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TUMOR HOMING PEPTIDES FOR DRUG DELIVERY AND IMAGING

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LIST OF ABBREVIATIONS

APN – Aminopeptidase N
CendR – C-end Rule
CRR – Colorectal Cancer
CT – Computed Tomography
DCE-MRI – Dynamic Contrast Enhanced MRI
ECM – Extracellular Matrix
EPR – Enhanced Permeability and Retention effect
F3-Cis-Np – Cisplatin-loaded Nanoparticles
GBM – Gliobalstoma
HCC – Hepatocellular Carcinoma
HMNG-2 – Human High Mobility Group Protein-2
KRIB – v-Ki-ras-oncogene Transformed Human Osteosarcoma
LCL – Long Circulating Liposomes
MPM – Malignant Pleural Mesothelioma
MRI – Magnetic Resonance Imaging
MTD – Maximum Tolerated Dose
NGR – Asparagine-Glysine-Arginine
NRP-1 – Neuropilin-1
PEG – Polyethylene Glycol
PET – Positron Emission Tomography
QD – Quantum Dots
RES – Reticuloendothelial System
RGD – Arginine-Glycine-Aspartic acid
SPIO – Superparamagnetic Iron Oxide
TNF-α – Tumor Necrosis Factor
TPP – Tumor Penetrating Peptide
TRAMP – Transgenic Adenocarcinoma of the Mouse Prostate Gland
VEGF – Vascular Endothelial Growth Factor

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H. Hunt

ABSTRACT

Tumor environment comprises of various signature molecules that are not expressed in such high levels in other organs and tissues in the body. This distinct feature of the tumor mass makes it an attractive target for anti-cancer drug delivery and molecular imaging. *In vivo* phage display has been used as a platform to exploit the tumor profile and various tumor homing peptides with different properties such as tumor and stage specificity have been discovered. Tumor homing peptides in conjugation with various anti-cancer agents and imaging probes have been validated in a variety of *in vitro* and *in vivo* tumor models. This review summarizes the recent progression in affinity-based cancer therapies with tumor homing peptides. Peptides with well documented homing specificities will be discussed and their applications will be described. We will also present current advances in the clinical development of the targeting peptides and introduce a novel class of tumor penetrating peptides.

1. INTRODUCTION

Cancer remains one of the leading causes of deaths worldwide (1). Current anti-cancer therapies are expected to target rapidly proliferating tumor cells, whereas common anti-cancer drugs are generally administered systemically and cause substantial side effects due to non-specific exposure of non-malignant cells (2). One way to increase drug selectivity is to use affinity ligands to specifically deliver the therapeutics to the tumor site and to differentiate between normal and tumor cells. Such physical targeting approach is one of the principal goals of modern anti-cancer research.

Tumors cannot evolve without a blood supply; tumor angiogenesis ensures a secure blood supply by initiating the sprouting of new blood vessels from existing ones to support tumor growth and metastatic spread (3). Tumor blood vessels differ from normal blood vessels. Apart from being leaky, twisted, irregular in their diameter, with loose inter-endothelial junctions, they also display distinct molecular markers such as cell surface and extracellular matrix proteins which are expressed by normal vessels at much lower levels or not at all (4). The signature set of molecules that distinguish angiogenic tumor blood vessels from resting blood vessels compose of certain integrins, endothelial growth factor receptors, cell surface proteoglycans and proteases (3, 5). Many of these molecules are functionally important for the process of angiogenesis (6, 7). Furthermore, published evidence demonstrates that vascular diversity extends beyond angiogenic status-related molecules, with distinct molecular patterns related to the tumor grade, location and metastatic status (8). In addition to blood vessels, tumor tissue contains lymphatic vessels that transport interstitial fluid and macromolecules back to the blood circulation from the tumor (9). Tumor lymphatics are dysfunctional and lymphatic endothelial

cells display a specific set of molecular markers that distinguish them from lymphatics of the non-malignant tissues (9, 10).

Affinity-based targeting (also known as synaphic or active targeting) utilizes systemically accessible tumor-specific molecular markers to guide systemically administered drugs to tumors (6). The intended outcome of this approach is zeroing-in on the target with a high local and low systemic exposure - comparable to topical administration (4). An important aspect is that unlike tumor cells, vascular cells are genetically stable and provide a steady target for targeted delivery (3). Antibodies and their fragments are widely used to deliver anti-cancer drugs and imaging agents into tumor lesions (11). Various monoclonal antibodies (e.g. Trastuzumab for breast cancer, Bevacizumab for colorectal cancer, Cetuximab for colorectal cancer) are already clinically used (11, 12). Nevertheless, antibody applications have disadvantages that limit their efficacy such as insufficient tissue penetration due to the large size and immunogenicity (13). As affinity ligands, peptides have several advantages over antibodies. The small size of peptides enables superior tissue penetrating properties and low immunogenicity (14). Compared to antibodies, peptides are cheap to manufacture. *In vivo* stability of the peptides can be increased by incorporation of unnatural amino acids and peptides can be readily conjugated with different diagnostic and therapeutic payloads (4, 14). *In vivo* biopanning of peptide libraries displayed on the surface of a bacteriophage is a powerful tool that allows identification and validation of molecular markers expressed in the lumen of the tumor vessels (3). Tumor homing peptides as targeting probes enable delivery of diagnostic material and drugs to tumor blood vessels and to tumor parenchyma thus improving tumor detection and increasing the efficacy of the treatment while reducing unwanted side effects (4).

