKQAHAHSKNHQQQNPHQPPM-NH_2	95

[a]*N*-terminal stearyl-modification; x – aminohexanoic acid (Ahx); [b]stearyl modification Lys^3; [c]Trifluoromethylquinoline based derivative (**Figure 3**) conjugated to succinylated lysine tree; [d]stearyl or 5(6)-carboxyfluoresceinyl modification at *N*-terminus; [e]doxorubicin; X – succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC)

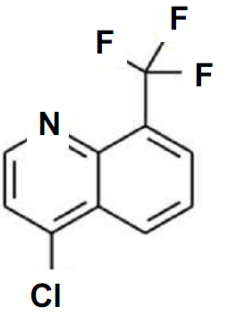

Figure 3. The structure of trifluoromethylquinoline

3.2. Prediction of CPP-s

The sequences of the peptides presented in Paper II were designed using the CPP prediction algorithm-based program VFREQ27, developed by Hällbrink *et al.,* in 2003.[225] The program utilizes QSAR descriptor scales, which describe a composite of the physical characteristics of the amino acids contributing to the cell-penetrating function of the peptides. The CPP prediction program enables to perform analysis of the CPP functionality of naturally occurring peptides and *de novo* designed CPP-s.

In Paper II, we generated short CPP fragments using the amino acid sequence of p53, available at NCBI Human Protein Database (http://www.ncbi.nlm.nih.gov/protein). The program searched for possible CPP sequences inside the defined search window. As a result a list of 100 peptide sequences total, with lengths ranging between 12–22, was generated. CPP efficacy was scored on a scale of 1–3, where score "1" represents the least and "3" the most potent CPP.

The peptide sequence, which is derived from the lysine-rich domain of C-terminus of p53 with the highest cell-penetrating prediction score, was chosen for further investigations.

3.3. Peptide synthesis

3.3.1. Solid-phase peptide synthesis (SPPS)

All peptides used in this thesis were synthesized using solid-phase peptide synthesis (SPPS) strategy. First described in 1986 by Bruce Merrifield,[226] SPPS is based on the repeated cycles of amino acid coupling and deprotection procedures, performed on a solid polymer resin. The side-chains of the amino acids are protected by special protective groups such as *t*-butyloxycarbonyl (*t*-Boc) or *t*-butyl (*t*Bu) in order to ensure that the activated carboxylic acid is reacting specifically with the α-amino group. The α-amino group of each amino acid is protected by either *t*-Boc or 9-fluorenylmethyl-oxycarbonyl (Fmoc) in order to prevent the non-coupled amino acids from reacting with each other. The α-amino protective group is removed subsequently before each coupling, while the side-chain protective groups remain on until the final cleavage of the peptide from the resin.

In current studies all peptides were synthesized in stepwise manner on a 0.1 mmol scale on an automated peptide synthesizer using Fmoc solid-phase peptide synthesis strategy[227] with Rink-amide MBHA (methylbenzyl-hydrylamine) resin as solid phase polymer to obtain C-terminally amidated peptides. The stearic acid was coupled manually to the *N*-terminus of the peptide by treatment of peptidyl-resins with 5 equivalent (eq.) stearic acid, 3 eq. HOBt and 3 eq. HBTU, 6 eq. DIEA in dimethylformamide/dichloromethane (1:1) overnight at room temperature. For synthesis of FAM-labelled peptides 5(6)-carboxyfluorescein monomer was used and the coupling was carried out

manually by treatment of peptidyl-resin with 4 eq. of a 5(6)-carboxyfluorescein, 4 eq. N-Hydroxybenzotriazole (HOBt) and 4 eq. N,N'-diisopropylcarbodiimide (DIC), 8 eq. N,N-diisopropylethylamine (DIEA) in DMF/DMSO (1:1; v:v) overnight at room temperature. After coupling the resin was washed 3 times with 20 % piperidine solution to remove the cross-linked carboxyfluorescein residues. The final cleavage was performed using standard protocol (95 % TFA/2.5 % TIS/2.5 % H_2O) followed by product precipitation using diethyl ether and lyophilization using a vacuum pump.

3.3.2. Purification and identification of synthesized peptides

Peptides were purified using preparative reversed phase high pressure liquid chromatography (RP-HPLC) with C4 column and a 5–100 % acetonitrile/water gradient containing 0.1 % TFA. The correct product-containing fractions were identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using α-cyano-hydroxycinnamic acid as the matrix.

3.3.3. Doxorubicin conjugation to CPP

For preparation of doxorubicin conjugates, cysteine amino acid was added to the *N*-terminus, followed by the cleavage and purification of the peptides as described above. Doxorubicin was conjugated according to Liang *et al*., with minor modifications (**Figure 4**).[228] Doxorubicin (2 mg) was dissolved in 500 µl of DMSO and then dispersed in 500 µl of PBS (pH 8.0) at the concentration of 2 mg/ml followed by addition of 20 µl of triethylamine (TEA) and 200 µl of succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC, 10 mg/ml). After incubation at a room temperature for 2 h the pH was adjusted to 5.5 using 2 M HCl and 300 µl of peptide solution (15 mg/ml) was added. Thereafter the mixture was incubated at room temperature for another 2 h. In order to remove non-conjugated doxorubicin, SMCC and TEA, the reversed-phase purification using a gradient of acetonitrile/water containing 0.1 % TFA (5–100 % for 45 min) was carried out. The identity and purity of the obtained doxorubicin conjugates were confirmed by using MALDI-TOF mass spectrometry and analytical HPLC respectively.

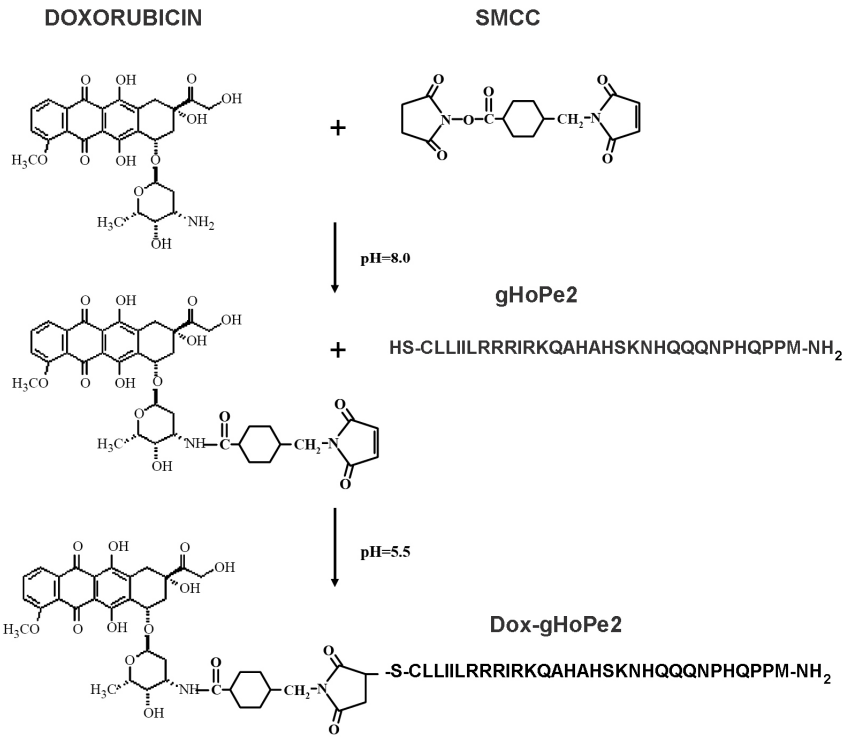

Figure 4. Doxorubicin conjugation to gHoPe2 drug delivery vector[228] (Paper III)

