



Seek & Destroy, use of targeting peptides for cancer detection and drug delivery



Vadim Le Joncour, Pirjo Laakkonen*

Research Programs Unit, Translational Cancer Biology, Biomedicum Helsinki 1, University of Helsinki, Haartmaninkatu 8, 00014 Helsinki, Finland

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ABSTRACT

Accounting for 16 million new cases and 9 million deaths annually, cancer leaves a great number of patients helpless. It is a complex disease and still a major challenge for the scientific and medical communities. The efficacy of conventional chemotherapies is often poor and patients suffer from off-target effects. Each neoplasm exhibits molecular signatures – sometimes in a patient specific manner – that may completely differ from the organ of origin, may be expressed in markedly higher amounts and/or in different location compared to the normal tissue. Although adding layers of complexity in the understanding of cancer biology, this cancer-specific signature provides an opportunity to develop targeting agents for early detection, diagnosis, and therapeutics. Chimeric antibodies, recombinant proteins or synthetic polypeptides have emerged as excellent candidates for specific homing to peripheral and central nervous system cancers. Specifically, peptide ligands benefit from their small size, easy and affordable production, high specificity, and remarkable flexibility regarding their sequence and conjugation possibilities. Coupled to imaging agents, chemotherapies and/or nanocarriers they have shown to increase the on-site delivery, thus allowing better tumor mass contouring in imaging and increased efficacy of the chemotherapies associated with reduced adverse effects. Therefore, some of the peptides alone or in combination have been tested in clinical trials to treat patients. Peptides have been well-tolerated and shown absence of toxicity. This review aims to offer a view on tumor targeting peptides that are either derived from natural peptide ligands or identified using phage display screening. We also include examples of peptides targeting the high-grade malignant tumors of the central nervous system as an example of the complex therapeutic management due to the tumor's location. Peptide vaccines are outside of the scope of this review.

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1. Introduction

Personified as widespread, resistant, and adapting disease that strikes regardless of age, gender or social status, cancer embodies one of the ultimate challenges of modern medicine. Cancer is the second cause of death in the U.S. (statistics from the CDC) and expected to surpass the current No 1, cardiovascular diseases, by 2030. Cancer patients suffer from insufficient specificity and severe side effects of the conventional chemotherapies. In the new era of personalized/precision medicine, goal of the therapeutic management is to use the tumor- and patient-specific genetic and molecular aberrations for the selection of specific targeted therapies for each patient.^{1–3} Inherent to this individualistic assessment of using genomic and molecular profiling of cancer, appropriate clinical management requires molecular probes capable of homing

specifically to the primary or metastatic tumor mass.⁴ The past decade has seen the emergence of numerous targeting agents providing the proof of concept of anticancer effects by targeted delivery. Aside of immunoglobulins, peptides or peptidomimetics have been developed. The cancer-targeting antibodies have exhibited excellent performance as vehicles to deliver radionuclides for imaging and cytotoxic agents for chemotherapies. Tested in the clinic and approved by the FDA, they unfortunately have also shown their limitations. For instance, the *fragment crystallizable* region of the antibody has a trend to non-specifically bind to the reticuloendothelial system thus causing notable toxicity towards tissues such as liver, spleen, and bone marrow.^{5,6} In addition, due to their high molecular weight (up to 160 kDa), they poorly diffuse into the tumor mass or do not reach the brain in case of central nervous system neoplasms, leading to the necessity of the transient opening of the blood-brain-barrier.^{7,8} Therapeutic antibodies while very specific and effective are rather difficult and particularly expensive to produce in mass scale. In the light

* Corresponding author.

E-mail address: pirjo.laakkonen@helsinki.fi (P. Laakkonen).

of these shortcomings, targeting peptides can be considered as an alternative vehicle for the delivery of diagnostic agents and/or anti-cancer drugs. Compared to antibodies, targeting peptides benefit from non-immunogenicity, fast blood clearance, better intratumoral diffusion due to their lower molecular weight, and excellent tolerability by patients. The short half-life of the peptides that may in some cases reduce the accumulation at the target is often considered as one of their limitations. Prolonged half-life of the peptides can be obtained by preventing the degradation by blood proteases through *i*) presence of a cycle, formed by for instance disulfide bonds between two cysteines, *ii*) blocking of the C- and N- terminus, *iii*) replacement of eukaryotic amino acids by their D-counterparts or *iv*) use of unnatural amino acids incompatible with endogenous proteases. However, in the case of peptide-coated quantum dots the prolonged half-life in circulation achieved via the polyethylene glycol (PEG) -coating that eliminated 95% of the non-specific uptake by the liver and spleen, did not increase the homing to the tumor tissue,⁹ suggesting that the receptor-mediated homing is very fast. Moreover, peptides are generally easy and relatively inexpensive to synthesize and allow myriad of possibilities for conjugation to imaging agents, therapeutic drugs, and nanodevices for targeted delivery. Thus, targeting peptides provide promising complementary tools for the modern personalized/precision medicine.

2. About targeting peptides

Modern molecular biology has dramatically facilitated the discovery of hundreds of cancer targets. Playing key roles in cellular functions and intercellular communication, peptide ligands are basically composed of a rosary-like assembly of amino acids connected by amide bonds containing usually less than one hundred monomers. Their low molecular weight allows a rapid clearance from the blood and non-specific binding sites, and their high specificity results in active concentration as low as nano-molar range. Interestingly, peptide ligands can be considered highly flexible regarding their chemical composition. Indeed, modifications such as cyclisation, unnatural amino acids or their combinations linked with chemical linkers can be easily achieved. However, such modifications must be carefully considered as they might result in a great diminution or total loss of affinity towards the target.

The targeting peptide sequence can be determined *via* different techniques. These include the development of derivatives inspired by the natural protein sequences *e.g.* vascular endothelial growth factor, VEGF¹⁰ and somatostatin (SST)¹¹ or screening of peptide libraries composed of billions of short random amino acid sequences ultimately displayed on viral particles.^{12,13} This phage display technique was first reported in 1985 using genetically engineered filamentous DNA-containing bacterial viruses (phage) that were modified to express foreign amino acid sequences as part of their protein coat.¹⁴ A decade later the first *in vivo* screening of peptides selectively homing to brain and lungs was performed.¹⁵ Since then the icosahedral T7 phage system has been introduced to display peptides as a fusion of its capsid protein.¹⁶ Within a library each phage clone displays one unique peptide in multiple copies and current libraries can account more than 10⁹ different peptides in total. The multiple display increases the avidity of binding and compensates for the possible low affinity of the peptides. The library is then introduced to targeting molecules embodied by isolated single proteins/receptors, cell cultures or extracts for the *in vitro* selections. *Ex vivo* selection can be performed on cell suspensions derived from organs or tumors of interest and *in vivo* selections are performed on live animals with administration of phage libraries via intravenous, intracardiac or intraperitoneal injections. A wash-off clears the unbound phage, leaving only the

ones exhibiting binding affinity to target(s) subsequently rescued and amplified.¹⁷

This review covers a selection of peptides, recently discovered or modified/enhanced versions of previously identified ones, summarized in the Table 1. Most of them are pre-clinically validated targeting moieties with some already transferred into the clinics.

