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ALLAN TOBI

Development of Smart Nanoparticles for Experimental Treatment of Cancer

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Laboratory of Precision and Nanomedicine, Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia.

The dissertation is accepted for the commencement of the degree of Doctor of Philosophy in Medicine on the 16th of October 2024 by the council of the Faculty of Medicine, University of Tartu, Estonia.

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II **Tobi, A**., Haugas, M., Rabi, K., Sethi, J., Põšnograjeva, K., Paiste, P., Jagomäe, T., Pleiko, K., Lingasamy, P., & Teesalu, T. (2024). Protease-activated CendR peptides targeting tenascin-C: Mitigating off-target tissue accumulation. *Drug Delivery and Translational Research*. https://doi.org/10.1007/s13346-024-01670-2

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AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS

Publication I

1. Assisted in the design of experiments and development of methodology relating to nanoparticles by attending meetings with T. Teesalu and P. Lingasamy.
2. Experimental part:
   - Silver nanoparticles (AgNP) and iron oxide nanoworms (NW) synthesis, functionalization, characterization.
   - Performed *in vitro* binding and internalization experiments with AgNPs, incl. cell culturing and confocal microscopy.
3. Participated in the writing of the manuscript.

Publication II

1. Designed all the experiments and developed the methodology together with T. Teesalu and M. Haugas.
2. Experimental part:
   - Silver nanoparticles (AgNP) synthesis, functionalization, characterization.
   - Performed or supervised all *in vitro* experiments, incl. culturing cells, binding and internalization assays.
   - Performed or supervised cell-free receptor binding AgNP experiments and measurements.
   - Performed immunofluorescence assays on tissue sections.
   - Performed or supervised confocal imaging.
   - Assisted M. Haugas with *in vivo* experiments.
3. Data analysis and interpretation: analyzed all the data in the article by using relevant software and interpreted the data. Had regular discussions with T. Teesalu and other co-authors about the data.
4. Wrote the manuscript draft.

Publication III

1. Designed all the experiments and developed the methodology together with T. Teesalu and A.M.A. Willmore.
2. Experimental part:
   - Silver nanoparticles (AgNP) synthesis, functionalization, characterization.
   - Performed all *in vitro* experiments, incl. culturing cells, binding and internalization assays, cytotoxicity assays, receptor immunochemistry assays.
   - Performed cell-free receptor binding experiments and measurements.
   - Prepared samples for HPLC-MS analysis.
   - Performed confocal and dark-field microscopy.
3. Data analysis and interpretation: analyzed all the data in the article by using relevant software and interpreted the data. Had regular discussions with T. Teesalu and other co-authors about the data.
4. Wrote the manuscript draft.

ABBREVIATIONS

^{107}Ag	silver isotope 107
^{109}Ag	silver isotope 109
2D	two-dimensional
3D	three-dimensional
7-AAD	7-aminoactinomycin D
ADC	antibody-drug conjugate
Ag^{+}	silver ions
$AgNO_3$	silver nitrate
AgNP	silver nanoparticle
Ahx	aminohexanoic acid
AI	artificial intelligence
ANOVA	analysis of variance
ATCC	the American Type Culture Collection
BBB	blood-brain barrier
BSA	bovine serum albumin
CF555	fluorescent dye, succinimidyl ester
CF647	fluorescent dye, succinimidyl ester
CendR	C-end Rule
CSC	cancer stem cell
$_{D}[KLAKLAK]_2$	proapoptotic peptide, sequence [KLAKLAKKLAKLAK]
DAB	3,3'-diaminobenzidine
DAPI	4`6-diamidino-2-phenylindole, fluorescent dye
DLS	dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EPR	enhanced permeability and retention
FAM	5(6)-carboxyfluorescein fluorescent dye
FDA	The Food and Drug Administration of the United States
FITC	fluorescein isothiocyanate
GBM	glioblastoma multiforme
G7	control peptide, sequence [GGGGGGG]
Gd	gadolinium
GFP	green fluorescent protein
GSH	glutathione
h	hour
H&E	hematoxylin & eosin
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HTS	high throughput sequencing
IC_{50}	half maximal inhibitory concentration
IO	iron oxide
IONP	iron oxide nanoparticle
iNGR	tumor targeting peptide, sequence [CRNGRGPDC]
iRGD	tumor penetrating peptide, sequence [CRGDKGPDC]
i.v.	intravenous

Kd	adsorption-desorption distribution coefficient
LA-ICP-MS	laser ablation inductively coupled plasma mass spectroscopy
LinTT1	linear TT1, tumor targeting peptide, sequence [AKRGARSTA]
LSTA1	brand name for the iRGD peptide
Lyp1	tumor targeting peptide, sequence [CGNKRTRGC]
M21	human melanoma cell line
mAbs	monoclonal antibodies
MeOH	methanol
min	minute
ML	machine learning
MMAE	monomethyl auristatin E
MMAF	monomethyl auristatin F
MQ	milli-Q (ultrapure water)
MRI	magnetic resonance imaging
MS	mass spectrometry
mut	mutant
NGS	next-generation sequencing
NCH421k	human glioblastoma cell line
NH_2	amino group
NHS	*N*-hydroxysuccinimide
Ni-NTA	nickel-nitrilotriacetic acid
NP	nanoparticle
NRP-1	neuropilin-1
NW	nanoworm
OPSS	orthopyridyl disulfide
P13	human glioblastoma cell line
P3	human glioblastoma cell line
p32	protein 32
PBS	phosphate buffered saline
PC3	human prostate carcinoma cell line
PEG	polyethylene glycol
pfu	plaque-forming unit
PL3	tumor targeting peptide, sequence [AGRGRLVR]
PL3uCendR	tumor targeting peptide, sequence [AGRGRLVRSAGGSVA]
PPC1	human primary prostate adenocarcinoma cell line
ROS	reactive oxygen species
RPARPAR	prototypic CendR peptide, sequence [RPARPAR]
RT	room temperature
RTK	receptor tyrosine kinase
SARS-CoV-2	severe acute respiratory syndrome related coronavirus 2
s.c.	subcutaneous
scrPL3	scrambled control peptide of PL3, sequence [RAGRGRLV]
SD	standard deviation
SEM	standard error of the mean
SKLG	tumor penetrating peptide, sequence [AGRGRLVRSKLG]
SPION	superparamagnetic iron oxide nanoparticle
SPR	surface plasmon resonance
TCEP	tris(2-carboxyethyl)phosphine hydrochloride
TEM	transmission electron microscopy

TME	tumor microenvironment
TNC	tenascin C
TNC-C	C-domain of tenascin C
TPP	tumor penetrating peptide
Tu	tumor
U87-MG	human glioblastoma cell line
uPA	urokinase-type plasminogen activator (a.k.a urokinase)
UV-Vis	ultraviolet-visible
Val-Cit	valine-citrulline
VEGF-KO-GBM	mouse glioblastoma cell line
VEGFR	vascular endothelial growth factor receptor
wt	wild-type
WT-GBM	mouse glioblastoma cell line
X	random amino acid

1. INTRODUCTION

The term “nanomedicine” can be defined as the application of nanotechnology in the context of medicine, and spans a broad range of diseases and conditions, including cancer (Jagaran & Singh, 2021; Satalkar et al., 2016). Nanoparticles (NPs) composed of various materials form the basis of nanomedicine, providing improved selectivity, reduced side effects, and longer circulation times (Yu et al., 2021). Although mainly used as drug delivery vehicles, NPs can exhibit beneficial intrinsic properties to offer enhanced diagnostic as well as therapeutic capabilities (Zahin et al., 2020). For example, the silver nanoparticle (AgNP) platform allows for a multitude of detection methods, robust tunability, and intrinsic therapeutic activity (Pasparakis, 2022). Clinically approved iron oxide NPs (IONPs) offer diagnostic and therapeutic applications as magnetic resonance imaging (MRI) contrast agents as well as drug carriers (Andrade et al., 2020; Soetaert et al., 2020). To further improve on the IONP platform, Park et al. developed iron oxide nanoworms (NWs) with superior MRI efficacy, longer circulation times and improved tumor accumulation when functionalized with affinity targeting ligands (Park et al., 2008).

Currently, over 50% of nanomedicines on the market or in clinical trials are indicated for cancer therapy (Shan et al., 2022). NPs excel as versatile and efficient drug delivery systems that can protect anticancer drugs and deliver them across biological barriers to cancer cells (Aghebati-Maleki et al., 2020). By taking advantage of characteristic molecular changes in tumor cells, vasculature and microenvironment, NPs can be constructed to target only diseased cells, release drugs at target tissues, and disrupt the tumor-supporting architecture (Awad et al., 2023; Roma-Rodrigues et al., 2019). To further improve selectivity and mitigate side effects, NPs carrying anticancer drugs can be decorated with affinity targeting ligands, such as antibodies and peptides (Bazak et al., 2015).

Tumor targeting peptides benefit from low immunogenicity, cost-effective production and small size, which makes them attractive candidates for active targeting (Lindberg et al., 2021). A subset of tumor targeting peptides, C-end-Rule (CendR) peptides not only home to tumors but also penetrate deep into the tumor tissue through a receptor-mediated transcytosis pathway, addressing a well-known limitation of solid tumor therapies (Pang et al., 2014; Teesalu et al., 2009). Moreover, it has been shown that CendR peptides can effectively deliver co-administered anticancer drugs into solid tumors in addition to conjugated cargo, simplifying the path to clinical application (Ruoslahti, 2017b; Sugahara et al., 2010). Indeed, a CendR peptide called iRGD (clinically developed as LSTA1) is currently in phase II clinical trials in combination with nab-paclitaxel and gemcitabine for the treatment of pancreatic ductal adenocarcinoma (Qian et al., 2023).

This thesis focuses on the preclinical development and application of AgNPs and NWs equipped with novel tumor targeting peptides to target and treat solid tumors. Through a series of *in vitro*, *in vivo* and *ex vivo* experiments we demon-

strated the utility of these NPs as versatile research tools as well as drug carriers. Additionally, by adapting a peptide-phage display method, we discovered a tumor targeting peptide PL3 (AGRGRLVR) specific for both the C domain of the extracellular matrix protein tenascin C (TNC-C) and b1 domain of the pleiotropic internalization receptor neuropilin-1 (NRP-1). Since the PL3 peptide exhibited unwanted accumulation in healthy tissues due to the exposed C-terminal arginine, we then developed enzymatically cleavable cryptic variants of the PL3 peptide. As a result, we propose two novel proteolytically activated cryptic variants of the PL3 peptide with reduced off-target accumulation in healthy tissues and retained tumor targeting properties: PL3uCendR (AGRGRLVRSAGGSVA) and SKLG (AGRGRLVRSKLG). This work paves the way for a strategy to develop cryptic tumor homing peptide variants with improved properties by utilizing a modified peptide-phage display method.

2. LITERATURE REVIEW

2.1. Nanomedicine

The term “nanomedicine” was first introduced in the year 2000 and has evolved to constitute a broad umbrella term with varying definitions, generally based on physical dimensions, the use of molecular tools, or the physical phenomena occurring within nanoscale objects in the context of medicine (Satalkar et al., 2016; Wagner et al., 2006). Here, we simply define nanomedicine as the application of nanotechnology in the field of medicine within a size range of 1–1,000 nm. Although there has been extensive debate on whether or not nanomedicine has lived up to its promises (Germain et al., 2020; K. Park, 2019), it cannot be denied that progress has been made over the years with about 70–90 approved nanomedicines on the market and many more in the clinical pipeline (Halwani, 2022; Jia et al., 2023).

Nanomedicine spans a broad range of diseases and conditions, such as cancer, viral and bacterial infections, and neurodegenerative disorders (Jagaran & Singh, 2021; Rubey & Brenner, 2021; G. Wei et al., 2021). To combat these maladies, nanoparticles (NPs) are the workhorse of nanomedicine with the aim to improve selectivity and accumulation at the target site, reduce side effects, protect therapeutic cargo from degradation and elimination, provide controlled or triggered release of drugs, improve avidity of affinity ligands, combine various therapeutic and diagnostic modalities, and introduce intrinsic beneficial properties (Sindhwani & Chan, 2021; Yu et al., 2021). There is a wide range of nanoparticles to choose from, each with their own unique advantages and disadvantages: metallic NPs, carbon nanotubes, silica-based NPs, liposomes, dendrimers, drug conjugates, protein NPs, micelles, exosomes etc. (Fig. 1) (Zheng et al., 2024). Most commercial NPs in medicine are deployed as drug delivery vehicles to improve bioavailability and solubility, increase circulation times, and provide targeting or higher localized accumulation (Zahin et al., 2020).

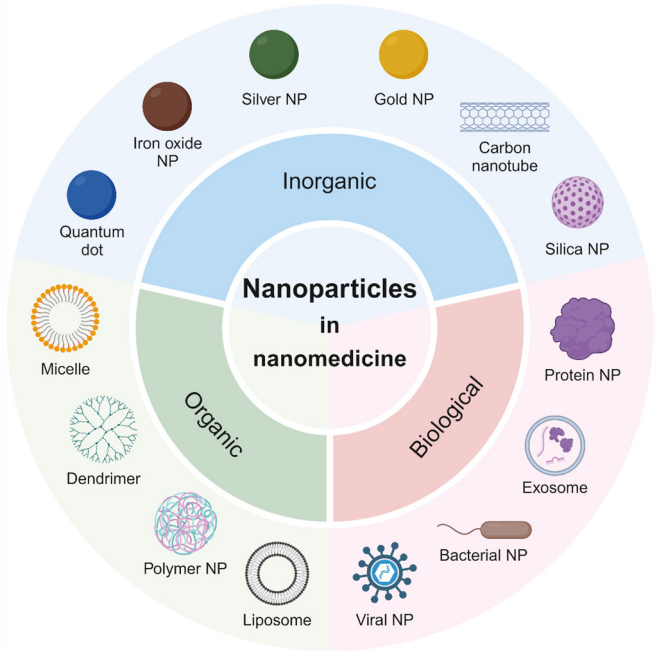

Figure 1. Examples of nanoparticles used in nanomedicine by category: inorganic, organic and biological/bio-derived nanoparticles. Schematic created with BioRender.com.

One aspect to consider is the formation of a protein corona around NPs upon entering biological environments as this could advantageously or detrimentally affect the fate of nanoformulations (Ren et al., 2022). This intricate, dynamic and not yet fully understood process of protein adsorption is influenced by a multitude of factors such as the composition, shape, size and charge of the nanoparticle as well as the route of administration and biological localization (Park, 2020). For example, it has been found that coating nanoparticles, including silver nanoparticles, with polyethylene glycol (PEG) – a common molecule for making nanoparticles "stealth" for *in vivo* use – can reduce the adsorption of proteins, such as bovine serum albumin (BSA) and human serum proteins (Ban & Paul, 2016; N. Liu et al., 2020). On the other hand, it has been shown that human serum albumin corona can actually enhance targeting effects of nanoparticles (Dai et al., 2015). One thing is for sure, it is important to keep in mind, and make use of, protein-nanoparticle interactions that occur upon coming into contact with biological medium, whether these mark nanoparticles for clearance, or help them reach the intended target (Mishra et al., 2021).

Recently, artificial intelligence (AI) and machine learning (ML) have come into the spotlight in the field of nanomedicine as promising tools to optimize drug development, study designs, dosing regimens, combination therapies, and much more (Ho et al., 2019; Serov & Vinogradov, 2022; Singh et al., 2020). The cost and availability of generating large datasets of molecular, genetic, epigenetic,

proteomic, metabolomic, and microbiomic information is rapidly decreasing, which fertilizes the ground for sophisticated algorithms to sypher through and analyze all this data as needed (Chen et al., 2012; Li et al., 2021). In the field of cancer theranostics, AI could assist with pre-screening, early detection of biomarkers, and patient stratification, which are also the prerequisites for personalized care and treatment (Tan et al., 2023). Related to the previous section, it has been proposed that AI can be used to accelerate, standardize and improve protein corona characterization as well as predict functional outcomes of a protein corona composition while lowering costs (Mahmoudi et al., 2023). Although promising, these approaches require reliable datasets and computational power to make full use of the potential of AI, at least for now, but the opportunities of exponential advancement in practically all aspects of nanomedicine seem closer than ever (Adir et al., 2020; Zaslavsky et al., 2023).

One of the main challenges in nanomedicine is the relative insufficiency of methods for assessing the toxicity and efficacy of nanoformulations along with lack of specific regulatory guidelines (Thapa & Kim, 2023). Accordingly, steps have been taken to move towards more robust and unified protocols for characterizing nanoformulations in regard to their interactions with biosystems, which could improve clinical translation (Mahmoudi, 2021). For the successful and safe application of nanoformulations in the clinical setting, it is important to first produce high quality preclinical research using model systems that are well characterized and imitate human (patho)physiology as closely as possible, although high costs may limit this pursuit in practice (Ray et al., 2021). Lastly, we need to better understand how the immune system reacts to and ultimately tries to eliminate foreign nanomaterials that mainly depends on the physicochemical properties of the nanoparticle, and affects the well-being of patients (Zolnik et al., 2010).

2.2. Silver nanoparticles

Although primarily known for their antimicrobial activity (Marambio-Jones & Hoek, 2010; Zheng et al., 2018), silver nanoparticles (AgNPs) are also amongst the most widely used platforms in biomedical research both for therapeutic and diagnostic applications (Pasparakis, 2022). AgNPs pose an attractive research tool because of their relatively high stability, tunability, and unique physicochemical and plasmonic properties (Tan et al., 2021). Noble metal-based NPs, including AgNPs, produce a nanoscale phenomenon called the surface plasmon resonance (SPR) effect, which is caused by the oscillation of conduction electrons on the surface of NPs upon light irradiation (Kelly et al., 2003; Wood, 1902). The SPR effect enables label-free detection, real-time monitoring, and enhances the fluorescence and luminescence signal of coupled reporter molecules (Kim et al., 2021; Su et al., 2021). Additional methods of detection for AgNPs include dynamic light scattering (DLS), dark-field microscopy, X-ray diffraction, UV-Vis spectroscopy, Fourier transform infrared spectroscopy, X-ray photoelectron

spectroscopy, and various electron microscopy techniques (Gao et al., 2021; Patil & Chougale, 2021).

Naturally occurring silver is composed of two stable isotopes, ^{107}Ag and ^{109}Ag isotopes (51,8% and 48,2%, respectively), which can be separated with high levels of purity, and used to produce isotopically labeled AgNPs for tracing studies (Laycock et al., 2014; Lu et al., 2016). Isotopically labeled AgNPs can be further functionalized with targeting moieties to carry out *in vitro* ratiometric phenotyping and *in vivo* quantitative biodistribution studies with laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) (Willmore et al., 2016; Pleiko et al., 2021; Toome et al., 2017). Coadministration of the isotopically labeled targeted and control AgNPs provides an internally-controlled method that has proven to be a valuable tool for preclinical cancer research (Lingasamy et al., 2019; Säälik et al., 2019), where inter- and intratumoral heterogeneity is a well-known limitation (Dagogo-Jack & Shaw, 2018).

To further enhance the versatility of the AgNP platform, Braun et al. have developed a biocompatible membrane-impermeable etching solution comprised of hexacyanoferrate and thiosulphate to remove the extracellular fraction of AgNPs (Braun et al., 2014). This allows to determine the fate of AgNPs after being taken up by cells, study internalization mechanisms and pathways, and conduct colocalization studies within cells (Hurtado de Mendoza et al., 2021; Lingasamy et al., 2021; Simonetti et al., 2022). The etching solution can also be used to regulate cytotoxic effects and alleviate non-specific toxicity *in vitro* when using AgNPs as drug carriers (Tobi et al., 2021).

The size, shape and surface chemistry of AgNPs can be customized relatively easily (Dawadi et al., 2021), which enables the use of AgNPs as "viral mimics" to study highly pathogenic viruses in a more safe and convenient manner. In 2020, Cantuti-Castelvetri et al. used AgNPs coated with the furin-cleaved S1 fragment of the spike protein to identify neuropilin-1 (NRP-1) as a host factor and potential drug target for the SARS-CoV-2 virus (Cantuti-Castelvetri et al., 2020). It has been further shown that, in addition to antimicrobial activity, AgNPs also hold promise as antiviral agents, including against SARS-CoV-2, via several potential mechanisms of action (Jeremiah et al., 2020; Salleh et al., 2020).

The precise mechanisms of AgNP toxicity are still not fully understood but the general consensus is that while AgNPs themselves have low toxicity in biological systems, the release of Ag^+ ions does have considerable negative impact (Akter et al., 2018; Rodriguez-Garraus et al., 2020). Ag^+ ions produce reactive oxygen species (ROS) leading to oxidative stress, although other toxicity mechanisms of AgNPs have been proposed, such as mitochondrial damage and endoplasmic reticulum stress (Nie et al., 2023). The extent of toxicity depends on size, dose, exposure time, synthesis method, and surface chemistry (Ferdous & Nemmar, 2020; Jaswal & Gupta, 2023). Conversely, if AgNPs are targeted to diseased cells (e.g., cancer cells) for therapeutic effect, some intracellular toxicity can even be considered beneficial as long as the side effects in healthy tissues are manageable (Gomes et al., 2021; Xu et al., 2020).