This review focuses on peptides with tumor homing characteristics discovered by *in vivo* phage display. We discuss tumor homing peptides with well-established specificity and receptor(s) and their applications for drug delivery and imaging.

IN VIVO PHAGE DISPLAY

In vivo peptide phage display was pioneered in 1996 by E. Ruoslahti´s lab (15). This technique has yielded unique peptide motifs to target angiogenic tumor vasculature and various normal and diseased tissues (15). Bacteriophages can be genetically modified to express random peptides as fusions with the coat proteins (14). There are two main phage display systems: Filamentous phage and T7 phage. The important distinction between them is that the T7 bacteriophage display system enables the expression of the exogenous peptide in the C-terminus of the phage coat protein whereas in the Filamentous phage system it is displayed in the N-terminal distal end (14). The difference in orientation is relevant when we will describe peptides exhibiting the C-end Rule (CendR) motifs because they must have a free C-terminal carboxyl group for activity and therefore can only be selected from the T7 system (14, 16). These peptides are considered to be a new class of position-dependant peptides for synaphic targeting (14). The phage library typically contains about a billion of unique peptides and the diversity of the library is an important determinant of the success of the screening. *In vivo* page display is similar to the conventional *in vitro* phage screening on purified target molecules, with the target changed to a systemically accessible tissue compartment in a live animal. In brief: The phage libraries are injected intravenously and after a short incubation time of around 5-15 minutes the animal is sacrificed and the phage bound the target organ (or tumor) is amplified and used for subsequent rounds of selection (3). After 3-5 rounds of selection, various peptide motifs specific

for the target tissue are identified. The DNA that encodes the displayed peptide is sequenced and the corresponding synthetic peptides are used for targeting *in vivo* (3). (Figure 1)

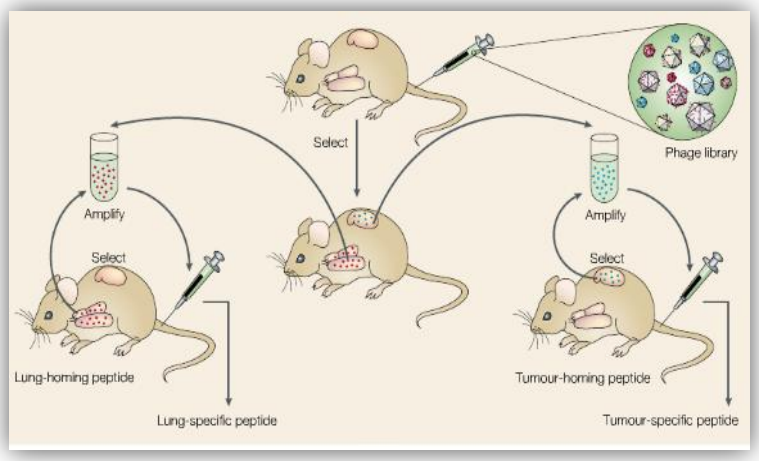

FIGURE 1 *IN VIVO* SCREENING OF PHAGE DISPLAY LIBRARIES TO IDENTIFY TUMOR HOMING PEPTIDE. Adopted from Ruoslahti et al. 2002 (3)

This unbiased exploratory technique has revealed a great number of peptides that home specifically to tumor vasculature. These unique molecular signatures of tumor vessels enable to target a variety of therapeutic and diagnostic agents into tumors and to provide new targets for drug discovery (3). In addition, some of the homing peptides recognize besides tumor vasculature also cancer cells, tumor lymphatics, and possess unique tumor penetrating properties (4, 10, 17).

2. PEPTIDES TARGETING TUMORS

2.1 HOMING PEPTIDES AND THEIR TARGET MOLECULES

As described in the introduction, *in vivo* phage display allows unbiased identification of the homing peptides to any systemically accessible target tissue. Homing peptides can be used to reveal its binding counterparts- "receptors". Besides being important for understanding of the homing process, tumor-specific peptide receptors may be functionally important for cancer biology and become targets of anticancer pharmacological intervention (18). For example, antibodies raised against receptor of homing peptide F3, nucleolin, have profound normalizing effect on the angiogenic tumor vessels (19, 20). The technique of choice for the receptor identification is affinity chromatography or peptide "pull-down" followed by the analysis of the bound peptides with mass spectrometry (4, 14).

The first generation of homing peptides revealed by *in vivo* phage display include arginine-glycine-aspartic acid (RGD) and asparagine-glycine-arginine (NGR) (21). After intravenous injection into tumor-bearing mice the RGD peptide was observed to home to melanoma and breast cancer lesions (22). The RGD4C peptide contains an angiogenic αvβ3 and αvβ5 integrin-

binding motif in the context of two internal disulfide bonds (18, 23, 24). Integrins are heterodimeric cell surface proteins that mediate close interactions with the extracellular matrix (ECM) molecules such as fibronectin and type I collagen and are important contributors to tumor cell growth, migration, invasion and survival (25, 26). The subset of integrins expressed on the surface of resting endothelial cells differs from that expressed on the angiogenic tumor endothelial cells (3, 18). Moreover, the integrins expressed on the luminal side of the vascular endothelial cells will be easily accessible for circulating compounds (3, 4). Internalization of the RGD containing peptides to the endothelial cells is mediated by receptor-mediated endocytosis or phagocytosis process (27).