3. Targeting peptides derived from natural ligands

The usage of a targeting ligand is generally motivated by the overexpression of tumor-specific receptors. The accumulation of targeting/homing peptide within tumors correlates with the receptor expression allowing the discrimination of the abnormal from the normal tissue. Therefore, peptides conjugated to imaging moieties as diverse as fluorescent dyes, radionuclides or iron-oxide particles are respectively used for the optical, positron emission tomography or single photon emission computed tomography (PET or SPECT) as well as magnetic resonance imaging (MRI). An ideal targeting peptide should accumulate in the target but not in the normal tissues and in case of imaging applications be cleared fast from the circulation to minimize the background and enhance the specific signal to noise ratio.¹⁸ In case of drug delivery, the accumulation of the peptide-drug conjugate at the target will increase the efficacy and decrease the side effects.

Moreover, the recent emergence of theragnostic tools suitable for use both in imaging and therapy transcends the borders between the two disciplines.¹⁹ The following is a non-exhaustive review of the peptides derived from natural ligands mainly used for imaging of peripheral cancers such as breast and prostate cancer and melanomas.

3.1. Somatostatin (SST) derivatives

Many solid cancers are frequently associated with aberrant overexpression of the G protein-coupled receptors (GPCR) activated by peptide ligands, including the somatostatin receptor (SSTR) family. The SSTR family comprises five receptors (SSTR1 to 5) widely distributed in the central nervous system, pituitary gland, and many peripheral organs. Binding of the natural ligand somatostatin peptide (SST) to the receptors leads to inhibition of proliferation and/or induction of apoptosis in cancer cells.¹¹

SSTR2 and 5 are specifically overexpressed in breast cancer, thus allowing their use as anti-cancer targets. SST exhibits high affinity towards the receptors but has a remarkably short half-life of only 1 to 3 min in plasma.¹¹ To overcome this challenge, SST analogue, a cyclic SSTR agonist octapeptide called octreotide (SMS 201-995; _DFCF_DWKTCT), which contains D-amino acids in the SST backbone and selectively binds SSTR2 and 5, was developed. Compared to the SST, octreotide has significantly increased plasma half-life up to 113 min.²⁰ Another example of natural ligand modifications is the incorporation of fatty acyl moieties in a process called lipophilization that may increase both the peptide stability and biological activity without causing conformational changes. For example, addition of 12, 14 or 16 carbons to another SST analogue RC-160 (_DFCY_DWKVCW) with short half-life in serum resulted in better stability and a 10-fold increase in potency over the RC-160 itself.²¹

The SSTR 2 and 5 were also used to visualize primary prostate neoplastic lesions and bone metastasis in PET/CT imaging of 20 patients.²² However, expression of SSTR 2 and 5 in the tumor tissue of the majority of the included patients was too low for the receptor-mediated delivery of therapies. Therefore, the authors suggest that SSTR subtypes 1 and 4 seem to be more prostate specific and thus should be considered for further investigations.²²

Table 1
Targeting peptides discussed in this review.

Peptide			Target	Ref.
Name	Sequence	Origin		
Octreotide	$_D$ FCF $_D$ WKTCT-ol	n	SSTR	[20]
RC160	$_D$ FCY $_D$ WKVCW	n	SSTR	[21]
Bombesin	YQLGNQWAVGHLM	n	GRPR	[27–32]
PSAP-peptide	DWLPK	n	apoptotic cascade, ?	[43]
NT21MP	LGASWHRPDKCCLGYQKRPLP	n	CXCR4	[46,47]
Nef-M1	NAACAWLEAQ	n	CXCR4	[48,49]
Peptide R	RACRFFC	n	CXCR4	[50]
Pentixafor	$_D$ Y-[NMe] $_D$ Orn-R-2Nal-G	n	CXCR4	[51]
pHLIP	ACEQNPIYWARYADWLFTTPLLDDALLVDADET	n	pH, cell membrane	[53,54]
l-zipper peptide	VSSLESKVSSLESKVSKLESKSKLESKVSKLESKVSSLESK	n	temperature	[56]
ELP	VPGXG	n	temperature	[57,58]
α -MSH mimics	Modifications of the α -MSH sequence([Ac-N]LDH $_D$ FRWGL)	n	MC1R	[60,61]
GZP	AGGIEFAD	n	granzyme B	[64]
cRGD	RGD $_D$ YK	s	$\alpha_v\beta_3$	[68,70–78]
EETI 2.5 F (knottin)	GCPRPGRDNPPLTCSQSDCLAGCVCGPNGFCG	s	integrins	[79–80]
NGR	CNGRC	s	APN (CD13)	[81–83]
SP2012	LRRFSTMPFMF-Abu-NINNV-Abu-NF	s	β_1 integrins	[84]
AARP	CTTHWGFTLC	s	MMP2/9 + blood vessels	[86]
CK	CVNHPAFAC-HTMYHHYQHHL	s	Sonic hedgehog + VEGFR2	[91]
LyP-1	CGNKRTRGC	s	p32	[94–102]
AGR	CAGRRSAYC	s	prostate cancer lymphatics	[103]
REA	CREAGRKAC	s	pre-malignant tumor lymphatics	[103]
LSD	CLSDGKRKC	s	tumor lymphatics	[103]
iRGD	CRGDKGPDG	s	$\alpha_v\beta_3$ +NRP-1	[71,106–109]
iPhage/pen	[M13]-RQIKIWFQNRRMKWKK	n	cell cytoplasm	[110]
M2pep	YEQDPWGVKWWY	s	M2/TAM	[112–115]
CooP	CGLSGLGVA	s	MDGI	[117–119]
CLT-1	CGLIQKNEC	s	fibrosis	[120–121]
Pep-1 L	CGEMGWVRC	s	IL13RA2	[122–123]
Angiopep-2	TFFYGGSRGKRNNFKTEEY	n	LRP-1	[124]
Angiopep-7	TFFYGGSRRNNFRTEEY	n	–	[125]
FHK	FHKHKSPALSPV	s	tenascin-c	[126]
tLyP-1	CGNKRTR	s	NRP-1	[127]
Cilengitide	cRGDF [N-Me]V	s	integrins	[130–134]

n, peptide sequence based on the natural ligand; s, synthetic sequence (isolated for instance from phage displayed peptides libraries); X in a sequence means any amino acid residue, ? means that the exact targeted protein/receptor is unknown. SSTR, somatostatin receptor; GRPR, gastrin releasing peptide receptor; PSAP, presenilin-associated protein; CXCR4, stromal-derived factor receptor; pHLIP, pH low insertion peptide; ELP, elastin-like peptide; α -MSH, α -melanocyte-stimulating hormone; MC1R, melanocortin 1 receptor; GZP, granzyme B peptide; APN (CD13), aminopeptidase N; Abu, l- α -amino-n-butyrac N; MMP, metalloprotease; VEGFR2, vascular endothelial growth factor receptor 2; p32, replication protein A; NRP-1, neuropilin receptor-1; pen, penetratin; TAM, tumor-associated macrophage; MDGI, mammary-derived growth inhibitor; LRP-1, low density lipoprotein receptor-related protein-1.

