Molecular Pharmacology Fast Forward. Published on April 18, 2017 as DOI: 10.1124/mol.116.108084
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Title page

Tumor Microenvironment Targeting and Responsive Peptide-based Nanoformulations for Improved Tumor Therapy

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Molecular Pharmacology Fast Forward. Published on April 18, 2017 as DOI: 10.1124/mol.116.108084
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Running Title page

Peptide nanoformulations against the tumor microenvironment

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Number of text pages: 57

Number of tables: 5

Number of figures: 4

Number of references: 78

Number of words in the abstract: 213

Number of words in the introduction: 557

Number of words in the discussion: 271

Abbreviations: Ang1, angiopoietin 1; Ang2, angiopoietin 2; ASC, adipose stromal cell; CAF, cancer-associated fibroblast; CPP, cell-penetrating peptide; CTL, cytotoxic T lymphocyte;

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CXCR4, CXC chemokine receptor 4; DEAP, 3-diethylaminopropylisothiocyanate; Dox, doxorubicin; ECM, extracellular matrix; ELP, elastin-like polypeptide; EPR, enhanced permeability and retention (EPR); FGF-12, fibroblast growth factor-12; GEM, gemcitabine; HMGN2, human high mobility group protein 2; HIF, hypoxia inducible factor; HSPG, heparin sulphate proteoglycan; HUVEC, human umbilical vein endothelial cell; K-FGF, Kaposi fibroblast growth factor; LCST, low critical solution temperature; MMP, matrix metalloproteinase; MSC, mesenchymal stromal cell; NLS, nuclear localization signal; NP, nanoparticle; NRP-1, neuropilin-1; PD-1, programmed cell death protein 1; PEG, poly(ethylene glycol); PFD, pirfenidone; pI, isoelectric point; ROS, reactive oxygen species; SPPS, solid phase peptide synthesis; SV40, simian virus 40; TAM, tumor-associated macrophage; TP10, transportan 10; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2.

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ABSTRACT

The tumor microenvironment participates in all stages of tumor progression and has emerged as a promising therapeutic target for cancer therapy. Rapid progress in the field of molecular self-assembly using various biological molecules has resulted in the fabrication of nanoformulations that specifically target and regulate microenvironment components to inhibit tumor growth. This inhibition process is based on differentiating between biophysicochemical cues guiding tumor and normal tissue microenvironments. Peptides and peptide derivatives, owing to their biocompatibility, chemical versatility, bioactivity, environmental sensitivity, and biological recognition abilities, have been widely utilized as building blocks to construct multifunctional nanostructures for targeted drug delivery and controlled release. Several groups of peptides have been identified with the ability to penetrate plasma membranes, regulate the essential signaling pathways of angiogenesis and immune reactions, and recognize key components in the tumor microenvironment such as vascular systems, stromal cells, and abnormal tumor biophysicochemical features. Thus, using different modules, various functional peptides and their derivatives can be integrated into nanoformulations specifically targeting the tumor microenvironment with increased selectivity, on-demand response, elevated cellular uptake, and improved tumor therapy. In this review, we introduce several groups of functional peptides, and highlight peptide-based nanoformulations that specifically target tumor microenvironment. We also provide our perspective on the development of smart drug delivery systems with enhanced therapeutic efficacy.

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Introduction

Tumors are composed of tumor cells and their microenvironment, including various stromal cells, extracellular matrix (ECM), soluble cytokines, and tumor vascular systems. The interaction between tumor cells and the surrounding microenvironment occurs at all stages of tumor progression. Due to hypoxia, tumor vascular endothelial cells are activated with aberrant expression of cell surface and secreted proteins, including integrins and matrix metalloproteinases (MMPs), that facilitate the construction of malformed and dysfunctional new blood vessels (Carmeliet, 2000). Cancer-associated fibroblasts (CAFs), the most abundant cell type in the tumor microenvironment, actively participate in ECM remodeling and in promoting tumor growth (Neri et al., 2016). Tumor-associated macrophages (TAMs) are the predominant inflammatory cells in malignant solid tumors. Most of the TAMs polarize towards M2-like macrophages, which are characterized as immunosuppressive and which play an important role in angiogenesis (Rogers and Holen, 2011). In addition, all stromal cells and ECM in the tumor microenvironment can form physiological barriers, sheltering tumor cells and contributing to drug resistance (Khawar et al., 2015).

Improved therapeutic outcomes can potentially be achieved by synergistically targeting tumor cells and regulating the tumor microenvironment. Compared to the normal tissue environment, the tumor microenvironment exhibits stromal cell abnormalities, aberrant protein expression, acidosis, and hypoxia. These differences provide multiple targets for the selective delivery of therapeutic agents to tumors (Ji et al., 2013).

The emergence and progression of nanotechnology provides a novel toolbox for designing

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the next generation of intelligent therapeutics with the design of well-defined structures with flexible composition. By carefully selecting the building blocks and modulating the physicochemical properties of different nanoformulations, elevated tumor accumulation, on-demand drug release, and enhanced therapeutic outcomes can be achieved. With the deeper understanding of the features of key components within the tumor microenvironment and the mechanisms by which the these components promote tumor progression, therapeutic nanoformulations have been designed to specifically target and regulate the tumor microenvironment (Danhier et al., 2010). In addition, the physicochemical differences between normal tissues and the tumor microenvironment, such as acidosis and hypoxia, have also been taken advantage of to construct well-controlled nanosystems with high therapeutic efficacy (Mura et al., 2013).

Endowed with low immunogenicity, facile modification, ample bioactivity, and the ability to penetrate tissues, peptides have served as a promising building blocks for the construction of advanced nanoformulations. Many peptides used in tumor therapy are derived from functional domains of proteins and exhibit specific bioactivities, such as receptor binding, structural sensitivity to changing local physiological or chemical conditions, penetration of the plasma membrane, and activation or inhibition of cellular pathways. Several research groups are screening more efficient functional peptides (Cieslewicz et al., 2013; Gautam et al., 2014; Laakkonen et al., 2002; Porkka et al., 2002). Owing to their salient bioactive functions, peptides hold great potential to become one of the most extensively used building blocks for the construction of nanoformulations for cancer treatment. Peptides can be incorporated into modules of nanoformulations that include targeting ligands, responsive cleavage sites,

internalization mediators, and therapeutic molecules. In addition, taking advantage of the solid phase peptide synthesis (SPPS) method, a sophisticated technique with high synthetic efficiency and moderate reaction conditions, one can easily obtain desired peptides with defined functions. In this review, we describe several groups of functional peptides with distinct bioactive properties and discuss recent progress focused on the design and fabrication of peptide-based nanoformulations specifically targeting the tumor microenvironment.