The NGR motif was determined by *in vivo* phage screen on human breast cancer xenografts and it is shown to home selectively to angiogenic vessels (22). Despite the capacity of NGR to home to αv integrins, the affinity for the receptors is much lower compared to RGD peptide (28, 29). The receptor for NGR motif is aminopeptidase N (APN) which is a membrane spanning surface protein overexpressed in angiogenic endothelial cells, in tumor cells, and in tumor stromal cells (29). Studies conducted with function-blocking antibodies against APN and APN null mice demonstrated impaired angiogenesis and tube formation (22, 29, 30). Whereas also normal epithelial cells express APN, i.v injected NGR peptide showed specific homing to vessels in tumors and in other angiogenic tissues (29). The disulfide-bridged cyclic conformation of the NGR peptide appeared to home to tumors more effectively than linear NGR peptides, possibly due to increased resistance to proteolysis and stability of the peptide in the bloodstream (21, 29).

Another peptide that homes to angiogenic vasculature is F3 (22). F3 is a 31-mer peptide that corresponds to N-terminal fragment of HMNG-2 (human high mobility group protein-2) (31). The peptide was isolated from a cDNA library after a combined screen first *in vitro* on progenitor cell-enriched bone marrow cells followed by *in vivo* screen to confirm the homing to human myeloid leukemia (HL-60) xenograft tumors (31). The binding partner for F3, a cell surface expressed nucleolin, was identified by affinity chromatography on the MDA-MB-435 breast carcinoma cell extracts on an F3 affinity matrix (31). Nucleolin is an abundant intracellular protein that is expressed on the surface of activated tumor cells and angiogenic tumor endothelial cells (19). A study conducted by Christian et al., 2003 confirmed that i.v. injected anti-nucleolin antibody selectively binds to non-malignant angiogenic blood vessels and to tumor blood vessels and in the same time showing anti-angiogenic effects (19). Importantly, different compartments within tumor tissue can be targeted with different homing peptides. For example, tumor lymphatic vasculature expresses a set of molecules different from tumor vascular endothelial cells that can be a used for the detection of new homing peptides of different specificities (9). For example, *ex vivo* and *in vivo* combined phage screening on a MDA-MB-435 breast cancer xenograft mice yielded in a cyclic peptide comprising of 9 amino acids (CGNKRTRGC) (10). This peptide, Lyp-1, is the first peptide to home to the tumor lymphatic vessels and hypoxic areas (10). Lyp-1 exhibits tumor-type specific binding patterns, apart from homing to MDA-MB-435 tumors, it shows affinity for transgenic prostate tumor (TRAMP), transgenic breast carcinoma and to KRIB osteosarcoma tumor xenografts. In contrast, it does not home to specific melanoma and leukemia cells and does not recognize certain transgenic cervical cancer models (9). This distinct binding pattern demonstrates that the molecular patterns expressed on tumor lymphatics depend on the tumor type. A set of peptides homing to tumor lymphatics identified by Zhang et al. revealed similar organ and stage-specific changes in

the lymphatics during the development of different tumors (32). The receptor for Lyp-1 is p32/gC1qR, a mitochondrial protein that shows cell surface expression in tumor lymphatics and macrophages (33). P32 has proven to play an important role in cell metabolism by shifting the balance from oxidative phosphorylation to glycolysis and increased glycose consumption is known to favor tumor growth (34). P32 knock-out mice show reduced tumor growth in an aggressive breast cancer model (34).

Tumors have been called "wounds that do not heal" and the walls of tumor blood vessels and interstitial tissue contain a meshwork of clotted plasma proteins that are not present in normal tissue (35, 36). Such products of blood clotting probably represent the aftermath of leaking tumor vessels. The leaked fibrinogen into the extravascular space is subsequently converted to fibrin meshwork by tissue procoagulant factors (37). Other leaked plasma proteins such as fibronectin forms a covalent bond with the fibrin meshwork (35). CREKA and CLT-1 (CGLIIQKNEC) are peptides characterized by the specific homing to fibrin-fibronectin complexes in tumors where they induce additional clotting and therefore extra binding sites (self-amplification) (37). CLT-1 is a 9-mer cyclic peptide and CREKA a pentamedric linear peptide. Since CREKA exhibits a sulphydryl group in the single N-terminal cystein residue, it is possible to couple different payloads to the peptide (37-39). It has been established that CREKA and CLT-1 is dependent on the presence of fibrinogen as the peptides fail to show any specific homing to tumors grown in fibrinogen-null mice and in plasma fibronectin deficient mice (38).

Most of cell surface target receptors of tumor homing peptides are expressed by both vascular endothelial cells and tumor cells (15). In theory, the same homing peptide could be used for targeting both tumor endothelial cells and tumor cells outside the blood vessels. However, an important challenge in the treatment of solid tumors is to achieve sufficient vascular exit and tissue penetration of the targeted payloads (40). The effect of many targeted anti-cancer drugs is hampered by the poor penetration into tumor tissue (2). It is known that drugs are typically capable of penetrating only three to five cell diameters outside the blood vessels while the distant tumor cells are subjected to very low drug concentration that can promote the development of resistance (2). The problem is exacerbated with high interstitial pressure due to leaky vasculature and poor lymphatic drainage (41). A novel class of peptides capable of inducing tissue penetration and cell internalization was recently identified using a combination of ex vivo and *in vivo* phage display on prostate tumor-bearing mice (40). These multifuntional peptides provide not only tumor homing but are also capable of exiting the vessels and penetrating into the tissue (17). The tumor penetrating peptide (TPP) iRGD (CRGDK/RGPD/EC) specifically homes to αv integrins that are expressed on tumor endothelium, tumor fibroblast and tumor cells (17). After binding to integrins, the peptide is proteolytically cleaved and the truncated peptide loses its integrin-binding capacity and gains affinity for tissue penetration receptor neuropilin-1 (NRP-1) due to the C-terminal exposure of the R/KXXR/K (x-random amino acid) CendR motif (40). NRP-1 is a trans-membrane co-receptor of vascular endothelial growth factor (VEGF) that is known to be involved in regulation of vascular permeability and angiogenesis (17). The binding of CendR to b1b2 domain of NRP-1 induces cell internalization and the activation of the trans-tissue transport pathway (Figure 2) (17). The pathway is an active transport pathway as it requires energy and is much faster than diffusion (42). Importantly, some of previously described tumor homing peptides such as Lyp-1 and F3 also contain (R/K)XX(R/K) CendR motifs (10, 31, 43).