It is noteworthy that a preclinical study reported that the SSTR expressing tumors exhibit higher uptake of the SST antagonists than agonists with similar binding affinities even though antagonists are not internalized by tumor cells like the agonists.²³ This higher tumor to normal tissue ratio of antagonists is probable due to the lower dissociation rate and the ability of the receptor antagonists to bind simultaneously more receptors than agonists.²³ The development of such antagonist-peptide probes was demonstrated to improve uptake and detection also of various human tumors over agonists in *in vitro* receptor autoradiography study with encouraging results suggesting that they should be tested *in vivo* in patients with wide range of tumors.²⁴ This feature is of importance as not all cancers overexpress SSTRs, and the expression level may be heterogeneous between patients.

3.2. Peptide-derivatives of gastrin releasing peptide (GRP)

Recently published pre-clinical and patient studies report that the gastrin releasing peptide receptor (GRPR), first associated with the prostate cancer progression, is also overexpressed in 62–96% of primary mammary lesions.²⁵ Interestingly, breast cancer derived lymph node metastases appear to display similar GRPR profiles than primary tumors,²⁶ which potentially allows follow-up of the disease progression. Among the gastrin releasing peptide (GRP) analogues, its amphibian counterpart bombesin (YQLGNQ-WAVGHLM) and its derivatives²⁷ with high affinity to the receptor

have been extensively studied in many human tumor cell lines and xenograft tumors, including breast cancer.^{28–30} These studies suggest that bombesin analogues could be beneficial in PET detection of human breast cancers expressing GRPRs.

Similar to the SSTR system, the antagonists appear superior to the agonists for *in vivo* imaging.^{24,31} However, opposite results have also been reported both *in vitro* and *in vivo*.^{32,33} The GRPR peptide antagonists, consisting of the C-terminal modification of the agonist bombesin have been successfully used for the contouring of prostate cancer masses *via* PET and CT in mice and men.³⁴ It was also suggested that bombesin itself could be used as targeting peptide for detection of prostate cancer and affected lymph nodes in a study conducted with 12 patients.³⁵ However, a study from 2013 conducted by Ananias et al. refuted this proposition due to very low metabolic stability of bombesin. Surprisingly, bombesin suffered from an unexpectedly rapid degradation in patients *in vivo*, even though exhibiting a robust half-life in human serum *in vitro*.³⁶ In another study, the GRPR antagonists detected the majority (62%) of the tumors of 16 men with recurrent prostate cancer that had given negative or inconclusive results in the conventional PET imaging.³⁷

3.3. Peptides targeting tumor microenvironment

Cancer, arising from a normal cell, is not only an agglutinate of malignant cells but rather a complex tissue including multitude of

recruited stromal cells as well as the extracellular matrix that affects communication between different cell types.³⁸ As a consequence, the infiltrates of normal or anti-tumor cells, such as macrophages, eventually end up actively participating in the tumorigenic process.^{39,40} Thus, the idea of targeting the microenvironment has raised great interest within the scientific and medical communities.

The glycoprotein prosaposin (PSAP) is cleaved by proteases in late endosomes, regulates lysosomal trafficking, and is a secreted factor exhibiting neuro/glioprotective properties.⁴¹ Once released to the microenvironment, it stimulates expression and production of the tumor suppressor thrombospondin-1 by macrophages, and is able to inhibit metastases from breast and lung cancer in preclinical models.⁴² Based on the PSAP sequence, a cyclic pentapeptide (DWLPK) called PSAP peptide was developed and tested on patient-derived xenografts (PDX) of ovarian cancer. *In vivo*, the PSAP peptide exhibited drug-like properties and could inhibit metastatic spread and restrain tumor development in general.⁴³

CXCR4, a chemokine receptor, that controls the metastatic invasion *via* an epithelial-to-mesenchymal transition (EMT) of primary tumors provides a notable example of the development of peptide-based targeting agents.⁴⁴ Blockade of the receptor or competition of the binding of its natural ligand CXCL12 could thus provide a remarkable tool to manipulate cancer evolution. Indeed, it has been shown that inhibition of the interaction between CXCR4 expressed by cancer cells and CXCL12, which is expressed in organs where metastatic lesions will form, leads to inhibition of breast cancer metastases in lymph nodes and lungs.⁴⁵

The synthetic antagonist of CXCR4 called NT21MP, a 21 amino-acid peptide, derived from the N-terminus of Kaposi's sarcoma associated herpesvirus macrophage inflammatory protein II, exhibits anti-tumor activities through decreased adhesion and migration of breast cancer cells and overall decrease in pulmonary metastasis in animals.⁴⁶ Moreover, NT21MP was shown to reverse the EMT and override the drug resistance of breast cancer cells in preclinical breast cancer models.⁴⁷ These promising results have been reproduced and confirmed in another study involving breast and colon cancers, by using another CXCR4 antagonist Nef-M1, a peptide corresponding the HIV-1 Nef protein amino acids 50–60,

that competes with the natural ligand for binding. In these studies, conducted with human cancer cells and xenografts, the Nef-M1 peptide induced apoptosis and inhibited tumor angiogenesis, growth, and metastases.^{48,49} Moreover, targeting of CXCR4 with yet another peptide antagonist, peptide R (RACRFFC), showed outstanding capacities to profoundly remodel the tumor stroma. In this study, Mercurio et al. demonstrated that intraperitoneally administered peptide R could reach intracranial xenografts of human glioblastoma. Peptide R tempered down the glioma-induced astrogliosis and microglia reactivity *via* polarization of the cancer promoting M2 glioma-associated macrophages to the anti-tumor M1 phenotype. This resulted in a significant slowdown of the neoplastic progression.⁵⁰ However, in a heterogeneous small patient cohort the CXCR4 radioligand, [⁶⁸Ga]-pentixafor, showed lower detectability of solid cancers than the [¹⁸F]-FDG PET.⁵¹