Functional Peptides

Tumor Microenvironment Targeting Peptides. Functionalization with tumor-targeting motifs is an excellent strategy for increasing the accumulation of therapeutic agents within tumor tissues. With the advancement in screening techniques such as the phage-displayed peptide library and the one-bead one-compound combinatorial library, peptides have been identified that possess tumor homing capacity. These peptides can specifically bind to various receptors that are specifically expressed or over-expressed in tumor tissues. Compared with antibodies, targeting peptides are much smaller in size, which makes them more efficient in tissue penetration and relatively easy to fabricate at much lower cost. In comparison to other small molecular targeting agents, peptides may be more straightforward for molecular design and more biocompatible in nature. A brief summary of some targeting peptides is provided in **Table 1**.

The tumor vasculature is the first checkpoint that therapeutic systems encounter when circulating into the tumor. This makes vascular constituents, including endothelial cells, pericytes, and blood components, attractive targets. RGD and NGR, the first two targeting

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peptides screened via phage-displayed peptide libraries (Arap et al., 1998; Pasqualini et al., 1997), are widely used in targeted delivery of therapeutic agents to tumor tissues. These short tripeptides bind to integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$, and aminopeptidase N, respectively, which are overexpressed on vascular endothelial cells of tumor angiogenic blood vessels, resulting in remarkable accumulation of cargoes within the tumor. Other than RGD and NGR, functional peptides such as IF7, F3, CTL, and CREAK also exhibit the ability to specifically target tumor blood vessels. The receptor for IF7 targeting is annexin1, a highly specific surface marker of tumor vasculature (Hatakeyama et al., 2011). F3, which is derived from the N-terminal fragment of human high mobility group protein 2 (HMGN2), possesses high affinity for nucleolin proteins expressed on the surface of tumor cells and endothelial cells (Porkka et al., 2002). The fibrin-fibronectin complex of plasma clots in tumor vessel walls and interstitial spaces, formed as a result of leaky blood vessels, provides another well-recognized target for tumor targeting. The cyclic nonapeptides CLT1 and CLT2 strongly accumulate in tumor blood vessels and stroma via their interaction with fibrin-fibronectin (Pilch et al., 2006). Another peptide, CREAK, binds to clotted plasma proteins and induces additional local clotting, thereby producing new targets for additional peptide binding (Simberg et al., 2007). This self-amplifying process is an excellent feature for enhanced targeting efficiency.

Tumor stromal cells with unusual protein expression are also good candidates for targeting. Lyp-1, a cyclic nonapeptide, has been verified to selectively bind to p32/gC1q, a receptor abundant on the surface of tumor-associated macrophages (TAM), as well as on tumor lymphatics and tumor cells (Fogal et al., 2008; Laakkonen et al., 2002; Uchida et al., 2011).

When expressed in normal tissues, p32 exists intracellularly as a mitochondrial protein and escapes peptide recognition. One group identified a unique M2-selective peptide, called M2pep, that specifically recognizes murine M2 cells, including TAMs, and has low affinity for other leukocytes (Cieslewicz et al., 2013). Another targeting molecule, WAT, is a cyclic peptide that homes to adipose stromal cells (ASC) (Daquinag et al., 2011), which belong to the mesenchymal stromal cell (MSC) lineage.

In addition to cytokines and enzymes, ECM presents other appealing therapeutic targets. MMPs, overexpressed by endothelial cells and tumor cells, play an important role in tumor growth, angiogenesis, and metastasis. Two peptides, CTTHWGFTLC and CRRHWGFEFC, have been shown to selectively target MMP-2 and MMP-9 (Koivunen et al., 1999). The two peptides display high affinity and also inhibit enzymatic activities of MMP-2 and MMP-9 (Koivunen et al., 1999). Specific targeting with simultaneous inhibition suggests the potential for enhanced targeting and antitumor efficacy.

Microenvironment Responsive Peptides. Since proteinases and peptidases are abundant in the tumor interstitial space, therapeutic nanosystems fused with peptides that are specific substrates of these enzymes can be designed to control the release of therapeutic agents within the tumor microenvironment. As described above, MMPs, with an elevated expression in tumor ECM, are crucial in tumor progression. Therefore, making use of MMP-cleavable sequences can readily achieve drug release or active site exposure. The substrate peptide for MMP-2 is GPLGIAGQ; this sequence is cleaved into GPLG and IAGO by MMP-2. One group developed a liposome modified with cell-penetrating peptide (CPP) and a

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PEG-conjugated antibody (Gao et al., 2013). PEG was linked to the liposome via a MMP-2 responsive sequence. In the presence of MMP-2, long PEG chains were removed from the liposomes. As a result, the exposed CPP mediated the internalization of the liposomes. In another example, the MMP-2 and MMP-9 sensitive sequence, PVGLIG, can be cleaved between glycine and leucine (Gao et al., 2013). This sequence can also be utilized for construction of tumor niche-responsive nanoformulations. FAP-α is another accessible protease that is specifically expressed on the surface of CAFs, a major cellular component in the tumor microenvironment. FAP-α selectively cleaves the sequence GPAX (X designates any amino acid) between proline, and alanine (Ji et al., 2016b). Microenvironment responsive peptides are listed in **Table 2**.

The sustained Warburg effect and limited clearance of metabolic acids leads to a more acidic pH within the tumor microenvironment than in normal tissues. The relative acidity has an important implication on specific responses of the tumor microenvironment. pHLIP is a peptide that has striking features of low pH sensitivity and tumor targeting (Andreev et al., 2007). This peptide, which is part of the bacteriorhodopsin C helix, inserts across the membrane as an α-helix at low pH. In a basic or neutral environment, the peptide is largely unstructured and has low affinity for cell membranes. Polyhistidines also possess the ability to respond to the acidic tumor environment. This is because an imidazole group on histidine, with a pKa of approximately 6.5, can be protonated under acidic conditions to make the peptide more hydrophilic (Zhao et al., 2016). The disturbed polarity usually leads to structural transformation of nanoformulations to exert favorable effects such as drug release and functional site exposure. Another group of pH-responsive peptides include sequences of

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carefully designed acidic and basic amino acids with isoelectric points (pI) between 6 and 7. One of the peptides includes an E_4K_4 sequence, which has a pI of approximately 6.4 and is negatively charged under physiological conditions; in an acidic tumor microenvironment, this peptide is uncharged or slightly positively charged (Huang et al., 2013a). The changes in hydrophilicity or net charge can be used to alter conformations supported by electrostatic attractions in order to induce functional domain exposure or cargo release. These microenvironment responsive peptides are described in **Table 2**.

