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Homing peptides for targeting of
brain diseases

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Cancer Biology Research Group, Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia.

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Supervisor:	Professor Tambet Teesalu Lab of Cancer Biology, Institute of Biomedicine and Translational Medicine, Faculty of Medicine, University of Tartu
Reviewers:	Professor Margus Pooga, Institute of Technology, Faculty of Science and Technology, University of Tartu
	Kalle Kilk, MD, PhD, Department of Biochemistry, Institute of Biomedicine and Translational Medicine, University of Tartu
Opponent:	Cornelis F.M. Sier, PhD, Department of Surgery, Leiden University Medical Centre, Leiden, Holland
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I   **Toome, K**; Willmore, AA.; Paiste, P.; Tobi, A.; Sugahara, KN.; Ruoslahti, E.; Braun, GB.; Teesalu, T.: **Ratiometric in vivo auditioning of targeted silver nanoparticles**. *Nanoscale*. 2017, 9(28)

II   Willmore, AA.; Simón-Gracia, L.; **Toome, K.**; Paiste, P.; Kotamraju, VR.; Mölder, T.; Sugahara; KN.; Ruoslahti, E.; Braun, GB.; Teesalu, T.: **Targeted silver nanoparticles for ratiometric cell phenotyping**. *Nanoscale*. 2016, 8(17)

III Säälik, P; Lingasamy, P.; **Toome, K**.; Mastandrea, I.; Rousso-Noori, L.; Tobi, A.; Simón-Gracia, L.; Hunt, H.; Paiste, P.; Kotamraju, VR.; Bergers, G.; Asser, T.; Rätsep, T.; Ruoslahti, E.; Bjerkvig, R.; Friedmann-Morvinski, D.; Teesalu, T.: **Peptide-guided nanoparticles for glioblastoma targeting.** *J Control Release.* 2019, 28(308)

IV   Mann, AP.; Scodeller, P.; Hussain, S.; Braun, GB.; Mölder T.; **Toome, K.**; Ambasudhan, R.; Teesalu, T.; Lipton, SA.; Ruoslahti, E.: **Identification of a peptide recognizing cerebrovascular changes in mouse models of Alzheimer's disease**. *Nat Commun.* 2017, 8(1)

My contribution to the articles referred to in this study is following:

Ref I
1. Designed all the experiments and developed the methodology by having discussions with T. Teesalu and A.A. Willmore.
2. Experimental part:
   - Ag nanoparticle coupling with linkers and relevant peptides
   - Performed all the animal experiments (including optimization experiments): injection of nanoparticles (including prior coupling of dye and peptides), termination of mice, extraction of relevant organs
   - Performed cryosectioning of tissues, antibody stainings and other necessary preparations for confocal microscopy, performed the confocal imaging of the tissue sections
   - Performed silver enhancement to visualize silver in the tissue sections, performed dark-field microscopy
   - Quantified fluorescent confocal images and dark-field microphotographs with proper software
   - Injected animals for ICP-MS/LA-ICP-MS analysis and conducted necessary tissue preparations
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 A.A. Willmore about the data and next steps.
4. Wrote the manuscript.

Ref II
1. Participated in the design of study and development of methodology by attending regular meetings with T. Teesalu, A. A. Willmore and L. Simón-Gracia.
2. Experimental part:
   a. Coupling of dye and peptides to AgNPs
   b. Cell culturing, seeding, AgNP binding experiments, antibody stainings and other necessary preparations for confocal microscopy, performed the confocal imaging
   c. Prepared samples for in vivo ICP-MS with 3 types of isotopic particles (injection of peptide, termination of mice and extraction of relevant organs followed by preparing samples for ICP-MS) – results were not included into the publication
3. Data analysis and interpretation: analyzed and interpreted the results from *in vivo* ICP-MS
4. Participated in writing of the manuscript.

Ref III
1. Participated in the design of study and development of methodology by attending meetings with T.Teesalu and P.Säälik
2. Experimental part:
   a. Established glioblastoma models in the Laboratory of Cancer Biology
   b. Performed phage display screens on GBM tumor bearing mice to identify new GBM homing peptides
   c. Tested multiple homing peptides in different GBM models to select the best suited one for subsequent experiments and treatment study – most of the results from 2a–2c were not included in this publication
   d. Induced GBM tumors for the experiments and experimental study (cell culturing for tumor induction in mice, monitoring of tumor growth in mice)
   e. Coupling of dye and peptides to AgNPs
   f. Preparing tissues for ICP-MS experiments
3. Participated in writing of the manuscript.

Ref IV
1. Experimental part:
   • Performed all the GBM experiments (including optimization experiments with different GBM models): Cell culturing for tumor induction in mice, monitoring of tumor growth in mice, injection of peptide, termination of mice and extraction of relevant organs followed by cryosectioning of the tissues, staining tissues with antibodies, and confocal microscopy.
2. Data analysis and interpretation: collected and interpreted confocal microscopy image from *in vivo* GBM homing experiments
3. Wrote the methodology and results part for GBM homing experiments and reviewed manuscript drafts.

ABBREVIATIONS

2D	two-dimensional
ABX	Abraxane
AD	Alzheimer's disease
Ag107	silver isotope 107
Ag109	silver isotope 109
AgNP	silver nanoparticle
APP	amyloid precursor protein
ATP	adenosine triphosphate
Aβ	β-amyloid peptide
B	bronchiole
B/biot	biotin
B-AgNP	silver nanoparticle blocked with biotin
BBB	blood-brain barrier
BBTB	blood-brain tumor barrier
Br	brain
BSA	bovine serum albumin
BSCB	blood-spinal cord barrier
CF555	succinimidyl ester, fluorescent dye
CendR	C-end Rule
CNS	central nervous system
CSF	cerebrospinal fluid
CTGF	connective tissue growth factor
DAPI	4`6-diamidino-2-phenylindole fluorescent dye
DMEM	Dulbecco's Modified Eagle Medium
DLS	Dynamic Light Scattering
EC	endothelial cell
EGF	epidermal growth factor
FAM	5(6)-carboxyfluorescein fluorescent dye
FDA	The Food and Drug Administration of the United States
FGF	fibroblast growth factor
FT	fibrillary tract
GBM	glioblastoma multiforme
GEMM	genetically engineered mouse model
GFAP	glial fibrillary acidic protein
GL	granular layer
GLUT1	glucose transporter 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Performance Liquid Chromatography
ICP-MS	inductively coupled plasma mass spectroscopy
IMAC	immobilized metal affinity chromatography
IONW	iron oxide nanoworm
IV	intravenous

K-AgNP	silver nanoparticle functionalized with SGKRK
LA-ICP-MS	laser ablation inductively coupled plasma mass spectroscopy
LB	lysogeny broth
LinTT1	linearTT1, p32-directed tumor penetrating peptide, sequence [AKRGARSTA]
LRP	low-density lipoprotein receptor-related protein
mAbs	monoclonal antibodies
MEM	Minimum Essential Medium
MeOH	methanol
ML	molecular layer
MR	magnetic resonance
NA	neutravidin
NHS	N-hydroxysuccinimide
NTA	nitrilotriacetic acid
NP	nanoparticle
NP40	nonyl phenoxypolyethoxylethanol
NRP-1	neuropilin-1
NW	nanoworm
OBOC	one-bead-one-compound
OCT	Optimal Cutting Temperature compound
p32	protein 32
PAGE	polyacrylamide gel electrophoresis
PD	Parkinson's disease
PDGC	patient-derived glioma cell
PDX	patient-derived xenograft
PEG	polyethylene glycol
PFU	plaque-forming unit
PV	pulmonary vessel
RAGE	receptor for advanced glycation end products
RPAR/R	prototypic CendR peptide, sequence [RPARPAR]
R-AgNP	silver nanoparticle functionalized with RPARPAR peptide
RT	room temperature
SC	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulfate
SM	smooth muscle
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
Tu/T	tumor
UV-vis	ultraviolet-visible
VEGF	vascular endothelial growth factor
Wt	wild type
X	aminohexanoic acid

1. INTRODUCTION

Already at the end of 19th century Paul Ehrlich discovered the vascular barrier between blood circulation and central nervous system as he observed that certain dyes when injected into vascular system stain all the organs except brain and spinal cord (Ehrlich 2013). Now it is well-known that protecting barriers exist in order to help maintain brain homeostasis and prevent the entrance of pathogens and toxic compounds (Engelhardt and Sorokin 2009). The same barriers limit the entrance of 98% of molecules, including most of the drugs into the brain tissue (Wolburg et al. 2009). This is the reason why improving diagnosis and treatment of brain disorders has remained a challenge compared to diseases in other parts of body. Enormous effort has been put into developing new drugs for CNS disorders but over 90% of drug candidates have proven inefficient, mainly because insufficient amount of drug was able to reach extravascular brain parenchyma (Cummings et al. 2015).

Actively targeted nanodrugs hold a great promise to improve the treatment of neurological disorders by precision delivery to disease site in CNS (Saraiva et al. 2016). Targeted nanomedicine approach requires excellent targeting ligands that would enable to transport adequate amount of cargo to lesion site. Phage display can be used for agnostic identification of peptide ligands that target receptors in the vascular beds in the CNS (Ghosh et al. 2018). Such homing peptides can be readily conjugated to drugs and nanoparticles to improve their biodistribution and activity. Using nanoparticles for the delivery has several advantages over the transport of free drugs, such as attachment of multiple copies of targeting peptides for enhanced avidity, prevention of the degradation of drugs during delivery and controlled release at disease site (VanDyke et al. 2016; Hua et al. 2018).

The thesis summarizes our work on identification and characterization of brain targeting peptides that could be used to improve the diagnosis and treatment of various neurological disorders. To enhance the validation process of homing peptides and to select the best performing peptides, an ultrasensitive quantification system based on isotopic silver nanoparticles and ICP-MS analysis was developed. Phage display on Alzheimer`s disease (AD) model was used to identify new peptides to target vascular changes in AD and studied suitability of a previously identified targeting peptide for malignant glioma nanodrug delivery.

2. LITERATURE REVIEW

2.1. Drug delivery challenge

Drug concentration at the biological receptor site determines the magnitude of the pharmacological response (Campbell and Cohall 2017). Even the agents with excellent bioavailability, long plasma half-lives and favorable receptor binding properties do not pass clinical testing if not able to reach the target at required concentration. Therefore, efforts to develop more efficacious drugs focus increasingly on optimizing drug biodistribution in tissues, cells, and on subcellular level. Encapsulating drugs into nanoparticles (NPs) can overcome some of the limitations of conventional medicine by promoting more desirable pharmacokinetics and biodistribution (Ventola 2017). The encapsulation in NPs can increase drug concentration at the intended site(s) of action (Vieira and Gamarra 2016). Loading a drug in nanocarriers can increase its stability, prolong the circulation time, extend the release of drug, and finally, decrease immunogenicity and systemic toxicity (Bobo et al. 2016).

NPs target tissues passively by taking advantage of longer circulation half-life and/or actively by using affinity ligands that bind to specific receptors expressed in a target tissue (Bertrand et al. 2014). Passive targeting to solid tumors relies on leaky vessels and poor lymphatic drainage that result in the extravasation and accumulation of drug in the tumor tissue (Maeda, Nakamura, and Fang 2013). Coating targeting ligands on NPs enables to fully exploit their potential as delivery vehicles. Active targeting improves accumulation of nano-therapeutics in target tissue and their uptake in target cells, leading to increased therapeutic efficacy and reduced off-target side effects (Danhier 2016; Ruoslahti 2012). By increasing the local drug concentration, active targeting helps to lower the risk of developing drug resistance (Andrieu et al. 2019).

The first nanodrug on the clinical market – Doxil (PEGylated liposomal doxorubicin) was approved by Food and Drug Administration (FDA) of the United States in 1995 for the treatment of Kaposi's sarcoma (Barenholz 2012; Harris et al. 2002). Compared with free doxorubicin, Doxil had fewer side effects. Approved nanodrugs are based on existing drugs that have been nano-formulated to improve their biodistribution or pharmacodynamic properties. Nanoparticle-based therapeutics have been approved for a variety of pathologies: cancer, hepatitis, multiple sclerosis, chronic kidney disease, macular degeneration, and hemophilia. All clinically approved nanoparticles are non-targeted passive drug delivery vehicles (Bobo et al. 2016).

2.1.1. Vascular heterogeneity and its targeting with affinity ligands

Blood and lymphatic vessels are morphologically, molecularly and physiologically diverse and specialized according to the site and physiological status of the tissue that they supply (Potente and Mäkinen 2017). In blood vessels, the luminal side of the vessels is covered by a single layer of endothelial cells (ECs), which form a continuous monolayer in arteries and veins, but can be fenestrated or discontinuous in capillaries. Endothelial layer permeability in the vasculature in different organs is not uniform. In the brain, ECs in conjunction with the supporting cells establish a highly selective blood-brain barrier with very low baseline permeability, whereas in the kidney glomerular endothelial cells enable rapid filtering of water and unbound drug molecules of less than 20 kDa. Above mentioned water and unbound drug molecules of less than 20 kDa are filtered through the glomerulus with the primary urine. Depending on tissue and its physiological status, endothelial cells also vary in their ability to mediate transport of nutrients and in expression of membrane receptors and transporters (Aird 2012). For example, neuronal cells need high levels of glucose and the ECs in the brain overexpress GLUT1 that transports glucose from blood to the brain parenchyma (Zhao et al. 2015). The ECs in the heart express high levels of the fatty acid transporter CD36, because cardiac muscles rely on fatty acid catabolism for ATP synthesis (Coppiello et al. 2015). Molecular heterogeneity of vascular endothelium has led to the identification of tissue and organ specific ligands that could be used for active targeting of drugs and nanoparticles into these tissues (Teesalu, Sugahara, and Ruoslahti 2012).

Normal vascular morphology, permeability, and gene expression are altered by many diseases, such as cancer, neurodegenerative disorders, cardiovascular disorders, and diabetes. Solid tumors depend on formation of new blood vessels to supply malignant tissue with nutrients and oxygen, leading to development of disorganized "leaky" vessels. Tumor neovasculature has molecular expression pattern different from the normal vessels in the same tissue; several molecular markers, like VEGF, integrins, and CD105, are upregulated on angiogenic vessels (Chen and Cai 2014). Alzheimer's lesions express elevated levels of RAGE receptor and decreased amount of LRP1 in their brain capillaries. Changes in RAGE and LRP1 expression promote disease progression by triggering accumulation of Aβ peptide and compromised BBB (Saraiva et al. 2016).

Molecular heterogeneity of blood vessels in normal tissues and elevated expression of systemically accessible molecular markers at disease sites allow affinity-based targeting to enhance drug concentration at disease-site, at the same time attenuating off-target side-effects (Ruoslahti 2004).

2.1.2. Classes of affinity ligands

A variety of molecules, such as antibodies, peptides, aptamers, vitamins and other low molecular weight compounds, can be used as affinity ligands (Liu et al. 2017).

Antibodies and antibody fragments are widely used as targeting ligands because of their high affinity and specificity. Several targeting antibodies have been approved by FDA for the delivery of radionuclides (e.g. Zevalin or Bexxar, anti-CD20 antibodies loaded with ^{90}Y or ^{131}I) and cytotoxic drugs (e.g. Trastuzumab emtasine) (Liu et al. 2017). However, most of the monoclonal antibodies (mAbs) penetrate poorly into tumor tissue because they are too big to extravasate and efficiently diffuse into tissue (Kue et al. 2016). Antibodies with high affinity to, e.g. tumor antigens, will stably bind to the first encountered antigen; this effect is called "binding site barrier", it will lead to uneven distribution inside the tumor tissue and suboptimal therapeutic effect. Thus, antibodies with moderate affinity are more favorable to be used as targeting ligands (Adams et al. 2001). The use of antibodies have several disadvantages; slow clearance of mAbs results in high normal tissue exposure, the Fc region of antibodies binds to reticuloendothelial system and can cause toxicities to liver, spleen and bone marrow (Liu et al. 2017). Their size makes large scale manufacturing process difficult and expensive (Liu et al. 2017). Antibody fragments can offer several advantages over the use of full-length mAbs; due to their smaller size antibody fragments can more effectively penetrate into tissues and access challenging epitopes and have potentially reduced immunogenicity (Bates and Power 2019).

Peptides are efficient alternative targeting ligands for selective delivery of drugs and nanoparticles. Several peptide hormones have already been used for tumor targeting, e.g. octreotide, an octapeptide that mimics natural somatostatin, has been used to target neuroendocrine tumor (Lopci et al. 2008). The size of targeting peptides falls in between the size of small molecule ligands and antibodies. Due to their larger size, they possess much higher binding affinity and specificity than small molecule ligands, but still being small enough to possess increased diffusion and tissue penetration (Jiang et al. 2019). The major problem of peptide ligands is their poor stability. Several modifications such as, cyclization, backbone/side chain modification, and unnatural residue substitution can be used to improve peptide stability against proteolysis (Chen et al. 2017). Peptide ligands are typically non-immunogenic, and the multivalent presentation on a NP provides high avidity for the target (Ruoslahti 2012). In addition, they can be readily conjugated to different drugs, nanoparticles, or radionuclides. Peptides can be synthesized on large scale at low cost (Zhao et al. 2007).

Aptamers are nucleic acid macromolecules with high affinity and specificity to their targets (Alshaer, Hillaireau, and Fattal 2018). DNA and RNA aptamers have been selected against different targets –small molecules, membrane receptors and whole living cells (Pereira et al. 2018). Aptamers can be readily

conjugated to drugs and nanoparticles and their chemical properties provide good solubility with low toxicity and immunogenicity. Additional advantage of aptamers is low cost of production. The main disadvantage of aptamers is sensitivity to degradation by nucleases and rapid clearance from the circulation (Alshaer, Hillaireau, and Fattal 2018). Additionally, aptamer-target interactions are strongly influenced by slight changes in the microenvironment that can alter the secondary and tertiary conformation of aptamers (Jiang et al. 2019).

Low molecular weight affinity ligands extravasate easily and quickly reach their target *in vivo*. Furthermore, small molecules are rapidly cleared from the circulation, if they fail to reach target cells, reducing the possibility of off-site toxicity (Srinivasarao and Low 2017; Vlashi et al. 2009). However, limited binding interfaces between low molecular compounds and their receptors restrict specificity of interaction (Jiang et al 2019). Small molecule ligands are amenable to chemical synthesis, making their production cost-effective (Kue et al. 2016). Some of the most promising small molecule ligands that have been used for tumor targeting are folate derivatives, glutamic acid urea derivatives, and low molecular weight analogues of peptide hormone somatostatin (Casi and Neri 2015).

2.1.3. Agnostic exploration of vascular heterogeneity by peptide phage display

Receptor-specific targeting peptides can be identified using various approaches: by using receptor's native ligands or their analogues (such as folic acid against folate receptor (Cheng et al. 2013)); by computer aided design based on the structure of the receptor (Chen et al. 2018); or by screening combinatorial libraries, such as OBOC combinatorial peptide library (Lam et al. 1991), peptide microarray (Xu and Lam 2003), phage-display peptide library (Krumpe et al. 2006), and other biological-display peptide libraries (Liu et al. 2017). Phage display is a powerful method that can be used to identify peptide ligands against simple and complex targets, including proteins, cells, and tissues. Phages are bacterial viruses that can be genetically engineered to express foreign peptides as fusions to the coat protein. Peptide phage library consist of a large number (typically about a billion) of bacteriophages, displaying different peptides on its surface (Smith and Petrenko 1997). Selection of phages that bind to specific target, "biopanning", can be performed *in vitro*, *ex vivo* and *in vivo* (Liu et al. 2017). Sequential biopanning rounds enrich the library with target-binding peptides, whereas the background phages are eliminated (Fig. 2). *In vivo* phage display can be used to identify peptides that target systemically accessible receptors that can be reached from the vasculature.
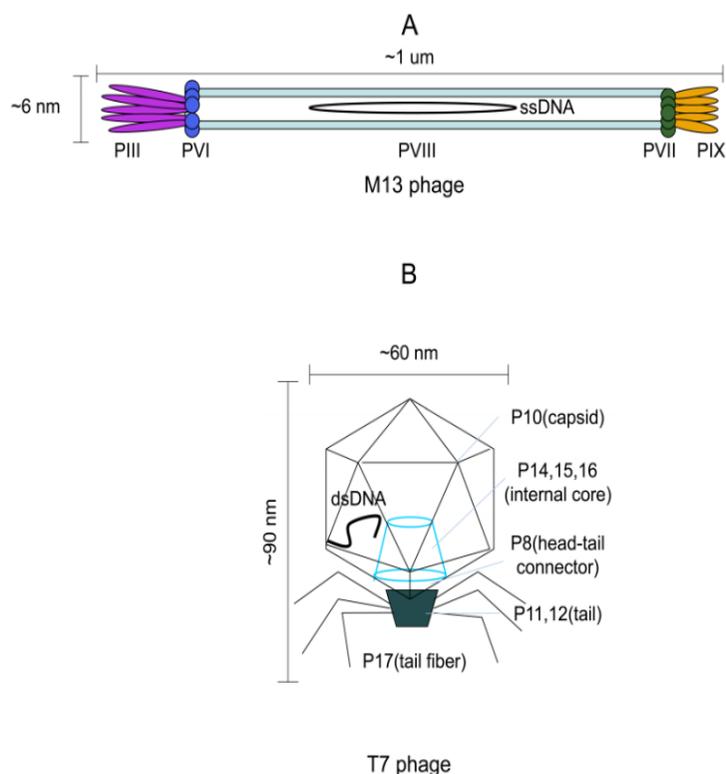
Figure 1: The schematic representation of (A) M13 phage and (B) T7 phage.
Adapted from Tan et al. (2016).