3.4. Peptides targeting the tumor pH and temperature

Within the tumor tissue, the dysregulated angiogenesis/lymphangiogenesis associated with a high metabolic rate of the neoplastic cells leads to insufficient clearance of metabolic acids and a gradual decrease in the pH in tumor microenvironment.⁵² This has led to development of pH-sensitive drug-delivery systems, such as the pHLIP (pH-Low Insertion Peptide).⁵³ pHLIP is a 36 residues long peptide derived from the bacteriorhodopsin C helix and able to insert into cell membranes as an α -helix only under low pH conditions whereas a basic or neutral pH environment results in a loss of the helical structure and decreased affinity towards the membranes (Fig. 1). In a recent paper pHLIP peptide was shown to significantly increase the uptake of peptide-coated gold nanoparticles to tumors compared to the naked particles when administered intra-tumorally or intravenously.⁵⁴

The intra-tumoral mild hyperthermia is another targetable characteristic of the tumor microenvironment. The aberrant endothelial cell proliferation results in malfunctioning blood vessel network that limits the overall heat exchange usually provided by a proper blood flow.⁵⁵ Thus, this local characteristic of the tumor microenvironment compared to the surrounding tissues can be used to trigger peptide formulations to respond accordingly.

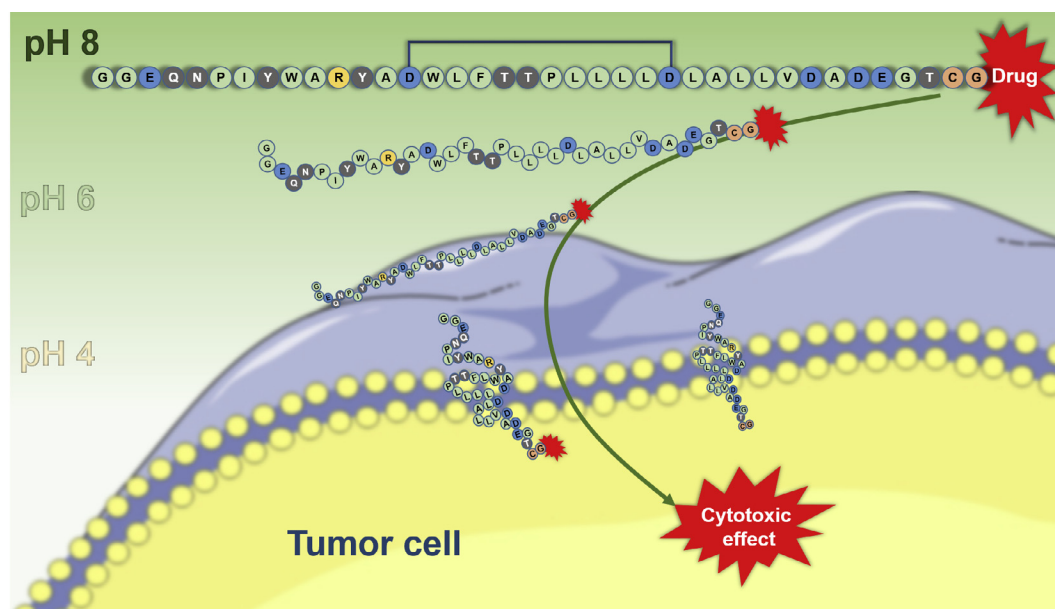


Fig. 1. Modality of the pHLIP peptide binding to the tumor cell membranes with subsequent local drug delivery. At basic/neutral pH, the peptide is unstructured and can be partly found associated but not inserted into biological membranes. At low pH, pHLIP will become fully inserted into the lipid membranes and exhibit an α -helical structure. This change in conformation is due to the binding of acid protons on the two aspartic acids (D) highlighted by a bridge on the peptide sequence. Moreover, the C-terminal C residues can be used for drug conjugation.

For instance, it has been demonstrated that a leucine zipper peptide (VSSLESKVSSLESKSKLESKSKLESKSKLESKSKLESK) can form spontaneous coiled-coil polymers composed of α -helix units that dissociate into non-structured monomers when the temperature is above 40 °C. During this temperature-sensitive process, loss of the original polymer structure can be used to deliver and release chemotherapies very locally at tumor site.⁵⁶ Other thermally responsive formulations have used elastin-like polypeptides (ELP) that consists of a repeated pentapeptide VPGXG motif (X can be any amino acid except proline). ELP peptides were conjugated to the cell penetrating peptide Bac (RRIRPRPRLPRPRPLPFRPG) for improved cellular uptake and shown to successfully deliver gemcitabine, the first line treatment for pancreatic cancer, into xenograft tumors.⁵⁷ In another study, Ryu and Raucher reported that when ELP-Bac and gemcitabine were administered to tumor-bearing animals a significant inhibition of the growth of pancreatic xenografts was observed compared to the monotherapies.⁵⁸

3.5. Other natural ligand-derived peptides

Melanocortin-1 receptor (MC1R) expressed by melanocytes in normal tissues plays a key role in the synthesis of epidermal melanin pigments and in photoprotective response *via* the activation of DNA-repair pathways and antioxidant defenses. As MC1R is highly overexpressed by primary melanomas and their metastasis, imaging techniques have been developed using several peptide moieties inspired by its natural ligand, the α -melanocyte-stimulating hormone (α -MSH).⁵⁹ During the last decade, MC1R ligand mimics have been developed for *i*) PET imaging such as radio-fluorinated metalloptides⁶⁰, *ii*) SPECT imaging, like the lactam bridge-cyclized α -MSH analogues that can be also used in *iii*) PET modality when this cyclic α -MSH is coupled to DOTA residues.⁶¹ All synthetic peptides exhibited better tumor uptake and stable retention in pre-clinical studies compared to the radiolabeled endogenous ligands.

More recently, melanoma imaging using molecular targets of the immune system have gained attention⁶² and its use over the classic FDG has been subjected for discussions.⁶³ For instance, a preclinical study conducted by Larimer et al.⁶⁴ brilliantly demonstrated that granzyme B, a serine protease that plays a major role in cancer cell death and is released by T-cells, can be used as an early biomarker of tumor response to immunotherapy. They developed a peptide (GZP) that is designed to irreversibly bind the granzyme B. When conjugated to [⁶⁸Ga]-DOTA for PET/CT imaging of melanoma-bearing immunocompromised mice treated with several immune checkpoint blockers, GZP allowed discrimination of treatment responsive tumors from the resistant ones based on the granzyme B levels in tumors.⁶⁴

4. Targeting peptides identified by combinatorial screens

Phage display is a very powerful selection tool that has been widely used to identify novel tumor homing peptides, most of which target the tumor-associated vasculature. One major advantage of the phage display screens is that it does not require any knowledge about the tumor-specific differences and selects peptides that home through the vasculature and bind to the receptors that are accessible for binding in an unbiased way.⁶⁵ These homing peptides have then been used to identify novel tumor-associated molecules and as leads to develop novel targeting agents.