Mild hyperthermia is another feature of tumor tissue that occurs as a result of dysfunctional tumor vascular systems and poor heat exchange. Slightly higher temperatures, as compared to the surrounding tissues, can potentially trigger particular peptides to respond to the tumor microenvironment. A leucine zipper peptide has been shown to form coiled-coil self-assembled aggregates composed of α-helix monomers; these dissociate into disordered, unstructured monomers at temperatures higher than 40°C. In this transformation, the peptide loses the ordered original structure, facilitating the release of drugs (Al-Ahmady et al., 2012). Elastin-like polypeptides (ELPs) are another group of temperature-responsive peptides that switch conformations in response to different temperature conditions (Macewan and Chilkoti, 2012). They possess low critical solution temperature (LCST), exhibit properties as soluble unimolecule below their LCST, and assemble into aggregates when the temperature is above the LCST. Amphiphilic ELPs can also self-assemble into micelles with a hydrophobic core and hydrophilic corona (Macewan and Chilkoti, 2012).

Tissue/Cell-Penetrating Peptides. Physiological barriers such as vascular endothelial cells,

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tumor stromal cells, and ECM in tumor tissues are known to hinder the efficient penetration of nanosystems to reach targeted cells. The selective permeability of the plasma membrane leads to insufficient internalization of therapeutic systems. One type of tissue penetrating motif, called the CendR motif, with a sequence R/KXXR/K and a second arginine or lysine residue on the C-terminal of the peptide, has shown to bind to neuropilin-1 (NRP-1), a membrane receptor expressed on endothelial cells, and to activate cell internalization and trans-tissue transportation (Wang et al., 2011). iRGD (CRGDKGPD) (Sugahara et al., 2010), one of the sequences within the CendR motif, exhibits tumor-specific penetrating ability as a result of the tumor homing RGD motif. The RGD sequence mediates binding of iRGD to the tumor vascular targets, integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Subsequent proteolysis generates the C-terminal R/KXXR/K motif, which activates the NRP-1-dependent penetrating pathway. Another type of CPP, which is varied in size and sequence, interacts with the lipid bilayer and internalizes into cells via clathrin- or caveolin-mediated endocytosis, micropinocytosis, or an endocytosis-independent mechanism such as the carpet model, inverted micelle model, barrel stave pore model, or toroidal model (Trabulo et al., 2010).

Most CPPs are derived from segments of natural translocating proteins with a large variety of sizes, sequences, and physiochemical properties. They can be classified into three main categories: cationic, hydrophobic, and amphipathic peptides. Cationic CPPs are positively charged and have a high affinity to negatively charged plasma membranes. The most commonly used cationic CPPs are poly-arginine and Tat-derived peptides (Takeshima et al., 2003; Walrant et al., 2011). Hydrophobic CPPs are comprised of nonpolar amino acids with low net charge and high lipid affinity. Signal sequence K-FGF (Kaposi fibroblast growth

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factor) and FGF-12 (fibroblast growth factor-12) belongs to the hydrophobic CPP category (Dokka et al., 1997; Nakayama et al., 2011). The remaining CPPs belong to amphipathic CPP class, which includes Antp, pVEC, penetratin, transportan 10 (TP10), M918, VP22, and SAP (Amand et al., 2008; Derossi et al., 1994; El-Andaloussi et al., 2007; Elliott and O'Hare, 1997; Elmquist et al., 2006; Fernandez-Carneado et al., 2004; Islam et al., 2014). Amphipathic CPPs contain both polar and nonpolar amino acids and are thus both hydrophobic and hydrophilic in nature. There is a special group of amphipathic CPPs, including MPG, Pep-1 and $S4_{13}$-PV, that are synthesized by fusing segments of the HIV GP41 protein, reverse transcriptase of HIV-1, or Dermaseptin S4 peptide with the nuclear localization signal (NLS) of simian virus 40 (SV40) large T antigen (Hariton-Gazal et al., 2002; Morris et al., 2008). The resulting peptides form stable complexes with their cargo via non-covalent interactions and penetrate into the cell with high efficiency. A summary of the most widely used CPPs is presented in **Table 3**.

Therapeutic Peptides. Peptides have received considerable attention as therapeutic agents due to their high specificity, low toxicity, good tissue penetration, cost effectiveness, and easy modification. Therapeutic peptides are commonly derived from chemokines, ECM proteins, growth factors, antigens, and other proteins, or are identified via screening of a phage-displayed peptide library. They selectively target particular receptors to either activate essential signaling pathways, or inhibit the receptors from interacting with their ligands. A great number of tumor therapeutic peptides have been identified, especially in the fields of anti-angiogenesis and immunotherapy. Some of the examples are summarized in **Table 4**.

Sustained formation of new blood vessels is an important hallmark of tumor progression. Inhibiting angiogenesis and normalizing tumor vascular systems are the strategies most emphasized in therapy targeted to the tumor vasculature. Many peptides have been reported to target tumor blood vessels and block angiogenesis; these include T4, C16Y and 6a-P (Rosca et al., 2011). T4 is a peptide identified by screening of a phage-displayed peptide library for high affinity to Tie2, an endothelial cell receptor kinase important in angiogenesis (Tournaire et al., 2004). Binding of T4 to Tie2 not only inhibits the interactions between Tie2 and angiopoietin 2 (Ang2) or angiopoietin 1 (Ang1), but also suppresses Ang1-dependent ERK activation and migration in human umbilical vascular endothelial cells (HUVECs). C16Y is an anti-angiogenic peptide derived from one of the most potent angiogenic sites, C16 in laminin-1. This peptide eliminates the attachment of endothelial cells to laminin-1, disrupts tube formation, and inhibits angiogenesis in the tumor site (Ponce et al., 2003). 6a-P is a 20 amino acid peptide derived from vascular endothelial growth factor (VEGF). It binds to heparin sulfate proteoglycan (HSPG), which is an important regulator in angiogenesis; the binding consequently inhibits the interaction between VEGF and HSPG (Lee et al., 2010).

Due to its potential for remarkable specificity and efficacy, tumor immunotherapy has emerged as a promising tumor therapeutic strategy. Peptides play a significant role in tumor immunotherapy. Examples include peptide vaccines and immune checkpoint blocking peptides. Tumor antigens that can be recognized by cytotoxic T lymphocyte (CTL) are usually expressed to a limited extent on tumor cells. However, peptides derived from tumor-associated antigens possess great potential to be recognized by CTL, and can be delivered to tumor cells. For example, peptides derived from Glypican-3 have been shown to

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efficiently stimulate CTL activity when loaded into tumors *in vivo* via intratumoral injection (Nobuoka et al., 2013). One of the mechanisms that allows tumor cells to escape from the immune system is over-expression of immune checkpoint proteins to inhibit T-cell attack. Utilizing exogenous antagonists to block immune checkpoints is a promising strategy to disturb immune-suppressing pathways and activate the anti-tumor immune response of T cells. ^{D}PPA-1, a D-peptide antagonist, targets programmed death-ligand 1 (PD-L1) and efficiently disrupts the interaction between PD-L1 and its receptor, programmed death 1 (PD-1), unleashing the anti-tumor immune reaction (Chang et al., 2015). ^{D}PPA-1 is the first reported proteolysis-resistant peptide antagonist targeting the immune checkpoint protein human PD-1/PD-L1 (Chang et al., 2015).