3.4. Covalent and non-covalent strategies for cargo conjugation

The conjugation of different cargos to the CPP can be performed following two main strategies: covalent conjugation based on the formation of covalent bond between the cargo and CPP, and non-covalent conjugation, which is possible due to electrostatic interactions. During the covalent conjugation the cargo can be coupled to the CPP using a reducible disulphide bridges. In the case of doxorubicin conjugation the coupling procedure with SMCC was performed beforehand. The SMCC contains two reactivities toward amines, through the succinimide group, and reactivity toward sulphhydryls, through the maleimide group. The chemical group *N*-hydroxysuccinimydyl (NHS) reacts in aqueous phase, optimally at neutral pH (7.5 (6.5–8.5)) or higher, on the amine functional group of doxorubicin, resulting in SMCC-doxorubicin formation. The maleimide group at neutral pH (6.5–7.5) reacts very specifically with the sulphhydryl group (-SH) of the cystein present in the CPP sequence. The reaction is rapid (a few minutes for cystein), but may require 1–2 h to be completed in certain conditions. The advantage of this approach is that the glutathione inside the cells cleaves the disulfide bridges and releases active

cargo from the conjugate. The main disadvantages of this strategy are its relative complexness, low synthesis yield and the necessity of extensive purification steps.

The non-covalent strategy involves a co-incubation of CPP with nucleic acid cargo (plasmid, oligonucleotides or siRNA) at a specified charge or molar ratios resulting in nanocomplex formation. The principle of this procedure is electrostatic interactions between the positive charges of CPPs and the negative charges of nucleic acid. The advantage of the non-covalent strategy is the simplicity of manipulations and short preparation time of the procedure.

In order to obtain PF/plasmid DNA complexes 0.5 μg of green fluorescent protein-expressing pEGFP-C1 plasmid was co-incubated with PF3 or PF4 at different plasmid/peptide charge ratios (CRs) 1:1 and 1:5 in MQ water in $1/10^{th}$ of the final treatment volume (i.e. 30 μl). CRs were calculated theoretically, taking into account the positive charges of the peptide and the negative charges of the plasmid. The final concentration of PF3 or PF4 was 0.75 μM at CR 1:1 and 3.75 μM at CR 1:5. Complexes were incubated for 1 h at room temperature.

In order to obtain PF/siRNA complexes PF6 (100 μM stock solution) was mixed with HPRT1 siRNA (10 μM stock solution) in MQ water in $1/10^{th}$ of final treatment volume (i.e. 30 μl), using peptide: siRNA molar ratios (MRs) 40 and 30, siRNA 50 nM and 100 nM respectively.

In order to obtain stearyl-$(RxR)_4$ or Arg9/plasmid DNA complexes, 0.5 μg of pGL3 luciferase expressing plasmid was mixed with CPP-s at different charge ratios (CRs) (1:1–1:5) in MQ water in $1/10^{th}$ of the final treatment volume (i.e. 50 μl). CRs were calculated theoretically, taking into account the positive charges of the peptide and negative charges of the plasmid. For instance, final concentration of stearyl-$(RxR)_4$ was 0.38 μM at the 1:1 CR.

2′-OMe SCO-s were mixed with stearyl-$(RxR)_4$ or Arg9 CPP-s at different molar ratios in MQ water in $1/10^{th}$ of the final treatment volume (i.e. 50 μl). Complexes were formed for 1 h at room temperature and meanwhile the cell medium was replaced in 24-well plates to fresh serum-free DMEM (450 μl). Thereafter, complexes were added to each well.

Complexes were incubated for 1 h at room temperature. In the case of Lipofectamine™ 2000 and Lipofectamine™ RNAiMAX (Invitrogen, Sweden), complexes were formed according to the manufacturer's protocol. Lipofectamine™ 2000 and Lipofectamine™ RNAiMAX were used as positive control in all experiments. Formation and stability of all the peptide/nucleic acid complexes in the presence or absence of serum was confirmed by agarose gel (2 %) stained with EtBr (0.5 μg/ml).

3.5. Cell culture

Several immortalized cell lines were used in this thesis. The cell lines chosen for the experiments are commonly used in the CPP field and enable comparisons with previously published data.

3.5.1. THP-1 cell line

Human acute monocytic leukemia cell line (THP-1) is commonly used as a model leukemia cell line in immunocytochemical analysis of protein-protein interaction, and immunohistochemistry. THP-1 cells are a monocyte-like cell line derived from a patient with acute monocytic leukemia and unlike other leukemic cell lines has a normal diploid karyotype. Derived from the peripheral blood of a one-year-old human male with acute monocytic leukemia, THP-1 cells have Fc and C3b receptors and lack surface and cytoplasmic immunoglobulins. In paper I, the THP-1 cell line was used as a model system for immune response studies *in vitro* as they are capable of IL-1 and TNF-α cytokine production when differentiated into macrophage-like cells using phorbol 12-myristate 13-acetate (commonly known as PMA).

3.5.2. PBMC cells

Peripheral blood mononuclear cells (PBMC) constitute an important part of human peripheral immune system. PBMC cells consist mainly of monocytes, T-cells and B-cells, and smaller amounts of natural killer cells (NK) and dendritic cells of both myeloid and plasmacytoid origin. The cells are derived from healthy human donors and processed using the Ficoll-Hypaque method. PBMC cells are suitable for assessment of the general immune activation of drug candidates or microorganisms, for which an immune stimulation is of interest (e.g. vaccine adjuvants and particles, immune stimulants for cancer treatment or immune stimulating natural extracts or micro organisms). The PBMC cells are suitable *in vitro* model for screening the compounds for immune-modulatory effects. In Paper I, the PBMC cells were used as a model non-cancer system for immune response studies *in vitro* as they are also capable of IL-1 and TNF-α cytokine production.