4.1. Peptides targeting tumor vascular systems

The constantly growing demand of nutrients and oxygen by increasing tumor mass, leads to recruitment of new blood vessels

to the neoplastic tissue. This angiogenesis (formation of new blood vessels from the existing ones) is affected by the aberrant tumor microenvironment, which contains unbalanced concentrations of the pro-angiogenic factors, such as the vascular endothelial growth factor A (VEGF-A), and gives rise to an aberrant, poorly functional and/or hemorrhagic blood vessel network.⁶⁶ In a similar manner, lymphangiogenesis, the growth of lymphatic vessels, is induced *via* release of the VEGF-C by the cancerous cells. Lymphatic vessels are not required for the tumor growth but allow the drainage of metabolites produced by the tumor and represent a wide-open door for the metastatic escape to draining lymph nodes.⁶⁷ In addition to the angiogenic/lymphangiogenic markers, the tumor vasculature also expresses tumor specific markers.

4.1.1. Tumor blood vessel targeting peptides

The rationale behind targeting angiogenesis lies on several aspects, *i*) control/decrease of the intra-tumoral blood vessel density to provoke tumor starvation, *ii*) use angiogenic blood vessel specific receptors as targets to deliver chemotherapies, and *iii*) for difficult-to-access cancers *i.e.* brain tumors, an endothelial-specific peptide with properties to enhance cell transcytosis to allow better passage of the drug conjugates through the blood-brain-barrier (BBB).

The first and the most widely used endothelial binding peptide is the tripeptide arginine-glycine-aspartic acid (RGD) with high specificity towards integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$,⁶⁸ which are specifically overexpressed during angiogenesis and nearly absent in the normal tissue.⁶⁹ *Sky is the limit* for the reported different RGD-derivatives: *i*) antagonist drugs based on the RGD sequence for anti-tumor and anti-angiogenic treatments (for review see⁷⁰), *ii*) tumor penetrating peptidomimetics to inhibit tumor metastasis,⁷¹ *iii*) conjugation to nanocargos (for review see⁷²), or *iv*) conjugation to cell death domain of the pro-apoptotic protein Bit1 to inhibit tumor growth⁷³ are just some selected examples of the use of this peptide as cancer therapy. Moreover, as the $\alpha_v\beta_3$ integrin overexpression is not limited to one tumor type, but it is rather universally expressed in the angiogenic vasculature, there are almost infinite possible applications for the RGD. It has been widely used as an imaging moiety for angiogenic tumors including the high-grade malignant brain tumors (glioblastomas), breast cancers, and associated brain metastases (for review see⁷⁴). To image brain tumors RGD can be conjugated to various vectors allowing enhanced penetrance through the BBB and better resolution imaging of tumors. For instance, radiolabeled, PEGylated RGD has been used as a PET-probe in mice to detect gliomas⁷⁵ and follow the anatomical variations during tumorigenesis and angiogenesis.⁷⁶ Preclinical studies indicate that RGD coupled to iron-oxide nanoparticles⁷⁷ or magnetosomes (biogenic iron-oxide particles engineered in bacteria⁷⁸), allow the MR imaging of brain tumors in mice.

Another promising integrin targeting peptide, called EETI 2.5 F, which was identified in a high-throughput screening of yeast-displayed knottin libraries,⁷⁹ with excellent specificity and stability *in vivo* has been shown to beautifully draw the shapes of medulloblastomas in tumor-bearing mice.⁸⁰

Another widely used targeting moiety homing to the angiogenic vasculature is the NGR peptide that binds to the aminopeptidase N, a metalloproteinase highly expressed in the angiogenic blood vessels.⁸¹ The various NGR-based drug delivery systems have been reviewed by Corti and Curnis.⁸² Like RGD, the NGR peptide was first discovered in *in vitro* screens to identify integrin-binding peptides and later validated *in vivo*.⁸³

Hijacking of the basement membrane proteins of the endothelial lamina using a peptide-peptide combination have recently been explored in a preclinical study by Koskimaki et al. using peptides identified by bioinformatics. A β_1 integrin binding peptide

derived from the collagen IV (LRRFSTMPFMF-Abu-NINNV-Abu-NF, Abu is a cysteine analogue L- α -amino-*n*-butyric acid) together with a somatotropin-derived peptide (LLRSSILLQGSWF) was capable of modifying both VEGF-A and focal adhesion kinase pathways resulting in strong inhibition of angiogenesis.⁸⁴ Another peptide-peptide combination is the AARP peptide, an assembly consisting of a MMP-2 and 9 metalloproteinase inhibitory peptide (CTTHWGFTLC, CTT for short⁸⁵) conjugated to two different anti-angiogenic agents (endostatin mimic and kringle 5). Thus, AARP comprises one single compound designed to block angiogenesis and inhibit MMP2 and -9. This conjugate was used to target various human solid tumors in murine models and shown to efficiently inhibit angiogenesis, tumor growth, and metastasis as well as being very well tolerated.⁸⁶

Some attempts have been made to target the vascular mimicry – a phenomenon where aggressive tumor cells generate channels for tumor perfusion.⁸⁷ This unconventional vascular modality is extremely resistant to anti-angiogenic drugs.⁸⁸ A recent study aimed at multi-targeted delivery of nanoparticles to vascular mimicry channels, angiogenic vessels, and glioma cells. The authors designed nanoparticles coated with the CK peptide that is a conjugation of the human sonic hedgehog targeting peptide (CVNHPAFAC)⁸⁹ and the VEGFR2 binding peptide, K237 (HTMYHHYQHHL),⁹⁰ both isolated by the phage display screens. This report demonstrated preferential accumulation of the nanoparticles in xenografted gliomas that correlated with higher survival rates compared to the non-targeted nanoparticles when conjugated to the cytotoxic drug Taxol.⁹¹

4.1.2. Tumor lymphatic vessel targeting peptides

Presence of cancer cells in the lymph nodes usually predicts poor outcome of patients. This metastatic colonization requires a physical connection between the primary tumor and the node via

lymphatic vessels. It has been recently discovered that neoplastic cells can induce formation of lymphatic vessels within and/or around the tumor and/or lymphatic enlargement/hyperplasia involving pre-existing vessels.⁹² Exploring and targeting the lymphatics presents one new breakthrough in the imaging and therapeutic management of metastatic cancers.⁹³ Several peptide-based tools have been recently engineered to target lymphatics. The LyP-1 peptide (CGNKRTRGC) was the first lymphatic vessel targeting peptide identified using the *in vivo* phage display technology.⁹⁴ LyP-1 homes tumor cells, tumor-associated macrophages, and tumor lymphatics in some tumors by binding to its cognate receptor p32⁹⁵ (Fig. 2).