2.3. Iron oxide nanoparticles

Extensively researched superparamagnetic iron oxide nanoparticles (SPIONs or IONPs) comprise a core structure magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3), which can be coated with various inorganic and organic coatings as well as targeting moieties (Alphandéry, 2020). IONPs have found significant success and clinical approval as magnetic resonance imaging (MRI) agents to possibly replace the mainstay gadolinium (Gd)-based contrast agents which suffer from severe side effects (Rogosnitzky & Branch, 2016). Although IONPs are more commonly used as T_2 MRI contrast agents, there is on-going research into IONPs with a more preferred T_1 relaxation (Jeon et al., 2021). Simply put, T_2 contrast agents such as most IONPs produce a dark signal on MRI images, whereas T_1 contrast agents such as Gd-based compounds produce a more distinguishable bright signal (Fernández-Barahona et al., 2020). IONPs have also been used to develop more quantitative detection methods, as opposed to relatively quantitative relaxation-based detection methods, such as susceptibility measurements with mass spectrometry which allows for accurate measurements of IONP concentration and enhanced tissue mapping when combined with MRI (Girard et al., 2012).

In addition to MRI imaging, iron oxide formulations have been approved for treating iron deficiency anemia and cancer, which highlights their translational potential and safety (Coyne, 2009; Iv et al., 2015; Soetaert et al., 2020). Recently, several studies have proposed repurposing IONPs for new indications and applications, such as antiviral therapy, immunotherapy, and multimodal imaging (Abo-zeid et al., 2020; Huang et al., 2022). Due to their unique properties, IONPs have prompted interest in the field of theranostics by combining therapeutic (photothermal and -dynamic therapy, localized hyperthermia) and diagnostic (MRI) modalities of IONPs (Ajinkya et al., 2020). IONPs have also been researched as drug carriers – in addition to acting as traditional (optionally targeted) drug vehicles, the magnetic properties of IONPs enable the use of magnetic fields to concentrate drug-loaded NPs to a target site (Andrade et al., 2020).

As an interesting development, Park et al. assembled IO cores into an elongated dextran-coated structures termed “nanoworms” (NWs) which showed superior MRI efficacy, improved binding to tumor cells when functionalized with affinity ligands, longer circulation times, and amplified passive accumulation in tumor tissues compared to IO nanospheres (Park et al., 2008). When functionalized with affinity ligands, elongated nanoparticles such as NWs benefit from higher avidity and selectivity to the target as well as longer circulation times compared to the spherical counterparts (Kolhar et al., 2013; Park et al., 2009). To date, NWs have been widely used for multimodal imaging, homing studies, and experimental targeted therapy (Hunt et al., 2017; Paasonen et al., 2016; Pemmari et al., 2020; Sharma et al., 2017).

Despite several US Food and Drug Administration (FDA) approved formulations, there is still some concern about the toxicity of IONPs (Malhotra et al., 2020). The toxicity of IONPs depends on various factors, such as concentration, composition, size, immunogenicity and tissue type, although it is thought that the

used coating materials and surface modifications affect toxicity the most (Wei et al., 2021). IONPs may induce toxicity mainly through the iron ion-mediated production of ROS that damages cellular machinery, or by high accumulation in specific tissues/organs where iron could act as a reactant and catalyst for disruptive processes, but more studies need to be conducted for a clearer understanding of the underlying toxicity mechanisms (Sengul & Asmatulu, 2020; Vakili-Ghartavol et al., 2020).

2.4. Nanoparticles in cancer therapy

Over 50% of nanoformulations currently on the market or in clinical trials are indicated for cancer therapy (Shan et al., 2022), which reflects the urgent need as well as continuing hope for advances in cancer therapy through the application of nanotechnology (Fig. 2). Abraxane and Doxil were the first nanotechnology-based drugs to have reached the anticancer pharmaceutical market, and since then many have followed suit (Raj et al., 2021). Nanoparticles have emerged as versatile and efficient drug delivery systems that can protect anticancer drugs and deliver them across biological barriers to target cancer cells with the added benefit of unique properties of nanomaterials (Aghebati-Maleki et al., 2020). NPs not only solve limitations of conventional cancer therapy but also strive to overcome multidrug resistance by simultaneously delivering synergistic drugs and inhibiting drug resistance mechanisms (Gavas et al., 2021).

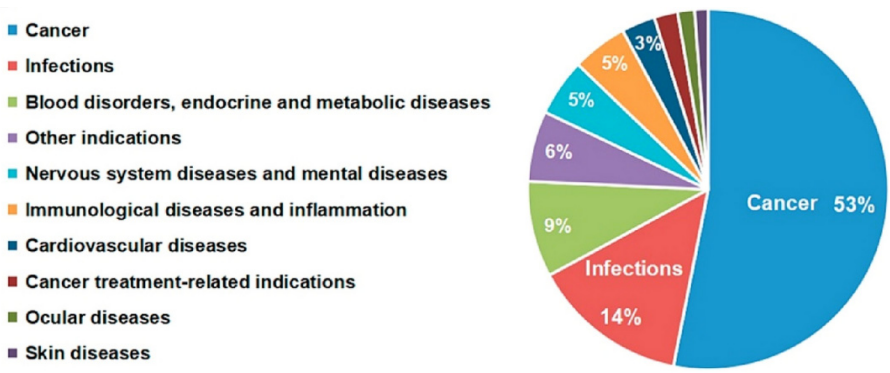

Figure 2. Overview of nanomedicines accessible in the market or in clinical trials by indication. Adapted from Shan et al. (2022).

The first NPs approved for cancer therapy in 1995 were liposomes carrying doxorubicin and several other liposomal anticancer drugs have been approved since (Salvioni et al., 2019). Liposome-encapsulated drugs have been shown to have superior pharmacological and pharmacokinetic efficiency compared to free drugs, and in the case of anthracyclines, such as doxorubicin and daunorubicin, liposomal encapsulation reduces cardiotoxicity (Beltrán-Gracia et al., 2019).

Currently, liposomes and lipid-based nanoparticles form the majority of NPs (33%) in clinical use or trials, many of them indicated for cancer therapy (Shan et al., 2022). Other promising types of NPs in clinical use or development for cancer therapy include polymeric NPs, micelles, protein-based NPs and metallic NPs (Kemp & Kwon, 2021).

Metallic NPs have shown promise in cancer drug delivery and theranostic fields due to their highly tunable and unique properties (Khursheed et al., 2022). For example, metallic NPs with magnetic properties have been extensively researched to induce localized hyperthermia and thermal ablation as cancer cells tend to be more susceptible to elevated temperature (Farzin et al., 2020; Hegyi et al., 2013). Since current hyperthermia methods lack selectivity, magnetic NPs have been proposed to solve this issue with promising *in vitro* and *in vivo* results, which have led to the first clinical trials with superparamagnetic IONPs (Pucci et al., 2022; Szwed & Marczak, 2024). Still, despite optimistic outlooks, metallic NPs in general have struggled to reach clinical use for cancer therapy, mainly due to safety concerns and a lack of uniform testing methods (Păduraru et al., 2022).

Another promising avenue of research pertains to stimuli-responsive NPs to target tumors based on characteristic changes in the tumor microenvironment (TME), specifically, acidic environment, hypoxic regions, high levels of ROS and overexpression of various enzymes (Kiran et al., 2021). For example, the antioxidant glutathion (GSH) regulates redox homeostasis and promotes resistance to anticancer drugs, which can be taken advantage of by using nanoformulations that deplete GSH and promote ROS to augment oxidative stress in tumor cells (Diaz-Vivancos et al., 2015; Hu & Liu, 2020). Enzymes overexpressed in tumors, such as cathepsin B, matrix metalloproteinases and heparanase, as well as hypoxia, one of the hallmarks of cancer, can be used to trigger the release of cytotoxic cargo (Hanahan, 2022; Tian et al., 2022). External stimuli (temperature, light, ultrasound, radiation, magnetic field) can also be used to guide "smart" nanoparticles to the tumor, activate prodrugs and control the release of drugs while mitigating side effects (Moradi Kashkooli et al., 2020; Yang et al., 2021).

Nevertheless, preclinical *in vitro* and *in vivo* cancer studies still translate to relatively poor clinical trial outcomes with less than 10% of promising preclinical candidates achieving approval (Gonzalez-Valdivieso et al., 2021; Metselaar & Lammers, 2020). Nanoparticles face many challenges along the way, including unpredictable side effects and immune system responses, endotoxin contamination, lacking knowledge on the cellular internalization processes and regulatory hurdles, but progress is being made every day (Mundekkad & Cho, 2022). More advanced cancer model systems, such as three-dimensional (3D) models and organoids, as well as earlier incorporation of humanized models or human cancer cell-based *in vivo* studies could also improve the current success rate of cancer nanomedicines (Boix-Montesinos et al., 2021). In conjunction with better model systems, it is essential to understand how the physical properties (e.g., size, surface charge and shape) of NPs affect biodistribution, penetration into tumors and toxicity (Zein et al., 2020). Lastly, the high costs of manufacturing complex nanosystems, environmental considerations, and outdated guidance and regulations

for nanotechnology continue to hinder their success in reaching markets and patients (Kemp & Kwon, 2021).

2.5. Affinity-targeted cancer therapy

Matsumura and Maeda showed already in 1986 that solid tumors exhibit enhanced vascular permeability and defective lymphatic drainage systems, which was coined the enhanced permeability and retention (EPR) effect (Matsumura & Maeda, 1986). The EPR effect allows for passive accumulation of nanoparticles as well as macromolecules in tumors by diffusion-driven extravasation through gaps in the endothelial lining – or so it was thought. Recently, it has become clear that the governing mechanisms behind the EPR effect are not so simplistic, and encompass several permeability mediators, active transport pathways as well as immune cells in the tumor microenvironment (Maeda et al., 2013; Nel et al., 2017; Shi et al., 2020). Furthermore, it has been found that there is significant heterogeneity between tumor types, tissue environments and host species, making the supposedly universal EPR phenomenon even more tricky to apply in cancer nanomedicine (Wu, 2021). Regardless of these limitations and somewhat underwhelming outcomes in the last 35 years, several strategies have been proposed to overcome EPR heterogeneity, such as patient stratification, priming treatments and active targeting (de Lázaro & Mooney, 2020; Subhan et al., 2021).

This central paradigm in cancer nanomedicine constituted that nanoparticles are mainly transported into tumors passively through inter-endothelial gaps of up to 2,000 nm until it was demonstrated that as much as 97% of nanoparticles enter solid tumors through active transport pathways (Maeda et al., 2013; Sindhwani et al., 2020). As mentioned above, one strategy to further promote active tumor targeting is to modify the surface of nanoparticles with high affinity targeting ligands, such as antibodies, peptides, aptamers, proteins, and small molecules (Bazak et al., 2015). Active targeting is generally based on molecular targets overexpressed or exclusively expressed on tumor cells, in the extracellular matrix (ECM) or tumor microenvironment (TME), and tumor vasculature (Awad et al., 2023; Roma-Rodrigues et al., 2019). For example, affinity ligands can be used to target a subpopulation of cancer cells called cancer stem cells that are notoriously resistant to treatment and cause tumor relapses (Walcher et al., 2020). Treatment strategies based on affinity-targeted NPs have been shown to overcome the increasing problem of multidrug resistance in cancers by targeting components of the multidrug resistance machinery, such as efflux transporters, defective apoptotic pathways and hypoxia (Yao et al., 2020).

Antibodies, especially monoclonal antibodies (mAbs), have had the most success in reaching the clinic since the antibody-drug conjugate (ADC) Mylotarg® (gemtuzumab ozogamicin) was first approved in the year 2000 (Norsworthy et al., 2018). By now, over 14 ADC-s have been approved for the treatment of cancers worldwide with over 100 candidates in clinical trials (Fu et al., 2022). Although mAbs alone can have intrinsic drug properties, the choice of target

receptor is a critical step in designing ADCs to ensure selectivity as well as internalization and, thus, optimal efficacy of the cytotoxic cargo delivered (Hafeez et al., 2020). Antibodies can generally carry only about 2–4 drug molecules per antibody, which necessitates the use of highly potent drugs, such as monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF) (Yaghoubi et al., 2020). To avoid aggregation, premature release of cargo, and establish stability, the linker employed in ADCs requires careful consideration (Su et al., 2021). Peptide-based linkers are a popular choice for ADCs due to their stability, biocompatibility, and options to incorporate drug release mechanisms, such as cleavage sites for the lysosomal protease cathepsin B overexpressed in several tumors (Balamkundu & Liu, 2023).

Targeted cancer therapy was first successfully applied for blood cancers (including multiple myeloma, leukemia, and lymphoma) that form in the bone marrow or in the lymphatic system (Shimada, 2019). By 2022, 46 of the 52 FDA-approved drugs indicated for blood cancers were targeted formulations (Sochacka-Ćwikła et al., 2022). One of these approved treatments, chimeric antigen receptor (CAR)-T cell therapy has conjured a lot of excitement with remarkable clinical responses in blood cancers (Haloupek, 2020; June et al., 2018), although the move to solid tumors has been limited by toxicities and subpar efficacy (Sterner & Sterner, 2021). For solid tumors, progress has been lagging behind blood cancers as NPs and anticancer drugs need to cross several biological barriers to reach cancer cells, subject to the route of administration, tumor type, size, stage and location (Fig. 3) (Huo et al., 2020; Zhao et al., 2020). Another consideration for targeted delivery is the abundance of target receptors which may become a limiting factor when saturated – for this, self-amplifying NPs, inducing more binding sites in the tumor, using higher affinity ligands, and deploying highly potent drugs may provide a solution (Ruoslahti et al., 2010).

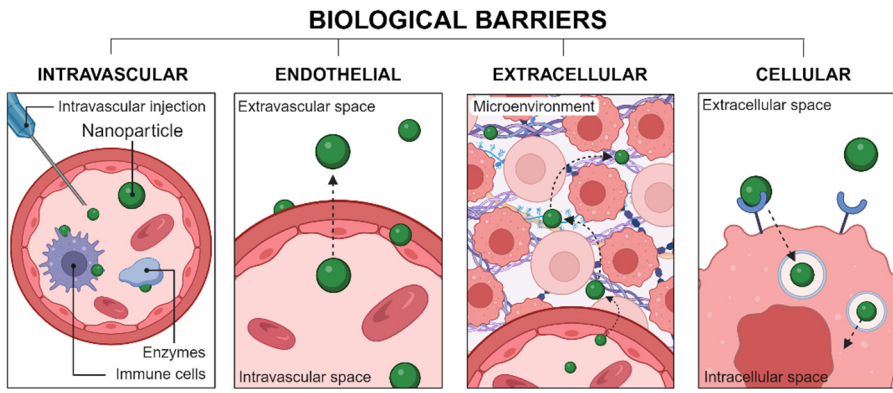

Figure 3. Biological barriers that nanoparticles need to cross upon intravenous administration. These include intravascular (e.g., mononuclear phagocyte system, enzymes), endothelial (e.g., the endothelial cell layer, blood-brain barrier), extracellular (e.g., dense matrix, high interstitial pressure) and cellular (e.g., cell membrane, endosomal escape) barriers. Schematic created with BioRender.com.

Wilhelm et al. published a controversial metastudy in 2016 stating that only 0.7% of administered nanoparticles reach solid tumors, wherein active targeted outperformed passive targeting (0.9% vs 0.6%, respectively) (Wilhelm et al., 2016). This finding has become under some scrutiny, saying that the study misrepresents the success of cancer nanotechnology by focusing on preclinical data as opposed to clinical progress and outcome measures (Lammers & Ferrari, 2020). Although there is definitely room for improvement, Lammers et al. have argued that the ultimate goal of targeted therapies is not to increase accumulation in the target tissues but benefit cancer patients by improving efficacy and reducing side effects (Lammers et al., 2016). As the cancer nanomedicine field matures, more focus should be set on biomarker-based patient stratification, prevention and elimination of metastases, addressing industrial and commercial aspects early on, and identifying and overcoming pitfalls in clinical trials as opposed to generating novel complex nanoparticles (Lammers, 2024; Zhang et al., 2023).

2.5.1. Tumor targeting peptides

Emerging as novel targeting moieties, tumor targeting/homing peptides share similarities with the already successful mAbs but have lower molecular weight, size and binding affinity, which can be beneficial for overcoming the affinity site barrier and penetrating deep into tumor tissue (Adams et al., 2001; Scodeller & Asciutto, 2020). Peptides are cost-effective, have low intrinsic immunogenicity, produce non-toxic metabolites, and are relatively easy to synthesize, making them attractive candidates for targeting NPs and anticancer drugs (Lindberg et al., 2021). The applicability of peptides in the clinical setting is further attested by over 15 peptide-containing drugs approved by the FDA (de la Torre & Albericio, 2020). Tumor targeting peptides are generally produced by three methods: 1) derivation from natural proteins, 2) chemical synthesis and rational design, or 3) screening of peptide libraries, wherein the phage display technology has been the most prevalent method (Wang et al., 2022).

The multitude of tumor-specific biological changes can be taken advantage of by tumor targeting peptides, such as the variety of overexpressed cell-surface receptors, including integrins, epidermal growth factor receptor (EGFR), G protein-coupled receptors (GPCRs), neuropeptide Y (NPY) receptor family, and mammalian bombesin (Bn) receptor family (Hoppenz et al., 2020). In addition to cancer cells that hide behind several biological barriers, the tumor microenvironment and extracellular matrix display tumor progression promoting abnormal changes that can be targeted with tumor targeting peptides (Lingasamy et al., 2019; Q. Zhang et al., 2023). Angiogenic solid tumors have aberrant and distinct vasculature, which provides easily accessible targets for tumor homing peptides upon intravenous administration, and can lead to activation of internalization pathways or opportunities to disrupt the blood supply of tumors (Li et al., 2020; Ruoslahti, 2000). Besides blood vasculature, the molecular changes in lymphatic vasculature of solid tumors can also be utilized by tumor targeting peptides with minimal accumulation in healthy tissues (Laakkonen et al., 2008). Furthermore,

homing peptides have been used to target the immune environment of tumors, specifically M2-like tumor-associated macrophages (M2 TAMs), which play an important role in the progression and immunotherapy resistance of tumors, with the intent to eliminate or reprogram them into antitumoral M1 macrophages (Han et al., 2021; Lepland et al., 2020).

2.5.1.1. C-end-Rule (CendR) peptides

Alongside off-target deposition and adverse effects on healthy cells, poor penetration into tumors is a major limiting factor for nanomedicines and anticancer drugs (Izci et al., 2021). A subgroup of tumor targeting peptides can guide cargo to tumors and subsequently trigger transcytosis pathways that penetrate deep into the tumor tissue – these are called tumor penetrating peptides (Teesalu et al., 2013). Teesalu et al. have demonstrated that peptides with a C-terminal motif R/KXXR/K (CendR peptides) activate a neuropilin-1- and neuropilin-2-dependent transcellular internalization pathway in solid tumors (Roth et al., 2012; Teesalu et al., 2009). The CendR pathway utilized by CendR peptides resembles micropinocytosis but differs by two main characteristics: it is receptor-dependent and regulated by nutrient availability via mTOR signaling cascades (Pang et al., 2014). Although the initial endocytosis process is fairly well studied (Zanuy et al., 2013), the molecular mechanisms for the cell-to-cell transport remain elusive. It has been proposed that CendR payloads may be transported through exosomes or intercellular tube-like conduits called micro-/nanotubes, although this is still speculative (Ruoslahti, 2017b). Simonetti et al. have shown that the retrograde endosomal sorting of neuropilin-1 (NRP-1) is mediated by the endosomal SNX-BAR sorting complex promoting exit 1 (ESCPE-1), the disruption of which perturbs the NRP-1-dependent endosomal trafficking process (Simonetti et al., 2022).

Activation of the CendR pathway also triggers a phenomenon called the “bystander effect” which enables to transport payloads co-administered with the CendR peptide, but not attached to it, in addition to covalently attached cargo (Fig. 4) (Ruoslahti, 2017a; Sugahara et al., 2010). In the context of drug delivery and therapy, this greatly simplifies the path to clinical application as there is no new chemical entity created and only the peptide needs to be validated when already approved drugs are used (Ruoslahti, 2017b). In the forefront of CendR peptides, co-administered iRGD (a.k.a LSTA1) is currently in phase II clinical trials in combination with nab-paclitaxel and gemcitabine for the treatment of pancreatic ductal adenocarcinoma, and showing promising results (Dean et al., 2022). Spurred by this success, further clinical trials with iRGD have been launched for other types of cancers, such as colorectal and appendix cancer, and other drug combinations (Qian et al., 2023).