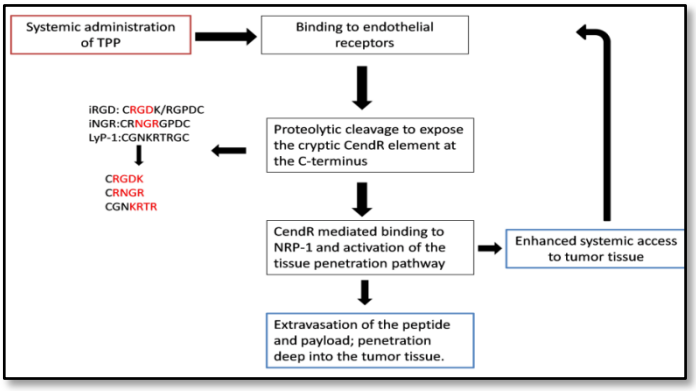

FIGURE 2 SCHEMATIC REPRESENTATION OF THE CendR MEDIATED MULTI-STEP TRANS-TISSUE TRANSPORT PATHWAY. The binding to NRP-1 mediates extravasation of the processed peptide and activates a bulk transport process to enhance the delivery of payloads and systemic access of compounds present in the blood, including TPP for continuous penetration into the tumor tissue. Partly adapted from Teesalu et. al 2009 and Sugahara et. al 2009 (17, 40)

Interestingly, the molecular signatures of angiogenesis have stage-specific components (8). A variety of peptides identified with *in vivo* phage display show that the vasculature of pre-malignant lesions and full-blown lesions have different molecular patterns (7). This was demonstrated in several transgenic murine tumor models (8, 9, 44). The corresponding receptors recognized by those peptides remain to be determined although the peptide probes can be already used to target tumors in a stage-specific manner.

The distinct expression of extracellular proteins in tumor vascular endothelial cells and in tumor cells appears to be a general principle and phage display has proven useful in mapping the differences.

3. TUMOR HOMING PEPTIDES AS DELIVERY SYSTEMS

3.1 HOMING PEPTIDES FOR DRUG DELIVERY

The two most widely used homing peptide motifs for drug delivery are RGD and NGR (4, 45). Both of the motifs have been successfully used to deliver tumor necrosis factor-α (TNF-α) which is considered to exhibit potent antitumor activity (46). The clinical use of TNF-α as an anti-cancer agent has been limited due to its general toxicity profile, but by fusing it with either RGD or NGR to constitute a new recombinant protein, anti-tumor activity was enhanced at a very low dose (0,3 μg) in a tumor model of mice (46). The dose was approximately 1000-fold lower than the typical dosage of a free TNF-α thus dramatically reducing the side effects of this highly toxic cytokine (46, 47). TNF-α coupled to RGD or NGR inhibited tumor growth in B-16 melanoma and RMA-T lymphoma bearing mice after i.v. injection compared to the free form of TNF-α (47, 48). NGR-TNF-α in combination with various chemotherapeutics such as doxorubicin, melphalan, paclitaxel and gemicitabine has shown to enhance the anti-tumor efficacy on various mouse tumor models compared to the drugs alone and a decrease in side-effects was observed (49). Furthermore, it was reported that in a prostate carcinoma murine model after pre-treating with

NGR-TNF-α followed by repeated cycles of doxorubicin, the therapeutic index of doxorubicin was increased and tumor growth was inhibited with less side effects (50). Similar results have been obtained with RGD-TNF-α in several tumor xenograft mouse models compared to mephalan and other chemotherapeutics (47). Besides TNF-α, various other inflammatory signaling molecules, such as interferon-γ and interleukin-12, have been genetically fused with RGD/NGR to increase their anticancer activity and to decrease side effects (47, 51, 52). TNF-α fused with NGR is currently under investigation in phase III clinical trials for malignant pleural mesothelioma (MPM) (53, 54).

RGD and NGR targeting probes have also been coupled to doxorubicin resulting in increased inhibition of tumor growth and angiogenesis in a mouse xenograft model of breast cancer (21). Furthermore, the targeted doxorubicin showed reduced toxicity profile thus effectively mitigating side effects in the heart and liver which are the main organs to encounter doxorubicin toxicity (21).

In addition to anti-cancer agents, apoptosis promoting peptide $KLAKLAK_2$ has also been used in conjugation with RGD or NGR to inhibit tumor growth in mice (55). $KLAKLAK_2$ is an antimicrobial proapoptotic sequence that is capable of disrupting the bacterial membranes and causes damage to mammalian cells only after internalization (56). Once inside the cell, it induces swelling of the mitochondria subsequently disrupting the membrane which leads to apoptosis (56). This can be explained by the fact that the cytoplasmic membrane of prokaryotes and mitochondrial membrane of eukaryotes share common characteristics such as high content of anionic phospholipids and therefore propose a target for the antimicrobial protein (56). RGD or NGR coupled $KLAKLAK_2$ displayed elevated toxicity on tumor endothelial cells and therefore we able to reduce tumor growth and prolong survival rate (55). Moreover, the same apoptotic peptide in conjugation with specific prostate homing peptide SMSIARL was able to cause tissue destruction, postponed cancer development and reduced prostate size which proposes an alternative non- invasive treatment for prostate hypertrophy (57). After the conjugation of another antimicrobial peptide-tachyplesin to RGD an induced apoptosis in cell culture was detected as well as tumor growth inhibition in mice (58).