3.5.3. Breast cancer cell lines

MCF-7 and MDA-MB-231 breast cancer cell lines used in this thesis are derived from tumor metastasis pleural effusions. MCF-7 is an invasive human ductal carcinoma and is the most common breast cancer cell line in cancer studies.[229] MDA-MB-231, a human breast adenocarcinoma cell line, was isolated from the patient with MTX resistance due to the lack of MTX transport protein while receiving chemotherapy. Both MCF-7 and MDA-MB-231 breast cancer cell lines are of adenocarcinoma origin but differ in the tumor suppressor protein p53 expression. MCF-7 is a wild-type p53 expressing cell line, while in MDA-MB-231 cells the p53 protein is inactivated due to mutations and these aspects were utilized in Paper II to investigate the effect of p53 C-terminal domain-derived CPP-s Peptide4 and Peptide5 on the restoration of the p53 functions and induction of apoptosis inside the tumor cells.

3.5.4. SK-N-AS cell line

SK-N-AS is an S-type human neuroblastoma cell line derived from the bone marrow of an eight-year-old girl. The primary site of the tumor was found to be localized in adrenal glands soon metastasing however to the bone marrow. The SK-N-AS cell line is adapted to serum-free conditions because it is constitutively expressing IGF-2. The ability of neuroblastoma cell line to grow in serum-free conditions in the absence of supplied growth factors has been used in Paper II in order to study the effect of p53-derived cell-penetrating protein analogues under serum withdrawal conditions. SK-N-AS cells have a deletion in chromosome 1p region, which is a common non-random genetic alteration resulting in several putative tumor suppressor genes (p53) dysfunction in many neuroblastoma and breast cancer cell lines.

3.5.5. HEK 293 cell line

The HEK 293 Human Embryonic Kidney 293 cell line was generated in the early 70s by adenoviral transformation of normal human embryonic kidney cells in Alex Van der Eb's laboratory in Leiden, The Netherlands.[230] HEK 293 cells are easy to transfect and have been widely used in cell biology research as well as biotechnology industry to produce therapeutic proteins and viruses for gene therapy. HEK 293 cell line is an experimentally transformed cell line and thus is not a suitable model for cancer studies. However, they are easy to handle and are commonly used in drug delivery and peptide uptake studies (Paper III).

3.5.6. U87 MG cell line

U87 is a human glioblastoma-astrocytoma cell line formally known as U87 MG (malignant glioma). Glioblastoma is the highest dedifferentiated form of astrocytic brain tumours and it is refractory to chemotherapy in most cases. U87 cells have epithelial morphology and were obtained from a stage IV cancer patient. The cell line was used in Paper III as an *in vitro* model of human glioblastoma to investigate tumor targeting and the cytotoxic effect of tumor targeted CPP-s towards the cancer cells.

3.5.7. HeLa cell lines

HeLa cell line derived from immortalized cervical cancer cells isolated in 1951 from Henrietta Lacks after her death. HeLa is one of the most widely used cell lines within various research fields and in particular in cancer studies. The cells are characterized as rapidly growing and robust. HeLa cells were used in Paper III as a common cancer cell line model for investigation of specific internalization mechanisms and cytotoxicity induced by novel designed CPP-s.

In Paper IV, the delivery efficiency of CPP/SCO complexes was evaluated in HeLa pLuc705 cell line, the widely used splice correction assay.[233] HeLa pLuc705 cell line is stably transfected with luciferase-encoding gene interrupted by a mutated intron 2 from β-globin gene carring a cryptic splicing site. Incomplete removal of the intron (during splicing) results in production of aberrant and non-functional luciferase. Shielding the cryptic site with SCO-s enables to restore the splicing producing functional luciferase. This splice-correcting assay provides a positive read-out over the low background while having a wide dynamic range.

3.5.8. CHO cell line

Chinese hamster ovary (CHO)-K1 cell line is the most commonly used cell line in studies of genetics, toxicity screening, and gene expression. It is a subclone of the parental CHO cell line, which was derived from the ovary of an adult Chinese hamster,[231] it contains a slightly lower amount of DNA than the original CHO. These cells have been widely used in CPP field. CHO cells were used in Paper IV for plasmid delivery assay

3.5.9. BHK 21 cell line

Baby Hamster Kidney fibroblast (BHK 21) is an adhesive cell line used in molecular biology. The cells were derived in 1961 by Macpherson and Stoker.[232] Nowadays, subclone 13, originally derived by single-cell isolation from the kidneys of 5 unsexed, 1-day-old hamsters, is widely used. The BHK 21 cells are mainly useful for transformations and for stable and temporary transfections. BHK 21 cells were used in Paper IV for plasmid delivery assay

3.6. Characterization of uptake of CPP-s

There are several methods for the characterization of peptide mediated uptake and drug delivery efficacy inside cells. The most widely used of them is the labelling of either the peptide or the nucleic acid polymer with a fluoresceinyl moiety. In the current thesis the 5(6)-carboxyfluoresceinyl moiety was covalently coupled to the peptide backbone in order to quantify the uptake and delivery efficacy of newly designed CPP-s into human cells.

3.6.1. Confocal microscopy

Fluorescence microscopy and confocal laser scanning microscopy enable the evaluation of fluorescein-labelled CPP-s uptake inside the cell or in tissue sections, providing valuable information about their intracellular localization and targeting. Live-cell imaging was used in all microscopy studies in this

thesis. Live-cell imaging was preferred to fixed and permeabilized cell visualization in order to avoid possible fixation-derived artefacts often interfering with correct image analyses. In Papers II, III and IV), the microscopy studies of fluorescein-labelled CPP-s were performed using an Olympus IX81 inverted microscope equipped with a FluoView 1000 confocal system with 60× water-immersion and 100× oil-immersion objectives and excitation at 488 nm (fluorescein). Images were captured using a digital camera (model DXM1200C) and processed with EZ-C1 software V.2.30 (Nikon, Japan).

3.6.2. Flow cytometry

Flow cytometry is a powerful technique for analysis of multiple parameters of individual cells within a heterogeneous population. Fluorescence-activated cell sorting (FACS) is used in various applications such as immunophenotyping, chromosomal ploidy analysis, cell counting and GFP expression analysis. FACS performs these analyses by passing thousands of cells per second through a laser beam and capturing the light that emerges from each cell as it passes through. The data are analyzed by flow cytometry software to obtain cellular characteristics such as size, complexity and viability. In Papers II and III, the flow cytometry was used in order to estimate the cell-penetrating activity of newly designed fluorescein-labelled peptides providing both qualitative (FITC mean) and quantitative (percentage of transfected cells) analyses.

3.7. Toxicity studies *in vitro*

The translocation of the CPP-s through cellular membrane can be accompanied by cytotoxic side effects derived from membrane perturbation especially at high peptide concentrations. In this thesis several methods were used to investigate the effect of these peptides on short-term membrane disturbance as well as long-term effects on cellular proliferation.