Numerous bacteriophage species have been employed in phage display systems, including f1, fd, T4, M13 and T7 (Deng et al. 2018).

T7 is a lytic coliphage with double-stranded DNA, which exhibits increased stability and low mutation rate during replication (Deng et al. 2018). Compared to filamentous phage-based systems, the peptide libraries constructed with the T7 display system retain fewer amino acid biases and have increased diversity of displayed peptides (Krumpe et al. 2006). Importantly, the size (~60 nm diameter) and shape of T7 particles and density of displayed peptides are similar to what is typically seen for (pre)clinical nanoparticles (Fig. 1b). Density of C-terminally exposed foreign peptides on T7 particle can be modulated across a wide range (1–415 peptides/particle) by amplifying peptide-phages in *E. coli* strains with different expression of wild-type p10 coat protein (T7Select® System Manual). As peptide density on phage particle is similar to the density on nanoparticles, it means that the novel peptides discovered using T7 display system are suitable for multivalent use on the nanoparticles. Multivalent presentation of peptides on biological phage particles and synthetic nanoparticles

increases the avidity and ensures strong target binding even with relatively low affinity (Ruoslahti 2012).
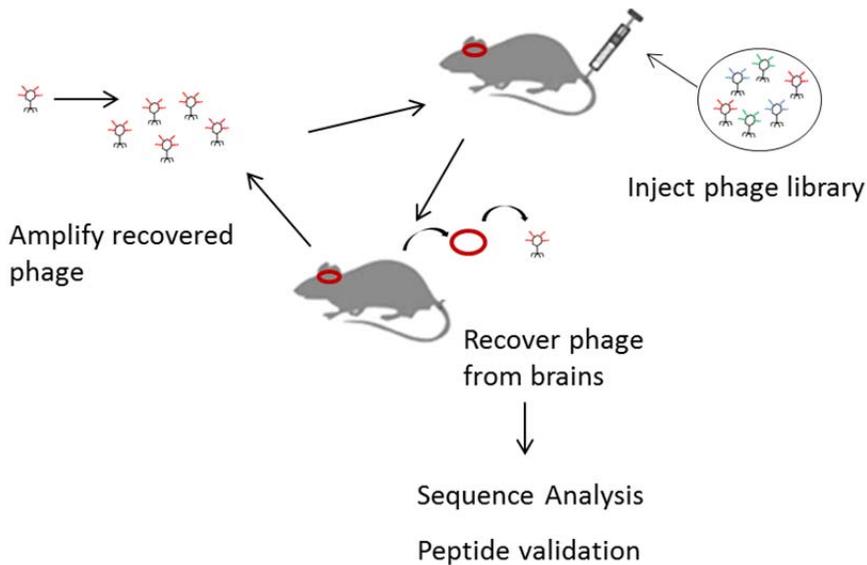
Figure 2: *In vivo* **phage display for brain homing peptides.** Peptide phage library is injected into blood circulation. After 10–60 min circulation, the brain and other organs are collected and homogenized in a buffered non-ionic detergent solution. The bound phage pool from the brain is amplified and used as input in the next biopanning round. After several rounds of selection, phages are recovered from brains and the peptide-encoding portion of the phage genome is sequenced. Enriched peptides are identified and validated further in individual phage homing assays and as synthetic peptides labeled with fluorophores or coated on nanoparticles.

The M13 phage is another widely used phage-display system with high capacity for replication and nonessential regions that allow exogenous gene insertions (Liu et al. 2017). M13 phage is a filamentous phage containing single-stranded DNA. M13 phage particles (Fig. 1a) are 6.5 nm in diameter and 1μm in length, and the peptides are displayed at one end of the phage particle (Tan et al. 2016). The use of filamentous phage systems have several disadvantages compared to lytic phage, filamentous phage vectors are associated with a limited cloning capacity, which in the case of M13 is < 1500 bp, and their genome exhibits markedly reduced stability following insertion of foreign DNA (Deng et al. 2018). Filamentous phage morphogenesis: phage assembly, secretion, and in-fection processes, limits significantly the diversity of peptides propagated in the M13 libraries as displayed peptides need to be compatible with *E.coli* secretory system (Krumpe et al. 2006).

2.1.4. Drug delivery to the brain. Blood-brain barrier

Already in the 1880s Paul Ehrlich observed that intravenously injected hydrophilic dyes stain all organs except the brain and the spinal cord (Dyrna 2013). It has become well established that the central nervous system has evolved different barriers that are important for protecting it from foreign substances and to maintain homeostasis. These protective structures include blood-brain barrier (BBB), blood-cerebrospinal fluid (CSF) barrier, blood-spinal cord barrier and blood-retinal barrier. The most selective of these barriers is the blood-brain barrier (Cipolla 2009). The BBB is crucial for protecting brain cells against circulating toxic or infectious agents and preserving the CNS homeostasis (Hawkins and Davis 2005). The BBB is impermeable to all large molecules and >95% of small molecules. Only a few molecules with specific characteristics are able to cross the barrier, such as water, some gases, and some lipophilic compounds (Saraiva et al. 2016). Small-molecule drugs need to have molecular mass under a 400- to 500-Da threshold, and high lipid solubility to cross the BBB in pharmacologically significant amounts (Pardridge 2003). Most low molecular weight drugs, mAbs, recombinant proteins, or gene therapeutics, do not cross the BBB (Pardridge 2005).

For most CNS diseases there are candidate drugs that show efficacy *in vitro* and when directly injected in the brain, however these compounds do not cross the BBB and are not effective upon systemic administration. For example, catalase could be potentially used to treat patients with chronic neurodegenerative diseases, as this enzyme binds to amyloid-β plaques and is a potent antioxidant able to fight oxidative stress seen at chronic inflammation (Singhal et al. 2013). However, inability of catalase to cross the BBB (and some other features such as a short half-life and poor cellular uptake) are not compatible with its therapeutic use (Jaffer et al. 2011). Curcumin, a polyphenol derived from turmeric herb, has a high affinity for the Aβ peptides and promotes the disaggregation of amyloid-β plaques. However, low bioavailability of curcumin excludes its uses for diagnosis and treatment of AD (Tang, Taghibiglou, and Liu 2017). Therapeutic hypothermia induced by neurotensin peptide has a neuroprotective effect and thereby could improve neurological outcome at severe liver failure, stroke, or neonatal ischemia. However, the clinical use of neurotensin is hampered because the peptide is not able to cross the BBB and enter the CNS to reach its target neurons in the lateral hypothalamus (Tang and Yenari 2010).

2.1.4.1. Anatomy and physiology of the BBB

Structurally, the BBB is composed of endothelial cells, astrocytes, pericytes and neurons (Fig. 3). Polarized endothelial cells lining the lumen of the cerebral capillaries are connected by tight junctions that form selective interface between blood and brain (Hawkins and Davis 2005).
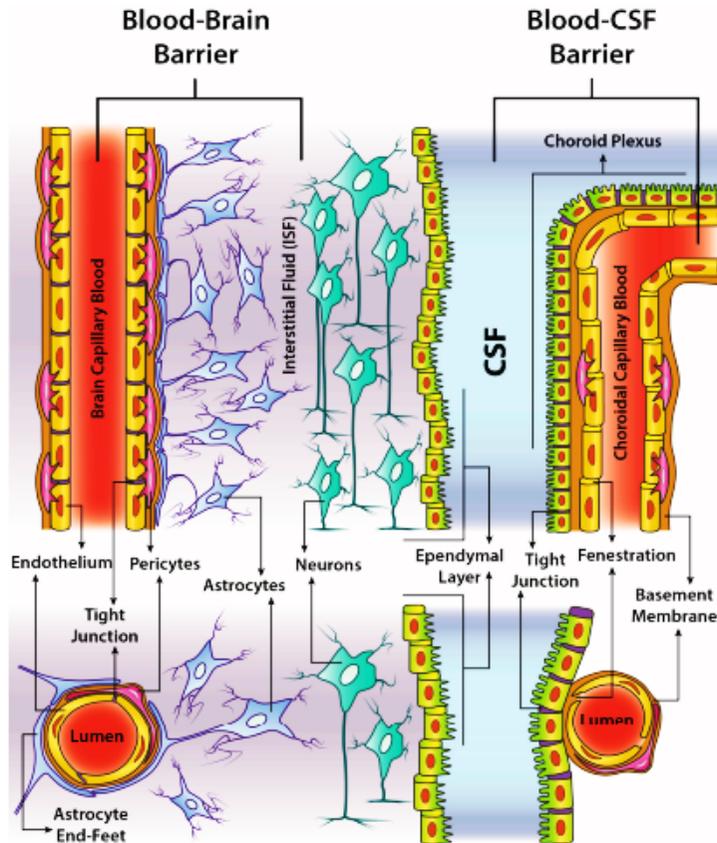
Figure 3: Structure of the BBB and the blood-CSF barrier. (Left) BBB is a highly selective barrier that separates blood from the brain parenchyma. BBB is composed of network of astrocytes, pericytes, neurons, and endothelial cells. (Right) The blood-CSF barrier is a more permissive barrier found in the choroid plexus of each ventricle of the brain. The blood-CSF barrier is composed of epithelium-like ependymal cells covering fenestrated capillaries. Tight junctions between ependymal cells are leakier to small molecules compared to the tight junctions forming the BBB. Adapted from D'Agata et al. (2018).
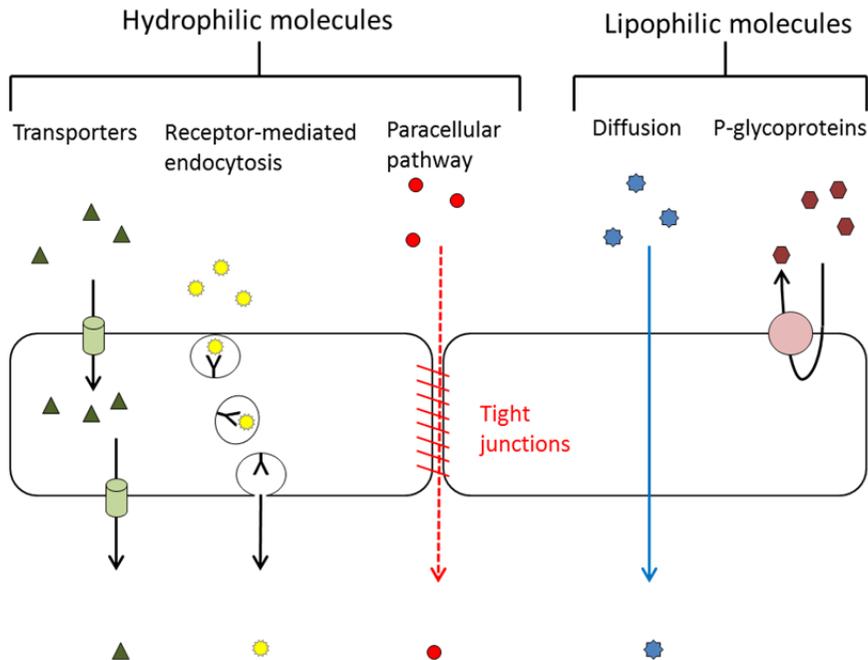
Figure 4: Pathways of transport across the BBB. Adapted from Gabathuler (2010).

Brain capillary endothelial cells express active transport mechanisms that ensure the transport of nutrients into the CNS and exclusion of blood-borne molecules (Fig. 4) (Engelhardt and Sorokin 2009). Small hydrophilic molecules that are essential for the brain cells to function, like amino acids and glucose, use transporters at the luminal and basolateral side of the endothelial cells. Larger and/or hydrophilic molecules such as hormones, transferrin and lipoproteins use specific receptors expressed in high numbers on the luminal side of the endothelial cells. These receptors facilitate endocytosis and transcytosis of compounds across the barrier. Small lipophilic molecules are able to diffuse passively across the BBB but are exposed to efflux pumps such as P-glycoprotein and Multidrug Resistance Proteins (Gabathuler 2010).

Some regions of the brain require a more permissive barrier for proper functioning, such as ventricular and circumventricular areas of the brain. Ventricular system is comprised of two lateral ventricles, and a third and fourth ventricle. Cerebrospinal fluid (CSF) is produced in the third and fourth ventricles by the choroid plexuses and capillaries. CSF protects the brain and spinal cord and provides them with important nutrients. Structurally, the choroid plexus is composed of capillaries covered by epithelium-like ependymal cells. Capillaries of the choroid plexus are fenestrated and allow direct passage from blood to the brain parenchyma. Tight junctions of the ependymal cells that cover the capillaries form the blood-CSF barrier (Fig. 3). Tight junctions between the

ependymal cells are leakier than between the cells of BBB (Cipolla 2009; Saraiva et al. 2016). Transport of compounds from blood into the CSF is similar to that into brain, with many of the transporters shared between the tissues. Due to the different properties of the two barriers, some compounds predominantly enter the brain by crossing the BBB, such as O_2, CO_2, glucose and amino acids, but others like Ca-ions and several hormones have choroid plexus as the major site of entry (Laterra et al. 1999). It has been shown that nanoparticles, which have been injected intravenously, will accumulate in these leakier areas irrespective of their cargo (Xin et al 2012).

Blood-spinal cord barrier (BSCB) protects and regulates the homeostasis of the spinal cord parenchyma. BSCB has similar structure as the BBB: it is composed of endothelial cells, basement membrane, pericytes, and astrocytic end-feet processes. However, the BSCB has several functional and morpho-logical differences when compared to the BBB. The microvessels in the spinal cord contain glycogen deposits not present in brain capillaries. Compared to the BBB, the BSCB is more permeable to variable tracers and cytokines (Pan, Banks, and Kastin 1997), potentially due to low expression of tight junction and adherens junction proteins in the spinal cord (Bartanusz et al. 2011).

BBB organization and function are severely altered in brain tumors and at neurodegenerative disorders, the disrupted BBB in case of brain tumors is called blood-brain tumor barrier (BBTB). BBTB limits the access of anticancer drugs to the brain tumor. In low grade diffuse gliomas the tumor cells are present as oligocellular aggregates in the brain parenchyma and the BBTB resembles structurally and functionally the BBB (Van Tellingen et al. 2015). In contrast, high grade gliomas are highly angiogenic tumors with leaky BBTB, as has been shown by contrast-enhanced MRI (Dhermain et al. 2010). BBTB shows elevated expression of receptors that mediate ligand-dependent drug delivery and could be targeted to improve drug delivery to tumor tissue (Ningaraj 2002). The BBB is formed by capillaries without fenestrations, but the BBTB can be comprised of non-fenestrated capillaries, capillaries with fenestrations and capillaries containing inter-endothelial gaps (Schlageter et al. 1999). BBTB capillaries present overexpression of drug efflux pumps and multidrug resistance-associated proteins, which function against the leaky vasculature and prevent the uptake of drugs by cancer cells (Ningaraj 2006). In addition, the tumor growth results in increased pressure to the surrounding tissue and leads to formation of intracranial edema that also limits the delivery of drugs to tumor (Wolburg et al. 2012).

2.1.4.2. Affinity targeting of the CNS

The presence of intact BBB is a major obstacle in treating CNS diseases. As less than 2% of the drug candidates are able to penetrate the BBB and to enter the cerebral tissue, more efficient CNS delivery strategies are needed to „by-pass" the protective BBB shield (Pardridge 2005). The concept of BBB shuttles –

application of molecular vectors for penetration from systemic circulation into brain parenchyma without disrupting the protective barrier – was introduced already more than three decades ago (Pardridge 1986). Most commonly used CNS delivery approach is receptor-mediated transport that takes advantage of highly expressed transport receptors on the luminal side of brain endothelial cells, such as transferrin receptor, insulin receptor, low-density lipoprotein receptor and LRP1 (Lajoie and Shusta 2014). Over the years, a number of BBB shuttle peptides have been published that bind to the receptors and thereby allow the transport of different cargoes, including small molecules, nano-particles and genetic material beyond the BBB (Table 1) (Urich et al. 2015; Zarebkohan et al. 2015). Despite the discovery of numerous BBB shuttle peptides, crossing the BBB to deliver drugs and diagnostic agents to the brain remains still a major hurdle. The shortage of brain selectivity and poor penetration capacity across the BBB are the two main problems current BBB crossing strategies are facing (Ross et al. 2018). Intensive research is ongoing in two main directions: firstly, to find new brain-specific markers to improve drug transport across the blood-brain barrier and more selectively to diseased cells, in order to minimize distribution into normal brain cells (Gao 2016). And secondly, to develop new nanocarriers with long plasma circulation that are nontoxic, biocompatible, and biodegradable, since the brain has very limited capability for elimination and metabolization of the translocated nanoparticles (Mi, Cabral, and Kataoka 2019).

Table 1: Brain homing peptides and evidence for the BBB penetration.

Peptide	Identification	Evidence for BBB penetration	Reference
CLSSRLDA	*In vivo* phage display for mouse brain homing peptides	Barrier penetration in an *in vitro* cell-based rat BBB model	(Pasqualini and Ruoslahti 1996)
LSSRLDAC	*In vivo* phage display for mouse brain homing peptides	Barrier penetration in an *in vitro* cell-based rat BBB model	(Pasqualini and Ruoslahti 1996)
CGHKAKG	*In vitro* phage display on extracellular domain of hTfR	Neuronal delivery of therapeutic payload in mouse AD model	(Xia et al. 2000)
THRPPMWSPVWP	*In vitro* phage display on cells that constitutively express the hTfR	Barrier penetration in an *in vitro* cell-based rat/bovine BBB model Demonstartion of the BBB penetration by transmission electron microscopy on rat brain sections	(Lee et al. 2001)
CAGALCY	*In vivo* phage display for mouse brain homing peptides	n.a.	(Fan et al. 2007)
TFFYGGSRGKRNNFKTEEYC	Neurotropic endogenous protein derived from the Kunitz domain of aprotinin	*In vivo* delivery of paclitaxel to mouse model of glioblastoma	(Demeule et al. 2008)
CMPRLRGCC	*In vitro* phage display on extracellular domain of human LDLR	Optical demonstration of BBB penetration by multiphoton microscopy in live mice	(Malcor et al. 2012)
RLSSVDSDLSGC	*In vivo* phage display for rat brain homing peptides	Peptide-phage recovered from the rat CSF	(Urich et al. 2015)
TPSYDTYAAELR	*In vivo* phage display for rat brain homing peptides	Peptide-phage recovered from the rat CSF	(Li, Feng, and Jiang 2015)

2.1.5. Brain diseases with unmet drug delivery challenge

2.1.5.1. Neurological disorders: Alzheimer's disease

The frequency of neurological diseases is expected to reach epidemic scale in the next decades as the average life expectancy is increasing globally. Neuropathies and neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) lead to adverse health outcomes in patients and put immense load on healthcare system (Heemels et al. 2016). Already now, the morbidity and mortality caused by brain-related disorders is higher than that of cardiovascular diseases and cancer (Sharma et al. 2017). Improved diagnostic tools and treatments need to be developed to decrease the burden on affected individuals and society (Kevadiya et al. 2018). Current treatments fail to cure the neurodegenerative diseases or even slow down their progression and only deal with the symptoms (Kevadiya et al. 2018). Treatment with neuroprotective agents is likely to be an effective strategy to prevent neuronal degeneration, but typically these bioactive compounds are not able to cross the BBB (Levi and Brimble 2004). There are clinically approved drugs that have been reported to be effective in treating neurodegenerative disorders on experimental models, but they lack the ability to cross the BBB or require very high doses causing severe off-target side effects. One of the examples is minocycline; it is a broad-spectrum antibiotic, which has been used over 30 years to treat infections caused by Gram-negative and Gram-positive bacteria. Several groups have shown it to have neuroprotective effect in various experimental models of CNS diseases. Whereas minocycline is able to penetrate the BBB to some degree, it must be administered at high doses (with serious side effects) to achieve therapeutic concentration in the brain (Sharma et al. 2017). Using targeted delivery strategies to transport minocycline and other neuroprotective agents selectively to the brain and across the BBB could be the way treat neurodegeneration in patients.

AD is the most common age-related neurological disorder. AD causes widespread neuronal and synaptic loss, amnesia, cognitive impairment, hallucinations, and social deficits. AD is characterized by progressive and irreversible damage to neurons (Kevadiya et al. 2018). The most prevalent hypothesis regarding AD pathogenesis suggests that the accumulation of β-amyloid peptide (Aβ) is the key mechanism causing the disease (de la Torre and Ceña 2018). Overproduction of Aβ peptide isoforms causes aggregation of peptide fragments into large fibrils that become deposited as insoluble plaques (Golde, Eckman, and Younkin 2000). The Aβ plaques form first in the basal cortex but are at later stages detected in most neocortical regions except in the hippocampus (Braunstein et al. 2016). Aβ plaque toxicity is caused by multiple mechanisms, including increased membrane permeability, oxidative stress, synaptic dysfunction and microglial activation (Lin et al. 2001, Butterfield et al. 2007, Parameshwaran et al. 2008).

Whereas in most cases the AD begins after the age of 65 years, approximately 6% of patients have early-onset AD, an autosomal-dominant genetic disease that manifests in patients at ages 30–50 (Bateman et al. 2011; Dzamba et al. 2016). The clinical diagnosis of AD is based on family history, symptoms of dementia, imaging by computerized tomography (or magnetic resonance imaging), and blood biomarkers (Hane et al. 2017). However, a conclusive AD diagnosis can only be obtained post-mortem by autopsy (Kelley et al. 2007).

Currently, there are only five medications approved for the treatment of AD; four cholinesterase inhibitors and N-methyl-D-aspartate receptor antagonist (Cummings et al. 2014). These drugs are used to treat cognitive symptoms but fail to stop the progression of neuronal damage and loss. Discovery of new drugs able to slow down or reverse the progression of the AD is challenging, and disappointingly most of the clinical studies using investigational new drugs have failed to demonstrate clinical efficacy (Salloway et al. 2014). New cellular pathways and molecules involved in AD pathogenesis need to be identified to improve diagnosis and treatment.