The tumor-homing CendR peptides described above improve parenchymal penetration of the coupled drugs (40). iRGD or LyP-1 attached to a drug are able to deliver a substantial amount into the tumor tissue compared to the drug alone (17). Interestingly, iRGD and LyP-1 both show a very distinct distribution pattern in the tumors, probably due to the different expression pattern of their primary recruitment receptors (angiogenic integrins and P32, respectively) within tumor tissue (40, 59). When iRGD and other CendR peptides were tested in different tumor models it appeared that a diverse set of therapeutics varying from small molecular weight doxorubicin, anti-Her2 antibody (Trastuzumab) to nanoparticle drugs such as Abraxane and Doxil could benefit from the iRGD-induced delivery (42). Besides targeting tumor blood vessels, the iRGD peptide is capable of penetrating deep into the tumor tissue - the outcome is delivery of 10 times more drug cargo into the tumor tissue compared to the conventional RGD peptide lacking a CendR motif (40).

Of great translational relevance, the CendR peptides have a unique ability to activate a bulk transport pathway able to sweep along any compound present in the blood when the system is activated (4, 42). Co-injection of iRGD with unmodified anticancer drugs resulted in enhanced tumor penetration and accumulation of each of the several compounds tested, and the

phenomenon has been observed in a large number of preclinical tumor models (transgenic models, xenograft and syngeneic implants of breast, pancreatic, prostate, and ovarian tumors), including metastases (42). iRGD combination treatment did not affect the extent of the side effects, such as cardiomyopathy caused by doxorubicin. Thus, iRGD broadens the therapeutic index of cancer drugs (42).

3.2 HOMING PEPTIDES FOR NANOPARTICLE DELIVERY

In addition to the delivery of various molecular therapeutics, amplified tumor homing of nanoparticles can also be achieved. T7 phage widely used for in vivo phage display is a 55 nm nanoparticle that is similar in size to clinically used nanoparticles, and peptides selected using this system may be particularly useful for targeted delivery of nanoparticles (4). Nanosize drug carriers such as micelles, liposomes or polymers can increase the targeting effect by having longer circulation times (45, 60). The clinically used liposome, albumin, or polymer-based nanoparticles loaded with anticancer drugs have higher therapeutic efficacy, improved solubility, and lower toxicity compared to respective free drugs (45). The nanoparticle drugs that have reached the market until now target tumors passively through enhanced permeability and retention (EPR) effect (61). EPR refers to the phenomenon that due to tumors' leaky vasculature molecules and particles on the tens to hundreds of nanometers scale can exit blood vessels and penetrate tumor interstitial spaces (45, 61). Due to the impaired lymphatic drainage in tumors the particles accumulate along with the chemotherapeutic they carry (62). However, it has been shown that EPR effect is not a constant feature in tumor vessels and even if present, high interstitial pressure and slow time frame propose additional limits to the entry of nanoparticles to the tumor interior environment (63, 64). Therefore, it has been proven that by adding a tumor penetrating peptide on the surface of nanoparticles it is possible to increase their tumor selectivity, tissue penetration and potency (40). For example, coating of abraxane, the clinically approved albumin-based nanoparticle of paclitaxel with either Lyp-1 or iRGD peptide increased the drug penetration into the tumor tissue and resulted in significantly higher inhibiton of tumor growth than the free drug (40, 65).

The conjugation of nanoparticles with different ligands has demonstrated potential therapeutic efficacy in targeted drug delivery (45). Achieving extravasation and deep infiltration of anticancer nanoparticles into the tumor mass remains a challenge that may be potentially circumvented by tumor penetrating peptides (40, 42). iRGD functionalized liposomes enter the cells via the clathrin-mediated pathway and are able to efficiently deliver antcancer agents and promote tumor suppression (66). Among other tumor types, tumor inhibition was seen on multidrug-resistant cells (66). Furthermore, iRGD targeted nanoparticles loaded with either doxorubicin or paclitaxel showed promising results on cellular toxicity and targeting evaluated by *in vivo* imaging compared to the conventional RGD peptide in a glioblastoma (GBM) model (66). To achieve a nanoparticle profile with controlled release, temperature- and pH-sensitive doxorubicin-loaded nanogels were used (67). The particles were able to maintain their properties *in vitro* therefore future *in vivo* stability studies are encouraged for more precise delivery systems (67). Another tumor penetrating peptide, Lyp-1, was coupled to abraxane and homing of fluoresein-labeled nanoparticles to extravascular p32 was observed (65)(65). When CREKA, the self-amplifying peptide specifically homing to fibrin-fibronectin complexes was coupled to the surface of abraxane, it accumulated in tumor blood vessels of mice bearing MDA-MB-435 human cancer xenografts, leading to formation of aggregates that contained red blood