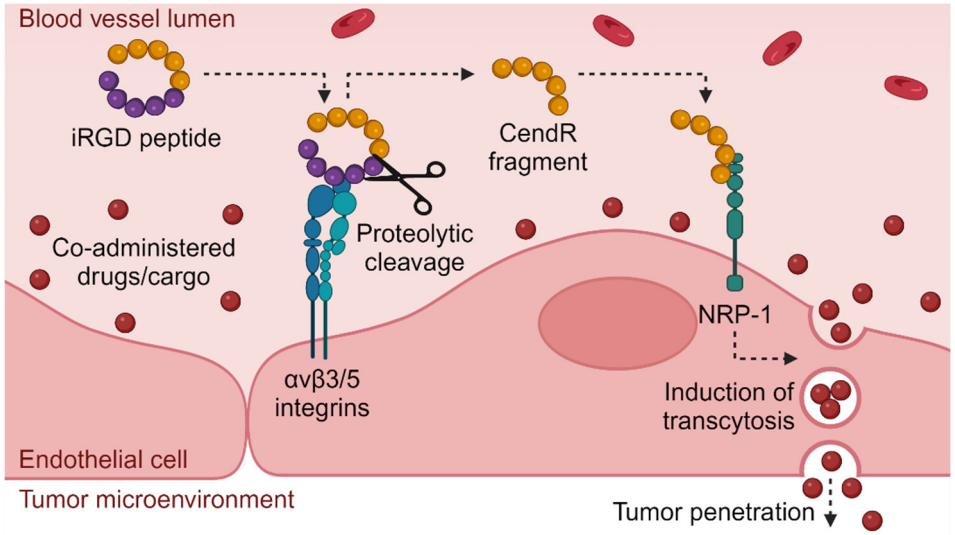

Figure 4. iRGD peptide triggers a by-stander effect to deliver not only conjugated but also co-administered drugs/cargo molecules deep into the tumor tissue. After binding to αv integrins on the endothelial cells of the tumor blood vessels, iRGD is proteolytically cleaved to generate a CendR fragment. The active CendR fragment is free to bind to neuropilin-1 (NRP-1), which triggers a bulk transcytosis pathway into the tumor tissue. Schematic created with BioRender.com.

Peptides with an exposed CendR motif have been shown to accumulate in the vascular beds of healthy tissues, mainly the lungs but also the heart (Teesalu et al., 2009). This has necessitated the development of cryptic CendR peptides that bind to primary target receptors overexpressed in the tumor vasculature, ECM, or on cancer cells, and reveal the neuropilin-binding CendR sequence only after proteolytic processing (Teesalu et al., 2013). Some examples of primary targets for cryptic CendR peptides include αvβ3 and αvβ5 integrins for iRGD, p32/gC1qR for linTT1 and Lyp1, and aminopeptidase N for iNGR, whereas the exact enzyme responsible for the subsequent cleavage may be unknown (Paasonen et al., 2016; Ruoslahti, 2022). There is evidence that any protease cleaving after a basic residue (R or K), such as furin, urokinase or trypsin, could reveal the CendR motif and activate the corresponding pathway (Balistreri et al., 2021; Braun et al., 2016).

2.5.2. Glioblastoma targeting

Glioblastoma is the most common malignant primary brain tumor with poor prognosis and survival rate as well as unique challenges compared to other solid tumors, such as the blood-brain barrier (BBB), and a unique tumor and immune microenvironment (Tan et al., 2020). Despite recent advances, standard of care for glioblastoma still consists of tumor resection followed by radiotherapy and

concomitant temozolomide, although significant efforts are being made in preclinical and clinical trials, especially with novel immunotherapeutics (Rong et al., 2022). Immunotherapies have had a head start in terms of progression through clinical trials but the recent shift towards targeted therapy approaches is illustrated by the fact that in 2022 targeted therapies accounted for 55% of the 136 on-going clinical trials, followed by cytotoxic chemotherapy (38%) and immunotherapy (34%) (Bagley et al., 2022). There are several targeting peptides under investigation as affinity ligands for GBM, led by a low-density lipoprotein receptor-related protein-1 (LRP-1) binding peptide Angiopep-2 that is currently in phase II clinical trials, and has been suggested to overcome the BBB to enhance the drug delivery for brain metastases (Kumthekar et al., 2020; Raucher, 2019).

Most emerging molecular targeted therapies are aimed at the epidermal growth factor receptor (EGFR; overexpressed in 50% of GBMs), tyrosine kinase receptors (RTKs), and the vascular endothelial growth factor receptor (VEGFR) (Cruz Da Silva et al., 2021). Unfortunately, many of these targeted therapies have suffered setbacks in late stage clinical trials, possibly due to low intratumoral drug levels, heterogeneity, signaling pathway redundancy, and inadequate patient stratification based on molecular signatures (Liu et al., 2020). A subpopulation of cancer cells called stem cell-like cells or cancer stem cells (CSCs) have recently attracted a lot of attention as targets for GBM therapy as they form the basis of tumorigenesis, have high plasticity and the ability to self-renew while interacting with the tumor microenvironment (Lathia et al., 2015). Several biomarkers have been identified for the GBM CSCs that allow for direct targeting of the essential protumoral signaling pathways, such as the Notch, Wnt/β-Catenin and RTK signaling pathways, along with indirect niches, such as targeting the perivascular, angiogenic, hypoxic and immune machinery (Tang et al., 2021).

Although cell-targeted approaches are promising, heterogeneity of GBM cells and molecular targets renders such monotherapies less-than-ideal (Nicholson & Fine, 2021). Another avenue being pursued is the GBM microenvironment which offers presumably more stable targets, such as ECM proteins, endothelial cells, microglia/macrophages and T cells, as well as better translatability from preclinical models (Muir et al., 2020). Overexpressed ECM proteins of GBM, such as tenascins, fibronectin and hyaluronic acid (HA), provide a target for antibody or peptide ligand-directed targeting that can be utilized to inhibit pathogenic signaling, deliver cytotoxic drugs and modulate the ECM (Mohiuddin & Wakimoto, 2021). One example of this is a novel bi-specific tenascin-C and fibronectin targeting peptide that has shown effective homing and drug delivery in preclinical GBM models (Lingasamy et al., 2019).

2.6. Summary of the literature

Nanotechnology has ushered in a new era of nanomedicines with seemingly endless possibilities. The fields of cancer diagnostics and therapy have spawned innovative nanoparticles with improved diagnostic capability, therapeutic efficacy, reduced side effects, and novel treatment options. Among a variety of nanomaterials, many of which display beneficial intrinsic properties, liposomal drug formulations were the first to hit the anticancer drug market in 1995. Since then, a steady flow of anticancer nanomedicines has reached approval for the complex and heterogeneous landscape of cancers. Passive targeting based on the leaky vasculature and faulty lymphatics of solid tumors has started to give way to a more precise form of targeting – active targeting. A myriad of newly discovered biomarkers and evolving tumor-specific hallmarks have enabled us to develop smart targeted nanodrugs with controlled release mechanisms, enzymatic triggers, immunomodulating capabilities, potent anticancer activity, and much more. Combined with a better understanding of the intricate interactions between nanomaterials and biological systems as well as regulatory streamlining, targeted cancer therapies could continue to introduce new and improved treatment options for cancer patients.

Antibody-drug conjugates have already proven the power of targeted cancer therapy, although the move from blood cancers to solid tumors has been hindered by the robust physical nature of solid tumors and protective biological barriers. Tumor penetrating peptides may provide a viable alternative as they not only specifically guide potent drugs to solid tumors but also penetrate deep into the hard-to-reach inner layers. The ability to cross biological barriers while bringing along cancer killing drugs could hold significant promise, even for the most challenging solid tumors. While the road from benchtop to patient is inevitably riddled with hurdles, promising results in late stage clinical trials with the tumor penetrating peptide iRGD (a.k.a LSTA1) could lead to a breakthrough of targeting peptides for cancer therapy.

3. AIMS OF THE STUDY

Hard-to-treat cancers, such as glioblastomas, pose a significant unmet challenge in oncology. Although the era of nanomedicine has paved a promising way forward, there are still hurdles to overcome to successfully target, discover and treat the heterogenous populations of solid tumors as well as alleviate side effects of these new therapies as much as possible. The development of targeted nanotherapeutics along with a better understanding of the underlying biological mechanisms may offer some much-needed relief.

This doctoral study demonstrates the development and applications of two nanosystems, silver nanoparticles and iron oxide nanoworms, equipped with tumor targeting peptides to specifically target solid tumors via overexpressed biomarkers. These "smart" nanoparticles can be used to specifically target cancer cells *in vitro* and *in vivo*, induce internalization, and deliver cytotoxic cargo to selectively eliminate target cancer cells as well as prolong the survival of tumor-bearing mice. Moreover, we propose a cell-free phage display method to modify existing NRP-1 targeting peptides with unfavorable biodistribution profiles. The modification of such targeting peptides allows to conditionally activate target receptor binding only after reaching the target site, thus vastly reducing accumulation in healthy tissues and potential side effects.

More specifically, the goals were as follows:

1. Identify tumor extracellular matrix (ECM) targeting peptides for therapeutic and diagnostic applications (I).
2. Develop a phage display method for identifying cryptic versions of NRP-1 targeting peptides with reduced accumulation in healthy pulmonary tissue (II).
3. Optimize nanoplatforms for targeted delivery of cytotoxic cargo molecules to cancer cells *in vitro* and *in vivo* (I, II, III).
4. Validate nanoplatforms functionalized with ECM targeting peptides in glioblastoma mouse models and clinical human patient samples (I, II).

4. MATERIALS AND METHODS

The detailed description of the materials and methods presented in this thesis are found in the original publications. This section provides a short summary of the methods used in the presented studies.

4.1. Peptides

All synthetic peptides used were ordered from TAG Copenhagen as powder with a purity of > 95%, and reconstituted in PBS. Biotinylation, free cysteine and/or FAM-label was added to the peptides as needed for conjugation to NPs or imaging, aminohexanoic acid (Ahx) was used as a spacer. Sequences of peptides used in the studies are shown in Table 1.

Table 1. Peptides used in the *in vitro* and *in vivo* studies.

Peptide sequence	Identification	Target site and receptor	Reference	Publication in thesis
RPARPAR	Prototypic CendR peptide	Lungs, heart, and angiogenic vasculature; receptors are NRP-1 and NRP-2	(Sugahara et al., 2009)	III
AGRGRLVR	Cell-free phage display on recombinant TNC-C	Tumor extracellular matrix; receptors are TNC-C and NRP-1.	(Lingasamy et al., 2020)	I, II
AGRGRLVRSAGGSVA	Cell-free phage display on recombinant NRP-1 (following uPA exposure)	Tumor extracellular matrix; receptors are TNC-C and uPA-conditionally NRP-1.	(Tobi et al., 2024)	II
AGRGRLVRSKLG	Cell-free phage display on recombinant NRP-1 (following uPA exposure)	Tumor extracellular matrix; receptors are TNC-C and uPA-conditionally NRP-1.	(Tobi et al., 2024)	II
$_{D}$(KLAKLAK)$_2$	*De novo* design as an antimicrobial peptide	Pro-apoptotic peptide disrupting mitochondrial membranes	(Ellerby et al., 1999; Javadpour et al., 1996)	III

4.2. Nanoparticles

Two types of nanoparticles were used in the studies presented. Silver nanoparticles (AgNP) with a wildtype, ^{107}Ag or ^{109}Ag core and NeutrAvidin for conjugating biotinylated peptides as well as separate NH_2 groups for attaching *N*-hydroxysuccinimide (NHS)-functionalized fluorophores or drugs were used in both *in vitro* and *in vivo* studies. Iron oxide nanoworms (NWs) with a crosslinked dextran and PEG coating as well as functional NH_2 groups for FAM-peptide conjugation were used for *in vivo* studies.

TEM imaging, DLS-based size measurements, UV-Vis spectrophotometry, and fluorescence- or HPLC-based quantification were used to characterize the nanoparticles.

4.2.1. Silver nanoparticles (AgNPs)

Silver nanoparticles were synthesized according to the Lee and Meisel citrate method (Lee & Meisel, 1982). Briefly, $AgNO_3$ or $^{107}AgNO_3$ or $^{109}AgNO_3$ was added to boiling milli-Q (MQ) water. Next, trisodium citrate hydrate in MQ was added to the solution to get naked AgNPs. Surface functionalization of AgNPs was done as previously published (Braun et al., 2014). NeutrAvidin functionalized with OPSS-PEG(5K)-linker was added to a solution of naked AgNPs. Next, 4-morpholineethanesulfonic acid hemisodium salt was added and the pH adjusted to 6.0. After overnight incubation at 37 °C, 10X phosphate buffered saline (PBS) and Tween® 20 were sequentially added. After centrifugation, reconstitution and sonication, tris(2-carboxyethyl)phosphine hydrochloride (TCEP) solution was added to the solution and incubated for 30 min at room temperature (RT). Then, lipoic acid-PEG(1K)-NH_2 was added, incubated for 2 h at RT, and filtered. NHS-functionalized fluorophores or OSu-Glu-Val-Cit-PAB-monomethyl auristatin E (linker-MMAE) was added to the AgNPs and incubated at 4 °C overnight. After washing, biotinylated peptide was added, incubated for 30 min at RT, washed, and filtered.

4.2.2. Iron oxide nanoworms (NWs)

NWs were prepared as previously described (Park et al., 2008). The synthesis was conducted in a sealed flask purged with N_2 on ice. Briefly, dextran was dissolved in MQ water and added with a syringe to the flask. $FeCl_3 \cdot 6H_2O$ and $FeCl_2 \cdot 4H_2O$ were separately dissolved in degassed MQ water and sequentially added to the flask with vigorous stirring. Next, 28% NH_3H_2O was slowly added with an automated pump. The solution was briefly warmed up and flask was heated at 80 °C. The resulting NW mixture was washed with ultracentrifugation. For dextran crosslinking, epichlorohydrin was added, and mixture stirred overnight. After washing with ultracentrifugation, 28% $NH_3 \cdot H_2O$ was added to achieve amination, and the suspension was dialyzed.

Aminated NWs were PEGylated with maleimide-PEG(5K)-NHS (JenKem Technology, TX, USA). Peptides were coupled to NWs through a thioether bond

between the thiol group of a cysteine residue added to the N-terminus of the peptide and the maleimide on the functionalized particles.

4.3. Cell-free peptide-phage biopanning

For PL3 peptide biopanning on recombinant human C-domain of tenascin C (TNC-C), we used NNK-encoded CX7C and X7 peptide T7 phage libraries. Throughout screens, the selected phages were amplified following a plate amplification protocol (Teesalu et al., 2012). The 1st and 4th rounds of biopanning were performed on TNC-C immobilized on ELISA plates. Briefly, the multiwell plates were coated with recombinant TNC-C, followed by blocking with 1% bovine serum albumin (BSA). The phage library was incubated overnight at 4 °C, followed by 6 washes, phage rescue and amplification in *E. coli* strain BLT5403. The subsequent rounds of selection were performed on nickel-nitrilotriacetic acid (Ni-NTA) magnetic agarose beads coated with His-6X-tagged TNC-C. After incubation with phages and 6 washes, the bound phages were eluted. The eluted phages were titered and amplified for a next round of selection. After 5 rounds of selection, peptide-encoding DNA from phage clones were subjected to Sanger sequencing.

To screen for urokinase-type plasminogen activator (uPA)-cleavable cryptic PL3 peptide variants, we constructed a constrained T7 phage library (configuration: AGRGRLVRXXXX, where X is a random amino acid) generated using partially randomized oligos. Generated phage library pool was divided into two groups: non-treated and uPA pretreated. See below for uPA treatment protocol. Ni-NTA magnetic agarose beads were coated with His-6X-tagged b1 or b1b2 domain of NRP-1 followed by incubation with phage library pools, 6 washes, and the phages eluted. Eluted phages were titered and amplified for a next round of selection. After 3 rounds of selection, peptide-encoding DNA phage clones were subjected to high-throughput sequencing (HTS).

4.4. Binding to recombinant proteins

For cell-free binding experiments, hexahistidine-tagged recombinant b1 or b1b2 domain of NRP-1 was coated onto Ni-NTA magnetic agarose beads. Tris buffer (50 mM, pH 7.0) containing 5 mM imidazole, 1 M NaCl and 0.05% Igepal CA-630 was used for the dilutions; the same buffer with 0.1% bovine serum albumin was used for washes after the protein coating step. Protein-coated magnetic beads were incubated with peptide-phage or peptide-AgNPs, followed by six washes with the washing buffer, and the release of protein–bound fraction with imidazole elution buffer (400 mM imidazole, 300 mM NaCl, 0.1% BSA and 0.05% Igepal CA-630 in PBS). Eluted phages were titered; eluted AgNPs were quantified by absorbance measurements.

4.5. Cell lines and cell culture

In this work we used cancer cell lines that originated from human or mouse tumors (Table 2). PPC1 cells were a gift from Erkki Ruoslahti (USA). U87-MG (HTB-14) and PC3 (CRL1435) cells were obtained from ATCC. M21 cells were a gift from David Cheresh (USA). NCH421k cells were obtained from CLS Cell Lines Service GmbH (Germany). P3 and P13 stem cell-like cells were a gift from Rolf Bjerkvig (Norway). Cell lines were cultured as described in the respective publications.

Table 2. Cell lines used in the *in vitro* and *in vivo* experiments.

Cell line		Application	Publication in thesis
PPC1	Human primary prostate adenocarcinoma	*In vitro*	I, II, III
PC3	Human prostate carcinoma	*In vivo*	I
M21	Human melanoma	*In vitro*	I, II, III
U87-MG	Human glioblastoma	*In vitro, in vivo*	I, II
NCH421k	Human glioblastoma	*In vitro, in vivo*	I, II
P13	Human glioblastoma	*In vivo*	I
P3	Human glioblastoma	*In vivo*	I
VEGF-KO-GBM	Mouse glioblastoma	*In vitro, in vivo*	I, II
WT-GBM	Mouse glioblastoma	*In vitro, in vivo*	I, II

4.6. *In vitro* experiments

4.6.1. Binding and internalization of peptide-AgNPs

For confocal microscopy imaging, semiconfluent cells cultured on coverslips were incubated with CF555-labeled AgNPs for 1 h. Cells were washed 3 times, fixed with −20 °C methanol (MeOH) for 1 min, washed 2 times, and counterstained with DAPI. Microscopy slides were coverslipped using an aqueous mounting medium Fluoromount-G (Electron Microscopy Sciences).

For flow cytometry, cells were detached with a non-enzymatic cell dissociation buffer, followed by incubation with AgNPs for 1 h. After washing the cells, samples were measured and analyzed with a BD Accuri C6 Plus flow cytometer (BD Biosciences, USA).

To eliminate extracellular AgNPs, we exposed the cells to a cell membrane impermeable biocompatible etching solution (10 mM), 1 : 1 solution of tripotassium hexacyanoferrate(III) ($K_3Fe(CN)_6$) and sodium thiosulfate pentahydrate ($Na_2S_2O_3{\cdot}5H_2O$) in PBS. After incubation with AgNPs, cells were treated with the etching solution for 3 minutes at RT, followed by washing. During etching, the AgNPs are dissolved through hexacyanoferrate-mediated oxidation, and the resulting Ag^+ ions form a complex with the thiosulfate. Only membrane-protected intracellular AgNPs remain intact and detectable.

4.6.2. Cytotoxicity experiments

Real-time cytotoxicity experiments were run on the xCELLigence® RTCA DP instrument (ACEA Biosciences, Inc., USA). Experiments were carried out in disposable 16-well xCELLigence® E-Plates (ACEA Biosciences, Inc., USA) with microelectrodes attached to the bottom of the wells for impedance measurements. First, complete medium was added to each well and background impedance was measured for each well. Next, cells were added and left to attach overnight. The next day, cells were incubated with the compounds for 1 h, washed once, and fresh medium was added. Measurements were terminated after 63 h.

For cytotoxicity analysis of a co-culture of GFP-expressing PPC1 cells and unlabeled M21 cells, cells were plated and left to attach overnight. The cells were washed once with warm Dulbecco's Modified Eagle Medium (DMEM), incubated with compounds for 1 h. After washing, the cells were grown for 72 h, and subjected to analysis with flow cytometry or microscopy.

For the FITC Annexin V (Biolegend, USA) / 7-AAD (BD Biosciences, USA) apoptosis assay, cells were seeded onto 12-well plates and left to attach overnight. After washing, the cells were incubated with the compounds for 1 h, washed, and grown for 48 or 72 h. Cells were dissociated by adding PBS-based non-enzymatic dissociation buffer. The resulting suspension of cells was resuspended in 1X Annexin V Binding Buffer. Finally, FITC Annexin and 7-AAD containing Cell Viability Solution were added. Data acquisition and analysis were carried out with a BD Accuri C6 Plus flow cytometer and the complementary software.