2.1.5.2. Gliomas

Malignant glial tumors are the most common primary brain tumors. Gliomas are characterized by heterogeneity inside the tumors and between the tumors of different patients (Parsons et al. 2008). Gliomas originate from the glial cells: astrocytes, oligodendrocytes or ependymal cells. Depending on the cell of origin gliomas are classified into 3 groups: astrocytomas (including glioblastomas), oligodendrogliomas, and ependymomas; some tumors display a mixed phenotype of these different cells (Louis et al. 2016). Glioblastomas (GBM) are the most frequent and aggressive of the glial tumors. Despite intense research, GBM remains highly malignant with very poor prognosis in the patients. The current median survival of the patients is less than 15 months after GBM diagnosis (Stupp et al. 2005). GBM treatment combines maximum surgical resection of the tumor tissue with the radiation therapy and/or chemotherapy. Despite the aggressive treatment, the prognosis remains low due to high recurrence rate. Complete resection of the tumor is impossible because of anatomical location and highly infiltrative nature of these tumors (Stupp et al. 2005; Shirahata et al. 2007). Radiation and chemotherapy kill actively proliferating glioma cells but are unable to reach to the quiescent glioma stem cells that are hiding in hypoxic regions and behind the blood brain barrier. Recurrent GBM that develops from the guerilla cells away from tumor bulk grows rapidly and spreads to more distant regions and is more resistant to chemotherapy than the initial tumor (Yip et al. 2009).

Although GBM is highly angiogenic with leaky blood vessels and BBB disruption in the core of the tumor, most of the approved anticancer therapeutics are not able to cross the BBB or the BBTB (Van Tellingen et al. 2015). Currently there are only two anticancer agents, temozolomide and nitrosourea, that are able to cross the BBB and reach the cerebral tissue. Neither of these

agents is highly effective against GBM and new strategies for GBM management are actively sought for with >400 glioblastoma studies ongoing all over the world in late 2019 (Argyriou and Kalofonos 2009; clinicaltrials.gov).

2.2. Preclinical modeling of glioma and neurodegenerative disease

2.2.1. Glioma modeling in mice

Tumor modeling in small animals has a crucial role for development of new therapeutic strategies, to study tumor biology, and predict efficacy and toxicities of potential new therapies. Implantable brain tumor models have been widely used and well characterized (Assi et al. 2012).

Commercially available tumor cell lines are extensively used for both *in vitro* and *in vivo* research. These cell lines were initially derived from patient GBM tumor tissue and are commonly cultivated in 2D in serum-containing medium (Kijima and Kanemura 2017). Human-derived glioma cells have to be xenografted into immunodeficient animals; transplantation of cancer cells into rodents by stereotactic injection enables to induce tumors at specific anatomical structures within the brain (Assi et al. 2012). Patient-derived glioma cells (PDGCs) give rise to tumors that grow rapidly, have high engraftment rate, and good reproducibility (Fig. 5b) (Huszthy et al. 2012). However, xenografts induced by PDGCs often fail to reflect important clinical characteristics of real patient tumors, as the cell-line derived tumors are often circumscribed and fail to exhibit the hallmarks of clinical glioma: the presence of necrotic areas and single-cell invasion into surrounding brain tissue (Mahesparan et al. 2003).

Patient-derived xenografts (PDX) are established by implanting fresh tumor tissue or cultured tumor spheroids into immunodeficient animals (Fig. 5b) (Bjerkvig et al. 1990; Tentler et al. 2012). PDX models are maintained by serial passaging in animals or by producing tumor spheroids, often under serum-free neurosphere-culture conditions (Bjerkvig et al. 1990). PDX models are widely used in translational research, as they preserve both the genetic and histological features of parental tumors. Array-comparative genomic hybridization analysis has shown that in contrast to cell line xenografts, which genetically do not resemble patient tumors, PDX spheroids retain the genetic profile of parental tumors even after multiple *in vitro*/*in vivo* passages. Importantly, the PDX tumors maintain tumor heterogeneity; they remain infiltrative and have necrotic areas, which are main characteristics of clinical GBM (De Witt Hamer et al. 2008). However, not all human gliomas are tumorigenic when injected to animals; and some tumors fail to produce tumor spheroids. The time needed to establish the tumor varies between passages and different PDX tumors, making experimental planning challenging (Huszthy et al. 2012). Despite the disadvantages, PDX models are the way to move towards personalized treatment of tumors (Kijima and Kanemura 2017).
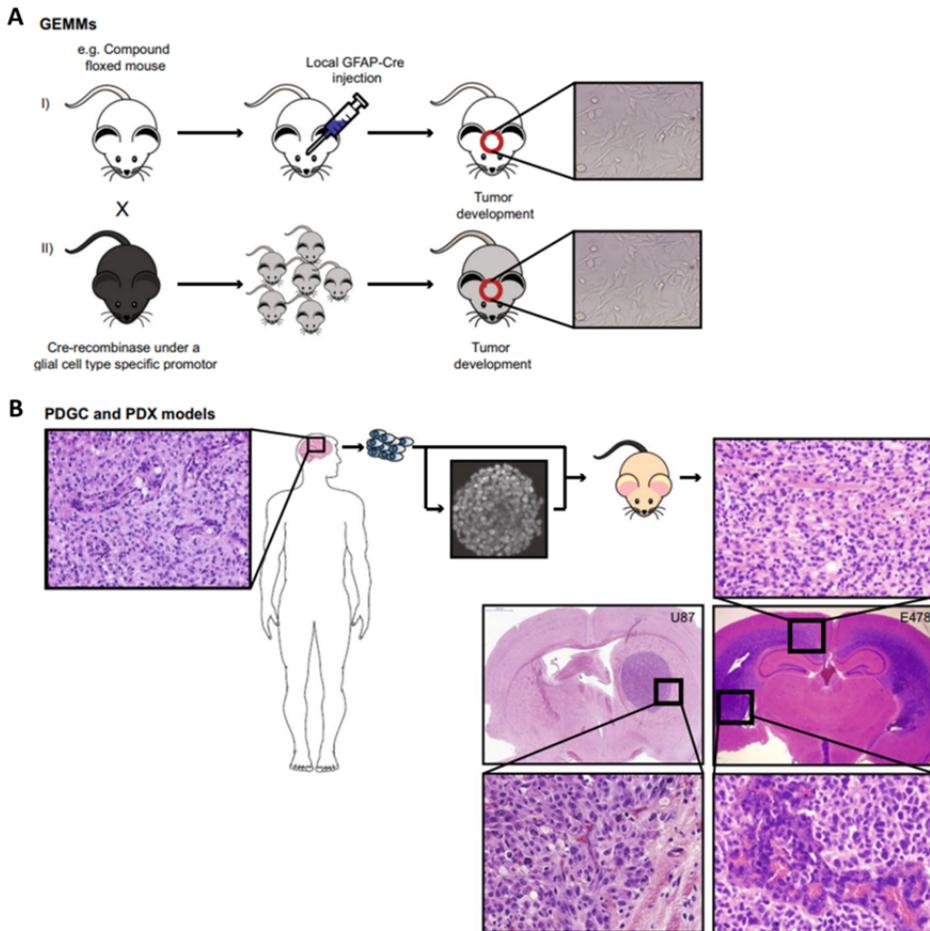
Figure 5: *In vivo* **GBM modeling.** (A) Genetically engineered mouse models are generated using Tet-regulation or Cre-inducible gene alleles. These GBM models are molecularly highly defined but fail to reflect biological heterogeneity seen in human GBM. (B) Patient-derived glioma cells (PDGCs) give rise to tumors that grow rapidly, have high engraftment rate, and good reproducibility. Tumors derived from PDGCs are often circumscribed without the presence of necrotic areas and single-cell invasion into surrounding tissue. Patient-derived xenografts (PDX) are generated by implanting fresh tumor tissue or cultured tumor spheroids into immunocompromised animals. Ideally, PDX models preserve both the genetic and histological features of parental tumors; florid microvascular proliferation (lower right panel) and diffuse infiltrative growth in the white matter (upper right image). Of note, as illustrated by a xenograft derived from U87 cells (lower left panel), PDGC models often fail to show diffuse infiltration in brain parenchyma. Adapted from Lenting et al. (2017).

Genetically engineered mouse models (GEMMs) resemble the histopathology and molecular features of human tumors (Fig. 5a). In genetically engineered models, gene expression has been manipulated often by using Tet-regulation or Cre-inducible gene alleles. GEMMs are especially useful to verify genetic alterations which are important for tumor initiation and progression (Kijima and Kanemura 2017). A disadvantage is that as the GEMM tumors are formed by cells with specific genetic changes, the tumors do not exhibit the biological heterogeneity seen in clinical GBM (Lenting et al. 2017).

2.2.2. Preclinical modeling of neurodegenerative diseases

To better understand AD pathology and progression, several human amyloid precursor protein (APP) transgenic models have been developed, these mouse lines express various mutations identified from patients with familial AD (Saraiva et al. 2016). hAPP-J20 model overexpresses human APP with Swedish mutation (K670N/ M671L) and Indiana mutation (V717F); Tg2576 model expresses human APP with the Swedish mutation (K670N/ M671L) (www.alzforum.org/research-models/j20-pdgf-appswind; Van Dam and De Deyn 2011). As these models rely on mutations from familial forms of human AD (5% of AD cases), they fail to replicate the complete neuropathology spectrum seen in patients. Nevertheless, AD animal models are crucial for developing new therapeutic strategies (Saraiva et al. 2016).

PD models are based on the administration of toxins that cause selective death of dopaminergic neurons (Saraiva et al. 2016). Two of the most promising models are the rotenone model and the combined paraquat/maneb model. Rotenone and paraquat are pesticides; maneb is a fungicide that has been linked to the development of parkinsonian symptoms in humans (Schapira and Jenner 2011; Thiruchelvam et al. 2000). PD neurotoxic models are essential for under-standing consequences of striatal dopamine loss and enable to develop current dopaminergic therapies (Bové et al. 2005; Schapira and Jenner 2011). Nume-rous genetic PD models have been developed using transgenes with mutations causing familial PD. Genetic models are mainly used for modeling familial PD but can also be used to study more common PD mechanisms. None of the current models recapitulates the complex and progressive pathology of human disease (Jagmag et al. 2016).

2.3. Nanomedicine

The use of nanoparticles has a surprisingly long history; already during Roman times gold nanoparticles were used by artisans to stain glass (Giljohann et al. 2010). The idea of using nanoparticles for medical purposes dates back more than a century ago, when German scientist Paul Ehrlich formulated a concept of

targeted delivery of drugs, that he called *Zauberkugeln* or magic bullets (Kreuter 2007).

Even today the nanomedicine is seen as the "the magic bullet" that could improve cancer therapy and ameliorate treatment of neurological disorders by developing noninvasive therapies that are more effective in reaching the target sites, have increased therapeutic efficacy, and cause less adverse side-effects (VanDyke et al. 2016; Hua et al. 2018). Multitude of nanoformulations of different composition, size, shape, and surface properties, have been designed for therapeutic, diagnostic and theranostic applications (Fig. 6), but only few of them have been approved for clinical use (Danhier 2016; Hua et al. 2018; L. Zhang et al. 2008).
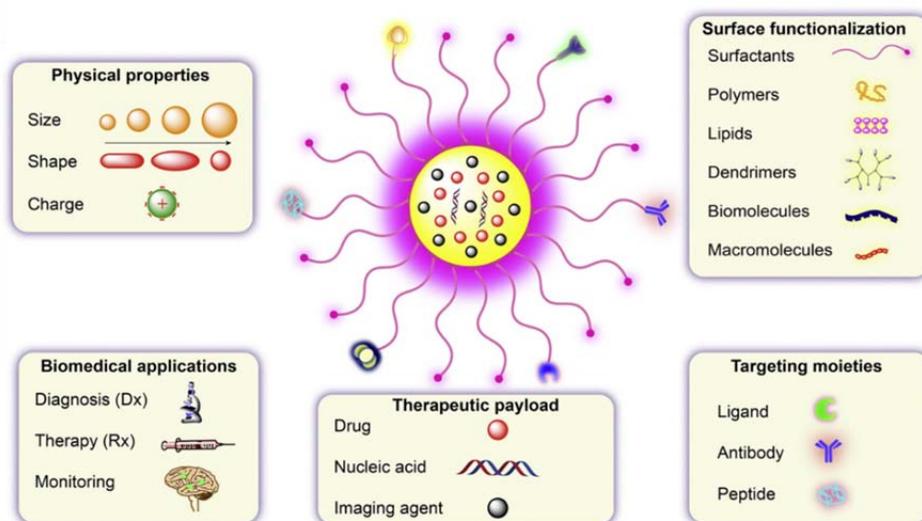
Figure 6: Design, physicochemical properties and biomedical applications of targeted nanoparticles. Adapted from Kevadiya et al. (2018).

2.3.1. Nanoparticle characteristics

The half-life of nanoparticles in the systemic circulation and how well they are able to accumulate in the target tissue is determined by their physicochemical properties. Very small particles, <5 nm, are not suitable for drug delivery, as they will be cleared quickly from the blood by kidneys and particles > 200 nm accumulate in the liver and spleen (Blanco, Shen, and Ferrari 2015; Ernsting et al. 2013). Nanoparticles with size between 8 nm and 100 nm are considered optimal for tumor targeting, as the circulation time is prolonged and they are still small enough to escape from the leaky vasculature present in tumors (Albanese, Tang, and Chan 2012). Bigger nanoparticles have longer half-life, but they would not be able to go through the fenestrations on the angiogenic blood vessels (Yue et al. 2013). Inside the tumor tissue, the correlation between

nanoparticle size and the penetration is reversed, with smaller nanoparticles able to accumulate deeper inside the tumor parenchyma, and bigger particles remaining close to the vasculature (Albanese, Tang, and Chan 2012). To take the advantage of the both phenomena, size-switchable nanoparticle systems have been developed that exploit the advantage of the longer circulation of larger particles and after extravasation particles shrink due to low pH or enzymatic cleavage, to penetrate more effectively (Ruan et al. 2015; Zhang et al. 2016).

The shape of nanoparticles has a strong effect on their distribution in the body and uptake by cells. Spherical nanoparticles are most often used in the preclinical studies, as it is relatively easy to synthesize them, but it has been shown that elongated nanoparticles have prolonged circulation and extravasate more effectively through the pores in tumor vessels (Geng et al. 2007; Stylianopoulos and Jain 2015). Targeted elongated particles have also stronger adsorption to their receptors; it has been shown that rod shape particles coated with transferrin receptor had seven times higher accumulation in the brain compared to spherical nanoparticles with the same targeting ligand (Kolhar et al. 2013).

The surface of nanoparticles is often coated with polyethylene glycol (PEG) to protect the particles from formation of protein corona, which would lead to aggregation and quick blood clearance by reticuloendothelial system (Jokerst et al. 2011). PEG is a water-soluble polymer that forms brush-like structure covering the particles. PEG has high hydration that enables to form water cloud around the particles protecting them from interactions with neighboring NPs, or with the plasma proteins. Nanoparticles with PEG coating have close to neutral surface charge independent of the molecules that have been PEGylated (Suk et al. 2016). Circulation time and delivery efficiency of the nanoparticles is strongly influenced by their zeta potential. Particles with neutral zeta potential have higher delivery efficiency compared to particles with negative or positive zeta potential (Wilhelm et al. 2016). Moreover, it has been shown that NPs with high zeta potential > 45 mV can damage the blood-brain barrier, and particles with low zeta potential are rapidly opsonized and cleared by macrophages (Honary and Zahir 2013; Lockman et al. 2004).

2.3.2. Silver nanoparticles

Silver nanoparticles (AgNPs) are used for various applications, including as antibacterial agents in medical device coatings, cosmetics, textiles and health-care products (Sukhanova et al. 2018). These nanosized metallic particles can possess very diverse physical, chemical and biological properties depending on their surface-to-volume ratio, shape, and surface chemistry, making them useful for various bio-applications such as antibacterial, antifungal, antiviral and anti-inflammatory agents (Zhang et al. 2016). The broad use of AgNPs has caused concerns regarding the possible risks for the human health and the environment.

Several studies have shown that the AgNPs are toxic by causing direct damage to cell membranes, by generation of active oxygen species, and by release Ag^+ ions (Sukhanova et al. 2018). Majority of these toxicity studies have been conducted using uncoated silver particles that can be oxidized and are able to release the ions. Coating the AgNPs with sodium citrate, polyvinylpyrrolidone, or polyethylene glycol molecules decreases their toxicity (Fahmy et al. 2019) .

AgNPs are well-suited for preclinical studies as the model payloads because these particles can be easily functionalized with targeting peptides and fluorescent dyes. AgNPs with surface-bound fluorophore have intense signals as the nanoparticles increase the fluorescence intensity of the dye by about an order of magnitude due to plasmonic enhancement. By using silver bio-compatible etchant to dissolve exposed AgNPs, it is possible to distinguish internalized particles from unbound and cell surface bound particles, allowing to study the cellular uptake of AgNPs (Braun et al. 2014).

2.3.3. Iron oxide nanoworms

Iron oxide nanoparticles are extensively used due to their magnetic properties that enables to exploit them for magnetic hyperthermia, magnetic separation and as contrast agent for MR imaging (Korchinski et al. 2015). As iron oxide NPs are reactive with oxidizing agents, the particles are usually coated with hydro-philic compounds (e.g. dextran) that do not affect the magnetic properties. Coated iron NPs are nontoxic and biocompatible and show prolonged systemic circulation (Ali et al. 2016). Dextran-coated ultrasmall superparamagnetic iron oxide ferumoxtran-10 nanoparticles used for MRI, have plasma half-life in humans up to 30 h (Arami et al. 2015).

Iron oxide nanoworms (IONWs) are approximately 100 nm strings of iron cores that are PEGylated and coated with dextran (Park et al. 2009). The elongated shape of IONWs extends their half-life in blood circulation and provides enhanced interaction between targeting ligands and their receptors. IONWs can be used for theranostic purposes, as their surface can be readily coated with targeting ligands and drugs, and their magnetic core is compatible with MR imaging (Park et al. 2009; Ruoslahti 2017). Peptide targeted cytotoxic IONWs loaded with proapoptotic peptide $_D$[KLAKLAK]$_2$ have been previously used to treat glioblastoma in orthotopic mouse models (Agemy et al. 2013).

2.4. Summary of the literature

Many people all over the world are diagnosed with age-related brain disorders, and as overall life expectancy is in rise, even more people will be affected by these pathologies. The drugs currently available for the treatment of AD and other neurodegenerative disorders do not slow down the progression of the diseases or cure the patients. Instead, they only temporarily alleviate the symptoms of neurodegeneration. To help the patients and to relieve the burden on health care system and society, early diagnosis methods and more efficient treatments need to be developed.

Treatment of CNS disorders is hampered by the presence of BBB – a highly selective protective barrier impermeable to most potential neuropharmaceuticals. Despite intense research the progress in development of new therapies for neurodegenerative disorders and brain tumors has been excruciatingly slow, mainly because large majority of the drug candidates have failed to reach the extravascular brain parenchyma. But most likely any effective treatment for neurodegenerative disorders would require therapeutic agent to be transported across the BBB, thereby the identification of efficient BBB targeting ligands are of utmost importance. Precision delivery of nanoscale carriers or drugs may allow for effective and site-specific transport of imaging agents, drugs, and other bioactive molecules across the BBB, to diagnose and treat brain tumors and neurodegenerative disorders.

3. AIMS OF THE STUDY

Brain parenchyma is shielded from systemic circulation by the BBB that allows penetration of only a limited number of agents with very specific characteristics. One strategy to improve delivery of drugs and nanoparticles across the barrier is to use homing peptides that interact with systemically accessible transport receptors in the brain. This doctoral thesis focused on the identification and pre-clinical validation of novel peptides, which could be used to target normal central nervous system and different neurological pathologies.

Validation of homing peptides is of critical importance for peptide development to establish whether the targeting takes place and on an adequate level. Targeting peptide validation typically involves *in vitro* and *in vivo* sample manipulation and is prone to artefacts. To improve the validation of homing peptides on cultured cells and *in vivo*, a highly sensitive and internally controlled AgNP-based cellular uptake/biodistribution quantification method was developed. This new method enables analysis of the tissues and cells with minimal prior sample processing.

More specifically the goals were:
1. Development of nanoparticle quantification system based on isotopically tagged AgNPs to study accessible affinity peptide receptors on cells (II)
2. Development of internally controlled AgNP quantification system for nano-particle homing studies (I)
3. Refinement of the AgNP quantification method for *in situ* analysis of tissues (I)
4. Targeting glioblastoma with nanoparticles functionalized with homing peptide (III)
5. Identification of novel peptides to target AD (IV).

4. MATERIALS AND METHODS

4.1. Peptides

Peptides (Table 2) were synthesized using Fmoc/t-Bu chemistry on a micro-wave-assisted automated peptide synthesizer (Liberty, CEM Corporation, NC, USA), purified by HPLC using 0.1% TFA in acetonitrile-water mixture to 90%–95% purity and validated by Q-TOF mass spectral analysis. Fluorescent peptides with blocked C-terminus were synthesized in-house by using 5(6)-carboxyfluorescein (FAM) with 6-aminohexanoic acid spacer attached to the N-terminus of the peptide, or ordered from a commercial supplier (TAG Copenhagen, Denmark).

Table 2: Previously identified peptides used in the *in vitro* and *in vivo* studies.