cells and fibrin (65). Despite, the study showed that about 20% of the vessels within the tumor were occluded, it was not sufficient to inhibit tumor growth (38). According to recent updates, the system has been improved, now resulting in approximately 60% of vessel occlusion along with tumor necrosis and significant growth inhibition (68). In addition, blood clotting was not detected in any other tissue or blood vessels in the RES (reticuloendothelial system) organs indicating that the targeted clotting system relies on the tumor environment. In a recent study the F3 peptide was coupled to cisplatin-loaded hydrogel nanoparticles (F3-Cis-Np) to target tumor vessels (69). The study revealed that although F3-Cis-Np bind with high specificity to both human ovarian tumor cells and tumor endothelial cells *in vitro*, the cytotoxic activity could only be detected on the tumor endothelial cells. After the treatment with F3-Cis-Np significant vascular necrosis was detected on a murine xenograft model suggesting a possible new treatment for human ovarian cancer (69). In addition, F3 was integrated onto the surface of a micelle containing fluorescent quantum dots, iron oxide nanoparticles and doxorubicin (70). Those multifuntional micelles increased intracellular delivery to the targeted MDA-MB-435 cells providing simultaneous imaging applications (70).

In another study the pro-apoptotic peptide $KLAKLAK_2$ was used as a drug for multifunctional theranostic nanoparticle for the treatment of GBM (71). It has previously been shown that $KLAKLAK_2$ is a toxic compound even with specific targeting (57, 72). Therefore, constructing it in a nanoparticle formulation resulted in a 100 fold lower dose and enabled to eliminate the toxicity problem (71). The nanosystem consisted of iron oxide nanoworms coated with a tumor-specific vascular homing element CGKRK (8) and $KLAKLAK_2$ serving as a drug. This system was later improved with the injections of iRGD which resulted in tissue extravasation and penetration of the blood-brain-barrier (BBB) increasing the survival of the GBM mice (71). The combined multifunctional targeting system to GBM tumors enables tissue targeting (vessels), subsequent internalization into the tumor cells and further penetration into the tumor tissue by iRGD, followed by a delivery of the payload to a subcellular organelle and prolonging the survival of GBM mice (71).

Liposomal formulations of doxorubicin such as Doxil/Caelyx have been clinically approved and various other lipid-based nanosystems are currently in clinical trials (60, 73). The formulation mainly relies on the EPR effect related to the leaky and dysfunctional vasculature. The coupling of liposomal doxorubicin to the NGR peptide, showed approximately 3 fold higher uptake and localization to the cell nucleus in a murine neurobalstoma model compared to non-targeted liposomes (74). Furthermore, RGD-peptides were incorporated to the distal end of poly-ethylene glycol (PEG) coated long-circulating liposomes (LCL) to obtain a stable long-circulating drug delivery system (75). The results indicate that cyclic RGD-peptide-coupled LCL displayed increased binding to endothelial cells *in vitro* and intravital microscopy revealed a specific interaction of these liposomes with tumor vasculature and this behavior was not observed for LCL (75). RGD-coupled LCL containing doxorubicin successfully repressed tumor growth in a doxorubicin-insensitive murine C26 colon carcinoma model, whereas the non-targeted LCL-doxorubicin did not succeed to inhibit tumor growth (75). In conclusion, superior efficacy was obtained through coupling of nanoparticles with tumor homing peptides and notable anti-tumor effects were detected possibly owing it to direct interaction with specific markers expressed on tumor endothelium (75).

3.3 HOMING PEPTIDES FOR IMAGING

Early and precise detection along with effective prevention is one of the keys to successful cancer treatment (13). So far, conventional anatomical imaging techniques such as computed tomography (CT) and Magnetic Resonance Imaging (MRI) enable tumor detection when tumors exhibit a size bigger than a centimeter in diameter (76, 77). Therefore, it is apparent that more sensitive imaging approaches are needed for early detection and increased performance for cancer diagnosis. Traditional imaging techniques rely on anatomical structures of organs whereas molecular imaging detects specific molecular probes and utilizes certain receptors unique in the tumor environment (77). Molecular imaging is considered to be a highly promising method for the early monitoring and detection of changes in key molecular behaviors and host responses related to early stage-specific events in disease development as well as progression in molecular and cellular levels (13, 76). Thus, peptides specifically homing to tumors with high binding affinity are a prosperous diagnostic approach for target specific cancer imaging.

Tumor homing peptides have been tested in various imaging approaches as molecular imaging probes (76). RGD and NGR peptides have been widely used to deliver different imaging agents (45). For example RGD and NGR peptides were coupled to quantum dots (QD) for tumor imaging purposes (78). The particles were injected to tumor bearing mice and the aim was to evaluate angiogenic activity in the tumors by the MRI system. The study showed that NGR-QD primarily located on the surface of tumor endothelial cells, less in the vessel lumen and did not extravasate into the tumor core (78). On the other hand, non-targeted QD were barely detected or not at all confirming the affinity for angiogenic tumor vasculature of the NGR-QD (78). Furthermore, Phase I trial with breast cancer patients has shown that PET imaging based on 18F-AH111585/RGD conjugate was retained in the tumor tissue thus, enabling PET imaging of breast cancer (79). Various other agents have been coupled to RGD peptide for imaging enhancement such as Ga, Cu and near infrared fluorescent (80, 81).

The self-amplifying peptide CREKA specifically recognizing clotted plasma proteins in tumors was also coupled to iron oxide particles. It demonstrated particle accumulation in tumor vessels where they induced additional clotting therefore providing new binding sites for more particles (38). It was shown that this clotting-based system significantly amplifies tumor imaging (38). Although, initially the targeting potential of CREKA-SPIO was impaired due to the fast uptake by RES and therefore the uptake by MDA-MB-435 cells was not effective compared to the peptide alone (38). To overcome the uptake by RES, a potential decoy particles were used which consisted of liposomes coated with Ni^{2+}. By injecting the coated liposomes, the half-life of CREKA-SPIO was increased approximately 5 fold as well as a notable increase in tumor homing mainly associated with blood vessels was detected (38).