Peptide toxins can also be utilized to regulate the tumor microenvironment by reducing the number of stromal cells to deteriorate the tumor-supporting stroma and break physiological barriers for better drug perfusion. Numerous peptide toxins have been used to kill tumor cells, such as pro-apoptotic peptide KLA, phalloidin, and amanitin (An et al., 2010; Jung et al., 2016; Moshnikova et al., 2013), and they can also be used to induce stromal cell toxicity for enhanced drug perfusion and anti-tumor therapeutic efficacy.

Tumor Microenvironment Targeting and Responsive Peptide-Based Nanoformulations

As shown above, peptides have exhibited various activities in the tumor microenvironment. For example, targeting peptides show high affinity to components of the tumor

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microenvironment, responsive peptides are sensitive to tumor physicochemical conditions, CPPs are able to enhance cellular uptake, and therapeutic peptides can regulate pathways related to tumor progression. Recently, a number of these attractive building blocks have been used to construct nanoformulations specifically targeting tumor microenvironment, and have achieved exciting treatment outcomes. In this section, we summarize recent work published by our group and others on the utilization of peptide-based nanoformulation strategies, especially for regulating tumor blood vessels and stromal cells.

Ligand Peptides Mediate Nanoformulations to Target Tumor Microenvironment

Herein, we review nanoformulations that are conjugated with targeting peptides against tumor vasculature, lymphatics, and stromal cells, and carry cargo such as chemotherapy or gene therapy agents, for improved tumor therapy and decreased systemic toxicity.

Targeting Tumor Vasculatures. The most studied peptides targeting tumor blood vessels are the derivatives of RGD, an integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ binding sequence present in various ECM proteins including fibronectin and vitronectin. Some recent studies suggest that modification of RGD ligands for use in nanoformulations carrying chemotherapeutic agents, siRNA, or small molecule inhibitors leads to enhanced inhibition of angiogenesis and tumor growth (Danhier et al., 2012). Interestingly, an anti-metastatic effect was observed was observed when integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were targeted alone (Gvozdenovic et al., 2016); further studies are needed to confirm this promising strategy for inhibition of tumor metastasis.

VEGF, also known as vascular permeability factor, plays a vital role in the angiogenic

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process by binding to specific VEGF receptor 2 (VEGFR2, also known as KDR/Flk-1), a tyrosine kinase receptor. The binding of VEGF and its receptor then activates downstream signaling pathways including FAK/Paxillin and RAS/ERK and results in the proliferation and migration of endothelial cells, consequently promoting angiogenesis and vascular growth (Liang et al., 2014). Therefore, the VEGF-VEGFR2 signaling pathway has been extensively explored as a target for tumor therapy. Recently, in order to enhance cellular uptake and anti-angiogenic acvities *in vitro* and *in vivo*, Fu and colleagues conjugated RGD peptide to selenium nanoparticles loaded with doxorubicin for targeting tumor vasculature. This nanosystem disassembled under acidic condition in lysosomes after internalization, triggering drug release. *In vivo* experiments showed inhibition of MCF-7 tumor growth and angiogenesis. The anti-angiogenesis effect resulted from apoptosis and cell cycle arrest in endothelial cells through down-regulation of the VEGF-VEGFR2 signaling pathway (Fu et al., 2016). Inhibition of VEGFR2 mRNA expression in new tumor blood vessels is also an attractive approach for antitumor therapy. For tumor-targeted VEGFR2-siRNA delivery, Liu amd collleagues (2014) designed a self-assembling peptide nanoparticle system, consisting of the targeting cyclopeptide c(RGDfk) with an 8-amino-3,6-dioxaoctanoic acid-β-maleimidopropionic acid modification (referred to as RPM) that is capable of encapsulating siRNA via intermolecular hydrogen bonding. The RPM/VEGFR2-siRNA showed high gene silencing efficiency and minimal cytotoxicity *in vitro*, and effectively suppressed neovascularization when delivered into zebra fish embryos. Administration of RPM/VEGFR2-siRNA to tumor-bearing nude mice significantly inhibited tumor growth and reduced the density of tumor vessels, accompanied by down-regulated VEGFR2 at both the

mRNA and protein levels in tumor tissues. No measurable immunogenicity of the nanoparticles was observed in mice (Liu et al., 2014).

In addition to RGD peptide derivatives, many other peptides can target tumor blood vessels. For example, Herringson and co-workers (2011) tested two targeting peptides, peptide WHSDMEWWYLLG, an antagonist for VEGFR-1, and peptide ATWLPPR that specifically binds to neuropilin-1, a VEGFR-2 co-receptor. When engrafted into doxorubicin-containing liposomes, the peptide WHSDMEWWYLLG promoted liposome aggregation and/or leakage of the encapsulated liposomal drug. ATWLPPR-liposomes showed significantly enhanced tumor targeting efficiency, particularly when PE-PEG750 was used as the stabilizing lipid instead of PE-PEG2000 in the construction of the liposomes. Moreover, ATWLPPR liposomes inhibited the growth of B16-F1 tumors in mice (Herringson and Altin, 2011).

Targeting Tumor Lymphatics and Tumor-Associated Macrophages (TAMs). Lymphatic vessels in tumors are morphologically distinct from normal lymphatic vessels. As shown in **Table 1**, LyP-1 is a ligand peptide that targets tumor cells, tumor lymphatics, and TAMs in tumor hypoxic regions (Sanchez-Martin et al., 2011). To improve the efficiency for targeted therapy of lymphatic metastatic tumors, Yan and colleagues (2016) developed LyP-1-decorated and doxorubicin-loaded PEGylated liposomes. Internalization of the LyP-1-decorated liposome was observed in tumor cells and metastatic lymph nodes, but not in normal lymph nodes. Peptide LyP-1 modification caused the liposomes to distribute adjacent to tumor lymphatics and TAMs in metastatic lymph nodes, and also enhanced growth inhibition of tumor cells *in vitro* and lymphatic metastatic tumors *in vivo* (Yan et al., 2012).

TAMs are the predominant inflammatory cell components in the tumor microenronment. Most of them polarize to become M2-like macrophages, which as immunosuppressive populations contribute to angiogenesis and tumor immune evasion, and subsequently promote tumor proliferation and metastasis. TAMs have also been reported to be the major players in chemoresistance and radioprotection in tumors (Jinushi and Komohara, 2015). In clinical studies, high densities and activation levels of TAMs are associated with poor treatment outcomes. Therefore, TAMs have become appealing targets for cancer therapy development. The most common strategies in TAM-targeted therapy include those that aim to inhibit macrophage recruitment, reduce TAM survival, enhance the M1-like tumoricidal activity of TAMs, or block their M2-like pro-tumor activity. Conde and colleagues (2015) designed nanoformulations composed of an RNA interference (RNAi)-peptide hybrid that specifically targeted murine lung TAMs using M2pep peptide, and delivered anti-VEGF siRNA into TAMs as well as tumor cells simultaneously. This approach achieved high targeting efficiency via M2pep and promoted effective VEGF down-regulation. Administration of the hybrid nanoparticle resulted in immunomodulation of the TAM population within the tumor microenvironment and efficient eradication of tumors with an increased overall survival (Conde et al., 2015).