4.7. Animal experiments

The Estonian Ministry of Agriculture, Committee of Animal Experimentation, approved all the experimental procedures that required animal usage under permits #42, #48 and #159. The Ethics Committee of the University of Tartu approved protocols for obtaining and using fresh surgical human tumor samples from Tartu University Clinics, Tartu, Estonia (permit #243/T27).

4.7.1. Tumor models

We used orthotopic or subcutaneous human and mouse xenograft tumor models. For orthotropic the induction of GBM tumor models, the cell lines were grown and maintained as adherent or non-adherent spheroids. The dissociated individual cells (700,000 for U87-MG, WT-GBM, VEGF-KO-GBM, 300,000 for NCH421k, P3 stem cell-like and P13) were intracranially implanted into the right striatum (coordinates: 2 mm right and 1 mm anterior to the bregma at 2.5 mm depth) of mice. The intracranial tumors were allowed to develop for 10–15 days (U87-MG), 6–7 days (WT-GBM), 12–14 days (VEGF-KO-GBM), 30–45 days (NCH421k), 30–45 days (P3) or 35 days (P13) before conducting experiments.

For the subcutaneous GBM and prostate carcinoma model, cells were grown as attached culture until 80% confluence. 2–9 × 10^6 (U87-MG, PC3) cells in 100 µl PBS were subcutaneously injected into the right flank of 11 to 15-week old male and female nude mice. The subcutaneous U87-MG GBM and PC3 prostate carcinoma tumors were allowed to grow until 100 mm^3 before starting the experiment.

4.7.2. *In vivo* peptide-phage playoff

The *in vivo* playoff auditioning of phage was performed for competitive systemic internally controlled peptide-phage homing study in mice bearing tumor xenografts models. The selected candidate phages and control peptides were individually amplified or pre-mixed in an equimolar ratio and purified. The peptide-phage was intravenously (i.v.) injected into tumor-bearing mice. After 30-min or 2-h circulation, mice were perfused under anesthesia, and the tumor along with control organs were collected. Tissues were homogenized, and the bound peptide-phage in the tissue lysates was amplified and purified. The phage genomic DNA was extracted and subjected to high throughput sequencing (HTS) using next-generation sequencing (NGS). The NGS data was used to evaluate the representation of different peptides in the input mixture, tumors, and control organs using a custom python script. The script identifies the length of the read, barcodes, and constant flanking residues of the peptide for identification and analysis.

4.7.3. Tumor homing and biodistribution studies

FAM-peptide, peptide-NPs or peptide-phage was injected i.v. into the tail vein of tumor-bearing mice. After 1–5 h of circulation and cardiac perfusion under anesthesia, the tumors and control organs were collected for further analysis. The organs were snap-frozen in liquid nitrogen and stored at −80 °C for further analysis.

4.7.4. Experimental tumor therapy

To test the *in vivo* efficacy of peptide-targeted NWs in tumor mice, U87-MG cells were implanted subcutaneously under the skin of the right dorsal flank of 11–15-week-old male nude mice. The tumor volume [calculated with the formula: length × (width × width)/2] and animal weight were recorded every other day until tumor volume reached ~100 mm^3. Animals were randomized into 4 groups (PBS, FAM-$_D$[KLAKLAK]$_2$-NWs, FAM-peptide-NWs, and FAM-peptide-$_D$[KLAKLAK]$_2$-NWs), 6 mice per group to achieve adequate statistical power (Charan & Kantharia, 2013). NWs (at 5 mg/kg body weight of iron) or PBS was i.v. injected into the tail vein every other day (10 injections). Tumor size, body weight, survival, and animal well-being (behavior, appearance, grooming) were recorded during treatment and post-therapy. When the tumor volume reached 2 cm^3 (or > 20% body weight), the mice were sacrificed, and organs and tumors were excised and snap-frozen for further analysis.

4.8. Immunohistochemistry analyses

For immunofluorescence analysis, cryosections were fixed, permeabilized, and blocked. Next, sections were incubated with relevant primary antibodies, washed, and incubated with fluorophore-labeled secondary antibodies. Cell nuclei were counterstained with DAPI. For hematoxylin & eosin (H&E) staining, fixed and permeabilized tissue sections were stained with Mayer's hematoxylin and eosin. For 3,3'-diaminobenzidine (DAB) staining, tissue sections were incubated with rabbit anti-FAM IgG as the primary antibody and donkey anti-rabbit horseradish peroxidase (HRP) as the secondary antibody. After washing, sections were incubated with ImmPACT DAB (Vector Laboratories, USA). Finally, the sections, they were mounted with aqueous mounting medium Fluoromount-G (Electron Microscopy Sciences, USA) or Depex (Merck Millipore, USA).

4.9. Microscopy-based imaging

Confocal microscopy imaging was done with the Olympus FV1200MPE confocal microscope (Germany), and data analyzed using the FV10–ASW4.2 viewer v. 4.2b, Imaris Viewer v. 10.2, and Fiji ImageJ v. 1.52t software tools. H&E- and DAB-stained tissue sections were imaged with a 10 × objective (HC PL

FLUOTAR 10 ×/0.32; Leica Microsystems, Germany) mounted to an Aperio VERSA 10 Brightfield, Fluorescence FISH Digital Pathology Scanner (Leica Biosystems, Germany). Images were analyzed with Aperio ImageScope v. 12.4.3.5008 software (Leica Biosystems Pathology Imaging, Germany).

4.10. Statistical analysis

All statistical analyses were performed using GraphPad Prism software v. 10.1.2 (GraphPad Software Inc., USA). The results were shown as mean with error bars indicating either ± SEM or ± SD, or as otherwise indicated. Student unpaired / paired t-test was used comparing two groups, and the ANOVA test with Tukey or Dunnett post-hoc was used for multiple groups comparison. All the statistical analyses with a p-value < 0.05 were considered significant. The p-values were shown as ns – not significant, * – $p \leq 0.05$, ** – $p \leq 0.01$, *** – $p \leq 0.001$ and **** – $p \leq 0.0001$. The details of the statistical methods used can be found in the experimental section of each publication.

5. RESULTS

5.1. Nanoparticle platforms

Two types of nanoparticle (NP) platforms were used in the presented studies (Fig. 5): silver nanoparticles (AgNPs) and iron oxide nanoworms (NWs). The AgNPs were used in both *in vitro* and *in vivo* experiments, whereas the NWs were mainly used for *in vivo* studies. Furthermore, isotopically enriched ^{107}Ag and ^{109}Ag nanoparticles were used in combination with laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) analysis for internally controlled *in vivo* homing studies. It should be noted that T7 phage, which could be considered a biological nanoparticle, was also extensively used in this thesis, and has many similarities to AgNPs in terms of size, shape, and amount of targeting peptides presented on the surface.

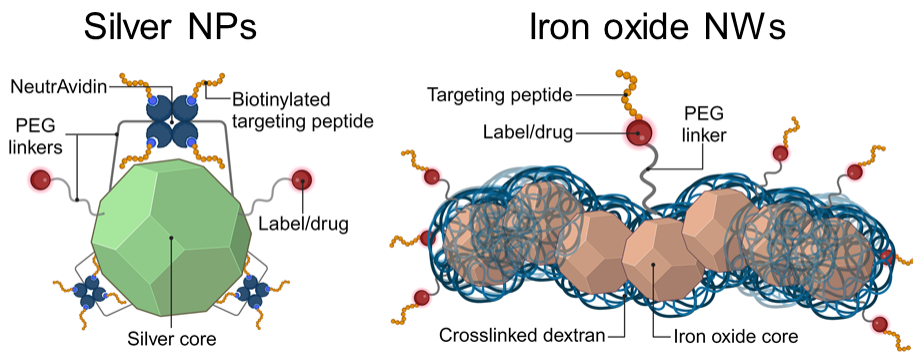

Figure 5. Schematic representations of nanoparticle platforms used in the thesis: silver nanoparticles (AgNPs) and iron oxide nanoworms (NWs). PEG = polyethylene glycol. Schematics created with BioRender.com.

5.1.1. Characterization of AgNPs

We synthesized AgNPs with an average core size of 62 ± 20 nm (Fig. 6A inset) and hydrodynamic size of 103 ± 40 nm (Fig. 6B) using the citrate method developed by Lee and Meisel (Lee & Meisel, 1982). The size and shape of the AgNPs showed some heterogeneity (Fig. 6A inset and 6B), as expected of this synthesis method (Braun et al., 2014). The UV-Vis peak absorbance of AgNPs at 415 nm (Figure 6A) and empirically determined extinction coefficient of 8.83×10^{-9} M^{-1} cm^{-1} were used to calculate the concentration of AgNPs. AgNPs were coated with NeutrAvidin and functionalized with biotinylated peptides. Fluorophores for fluorescence-based detection methods, or monomethyl auristatin E (MMAE), a potent inhibitor of tubulin polymerization, were conjugated to the AgNPs via separate polyethylene glycol (PEG) linkers. To allow for the release of MMAE upon cellular internalization, we integrated an additional commercial linker OSu-Glu-Val-Cit-PAB that includes a lysosomal cathepsin B sensitive

valine-citrulline (Val-Cit) motif, which is also used in several clinical MMAE-antibody conjugates (Balamkundu & Liu, 2023).

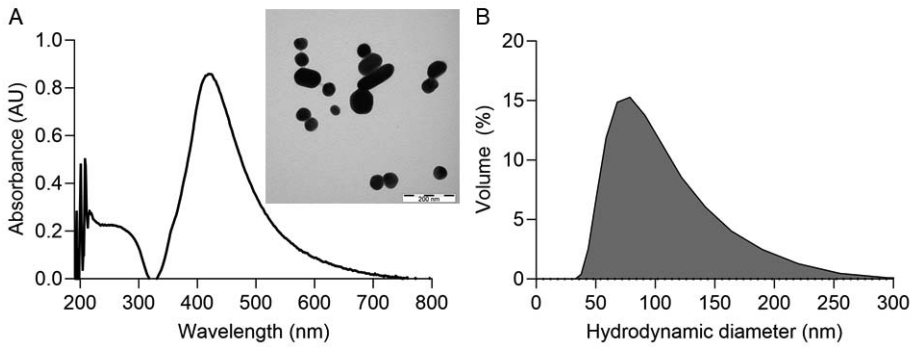

Figure 6. Characterization of AgNPs. (**A**) UV-Vis spectral analysis of AgNPs in PBST demonstrated peak absorbance at 415 nm. AU = absorbance units. Inset in (A) shows a representative transmission electron microscopy (TEM) image of the AgNPs: core size of the AgNPs was 62 ± 20 nm (n = 99) based on analysis of TEM images; scale bar: 200 nm. (**B**) Hydrodynamic diameter of AgNPs. Hydrodynamic diameter in MQ water was measured by dynamic light scattering (DLS) at room temperature (RT). The average hydrodynamic diameter was 103 ± 40 nm.

The number of NHS-sites on the AgNPs that are used to conjugate fluorophores or MMAE was determined directly by high performance liquid chromatography-mass spectrometry (HPLC-MS), and confirmed indirectly with a fluorescein-based standard curve method. There were 15 ± 2 available NHS-sites per AgNP. The number of peptides per AgNP was determined indirectly by using a fluorescent reporter (biotin-fluorescein) instead of a targeting peptide. There were 280 ± 20 available biotin binding sites per AgNP. The zeta potential of the functionalized AgNPs was −0.6 ± 0.9 mV as determined by dynamic light scattering (DLS), wherein negative zeta potential is considered optimal for cellular interactions as there are less charge-related interactions with the negatively charged cell membrane (Shao et al., 2015).

5.1.2. Characterization of NWs

We synthesized dextran-coated PEGylated paramagnetic iron oxide NWs – a dual-use model nanoplatform that can be utilized as a carrier for drugs and contrast agent for MRI applications due to intrinsic T_2 contrast properties. The synthesis procedure was based on a method published by Park et al. (Park et al., 2008). The NWs exhibited a worm-like branching iron oxide core structure (Fig. 7A, inset) with a heterogeneous distribution of size and shape. Determined by DLS, the NWs had an average hydrodynamic size of 88.8 ± 5 nm (Fig. 7B) and a zeta potential of −7.8 ± 2 mV (Fig. 7C). It should be mentioned that the DLS method for hydrodynamic size is best suited for spherical NPs due to the nature

of the embedded algorithm, which assumes spherical nanoparticle shape (Filippov et al., 2023), and should therefore be applied with certain amount of skepticism in the case of elongated structures, such as NWs. The concentrations of NWs provided throughout this thesis are based on the iron (Fe) content of NWs in solution, which was measured spectrophotometrically and calculated based on a standard curve.

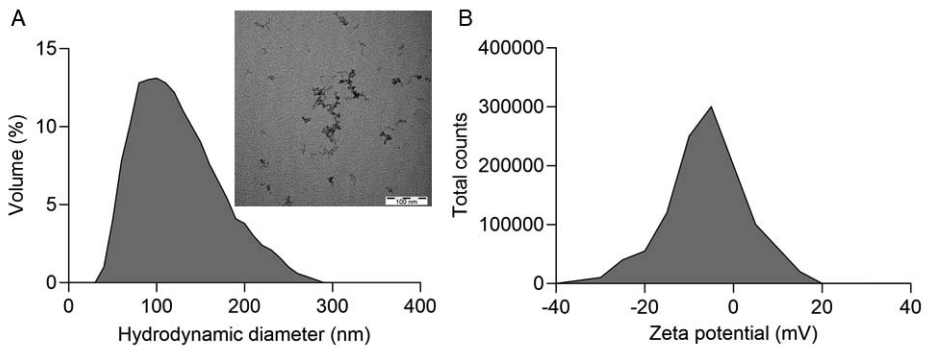

Figure 7. Characterization of NWs. (**A**) Size distribution of NWs. Hydrodynamic diameter of NWs in MQ water was measured by DLS at RT. The average hydrodynamic diameter was 88.8 ± 5 nm. Inset in (A) shows a representative TEM image of NWs; scale bar: 100 nm (**B**) Zeta potential of NWs. Apparent zeta potential of NWs was measured by DLS at RT. The average zeta potential was −7.8 ± 2 mV.

5.2. Identification of tumor ECM targeting peptides

We developed a T7 phage-based cell-free phage display method to screen for TNC-C and conditional NRP-1 binding peptides using CX7C and PL3X4 peptide-phage libraries, respectively (Fig. 8A). Bound peptide-phage genomes were sequenced with Sanger sequencing or HTS to determine the most enriched peptide sequences through the biopanning rounds. Top candidate peptides from a selected round were then subjected to further testing and functional validation.

Thereafter, we studied the biodistribution of the candidate peptide-phages using *in vivo* peptide-phage playoff (Fig. 8B), a technique that allows comparative parallel evaluation of multiple candidate peptides in the same animal. It is important to audition candidate peptides *in vivo* as systemic administration exposes nanoparticles and peptides to a milieu of factors absent in controlled cell-free or *in vitro* conditions – namely, plasma and tissue proteins, including a plethora of proteases and their regulatory molecules, alongside various other potentially interacting macromolecules.

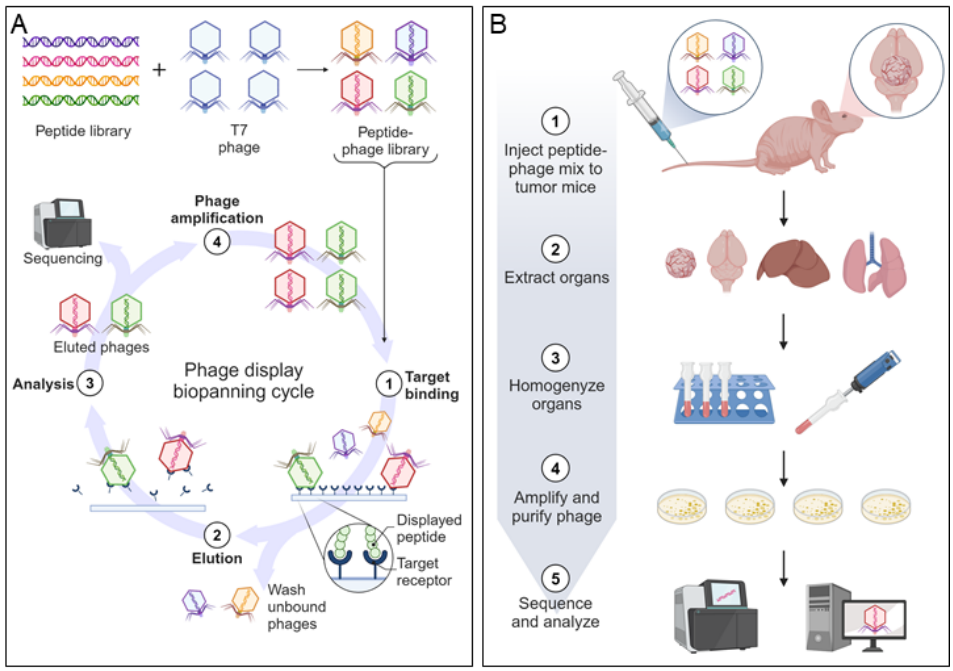

Figure 8. (**A**) Schematic of peptide-phage display biopanning screen design. (**B**) Schematic of auditioning candidate peptides by *in vivo* peptide-phage playoff. Schematics created with BioRender.com.

5.2.1. Identification of the PL3 peptide

To identify TNC-C-interacting peptides, we performed a 5-round biopanning screen with CX7C peptide T7 phage libraries using 6x-His-tagged TNC-C coated on multiwell plates in rounds 1 and 4 or TNC-C immobilized on nickel-nitrilotriacetic acid (Ni-NTA) magnetic beads in rounds 2, 3 and 5 as the target. Selection on TNC-C in multiwell plates was included to avoid enrichment for histidine-containing peptides on the Ni-NTA beads. By round 5, ~1000-fold enrichment in the binding of the selected phage pool to TNC-C was observed. Phage-displayed peptide sequences with the highest representation were selected for further studies.

For evaluating systemic tumor homing of the candidate TNC-C-targeting peptides, we used *in vivo* peptide-phage playoff auditioning (Teesalu et al., 2012). Tumor-bearing mice were i.v. injected with a phage pool of equally represented candidate TNC-C-binding and control phages, followed by 2-h circulation, perfusion, and assessment of the representation of phage clones in malignant tissues (4 glioblastoma models and 1 prostate cancer model) and in normal brain tissue (Table 3). We found that a T7 clone displaying the AGRGRLVR octapeptide was overrepresented in tumor tissues across the tested models while showing low levels of accumulation in normal brain tissue. In the following studies, we focused on the AGRGRLVR peptide that we codenamed PL3.

Table 3. *In vivo* playoff auditioning of TNC-C-selected peptide-phages. An equimolar mix of TNC-C-selected phages was i.v. injected into mice bearing WT-GBM, P3 stem cell-like, P13, and U87-MG glioblastoma, or PC3 prostate carcinoma xenografts at 1×10^{10} pfu/mouse. After 2 h of circulation, background phages were removed by perfusion. Representation of each peptide-phage in tumor tissue or in normal brain was assessed by HTS. In the tumor tissue, clone 5-derivative phage PL3 (AGRGRLVR) showed the highest representation across tumor models tested. Mean values are presented; n = 3 mice for each model.

Phage-displayed peptides in the playoff mix			Representation of peptide-phage in tumor/brain (fold over control G7)					
			WT-GBM	P3	P13	U87-MG	PC3	Normal brain
Control	GGGGGGG	Control	1.0	1.0	1.0	1.0	1.0	1.0
TNC-C-selected peptides from round 5	AGVGRLRR AKLAAALE	Clone 1	2.3	0.9	0.9	1.0	1.4	0.2
	CRGVLRRA KLAAALE	Clone 2	1.6	0.4	0.4	0.7	0.9	0.1
	AVRGRLRV AKLAAALE	Clone 3	1.3	0.6	0.6	0.8	1.0	0.1
	CSRRGILRA KPAAALE	Clone 4	1.5	0.9	0.8	1.1	1.2	0.1
	ARGRLVRA KLAAALE	Clone 5	23.9	0.4	0.1	0.2	0.3	0.0
	VGRVRFSR KLAAALE	Clone 30	2.5	0.4	0.5	0.7	1.2	0.1
	RRLVRVA	Clone 35	1.4	0.3	0.4	0.6	0.9	0.1
	RGRLVRA	Clone 45	3	0.3	0.4	0.8	1.8	0.1
	GRLTRVR	Clone 46	1.9	0.5	0.5	0.9	0.9	0.1
Clone 5 derivatives	**AGRGRLV R (PL3)**	**Modified clone 5**	**24.1**	**2.1**	**4.7**	**2.1**	**3.9**	**0.4**
	CAGRGRLV RC	Modified clone 5	0.9	0.2	0.4	0.1	0.4	0.0
	RGRLVRAK	Modified clone 5	23.8	0.3	0.1	0.5	3.0	0.2

5.2.2. Identification of cryptic PL3 derivatives

We used peptide-phage biopanning screens to identify conditional uPA-dependent cryptic PL3 derivative peptides. For this, a peptide-phage library in which the PL3 sequence was C-terminally followed by four random amino acids (configuration: AGRGRLVRXXXX, where X is a random amino acid) was constructed. The resulting library was divided into two parallel pools for biopanning. One pool was treated with uPA, followed by incubation with Ni-NTA magnetic beads coated with the recombinant b1 domain of NRP-1 to capture phages displaying conditionally active CendR peptides. The other pool was left non-treated to account for CendR peptides binding to the b1 domain of NRP-1 regardless of uPA activation. The evolution of the peptide landscape throughout the screening was monitored by titering the bound phage and analyzing the peptide encoding region of the phage genome by HTS. HTS-based assessment of the representation of peptides in the third round of selection led to 10 candidate peptides with the highest relative enrichment in the uPA-treated pool (Fig. 9A). These candidate peptides were selected for individual testing. Additionally, we designed a cryptic PL3 peptide that integrates a previously published uPA cleavage site compatible with CendR activation (Braun et al., 2016), which we named "PL3uCendR" (AGRGRLVR↓SAGGSVA, where ↓ denotes the cleavage site).