In addition, Lyp-1 has been used in optical imaging in tumors. It showed that fluorescein-conjugated LyP-1 strongly accumulated in primary MDA-MB-435 breast cancer xenografts and their metastases from i.v. peptide injections (59). This method enabled visualization of orthotopic tumors in intact mice by concentrating the fluorecein to the nuclei of target cells. In addition, the LyP-1 peptide accumulation corresponded with hypoxic areas in tumors (59). Moreover, QD have also been coupled to Lyp-1 and F3 peptides to evaluate their tumor imaging capacity (31, 82). Peptide coupled QD showed successful homing specificity to their corresponding receptors, namely LyP-1-QD homed to tumor lymphatic vessels and F3-QD to the blood vessels (10, 31, 82). Interestingly, the fluorescein coupled Lyp-1 showed tissue

penetration properties whereas peptide coated QD only homed to the vasculature and were not found in the parenchyma which can be due to the rather large size of the peptide coupled QD (82).

The newly discovered tumor penetrating peptides proved to amplify tumor imaging, as was shown by coating iron oxide nanoparticles with iRGD for MRI (40). The data demonstrated that particles coated with iRGD enhanced the contrast on the MRI images compared to conventional RGD peptide. iRGD-coated particles displayed a wide distribution pattern in the tumor tissue determined by histopathological staining whereas RGD was only able to delineate tumor vasculature (40). These suggest the great potential of iRGD peptide conjugates for diagnostic imaging.

These data demonstrate the high potential of peptide-targeted delivery of imaging probes with different imaging techniques after systemic delivery.

4. HOMING PEPTIDES IN OR ON THE WAY TO THE CLINIC

In order to understand the potential relevance of tumor homing peptides discovered by *in vivo* phage display, it is important to mention the current state and recent updates in the clinical phase. The first generation of tumor homing peptides such as RGD and NGR have already entered clinical trials as a single and combination therapy and results from Phase I and II trials have been reported (83, 84). NGR-TNFα as a single agent underwent a phase I trial to determine the toxicity profile and the maximum tolerated dose (MTD) (85). Results showed that the compound was well-tolerated with a MTD of 45 μg/m^2. Furthermore, vascular effect of NGR-TNFα was determined with Dynamic Contrast Enhanced MRI (DCE-MRI) (85). Notable results have been obtained with NGR targeted TNFα to evaluate its safety in combination with doxorubicin to treat refractory/ resistant solid tumors. 15 patients were given an i.v. injection of different combinations of NGR-TNFα and doxorubicin during 3 weeks (86). The results indicated that no dose-limiting toxicity was detected and the combination was well tolerated. 11% of the side effects were related to NGR–TNFα and were short-lasting and mild-to-moderate in severity (86). NGR-TNFα plus doxorubicin was administered safely and showed promising activity in patients pre-treated with anthracyclines (86). Another Phase I trial was conducted with a low-dose of NGR-TNFα in combination with Cisplatin for the treatment of refractory solid tumors (84). The results were comparable to combination therapy with doxorubicin showing induced response to therapy and disease stabilization overcoming serious side effects of the cytokine (84). No patients were reported to develop anti- NGR-TNFα antibodies (84). This encouraged proceeding with Phase II development. Single-agent phase II studies with low-dose NGR-TNF (0.8 lg/m2, 1 h infusion, every 3 weeks or weekly) were performed in several tumors such as malignant pleural mesothelioma (MPM), hepatocellular carcinoma (HCC), and colorectal cancer (CRC) (53, 83). At low doses of NGR–TNFα a significant disease control and radiologically detected anti-vascular effect was determined. NGR–TNFα was well tolerated and the study in MPM patients showed disease control which was maintained up to 9 months. These results are noteworthy since currently there are no standard options for MPM patients who are unresponsive to pemetrexed-based regimen and considering the mild toxicity profile of NGR–TNFα (83). Based on these results of NGR–TNFα on patients with MPM and HCC, the compound

was granted an Orphan Drug status for the above mentioned indications in the European Union and the United States of America (83). Currently, NGR–TNFα is being evaluated in phase III trials for the treatment of MPM as well as Phase II studies in various solid tumors as a single agent and combination therapy (54).

Cilengitide (EMD 121974, Merck) is a derivative of the RGD peptide, a cyclic pentapeptide and the only small molecule exhibiting antagonistic effects for αvβ3 and αvβ5 integrins and therefore inducing apoptosis and impairing tumor growth in human xenografts in mice and other animal models (87). It is currently under investigation in clinical trials for the treatment of glioblastomas which are the common type of primary brain tumors (87, 88). Cilengitide monotherapy demonstrated an unexpected anti-tumor activity and no dose limiting toxicities in the first Phase I trial in patients with recurrent glioblastomas (89). A multicenter Phase II study was performed to investigate and evaluate the efficacy and tumor delivery (88). Patients were treated with Cilengitide prior to undergoing tumor resection followed by blood collection for plasma and tumor comparison (88). Cilengitide was detected in all tumor specimens in the high dosage group while corresponding plasma concentrations were low (88, 89). The study showed that Cilengitide as monotherapy exhibited moderate efficacy in recurrent glioblastoma, but was successfully delivered and retained in tumors with the established dosing (90). Several other Phase II and Phase III trials are ongoing where Cilengitide is administered in combination with the standard chemoradiotherapy agent Temozolomide for glioblastoma patients and for various other cancers with promising therapeutic outcomes to date (91-93).