Peptide sequence	Identification	Target site and receptor	Reference	Publica-tion in thesis
RPARPAR	Prototypic CendR peptide	Lungs, heart, and angiogenic vasculature; receptor are NRP-1 and NRP-2	Teesalu et al. 2009	I-II
CGKRK	*In vivo* phage display on epidermal carcinogenesis mouse model	Angiogenic blood vessels and tumor cells; receptors are p32 and calreticulin	Hoffman et al. 2003	II
AKRGARSTA (LinTT1)	*In vitro* phage display on recombinant human p32 protein	Tumor lymphatics, tumor-associated macrophages, tumor endothelial cells, and tumor cells; receptor is p32	Paasonen et al. 2016	III
CAGALCY	*In vivo* phage display on healthy mice	Brain microvessels; receptor is unknown	Fan et al. 2007	V

4.2. Targeted nanoparticles

Three different nanoparticle platforms were used in the experiments presented in this thesis: AgNPs, IONWs and albumin-paclitaxel NPs (Fig. 7).

Silver nanoparticles
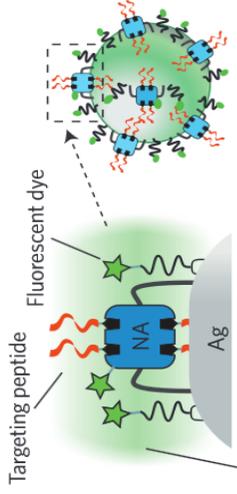
Iron oxide nanoworms

Albumin-paclitaxel nanoparticles

Targeting peptide   Fluorescent dye

- Dextran-coated iron oxide core
- 5 kDa PEG linker
- FAM-LinTT1

nab-Paclitaxel nanoparticle

Cross section
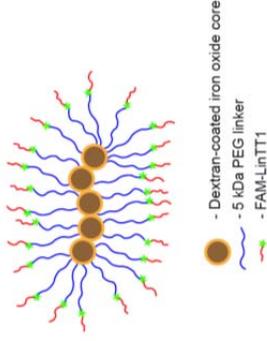
Paclitaxel

Albumin

Paclitaxel
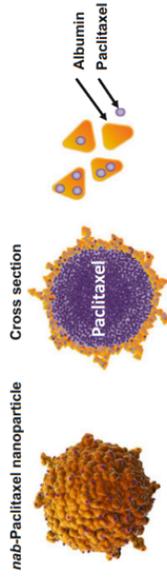
Figure 7: Nanoparticle platforms used in this thesis. Adapted from Braun et al., (2014); Säälik et al., (2019) and Desai (2016).

Ultraviolet-visible (UV-vis) absorption spectrum was analyzed with a Nanodrop 2000c spectrophotometer (Thermo Scientific Inc., Washington, USA) to quantify molarity of AgNPs. Dynamic Light Scattering (DLS; Zetasizer Nano ZS, Malvern Instruments, UK) was used to assess the polydispersity and hydrodynamic radius of the particles. Transmission electron microscopy (TEM; Tecnai 10, Philips, Netherlands) was used to characterize the morphology of the nanoparticles.

4.2.1. Synthesis of nanoparticles

4.2.1.1. Silver nanoparticles

Silver nanoparticles (AgNP) with four different compositions were used in the experiments: wtAg, Ag107, Ag109 and wtAg-Pd (Table 3). AgNP were synthesized in-house using Lee and Meisel citrate method or its modified version (Dadosh 2009; Lee and Meisel 1982). Synthesis of the particles was conducted using natural silver (composed of 51.8% Ag107 and 48.2% Ag109 isotopes) in case of wtAgNPs and wtAg-Pd, or by using isotopically pure silver (Ag107 or Ag109) in case of isotopic AgNPs. WtAg-Pd nanoparticles contained 1% naturally occurring Pd that was added in the form of $Pd(NO_3)_2$ at the end of synthesis process.

Synthesized particles had the Ag core size in the 20–70 nm range. Nanoparticles were functionalized with NeutrAvidin (NA) for biotinylated-peptide conjugation, and with lipoic acid-polyethylene glycol (1k)-NH_2 (PEG) molecules (Braun et al 2014). The terminal amines of PEG were used for coupling of CF555-N-hydroxysuccinimide-dye (NHS-dye). Nanoparticles with targeting ligand were prepared by attaching biotinylated peptides to the particles; in case of control particles the biotin binding pocket was blocked with free D-biotin.

Nanoparticles made of naturally occurring silver were used for optical imaging of NP distribution on tissue sections; fluorescence signal of CF555 dye was detected by confocal microscopy. Silver enhancement procedure that was conducted on tissue sections enabled to visualize silver grains using dark-field microscopy. Atomically distinct nanoparticles Ag107, Ag109 and wtAg-Pd were used for analysis of cells and tissue homogenates by ICP-MS. Ag107 and Ag109 particles were used in homing experiments where silver was quantified from tissue lysates by ICP-MS and tissue sections by laser ablation-ICP-MS.

Extracellular AgNPs can be "etched", or dissolved, using a mild biocompatible solution to differentiate between internalized and surface-bound exposed particles (Braun et al 2014). Etching solution was prepared fresh before each use from 0.2 M stock solutions of $Na_2S_2O_3$ and $K_3Fe(CN)_6$ stored in the dark. A working solution, 10 mM in each component diluted in PBS, was applied to cells for five minutes followed by PBS washes.

Table 3: Nanoparticles used in the experiments.

Nanoparticle type	Size	Synthesis protocol	Applications		
WtAgNP	20–60 nm	Ag-citrate (Lee and Meisel 1982)	*In vitro*	Confocal microscopy	Flow cytometry
			In vivo	Confocal microscopy	Silver enhancement and dark field microscopy
WtAgNP+1% Pd	25 nm	Tannic acid (Dadosh 2009)	*In vitro*	Confocal microscopy	ICP-MS
Ag107NP	25 nm	Tannic acid (Dadosh 2009)	*In vitro*	Confocal microscopy	ICP-MS
			In vivo	ICP-MS	LA-ICP-MS
Ag109NP	25 nm	Tannic acid (Dadosh 2009)	*In vitro*	Confocal microscopy	ICP-MS
			In vivo	ICP-MS	LA-ICP-MS
IONW	100 nm	(Fogal et al. 2015; Miao et al. 2014)	*In vitro*	Flow cytometry	
IONW	100 nm	(Fogal et al. 2015; Miao et al. 2014)	*In vivo*	Confocal microscopy	Pre-clinical therapy
Albumin-paclitaxel NP	130 nm		*In vivo*	Confocal microscopy	

4.2.1.2. Iron oxide nanoworms

NWs were synthesized as previously described (Fogal et al. 2015; Miao et al. 2014). Briefly, aminated NWs were PEGylated with maleimide-5k-PEG-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.

4.2.1.3. Albumin-paclitaxel nanoparticles

Albumin-paclitaxel nanoparticles (Abraxane) were purchased from producer (Celgene). For peptide coupling, 5 mg of albumin-paclitaxel nanoparticles were dissolved in 1 mL of PBS (previously purged with nitrogen) and combined with 0.3 mg (0.6 μmols) of linker sulfosuccinimidyl 4-(N-maleimidomethyl)cyclo-hexane-1-carboxylate in 0.2 mL of PBS. The mixture was stirred for 30 min at RT and modified albumin-paclitaxel nanoparticles were purified using a NAP-10 column (GE Healthcare Life Sciences). 0.25 μmol of LinTT1 peptide with N-terminal extra cysteine residue dissolved in 0.2 mL of PBS was slowly added to the purified albumin-paclitaxel nanoparticles, the mixture was stirred for 1 h at RT and purified by gel permeation chromatography using Sepharose 4B columns (GE Healthcare Life Sciences).

4.2.2. Cell lines

Nine different tumor cell-lines derived from human or mouse tumors (Table 4) were used in the experiments. PPC-1 cells were obtained from the Ruoslahti laboratory at Sanford-Burnham-Prebys Medical Discovery Institute (SBPMDI), and M21 cells were a gift from David Cheresh at University of California San Diego (UCSD). U87-MG GBM and U251 cells were acquired from ATCC, and NCH421K cells from CLS Cell Lines Service GmbH (Eppelheim, Germany). WT-GBM and VEGF-KO GBM cells were a gift from Gabriele Bergers (Leuven, Belgium), P13 spheroids were a gift from Rolf Bjerkvig (Bergen, Norway), and 005 cells were established from murine GBM as previously described in Marumoto et al 2009.

Table 4: Cell-lines used in the *in vitro* and *in vivo* experiments.

Cell-line		Application	Publication in thesis
PPC1	Human prostate adenocarcinoma	*In vitro*	II
M21	Human melanoma	*In vitro*	II
U87-MG	Human glioblastoma	*In vitro* and *in vivo*	III
WT-GBM	Mouse glioblastoma	*In vitro* and *in vivo*	III
VEGF-KO-GBM	Mouse glioblastoma	*In vitro* and *in vivo*	III
005	Mouse glioblastoma	*In vivo*	III
NCH421k	Human glioblastoma	*In vitro* and *in vivo*	III
U251	Human glioblastoma	*In vitro*	IV
P13	Human glioblastoma	*In vivo*	IV

4.2.3. *In vitro* experiments

4.2.3.1. AgNP binding to immobilized proteins

His-tagged NRP-1 b1b2 domain (wt and mutant) were expressed and purified as described (Teesalu et al. 2009). 6× His-Tagged p32 was expressed in Rosetta-gami-2 cells (Novagen). The protein was purified by IMAC using HIS-select resin (Sigma) with an imidizole gradient from 20–300 mM and eluted fractions were analyzed on analytical SDS-PAGE. Sedimentation velocity assay was used to confirm the trimeric state of the recombinant p32 protein. AgNP binding experiments to magnetic beads coated with recombinant proteins were carried out with wtAgNPs. Ni-NTA magnetic agarose beads (Qiagen) in Tris buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.05% NP40, 5 mM imidazole) were loaded, as suggested by the manufacturer, with either the b1b2 binding domain of NRP-1, with p32, or with a b1b2 mutant, followed by multiple washes. NPs were incubated for one hour with the protein-loaded beads in Tris buffer with 1% BSA at room temperature with gentle agitation, followed by washes, and elution with high imidazole concentration buffer (PBS, 400 mM imidazole, 150 mM NaCl, 1% BSA, 0.05% NP40). The eluted samples were analyzed by UV-vis spectrophotometer (NanoDrop 2000c, Thermo Scientific). The spectrum and maximum absorbance at 400 nm were recorded for each sample.

4.2.3.2. Receptor-dependent binding and uptake of AgNPs

Receptor-dependent binding of AgNPs by two cancer cell-lines, M21 and PPC-1, was assessed by flow cytometry and uptake of AgNPs was determined by confocal microscopy. Both are well studied cell-lines with known receptors (Sugahara et al. 2009; Teesalu et al. 2009). PPC-1 cells express high levels of

NRP-1, whereas M21 cells lack the extracellular NRP-1 expression (Teesalu et al. 2009). Both cell lines have p32 protein exposed at the cell surface.

To study cellular uptake, three different types of wtAgNPs were prepared by conjugating biotinylated peptides: biotin-X-RPARPAR or biotin-X-SGKRK-NH_2, or by blocking the biotin binding pocket with free biotin. M21 and PPC1 cells were cultured in DMEM high glucose medium containing 100 IU/mL of penicillin, streptomycin, and 10% of fetal bovine serum (GE Healthcare, UK) in 5% CO_2 atmosphere.

For fluorescence confocal imaging the cells were washed twice with warm medium after which fresh medium with AgNP at 0.2 nM was added. The cells were incubated with NPs for one hour at 37 °C. After incubation the NP containing medium was removed, the cells were washed and fixed with -20 °C methanol for 60 sec. The cells were blocked (PBS, 1% BSA, 0.05% Tween-20) for 30 min at room temperature and co-stained with NRP-1 and p32 antibodies, and DAPI. The cells were imaged using fluorescence confocal microscopy (Zeiss LSM 510; Olympus FV1200MPE, Germany).

For flow cytometry experiments the cells were washed twice with warm medium, and fresh medium with AgNP at 0.2 nM was added. The cells were incubated with NPs for one hour at 37 °C. After incubation the AgNP containing medium was removed and the cells were washed with PBS. Cells were dissociated using Cell Dissociating Buffer (Gibco) and suspended in 300 µl PBS. Analysis was done using BD Accuri flow cytometer by monitoring the 555 channel FL2.

4.2.4. Animal experiments

Animal experimentation protocols were approved by Committee of Animal Experimentation of Estonian Ministry of Agriculture (Permit #48), by the Tel Aviv University Institutional Animal Care and Use Committee, and by the Institutional Animal Care and Use Committee of Sanford Burnham Prebys Medical Discovery Institute.

Athymic nude mice were purchased from Harlan and Balb/c mice were purchased from Charles River. Animal experiments were conducted using healthy mice or mouse models of various CNS disorders. Six different glioblastoma models (Table 5), two AD models, PD model and acute brain injury model were used in the experiments.

4.2.4.1. GBM tumor models

Cell culture reagents were from Gibco (USA) if not stated otherwise. WT-GBM and VEGF-KO-GBM cell lines (Blouw et al. 2003) were cultured in MEM with Earl's salts (Capricorn Scientific, Germany) supplemented with 100 IU/mL of penicillin/streptomycin, 1% sodium pyruvate, 0.01 M HEPES, 0.6% glucose (Applichem, USA) and 5% of fetal bovine serum (GE Healthcare, UK). NCH421k cells (Campos et al. 2010) were cultivated in neurobasal medium complemented with B27 neurobasal supplement (Thermo Fisher Scientific, USA), 100 IU/mL of penicillin/streptomycin, 0.4 mM Glutamax, 20 ng/ml basic FGF, 20 ng/ml EGF and 40 µg/ml of heparin (Sigma-Aldrich, USA). U87-MG cells were grown in DMEM (Lonza, Belgium) containing 100 IU/mL of penicillin, streptomycin, 10% fetal bovine serum, 1% sodium pyruvate and 1% non-essential amino acids (Sigma-Aldrich, USA). P13 spheroids (Fack et al. 2015) were grown in DMEM (Lonza, Belgium) containing 100 IU/mL of penicillin/ streptomycin, and 10% of fetal bovine serum (GE Healthcare, UK) as 3D culture on 0.75% agar coated flasks. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

For induction of orthotopic GBM xenografts in nude mice, 7×10^5 (WT-GBM, VEGF-KO-GBM), 3×10^5 (NCH421k) cells, 3×10^5 (005) cells or 3×10^5 (P13) cells from dissociated spheroids were implanted intracranially in the right striatum of the brain (coordinates: 2 mm laterally and 2 mm posteriorly from bregma and at 2.5 mm depth). For induction of s.c. U87-MG tumors, 4×10^6 cells were injected under the skin of the right flank of nude mice. Intracranial tumors were allowed to develop for 6–7 (WT-GBM), 12–14 (VEGF-KO-GBM), ~30 (NCH421k), 10 (005) or ~35 (P13) days before performing experiments. U87-MG s.c. tumors were allowed to grow until tumors had reached ~ 1 cm^3.

Table 5: GBM tumor models used in the experiments.

Tumor model	Type	Culture conditions	Tumor location	Time of tumor formation	Characteristics
U87-MG	Cell-line xenograft	2D culture	Subcutaneous	~30 days	Poorly invasive and angiogenic
WT-GBM	Genetically engineered mouse model	2D culture	Orthotopic	~7 days	Angiogenic and poorly invasive
VEGF-KO-GBM	Genetically engineered mouse model	2D culture	Orthotopic	~14 days	Infiltrative
005	Genetically engineered mouse model	2D culture	Orthotopic	30–40 days	Invasive and angiogenic
NCH421k	Patient-derived xenograft	3D in neurobasal medium	Orthotopic	~21 days	Moderately invasive and slightly modified vasculature
P13	Patient-derived xenograft	3D in agar-coated flask	Orthotopic	30–40 days	Poorly invasive and angiogenic with areas of pseudo-palisading necrosis

4.2.4.2. Models of CNS disorders

Three transgenic mouse models of neurodegenerative disorders were used: AD models hApp-J20 and Tg2576, and PD model mTHY-1-α-synuclein. All the three transgenic mouse models were derived from C57BL/6 mice. The acute brain injury model was set up as previously described in Mann et al. 2016.

4.2.4.3. *In vivo* phage display

To find AD targeting peptides, CX7C (1e10 pfu) naïve phage library was injected intravenously to hAPP-J20 mice and allowed to circulate for 30 minutes. After circulation, the animals were anesthetized with 2.5% avertin and perfused intracardially with PBS. Hippocampus and control organs were collected and homogenized in LB-NP40 (1%). Bound phages were rescued by amplification in *E. coli* and their genomic DNA was sequenced by Ion Torrent next generation DNA sequencing system.

4.2.4.4. Peptide homing studies

To study AD peptide homing, mice were intravenously injected with 50 nmoles of peptide dissolved in PBS and allowed to circulate for 30 minutes. Mice were then anesthetized and perfused with PBS and all major organs were fixed in 4% paraformaldehyde at pH 7.4 overnight. The organs were washed with PBS and placed in graded sucrose solutions before embedding into optimal cutting temperature (OCT) medium. 10 μm thick sections were prepared by cryosectioning and subjected to immunostaining.

4.2.4.5. Homing of nanoparticles

To study homing of AgNPs, four different types of particles were prepared by attaching the following biotinylated peptides: biotin-X-RPARPAR-OH; biotin-X-RPARPAR-NH$_2$; biotin-X-CAGALCY (X = aminohexanoic acid), or by blocking the biotin binding pocket with free biotin. AgNPs at O.D.50 were suspended in 200 µl of PBS and injected intravenously to Balb/c mice. 1 h or 5 h after i.v. administration the mice were perfused intracardially with PBS. Organs were snap-frozen in liquid N$_2$ for cryosectioning.

For microscopic imaging, 10 μm cryosections were fixed in 4% paraformaldehyde in PBS and stained with DAPI. For silver enhancement, cryosections were fixed in MeOH (-20 °C for 60 s), washed in PBS, and treated with Silver Enhancement kit (Molecular Probes) for 30–40 min with fresh solution added every 13 min. The reaction was stopped in 75 mM Na$_2$S$_2$O$_3$ in water. The nuclei of cells were stained with DAPI, followed by dehydration of sections through graded ethanol series, xylene, and mounting in DPX mounting

medium. The signal in fluorescent confocal images and dark-field microphotographs was quantified with ImageJ freeware.

FAM-labeled NWs were injected intravenously (7.5 mg/kg Fe), and 1 or 5 h post i.v. administration the animals were perfused with DMEM-1% BSA. The organs were collected and snap-frozen in liquid nitrogen for cryosectioning. For confocal microscopy, the tissues were sectioned at 10 μm and stained with antibodies and DAPI.

Iron oxide nanoparticles (NWs) conjugated with DAG peptide (DAG-IONWs) were injected i.v. to 9-month-old hPPP-J20 mice and age-matched WT mice. NWs were allowed to circulate for 5 h before perfusion with PBS. The organs were collected and snap-frozen in liquid nitrogen for cryosectioning. NWs in tissue sections were stained with Prussian blue and the sections were counterstained with nuclear fast red.

4.2.4.6. ICP-MS experiments

Inductively coupled plasma mass spectrometry (ICP-MS) allows extremely high sensitivity analysis of elements and even enables discriminations of different isotopes of the same material (Agilent 2015). Two distinct ICP-mass spectrometry methods were applied for ratiometric analysis of cells and organs: ICP-MS analysis on cells and tissue extracts and laser ablation ICP-MS (LA-ICP-MS) on frozen tissue sections.

For ratiometric cell phenotyping, three atomically different AgNPs: Ag107, Ag109 and wtAg-Pd, and two different cell lines: M21 and PPC1 cells, were used. After incubating cells with AgNPs, the medium was removed, and cells were washed to remove the unbound particles. Cells were dissociated using non-enzymatic Cell Dissociation Buffer (Gibco), transferred to 1.5 mL tubes and disrupted with 2% SDS solution. The samples were analyzed using an Agilent 8800 QQQ ICP-MS.

For ratiometric ICP-MS-based biodistribution experiments, Ag107 and Ag109 isotopic nanoparticles were injected to healthy Balb/c mice. 200 μL of a mixture of peptide–AgNPs (prepared from Ag107) and control biotin-blocked AgNPs (prepared from Ag109) was administered i.v. and allowed to circulate for 1 or 5 hours. Animals were perfused with PBS and organs were collected and snap-frozen. Frozen tissue samples were thawed, digested and analyzed using Agilent 8800 ICP-MS.

For ratiometric LA-ICP-MS-based biodistribution studies, mice were injected i.v. with 200 μL of a mixture of peptide–AgNPs (prepared from Ag107) and control biotin-blocked AgNPs (prepared from Ag109). At indicated time points, the mice were perfused with PBS and the organs were collected, snap-frozen, sectioned at 30 μm on Superfrost+ slides, and air-dried. Determination of Ag107/109 ratio in tissue sections was performed using Cetac LSX-213 G2+ laser ablation system (Teledyne Cetac Technologies, USA) using a HelEx 2-volume ablation cell on Agilent 8800 ICP-MS system.

4.2.4.7. Experimental tumor therapy

For experimental therapy of mice bearing s.c. U87-MG tumors, 1×10^6 cells were injected under the skin of dorsal flank of 11–15-week old male nude mice. Tumor volume [calculated with the formula: V = (width × width × length)/2] and mouse weight was recorded every other day. When tumors reached 100–200 mm^3 in size, the mice were divided in 4 groups (n = 6) so that the total volume of tumors in all groups were equal. Treatment with intravenous FAM-LinTT1-NW, FAM-$_D$(KLAKLAK)$_2$-NW, or FAM-LinTT1- $_D$(KLAKLAK)$_2$-NWs (at 5 mg/kg Fe per injection), or 100 μl of PBS, was started on day 36 after tumor induction. Eight injections in total were performed on every other day. The study was terminated when the first tumor reached 1.5 cm^3. Mice were sacrificed by cardiac perfusion and tissues were snap-frozen for further analysis.