Even though LyP-1 has been shown to possess cytotoxic activity by itself,⁹⁶ various combinations of LyP-1 have been evaluated in preclinical studies: *i*) to target clinically approved paclitaxel-albumin nanoparticles to inhibit tumor growth,⁹⁷ *ii*) conjugated to PEGylated nanoparticles for efficient drug delivery to metastatic lymph nodes of pancreatic adenocarcinoma xenografts,⁹⁸ *iii*) conjugated to doxorubicin (DOX) loaded liposomes to destroy tumor lymphatics of melanoma xenografts and prevent their metastatic spread,⁹⁹ *iv*) bound to PEGylated liposomes containing DOX to target lung adenocarcinoma cells, lymphatics, and tumor-associated macrophages,¹⁰⁰ and *v*) conjugation to micelles for the delivery of artemisin, a natural anti-cancer and anti-lymphangiogenic compound, showed enhanced anti-tumor efficacy compared to non-targeted micelles in a metastatic breast cancer model.¹⁰¹ In addition to tumors, LyP-1 has been used to target macrophages within atherosclerotic plaques.¹⁰² Very interestingly, other lymphatics homing peptides identified by using phage display exhibit specific binding to either premalignant lesions or the tumors. For instance, the AGR (CAGRRSAYC) peptide recognizes lymphatic vessels in fully developed prostate tumors but not in the pre-malignant lesions while the REA (CREAGRKAC) peptide homes to lymphatics

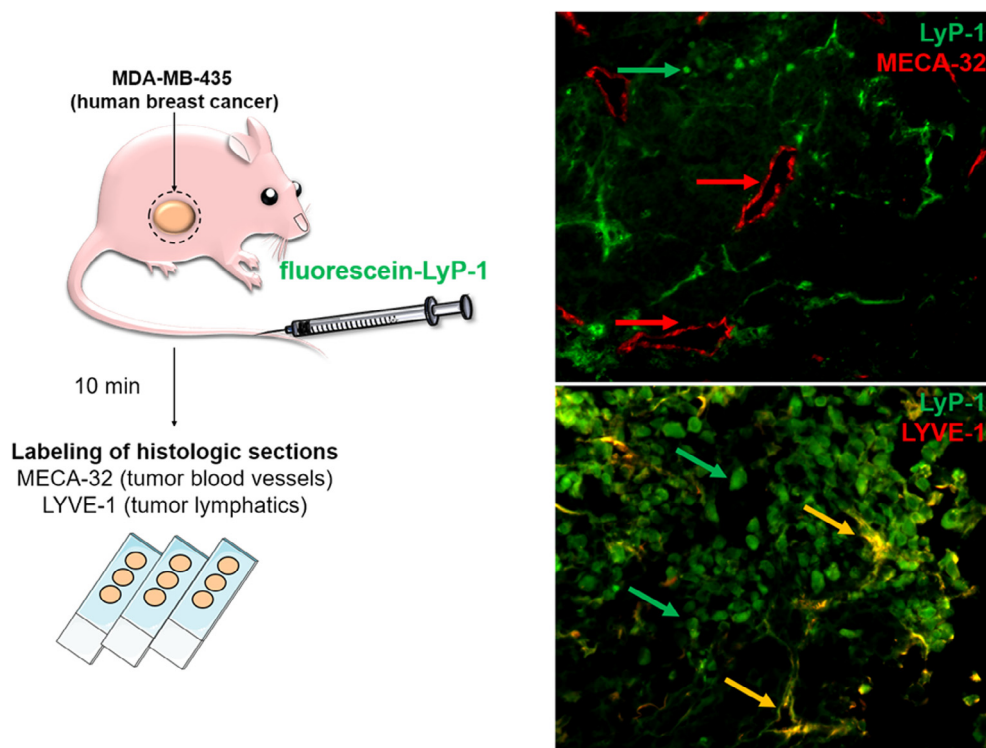


Fig. 2. Illustration of the LyP-1 peptide binding to human breast cancer cells and the associated lymphatics in tumor xenografts. Fluorescein-labeled LyP-1 peptide was injected into athymic nude mice bearing human breast cancer xenografts. Immunofluorescence staining of blood vessels (MECA-32, red, top panel) shows absence of LyP-1 homing/binding to tumor blood capillaries (red arrows). Staining of tumor lymphatics (LYVE-1, red, bottom panel) reveals avid homing/binding of the LyP-1 peptide to the lymphatic network (orange arrows). Interestingly, LyP-1 also homes to tumor cells (green arrows). Laakkonen et al. unpublished figures.

of a premalignant stage, but not to the tumor lymphatic vessels.¹⁰³ These peptides are not only excellent targeting moieties but have shown that lymphatic vessels in different tumors express different markers. As an example, the LyP-1 peptide homes to lymphatics of the MDA-MB-435 xenografts but not to those of the C8161 melanomas or the transgenic TRAMP prostate carcinomas while the LSD peptide (CLSDGKRRK) shows the opposite homing pattern.¹⁰³ This phenomenon is explained by the molecular *zip coding*, which is a result of the tissue- and stage-specific modifications occurring during tumor progression.¹⁰⁴ It also should be noted that none of the lymphatic homing peptides bind the blood vascular endothelial cells.

4.3. Tissue-penetrating peptides

Some of the tumor targeting peptides, like the LyP1, exhibit cell-penetrating properties and are able to internalize cells in a cell-type specific manner.¹² Recently, it was reported that peptides sharing the R/K/XXR/K (CendR) motif induce both cellular uptake and tissue penetration through interaction with neuropilin-1 (NRP-1).¹⁰⁵ The prototypic tumor-penetrating, cyclic peptide iRGD (CRGDKGPDC) binds both α_v integrins and NRP-1 and has been shown to significantly inhibit tumor metastasis *in vitro* by provoking the detachment and subsequent death of human prostate cancer cells anchored to fibronectin.⁷¹ Moreover, the presence of the iRGD peptide drastically increased the tumor tissue penetration and the delivery of various attached reagents such as nanoparticles, viruses, or antibodies *in vivo*.^{106–109}

With the rationale of performing the screening on proteins located in the cell cytoplasm by using a phage displayed peptide library, engineered M13 filamentous phage, called iPhage, was very recently developed. For this purpose, the cell penetrating penetratin (pen) sequence RQIKIWFQNRRMKWKK was ligated to the pVIII capsid protein of the phage to allow the internalization of the iPhage.¹¹⁰ Interestingly, it appears that the two tryptophan residues of the pen sequence are essential for the intracellular translocation, since their replacement by alanine residues resulted in a total loss-of-function.¹¹⁰ In the same paper, the authors inserted either a mitochondrial localization signal of the cytochrome *c* oxidase or a peptide sequence YKWYYRGAA in the iPhage to target the mitochondria or the endoplasmic reticulum, respectively. As a consequence of the homing of the viral particles to their intracellular targets, ultrastructural alterations such as cytoplasmic vacuoles, swollen mitochondria, and chromatin condensation, indications of an ongoing cell death, were observed.¹¹⁰