3.7.1. LDH leakage assay

The LDH leakage assay is based on lactate dehydrogenase (LDH), a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity resulting from either apoptosis or necrosis. LDH activity can be used as an indicator of cell membrane integrity and serves as a general means to assess cytotoxicity resulting from chemical compounds or environmental toxic factors. In the first step, LDH catalyzes the reduction of NAD^+ to NADH and H^+ by oxidation of lactate to pyruvate. In the second step of the reaction, diaphorase uses the newly formed NADH and H^+ to catalyze the reduction of a tetrazolium salt (INT) to highly coloured formazan, which absorbs strongly at 490–520 nm. In Paper III and IV LDH leakage assay was

performed in order to estimate the effect of the cell-penetrating vector gHoPe2 on cellular membrane integrity before conjugation of the vector with the chemotherapeutic molecules.

3.7.2. Cell proliferation assay

For investigation of long-term effects of CPP-s and CPP/cargo complexes on cell proliferation and survival a tetrazolium salt-based colorimetric MTS proliferation assay was performed (Paper I, III and IV)). The principle of the assay is to detect the metabolizing cells by measuring the activity of the intracellular enzyme mitochondrial dehydrogenase that in the presence of phenazine methosulphate reduces tetrazolium to formazan dye giving a purple colour with absorbance maximum at 490–500 nm in phosphate-buffered saline. In Paper II, long-term toxicity of cell-penetrating protein analogues was evaluated using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Sweden). The CellTiter-Glo® Luminescent Cell Viability Assay is a similar method to determine the number of viable cells in culture based on quantification of the ATP, which signals the presence of metabolically active cells. Luminescent Cell Viability Assay results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of cells in culture.

3.8. ELISA assay

Enzyme-linked immunosorbent assay (ELISA) is a plate-based immunoassay for detection and quantification of substances such as peptides, proteins, antibodies and hormones. The main principle of the assay is the immobilization of the antigen of interest that can be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody that has been attached to the plate. The antigen is detected either directly (labelled primary antibody) or indirectly (labelled secondary antibody). The most powerful ELISA assay format is the sandwich assay where the antigen is bound between two primary antibodies-the capture antibody and the detection antibody. The sandwich format has high selectivity and sensitivity as the key element of the detection strategy is a highly specific antibody-antigen interaction. In Paper I, the ELISA assay was used in order to analyze the influence of novel CPP-s PF-s and peptide/nucleic acid complexes on innate immune response by measuring IL-1β, IL-18 and TNF-α cytokine release in cell culture supernatants and mouse serum.

3.9. Apoptosis detection assays

Apoptosis is the best-characterized type of programmed cell death. Because of its importance in development, homeostasis and pathogenesis of cancer, apoptosis has undergone extensive investigation over the past few decades.

Several changes affecting the plasma membrane, mitochondria, cytoplasm and nucleus during apoptosis have been measured and analyzed. One of the earliest events in apoptosis is the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane that can be detected with annexin V. Mitochondrial dysfunction also occurs early in apoptosis and is accompanied by a decreased membrane potential and the release of mitochondrial proteins such as Cyt C and Smac/DIABLO. This leads to the activation of caspases-3 and 7 and DNA fragmentation.

3.9.1. Annexin V assay

The annexin V assay is a rapid and convenient method for detection of apoptosis based on flow cytometry. The human anticoagulant, annexin V, is a 35–36 kDa Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for phosphatidyl serine (PS). PS is normally located on the inner cytoplasmic surface of the cell membrane, while during apoptosis it is translocated to the outer leaflet of the membrane exposing itself to the extracellular environment. Annexin V is labelled with a fluorophore that can identify apoptotic cells by binding to PS exposed on the outer leaflet. After staining a cell population with annexin V in the provided binding buffer, apoptotic cells show green fluorescence that can easily be distinguished using a flow cytometer with the 488 nm line of an argon-ion laser for excitation. An Alexa Fluor® 488 annexin V assay was performed in Paper II in order to characterize the apoptotic activity of the newly designed p53-derived cell-penetrating protein analogues (Peptide4 and Peptide5) in breast cancer cell lines.

3.9.2. Caspase activity assay

Caspases, essential proteins in cells for apoptosis and inflammation, were first implicated when *Caenorhabditis elegans* protein CED-3 was found to have close homology with the mammalian interleukin-1-converting enzyme (ICE or caspase 1) which when over-expressed induces apoptosis. Caspases are cysteine proteases, which use a cysteine residue to cleave their substrate proteins at the aspartic acid residue. The generic name for all members is caspase with the "c" denoting a cysteine protease and "aspase" referring to the aspartate specific cleaving ability of these enzymes. The individual members are then numbered according to their chronological order of publication. Currently 14 caspases have been identified: 12 human and 2 murine. In Paper I, PF peptide`s possible apoptotic and immunogenic effects were analyzed by measuring caspase-1 and caspase 3/7 activities *in vitro*. Caspase-specific peptide conjugated to fluorescent reporter molecule (AFC) was used as a cleavage substrate for caspase-1 and caspase 3/7. The cleavage of this caspase-specific peptide by the caspases releases the fluorochrome that, when excited by light at 400 nm wavelength, emits fluorescence at 505 nm. The level of caspase enzymatic activity in the cell

lysate is directly proportional to the fluorescence signal detected with a fluorimeter or a fluorescent microplate reader.

3.10. *In vivo* studies

In Paper III, subcutaneous and intracranial glioblastoma xenograft bearing mice were utilized for *in vivo* studies.

3.10.1. U87 MG tumor bearing mice

In Paper III, homozygous female nude mice (C.Cg/AnNTac-Foxn1nu, Taconic), bearing tumor xenografts either intracranially or subcutaneously were established in order to evaluate the localization of the fluorescein-labelled peptide vectors. 8×10^5 U87 cells were resuspended in 100 µl (subcutaneous tumor model) or in 5 µl (intracranial tumor model) of fresh DMEM medium, without serum and antibiotics. The xenografting was performed by implanting the cell suspension subcutaneously or intracranially into the striatum.

3.10.2. Biodistribution and tumor reduction studies

In Paper III, biodistribution of gHoPe2 in U87 mouse xenografts was analyzed. At 3 h post-injection of carboxyfluorescein labelled peptide (4 mg/kg), the tissues were collected, fixed for 2 h in the fixation solution, cryoprotected in sucrose and snap frozen in 2-methylbutane (AppliChem) kept on dry ice. The tissues were cut on a cryostat with a thickness of 10–15 µm, counterstained with DAPI (AppliChem) and mounted using Vectashield (Vector Labs) mounting medium. The tumor treatments were performed using 1 mg/kg doxorubicin (369 µM, 100 µl, for an animal of 20 g) and an equivalent dose (8.3 mg/kg) of dox-gHoPe2 (369 µM, 100 µl) i.v. 2 times per week. The subcutaneous tumor dimensions were measured using caliper; tumor volume was calculated as $(\text{length} \times \text{width}^2)/2$.