Enhanced Tumor Penetration via Tumor Microenvironment Responsive Peptides

Based on the pathophysiological variations in tumor microenvironment, many on-demand responsive nanoformulations have been designed for the spatially and temporally controlled release of therapeutic agents in response to specific stimuli. Some excellent review articles

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have already extensively discussed the molecular design and development of nanoscale systems for tumor microenvironment response (Mura et al., 2013; Wang et al., 2014). In this section, we focus on the peptides that are responsive to the tumor microenvironment responsive as tools to enhance the tumor penetration ability of nanoformulations.

CPPs have received tremendous attention for their abilities to improve drug penetration *in vivo* due to their capability to mediate the internalization of a variety of cargo molecules into cells (Fonseca et al., 2009). However, the use of CPPs are limited *in vivo* due to cell damage and low tissue specificity, and to their positively charged nature (Zorko and Langel, 2005). Great efforts have been made to improve the selectivity of CPPs. Generally, CPPs are suppressed via electrostatically interacted shields or steric hindrance under physiological conditions, and are exposed to cells when they are circulating into tumor tissues. Based on this strategy, many nanosystems have been designed, including, (a) recovery of CPP functions via pH-activatable systems, (b) recovery of CPP functions via protease-activatable systems, and (c) combination of above two strategies (Huang et al., 2013c).

pH-activatable Tumor Penetration. Owing to the excess metabolic acid secreted from fast-growing tumors, the pH in tumor microenvironment is more acidic than that in normal tissue, which makes the design of systems responsive to low pH an effective tumor targeting strategy. Several pH responsive systems have been developed using the amino acid histidine, which has a pKa value of 6.5 and can be protonated from a net negative charge to a net positive charge when translocated into the acidic tumor microenvironment. Zhang and co-workers designed a pH-responsive cell penetrating carrier based on histidine-rich peptide

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via a pH-triggered charge conversion strategy (Zhang et al., 2011). Yeh and colleagues reported another pH-responsive CPP, in which pH sensitivity was controlled by recombinant fusion to a histidine-glutamine (HE) oligopeptide (Yeh et al., 2016). Zhao and co-workers found that polyhistidine could respond to the acidic tumor microenvironment by exposing a CPP R4 sequence (Zhao et al., 2016). In the above-mentioned literature, cell internalization of nanocarriers can be promoted by means of pH-triggered CPP surface-charge reversal from negative to neutral or positive.

CPPS that are pH-responsive can facilitate the active targeting and uptake of tethered nanoparticles. Shi and colleagues (2015) designed a novel peptide containing a targeting peptide c(RGDfK) and a pH-responsive CPP (AGYLLGHINLHHLAHL(Aib)HHIL-NH2). Nanoparticles conjugated with this RGD-CPP peptide were not only able to actively target $\alpha_v\beta_3$-overexpressing cells, compared with CPP-NPs, but also increased cellular uptake as compared with RGD-NPs. RGD-CPP-NPs loaded with paclitaxel showed significantly higher survival rates in B16F10 tumor-bearing mice compared with other groups (Shi et al., 2015).

Enzyme-activatable Tumor Penetration. Expression of MMPs is often up-regulated in tumor ECM, and MMPs can cleave specific sequences via enzymatic catalysis. Thus, MMPs may serve as a better target for broad-spectrum theranostic applications. Nanoparticles incorporated with CPPs can be modified with PEG via an MMP-2 cleavable spacer to mask positively charged CPPs. Linkage with PEG was able to enhance the stability of nanoparticles in serum and avoid nonspecific interactions (Harada et al., 2010). For example, one group of investigators developed a liposome modified with CPP and a PEG-conjugated antibody. PEG

Molecular Pharmacology Fast Forward. Published on April 18, 2017 as DOI: 10.1124/mol.116.108084
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was linked to the liposome via a MMP-2 responsive sequence (Zhu et al., 2012). Through the EPR effect, these liposomes could selectively accumulate at the xenograft tumor site, where PEG was subsequently detached due to cleavage of the linker by endogenous MMP-2. In this manner, CPP-mediated cell penetration of the liposomes was achieved.

Recently, Wang and co-workers developed a tumor specific drug delivery system by encapsulating nanovesicles with an MMP-responsive polymeric network. This polymeric network cover could effectively prevent the cargo from non-specifically leaking from the nanoparticles, with enhanced drug bioavailability. Tumor over-expressed MMP-2 specifically triggered the disassembly of polymeric networks and released the functional nanoparticles in tumor tissues. By using a tumor-homing peptide iRGD conjugated to the nanoparticles as a target ligand, the investigators were able to facilitate tumor targeting and penetration of the drug both *in vitro* and *in vivo*. These results demonstrate that synergistic therapeutic efficacy can be achieved based on improved tumor accumulation, penetration, and MMP-responsive drug release in the tumor microenvironment (Liu et al., 2015).

pH and Enzyme Double-activatable Tumor Penetration. Another strategy reported for tumor microenvironment-specific recovery of the CPP effect is based on pH and protease double-activatable systems. Polyanionic peptides with pH-responsive properties are used to neutralize the CPP charge. In the tumor microenvironment the charges of these masking peptides is altered to result in CPP dissociation. For example, Jiang's group devised a pH-sensitive masking peptide with an iso-electric point (pI) of approximately 6.4 (Huang et al., 2013a; Huang et al., 2013b). This peptide is negatively charged under physiological pH,

but becomes predominantly uncharged or positively charged in the acidic tumor microenvironment. Since the conjugation of this masking peptide with CPP is weak, a MMPs-cleavable peptide linker was inserted between the masking peptide and CPP to form an intramolecular hairpin structure. When administrated *in vivo*, the hairpin structure remains intact in the circulation with the CPP blocked, maintaining the delivery system cell-impermeable and pharmacologically inactive in plasma. In the tumor microenvironment the enzyme-responsive linker is cleaved by MMPs, and the hairpin structure disassociates. The exposed CPP can then penetrate the tumor. Based on this strategy, Jiang's group developed a series of tumor targeting nanosystems with enhanced drug delivery and antitumor efficacy (Huang et al., 2013a; Huang et al., 2013b).