For experimental therapy of mice bearing orthotopic syngeneic 005 tumors, 3×10^5 cells were injected in the hippocampus (coordinates: 1.5 mm laterally and 2 mm posteriorly from bregma and at 2.3 mm depth) of 8–12 weeks old C57BL/6 female mice. Ten days after transplantation of tumor cells, mice were divided in 5 groups (n = 6) and treated intravenously with either FAM-NW, FAM-LinTT1-NW, FAM-$_D$(KLAKLAK)$_2$-NW, or FAM-LinTT1-$_D$(KLAKLAK)$_2$-NWs (at 5 mg/kg Fe per injection), or 100 ul of PBS. Mice were treated for 2 weeks, 7 injections in total performed on every other day. Mice showing symptoms of disease were sacrificed by perfusion and tissues collected for further analysis.

5. RESULTS

## 5.1. AgNP binding to immobilized receptor proteins

Affinity targeted nanoparticles need to bind to their receptors with high specificity. For proof-of-concept experiment, well-established peptide/receptor combinations were used. RPARPAR peptide binds to b1b2 domain of NRP-1 and p32 protein is the known receptor for CGKRK peptide. SGKRK was used instead of CGKRK as replacing the cysteine with a serine avoids potential peptide-dimer formation and cysteine is not required for the binding of the peptide (Agemy et al. 2011). NRP-1 b1b2 with mutated binding pocket that prevents RPARPAR peptide binding (Teesalu et al. 2009) was used as a negative control. Nanoparticles functionalized with RPARPAR peptide (R-AgNPs) bound to wild-type NRP-1 protein but not to mutant NRP-1, or to p32 (Fig. 8). SGKRK-AgNPs (K-AgNPs) bound to immobilized p32 and not to the other two proteins. In this assay, both peptide-guided AgNPs showed very low background binding.
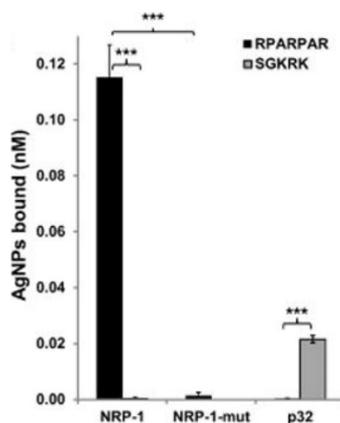
Figure 8: Cell free binding of peptide-AgNPs to recombinant receptor proteins. AgNP binding experiments to recombinant proteins were carried out with wtAgNPs. Ni-NTA magnetic agarose beads were loaded with either the b1b2 binding domain of NRP-1, with p32, or with a b1b2 mutant. NPs were incubated for 1 h with the protein loaded beads, followed by washes, and elution with high imidazole concentration buffer. The eluted samples were analyzed by UV-vis spectrophotometer. The spectrum and maximum absorbance at 400 nm were recorded for each sample. Data represent mean values $\pm$ SD (n=3); *** $p < 0.001$ by Student's t-test.

5.2. Receptor-dependent binding and uptake of nanoparticles by cultured tumor cells

NRP-1 and p32 are commonly present on the surface of cancer cells. Binding and internalization of peptide-functionalized AgNPs was tested using two tumor cell lines: PPC1 prostate carcinoma cells, which express both receptors, and M21 melanoma cells, which express only p32. To enable the detection by flow cytometry and fluorescence microscopy the AgNPs were labeled with CF555 fluorescent dye before adding the peptide. Flow cytometry-based assay showed that R-AgNPs bound specifically to NRP-1 expressing PPC1 cells ($66 \pm 1\%$ positive), but not to M21 cells that do not express NPR-1 (Fig. 9). As expected, both cell lines bound K-AgNPs ($7 \pm 1\%$ positive for M21 and $8 \pm 1\%$ for PPC-1 cells) because p32 is present on the surface of both cell lines. Control particles (B-AgNPs) showed only background binding in both cell lines.
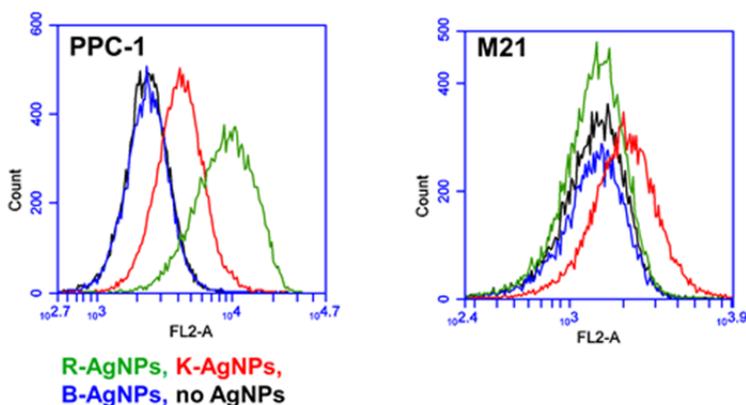
Figure 9: Receptor-dependent binding and uptake of nanoparticles in PPC-1 and M21 cells. For flow cytometry experiments, AgNPs were labeled with the fluorescent dye CF555. The AgNPs were tested individually for binding against two cancer cell lines: PPC-1 and M21. Cells were incubated for 1 h with R-, K-, or B-AgNPs at 0.2 nM. After incubation with AgNPs, the cells were washed, dissociated from the culture dish and analyzed for AgNP binding with a flow cytometer by monitoring the 555 channel: FL2. (n=3).

Next, the uptake of nanoparticles in M21 and PPC1 cells in co-culture was tested to assess whether the two cell lines can be phenotypically distinguished based on their nanoparticle uptake pattern (Fig. 10). R-AgNPs co-localized with NRP-1 on the surface and in endocytic vesicles of PPC-1 cells, whereas M21 cells did not display any signal from the R-AgNPs. In contrast, both cell lines bound K-AgNPs in agreement with the fact that both express p32. Control B-AgNPs showed only weak background signal in both cell lines. These results demonstrate that AgNP tropism could be accurately directed by attaching targeting peptide ligands to the NP surface.
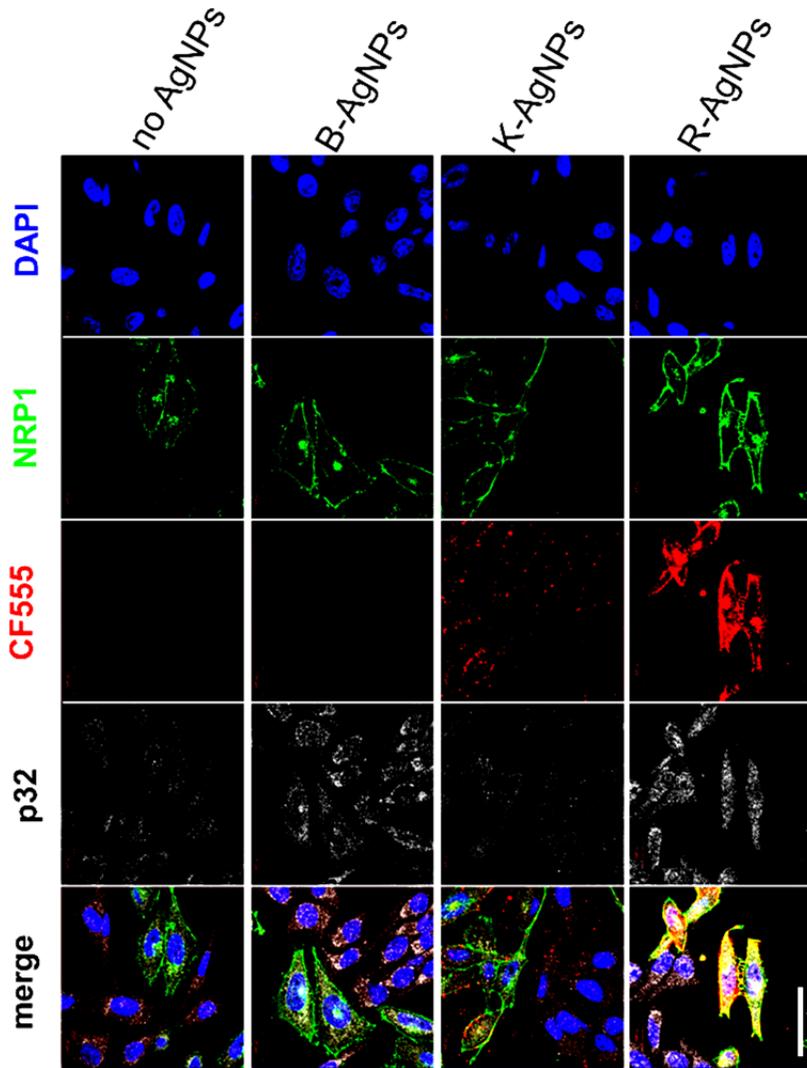
Figure 10: Receptor-dependent binding and uptake of nanoparticles by cultured PPC-1 and M21 cells. Fluorescence confocal microscopy of M21 and PPC-1 co-cultured cells incubated with R-, K-, or B-AgNPs at 0.2 nM. Attached cells were incubated with AgNPs for 1 h. The cells were stained with anti-NRP1-1 and anti-p32 antibodies and DAPI. AgNPs were labeled with CF555 fluorescent dye. The cells can be distinguished by the high amount of NRP-1 protein on the surface of PPC-1 cells and absence of NRP-1 in M21 cells. Note uptake of R-AgNPs in NRP-1-positive PPC-1 cells and binding of both PPC-1 and M21 cells by K-AgNPs. Scale bar = 50 μm.

5.3. Identification of Alzheimer's disease targeting peptide

To identify homing peptides for targeting AD, *in vivo* T7 phage screens on AD mouse model hAPP-J20 were conducted (Fig. 11a). T7 CX7C library was intravenously injected to transgenic mouse and age-matched wild-type mice in four different age groups: 3, 5, 7 and 9 months. Significant difference in phage counts recovered from AD brain compared to wt brain was seen only for 9-month-old age group, where AD brains with full-blown disease had four-fold higher phage titers than normal brains from wt mice (Fig. 11b).

Recovered phages were analyzed by high-throughput DNA sequencing to map the landscape of phage displayed peptides in AD and control brains in all the age groups. Data mining revealed a consensus motif (A/G)BB(N/Q) (where B is a basic amino acid) that was enriched in all the age groups of hAPP-J20 mice, but absent in the wt controls (Fig. 11c). One of the peptides from 9-month AD selected phage pool, CDAGRKQKC ("DAG"), was selected for subsequent studies as it agreed with the consensus sequence and contained the most commonly occurring amino acids in the adjacent positions.
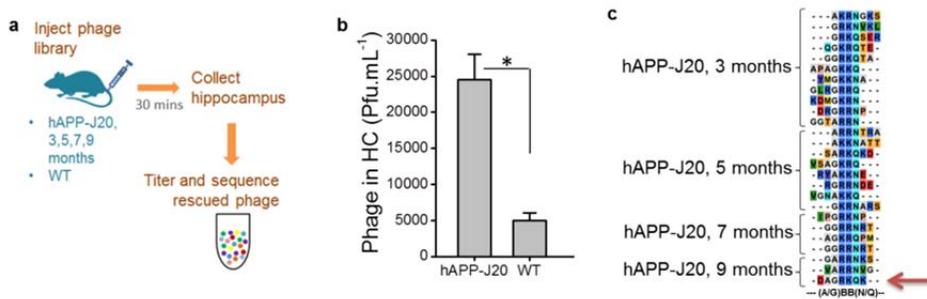
Figure 11: Identification of AD-homing DAG peptide by *in vivo* phage display. Schematic representation of phage screening done in transgenic hAPP-J20 mice. (a) A CX7C library (10^9 pfu) was injected intravenously in hAPP-J20 mice and wild-type (wt) littermate controls of different ages. After 30 minutes of circulation and perfusion to remove unbound phage, the hippocampus (HC) was excised, and phages were recovered and quantified. (b) The phage DNA was subjected to high-throughput sequencing, and the sequences present in the hAPP-J20 brains were compared to sequences from wt brains. (c) Analysis of the hAPP-J20-specific sequences revealed a consensus motif, (A/G)BB(N/Q) (where B is a basic amino acid). A cyclic 9-amino acid consensus peptide (red arrow) containing this motif (sequence CDAGRKQKC; abbreviated "DAG") was chemically synthesized.

### 5.3.1. DAG peptide homing to the AD lesions modeled in mice

To see whether the new cyclic DAG peptide is able to target AD lesions, synthetic fluorophore-labeled peptide was injected intravenously in hAPP-J20, Tg2576, and age-matched wt mice (Fig. 12). DAG peptide homed specifically to the brains of both AD models, whereas the brains from wt mice remained negative. In AD lesions, DAG accumulated in GFAP-positive activated astrocytes surrounding the Aβ plaques and blood vessels with adjoining glial cells (Fig. 12a). In the Tg2576 model, peptide showed co-localization with endothelial cells. Besides senile plaques, Aβ peptides are known to accumulate in small blood vessels in the AD brain; this phenomenon is particularly pronounced in aged Tg2576 mice (Fig. 12b) (Milner et al. 2014).
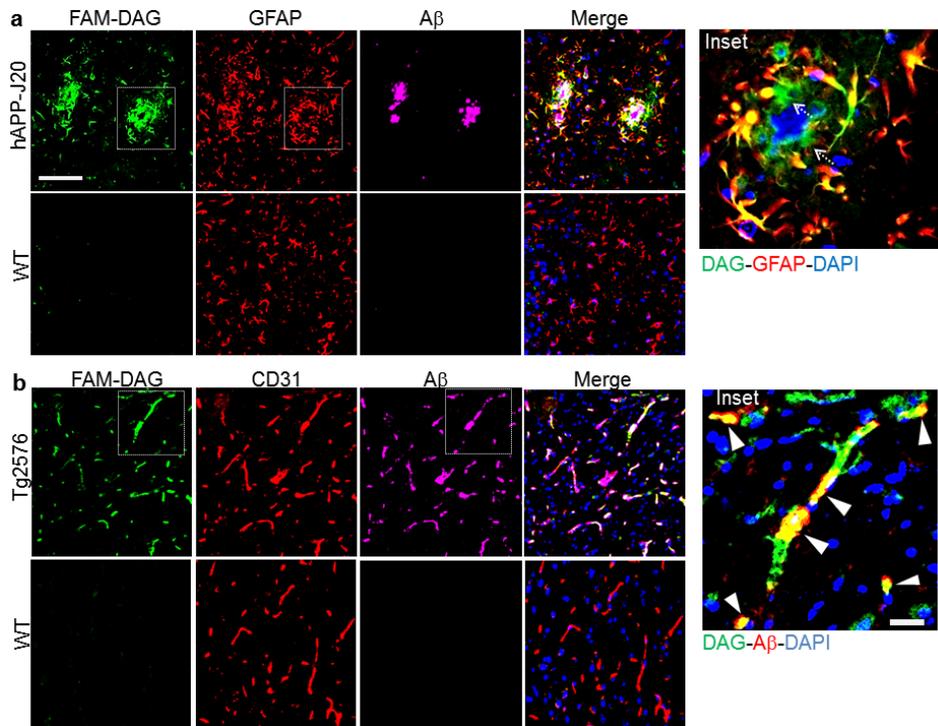
Figure 12: Homing of DAG peptide to the brains of AD model mice. (a) DAG targets activated astrocytes in AD mouse brain. FAM-DAG was intravenously injected to 9-month-old hAPP-J20 and wt mice and allowed to circulate for 30 minutes. The mice were perfused, and the brains were fixed, sectioned and stained for FAM in DAG (green), GFAP (red) and Aβ (magenta). The region shown is the hippocampus. The inset shows co-localization between DAG and GFAP, and also DAG⁺ and GFAP⁻ regions (arrows). (b) DAG targets vessels positive for Aβ in Tg mice. DAG was injected intravenously to 20-month-old Tg2576 wt mice, and processed as in panel a. The brain sections were stained for FAM (green), CD31 (red) and Aβ (magenta). The region shown is the cerebral cortex. The inset shows co-localization of DAG and Aβ (yellow, arrowheads). Scale bars, 50 μm (a and b), 20 μm (insets).

5.3.2. DAG homing to other models of neuroinflammation

Since DAG peptide in AD models showed co-localization with activated astrocytes, it was decided to test the homing in other models of neuroinflammation where reactive astrocytes are similarly present. DAG peptide showed high accumulation in P13 PDX glioblastoma, where it co-localized with GFAP-positive cells (Fig13a). For the brain injury model, DAG peptide homed to perilesional area containing activated astrocytes (Fig13b). Lastly, also in PD model DAG peptide showed preferential accumulation in GFAP-positive astrocytes (Fig13c). These experiments demonstrated that DAG targets activated astrocytes in both acute and chronic models of neuroinflammation.
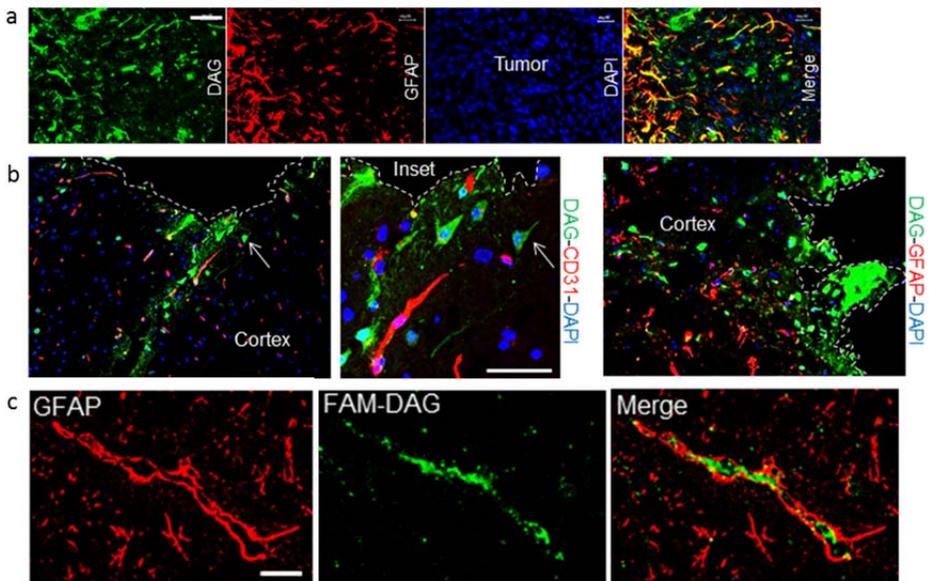

Figure 13: DAG accumulates in neuroinflammatory lesions of different etiologies.
(a) DAG homes to glioblastoma. FAM-DAG was i.v. injected in mice bearing an angiogenic glioblastoma derived from a patient (P13), and allowed to circulate for 30 minutes, after which the mice were intracardially perfused, the tumors were fixed, sectioned and immunostained for FAM (green), and GFAP (red). Scale bar, 40 μm. (b) DAG homes to acute brain injury. FAM-DAG was injected intravenously in mice with penetrating brain injury, processed as in panel a. Representative immunofluorescence images of brain sections from the injury area stained for FAM (green), and CD31 (red; left and middle panels) or GFAP (red; right panel) show stellate-shaped cells positive for DAG (see arrow). Scale bar, 40 μm. (c) DAG homes to the brain in a Parkinson's disease mouse model. FAM-labeled DAG or control peptide was intravenously injected to Parkinson's disease mice (mTHY-1-α-synuclein model, 1-year old) and processed as in panel a. Brain sections from DAG-injected mice were stained for FAM (green) and GFAP (red). DAG signal associates with activated astrocytes. Scale bar, 40 μm. *P<0.05.

5.4. Receptor-dependent homing of nanoparticles

5.4.1. Lung targeting in healthy mice

Systemically injected RPARPAR peptide homes to lung microvasculature in healthy animals (Teesalu et al. 2009). To test whether AgNPs functionalized with RPARPAR peptide target lungs as expected, polydisperse AgNPs particles with CF555 fluorescent label were used (Fig. 14). Wt-AgNPs were functionalized with either RPARPAR-OH or RPARPAR-NH_2 peptides. Amidated RPARPAR peptide is a control that does not bind to NRP-1 and thereby does not home to the lungs. Animals were injected with AgNPs and 5 hours after administration, the animals were perfused intracardially to remove unbound circulating particles. Fluorescence confocal imaging was used to study biodistribution of the AgNPs. Signal from the particles was quantified using ImageJ freeware.

RPARPAR-OH-AgNPs accumulated in lungs as expected, showing 7 times higher fluorescence signal compared to control particles (Fig. 14). This result was replicated by silver enhancement and imaging by dark-field microscopy (not shown).
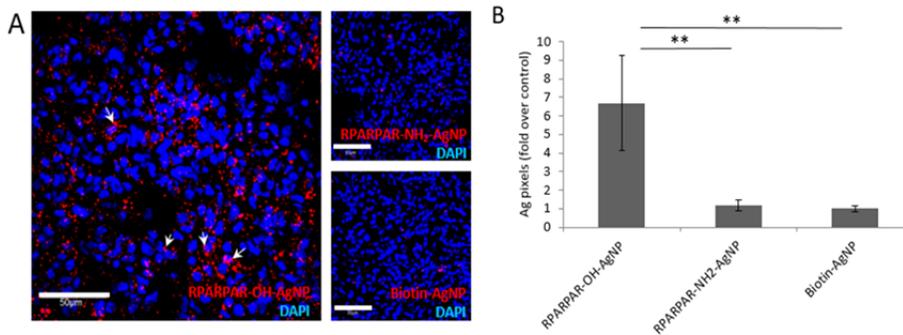
Figure 14: Systemic CF555-labeled RPARPAR-OH-AgNPs home to lung tissue.
CF555-labelled RPARPAR-OH-AgNPS and biotin-AgNPs in 200 µl PBS were intravenously injected to balb/c mice. After 5-h post i.v. administration, the animals were intracardially perfused with 15 ml PBS to wash out blood and to remove unbound AgNPs, and the organs of interest were snap-frozen. (A) Confocal imaging of lung cryosections from mice injected with CF555-AgNPs, 5 h post i.v. administration. Arrows point at areas of accumulation of CF555-positive RPARPAR-OH AgNPs. N=3; scale bars = 50µm. (B) Quantitation of the CF555 fluorescence in lung tissue sections. AgNP signal (red in panel A) expressed as percent of pixels with intensity above threshold. Five randomly chosen fields from 3 animals/group were analyzed. Data represent mean ± SD, ** $p < 0.01$.