3.10.3. Toxicity analysis *in vivo*

In Paper I, possible toxic and immunogenic effects of PF peptides were analyzed. The peptides were administered to immunocompetent C57BL/6 male and female mice *via* i.v. injection followed by collection of the blood samples. Cytokine levels in serum were analyzed at 24 and 48 h after i.v. injection of 1 and 5 mg/kg of TAT (48–60) and their equimolar doses of 0.01 and 0.06 µmol per animal of TP10, stearyl-$(RxR)_4$, PF3, PF4, and PF6 respectively. Some animals received LPS (10 µg i.p.). Blood samples were collected *via* heart puncture and serum was separated using serum gel-separation tubes (Terumo). Ketamine (75 mg/kg (Vetoquinol, Bioketan)) and dexmedetomidine (1.0 mg/kg

(Laboratorios SYVA S.A.U., Dorbene)) in saline solution were applied *via* i.p injection and used for anaesthesia. Liver, kidney, lung and spleen were dissected immediately after blood samples collection and rapidly frozen in 2-methylbutan at –60ºC. Cryosections were fixed in formalin, stained with hematoxylin and eosin and used for histopathological analysis. Two mice were used per group in three independent experiments.

4. RESULTS AND DISCUSSION

The articles included in this thesis describe the cytotoxic and immunogenic activity of PF CPP-s (Paper I); analyze the apoptotic activity of newly designed cell-penetrating p53 analogues (Paper II) and evaluate the tumor targeting and antitumor activity of the tumor-specific peptide-based drug delivery vector gHoPe2 (Paper III); estimate the nucleic acids delivery efficacy of *N*-terminally stearylated $(RxR)_4$ and Arg9 CPP-s *in vitro* (Paper IV).

4.1. Immunogenic and cytotoxic characteristicts of PF-s peptides (Paper I)

CPP-s have been successfully used for the delivery of different payloads *in vitro* and *in vivo*. However, undesired side effects such as cytotoxicity and the ability to induce innate immune response, limit their application as drug delivery vehicles.

In Paper I, we evaluated the influence of PF peptides and PF/nucleic acid complexes on the immune activity *in vitro* and *in vivo*. During the analyses the PF/plasmid and PF/siRNA complexes with the highest charge and molar ratios, that indicated to be stable in serum and showed the best transfection effectiveness in different cell lines, received the greatest attention. The cytotoxic and immunologyc response of PF3, PF4 and PF6 peptides was analyzed in monocytic leukaemia (THP-1) and peripheral blood mononuclear cells (PBMC). Well-established CPP-s such as transportan 10 (TP10), TAT (48–60), stearyl-$(RxR)_4$ peptides and the most widely used transfection reagents Lipofectamine™ 2000 and Lipofectamine™ RNAiMAX were analyzed in order to provide a comparison with PF in the study. Aiming to analyze the immunogenic activity of the peptides and peptide/nucleic acid complexes, the release of cytokines IL-1β, IL-18 and TNF-α in cell culture medium and in mouse serum was detected using highly sensitive enzyme-linked immunosorbent assay (ELISA). For the estimation of possible toxic effects of PF3, PF4 and PF6 peptides and their complexes with nucleic acids, the viability of THP-1 cells was assessed using MTS viability assay. The viability of the cells correlates with the activity of intracellular enzymes and their ability to reduce water-soluble tetrazolium salts to formazan dyes. In order to estimate possible apoptotic effects of the PF peptides in THP-1 cells, total cellular caspase-1 and caspase-3/7 activity was analyzed. As a result, PF peptides as well as peptide/nucleic acid complexes were found to be non-toxic (non-apoptotic) and non-immunogenic *in vitro* at the peptides concentrations of 10 μM and 5 μM, respectively and at peptide/nucleic acid complexes charge ratios 1 and 5 (0.5 μg plasmid DNA) and molar ratio 40 (50 nM siRNA). PF peptides and peptide/nucleic acid complexes also showed minimal influence on host immune system at a peptide dose of 5 mg/kg *in vivo*.

CPP-s contain fewer than 30 amino acids[234] and are expected to have no indications for oncogene activation.[214] Nevertheless, biological side effects of cationic and amphipathic cell-penetrating peptides has been thoroughly studied. The results of several studies suggest that amphipathic peptides, such as transportans, which have polar and non-polar portions in their structures, affect the normal metabolism of the cells inducing toxicity already at a concentration of 5 μM.[142, 235] Design of PF peptides is based on the TP10 sequence with incorporated stearyl and/or trifluoromethylquinoline (chloroquine) groups. Chloroquine possesses immunosuppressive activity and has been utilized for the treatment of autoimmune disorders.[236] Recently, chloroquine was found to enhance the endosomal release of peptide-based delivery vectors providing higher intracellular activity of delivered cargos. Nevertheless, chloroquine has been associated with several toxicity cases, including retinal injury and skin irritations particularly when provided at high doses for a long duration.[237, 238] These data indicate the possibility that transportan derived PF peptides may trigger the undesired toxic or inflammatory effects. We investigated possible toxic and immunogenic activity of the PF peptides and compared it with activity of the widely used cationic CPP-s such as TAT (48–60), stearyl-$(RxR)_4$ and amphipathic TP10. The results of our study did not show any cytotoxic and immunogenic activity of PF peptides *in vitro* and these effects were similar with TP10, and also with TAT (48–60) and stearyl-$(RxR)_4$ peptides. *In vivo,* systemically administered, PF peptides did not induce inflammation or histopathological changes in liver, kidney, lung and spleen of the immunocompetent mice, even at the peptide much higher than the doses used for *in vivo* nucleic acid delivery.[239] We concluded that the peptides have no influence on the inflammatory response suggesting that introduced stearyl and chloroquine groups do not interfere with cellular viability and immunogenicity.

Although the novel TP10 analogues used in this study were found to be non-toxic and non-immunogenic *in vitro* and *in vivo* long-term toxicity and immunogenicity studies are needed to confirm the data. In conclusion, our studies demonstrate that PF peptides are efficient non-toxic and non-immunogenic peptide-based vectors for nucleic acid delivery both *in vitro* and *in vivo,* showing potential for their future therapeutic application without risk of inflammation.

4.2. Induction of apoptosis by p53-derived CPP-s (Paper II)

Cell cycle arrest, DNA repair, and apoptosis are the most common responses to DNA damage in normal mammalian cells. Genetic instability and malignant transformation are consequences of improper DNA damage responses.[240–242] Tumor suppressor proteins play an important role in the regulation of tissue homeostasis and in the prevention of the genetically damaged cell proliferation.