The candidate peptide-phages were studied individually to determine the uPA-conditional binding to the recombinant b1 domain of NRP-1. After incubation with uPA, the phage displaying SKLG peptide exhibited a 255-fold mean increase in NRP-1 binding, while the PL3uCendR phage showed a 122-fold mean increase, ranking sixth among the tested peptide-phages (Fig. 9B). As anticipated, candidate peptides SPDR, EASR and ADVR with C-terminal arginine (R) residues showed the highest NRP-1 binding in the absence of uPA treatment and the least enhancement following uPA treatment. Consequently, these peptides were excluded from further studies.

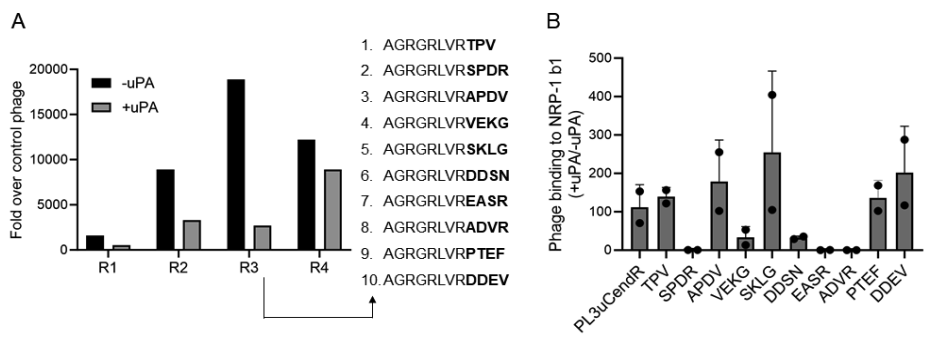

Figure 9. Discovery of uPA-dependent NRP-1 b1-binding PL3 derivative peptides. (**A**) Library enrichment through biopanning rounds 1–4 (R1–4). Ni-NTA magnetic agarose beads were coated with recombinant 6x-His-tagged b1 domain of NRP-1, incubated with phage pools with or without uPA pretreatment, washed, eluted, titered, and sequenced with HTS. Results are displayed as fold over control G7 phage (n = 1). Peptide sequences of top 10 phage candidates from round 3 based on +/– uPA enrichment ratios are listed.

(**B**) Conditional NRP-1 b1 domain binding of individual phage candidates. Experiment was conducted as in (A), except individual phages were used and phage titering was used instead of HTS. Names on the x-axis refer to the C-terminal part of the homing peptide on phage with reference to (A); PL3uCendR = AGRGRLVRSAGGSVA. Results are displayed as a ratio of +/– uPA normalized to control insertless phage. Error bars show standard deviation (SD), scatter symbols individual measurements (n = 2).

Next, we studied the biodistribution of the full panel of candidate peptides using *in vivo* peptide-phage playoff. An equimolar mixture of 10 candidate peptides from the biopanning screen, PL3uCendR, PL3 and G7 negative control was i.v. injected into athymic nude mice bearing orthotopically implanted SV40 large T-antigen and H-ras transformed mouse astrocytoma cell line (WT-GBM) xenografts expressing high levels of VEGF (Lu et al., 2012). After 30 min of circulation the mice were perfused, and retained phages in target and control tissues (tumor, brain, lungs, liver) were amplified and quantified with HTS.

In the lungs, PL3-phages were ~39 fold overrepresented compared to the cryptic candidate peptides (Fig. 10A). Phages displaying candidate peptides SPDR, EASR and ADVR also showed slightly higher pulmonary accumulation, which can be attributed to the presence of C-terminal arginine in the sequence of these peptides. This observation aligns with prior research indicating that whereas the presence of a C-terminal R/KXXR/K is required for optimal NRP-1 binding activity, even a single arginine residue can confer binding (Haspel et al., 2011; Teesalu et al., 2009). When expressing the *in vivo* playoff data as a ratio of tumor over lung, SKLG and PL3uCendR were confirmed as the leading peptides with p-values of 0.0048 and 0.0274, respectively (Fig. 10B).

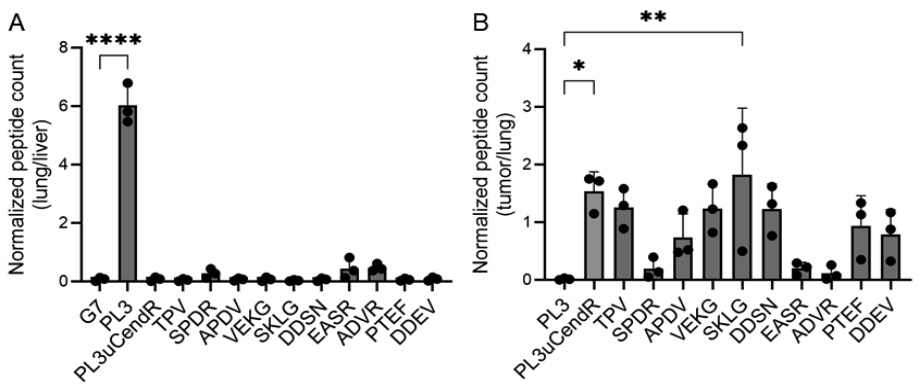

Figure 10. *In vivo* playoff with selected peptide-phage candidates. Phages expressing peptide of interest or control peptide were mixed in an equimolar ratio and i.v. injected into orthotopic WT-GBM-bearing female nude mice. After 30 min, mice were anesthetized and perfused, organs were harvested, homogenized, and tissue lysates were amplified, purified and sequenced with HTS. Results are shown as (**A**) peptide count in lung normalized to liver and (**B**) peptide count in WT-GBM tumor as a ratio of tumor over lung normalized to liver and control G7 phage. Error bars show standard deviation (SD), scatter symbols individual measurements (n = 3); * = p-value < 0.05, ** = p-value < 0.01, **** = p-value < 0.0001, one-way ANOVA with Dunnett post-hoc.

5.3. PL3 and its cryptic derivatives trigger receptor-dependent uptake

The specificity profile of each tumor penetrating peptide is defined by an interplay of its primary recruitment receptor and proteolysis-dependent engagement of the CendR element of a tumor penetrating peptide (TPP) with NRP-1 (Paasonen et al., 2016; Sugahara et al., 2009). To study the peptide-receptor interactions of PL3 and its cryptic derivatives, we performed a series of cell-free binding experiments with the recombinant target proteins, followed by *in vitro* binding and internalization experiments. In this thesis, only internalization data is shown for the sake of brevity; refer to the original publication (II) for binding data. For *in vitro* studies, we used three cancer cell lines of known receptor expression status: U87-MG glioblastoma cells (TNC-C$^+$, NRP-1$^+$), PPC1 prostate cancer cells (TNC-C$^-$, NRP-1$^+$) and M21 melanoma cells (TNC-C$^-$, NRP-1$^-$). All three cell lines were shown to express uPA.

5.3.1. PL3 peptide binds target receptors

Fluorescent anisotropy is a widely used solution-based method for characterizing interactions between small fluorescent ligands and their larger partners. We applied this method for studying the binding of FAM-PL3 (MW: 1.46 kDa) to the b1b2 domain of NRP-1 (MW: 37.8 kDa) and TNC-C (MW: 12.3 kDa). Binding of FAM-PL3 to both targets was saturable, although at different levels, which is likely caused by the difference in rotational mobility of bound ligand due to the ~3-fold difference in molecular weight of TNC-C and NRP-1 (Lakowicz, 1999). The Kd values were obtained by global fitting data to a binding isotherm, assuming single binding site with ligand depletion (Veiksina et al., 2014). For PL3/TNC-C, the fluorescent anisotropy assay and calculations yielded a Kd of 51 ± 19 μM (Fig. 11A). For the PL3/NRP-1 interaction, the Kd was 1.1 ± 0.2 μM (Fig. 11B).

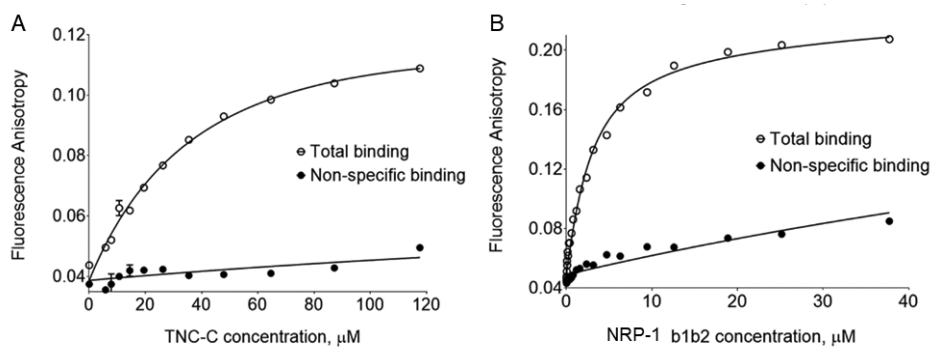

Figure 11. PL3 peptide binding to target receptors. Saturation curve for the binding of FAM-PL3 to TNC-C (**A**) and NRP-1 (**B**). FAM-PL3 (0.66 μM) was incubated with different concentrations of proteins in the absence (total binding, open circles) or in the presence (nonspecific binding, filled circles) of 0.5 mM biotin-Ahx-PL3. After 24 h of

incubation at 25 °C, fluorescence anisotropy values were calculated according to the formula $FA = (I|| - G \cdot I\perp)/ (I|| + 2 \cdot I\perp)$ and fitted globally (Veiksina et al., 2014). The data represent mean ± standard deviation (SD); n = 3.

5.3.2. Cryptic PL3 derivatives are proteolytically activated

We studied whether uPA-actuated increase in NRP-1 binding can be also observed for silver nanoparticles (AgNPs) functionalized with synthetic SKLG peptide. uPA pretreatment of AgNPs coated with SKLG peptide (SKLG-AgNPs) activated the engagement of the nanoparticles with the recombinant b1 domain of NRP-1 in uPA concentration-dependent manner (Fig. 12A). To study the specificity of the enzymatically actuated NRP-1 binding, the SKLG-AgNPs were pre-incubated with cathepsins B and K (cysteine proteinases), hyaluronidase (endoglycosidase), and TrypLE™ (serine proteinase), besides uPA. Cathepsins B and K as well as hyaluronidase failed to increase NRP-1 binding of the SKLG-AgNPs, whereas treatment with uPA and trypsin-like TrypLE™ reagent induced robust NRP-1 binding (Fig. 12B). Trypsin, and by extension TrypLE™, is a broad-spectrum proteinase with cleavage specificity at the C-terminal side of basic amino acid residues, arginine and lysine (Tsuji et al., 2017). Trypsin processing has been used to expose cryptic CendR elements in the past (Sugahara et al., 2009; Teesalu et al., 2009), and thus enhancement of binding of SKLG-AgNPs to NRP-1 was expected.

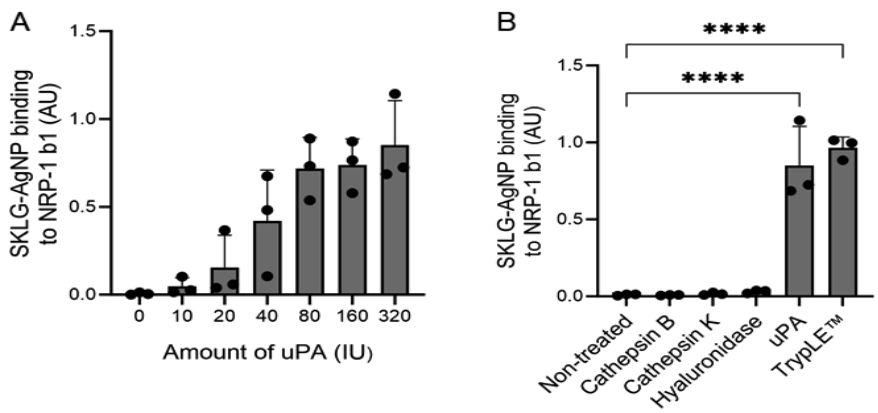

Figure 12. uPA treatment enhances binding of SKLG-AgNPs to NRP-1 b1 domain. (**A**) Dose-dependent cleavage of SKLG peptide. Ni-NTA magnetic beads were coated with recombinant 6x-His-tagged b1 domain of NRP-1, incubated with SKLG-AgNPs, washed, and eluted. AgNP absorbance of bound SKLG-AgNPs was measured at 415 nm. (**B**) uPA specifically cleaves SKLG peptide at target site to induce NRP-1 b1 binding along with trypsin-like TrypLE™. Experiment was conducted as in (A). AU = absorbance units; IU = international unit for enzymes; error bars show standard deviation (SD), scatter symbols individual measurements (n = 3); **** = p-value < 0.0001, one-way ANOVA with Dunnett post-hoc.

5.3.3. Cryptic PL3-AgNPs are conditionally taken up by target cancer cells

PL3, PL3uCendR and SKLG peptide-functionalized CF555-labeled AgNPs were incubated with cultured cells and used in confocal microscopy-based experiments. To assess cellular internalization, AgNP-treated live cells were exposed to a biocompatible etching solution that dissolves extracellular AgNPs leaving intracellular nanoparticles intact (Braun et al., 2014). Additionally, the nanoparticles were subjected to uPA pretreatment to activate the cryptic CendR peptides. Three model cancer cell lines with different target receptor expression statuses were used: PPC1 (TNC-C^{-}, NRP-1^{+}), U87-MG (TNC-C^{+}, NRP-1^{+}), and M21 (TNC-C^{-}, NRP-1^{-}).

We observed that whereas the NRP-1-positive PPC1 cells showed robust internalization of the parent PL3 peptide-functionalized AgNPs, the internalization of cryptic PL3uCendR- and SKLG-AgNPs was dependent on uPA pretreatment (i.e., CendR activation), and no uptake was evident in the case of non-peptide control AgNPs (Fig. 13A). In U87-MG cells positive for both receptors, PL3-, PL3uCendR- and SKLG-AgNPs all showed similar internalization, whereas control AgNPs remained negative (Fig. 13B). AgNPs decorated with PL3uCendR and SKLG peptides were retained by the U87-MG cells regardless of uPA treatment. We hypothesize that this may be due to turnover of TNC-C in these cells and processing by endogenous uPA expressed in all tested cell lines. Control M21 cells, negative for both TNC-C and NRP-1, did not internalize any of the tested AgNPs (Fig. 13C).

These results show that cellular engagement of cryptic PL3 derivative-equipped AgNPs can be modulated by proteolytic switching with uPA. In NRP-1-high PPC1 cells, AgNPs functionalized with cryptic PL3 derivatives PL3uCendR and SKLG were internalized in a strictly uPA-dependent manner. In contrast, in TNC-C-expressing U87-MG cells, promiscuous binding and internalization was observed for both nanoparticles coated with the parent PL3 peptide as well as its cryptic uPA-sensitive derivatives.

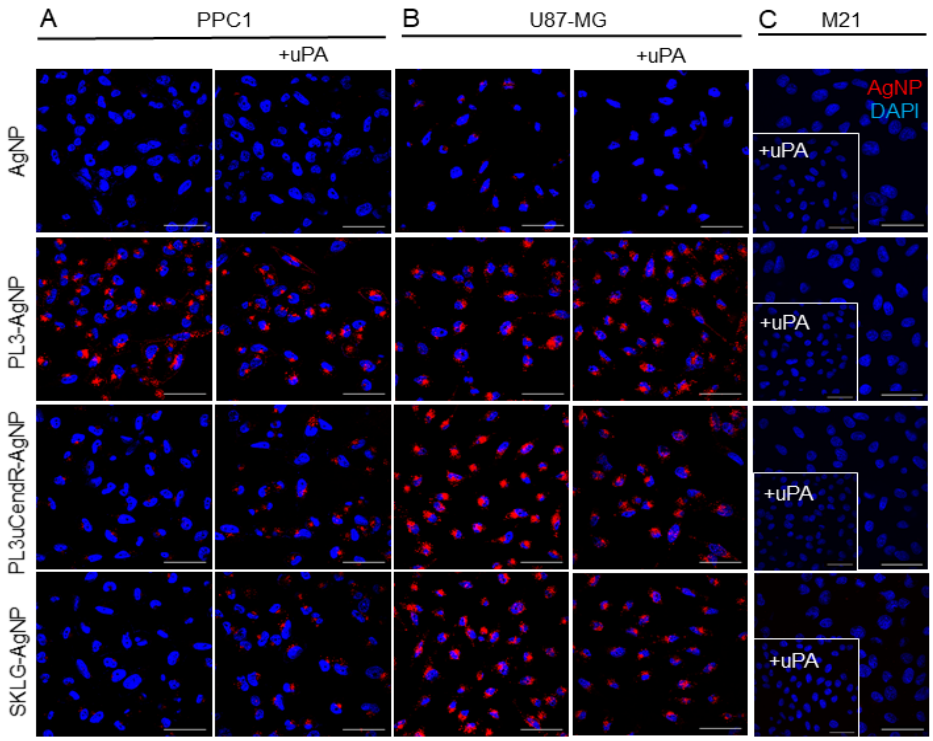

Figure 13. Internalization of peptide-targeted AgNPs *in vitro*. (**A**) TNC-C-negative NRP-1-positive PPC1 prostate carcinoma cells, (**B**) TNC-C-positive NRP-1-positive U87-MG glioblastoma cells, or (**C**) TNC-C-negative NRP-1-negative M21 melanoma cells were grown as a 2D culture, incubated with CF555-labeled AgNPs (red) optionally pretreated with uPA, etched to remove extracellular AgNPs, washed, fixed with –20 °C MeOH, counterstained with DAPI (blue; nuclei), and imaged. Representative images are shown (n = 3). Scale bars: 100 μm.

5.4. CendR-AgNPs as *in vitro* drug delivery vehicles

We set out to determine if conjugating a potent cytostatic drug, monomethyl auristatin E (MMAE), to CendR peptide-targeted AgNPs affects their binding to NRP-1 and subsequent uptake by target cancer cells. For this, we used a known prototypic CendR peptide RPARPAR (Teesalu et al., 2009). Next, we validated the RPARPAR-targeted MMAE-AgNPs in a panel of cytotoxicity assays. Two model cell lines were used in these studies: PPC1 (TNC-C^{-}, NRP-1^{+}) as well its GFP-expressing subline, and M21 (TNC-C^{-}, NRP-1^{-}). Importantly for MMAE-AgNP cytotoxicity studies, PPC1 and M21 were also shown to express lysosomal cathepsin B needed to transform MMAE into its active form. As MMAE occupies the same conjugation sites on the AgNPs as the fluorescent labels, we utilized label-free detection methods based on the intrinsic properties of AgNPs: dark-field imaging, spectrophotometric absorbance profile (415 nm), and enhancement of light scattering.

5.4.1. CendR-AgNPs retain targeting while carrying cytotoxic cargo

Ni-NTA magnetic beads coated with recombinant 6x-His-tagged b1b2 domain of NRP-1 were used to study the effect of conjugating MMAE to RPARPAR-targeted AgNPs in terms of binding capacity. Conjugation of MMAE did not hinder the binding of RPARPAR-AgNPs to the b1b2 domain of NRP-1 (Fig. 14A). Furthermore, the interaction between RPARPAR-MMAE-AgNPs and NRP-1 was shown to be specific, and mediated by the CendR binding pocket, as no binding of the RPARPAR-MMAE-AgNPs to the mutated b1b2 domain of NRP-1 was observed. In cellular studies, RPARPAR-AgNPs with and without MMAE exhibited practically identical levels of binding to the NRP-1-positive PPC1 cells (Fig. 14B), whereas no binding was seen to the NRP-1-negative M21 cells (refer to publication III). Dark-field imaging of cells after 1-h incubation with RPARPAR-MMAE-AgNPs and subsequent etching to remove the extracellular fraction of AgNPs revealed accumulation of the NPs inside target PPC1 cells (Fig. 14C), but not in the NRP-1-negative M21 cells (refer to publication III).