5.4.2. Brain targeting in healthy mice

To verify that our AgNP platform is also suitable for targeting central nervous system, AgNPs functionalized with previously identified brain targeting peptide CAGALCY (Fan et al. 2007) were used. CAGALCY-AgNPs were injected intravenously and 5 h post administration biodistribution of AgNPs was assessed. CAGALCY-AgNPs accumulated in discrete areas in the brain parenchyma, quantitative analysis demonstrated ~9-fold increase of the fluorescence signal in the brain regions positive for these NPs than of control biotin-AgNPs (Fig. 15).
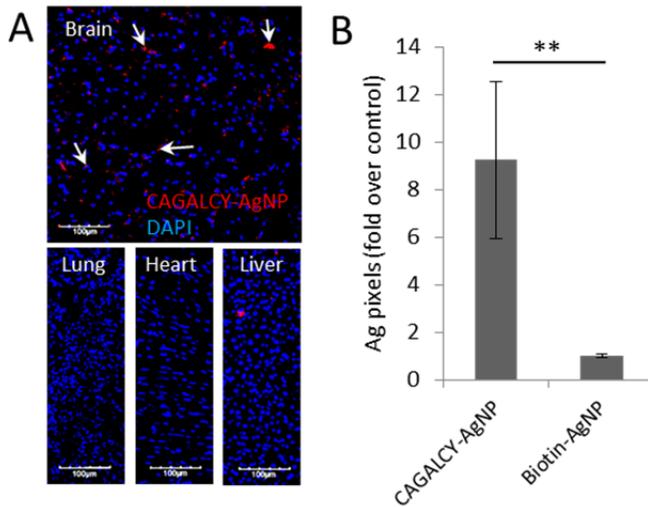

Figure 15: Biodistribution of systemically administered CAGALCY-AgNPs.
(A) Confocal imaging of tissues from mice injected i.v. with CAGALCY-functionalized AgNPs labeled with CF555 fluorescent dye, 5h post i.v. administration. Arrows point at areas of accumulation of CAGALCY AgNPs (red). Scale bars = 100μm. (B) Quantitation of CF555 fluorescence in tissue sections. Ag-positive pixels/field were plotted for each type of nanoparticle as fold over control. N=3; 5 fields were analyzed per tissue. Data represent mean ± SD, **p < 0.01.

5.4.3. DAG-mediated payload delivery to AD lesions

To test if previously identified DAG peptide could be used for site-specific delivery to AD lesions, iron oxide nanoworms (IONW) were used. DAG-coated IONWs were administered intravenously in 9-month old hAPP-J20 and wt mice, and 5 h post administration NW homing to different parts of brain was assessed (Fig. 16). NW accumulation was seen in the hippocampus and the cortex of AD mice but no nanoparticle signal was seen in wt control mice. This result demontrated the utility of DAG peptide to carry payload to AD brains.
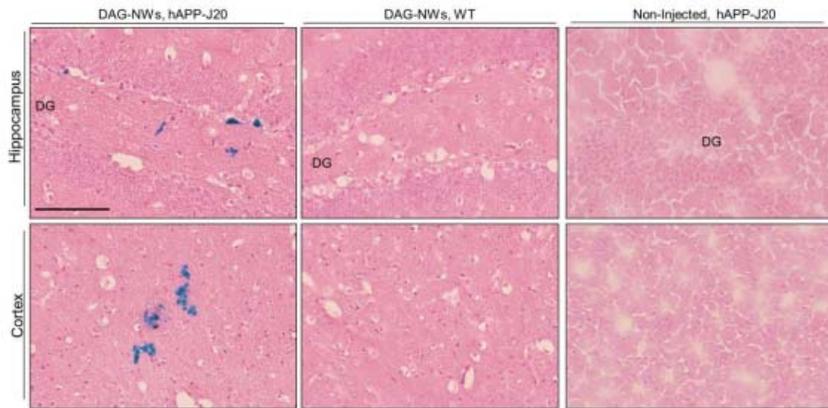
Figure 16: DAG-mediated delivery of nanoparticles to AD lesions. NWs conjugated with DAG (DAG-NWs) were injected i.v. into 9-month-old hAPP-J20 mice and allowed to circulate for 5 hours before perfusion (n=2). In the brain sections shown, NWs were visualized by Prussian blue staining and the sections were counterstained with nuclear fast red. Shown are representative sections with DAG-NWs in the hippocampus (top panel) and cortex (bottom panel). The controls include DAG-NWs injected into age-matched wt mice and non-injected hAPP-J20 mice. DG: dentate gyrus. Scale bar – 100 µm. DG: dentate gyrus.

5.4.4. p32-dependent precision targeting of glioblastoma

5.4.4.1. p32 expression in glioblastoma mouse models

Mitochondrial protein p32 is intracellular in normal cells, but in highly activated cells in tumors and atherosclerotic plaques, it is also exposed on the cell-surface. Extracellular presentation of the p32 has been detected both in tumor macrophages and in cancer cells (Fogal et al. 2008). It has been demonstrated that compared to the normal brain, malignant cells and tumor macrophages in lentivirally-induced 005 transgenic GBM model show upregulated cell surface expression of p32 (Agemy et al. 2013). To assess the relevance of p32 protein for GBM targeting, the distribution of total (Fig. 17a) and systemically accessible p32 (Fig. 17b) in a panel of four GBM models (orthotopic WT-GBM, VEGF-KO, NCH421k and subcutaneous U87-MG) was studied. Whereas in orthotopic tumors, the strongest signal was seen in the center of the tumor, in s.c.U87-MG tumors the p32 immunoreactivity was more pronounced at the tumor rim. To assess systemically accessible p32 rabbit polyclonal anti-p32 antibody was administered intravenously (Fig. 17b). In mice bearing WT-GBM, stronger signal was visible in the tumor periphery and for U87-MG the p32 antibody was predominantly seen at the rim of the tumor.

These results demonstrated upregulation of total p32 and presence of systemically accessible p32 in GBM models and suggested that p32 targeting peptides may be relevant for targeting glial tumors.
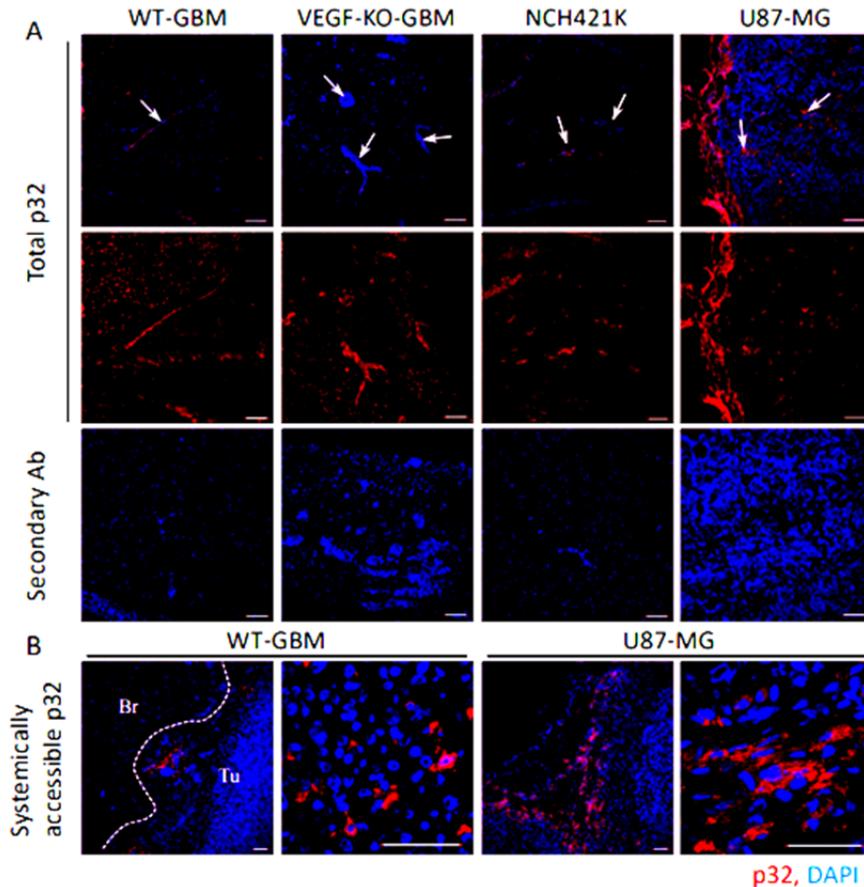
Figure 17: Expression of total and systemically accessible p32 in GBM models. (A) Mapping of total p32 in GBM tissue sections. Cryosections of orthotopic (WT-GBM, VEGF-KO- GBM, NCH421k) and subcutaneous tumors (U87) were stained with rabbit anti-p32 antibody and the nuclei were counterstained with DAPI. Arrows in the upper panel indicate p32-positive areas that colocalize with tumor nodules in GBM lesions. Middle panel – confocal planes with p32 staining only. Lower panel – staining with secondary antibody only. (B) Mapping of systemically accessible p32. Mice bearing orthotopic WT-GBM or subcutaneous U87 tumor were injected i.v. with 200 µg of anti-P32 antibody and let circulate for 20 minutes. Cryosections from tumors were stained by secondary anti-rabbit Alexa 546 antibody to visualize the tissue-retained anti-p32 antibody. Br – brain, Tu – tumor. Scale bars: 100 µm and 50 µm in low and high magnification fields, respectively.

5.4.4.2. LinTT1-guided NWs home to GBM tumors

Elevated expression of total p32 and presence of extracellular p32 on GBM tumors demonstrated feasibility of targeting p32 in brain tumors. LinTT1 is a tumor homing peptide that was identified from *in vitro* phage display on immobilized p32 protein (Paasonen et al. 2016). LinTT1 is a tumor penetrating peptide that contains a cryptic CendR motif. After binding to p32 cell surface proteases cleave the peptide exposing C-terminal RPAR peptide that interacts with NRP-1, leading to vascular leakage and tissue penetration (Sugahara et al. 2009; Teesalu et al. 2009) LinTT1 peptide targets tumor endothelial cells, macrophages, tumor lymphatics and tumor cells (Paasonen et al. 2016).

FAM-LinTT1 or FAM alone was conjugated to iron oxide nanoworms. Functionalized NWs were administered in mice with phenotypically diverse GBM xenograft tumors. Peptide-guided NWs were accumulating in all the different tumors, whereas control particles demonstrated only background signal (Fig. 18). Increased uptake of peptide-guided NWs was seen only for malignant tissue; in control organs, including the brain, the fluorescent signal of LinTT1-NWs was at the same level with non-guided NWs.
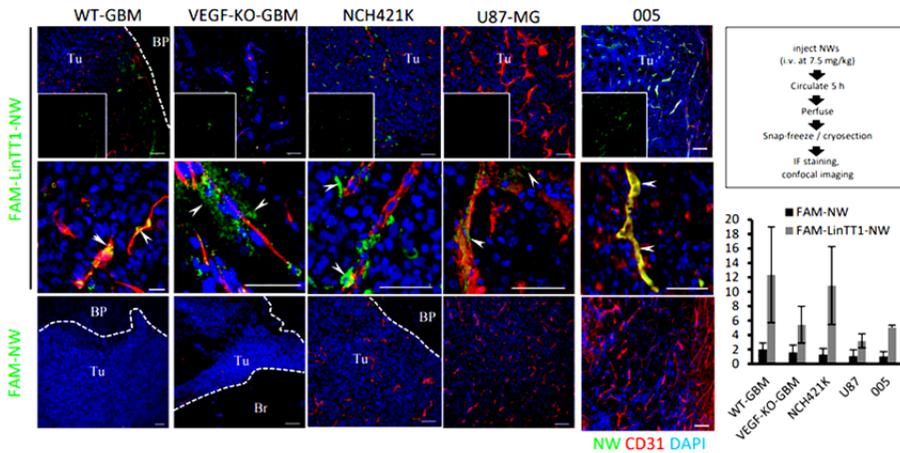
Figure 18: Systemic FAM-LinTT1-iron oxide nanoworms home to mouse and human GBM tumors implanted in nude mice. Mice bearing GBM xenografts of mouse (005, WT-GBM, VEGF-KO-GBM – intracranial) or of human origin (NCH421k – intracranial, U87-MG – subcutaneous) were injected intravenously with 7.5 mg/kg FAM-LinTT1-NW or FAM-NW, and allowed to circulate for 5 h followed by cardiac perfusion of the animals. Cryosections from subcutaneous tumor or coronal cryo-sections from brain with GBM were stained by anti-FAM (NWs), anti-CD31 (blood vessels), and DAPI and visualized by confocal microscopy. Tu – tumor, BP – brain parenchyma. Insets show the FAM channel alone. Arrowheads indicate LinTT1-NW signal. Scale bars – 100 µm in low magnification panels; 50 µm in high magnification panel. FAM-LinTT1-NW and FAM-NW signal intensity in GBM tissue was quantified from 6–9 confocal images and analyzed by ImageJ. Statistical analysis was performed by one-way ANOVA. Error bars: standard deviation.

5.4.4.3. LinTT1 guided Abraxane homes to GBM

To demonstrate that GBM homing by LinTT1 guided NPs is not dependent on nanoparticle platform, LinTT1 peptide was conjugated to nanoformulated paclitaxel-albumin. Mice bearing WT-GBM tumors were administered FAM-LinTT1-Abraxane or control FAM-Abraxane particles without the peptide. Fluorescence signal of LinTT1-Abraxane was detected in tumor blood vessels and tumor parenchyma, whereas non-guided Abraxane showed no accumulation in the GBM tissue (Fig. 19).
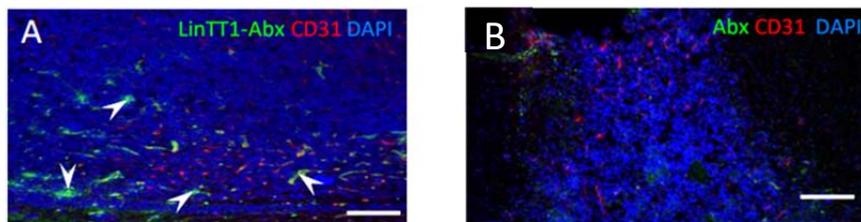
Figure 19: LinTT1-guided Abraxane home to GBM lesions. Systemic LinTT1-functionalized Abraxane nanoparticles home to WT-GBM glioma. Mice bearing ortho-topic WT-GBM glioma were injected intravenously with 0.5 mg of FAM-labeled LinTT1-Abraxane conjugate (A) or FAM-Abraxane (B), after 5h animals were per-fused, and tissue snap-frozen for microscopy. Coronal cryosections from PFA-fixed brain with tumor were stained with anti-FAM (green, FAM-LinTT1-abraxane or FAM-Abraxane) and anti-CD31 (red, blood vessels) antibodies, nuclei were counterstained with DAPI (blue), and sections were imaged by confocal microscopy. Tumor is seen as an area of high nuclear density. Scale bar: 100 μm.

5.5. ICP-MS-based quantification of AgNPs

5.5.1. Phenotyping of cells using isotopically barcoded AgNPs

As shown before with co-culture of PPC1 cells and M21 cells, binding of targeted nanoparticles can be used to phenotypically distinguish cells (Fig. 10). To audit targeting peptide receptors on cells a highly sensitive tool based on isotopic AgNPs and ICP-MS quantification was developed. AgNPs were prepared from >99% pure silver isotopes (Ag107 or Ag109), or from wtAg-Pd particles that contained naturally occurring wild-type silver (wtAg), made up of 51.8% Ag107 and 48.2% Ag109 isotopes, doped with 1% Pd. Cells were incubated with a mixture of homing peptide-functionalized isotopically bar-coded AgNPs, uptake of particles was quantified by ICP-MS (Fig. 20).
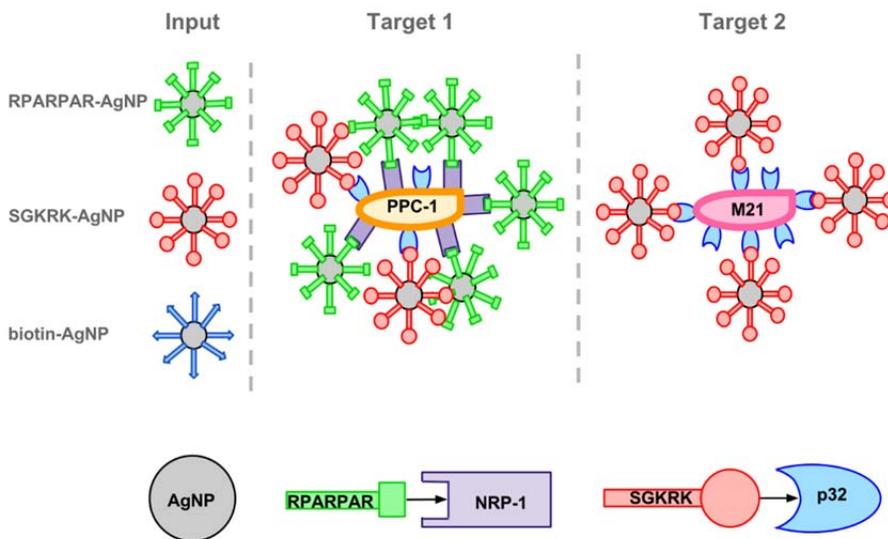
Figure 20: The principle of multiplexed ratiometric AgNP-based test system. The triplex system shown here is based on two targeting peptides: RPARPAR and SGKRK, and two biological targets: NRP-1 and p32. M21 cells express cell surface p32, whereas PPC-1 cells expose both p32 and NRP-1 on the cell surface. Control biotin-AgNPs do not bind to either cell line and serve as a negative control. When an input of three different AgNPs is applied to the cells, their cell binding and uptake pattern will correlate with availability of accessible cell surface receptor.

To make sure that composition of NP core does not affect the functionality of targeted AgNPs, all the three isotopically-tagged AgNPs were functionalized with RPARPAR peptide and cells were incubated with the particles. Independent of core type, all RPARPAR functionalized AgNPs bound specifically to PPC1 cells and not to M21 cells. ICP-MS was able to correctly identify different Ag isotopes and Pd doping (Fig. 21a).

As a next step, isotopic AgNPs were functionalized with SGKRK and RPARPAR peptides and biotin-AgNPs were used as non-targeted control. Three types of AgNPs were mixed and applied to M21 and PPC1 cells. Uptake of AgNPs was quantified, after removing unbound and extracellular AgNPs by etching and washing (Fig. 21b). $75 \pm 5\%$ of AgNPs internalized by PPC1 cells were RPARPAR particles and the remaining $25 \pm 5\%$ were SGKRK particles. M21 cells were preferentially internalizing SGKRK particles ($89 \pm 9\%$) and only a small amount of RPARPAR particles ($11 \pm 1\%$). The amount of biotin-AgNPs remained under detection limit, low background binding expected based on previous experiments was further reduced by etching of the extracellular particles. These results demonstrated the potential of multiplexing isotopic particles for assessing simultaneously the presence of multiple receptors on cultured cells.
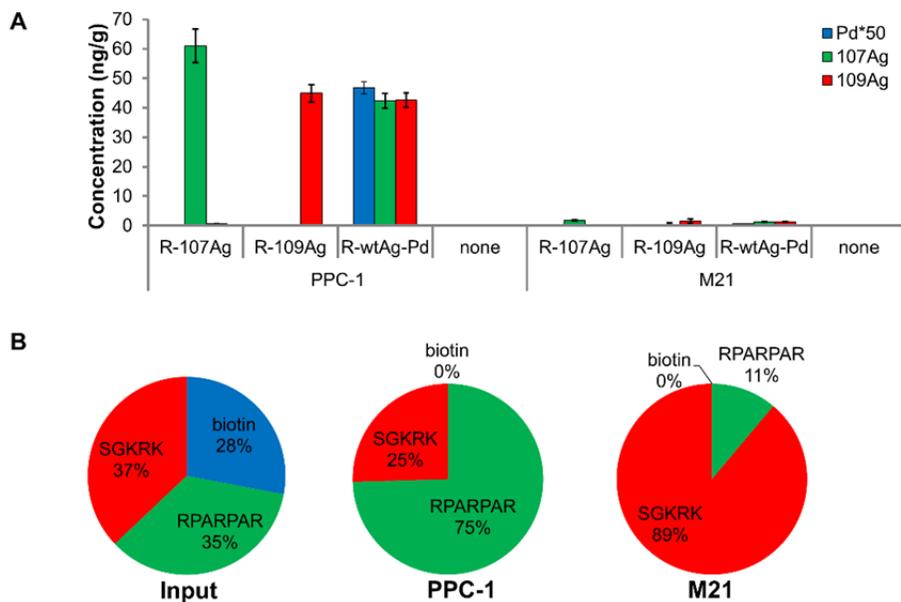
Figure 21. Isotopic multiplexing of peptide-AgNPs after incubation with M21 and PPC-1 cells. A) Three isotope-tagged RPARPAR-coupled silver cores were incubated with PPC-1 or M21 cells for 1 hour and total bound AgNPs were quantified by ICP-MS. Each of the three silver cores functionalized with RPARPAR peptide showed robust PPC-1 binding and low M21 binding. The Pd concentration is multiplied by 50 to visualize more clearly; the R-wt-Ag-Pd sample's Pd content of 0.94 ± 0.04 ng/g is 100 times above the detection limit. Data represent mean values $\pm$ SD (n=3). B) Cells incubated with isotopically coded AgNPs (R-107AgNPs, K-109AgNPs, B-wtAg-Pd), followed by etching to remove the extracellular nanoparticles and quantification of the internalized nanoparticles by ICP-MS. PPC-1 cells demonstrated preferential uptake of R-AgNPs and M21 cells took up mainly K-AgNPs (n=3).