It has been previously shown that the lysine-rich domain of the C-terminus of p53 is able to induce apoptosis by activating wild-type p53 protein and restoring its normal functions in cancer cells.[213, 215] In Paper II, we developed novel human p53 C-terminal domain-derived stearyl-modified peptides (Peptide4 and Peptide5), which showed specific apoptotic activity in breast cancer cell line where the p53 activity is suppressed. Applying the CPP prediction algorithm the p53 protein-derived CPP-s were designed and synthesized by standard Fmoc chemistry. Peptide4 and Peptide5 peptide sequences were derived from the primary amino acid sequence of p53 available from the NCBI Human Protein Database. In order to investigate the cell-penetrating properties of newly designed p53-derived analogues the peptides were labelled with carboxyfluorescein and the uptake was analyzed in SK-N-AS cells using confocal microscopy. Detection of a green fluorescent signal inside the cells confirmed the intracellular translocation of the p53-derived peptides. The introduction of a stearyl group in the structure of CPP-s was used to overcome the problem of endosomal entrapment of drug delivery agents (Paper IV).[59, 222, 243] As it was shown in Paper I and IV, the incorporation of the stearyl group in the peptide sequence does not induce the non-specific toxicity and immunogenicity and will provide no interference with the apoptotic activity of the novel peptides.[244] The orthogonal addition of the stearic acid moiety via lysine 3 improved the intracellular penetration activity of the peptides. The quantitative uptake of the stearylated Peptide4 and Peptide5 in breast cancer cell lines MDA-MB-231 and MCF-7 was estimated using flow cytometry (FACS). The results from FACS analyses showed higher cellular uptake of the stearylated peptides compared to unmodified peptides. The effect was observed in both cell lines, especially under the condition of foetal bovine serum withdrawal. The penetration ability of Peptide4 and Peptide5 was strongly influenced by the presence of foetal bovine serum in the incubation medium: up to 10-fold higher uptake of stearylated peptides was estimated in both MDA-MB-231 and MCF-7 cell lines in serum free medium. On the other hand, unmodified peptides showed only minor cell-penetrating activity in serum free and complete media in both breast cancer cell lines.

The Peptide4 and Peptide5 were also found to be capable of specific induction of cytotoxic and apoptotic effects in p53-non-active breast cancer cell lines in the absence of serum. The ability of the Peptide4 and Peptide5 to induce cytotoxicity and apoptosis was analyzed in p53-non-active and p53-intact breast cancer cell lines. Long-term toxicity measurements were performed using Luminescent Cell Viability Assay and apoptotic effects in breast cancer cells were analyzed using an annexin V apoptosis kit. Despite intracellular translocation, Peptide4 and Peptide5 showed no cytotoxic effect in MDA-MB-231 cell line after 24 h of incubation. The peptides remained non-toxic even at a concentration of 40 μM, which is the highest concentration used in this study. In contrast, MCF-7 cells showed mild reduction in viability already after 24 h of incubation with the peptides, but this result was not confirmed by the further apoptotic analyses. Prolonged incubation (48 h) with Peptide4 and Peptide5

(20 μM) specifically reduced the viability of the cells up to 60 %. The induction of apoptosis in MDA-MB-231 cells, however, was observed already after 24 h of incubation under serum free conditions: 67 % of the MDA-MB-231 population had exhibited early apoptotic activity. At the same time Peptide4 and Peptide5 did not influence the viability of wild-type p53 bearing MCF-7 cells even after 48 h of incubation under similar conditions. It is important to note that Peptide4 and Peptide5 peptides failed to induce apoptosis in MDA-MB-231 cells when incubated with the cells in the presence of serum. The reduced activity of Peptide4 and Peptide5 can be explained by the enhanced degradation of the p53 inside the cancer cells.[215, 245, 246] Therefore, higher concentrations of the peptides might be needed to obtain the significant effect *in vitro*. The obtained data also suggest that further *in vivo* studies are required in order to investigate the influence of blood serum on the p53-derived CPP-s activity. Additionally, the anti-tumor properties of the Peptide4 and Peptide5 can be enhanced by the conjugation of the peptides to the pro-apoptotic agents achieving thereby the synergistic effect on tumor cell viability.

In summary, our study demonstrated that Peptide4 and Peptide5, the peptide sequences of which were generated by using QSAR-based algorithm, are prone to penetrate inside the cells inducing specifically apoptosis in p53- non-active breast cancer cell line. The introduction of stearic acid moiety in the Peptide4 and Peptide5 backbone at *N*-terminal or lysine 3-orthogonal positions similarly enhanced the cellular uptake of these peptides, improving their cytotoxic and apoptotic activities in a serum free environment. Being capable of cellular translocation and having an apoptosis inducing effect, Peptide4 and Peptide5 might be implemented together with various cytotoxic agents for anticancer treatment. These findings suggest that novel p53-derived CPP-s have a promising potential in the anticancer therapy.

4.3. Development of the glioma-targeted drug delivery vector gHoPe2 (Paper III)

Cancer is the leading cause of death in economically developed and developing countries.[247–249] In particular the treatment of gliomas is therapeutically challenging with drug delivery being limited due to either insufficient penetration through the blood-brain barrier or the overly fast clearance.[250]

Paper III describe the development of the tumor-targeted delivery vector gHoPe2 based on a glioma-targeted peptide sequence gHo, which was identified by using *in vitro* phage display technology.[113]

The main aim of the study was to create an efficient drug delivery vector capable of penetration through the blood-brain barrier and selective targeting of the tumor tissue/cells. The novel glioma-targeted delivery vector was generated by a covalent conjugation of well-established CPP pVEC and glioma-targeted sequence gHo. The incorporation of gHoPe2 into a widely used chemotherapeutic drug doxorubicin resulted in the formation of the conjugate dox-

gHoPe2. The gHo sequence tumor targeting properties were verified by analysing the binding activity of the carboxyfluorescein (FAM) labelled gHo sequence to the membrane of malignant glioblastoma cells (U87 MG) using confocal microscopy. The detection of green fluorescence signal on the membrane of the U87 MG cells but not the HeLa and HEK 293 cells after incubation even with high concentrations (10 and 20 μM) of the peptide confirmed specific association of the gHo with glioblastoma.

In order to evaluate the cellular uptake of glioma-targeted delivery vector gHoPe2, the peptide was labelled with carboxyfluorescein and assessed qualitatively using confocal microscopy and quantitatively using flow cytometry. gHoPe2 showed high cellular uptake inside the U87 MG cells, while gHo sequence not conjugated to CPP did not internalize into the cells. The characterization of the gHoPe2 proceeded in *in vivo* studies, where the biodistribution of FAM-labelled gHoPe2 was determined. Precise examination of different tissues confirmed specific localization of gHoPe2 in the tumor. The gHoPe2 was internalized into the intracranial tumor but not into intact brain, kidney and liver. At the same time the non-conjugated pVEC and gHo were not detected in any of the examined organs.