The specific contribution of NRP-1 to the interaction of RPARPAR-MMAE-AgNPs with PPC1 cells was studied by pre-incubating the cells with a function-blocking polyclonal α-NRP-1 antibody (Fig. 14D). The extracellular fraction of AgNPs was eliminated with a cell membrane-impermeable etching solution prior to the flow cytometry analysis. Pre-incubation of PPC1 cells with α-NRP-1 antibody blocked the internalization of RPARPAR-MMAE-AgNPs (Fig. 14D). Although some extracellular binding was still evident, we attributed this to competitive binding due to the multivalency-based increase in the effective affinity of the AgNPs (Sugahara et al., 2010). In contrast, the control IgG antibody had no effect on the cellular binding or internalization of the AgNPs (refer to original publication III).

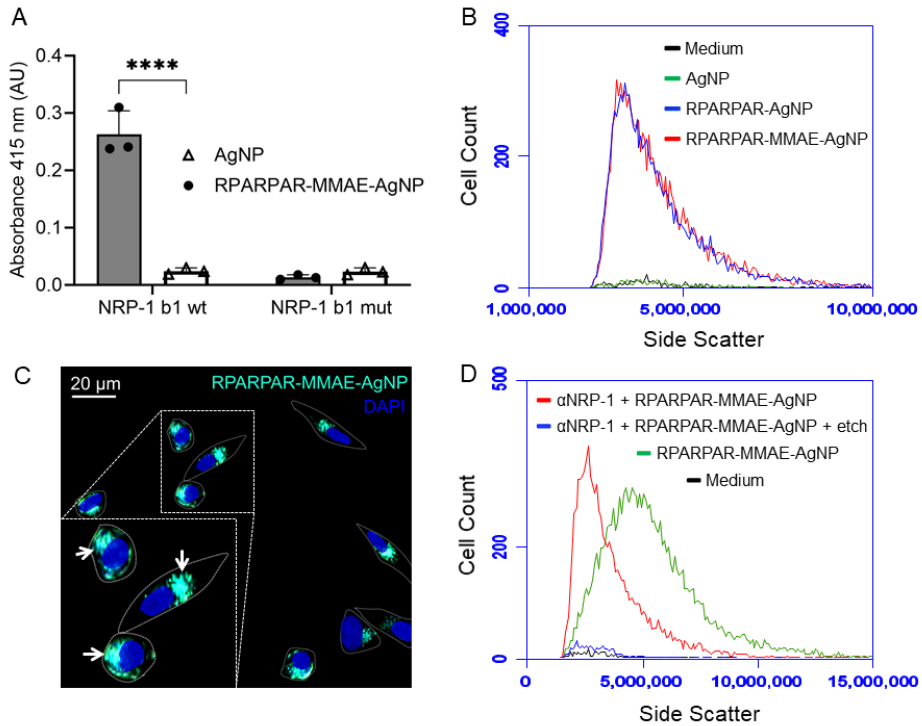

Figure 14. NRP-1-dependent binding and internalization of RPARPAR-MMAE-AgNPs. (**A**) RPARPAR-MMAE-AgNPs bind to the b1b2 domain of NRP-1. Recombinant wild type (wt) and mutant (mut) b1b2 domains of NRP-1 coupled to Ni-NTA magnetic agarose beads were incubated with AgNPs, washed, eluted, and absorbance of the eluate at 415 nm was measured. AU = absorbance units; error bars show standard deviation (SD), scatter symbols individual measurements (n = 3); **** = p-value < 0.0001, two-way ANOVA with Tukey post-hoc. (**B**) Conjugating MMAE to RPARPAR-AgNPs does not affect NRP-1 binding. PPC1 cells in suspension were incubated with AgNPs, washed, and analyzed with flow cytometry. Representative data are shown (n = 3). (**C**) Representative dark-field image (with DAPI overlay) showing internalization of RPARPAR-MMAE-AgNPs in PPC1 cells. Attached PPC1 cells were incubated with RPARPAR-MMAE-AgNPs (green) for 1 h, extracellular AgNPs were removed by etching. Dotted lines outline the cells, arrows point to internalized AgNPs; scale bar: 20 µm. (**D**) NRP-1-dependent internalization of RPARPAR-MMAE-AgNPs by PPC1 cells. PPC1 cells in suspension were incubated with AgNPs, optionally etched, washed, and analyzed with flow cytometry. To study the role of the NRP-1 in the cellular interaction of particles, the cells were optionally incubated with a function-blocking polyclonal rabbit α-NRP-1 antibody prior to incubation with AgNPs. Representative data are shown (n = 3).

5.4.2. CendR-AgNPs deliver cytotoxic cargo into cancer cells

We used HPLC-MS analysis to determine if MMAE is internalized along with the RPARPAR-AgNPs by measuring the concentration of MMAE in PPC1 cells after incubation with RPARPAR-MMAE-AgNPs, non-targeted control MMAE-AgNPs, MMAE-linker or free MMAE (Table 4). Cathepsin B treatment was used to convert all of the MMAE in the cell lysate to a detectable active form. After 1-h incubation with 1.5 nM (by Ag) RPARPAR-MMAE-AgNPs the concentration of MMAE in the cleared cell lysate was 19.1 ± 4 nM, ~85% of the input of MMAE as there are ~15 MMAE molecules per AgNP. In contrast, after incubation with the non-targeted MMAE-AgNPs, the MMAE concentration in the cell lysate was about 16-fold lower than for RPARPAR-guided nanoparticles (1.2 ± 0.2 nM; ~5% of input). Furthermore, the uptake of free MMAE was 4.5 ± 3 nM (~20% of input) and for MMAE-linker 2.4 ± 0.5 nM (~11% of input). Thus, conjugating MMAE to a linker or onto AgNPs reduces uptake by cells while targeting MMAE-AgNPs with RPARPAR greatly increases uptake by NRP-1 expressing cancer cells.

Table 4. Quantification of PPC1 cell-associated MMAE after incubation with RPARPAR-MMAE-AgNPs or controls. 5.7 × 105 cells in suspension were incubated with AgNPs (1.5 nM) or MMAE (22.5 nM) at 37 °C for 1 h, washed, lysed, and the lysate was treated with cathepsin B to release the detectable active form of MMAE. The lysate was cleared of cell debris by centrifugation, and the supernatant was used in the HPLC-MS analysis (n = 3–4).

Compound	C_{MMAE} ± SD* (nM)	Uptake (%)**
MMAE	4.5 ± 3	20
MMAE-linker	2.4 ± 0.5	11
MMAE-AgNPs	1.2 ± 0.2	5
RPARPAR-MMAE-AgNPs	19.1 ± 4	85

*Standard of deviation
**Percentage of input

These studies show that conjugating cargo, such as MMAE or fluorophores, to AgNPs functionalized with targeting peptides (e.g., RPARPAR), does not interfere with the ability of the AgNPs to interact with the target receptor. Importantly, the entire RPARPAR-MMAE-AgNP complex, including the MMAE payload, is effectively internalized into cells via a NRP-1-dependent mechanism suggesting that peptide-functionalized AgNPs can serve as targeted drug carriers.

5.4.3. MMAE-loaded CendR-AgNPs selectively eliminate target cancer cells *in vitro*

Next, we studied whether RPARPAR-targeted AgNPs can be used to potentiate the cytotoxic activity of MMAE. The xCELLigence® real-time cell viability assay, which measures cell viability via the impedance of adhered cells, was used to study the effects of RPARPAR-MMAE-AgNPs and control compounds (RPARPAR-AgNPs, MMAE-AgNPs, AgNPs, free RPARPAR peptide, and MMAE-linker) on the viability of PPC1 and M21 cells. A robust cytotoxic effect on PPC1 cells was observed at a RPARPAR-MMAE-AgNP concentration of 0.45 nM, whereas the control compounds (MMAE-AgNPs, AgNPs, RPARPAR, MMAE-linker) at an equivalent dose had no effect (Fig. 15A). Interestingly, RPARPAR-AgNPs had a modest (statistically insignificant) effect on the xCELLigence® cell index, possibly due to the anti-adhesive effects of CendR peptides (Sugahara et al., 2015), potentiated by the multivalent presentation on AgNPs. At this concentration, no toxic effect was evident on the control M21 cells (refer to publication III).

To investigate the ability of the accompanying biocompatible etching solution to regulate the toxic effects of drug-carrying AgNPs, we compared the incidence of apoptosis in target PPC1 vs. control M21 cells by calculating the cytotoxicity selectivity index (ratio of PPC1/M21 cells in late apoptosis). Cells were incubated with free MMAE, MMAE-AgNPs, or targeted RPARPAR-MMAE-AgNPs for 1 h, subjected to optional etching, cultured for 72 h, and assessed for the markers of late apoptosis. Treatment with RPARPAR-MMAE-AgNPs caused 3.1-fold more PPC1 cells to enter late apoptosis than M21 cells (Fig. 15B). However, etching increased the selectivity index to 7.2, a marked improvement in the ability to kill target cells while decreasing off-target toxicity in cells negative for target receptors. As expected, etching had no statistically significant effect on the PPC1/M21 selectivity of untargeted MMAE-AgNPs or the free drug (MMAE). These results show that removal of extracellular (free and plasma membrane-bound) particles by mild biocompatible etching can be used to reduce non-specific activity of affinity-targeted therapeutic AgNPs.

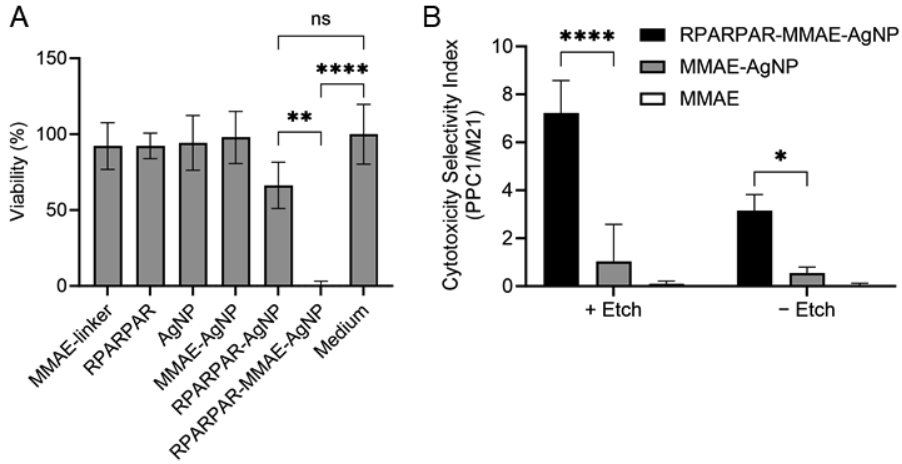

Figure 15. Effect of RPARPAR-MMAE-AgNPs on viability of cultured PPC1 cells. (**A**) Percentage viability of PPC1 cells treated with 0.45 nM RPARPAR-MMAE-AgNPs or control compounds at corresponding concentrations for 1 h, washed, left to grow for 63 h, and measured with xCELLigence® system. Error bars show standard deviation (n = 3). One-way ANOVA with Tukey post-hoc was used to calculate p-values: ns = not significant, ** = p-value < 0.01, **** = p-value < 0.0001. (**B**) Etching increases cytotoxicity selectivity index of RPARPAR-MMAE-AgNPs. Attached target PPC1 or control M21 cells were incubated with 0.45 nM targeted or non-targeted MMAE-AgNPs or free MMAE (9 nM) for 1 h, washed, and left to grow for 72 h. For flow cytometry analysis, cells were dissociated, stained with 7-AAD viability solution, and measured. Signal was normalized by untreated cells. Error bars show standard deviation (n = 3). Two-way ANOVA with Tukey post-hoc was used to calculate p-values: * = p-value < 0.05, **** = p-value < 0.0001.

Finally, we studied the effect of RPARPAR-MMAE-AgNPs on a mixed culture of GFP-expressing PPC1 (PPC1-GFP) and non-fluorescent M21 cells (Fig. 16). The cells were mixed in a 1 : 1 ratio, incubated with 0.45 nM RPARPAR-MMAE-AgNPs or control compounds in equivalent concentrations for 1 h, washed, and left to grow for 72 h. RPARPAR-MMAE-AgNPs induced a marked reduction in the GFP-positive PPC1 cell population compared to non-treated cells and control compounds (Fig. 16A). Quantitative flow cytometry analysis revealed that the GFP-positive cell fraction dropped from 50% to 9.6 ± 0.8% when the cells were treated with RPARPAR-MMAE-AgNPs, whereas the GFP-positive fraction remained at approximately 50% (50.5 ± 3.3%) when the cells were left untreated (Fig. 16B). The removal of extracellular RPARPAR-MMAE-AgNPs by etching reduced the cytotoxic effect on PPC1-GFP cells (36.0 ± 1.9%) (Fig. 16C). Control compounds did not have a significant impact on the PPC1-GFP/M21 ratio with or without etching. These results suggest applications for affinity-targeted therapeutic AgNPs for selective elimination of target receptor-positive cells in complex systems.

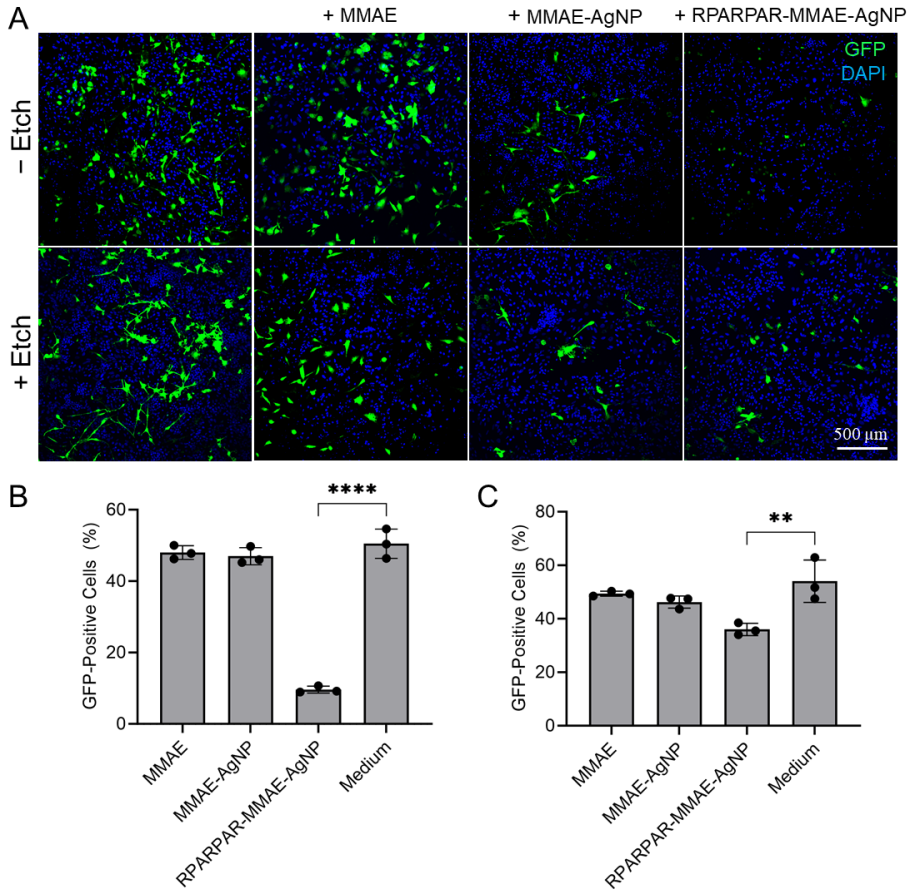

Figure 16. RPARPAR functionalization renders MMAE-AgNPs selectively cytotoxic to NRP-1-positive PPC1 cells in a co-culture of PPC1-GFP and M21 cells. (**A**) Microscopy-based assay: PPC1-GFP (green) and M21 cells were pooled in a 1 : 1 suspension and grown as attached culture. Co-cultures were incubated with targeted or non-targeted 0.45 nM MMAE-AgNPs or free MMAE (9 nM) for 1 h, optionally etched to remove extracellular AgNPs, washed, and left to grow for 72 h. Cells were fixed with MeOH at −20 °C, counterstained with DAPI (blue; nuclei), and imaged. Representative confocal images are shown (n = 3). Scale bar: 500 µm. (**B, C**) Flow cytometry-based assays were performed as in (A), except that cells were detached for analysis with flow cytometry. Panel (B) shows results without etching and panel (C) with etching. Error bars show standard deviation (n = 3). One-way ANOVA with post-hoc Tukey was used to calculate p-values: ** = p-value < 0.01, **** = p-value < 0.0001.

5.5. Peptide-targeted NPs home to GBM models *in vivo* and *ex vivo*

Animal models play an important role in translational research as the complexity of an organism could significantly impact and potentially alter the journey of nanosystems on their way to the target, and in some cases even the composition of nanosystems, compared to *in vitro* and cell-free models. Consequently, it is important to assay any and all entities with a clinically relevant objective in animal models to better understand their effects, biodistribution, and mechanisms. We tested NWs and AgNPs decorated with PL3 or its cryptic variants in three glioblastoma models (U87-MG, WT-GBM, NCH421k) and one prostate cancer model (PC3) as mouse xenograft models. Tumors were induced either subcutaneously (s.c.) or orthotopically, and the NPs were administered systemically via the tail vein in all cases. Additionally, the binding of PL3-NWs to *ex vivo* human patient glioblastoma samples was studied to determine translational relevance.

5.5.1. Peptide-targeted NPs target GBM in mice

We first tested the effect of PL3-functionalization on tumor targeting of dextran-coated PEGylated paramagnetic nanoworms (NWs). NWs were coated with FAM-labeled PL3 peptide or FAM-control, purified, and i.v. administered at 7.5 mg/kg of NWs into mice bearing prostate cancer xenografts (PC3 s.c. tumors), or glioblastomas (s.c. U87-MG and orthotopic WT-GBM), both known to overexpress TNC-C and NRP-1 (Lingasamy et al., 2019; Säälik et al., 2019). After 5 h of circulation we observed accumulation of PL3-NWs, but not control NWs, in all 3 tested tumor models (Fig. 17). Confocal analysis demonstrated that the localization of PL3-NWs often overlapped or associated with CD31-positive vascular structures (Fig. 17, arrows), although in some regions the PL3-NWs had also extravasated and accumulated in the tumor parenchyma (Fig. 17, arrowheads). In control organs, PL3-NWs and control NWs showed similar background signal.

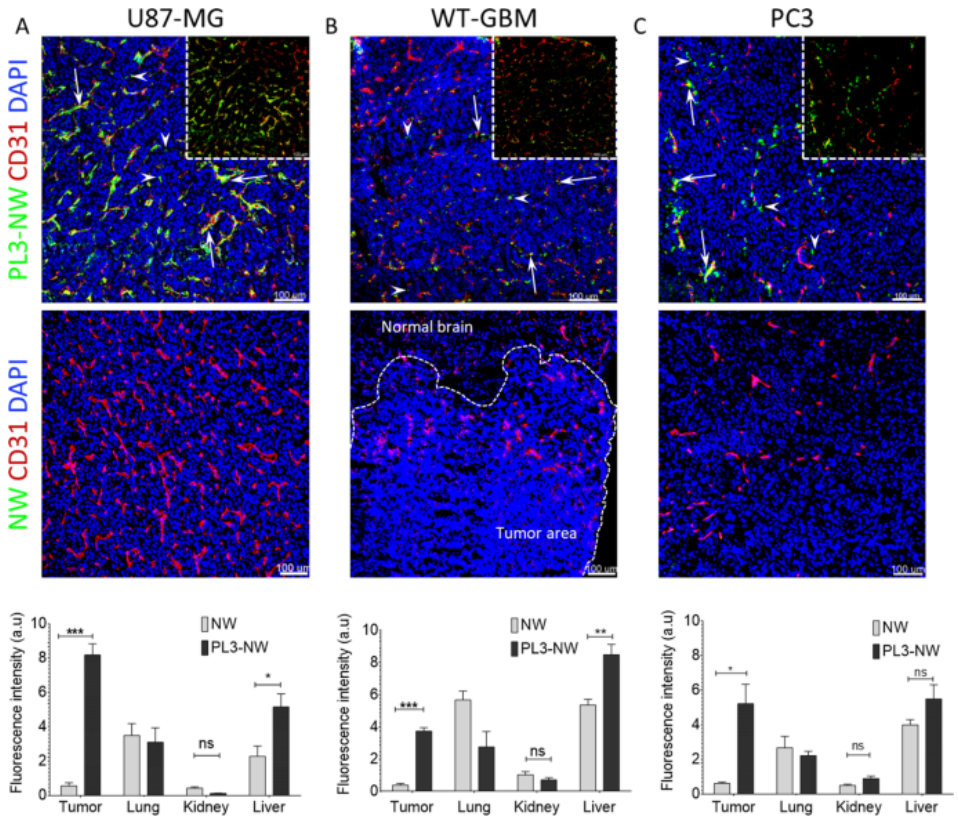

Figure 17. Systemic PL3-NWs accumulate in solid tumors. PL3-NWs or control FAM-NWs were injected i.v. at 7.5 mg/kg into mice bearing s.c. U87-MG glioblastoma (**A**), orthotopic WT-GBM glioblastoma (**B**), or s.c. PC3 prostate carcinoma (**C**). After 5-h circulation, the animals were intracardially perfused, and the tumors and control organs were snap-frozen, sectioned, immunostained with rabbit anti-FAM (green) and rat anti-CD31 (red; blood vessels) antibodies, counterstained with DAPI (blue; nuclei) and imaged. Arrows point to PL3-NWs in the CD31-positive vessels and arrowheads point to extravasated PL3-NWs in the tumor parenchyma. Insets: confocal images without DAPI channel. Bar charts: quantitative analysis of FAM fluorescence (NWs) in images of the tissue sections. Error bars: mean ± SEM (n = 3–6). Scale bars: 100 μm. Unpaired Student's t-test was used to calculate p-values: ns = p-value > 0.05; * = p-value < 0.05; ** = p-value ≤ 0.01; *** = p-value ≤ 0.001.