Peptide Self-assembled Nanoformulations Targeting CAFs to break Stromal Barriers

Despite the promising therapeutic potential exhibited by numerous anti-tumor nanoformulations, the heterogeneity among tumor cells and the presence of complex stromal cell barriers still present great challenges that limit tumor-targeting and cell-penetrating performance. Therefore, strategies to overcome tumor heterogeneity and to break stromal barriers are urgently needed. CAFs, the major stromal cell type in tumor microenvironment, play a key role in the formation of stromal barriers, leading to poor penetration for particulate therapeutics and also limiting access of pharmacologic drugs. Nanoformulations specifically targeting CAFs have shown promising results, as discussed below.

Our group developed peptide-based nanoformulations targeting and depleting CAFs in order to overcome the aforementioned obstacles. For example, a dual-mode nanomaterial that utilized CAF targeting combined with increased cellular uptake coordinated by CPP and cholesterol, improved the tumor penetration of chemotherapeutic drugs. This was achieved by depletion of CAFs and disruption of stromal barriers for the treatment of CAF-rich solid tumors (Ji et al., 2015). In another study, we reported a novel CAF-targeting drug delivery nanosystem based on a cleavable amphiphilic peptide (CAP) designed to be specifically responsive to FAP-α, a membrane-bound serine protease specifically expressed on CAFs (Ji et al., 2016b). This CAP nanocarrier transformed from self-assembled nanofibers to spherical nanoparticles when loaded with hydrophobic drugs. The disassembly of these NPs upon FAP-α cleavage resulted in efficient release of the encapsulated drugs specifically at tumor sites. This "transformer"-like drug nanocarrier could also disrupt the stromal barrier, and enhance local drug accumulation (Fig. 1).

Since CAFs encompass a multifunctional stromal cell type, we found that selective inhibition rather than completely depletion of CAFs, may be more appropriate for a safer long-term effective strategy. In one recent study, we developed a β-cyclodextrin (β-CD) modified MMP-2 responsive liposome loaded with the anti-fibrotic and anti-inflammatory agent pirfenidone and the chemotherapeutic drug gemcitabine for CAF regulation; this formulation was used for targeted delivery of gemcitabine in pancreatic cancer therapy (Ji et al., 2016a). When this nanoformulation reached the tumor site, the pirfenidone loaded β-CD was able to discharge and accumulate in the stroma following cleavage of the MMP-2

substrate peptide. The initial release of pirfenidone effectively down-regulated fibrosis and decreased the stromal barrier. Subsequently, the RGD containing liposomes loaded with gemcitabine recognized tumor cells and penetrated into the tumor tissue, resulting in enhanced therapeutic efficacy. This combined anti-fibrosis and anti-tumor strategy may increase drug penetration and enhance chemotherapeutic efficacy, providing a potential strategy for the design of nanoformulations to improve the pancreatic tumor therapy (Fig. 2).

Therapeutic Peptide Self-assembled Nanoformulations Targeting Tumor Vasculatures

The short circulating half-life of small therapeutic peptides *in vivo* may limit their use in human clinical applications for tumor therapy (Talmadge, 1998). Strategies are needed for increasing the stability and activity of peptide drugs to improve their therapeutic outcomes. Apart from chemical modification, incorporating therapeutic peptides into nanosystems with adequate size, morphology, and surface properties can improve their stability and pharmacokinetics. Mediated by intermolecular forces, amphiphilic peptides composed of distinct hydrophobic and hydrophilic segments are able to self-assemble into particular nanostructures. A therapeutic peptide with the ability to inhibit CXCR4 (CXC chemokine receptor 4) function was demonstrated to form self-assembled nanoparticles that hindered CXCR4 mediated tumor metastasis (Tarasov et al., 2011).

Inspired by the peptide self-assembly concept, we designed a tumor microenvironment-responsive nanoformulation based on a tailor-designed amphiphilic peptide through conjugation of functional 3-diethylaminopropylisothiocyanate (DEAP)

Molecular Pharmacology Fast Forward. Published on April 18, 2017 as DOI: 10.1124/mol.116.108084
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molecules to a short peptide (Zhao et al., 2014). The apparent pK_b of peptide conjugated with DEAP was approximately 6.9, which is close to the pH range within the tumor microenvironment. Protonation of the amphiphilic peptide at a weakly acidic pH led to a reduction in hydrophobicity, which triggered a pH-induced "intelligent switch" for the peptide self-assembled nanostructures, subsequently leading to their disassembly in the tumor microenvironment (Zhao et al., 2014). Furthermore, we recently developed a smart self-assembled nanoformulation using a therapeutic peptide known as C16Y (DFKLFAVYIKYR). This peptide, with a hydrophilic head and DEAP as a hydrophobic tail, inhibits angiogenesis and tumor growth by targeting integrin $\alpha_v\beta_3$ and $\alpha_v\beta_1$ (Ding et al., 2015). DEAP-C16Y peptides self-assembled into spherical nanostructures under physiological conditions, and subsequently dissociated to release individual peptide molecules in weakly acidic tumors. Mechanistic investigation revealed that dissociated DEAP-C16Y peptides inhibited vascular endothelial cell migration and tubule formation through suppression of the focal adhesion kinase and PI3K-Akt signaling axis, and also restrained tumor cell invasion via down-regulation of invadopodia formation. The systemically administered DEAP-C16Y nanoformulations significantly decreased microvessel density, tumor growth, and distant metastasis formation in nude mice, with effectively prolonged blood circulation of the therapeutic peptide *in vivo*. Additionally, the DEAP-C16Y nanostructures can be a smart and effective drug delivery system of antineoplastic agents for targeted combination therapy. In this study, for the very first time, we developed a self-assembled nanoformulation by using a therapeutic peptide as a building block. This peptide showed intrinsic anti-tumor activity and also provided a platform for combination therapy by encapsulating chemotherapeutic drugs in

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the nanoformulation, demonstrating a potential strategy for the future design of anti-tumor nanotherapeutics (Fig. 3) (Ding et al., 2015).

Numerous tumor microenvironment targeted nanosystems have been constructed and optimized using functional peptides to mediate cell penetration, target the tumor microenvironment, respond to tumor specific enzymes for controlled drug release, or regulate cellular signaling transduction pathways. According to their functionalities, building blocks in nanoformulations can be classified into different categories and regarded as modules for nanosystem construction. For more efficient design and implementation of peptide-based drug delivery, controlled release and targeting, and regulation of tumor microenvironment, we propose a modularized concept for peptide nanosystem construction (Fig. 4A). The self-assembled micelles, vesicles, and nanofibers are assembled from amphipathic monomers in a process that is generally mediated by various intermolecular forces. Typically, functional peptides are used as the hydrophilic heads, and lipophilic molecules such as cholesterol, alkyl chains, polymers, and lipids serve as the hydrophobic tails. Such monomers, constructed with multiple modules, then self-assemble into nanostructures with diverse functions and physicochemical features. Apart from this self-assembled system, there is another common type of peptide-based nanosystem that employs pre-formed nanostructures, such as liposomes, polymeric particles, or inorganic nanostructures (e.g. gold, silicon, and ferric oxide), onto surfaces to achieve improved biocompatibility, active targeting, controlled release, or enhanced tissue penetration (Fig. 4B).