Recently, the tumor homing and tissue penetrating peptide iRGD entered an open-label Phase I safety and efficacy study. The study has a diagnostic purpose in patients with various tumors as well as metastasis. The study is set up to investigate and measure the patients' response to treatment with DCE-MRI and to explore whether iRGD is capable to elicit changes in cancer vascular permeability. Furthermore, the pharmacokinetics of iRGD peptide will be evaluated (NCT01741597) (http://clinicaltrials.gov/ct2/show/NCT01741597).

5. CONCLUSIONS & FUTURE CHALLENGES

This review focused on peptides specifically homing to tumors, their receptors and their applications. Remarkably, the tumor homing peptides described do not home to the corresponding normal tissue, but recognize and have strong affinity for vascular markers upregulated during tumor development. Therefore, the target molecules for tumor homing peptides can also be considered as biomarkers of a disease that are intracellular in a normal tissue, but quickly become upregulated in the tumor environment. As the molecular pattern in the tumor environment seems to be very distinct, it is no surprise that some conditions such as inflammation, stroke and arthrosclerosis are also accompanied with various markers associated with angiogenesis and thus shared with tumors. Surely, it will further enrich the application of those peptides, but if in certain circumstances a tumor coexists with one of the conditions, targeted therapy with tumor-homing peptides might be excluded due to possible undesired vascular destruction in the non-malignant tissue. For example, peptide Lyp-1 originally homing to tumor cells and tumor lymphatics was tested in a mouse model of atherosclerosis where it showed significant penetration and accumulation in the plague interior (94). Alternatively, the discovery of markers with more focus would enable to overcome this problem.

As the collection of tumor homing peptides increases, the receptor identification has proven to be a bottleneck of the system. Although several methods exist, adequate and successful receptor identification depends on the characteristics of the peptide and the corresponding receptor along with the affinity between them (22). Receptors could as well be motifs of lipids or carbohydrates located on the cell surface which is a possibility that cannot be excluded (13).

Until now, the most reoccurring model in cancer related studies is the subcutaneous inoculation model. However, it is uncertain whether this model might really mimic the real microenvironment where the cancer originates and might therefore modify the natural progression of the tumor. Growing evidence show that cancers interplay with their surroundings and pro- and anti-angiogenic cytokines are a vital element in tumor microenvironment which in turn can have an impact on angiogenesis, apoptosis, proliferation, metastasis (95). For this reason orthotopic tumor models are more suitable as they provide cancer cells the growth in their natural location and environment thus mimicking the human disease to a greater extent.

Over the years, a variety of therapeutic molecules have been conjugated to tumor homing peptides to increase their tumor selectivity. This method has revealed a targeted delivery system capable of increasing the efficacy while decreasing the side effects of the corresponding therapeutic agents. Peptide-based delivery of compounds has many advantages such as small size which enables efficient tissue penetration and is of non-immunogenic nature. Moreover, they are very versatile and exhibit high specificity towards the target tissue. Although their short half-life and rapid clearance prevents them accumulating in tissues, it can also substantially reduce the efficacy of the targeting probe. Short *in vivo* half-life is very beneficial in imaging where the circulating probe is rapidly removed preventing background noise on the images. Nonetheless, in drug delivery increasing the half-life of a peptide probe is desired to secure an effective penetration into the target tissue. To increase the circulation time and decrease the elimination rate the protein probes are usually coated with polyethylene glycol (60). This strategy is also applied for nanoparticle delivery where the non-specific uptake by RES is a common problem.

The discovery of a specific class of peptides called the CendR peptides has led to the identification of a new trans-tissue transport pathway, the CendR pathway. The activation of the pathway in a tumor specific manner with peptides containing the CendR motif has shown to significantly increase the efficacy and activity of anti-cancer drugs and improve tumor imaging. Therefore, the tumor penetrating CendR peptides propose a substantial advance in current cancer treatment and diagnosis methods. This class of peptides can also overcome the major restriction related to synaphic delivery- the limited capacity of the receptors the targeting probe recognizes and binds to. It has been calculated that a gram of tumor tissue only has approximately a few picomoles of any given receptor available (4). These peptides are not dependent on the number of receptors displayed on the cell surface. It is also known that numerous drugs require larger amounts of receptors to be effective, therefore the co-administration method provides a way of delivering sufficient amounts of drugs to the target tissue because it only requires triggering the CendR pathway. In addition, there is no need for chemical conjugation of the peptide to the drug as it might modify the conjugated compounds` activity.

Homing peptide selection via *in vivo* phage display provides with excellent opportunities to isolate peptides specific to tumor vasculature. Since its initiation in 1996 creatively developed by the laboratory of Erkki Ruoslahti, a variety of vascular homing peptides have been identified and evaluated in different tumor models for various applications. Moreover, the discovery of the homing peptides also provides proof about the molecular heterogeneity of the vascular system in health and disease. The identification of tumor homing peptides by *in vivo* phage display expands the scope of affinity-based tumor targeting and diagnosis methods as it produces novel targeting probes for efficient and specific delivery of drugs and imaging agents to tumor sites. Furthermore, the discovery of peptides with tumor penetrating properties and the identification of a new trans-tissue transport pathway further extends their potential for tumor targeted diagnosis and therapy therefore presenting a promising advance in current cancer treatment.

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