5.5.2. Application of ratiometric ICP-MS for
AgNP *in vivo* homing studies

Cell phenotyping with isotopically barcoded AgNPs suggested applications of the same technology for *in vivo* homing studies. For proof of concept animal experiments with Ag107NPs and Ag109NPs were used. Ag107NPs were functionalized with targeting peptides and Ag109NPs were blocked with biotin. To determine ratio of Ag107/109 from tissue extracts ICP-MS was used that provides information on the average representation of the isotopic particles in the tissue (Fig. 22a). LA-ICP-MS analysis of frozen tissue sections was used to study spatial distribution of AgNPs in tissues (Fig. 22b).
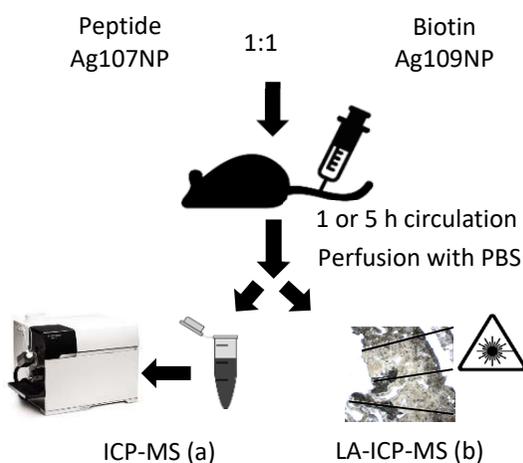
Figure 22: ICP-MS-based ratiometric analysis of homing experiments with isotopically-barcoded AgNPs. Balb/c mice were injected in tail vein with an equimolar mix of peptide-AgNPs (Ag107) and biotin-AgNPs (Ag109) in 200 µl PBS. 1 h or 5 h post i.v. administration, the animals were perfused with 15 ml PBS to remove free plasma AgNPs, and the organs of interest were snap-frozen. Tissues were subjected to HNO_3/H_2O_2 extraction protocol for ICP-MS or cryosectioned for Laser Ablation ICP-MS (LA-ICP-MS).

5.5.2.1. ICP-MS analysis of whole organs

To study biodistribution of targeted and non-targeted isotopic AgNPs in healthy animals, Ag107 particles were functionalized with either lung-homing RPARPAR or brain-homing CAGALCY peptide. Equal amount of targeted Ag107-AgNPs and control biotin-AgNPs were i.v. injected in healthy animals. Preferential accumulation of RPARPAR-AgNPs was detected in lungs, ~9 fold higher amount of targeted particles were found in lungs at the one hour time point, after five hours the ratio over control particles had been reduced to ~5 (Fig. 23a), which agreed well with the results from confocal imaging (Fig. 15b). CAGALCY-AgNPs were overrepresented in the brain but the difference was lower, than seen by optical imaging, this phenomenon was likely caused by heterogeneous homing pattern of CAGALCY and averaging of the whole brain extract (Fig. 23b).
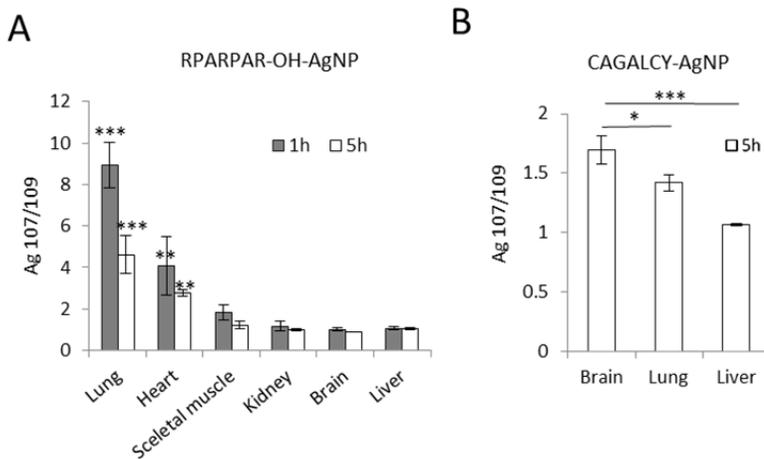
Figure 23: ICP-MS analysis of tissue distribution of homing peptide-functionalized AgNPs. (A) ICP-MS analysis of tissue distribution of RPARPAR-OH-AgNPs after 1 or 5 h post i.v. administration. Data represent mean values ± SD (n=3). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. (B) ICP-MS-based analysis of tissue distribution of CAGALCY AgNPs. Balb/c mice were intravenously injected with an equimolar mixture of targeted CAGALCY AgNPs and biotin-AgNPs. 5-h post i.v. administration the animals were intracardially perfused with PBS, and the extracts of organs were subjected to ICP-MS to for relative quantitation of silver isotopes. Data represent mean ± SD (n=3), *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

5.5.2.2. LA-ICP-MS based analysis of spatial homing

5.5.2.2.1. *In situ* analysis of of RPARPAR-AgNPs in the lungs

Homing pattern of targeted nanoparticles can be very heterogeneous and if averaged, the difference compared to non-targeted payloads is strongly diminished. To study heterogeneous accumulation in tissues, LA-ICP-MS based spatial analysis of cryosections was used. Heterogeneous homing of RPARPAR-AgNPs in the lungs was observed, more particles were accumulating in regions close to pulmonary vasculature and bronchioli with Ag107/109 ratio over 8 (Fig. 24a). When Ag107/109 ratio from multiple ablation paths was averaged, the resulting Ag107/109 value from lungs was similar to the value obtained from tissue extract: ~6.1 with LA-ICP-MS vs ~5 with ICP-MS. In contrast, the ratio of Ag107/109 in the liver, a control organ, was ~1 with both analysis methods.
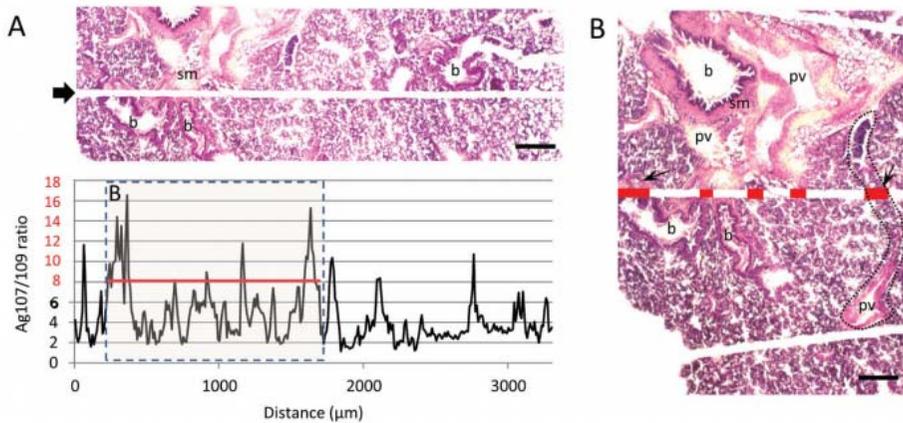
Figure 24: Ratiometric LA-ICP-MS profiling in lung cryosections. (A) Balb/c mice were injected i.v. with 200 μL of a mixture of RPARPAR-OH-Ag107NPs and control biotin-Ag109NPs. At 5 h time point mice were perfused, and organs collected. Tissues were snap-frozen, sectioned at 30 μm, and laser ablation line scans using 40 μm spot were performed. Hematoxylin–eosin staining of a lung cryosections used for LA-ICP-MS. The laser ablation path is indicated by arrow. Note the microanatomical heterogeneity of the lung section. The region of the graph indicated by the dashed box and pink shading correspond to the inset showing the laser ablation path in panel B. (B) Areas along the ablation path with Ag107/109 > 9 are indicated in red. Elevated Ag107/109 ratios are associated with vascular and bronchial structures. Data are representative of 16 laser ablation paths. Scale bars: (A) 200 μm; (B) 100 μm. b: bronchiole, sm: smooth muscle, pv: pulmonary vessel.

5.5.2.2.2. *In situ* analysis of of CAGALCY-AgNPs in the brain

To study heterogeneous accumulation of CAGALCY AgNPs in brain tissue LA-ICP-MS-based spatial analysis of cryosections was used. Mice injected with a mixture of CAGALCY and control AgNPs demonstrated overrepresentation of CAGALCY AgNPs in several areas in the brain, such as fibrillary tracts and pontine microvessels (Fig. 25). In these areas, the ratio of Ag107/109 was remarkably higher, between 15 and 30, compared to result from ICP-MS where ratio remained under 2.

These results demonstrate the importance of tissue heterogeneity in the homing of targeted NPs to tissue. Spatial distribution analysis provides valuable information to validate new affinity ligands and study targeted therapies.
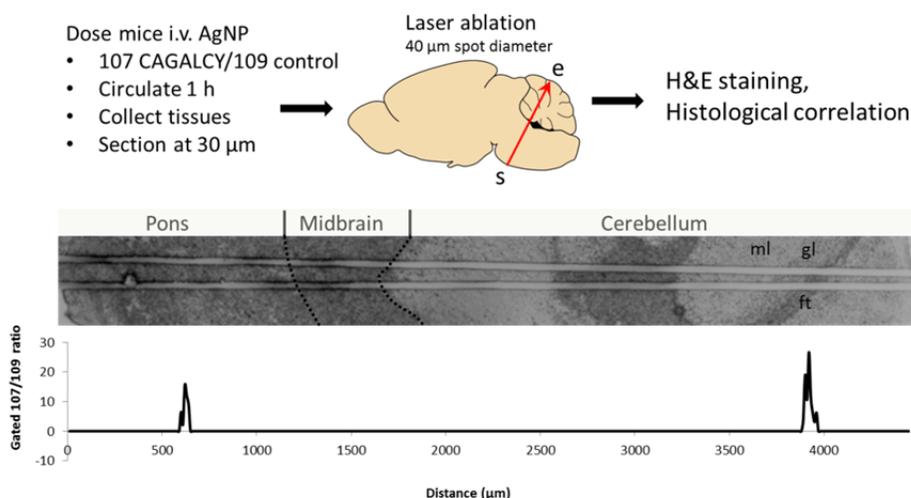
**Figure 25: Ratiometric LA-ICP-MS profiling on brain cryosections.** Balb/c mice were injected i.v. with 200 µL of a mixture of CAGALCY-AgNPs (prepared from Ag107) and control biotin-AgNPs (prepared from Ag109). At 5 h time point, tissues were snap-frozen, sectioned at 30 µm, and laser ablation line scans using 40 µm spot were performed. ft: fibrillary tract, ml: molecular layer, gl: granular layer.

5.5.2.2.3. Spatial distribution analysis of LinTT1-AgNPs in GBM lesions

To study accumulation of LinTT1-AgNPs in GBM tissue, Ag109 isotopic particles were functionalized with linTT1 peptide and injected in equal amount with biotin-Ag107-NPs to s.c. U87-MG tumor bearing animals. Spatial analysis of tissues demonstrated elevated ratios of Ag109/107 (up to 30) in tumor areas rich in tumor vessels (Fig. 26) whereas isotopic ratio in liver was homogeneous all over the tissue.
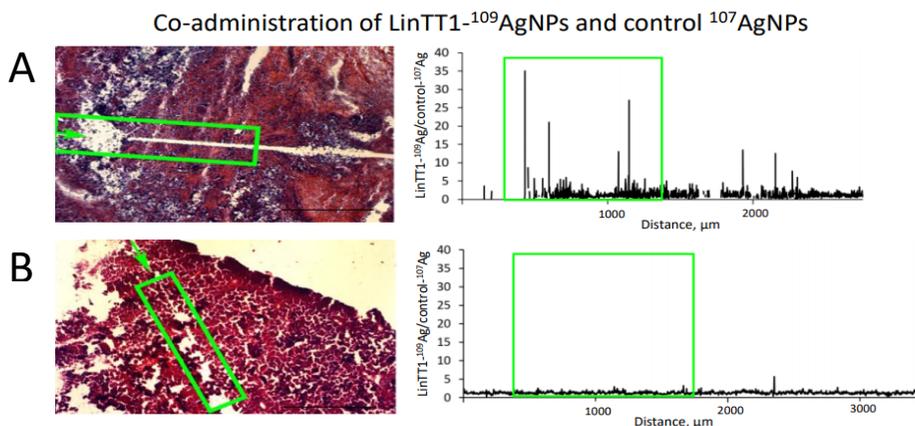
Figure 26: Lin-TT1-targeted silver nanoparticles home to GBM lesions. (A, B) Profiling of LinTT1-coupled AgNPs in U87-MG tumor by laser ablation ion-coupled mass spectrometry (ICP-MS). Mice bearing s.c. U87-MG tumor were injected i.v. with equimolar ratio of neutravidin-coated Ag109 nanoparticles coupled to biotinylated linTT1, and Ag107 nanoparticles coupled to biotin only. Mice were subjected to cardiac perfusion after 5 h of circulation of the particles, and tissue sections from snap-frozen organs were applied for laser ablation ICP-MS and subsequent staining with H&E. Left panel – tissue sections of H&E-stained tumor and liver from mouse bearing U87-MG subcutaneous tumor. Right panel: ratio of Ag109/107 isotopes detected from the laser ablation line shown in the left panel. Areas from the representative tissues and the respective areas on silver ratio profile are depicted with green boxes on both panels. Note the uniform ratio of Ag109/107 in the liver. Scale bar in H&E stained tissue sections: 500 μm.

5.6. LinTT1-guided experimental therapy of GBM tumors

To evaluate the effect of linTT1 functionalization on anti-GBM activity of therapeutic NWs two different tumor models were used: s.c. U87-MG and orthotopic 005. Nanoworms were loaded with amphipilic proapoptotic $_D$(KLAKLAK)$_2$ peptide that destabilizes mitochondrial membranes leading to apoptosis. $_D$(KLAKLAK)$_2$ has been used in several experimental studies (Agemy et al. 2011; Hunt et al. 2017). In mice with s.c. U87-MG tumors, treatment with LinTT1-$_D$(KLAKLAK)$_2$-NWs suppressed tumor progression and tumor size at the end of treatment was significantly reduced compared to PBS group (Fig. 27). In mice with orthotopic 005 tumors, LinTT1-$_D$(KLAKLAK)$_2$-NWs delayed the tumor progression and improved survival of the animals (Fig. 28).
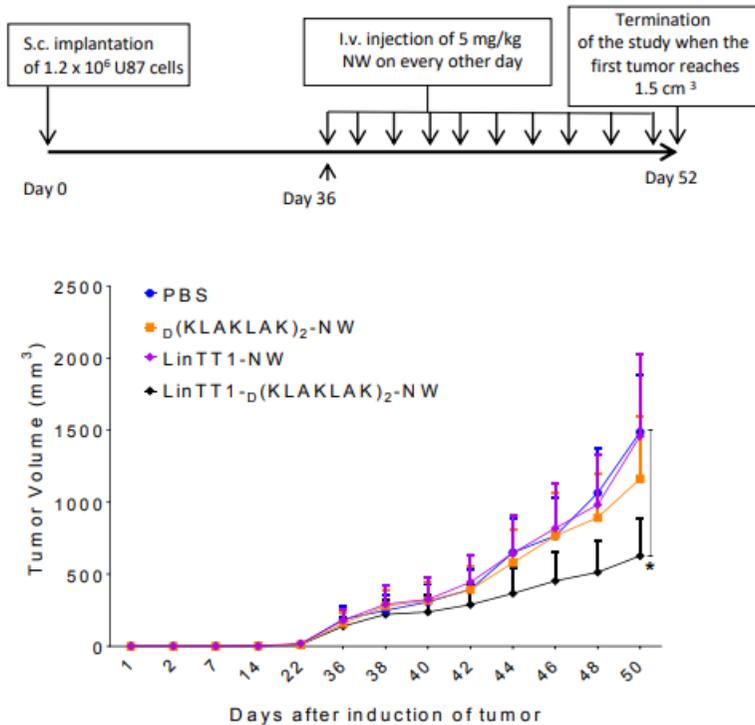
Figure 27: Experimental therapy of U87-MG subcutaneous tumor-bearing mice with LinTT1-$_D$(KLAKLAK)$_2$-NW inhibits the tumor growth.
The treatment of U87-MG-bearing mice with LinTT1-D(KLAKLAK)$_2$-NW and control agents [$_D$(KLAKLAK)$_2$-NW, LinTT1-$_D$(KLAKLAK)$_2$-NWs, and PBS] was started on day 36 after tumor induction. The therapy consisted of 8 intravenous injections of NWs (5 mg/kg Fe in 100 µl) every other day. Tumor size was recorded daily, and the study was terminated when the first tumor reached 1.5 cm^3, the maximum size allowed by the animal care committee. * – $p < 0.01$. Error bars – standard deviation.
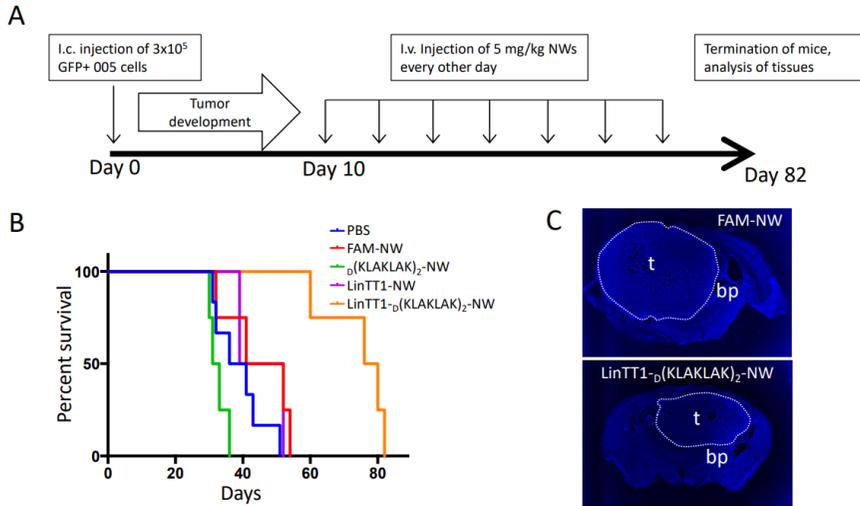
Figure 28: Experimental therapy of 005 GBM with LinTT1-$_D$(KLAKLAK)$_2$-NW inhibits the tumor growth. (A) – Starting on day 10 post tumor induction, the 005-bearing mice received 7 i.v. injections of 5 mg/kg of LinTT1-$_D$(KLAKLAK)$_2$-NW, or control compounds [$_D$(KLAKLAK)$_2$-NW, LinTT1- $_D$(KLAKLAK)$_2$-NWs, and PBS] every other day. (B) – Body weight and motor functions of mice were monitored daily and the animals were sacrificed when they reached the limits allowed by the animal ethics committee, and the survival data were expressed as Kaplan-Meyer plots. (C) – Representative images DAPI-counterstained (blue) sections of orthotopic GBM (indicated by dotted line) collected on day 82 from mice treated with FAM-$_D$(KLAKLAK)$_2$-NW and FAM-LinTT1- $_D$(KLAKLAK)$_2$-NWs. t: tumor, bp: brain parenchyma.

6. DISCUSSION

6.1. Significance

The identification of new affinity ligands to target CNS is important for the development of novel strategies to diagnose and treat nervous system disorders – as most brain disorders have no effective cure. Brain is a difficult organ to target as it is shielded by different protective barriers against invading pathogens and neurotoxic molecules. These barriers also prevent access to the brain of over 90% of potential neuropharmaceuticals. Nanoparticles are considered one of the most promising strategies to deliver drugs to the CNS. Several NP formulations have been reported to successfully reach the brain tissue, mainly when modified with surfactants or affinity ligands (De la Torre and Ceña 2018). Functionalizing nanoparticles with brain targeting ligands could enable therapeutics and imaging agents to effectively cross the blood-brain barrier and reach to disease sites in the brain. Targeted therapies have improved sensitivity and reduced off-target side effects compared to untargeted nanomedicines and conventional drug formulations. To develop novel targeted nanomedicines with a high potential to reach to clinical settings, it is important to make the right decisions over key features, like NP size, shape, coating and affinity ligands. Making these decisions is often difficult due to the lack of quantitative assays to evaluate *in vivo* homing of nanoparticles.

6.2. Main findings

The current study focused on identification and characterization of brain-homing peptides that could be used to deliver cargo to brain tissue and target various nervous system disorders. To improve validation process of novel affinity ligands a highly sensitive ICP-MS-based internally controlled system was developed that enables silver nanoparticle quantification from cell and tissue lysates, and *in situ* analysis of tissue sections. It was demonstrated that previously described tumor homing peptide linTT1 increases selectivity of nanoparticles to malignant brain tumors and improves therapeutic efficacy of NPs functionalized with proapoptotic peptide. In addition, a novel homing peptide was identified, that recognizes cerebrovascular changes in Alzheimer`s disease models and homes to activated astrocytes also in other models of neurological disorders.