By proposing the concept of modularized construction, our intention is to highlight a

strategy that categorizes the building blocks into different modules depending on their functions. The desirable modules suitable for targeting environment and encapsulated therapeutic molecules can be selected and then combined together to construct intelligent and well-controlled nanoformulations. Modularized construction makes it easier to construct nanoformulations with promising specificity and therapeutic efficacy.

Conclusions

Compared to tumor cells, the surrounding tumor microenvironment is a more accessible target for antitumor therapy. The specifically expressed receptors and unique physicochemical conditions within the tumor microenvironment provide many opportunities for nanoformulations to target tumor tissues. Many nanosystems have been developed to target the tumor microenvironment with the goal of achieving enhanced targeting efficacy and therapeutic outcomes. As discussed in this review, tumor progression is promoted by the sustained influence of the tumor microenvironment. In addition, stromal cells and ECM in the tumor microenvironment generate a natural sanctuary for tumor cells. Therapies that regulate the components in the tumor microenvironment, such as tumor blood vessels and CAFs, have been reported to efficiently suppress tumor growth with reduction of several underlying threats such as invasion and metastasis. Moreover, the novel therapeutic strategy based on the dual targeting of tumor microenvironment and tumor cells has become increasingly attractive, and has exhibited increased drug accessibility and elevated treatment efficacy. In particular, incorporated functional peptides are able to endow anti-tumor nanoformulations with high specificity, cell penetrating ability, sensitivity to slight change in conditions, and potential for

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cellular pathway regulation, and have achieved excellent therapeutic efficacy. In this review, we have proposed a modularized construction strategy for the assembly of peptide-based nanoformulations, and have discussed its potential advantages and utility in anti-cancer nanosystem development. It should be noted that, despite the great advances in tumor microenvironment targeting and therapy, additional functional peptides need to be designed. A deeper understanding of the tumor microenvironment can also help us to make full use of its properties to develop anti-tumor nanoformulations with better specificity and stronger therapeutic efficacy.

Authorship Contributions:

Wrote or contributed to the writing of the manuscript: Hao Qin, Yanping Ding, Ayeesha Mujeeb, Ying Zhao, Guangjun Nie.

Molecular Pharmacology Fast Forward. Published on April 18, 2017 as DOI: 10.1124/mol.116.108084
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Molecular Pharmacology Fast Forward. Published on April 18, 2017 as DOI: 10.1124/mol.116.108084
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Footnotes:

The writing of this review was supported by the grants from Ministry of Science and Technology of the People's Republic of China [2013CB932701], National Natural Science Foundation of China [21373067, 51673051, 31325010 and 11621505], Beijing Municipal Science & Technology Commission [Z161100000116035], Youth Innovation Promotion Association of Chinese Academy of Science [2017056], and the Frontier Research Program of Chinese Acadamy of Science [QYZDJ-SSW-SLH022].

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Legends for figures:

Figure 1. FAP-α responsive nanocarriers based on a cleavable amphiphilic peptide. (A) The structure of peptide CAP (cleavable amphiphilic peptide). CAP contains a TGPA sequence that can be cleaved by FAP-α. (B) Proposed mechanism of peptide self-assembly, drug induced reassembly, and peptide and drug co-assembly in the hydrophobic drug and amphiphilic peptide mixed solution. The components form the stable nanoparticles. The morphology of peptide assembly during Dox loading was observed by TEM. The assemblies transformed from mace-like (1) to spherical (2) with prolonged ultrasonication. (C) Drug release profiles of CAP-Dox and UAP-Dox (uncleavable amphiphilic peptide-Dox) in the presence or absence of FAP-α. (D) Penetration of Dox into prostate tumor (PC-3 and CAF co-implanted) tissues after intravenous injection of different Dox formulations. Frozen tumor sections were stained with DAPI (blue) to label nuclei and CD31 (green) antibody to label tumor vasculature. Red: Dox. (E) The growth curves of PC-3 and CAF co-implanted prostate tumors in mice treated with different Dox formulations. (Taken with permission from *Angew. Chem. Int. Ed.,* John Wiley and Sons; Ji et al., 2016.)

Figure 2. A β-cyclodextrin (β-CD) modified MMP-2 responsive liposome loaded with the anti-fibrotic and anti-inflammatory agent pirfenidone (PFD) and the chemotherapeutic drug gemcitabine (GEM). (A) Illustration of the nanomedicine LRC-GEM-PFD (liposome with

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RGD and β-cyclodextrin containing GEM and PFD). (B) IHC staining of collagen I and TGF-β in pancreatic tumor tissue slices after LRC, PFD, and LRC-PFD treatment. (C) Penetration of rhodamine into pancreatic tumor (Panc-1 and PSCs co-implanted) tissues after intravenous injection of different PFD formulations. Red: rhodamine. (D) Tumor growth curves of PSCs/Panc-1 pancreatic tumors in mice treated by different GEM formulations. (Taken with permission from *ACS Appl. Mater. Interfaces*, American Chemical Society; Ji et al., 2016.)

Figure 3. A smart therapeutic peptide for inhibiting angiogenesis and tumor growth. (A) Schematic structure of the DEAP-C16Y peptide. (B) Proposed anti-tumor mechanism of DEAP-C16Y nanostructures. (C) Migration of HUVECs treated by peptides in a Transwell migration assay, the cells were chemoattracted by 5% FBS/RPMI-1640 medium. The number of migrated cells in each group was quantified. Tubule formation was assessed in HUVECs treated by C16Y or DEAP-C16Y. The total network length in each treatment group was quantified. (D) Growth curves and metastatic foci number of 4T1 tumors. Mice bearing 4T1 tumors were treated with DEAP-HSAF nanostructures (negative control), or with C16Y or DEAP-C16Y nanostructures for indicated period. Tumor volume was calculated every other day. Lung sections from above 4T1-bearing mice were stained with H&E or PCNA. The number of metastatic foci was quantified. (E) Antitumor efficacy of DEAP-C16Y-Dox nanostructures. Mice bearing 4T1 tumors were treated with the indicated formulations every third day. Tumor volume was calculated every other day. The number of metastatic foci per

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lung was quantified. (Taken with permission from *Mol Cancer Ther.,* American Association for Cancer Research; Ding et al., 2015.)

Figure 4. Modularized construction of nanoformulations. (A) Amphipathic peptide consisting of a hydrophilic head and hydrophobic tail, based on various modules that self assembles into nanostructures such as micelles, vesiclse, and fibers in response to intermolecular forces. (B) Pre-formed nanostructures can be modified with functional modules for enhanced therapeutic efficacy.