4.4. Peptides targeting macrophages

M2 macrophages participate in the progression of various diseases including cancer.¹¹¹ In order to inhibit tumor growth by depletion of the M2 macrophages, a targeting peptide YEQDPWGVKWWY (M2pep) was recently identified from the biopanning of a phage displayed peptide library on isolated M2 macrophages.¹¹² Mice bearing fibroblastic colon carcinomas were injected with a fluorescently-labeled Alexa660-M2pep to evaluate the intra-tumor homing and binding to the M2 macrophages. Addition of a cytotoxic (KLAKLAK)₂ sequence to the M2pep prolonged the survival of the treated animals compared to the untreated ones. Moreover, specific reduction in the M2 number within the tumor supported the targeting efficiency of M2pep.¹¹³ Interestingly, divalent M2pep linked to a divalent (KLAKLAK)₂ reinforced the depletion of M2 macrophages *in vivo* with increased selectivity and toxicity towards M2 but not the M1 subtype macrophages in the tumor-bearing animals.¹¹⁴ Other peptide modifications such as cyclisation also increased M2pep stability in serum and homing to the M2 macrophages.¹¹⁵

4.5. Peptides targeting malignant brain tumors

The central nervous system is a closed territory, delimited by a physical border *i.e.* the skull bone and meninges tissue, and a cellular/molecular limitation personified by the BBB that rigorously selects the nutrients, macromolecules, and drugs able to diffuse into the brain parenchyma.¹¹⁶

Recently, novel targets have been discovered for malignant brain tumor imaging and drug delivery. A novel 9 amino acid long, linear targeting peptide called CooP (CGLSGLGVA) that binds the mammary-derived growth inhibitor (MDGI) highly expressed by various cancers including a subset of breast cancers and invasive brain tumors was identified by using the phage display screen. The MDGI, a fatty acid binding protein, was found highly expressed at the cell membrane of malignant glioma cells and their associated vasculature (Fig. 3A). CooP peptide has been demonstrated to be an excellent homing peptide targeting in addition to glioma cells, tumor-associated blood vessels and invasive glioma cells that have co-opted the existing brain vasculature.¹¹⁷ The radiolabeled CooP was shown to provide an outstanding tool to visualize the intracranial malignant mass¹¹⁷ (Fig. 3B). In addition, CooP peptide conjugated to a chemotherapeutic drug was able to prolong the survival of the intracranial xenograft bearing mice and reduce the number of invasive tumor cells compared to the free drug.¹¹⁷ Moreover, the CooP-coated nanoparticles showed significantly better accumulation to the MDGI-expressing tumors than the naked particles^{118,119} and the glioma-bearing mice treated with paclitaxel-loaded CooP-coated nanoparticles showed increased survival compared to the paclitaxel-loaded non-targeted nanoparticles.¹¹⁸

Also, the extracellular matrix components can be used for targeting purposes. Fibronectin plays a key role in tumor expansion and formulations of nanoparticles conjugated to a fibrin/fibronectin-binding peptide (CLT-1 – CGLIQKNEC) identified by phage display¹²⁰ were successfully used for brain tumor imaging and/or drug delivery purposes in pre-clinical rat model of human glioma.¹²¹

Sai et al. identified overexpression of the interleukin receptor 13 RA2 (IL13RA2) as a glioma specific signature that is nearly absent in the normal brain.¹²² Using a cyclic hepta-peptide phage displayed library, they isolated a peptide called Pep-1L that specifically binds to IL13RA2.¹²³ [⁶⁴Cu]-Pep-1L bound to intracranial tumors in mice and showed clear contouring of the glioma.¹²²

Similarly, the 19-amino acid long peptide, angioprep-2 (TFYGGSRGKRNNFKTEEY), which binds to the low-density lipoprotein receptor-related protein-1 (LRP1) expressed by the BBB and even more by glioma cells, may provide deeper but non-invasive penetration of imaging probes due to the enhanced transcytosis by the BBB.¹²⁴ Interestingly, almost identical peptide, Angioprep-7, that contains arginines instead of lysines in positions 10 and 15 was not able to penetrate the BBB.¹²⁵

In another recent study with aim to target gliomas, a double targeting peptide for tenascin C and neuropilin-1 receptor was engineered by coupling the FHK peptide (FHKHKSPALSPV) isolated from phage displayed library¹²⁶ with tLyP-1 (CGNKRTR),¹²⁷ a truncated derivative of the tumor lymphatic targeting peptide (CGNKRTRGC, see chapter 4.1.2).⁹⁴ Conjugated with Taxol, this therapeutic peptide could penetrate the brain parenchyma and prolong the survival of immunocompromised mice bearing intracranial gliomas.¹²⁸

5. Clinical trials evaluating the peptide-based therapeutics

An immense majority of the targeting peptides used in the clinics nowadays consists of hormone analogues that are also used as imaging agents. However, some of the other peptide-based