For biodistribution studies of cryptic PL3 peptides in orthotopic glioblastoma models, orthotopic GBM-bearing mice were i.v. injected with AgNPs coated with either PL3, PL3uCendR or SKLG peptides. After 3-h circulation, the animals were perfused and tissues were collected, sectioned, and used for confocal microscopy imaging. Confocal microscopy imaging of CF555-labeled AgNPs decorated with targeting peptides showed that in all three tested tumor models (U87-MG, WT-GBM, NCH421k) the targeted AgNPs accumulated after 3 h of

circulation near the CD31-positive tumor blood vessels (Fig. 18, arrows), and also appeared to extravasate into the tumor parenchyma (Fig. 18, arrowheads), as was previously seen with PL3-NWs. Although control AgNPs without targeting peptide accumulated less in the tumor tissue, areas with higher accumulation could still be seen, especially for the leaky WT-GBM and U87-MG models, possibly due to the enhanced permeability and retention (EPR) effect (refer to publication II).

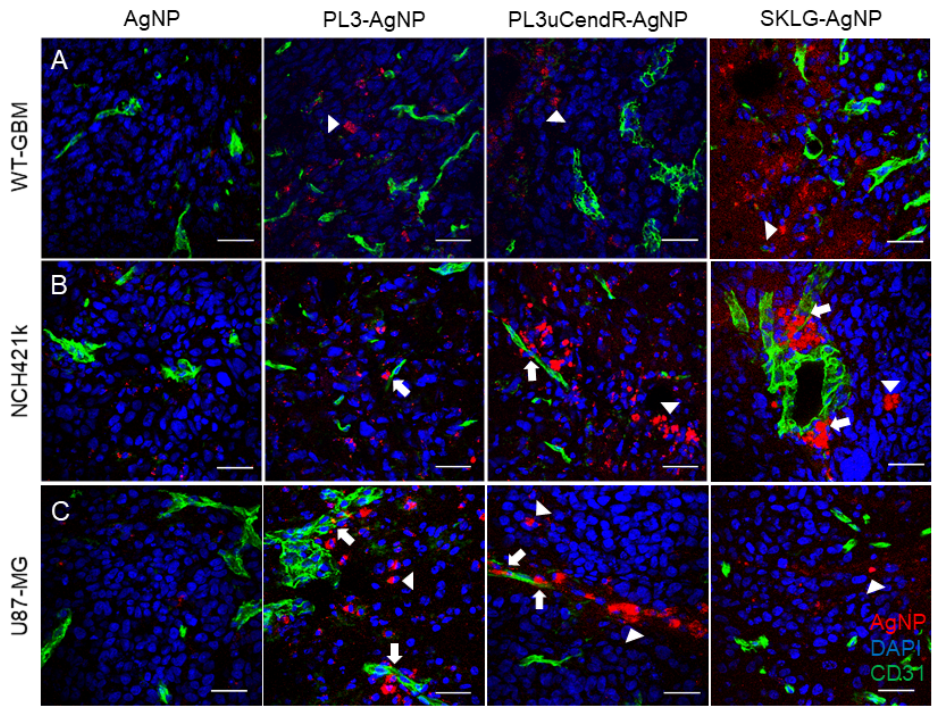

Figure 18. Biodistribution of peptide-AgNPs in (A) WT-GBM, (B) NCH421k and (C) U87-MG GBM models. Orthotopic GBM-bearing nude mice were i.v. injected with CF555-labeled AgNPs (red). After 3 h of circulation, mice were anesthetized, and perfused. Organs were harvested, sectioned, immunostained with anti-CD31 antibody (green; blood vessels), counterstained with DAPI (blue; nuclei). Representative images are presented (n = 3). Arrows point to AgNPs in or near CD31-positive blood vessels, arrowheads to extravasated AgNPs. Scale bar: 100 µm.

5.5.2. PL3-NWs bind human glioblastoma tissue samples *ex vivo*

We also studied the binding of PL3-NWs and control NWs to 6 clinical glioblastoma samples from human patients in an overlay assay. The tissue samples used were classified as glioblastoma, diffuse astrocytoma, anaplastic oligodendroglioma or oligodendroglioma, and stained for the target receptor (TNC-C) expression status. The NWs were overlaid on GBM cryosections, washed, and subjected to confocal imaging. PL3-NWs showed co-localization with TNC-C-positive regions in the tumor perivascular space and parenchyma, whereas control FAM-NWs did not show any binding to the human GBM cryosections (Fig. 19). No binding of PL3-NWs was seen in the case of patient 11 with diffuse astrocytoma due to the lack of detectable TNC-C expression. This assay demonstrates the translational potential of PL3-NPs from animal models to human patients as well as the importance of patient stratification based on target receptor expression for targeted therapy approaches.

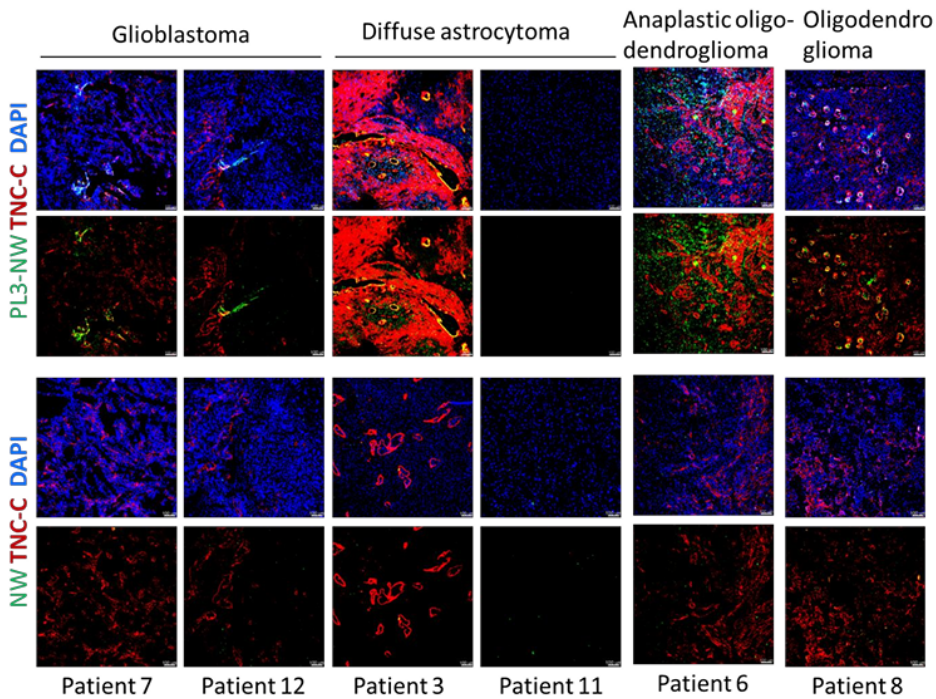

Figure 19. PL3-NWs bind to surgical explants of human glioblastoma tissues. The snap-frozen clinical glioblastoma tissues were sectioned and incubated with FAM-labeled PL3-NWs or non-targeted NWs, stained with anti-FAM (green; NWs) and anti-TNC-C (red) antibodies. Nuclei were counterstained with DAPI (blue). Upper row of each panel shows merge, bottom row is without the DAPI channel for clarity. Scale bars: 100 μm.

5.6. Cryptic PL3 derivatives eliminate accumulation in healthy lung tissue

We employed isotopically barcoded peptide-AgNPs and control AgNPs for internally-controlled laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)-based quantitative mapping studies (Willmore et al., 2016; Toome et al., 2017). Such internally-controlled experimental analysis methods could provide a solution for bypassing the well-known limitation of inter- and intratumoral heterogeneity (Dagogo-Jack & Shaw, 2018). Here, an equimolar mixture of ^{109}AgNP-peptide and biotin-blocked control ^{107}AgNPs was i.v. injected into orthotopic WT-GBM-bearing mice. Following tissue collection and sectioning, LA-ICP-MS was used to quantitatively map the 2D distribution of both targeted and control AgNPs. These individual maps were then combined to provide a ratio of ^{109}Ag/^{107}Ag (i.e., targeted/non-targeted).

In the lungs, PL3-AgNPs with an exposed CendR element exhibited approximately 2.5 times higher accumulation compared to co-injected control AgNPs. Conversely, the cryptic PL3uCendR- and SKLG-AgNPs showed reduced accumulation in healthy pulmonary tissue as the ^{109}Ag/^{107}Ag ratio in the lungs approached 1 (Fig. 20). This further demonstrates that the newly identified cryptic PL3 derivatives exhibit decreased off-target binding to healthy lungs. In the WT-GBM tumor, the accumulation of PL3-, PL3uCendR- and SKLG-AgNPs appeared similar to control AgNPs (Fig. 20). Here, CendR peptide-AgNPs may have triggered tumor transport of co-circulating control AgNPs – CendR peptides are known to trigger the "bystander effect", activating a bulk transport pathway to tumors (Pang et al., 2014), which could potentially limit the applicability of coadministration-based ratiometric biodistribution analyses. WT-GBM tumors are also highly angiogenic and contain dysfunctional and leaky neovessels (Du et al., 2008), a factor that may have contributed to increased nonspecific retention of AgNPs in the tumor tissue.

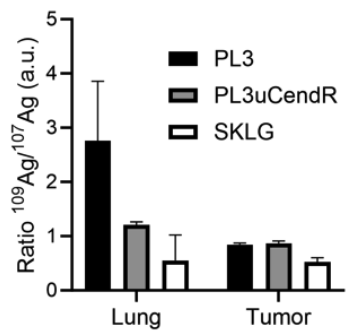

Figure 20. Internally-controlled LA-ICP-MS-based quantitative mapping of isotopically labeled AgNPs. Orthotopic WT-GBM-bearing nude mice were i.v. injected with an equimolar mixture of peptide-^{109}AgNPs and biotin-blocked control ^{107}AgNPs. After 3 h of circulation, mice were anesthetized, and perfused. Organs were harvested, sectioned, and subjected to laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) analysis. Error bars show standard deviation (SD) (n = 3); a.u. = arbitrary units.

5.7. Monomeric PL3 derivative targets tumor and reduces off-target accumulation in GBM mice

Whereas polyvalent targeting systems, such as phages, NWs and AgNPs used in previous sections, exploit multivalent interactions to enhance specificity and binding efficiency, monovalent targeting systems rely on single ligand-receptor interactions for targeted drug delivery (Montet et al., 2006). The choice between these two approaches depends on affinity of the targeting ligand and the pharmacokinetic properties of the therapeutic agent. To probe the potential for single ligand delivery, we studied the *in vivo* biodistribution of monomeric FAM-labeled SKLG, PL3, and scrambled PL3 control (scrPL3, sequence RAGRGRLV) peptides in WT-GBM tumor-bearing mice. The mice were dosed with FAM-labeled peptides followed by perfusion, tissue collection, sectioning, and detection of the peptides using immunohistochemistry with anti-FAM antibodies. Hematoxylin and eosin (H&E) staining of parallel cryosections was used to locate and outline the orthotopic tumors in the brains of mice (Fig. 21A) and pulmonary tissue structures (Fig. 21C).

In the tested tumor tissues, somewhat ambiguous preferential accumulation of PL3 and SKLG compared to scrambled PL3 (scrPL3) could be seen (Fig. 21B). Although the employed method of detection is qualitative rather than quantitative, it seems that SKLG exhibits a lower signal compared to PL3, which may suggest that the SKLG peptide is better suited for multivalent targeting approaches. In the healthy pulmonary tissue, a robust and consistent accumulation of the PL3 peptide was evident, whereas cryptic SKLG and scrambled PL3 control peptides exhibited only a background signal comparable to that of the secondary antibody alone (Fig. 21D).

These findings align with the phage- and nanoparticle-based data previously presented, and underscore the importance of minimizing off-target binding of non-cryptic CendR-peptides, particularly in the lungs. Based on these preliminary results, the SKLG peptide may be better for multivalent targeting, whereas PL3 may additionally be suited for monomeric applications where pulmonary accumulation is not a concern.

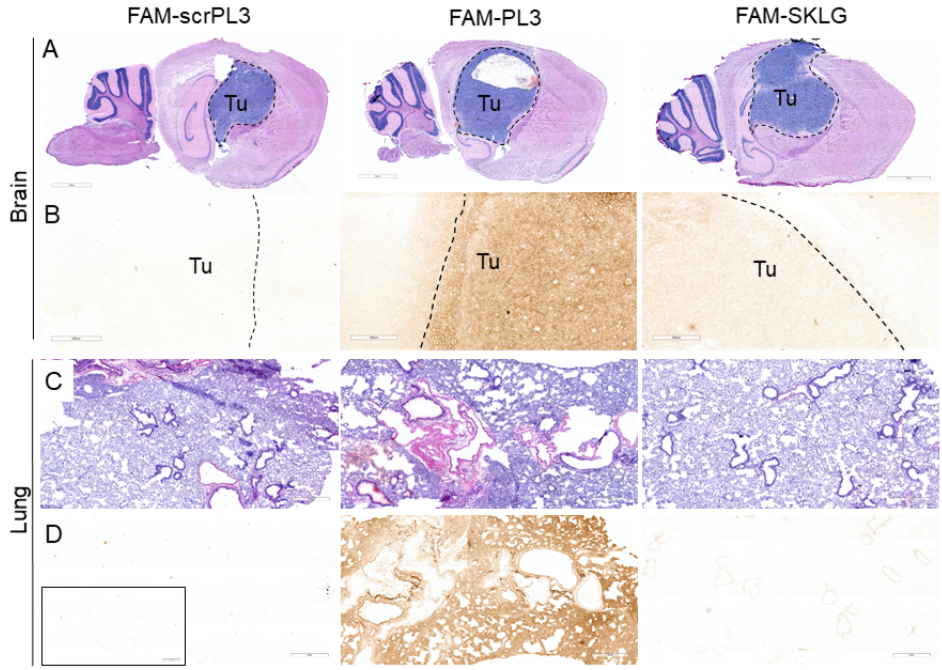

Figure 21. Biodistribution of monomeric FAM-labeled peptides in WT-GBM mice. Orthotopic GBM-bearing nude mice were i.v. injected with FAM-labeled scrambled PL3 control (scrPL3), PL3 or SKLG peptides. After 1 h of circulation, mice were anesthetized, and perfused. Organs were harvested, sectioned, and parallel sections were immuno-stained with hematoxylin & eosin (H&E) staining or anti-FAM DAB staining. (**A**) H&E of WT-GBM tumor bearing brain tissue sections. (**B**) DAB staining of WT-GBM tumor region in the brain. (**C**) H&E staining of lung tissue sections. (**D**) DAB staining of lung tissue sections. Representative images are presented (n = 3). Dashed lines outline tumor; Tu = tumor. Inset in (D) shows secondary antibody control for DAB staining. Scale bars: 3 mm for (A), 500 µm for (B), 1 mm for (C, D).

5.8. PL3-targeted proapoptotic NWs inhibit tumor growth in mice

Lastly, we tested the effect of PL3-functionalization on the anticancer efficacy of nanoparticles loaded with the proapoptotic peptide $_D$(KLAKLAK)$_2$ (Arap et al., 2002). For these studies we used the s.c. U87-MG tumor model in order to monitor tumor size, rather than survival, as the endpoint. Starting on day 36 after tumor induction (when the tumors had reached 100 mm^3), the tumor mice were treated with i.v. injected PL3-$_D$(KLAKLAK)$_2$-NWs, $_D$(KLAKLAK)$_2$-NWs, PL3-NWs or PBS every other day for a total of 10 injections, and the tumor volume as well as survival of the mice was recorded (Fig. 22A). Over the treatment period, the volume of tumors in PBS, PL3-NW, and $_D$(KLAKLAK)$_2$–NW-treated mice increased rapidly while tumor growth in the $_D$(KLAKLAK)$_2$-PL3-treated

group was significantly delayed (Fig. 22B). The median survival of PBS, $_D$(KLAKLAK)$_2$-NW, PL3-NW, and PL3-$_D$(KLAKLAK)$_2$-NW groups was 55, 58, 54, and 70 days, respectively. In the $_D$(KLAKLAK)$_2$-PL3 group, 50% of the mice demonstrated prolonged survival compared to the animals in the other treatment groups (Fig. 22C). Immunostaining of post-treatment tumor tissue with anti-CD31 antibody to visualize tumor vasculature showed that PL3-$_D$(KLAKLAK)$_2$-NW-treated tumors had a significant reduction in CD31-positive area, and the apoptosis marker cleaved Caspase-3 was significantly increased compared to controls, highlighting the successful delivery of the proapoptotic cargo (refer to publication I).

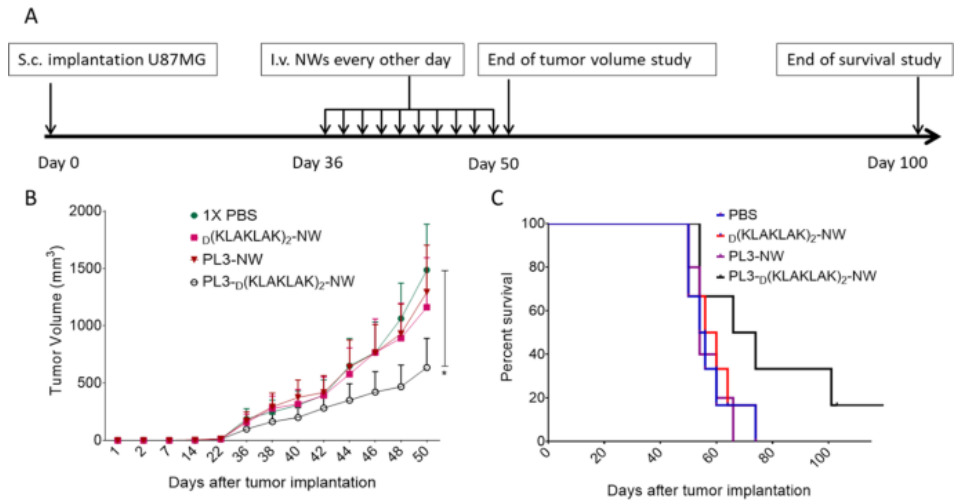

Figure 22. Experimental therapy with PL3-$_D$(KLAKLAK)$_2$-NWs suppresses glioblastoma growth. (**A**) Experimental design of the tumor treatment study. Treatment of mice bearing s.c. U87-MG tumors with systemic injections of NWs were initiated on day 36 when the tumors had reached 100 mm^3; groups of 6 randomized mice were treated every other day for a total of 10 injections. The body weight, survival, behavior, and tumor volume were monitored every two days until the end of the treatment. (**B**) Tumor size dynamics of mice treated with 5 mg/kg of $_D$(KLAKLAK)$_2$-NWs, PL3-NWs and PL3-$_D$(KLAKLAK)$_2$-NWs, or control PBS (n = 6 mice/group). Tumor volume was measured with a digital caliper and calculated using the formula: volume (V) (mm^3) = [length × (width × 2)]/2. Data were analyzed with two-way ANOVA and log-rank test. Error bars: mean ± SEM; * = p-value < 0.05. (**C**) Kaplan-Meier survival analysis. At the endpoint of the study the mice were sacrificed by perfusion, and organs and tumors were collected. Tumor volume, Kaplan–Meier survival curve and body weight curve were calculated for each group; p-values < 0.05 were considered significant.