Thereafter the treatment efficacy of novel glioma-targeted delivery vector was assessed. The gHoPe2 was conjugated to chemotherapeutic drug doxorubicin and the cytotoxic effect of free doxorubicin and doxorubicin assembled into delivery vector dox-gHoPe2 was validated. Both free and complexed doxorubicin reached the cell interior. The ability of dox-gHoPe2 to reduce the viability of the glioblastoma cells *in vitro* was examined. As a result dox-gHoPe2 exhibited similar cytotoxic efficiency as free doxorubicin. Although doxorubicin is one of the most potent chemotherapeutic drugs and has shown a high antitumor effect *in vitro*, its activity against brain tumor *in vivo* remains quite low.

In order to investigate if dox-gHoPe2 would have an advantage over free doxorubicin *in vivo* the efficacy of both compounds was analyzed in the models of subcutaneous and intracranial gliomas. The dox-gHoPe2 and free doxorubicin were administrated via i.v injection two times per week within 17 days of the experiment. Subcutaneous U87 tumor growth was monitored by recording tumor size 2 times per week. Tumor growth dynamics revealed a significant effect of the treatment with dox-gHoPe2 as the tumors in this group were smaller then the tumors in the untreated group. Also the body weight loss of the intracranial glioma bearing mice was less in the group that received dox-gHoPe2 compared to the free doxorubicin-receiving group although the observed effect was statistically not significant. Unfortunately, the administration of both dox-gHoPe2 and free doxorubicin did not prolong the survival of the animals probably because the dose of 1 mg/kg used in this study was too low to induce the reduction of tumor volume. Yet the inhibition of tumor growth was observed only in subcutaneous tumors suggesting that higher amount of dox-gHoPe2 is required to achieve tumor regression and an effect on

survival in intracranial tumor model. Therefore, more experiments are needed in order to verify the effect of the dox-gHoPe2 vector *in vivo*.

There are not many reports demonstrating the development of a vector system for the effective systemic treatment of gliomas. Recently, Ruoslahti *et al*., have developed a glioblastoma-targeting tumor-penetrating peptide-based nanosystem that increased the survival of glioblastoma 005[251] xenografts from 32 to 52 days. However, the extended treatment did not provide further survival of the animals.[252] These data confirm that the treatment of glioblastoma is likely a very challenging process. Our study demonstrates for the first time the applicability of the combined approaches for tumor treatment, utilizing the *in vitro* phage display for the detection of novel peptide sequences capable of selective binding to certain tissues or cell types and CPP-s to provide the intracellular delivery of the cytotoxic drugs. Effective and specific localization of the vector to the glioma demonstrated in this study confirmed the successful application of the *in vitro* phage display for tumor targeting. Nevertheless the additional experiments are still needed to improve the activity of the novel tumor-targeted drug delivery vector.

In summary, Paper III demonstrates the potential application of CPP-s for the treatment of the invasive tumors. The strategy successfully combines the *in vitro* phage display for the detection of novel tumor-selective peptide sequences and the conjugation of these sequences to the CPP-s in order to develop highly efficient tumor-targeted drug delivery system.

4.4. Nucleic acids delivery with stearyl-$(RxR)_4$ peptide by non-covalent co-incubation strategy (Paper IV)

The $(RxR)_4$ peptide has been utilized for the delivery of covalently conjugated splice-correcting oligonucleotides (SCO) and phosphorodiamidate morpholino oligonucleotides (PMO),[253, 254] and has been already applied in pre-clinical trials.[255] The aim of this study was to modify $(RxR)_4$ peptide with stearic acid moiety and to utilize it for SCO-s and plasmid DNA delivery using non-covalent conjugation strategy.

The size of the nanoparticles of stearyl-$(RxR)_4$/pDNA complexes was around 370–450 nm depending on charge ratio 1:5 and 1:3, respectively. The nanoparticles were capable to mediate efficient intracellular delivery of pGL3 luciferase expressing plasmid. The plasmid delivery experiments were performed in CHO, BHK 21 and HEK 293 cell lines. The obtained data were compared with the data observed by using stearyl-Arg9, which has been showen to be able to transport plasmids into the cell.[256] Although stearyl-Arg9 demonstrated some capacity for intracellular plasmid delivery, it was less efficient compared to stearyl-$(RXR)_4$ and Lipofectamine™ 2000 transfection agent, which was used as a positive control in this experiments. Although the transfection efficacy of Lipofectamine™ 2000 was significantly higher compared to

stearyl-$(RxR)_4$, also high cytotoxic effect of Lipofectamine™ 2000 was observed: the viability of the cells was reduced more than 30 % as compared to untreated cells.[257] At the same time stearyl-$(RxR)_4$ did not show any toxicity in similar transfection conditions. To assess cellular uptake of stearyl-$(RxR)_4$/ plasmid complexes, CHO cells were transfected with EGFP expressing plasmid and analyzed by confocal microscopy. Stearyl-$(RxR)_4$/plasmid complexes as well as stearyl-$(RxR)_4$/SCO-s complexes transfected uniformly most of the cell population. Lipofectamine™ 2000/pDNA complexes failed to transfect the entire cell population.

The $(RxR)_4$ peptide conjugated to SCO-s has been successfully utilized *in vitro* and *in vivo*.[258–260] However, using a non-covalent co-incubation strategy, the peptide was not able to mediate any splice correction in HeLa pLuc705 cells.[233] In this study we analyzed the ability of stearyl-$(RxR)_4$ to deliver SCO-s into the cells using non-covalent approach. As a result stearyl-$(RxR)_4$ promoted efficient splice correction. Splice correction efficiency as high as 20-fold was observed at SCO-s concentration of 200 nM at molar ratio 3. The SCO concentrations needed to obtain efficient splice correction with stearyl-$(RxR)_4$/SCO-s complexes were much lower compared to the concentrations necessary when utilizing the covalent conjugation strategy of $(RxR)_4$-PMO, which are in μM concentration range.[254]

The hydrophobicity might also play an important role in the CPP-s mediated plasmid and oligonucleotide delivery. Introduction of a hydrophobic stearyl moiety to the $(RxR)_4$ structure significantly increased both pDNA and SCO-s delivery, while the same modification of Arg9 has significantly lower effect. Stearyl-$(RxR)_4$ showed to be an effective nucleic acid delivery vector, although its transfection efficiency did not reach levels of Lipofectamine™ 2000. At the same time stearylation of Arg9 did not have any effect on the nucleic acid delivery, indicating that the successful implementation of stearylation is dependent on the properties of the used CPP. It has been showen that stearylation of TP10 had higher effect on oligonucleotides delivery,[222] than stearylation of $(RxR)_4$. This can be explained by the amphipathic/hydrophobic origin of TP10. $(RxR)_4$ and stearyl-$(RxR)_4$ complexed with Cy5-labelled SCO-s were taken up in comparable quantities suggesting that small differences in uptake could not account for the large differences in biological activity, indicating the central role of the stearic acid moiety. Moreover, we can assume that these complexes were taken up by endosomal pathway, indicating that stearic acid promotes endosomal escape of stearyl-$(RxR)_4$ peptide.