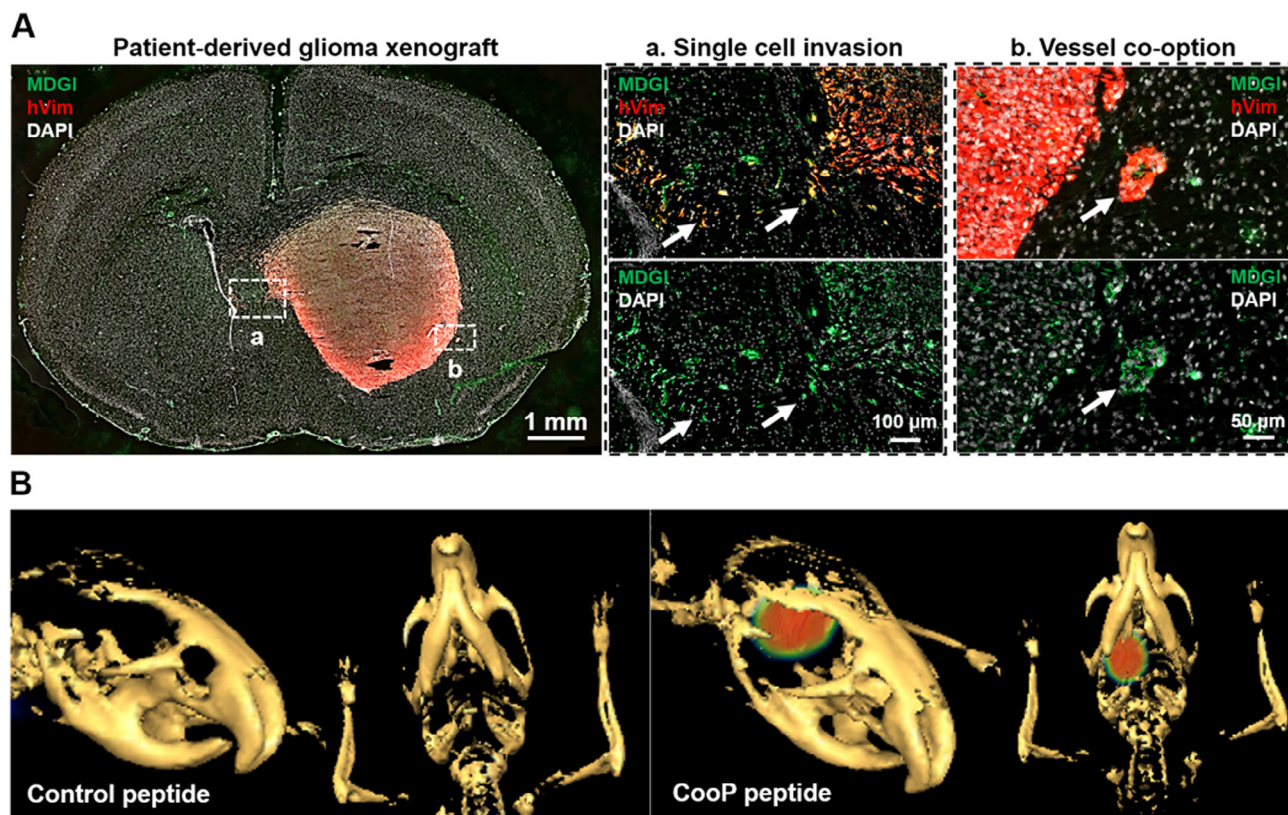


Fig. 3. CooP peptide homes to invasive glioblastoma cells. (A) Patient derived glioblastoma xenografts exhibit the hallmarks of the disease such as highly proliferative cells (human Vimentin labeling, in red), single cell invasion (frame a) and co-option of existing blood capillaries by tumor cells (frame b). The mammary-derived growth inhibitor (MDGI, green) is specifically overexpressed by the invasive and co-optive tumor cells (white arrows). *Le Joncour et al. unpublished figures.* (B) Radiolabeled CooP peptide can be used to deliver imaging agents to detect the intracranial development of glioblastomas in animals via microSPECT. Specific homing of CooP was confirmed by the absence of radioactive signal in the brain when a control radiolabeled peptide was injected to animals. *Hyvönen et al. unpublished figures.*

therapeutics have been evaluated in clinical trials. Unfortunately, like so many other clinical trials, also peptide-based therapeutics often exhibit great potential in theory or in pre-clinical studies but appear less effective in patients.

Somatostatin (SST) analogues provide an example of peptides derived from the natural ligand of the somatostatin receptors that has been used in the clinics. Since 1990's radiolabeled SST analogues have been evaluated in the peptide receptor radionuclide therapy (PRRT) in several clinical trials and shown promising results in the treatment of advanced, well-differentiated neuroendocrine tumors (reviewed recently in¹²⁹).

Of the other peptides reviewed here the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin binding peptide RGD has been most often evaluated in clinical trials of solid tumors. The safety and efficacy of Cilengitide (EMD121974, Merck), a cyclic RGD containing peptide, in combination with radiation and the drug-of-reference temozolomide (TMZ) was validated in a randomized phase II trial with 112 patients suffering from recurrent malignant glioma. Authors of this study concluded that Cilengitide was well tolerated and may improve survival of patients with newly diagnosed glioblastoma.¹³⁰ This was followed by a large scale multi-centered phase III clinical trial (CENTRIC EORTC 26071-22072) including 3471 patients suffering from glioblastoma. Patients received combination therapy of TMZ radiochemotherapy and Cilengitide. Median overall survival was 26,3 months in the Cilengitide group and 26,3 months in the control group.¹³¹ Unfortunately, despite extremely promising results in preclinical tests and in the phase I/II trials,¹³² the clinical trials failed with insignificant effect on the progression-free survival compared to the TMZ and as a result the development of Cilengitide for treatment of glioblastoma was stopped.^{131,133} However, a

recent phase I study used Cilengitide in combination with paclitaxel to treat 12 patients with advanced solid tumors. Again, the drug was well tolerated and showed some efficacy. Thus, the authors concluded that further studies evaluating drugs targeting this pathway would be warranted.¹³⁴

In addition, several phase I and II clinical studies have been conducted using a fusion molecule consisting of the NGR peptide conjugated to an anti-tumor cytokine, the human tumor necrosis factor (hTNF). These studies have shown good tolerability and encouraging results as therapy for resistant metastatic colorectal cancer and various other refractory or metastatic solid tumors.^{135–137}

6. Concluding remarks

Identification of targeting peptides have been inspired by the natural ligands as well as by using combinatorial screening of various libraries. Specific homing to any cancer marker is hypothetically possible using synthetic moieties, modified agonists/antagonists in addition to the new peptides that are discovered using for instance the phage display technology. The phage display screening technique has provided many excellent peptides targeting different tumor-specific markers in an unbiased manner. Many of the receptors of the targeting peptides are proteins whose localization is different in tumors than in normal cells. For example, the p32 protein, a receptor of the tumor lymphatics homing peptide LyP-1, resides normally in mitochondria but is found on the cell surface in tumors.⁹⁵ Due to their high specificity, low antigenicity, flexibility, and rather simple production, peptides represent possi-

bilities for the development of better targeted imaging and therapy options for various solid tumors in the field of personalized medicine of cancer.

Conflicts of interest of the authors

None.

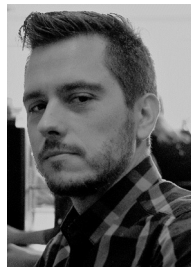
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Since 2015, Dr. Vadim Le Joncour has worked in Prof. Laakkonen's group on the preclinical validation of molecular nano-cargos displaying targeting peptides previously identified via the phage display technique. More specifically, his work focuses on different methods to increase the permeability through the blood-brain-barrier and the specific targeting of invasive glioblastoma cells. He is also interested in the fine molecular mechanisms involved in the metastatic spread of peripheral neoplasms that could constitute key targets for new drug development.



After completing her Ph.D. in 1996 at the University of Helsinki, on membrane association and intracellular localization of virus methyltransferase, Prof. Pirjo Laakkonen joined Prof. Erkki Ruoslahti's lab at the Burnham Institute (La Jolla, CA, USA) as a postdoctoral fellow. During the years in the Ruoslahti lab, she focused her research on developing and perfecting the phage display technology and successfully discovered novel targeting peptides specific to the tumor tissue and tumor lymphatics network. Back to Finland she founded her own research group at the Translational Cancer Biology Program of the Faculty of Medicine, University

of Helsinki, Finland. She currently leads key research projects on the identification of new molecular targets involved in the tumorigenesis and invasive spread of peripheral and central nervous system cancers.