6.2.1. ICP-MS-based ratiometric system for quantitative and internally controlled receptor profiling

Novel targeting ligands identified by screening combinatorial libraries, e.g. phage display peptide library, need to be validated. Assessment of expression and accessibility of the target receptors on the cell surface can be conducted by assessing binding and internalization of peptide-nanoparticles in the cells. A multiplex ultrasensitive ICP-MS-based assay was developed to quantitate nanoparticles prepared from pure silver isotopes and palladium. In proof of concept experiments, isotopically-barcoded AgNPs were functionalized with two different homing peptides (RPARPAR and GKRK) and used in internalization studies using cell lines with different homing peptide receptor expression. Amount of internalized particles was quantified by ICP-MS. It was established, that uptake of peptide-functionalized particles was dependent on the expression of the relevant receptors on the cell surface and was not affected by the exact composition of the nanoparticles. This highly sensitive method enables to measure relative ratios of internalized differentially functionalized nanoparticles and thereby provides information on the expression and availability of the relevant receptors.

6.2.2. Ratiometric *in vivo* comparison of AgNPs is a highly sensitive internally controlled assay for homing studies

Isotopically barcoded AgNPs can be used for simultaneous evaluation of the affinity targeting ligands in the same animal to minimize artefacts due to inter-animal variability. Quantitative biodistribution studies using multiplexed nanoparticles would facilitate optimization of affinity ligands to target disease sites or specific organs. Firstly, *in vivo* homing of AgNPs functionalized with non-pathogenic organ homing peptides RPARPAR (selective for lungs in normal mice) and CAGALCY (brain microvessels) was evaluated. Equimolar amount of peptide-guided Ag107 and control Ag109 particles was injected intravenously in the animals. Ag107/109 ratio was determined from tissue extracts by ICP-MS and from cryosections by LA-ICP-MS. Both methods showed overrepresentation of RPARPAR-NPs in the lung and CAGALCY-NPs in the brain, but LA-ICP-MS provided additional information about the homing within the tissue. Later, the LA-ICP-MS method was also used to study linTT1-AgNP homing to GBM lesions, showing accumulation of functionalized particles in tumor areas rich in blood vessels.

Several internally controlled multiplexing approaches have been reported in the literature (Dahlman et al. 2017; Kunjachan et al. 2014), but they possess several disadvantages compared to ICP-MS based quantification. DNA-barcoded NPs have high sensitivity and can be used across a wide dynamic range, but for several reasons: these particles cannot be readily distinguished on tissue sections for spatial mapping, barcodes need to be protected from degradation,

and barcode sequences can affect PCR amplification, or NP behavior (Dahlman et al. 2017). NPs with different fluorophores allow noninvasive longitudinal homing studies, but optical imaging is not quantitative (Kunjachan et al. 2014).

6.2.3. LinTT1 peptide guided nanoparticles accumulate in GBM lesions

Precision delivery of anticancer drugs could potentially improve the prognosis of GBM patients. Currently, there are only two approved anticancer compounds, temozolomide and nitrosourea, that are able to cross the BBB and reach to the cerebral tissue. Temozolomide, the standard chemotherapeutic drug of GBM, has only modest anticancer effect and it fails to penetrate the GBM lesions (Chamberlain 2010). Affinity targeted delivery of anticancer compounds could increase the concentration of drugs at disease site and enable to deliver drugs that do not readily cross the BBB.

A novel tumor penetrating peptide, linTT1, was evaluated as an affinity ligand on five phenotypically diverse GBM tumor models. Functionalization with linTT1 peptide increased accumulation of nanoparticles at tumor site in all the tested models. LinTT1-NWs accumulated preferentially in stromal cell populations: in endothelial cells and in tumor associated macrophages (TAM). Experimental therapy with linTT1 guided proapoptotic iron NPs inhibited tumor growth and markedly improved survival of the mice compared to control groups. Increased amounts of TAMs have been found in stroma of various human malignancies (Chanmee et al. 2014). TAMs are the main cell population promoting tumor growth and drug resistance (Yang and Zhang 2017). Targeting TAMs could be a potential strategy to treat tumors that are unresponsive to conventional anticancer therapies. Thus, linTT1 could be used to develop targeted therapies to selectively kill or reprogram TAMs, and thereby improve the prognosis of cancer patients. Furthermore, elevated expression of p32 is associated with worse prognosis in GBM patients and knockdown of p32 has been demonstrated to slow the progression of tumor growth (Laakkonen et al. 2004; Yenugonda et al. 2017). Future studies will show whether peptidic p32 targeting ligands possess intrinsic antitumor activity and act in synergy with payload drugs promoting their therapeutic efficacy.

6.2.4. AD peptide targets activated astrocytes in AD and other models of neuroinflammation

AD is the most common age-related progressive neurodegenerative disorder with no effective cure. Detailed mechanistic understanding of the pathogenesis of AD is still missing (Du, Wang, and Geng 2018). Changes in the brain vasculature that lead to disruption of BBB have been reported in AD animal models as well as in patients with late-stage AD. Morphological and functional

changes in the vessels could contribute to neuronal dysfunction and neuro-degeneration (De la Torre 2002; Zlokovic 2011). *In vivo* phage display screens on AD animal model were conducted to identify peptides that bind to molecular changes in the vasculature associated with AD. The novel peptide, DAG, bound selectively to activated astrocytes present in early stages of AD and in other models of neuroinflammation. DAG receptor was identified using affinity chromatography separation; and found to be connective tissue growth factor (CTGF), a matricellular protein with elevated expression in various types of brain injuries and in small blood vessels in the brains of human AD patients (Conrad et al. 2009; Spliet et al. 2003; Ueberham et al. 2003).

DAG peptide accumulation was also seen in GBM lesions, in patient-derived GBM model (P13). It is worth noting that P13 model has been reported to have elevated CTGF expression (Bougnaud et al. 2016). P13 tumors are angiogenic with areas of pseudopalisading necrosis, features characteristic of clinical GBM. Our results suggest the potential uses for DAG peptide as a targeting ligand for improving imaging of neurological disorders, especially early AD and for delivery of drugs to brain for the treatment of neuroinflammatory conditions.

6.3. Future directions

Precision nanomedicine is a rapidly developing field with potential to overcome challenges associated with diagnosis and treatment of brain disorders. Nano-scale systems can potentially improve imaging and treatment of neurodegene-rative disorders and brain tumors. Nanomedicines conjugated with targeting ligands provide platforms for selective delivery of drugs and imaging agents to the CNS. Encapsulating drugs into affinity guided nanocarriers protects the drugs from degradation, increases their circulation time, enables crossing the BBB and provides controlled release at the disease site. Targeted therapies increase local drug concentration and protect peripheral system and surrounding brain tissue from toxicities.

7. CONCLUSIONS

1. ICP-MS-based quantification of peptide-coated isotopically tagged AgNPs can be used to determine peptide receptor phenotype of cultured cells.
2. Internally controlled ICP-MS-based AgNP quantification system makes it possible to study homing of targeted NPs and compare specificity of different homing peptides in the same animal.
3. LA-ICP-MS based AgNP quantification method allows internally controlled mapping of peptide-AgNP distribution within the tissue.
4. LinTT1 peptide guided NPs accumulate in GBM lesions and exert therapeutic activity in case the particles carry anticancer compounds.
5. Phage display screens on AD model led to the discovery of homing peptide, DAG, which recognizes connective tissue growth factor (CTGF) in Alzheimer's brains. DAG peptide homes to the brain also in GBM, PD and traumatic brain injury animal models.

8. SUMMARY IN ESTONIAN

Kullerpeptiidide kasutamine ajuhaiguste ravis

Ühiskonna keskmise eluea suurenemise tulemusel kasvab vananemisega seotud kesknärvisüsteemi haiguste, näiteks Alzheimeri ja Parkinsoni tõve, sagedus. Nende haiguste puhul puudub tõhus ravi haiguse kulu peatamiseks või patsientide terveks ravimiseks. Neurodegeneratiivsed haigused pole iseenesest letaalsed, kuid haiguse progresseeruv kulg muudab patsiendid kõrvalabist sõltuvaks. Seevastu ajukasvajad on väga kehva prognoosiga – hoolimata ravist ei ületa agressiivsemate ajukasvajate korral patsiendide elulemus 1,5 aastat.

Kesknärvisüsteemi haiguste ravi on keeruline, kuna seda kaitsevad erinevad füüsilised ja funktsionaalsed barjäärid, mille eesmärgiks on takistada patogeenide, toksiinide ja vererakkude sisenemist ajukoesse. Olulisimad nendest mehhanismidest on vere-aju barjäär ning vere-tserebrospinaalvedeliku barjäär, millest vaid väga kindlate omadustega molekulid on võimelised läbi tungima. Enamikele molekulidele ning ka 99% ravimitele on need tõkked läbipääsmatud.

Vananemisega seotud neuroloogiliste haiguste ravi parandamiseks on oluline parendada nende haiguste diagnoosimist. Selleks on vaja identifitseerida ja valideerida molekulaarseid markereid, mis võimaldavad haigusi diagnoosida varasemas staadiumis. Eelistatult juba enne kliiniliste sümptomite avaldumist. Järgmine oluline samm neuroloogiliste haiguste ravis on leida molekulaarsed transportsüsteemid, mille abil saaks ravimeid viia läbi kaitsva barjääri, et need jõuaksid haigusest haaratud koeni. Üks strateegia, mis võiks parandada ajuhaiguste ravi, on nanotehnoloogia rakendamine. Vere-aju barjääri läbivad kullermolekulid on võimalik kinnitada ravimitega laaditud nanoosakeste pinnale. Sel viisil oleks võimalik ajukoesse transportida ka ravimeid, mis on koekultuuri süsteemis näidanud efektiivsust kasvajarakkude hävitamisel või neuronite kaitsmisel, kuid vereringesse süstituna ei suuda iseseisvalt aju kaitsvaid tõkkeid läbida.

Käesoleva prekliinilise töö eesmärgiks oli leida peptiidid, mis akumuleeruvad ajus ning mida saab rakendada kullermolekulidena kontrastainete ja/või ravimite transportimiseks. Parimate kullermolekulide välja valimiseks töötasime välja väga täpse kvantitatiivse metoodika, mis võimaldab erinevaid kullerpeptiide raku- ja loomkatsetes omavahel võrrelda.

Uurimistöö eesmärgid

1. Töötada välja kvantitatiivne meetod, mis võimaldab võrrelda kullerpeptiididega funktsionaliseeritud nanoosakeste võimet rakku siseneda;
2. Töötada välja kvantitatiivne meetod, mis võimaldab võrrelda süsteemselt manustatud kullerpeptiididega nanoosakeste võimet sihtmärkkoes akumuleeruda;
3. Kohandada kvantitatiivne meetod sobivaks koelõikude analüüsimiseks;
4. Hinnata kullerpeptiididega kaetud nanoosakeste võimet siseneda ajukasvaja koesse ning võimendada nanoravimite efektiivsust;
5. Identifitseerida Alzheimeri tõve spetsiifiliste molekulaarsete markerite suhtes selektiivsed kullerpeptiidid.

Materjal ja metoodika

Käesolevas uurimistöös oli kasutusel kokku üheksa erinevat kasvajarakuliini, millest kolm pärinesid hiirtelt ja ülejäänud kuus inimestelt. Loomkatsetes kasutati Balb/c hiiri ja atüümseid karvutuid hiiri. Kokku kasutati kuut erinevat multiformse glioblastoomi mudelit, kahte Alzheimeri tõve mudelit, ühte Parkinsoni tõve mudelit ning ühte traumaatilise ajukahjustuse loommudelit. Kõik loomkatsed olid kooskõlastatud vastavalt Eesti Põllumajandusministeeriumi, Tel Avivi ülikooli ja Sanford Burnham Prebyse meditsiiniuuringute instituudi vastava komisjoni poolt. Töös kasutati erinevaid varasemalt kirjeldatud kullerpeptiide (RPARPAR, CGKRK, CAGALCY, linTT1), mis kinnitati nanoosakeste pinnale. Nanoosakestest kasutati kahe erineva suurusega hõbeosakesi, raudoksiidi osakesi ja albumiin-paklitakseel nanoosakesi (Abraxane). Hõbe- ja raudosakesed sünteesiti vastavalt varem kirjeldatud protokollidele, Abraxane'i osakesed soetati valmiskujul. Peptiidid kinnitati hõbeosakestele biotiin-neutravidiin interaktsiooni abil ning raudoksiidi ja Abraxane'i osakestele NHS-PEG-maleimiidlinkeri abil. Nanoosakeste seondumist retseptorvalgule analüüsiti spektrofotomeetriat kasutades. Seondumiskatsed kasvajarakkudele viidi läbi kasutades voolutsütomeetriat, konfokaalmikroskoopiat ja induktiiv-sidestatud plasma mass-spektromeetriat (ICP-MS). Alzheimeri tõve (AD) peptiidi koejaotumise uurimiseks kasutati AD, Parkinsoni tõve, P13 ajukasvaja ja traumaatilise ajukahjustuse loommudeleid, hiirtele süstiti FAM märgisega peptiidi ja hiljem värviti ajude ja kontrollkudede koelõike antikehadega ning koed visualiseeriti konfokaalmikroskoopia abil. Hõbeosakeste biojaotumise uurimiseks süstiti peptiidiga osakesed ja kontrollosakesed tervetesse loomadesse ning hiljem teostati immunohistokeemilised värvingud ja visualiseeriti konfokaalmikroskoopia abil koelõigud. Kvantitatiivse analüüsimeetodi väljatöötamiseks süstiti tervetele loomadele peptiidiga osakeste ja kontrollosakeste segu ning postmortaalselt eemaldatud koed jagati kaheks: üks osa külmutati ning teine osa kudedest lüüsiti. Kudede lüsaate analüüsiti ICP-mass-spektromeetriaga ning külmutatud koest tehti lõigud, mida analüüsiti laser-ablatsioon ICP-

mass-spektromeetria abil. Kasvajaspetsiifilise peptiidiga nanoosakeste biojaotumise kvantitatiivseks analüüsiks indutseeriti hiirtele kas ortotoopsed või nahaalused kasvajad ning loomadele süstiti peptiidiga raudoksiidi ja Abraxane'i osakesi. Pärast ohverdamist tehti kudedest krüolõigud, mida värviti sobivate antikehadega ning visualiseeriti konfokaalmikroskoopia abil. Kasvajaspetsiifilise peptiidiga nanoosakeste biojaotumise kvantitatiivseks analüüsiks süstiti nahaaluste kasvajatega loomadele peptiidiga osakeste ja kontrollosakeste segu, koed külmutati ning tehti krüolõigud, mida kuivatati vaakumis ning seejärel analüüsiti laser-ablatsioon ICP-MS analüsaatoriga. Hindamaks kullerpeptiidi spetsiifilisust kasvajakoe suhtes ja proapoptootilise peptiidiga nanoosakeste efektiivsust kasvajarakkude hävitamisel viidi läbi ravikatse kahe erineva GBM loommudeliga. Selleks tekitati hiirtel ortotoopsed ja nahaalused kasvajad ja kui kasvajad olid saavutanud vajaliku suuruse, süstiti nanoosakesi sabaveeni ning katse lõpus võrreldi kasvajate suurust ja koehistoloogiat kontrollosakestega süstitud loomade kasvajatega.

Uurimistöö peamised tulemused ja järeldused

1. Töötati välja hõbedananoosakeste platvormil põhinev süsteem, mis võimaldab kvantiseerida erinevate peptiididega nanoosakeste võimet rakkudesse siseneda. Metoodika võimaldab hinnata nanoosakeste rakku sisenemiseks vajalike retseptorite ekspressioonitaset ja ligipääsetavust. Lisaks võimaldab see metoodika fenotüpiseerida segukultuurina kasvavaid rakke tänu varieeruvale retseptorite ekspressioonimustrile erinevatel rakuliinidel.

2. *In vitro* analüüsi süsteem kohandati sobivaks *in vivo* katsete jaoks nii, et see võimaldab võrrelda erinevate kullerpeptiididega nanoosakeste võimet sihtmärkkoes akumuleeruda. Välja töötatud analüüsimeetodi abil saab mitme erineva peptiidiga nanoosakesi testida seguna samas loomas, lisades meetodile nn sisemise kontrolli ning vähendades kõrvalekallete esinemise tõenäosust.

3. Laser-ablatsioon ICP-MS abil on võimalik sihtmärkkoe külmlõikudel kullerpeptiidiga nanoosakeste biojaotumist kvantitatiivselt analüüsida.

4. LinTT1 kasvajaspetsiifilise peptiidiga nanoosakesed akumuleerusid kasvajakoes kõigi viie testitud GBM mudeli puhul sõltumata nanoosakeste tüübist. LinTT1 nanoosakeste peamiseks sihtmärgiks olid kasvaja veresooned ja kasvajaseoselised makrofaagid. LinTT1 peptiidi ja proapoptootilise peptiidiga raudoksiidi nanoosakesed olid eksperimentaalteraapia katses võrreldes kontrollosakestega efektiivsed, pidurdades tuumorite kasvu ning pikendes loomade elulemust.

5. *In vivo* faagidisplei AD mudelil viis sidekoe kasvufaktor CTGFga seonduva DAG peptiidi avastamiseni, ning me näitasime, et DAG peptiid akumuleerub hiirtes glioblastoomi mudelites.

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PUBLICATIONS

CURRICULUM VITAE

Name: Kadri Toome
Date of birth: September 29, 1984
Citizenship: Estonian
Contact: Cancer Biology Group, Institute of Biomedicine and
                 Translational Medicine,
                 University of Tartu, Ravila 14b 50411, Tartu
Phone: +372 737 4268
E-mail: kadri.toome@gmail.com

Education:
1991–2003   Kadrina Secondary School
2003–2006   University of Tartu, Faculty of Science and Technology, Gene
                technology, BSc
2006–2008   University of Tartu, Faculty of Science and Technology,
                Biotechnology and biomedicine, MSc
2012–   University of Tartu, Faculty of Medicine, PhD

Professional employment:
2007–2008   Asper Biotech Ltd., Laboratory specialist
2008–2014   Asper Biotech Ltd., Project manager
2012–2019   University of Tartu, Cancer Biology Group, Institute of
                Biomedicine and Translational Medicine, Laboratory specialist
2016–   Tartu University Hospital, United Laboratories,
                Clinical Genetics Center, Laboratory specialist

Professional organization:
Member of the Estonian Society of Medical Genetics

Special courses:
2014   EMBO Young Investigator PhD Course, Heidelberg, Germany

List of publications:
1. Säälik P, Lingasamy P, **Toome K**, Mastandrea I, Rousso-Noori L, Tobi A, Simón-Gracia L, Hunt H, Paiste P, Kotamraju VR, Bergers G, Asser T, Rätsep T, Ruoslahti E, Bjerkvig R, Friedmann-Morvinski D, Teesalu T. (2019) Peptide-guided nanoparticles for glioblastoma targeting., *J Control Release*
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ELULOOKIRJELDUS

Nimi: Kadri Toome
Sünniaeg: 29. september 1984
Kodakondsus: Eesti
Kontaktandmed: Vähibioloogia töögrupp, Bio- ja Siirdemeditsiini instituut, Tartu Ülikool, Ravila 14b 50411, Tartu
Telefon: +372 737 4268
E-mail: kadri.toome@gmail.com

Hariduskäik:
1991–2003   Kadrina Keskkool
2003–2006   Tartu Ülikool, Geenitehnoloogia, BSc
2006–2008   Tartu Ülikool, Biotehnoloogia ja biomeditsiin, MSc
2012–   Tartu Ülikool, arstiteaduse doktoriõpe

Teenistuskäik:
2007–2008   Asper Biotech Ltd., Laborispetsialist
2008–2014   Asper Biotech Ltd., Projektijuht
2012–2019   University of Tartu, Laborispetsialist
2016–   Tartu Ülikooli Kliinikum, Ühendlabor, Kliinilise geneetika keskus, Laborispetsialist

Erialaorganisatsioonid:
Eesti Meditsiinigeneetika Seltsi liige

Erialane enesetäiendus:
2014   *EMBO Young Investigator PhD Course*, Heidelberg, Saksamaa

List of publications:
1. Säälik P, Lingasamy P, **Toome K**, Mastandrea I, Rousso-Noori L, Tobi A, Simón-Gracia L, Hunt H, Paiste P, Kotamraju VR, Bergers G, Asser T, Rätsep T, Ruoslahti E, Bjerkvig R, Friedmann-Morvinski D, Teesalu T. (2019) Peptide-guided nanoparticles for glioblastoma targeting., *J Control Release*
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DISSERTATIONES MEDICINAE
UNIVERSITATIS TARTUENSIS

1. **Heidi-Ingrid Maaroos**. The natural course of gastric ulcer in connection with chronic gastritis and *Helicobacter pylori*. Tartu, 1991.
2. **Mihkel Zilmer**. Na-pump in normal and tumorous brain tissues: Structural, functional and tumorigenesis aspects. Tartu, 1991.
3. **Eero Vasar**. Role of cholecystokinin receptors in the regulation of behaviour and in the action of haloperidol and diazepam. Tartu, 1992.
4. **Tiina Talvik**. Hypoxic-ischaemic brain damage in neonates (clinical, biochemical and brain computed tomographical investigation). Tartu, 1992.
5. **Ants Peetsalu**. Vagotomy in duodenal ulcer disease: A study of gastric acidity, serum pepsinogen I, gastric mucosal histology and *Helicobacter pylori*. Tartu, 1992.
6. **Marika Mikelsaar**. Evaluation of the gastrointestinal microbial ecosystem in health and disease. Tartu, 1992.
7. **Hele Everaus**. Immuno-hormonal interactions in chronic lymphocytic leukaemia and multiple myeloma. Tartu, 1993.
8. **Ruth Mikelsaar**. Etiological factors of diseases in genetically consulted children and newborn screening: dissertation for the commencement of the degree of doctor of medical sciences. Tartu, 1993.
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