In conclusion, stearyl-$(RxR)_4$ enables efficient pDNA and SCO-s delivery in non-toxic manner using non-covalent co-incubation strategy. Moreover, dose-dependent splice correction provided by non-covalent approach was comparable with $(RxR)_4$-PMO covalent conjugation, but using at least 10-times lower peptides concentration. These properties of stearic acid modified $(RxR)_4$ in combination with the simplicity of experimental set-up make it a promising delivery vector for future *in vivo* application.

5. CONCLUSIONS

The findings of this thesis provide information about the cytotoxic and immunogenic activity of TP10-derived CPP-s PF3, PF4, PF6, the apoptotic activity of p53-derived short synthetic CPP-s Peptide4, Peptide5, tumor-targeted delivery by novel gHoPe2 drug delivery vector and transfection efficacy of stearylated CPP $(RxR)_4$. The main conclusions of the Papers I, II, III and IV included in the thesis are the following:

Paper I
The analysis of cytotoxic and immunogenic activity of PF3, PF4 and PF6 peptides in THP-1 cells, peripheral blood mononuclear cells and in mice serum showed no cytotoxic and immunogenic effect for the examined peptides *in vitro* at concentrations of 10 μM and 5 μM respectively and at dose of 5 mg/kg *in vivo*. The current study confirms that PF3, PF4 and PF6 peptides have a promising potential in plasmid and siRNA delivery with no evidence of undesired toxicity and inflammation.

Paper II
Novel human p53-derived Peptide4 and Peptide5 are capable of translocation inside the neuroblastoma and breast cancer cell lines. Moreover these peptides exhibit specific apoptotic activity towards the p53-non-active MDA-MB-231, but not the MCF-7 cells, where p53 is intact. The observed apoptotic effect was more pronounced under the condition of serum withdrawal *in vitro*. Novel p53-derived CPP-s Peptide4 and Peptide5 may have a promising role in future anticancer strategy.

Paper III
The gHoPe2 vector demonstrated effective and specific localization to the glioma *in vivo.* The chemotherapeutic drug conjugated to the gHoPe2 vector proved to be efficient *in vitro* and *in vivo*. Development of the delivery vectors based on the conjugation of targeting moiety and CPP-s is a promising strategy for future antitumor applications.

Paper IV
The stearylation of *N*-terminus of $(RxR)_4$ significantly increases plasmid DNA and oligonucleotides delivery showing no cytotoxicity *in vitro*. Stearyl-$(RxR)_4$ was significantly more efficient than non-modified $(RxR)_4$ and stearyl-Arg9, but not as effective as lipofectamine transfection reagent. The splice correction effect of stearyl-$(RxR)_4$, using non-covalent co-incubation strategy was comparable to that of the pre-clinically used $(RxR)_4$-PMO. The versatility of the stearyl-$(RxR)_4$ in combination with its non-toxic properties makes it a promising vector for further *in vivo* applications.

SUMMARY IN ESTONIAN

Rakku sisenevad peptiidid: tsütotoksilisus, immunogeensus ning rakendamine tuumor-spetsiifilise transportvektorina

Terapeutilise toimega ravimite kasutamine kasvajate ravis on takistatud peamiselt nende ebaefektiivse ja mitteselektiivse toime tõttu süsteemsel organismi viimisel. Uued strateegiad, mis tõhustaksid ravimi manustamise efektiivsust ning sisseviimise spetsiifilisust, on seega väga vajalikud. Viimastel aastatel on suur tähelepanu pööratud mitmesugustele ravimainete sisestamise mehhanismidele, mis ühendavad endas nii ravimite spetsiifilise kui ka vähivastase kemoteraapia strateegiaid.[95] Võttes arvesse neid aspekte on rakku sisenevad peptiidid (RSPd või *CPP-s*, *cell-penetrating peptides*) osutunud väga tõhusateks vektoriteks terapeutiliste ravimite sisseviimisel. RSPde kaudu on võimalik nii rakukultuuri kui ka elusasse organismi transportida erinevaid bioloogiliselt aktiivseid aineid nagu proteiine ning nukleiinhappeid (plasmiidne DNA (pDNA) ja splaissingut korrigeerivaid oligonukleotiide (SKO)). Muude (näiteks viiruslike) vektorite kasutamine on piiratud nende poolt põhjustatud kõrvaltoimete tõttu: tsütotoksilised efektid ning immunvastuse esilekutsumine.

Käesoleva töö peamiseks eesmärgiks oli analüüsida uute RSPde, PepFect-ide immunoloogilist ja tsütotoskilist aktiivsust *in vitro* ja *in vivo*; uurida p53 analoogsete peptiidide tsütotoksilisust ning apoptootilist toimet rinnavähi rakumudelil ning välja töötada uus RSP, mis on võimeline sisenema ja spetsiifiliselt ära tundma peaajuvähi rakke gliobalstoomi (U87 MG) rakumudelil nii *in vitro* kui ka *in vivo*.

Esimeses töös näidati, et uued RSPd PepFect-id, mis põhinevad peptiidi transportaan järjestusel, ei avaldanud tsütotoksilist ega immunogeenset toimet immuunsüsteemi rakkudele peptiidi kontsentratsioonidel 5 μM ja 10 μM *in vitro* tingimustes. Sarnane tulemus saadi ka immunokompetentsete hiirte süstimisel, kui kasutatud annused olid suuremad kui 5 mg/kg kohta.

Teises töös disainiti ning sünteesiti lühikesi p53-analoogseid peptiide, mis lisaks efektiivsele rakku sisenemisele avaldasid ka apoptootilist toimet rinnavähi rakkudele *in vitro* (seerumivabas keskkonnas). Lühikesed p53-analoogsed peptiidid omavad head perspektiivi vähivastase strateegia arendamiseks.

Kolmandas töös kirjeldati uue transportvektori, gHoPe2, väljatöötamist, mis on võimeline spetsiifiliselt sihtmärkrakkudesse sisenema. Demonstreeriti ka selle potentsiaalseid kasutusvõimalusi ning toimet ainete transpordil vähirakkudesse.

Neljandas töös näidati, et mitteamfipaatse ja sünteetilise $(RxR)_4$ peptiidi stearüülhappega *N*-terminaalse modifitseerimine tagab efektiivse ning mitte toksilise nukleiinhappete transporti rakkudesse.

Käesolev töö annab täiendava ülevaate vähispetsiifilistel RSPdel põhinevatest transportsüsteemidest, hinnates nende toksilisust, immunogeenset aktiivsust ning spetsiifilist transporti kasvajarakkudesse.

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List of publications

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DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS

1. **Imre Mäger.** Characterization of cell-penetrating peptides: Assessment of cellular internalization kinetics, mechanisms and bioactivity. Tartu 2011, 132 p.
2. **Taavi Lehto.** Delivery of nucleic acids by cell-penetrating peptides: application in modulation of gene expression. Tartu 2011, 155 p.
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