6. DISCUSSION

6.1. Significance

Glioblastoma is the most common malignant primary brain tumor with poor prognosis and survival rate as well as unique challenges compared to other solid tumors (Tan et al., 2020). Standard of care for glioblastoma still consists of tumor resection followed by radiotherapy and concomitant temozolomide, although a lot of effort is being put into development of novel treatment strategies (Rong et al., 2022). Already in 2022, targeted therapies accounted for 55% of the 136 ongoing clinical trials, followed by cytotoxic chemotherapy (38%) and immunotherapy (34%) (Bagley et al., 2022). In an attempt to improve the current fate of glioblastoma patients, we identified a novel tumor targeting peptide PL3 that was validated across several glioblastoma mouse models as well as patient samples. To further mitigate off-site binding of PL3-targeted nanoparticles, we identified cryptic variants of the PL3 peptide that are proteolytically activated after reaching the tumor. These targeting peptides were mounted on silver and iron oxide nanocarriers along with cytotoxic cargo to affect targeted anticancer activities in model systems of cancer.

6.2. Main findings

The studies presented in this thesis advanced and characterized two metallic nanoparticle platforms as preclinical cancer research tools and drug delivery vehicles: silver nanoparticles (AgNPs) and iron oxide nanoworms (NWs). We demonstrated that these nanoparticles can be functionalized with tumor targeting peptides and used as vehicles for targeted delivery of cytotoxic and proapoptotic cargo. Due to their beneficial intrinsic properties, the AgNP and NW platforms also enhance and expand the choice of analysis methods for *in vitro* and *in vivo* preclinical research of targeting moieties. Furthermore, we identified a tumor targeting peptide PL3 (AGRGRLVR) with bispecific affinity for tenascin-C and neuropilin-1, and demonstrated its efficacy for tumor targeting and therapy. To mitigate off-site binding to healthy tissues, mainly the lungs, of the PL3 peptide with an exposed CendR motif, we set out to find cryptic version of the PL3 peptide with a modified cell-free peptide-phage display method. As a result, we identified two candidate PL3 variants, SKLG (AGRGRLVRSKLG) and PL3uCendR (AGRGRLVRSAGGSVA), with a urokinase-dependent trigger mechanism to activate neuropilin-1 binding upon reaching the tumor, which eliminated off-site binding to healthy lung tissue in mice.

6.2.1. Identifying a bispecific TNC-C and NRP-1 binding tumor targeting PL3 peptide for experimental cancer therapy

We identified a bispecific tumor targeting peptide PL3 (AGRGRLVR) that is able to bind to the C-domain of the extracellular matrix protein tenascin-C (TNC-C) as well as the cell and the tissue penetration receptor neuropilin-1 (NRP-1). The CendR motif of PL3 (RLVR) binds to NRP-1 and activates a transcytosis pathway that delivers the peptide along with cargo into the tumor (Pang et al., 2014). In cell-free protein binding studies, we demonstrated that the PL3 peptide binds specifically to TNC-C and NRP-1. When conjugated to nanoparticles, the PL3 peptide was able to induce specific NRP-1-dependent internalization in NRP-1-positive cancer cells.

We used xenotransplanted tumor models in mice to show that systemically administered NWs and AgNPs equipped with the PL3 peptide improved tumor targeting and accumulation in tumors. In a proof-of-concept experimental treatment study, PL3-NWs carrying the proapoptotic peptide $_{D}[KLAKLAK]_2$ significantly reduced tumor volume, and prolonged the survival rate of mice. The PL3 peptide targeted NWs, but not control nontargeted NWs, also bound to TNC-C- and NRP-1-positive regions of cryosections of human glioblastoma samples, which suggests translational relevance. Overall, the studies indicate that PL3 targeting may have applications for delivering diagnostic and therapeutic payloads to solid tumors.

6.2.2. Identifying and validating a proteolytically actuated cryptic version of PL3

We demonstrated that the cell-free peptide-phage display method can be used to identify novel proteolytically activated tumor penetrating peptides. Two PL3 peptide derivatives, PL3uCendR (AGRGRLVRSAGGSVA) and SKLG (AGRGRLVRSKLG), that showed uPA-dependent activation of binding to cell-free and cell surface-expressed NRP-1 were identified in this study. *In vitro* studies with AgNPs showed that these cryptic peptides activate NRP-1-mediated internalization pathways in cancer cells only after being processed by proteases that cleave after arginine, such as uPA and trypsin.

In vivo, both monomeric and nanoparticle-tethered uPA-actuated PL3-derivative peptides accumulated in glioblastoma mouse models that overexpressed TNC-C, NRP-1 and uPA. Furthermore, NPs equipped with PL3uCendR and SKLG exhibited a robust reduction of pulmonary accumulation compared to the parent PL3 peptide. Taken together, these results show that the cell-free peptide-phage display method can be used to modify certain tumor targeting peptides with a proteolytic trigger, and thus eliminate off-site binding to healthy tissues while preserving tumor targeting properties.

6.2.3. CendR peptide-targeted AgNPs as drug delivery vehicles

We developed a prototypic CendR peptide-targeted AgNP nanocarrier system that potentiates the cytotoxic activity of a potent anticancer drug, monomethyl auristatin E (MMAE). It was shown that conjugating MMAE to the peptide-AgNPs does not affect their ability to bind the target receptor NRP-1 and trigger the subsequent transcytosis mechanism. As a secondary tumor-specific trigger, we used a peptidic Val-Cit linker module to conjugate MMAE to the AgNPs, which releases cytotoxic cargo within cancer cells after cleavage by the lysosomal cathepsin B overexpressed in several types of cancer.

In *in vitro* experiments, we determined the IC_{50} value and therapeutic window for peptide-targeted MMAE-AgNPs. Cytotoxicity experiments with a mixed culture of NRP-1-positive target and NRP-1-negative non-target cells showed that therapeutic peptide-AgNPs can be used to improve targeting selectivity in complex systems. Furthermore, treatment with a biocompatible etching solution enabled to control the colloidal status of the extracellular AgNPs in order to improve the targeting selectivity and regulate the therapeutic effect of MMAE-loaded peptide-AgNPs.

6.3. Future directions

In the context of targeted cancer therapy, patient stratification based on biomarker expression is a paramount necessity. As shown in this thesis, expression of target receptors and enzymes responsible for prodrug or secondary targeting moiety activation can make the difference between a successful and unsuccessful therapeutic or diagnostic approach. Here, we based our choice of cancer cell lines and model systems on the biomarker expression profiles, and excluded non-expressing cell lines after pilot experiments as no homing or very little of it was seen. We encourage all future studies to take into careful consideration the biomarker profiles of chosen experimental systems and models as well as patient samples. By extension, this principle should also be applied more rigorously in clinical trials and clinical applications of targeted cancer therapies.

We have demonstrated that targeting tumor ECM proteins as the primary target with peptides is a viable strategy for drug delivery, and especially useful when combined with tumor penetrating modalities, such as the activation of the CendR pathway. For discovering such peptides, cell-free peptide-phage display method is an excellent option with the added benefit of knowing the target receptor due to the design of the experiment, which has been a downside for the more common *in vivo* phage display method. In regard to identifying cryptic versions of targeted peptides, we envision the application of the constrained peptide-phage display method not only for modifying existing dual-binding homing peptides plagued by off-target accumulation but also discovering fully new target receptor binding protease-activated peptides, where the exact cleavage site may be initially unknown. The overexpression of various proteases in

gliomas or other types of solid tumors enables the application of such protease-sensitive peptide targeting moieties for targeted delivery of cargo molecules and nanosystems.

For future developments of this thesis, we propose the use of cryptic PL3 peptides in combination with MMAE-AgNPs for *in vivo* treatment studies on glioblastoma mice. The more potent MMAE compared to the proapoptotic $_{D}$[KLAKLAK]$_{2}$ peptide could induce a more striking antitumoral effect, and using cryptic versions of PL3 should mitigate potential side effects in healthy tissues. It should be noted that the Val-Cit motif within the OSu-Glu-Val-Cit-PAB linker used here for conjugating MMAE is susceptible to cleavage by carboxylesterase 1C in mouse and rat plasma, so alternatives have to be considered for *in vivo* experiments in these species (Balamkundu & Liu, 2023). The potential toxicity of AgNPs should also be studied in more depth to ensure sufficient biocompatibility, wherein the linking molecule NeutrAvidin – a modified version of streptavidin – applied in the design of the AgNPs used here has raised some concerns about immunogenic responses (Jain et al., 2017). Although convenient and robust, NeutrAvidin can easily be substituted for *in vivo* treatment studies in favor of some other type of linker chemistry, such as thiol-maleimide click chemistry. We hypothesize that the potential off-site toxicity of AgNPs *in vivo* could be even more reduced with the use of the biocompatible etching solution, which breaks down and complexes the circulating particles that did not reach target tissue, thereby introducing different routes of elimination from the body based on size and dissipating the toxicity burden for the liver associated with the clearance of NPs.

7. CONCLUSIONS & SUMMARY

1. Tumor penetrating peptide PL3 with dual affinity towards the extracellular matrix protein TNC-C and the tissue penetration receptor NRP-1 can be used to functionalize different nanocarriers for targeting glioblastoma and prostate cancer cells *in vitro* and *in vivo*.

2. A modified peptide-phage display method was developed to identify cryptic versions of the PL3 peptide that reduce off-target accumulation in healthy tissues, mainly pulmonary tissue, while incorporating a proteolytic uPA-dependent activation mechanism for restoring the parent PL3 peptide configuration.

3. Modified iron oxide nanoworm (NW) and silver nanoparticle (AgNP) platforms are well-suited for preclinical research of peptide-targeted nanodrugs, acting as carriers with useful multimodal intrinsic properties.

4. Targeted NWs and AgNPs can be equipped with cytotoxic and proapoptotic cargo to selectively eliminate target cancer cells *in vitro* as well as inhibit tumor growth *in vivo*.

Nanocarriers as tunable drug delivery vehicles that can be easily targeted with affinity ligands continue to be of great interested in nanomedicine. Compared to drug conjugates, NPs can often carry more anticancer drugs which reduces the concentrations needed for comparable antitumoral effects and decreases side effects. NPs can be decorated with more targeting ligands, increasing avidity through multivalency, and this is especially useful in the case of targeting peptides. Metallic NPs with a wide range of diagnostic and potentially therapeutic characteristics, such as AgNPs and NWs, are an attractive option for further research, although a more systematic inquiry of the possible toxicities needs to be carried out if the aim is to reach the clinics. Nevertheless, they still offer a great model platform for preclinical research to validate targeting ligands and drug delivery.

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9. SUMMARY IN ESTONIAN

Tarkade nanoosakeste väljatöötamine eksperimentaalseks vähiraviks

Glioblastoom on kõige sagedasem pahaloomuline ajukasvaja, mis on ebasoodsa prognoosi ja kõrge suremusmääraga. Glioblastoomi standardravi koosneb peamiselt kasvaja kirurgilisest eemaldamisest, millele järgneb kiiritusravi ja samaaegne keemiaravi temosolomiidiga. Üha suuremat tähelepanu pööratakse uudsete täppis-suunatud nanoosakestel põhinevate ravimeetodite väljatöötamisele, et parandada vähiravi tõhusust ja vähendada kõrvalmõjusid. Nanotehnoloogilised ravimid on osutunud tõhusaks mitmete vähitüüpide puhul, sh glioblastoomi prekliinilistes ja kliinilistes uuringutes. Sellest hoolimata ei ole need veel suurel määral patsientideni jõudnud. Üks viis suunatud raviks on kasutada ravimiga konjugeeritud ja kullerpeptiidide abil afiinsus-suunatud nanoosakesi, et suurendada ravimi kontsentratsiooni kasvajakoes ja vähendada kõrvalmõjusid tervetes kudedes. Teatud tüüpi kasvajaspetsiifilised peptiidid, CendR-peptiidid, aktiveerivad kasvajarakkudeni jõudes transportmehhanismi, mis viib peptiidid koos ravimimolekulide ja nanoosakestega läbi rakkude sügavale kasvajakoe parenhüümi.

Käesolevas doktoritöös esitatud uuringutes kasutati prekliiniliste vähktõve uuringute tööriistade ja ravimikandjatena kahte nanoosakeste platvormi: hõbeda nanoosakesi (AgNP, ingl *silver nanoparticles*) ja raudoksiid-nanousse (NW, ingl *nanoworms*). Demonstreerisime, et neid saab funktsionaliseerida kasvajaspetsiifiliste kullerpeptiididega ning kasutada tsütotoksiliste ja proapoptootiliste ravimite suunatud kohaletoimetamiseks. AgNP ja NW platvormid võimendavad ja laiendavad *in vitro* ja *in vivo* analüüsimeetodeid, mis hõlbustavad suunamisligandide uurimist ja valideerimist. Lisaks nanokandjate väljatöötamisele ja iseloomustamisele tuvastasime kasvajaspetsiifilise kullerpeptiidi PL3 (AGRGRLVR), mis seondub valkudega tenastsiin-C ja neuropiliin-1. PL3-peptiid suurendas nanoosakeste akumuleerumist kasvajakoes ja tõhustas eksperimentaalset vähiravi hiiredmudelites. PL3-peptiid omab vaba CendR-motiivi, mistõttu kaasneb sellega mõningane akumulatsioon tervetes kudedes, peamiselt kopsudes. Selle probleemi lahendamiseks kasutasime modifitseeritud faagidisplei meetodit, et tuvastada krüptilised proteolüütiliselt aktiveeritavad PL3 derivaatpeptiidid: SKLG (AGRGRLVRSKLG) ja PL3uCendR (AGRGRLVRSAGGSVA). Krüptiliste derivaatide lõhustamisel kasvajates üleekspresseeritud urokinaasiga (uPA) aktiveerus neuropiliin-1-st sõltuv penetratsioonimehhanism, kuid akumulatsioon terves kopsukoes vähenes olulisel määral. Töö tulemuste põhjal saab edasi arendada tõhusamaid suunatud vähiravimeetodeid glioblastoomile, vähendades ravi kõrvalmõjusid.

Uurimistöö eesmärgid

1. Tuvastada kasvaja rakuvälisele maatriksile (ECM, ingl *extracellular matrix*) suunatud kullerpeptiidid, mida saab rakendada glioblastoomi diagnoomisel ja ravimisel.
2. Välja töötada faagidisplei meetod neuropiliin-1-le suunatud kullerpeptiidide krüptiliste derivaatide leidmiseks, mis akumuleeruksid vähemal määral terves kopsukoes.
3. Optimeerida nanokandjate platvorme tsütotoksiliste ravimolekulide toimetamiseks vähirakkudeni *in vitro* ja *in vivo*.
4. Valideerida ECM-ile suunatud kullerpeptiididega funktsionaliseeritud nanokandjad glioblastoomi hiiremudelites ja kliinilistes proovides.

Materjalid ja metoodika

Doktoritöös sünteesiti, funktsionaliseeriti ja iseloomustati kahte nanoosakeste platvormi: hõbeda nanoosakesed (AgNP) ja raudoksiid-nanoussid (NW). Nanoosakesed funktsionaliseeriti kullerpeptiididega ja kuvamiseks fluorofooride või eksperimentaalteraapiaks ravimolekulidega (monometüülauristatiin E või proapoptootiline peptiid $_{D}$(KLAKLAK)$_2$). Tsirkulatsiooniaja pikendamiseks ja ühenduslülina kasutati mõlema platvormi puhul polüetüleenglükooliga (PEG) katmist. Uudsete kasvajaspetsiifiliste kullerpeptiidide leidmiseks kasutati modifitseeritud rakuvaba T7-faagidisplei meetodit valkude tenastsiin-C (TNC) ja neuropiliin-1 (NRP-1) vastu, mis kinnitati magnetiliste Ni-NTA agarooskerakeste külge. Krüptiliste PL3 derivaatide leidmisel eeltöödeldi peptiid-faage valikuliselt urokinaasiga (uPA), et leida konditsionaalselt NRP-1-ga seonduvad peptiid-faagid. Enim esindatud kandidaatidega viidi läbi *in vivo playoff* katse kasvajaga hiirtes. Homogeniseeritud sihtkudedes faagide genoomi sekveneerimise tulemusena saadud andmeid analüüsiti, et leida suurima rikastumisega peptiid-faagid. Välja valitud kullerpeptiidide sünteetilisi variante kasutati nanoosakeste suunamiseks. Teostati *in vitro* katsed erinevate vähirakuliinidega (peamiselt glioblastoomi ja eesnäärmevähi rakuliinid), määramaks sihtretseptorist sõltuv seondumine vähirakkudega ja nendesse sisenemine. Lisaks viidi läbi *in vitro* tsütotoksilisuse katsed ravimiga varustatud AgNP-dega. Ortotoopsete ja subkutaansete kasvajamudelite abil analüüsiti kullerpeptiidiga suunatud nanoosakeste biodistributsiooni hiirtes. PL3-peptiidiga suunatud ja proapoptootilise peptiidiga $_{D}$(KLAKLAK)$_2$ varustatud NW-dega tehti eksperimentaalne raviuuring glioblastoomi hiiremudelites. Lisaks testiti PL3-peptiidi seondumist glioblastoomi patsientidelt saadud kasvajaproovidega *ex vivo* ning uuriti monomeersete krüptiliste PL3 derivaatide SKLG ja PL3uCendR biodistributsiooni glioblastoomiga hiirtes. Kõik loomkatsed kiideti heaks Eesti Regionaal- ja Põllumajandusministeeriumi loomkatsete komitee poolt lubade nr 42, 48 ja 159 alusel. Tartu Ülikooli Kliinikumist saadud patsientide kasvajaproovide hankimise ja kasutamise protokollid kiitis heaks Tartu Ülikooli eetikakomitee loa nr 245/T27 alusel.

Uurimistöö peamised tulemused ja järeldused

1. Kasvajaspetsiifilist kullerpeptiidi PL3, mis seondub nii rakuvälise maatriksi valguga tenastsiin-C kui ka koepenetratsiooni aktiveeriva valguga NRP-1, saab kasutada erinevate nanokandjate funktsionaliseerimiseks, et viia need spetsiifiliselt glioblastoomi ja eesnäärmevähirakkudesse *in vitro* ja *in vivo*.
2. PL3-peptiidi krüptilised derivaadid vähendavad akumulatsiooni tervetes kudedes, peamiselt kopsukoes, omades seejuures kasvajates üleekspresseeritud urokinaasist sõltuvat aktiveerimismehhanismi PL3-lähtepeptiidi konfiguratsiooni taastamiseks.
3. Modifitseeritud hõbeda nanoosakeste (AgNP) ja raudoksiid-nanousside (NW) platvormid sobivad peptiidsuunatud nanoravimite prekliiniliseks uurimiseks, toimides kasulike täiendavate omadustega kandjatena.
4. Suunatud AgNP ja NW platvorme saab varustada tsütotoksiliste ja proapoptootiliste ravimolekulidega, et selektiivselt kõrvaldada sihtmärgiks olevaid vähirakke *in vitro* ning inhibeerida kasvajate proliferatsiooni *in vivo*.

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PUBLICATIONS

CURRICULUM VITAE

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09.2017–…	PhD studies (medicine) – University of Tartu, Faculty of Medicine
09.2014–06.2016	MSc (gene technology) – University of Tartu, Faculty of Science and Technology
09.2011–06.2014	BSc (chemistry) – University of Tartu, Institute of Chemistry
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Professional employment:

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09.2019–12.2020	Junior research fellow – University of Tartu, Institute of Biomedicine and Translational Medicine
03.2018–09.2018	Visiting Graduate Student – Sanford Burnham Prebys Medical Discovery Institute (USA)
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List of publications:

1. Tobi, A., Haugas, M., Rabi, K., Sethi, J., Põšnograjeva, K., Paiste, P., ... & Teesalu, T. (2024). Protease-activated CendR peptides targeting tenascin-C: mitigating off-target tissue accumulation. Drug Delivery and Translational Research, 1–17.

2. Simonetti, B., Daly, J. L., Simón-Gracia, L., Klein, K., Weeratunga, S., Antón-Plágaro, C., ... & Cullen, P. J. (2022). ESCPE-1 mediates retrograde endosomal sorting of the SARS-CoV-2 host factor Neuropilin-1. Proceedings of the National Academy of Sciences, 119(25), e2201980119.
3. Simón-Gracia, L., Kiisholts, K., Petrikaitė, V., Tobi, A., Saare, M., Lingasamy, P., ... & Teesalu, T. (2021). Homing Peptide-Based Targeting of Tenascin-C and Fibronectin in Endometriosis. Nanomaterials, 11(12), 3257.
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10. Lingasamy, P., Tobi, A., Haugas, M., Hunt, H., Paiste, P., Asser, T., ... & Teesalu, T. (2019). Bispecific tenascin-C and fibronectin targeted peptide for solid tumor delivery. Biomaterials, 219, 119373.
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1. Tobi, A., Haugas, M., Rabi, K., Sethi, J., Põšnograjeva, K., Paiste, P., ... & Teesalu, T. (2024). Protease-activated CendR peptides targeting tenascin-C: mitigating off-target tissue accumulation. Drug Delivery and Translational Research, 1–17.
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