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Tables:

TABLE 1. Brief summary of targeting peptides

Targeting	**Names**	**Sequence**	**Receptors**	**References**
Position				
	RGD	RGD	Integrin $\alpha_v\beta_3$ $\alpha_v\beta_5$	Pasqualini et al., 1997
	NGD	NGD	aminopeptidase N	Arap et al., 1998
	IF7	IFLLWQR	Annexin 1	Hatakeyama et al., 2011
	F3	KDEPQRRSARLSAK	Nucleolin	Porkka et al., 2002
Tumor		PAPPKPEPKPKKAPA		
Vasculature		KK		
	CTL1	CGLIIQKNEC	Clotted plasma protein	Pilch et al., 2006
	CTL2	CNAGESSKNC	Clotted plasma protein	Pilch et al., 2006
	CREAK	CREAK	Clotted plasma protein	Simberg et al., 2007
TAM	Lyp-1	CGNKRTRGC	P32/gC1q	Uchida et al., 2011
	M2pep	YEQDPWGVKWWY	Unclear	Cieslewicz et al., 2013
MSC	WAT	CSWKYWFGEC	Unclear	Daquinag et al., 2011
ECM	*	CRRHWGFEFC	MMP-2/9	Koivunen et al., 1999
	*	CTTHWGFTLC	MMP-2/9	Koivunen et al., 1999

*: No specific names.

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TABLE 2. Brief summary of the stimuli-responsive peptides and their applications

Stimuli	**Peptide Sequence**	**Therapeutic Strategy**	**Result**	**References**
Enzymes	GPLG↓IAGQ (MMP-2) PVG↓LIG (MMP-2/9) GP↓AX (FAP-α)	Linked protective PEG to CPP modified liposome via MMP-2 responsive sequence	Enhanced target ability and internalization of nanocarriers in cancer cells	Zhu et al., 2012 Gao et al., 2013 Ji et al., 2016b
	ACEQNPIYWARY ADWLFTTPLLLL DLALLVDADEGT G	Combined pHLIP with a microRNA miR-155 via disulfide bond	Delivery of miR-155 specifically to tumor site and efficient uptake by tumor cells	Andreev et al., 2007
Acidosis	H_7	Conjugated cell-penetrating peptide $(R_2)_2$ and H7 modify to polymeric micelle	Activation of cell-penetrating peptide by response of H7 to the acidic tumor microenvironment	Zhao et al., 2016
	E_4K_4	Mask to a cell penetrating peptide via linking by an enzyme responsive cleavable sequence	Cell-penetrating peptide exposed via E_4K_4 charge transforming and linker cleaving	Huang et al., 2013a

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	VSSLESKVSSLES KVSKLESKKSKL ESKVSKLESKVSS LESK	Leucine zipper peptide-lipid hybrid nanovesicles loaded with doxorubicin inside	Superior serum stability at physiological temperature and increased drug accumulation in tumor	Al-Ahmady et al., 2012
Hypertherm **ia**	$(VPGVG)_{40}(VPGX G)_{60}$ X=A:G; 1:1	Temperature triggered activation of the cell penetrating ability of ELP-pentaarginine copolymer	Thermal triggered copolymer assembly increased the local density of arginine and the cell penetrating activity at higher temperature	Macewan and Chilkoti, 2012

X: any amino acid; ↓ : cleavable site.

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TABLE 3. Sequences of several widely used CPPs

Categories	Names	Sequences	Origin	References
CenR motif	iRGD	CRGDKGPD	ECM proteins	Sugahara et al., 2010
Cationic	Nonaarginine	R_9	Synthetic	Walrant et al., 2011
	TAT	YGRKKRRQRRR	Human immunodeficiency virus type 1 (HIV-1)	Takeshima et al., 2003
	K-FGF	AAVALLPAVLLALLAP	K-FGF	Dokka et al., 1997
Hydrophobic	FGF-12	PIEVCMYREP	FGF-12	Nakayama et al., 2011
	Antp	RQIKIWFQNRRMKWKK	Third helix of the antennapedia homeodomain	Derossi et al., 1994
	pVEC	LLIILRRRIRKQAHAHSK	Murine vascular endothelial-cadherin protein	Elmquist et al., 2006
	Penetratin	RQIKIWFQNRRMKWKK	Antennapedia homoeodomain in Drosophila	Amand et al., 2008
Amphipathic	TP10	AGYLLGKINLKALAALAKKIL	Synthetic peptide	Islam et al., 2014
	M918	MVTVLFRRLRIRRACGPPRVRV	Tumor suppressor protein p14RF	El-Andaloussi et al., 2007

VP22	NAKTRRHERRRKLAIER	Herpesvirus structural protein	Elliott and O'Hare, 1997
SAP	VRLPPPVRLPPPVRLPPP	Synthetic	Fernandez-Carneado et al., 2004
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	Combination of hydrophobic domain from HIV GP41 and NLS of SV40 large T antigen	Morris et al., 2008
Pep-1	KETWWETWWTEWSQPKKKRKV	Combination of reverse transcriptase of HIV-1 and NLS of SV40 large T antigen	Morris et al., 2008
$S4_{13}$-PV	ALWKTLLKKVLKAPKKKRKV	Combination of dernaseptin S4 peptide with NLS of SV40 large T antigen	Hariton-Gazal et al., 2002

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TABLE 4. Some examples of therapeutic peptides

Names	Sequences	Origin	Receptors	Mechanism	References
T4	NLLMAAS	Phage-displayed peptide library	Tie 2	Blocking the interaction between Ang1 and Tie2	Tournaire et al., 2004
C16Y	DFKLFAVYIKY R	Scrambled laminin-1 C16 sequence	Integrin $\alpha_v\beta_3$ and $\alpha_v\beta_1$	Antagonistically binding to integrin, blocking laminin-1 induced angiogenesis	Ponce et al., 2003
6a-p	KSVRGKGKGQ KRKRKKSRYK	Exon 6a-encoded domain of VEGF	HSPG	Inhibiting the binding of VEGF and HSPG, blocking the angiogenesis pathway	Lee et al., 2010
Glypican-3 **peptide**	FVGEFFTDV	Glypican-3	CTL	Loading to tumor cells and enhancing the recognition of tumor cells by CTL	Nobuoka et al., 2013
^{D}PPA-1	NYSKPTDRQYH F	Phage-displayed peptide library	PD-1	Binding to immune checkpoint protein PD-1 and inhibiting the interaction between PD-1 and PD-L1	Chang et al., 2015
KLA	KLAKLAKKLA KLAK	Natural antibacterial peptide	Mitochon dria membrane	Disrupting mitochondrial membrane and induce apoptosis (can be used in regulating stromal cell	An et al., 2010

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number in future)

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TABLE 5. Nanoformulations targeting tumor microenvironment via conjugated ligand peptide

Targets	**Therapeutic agents**	**Targeting ligands**	**Cell lines**	**Results**	**References**
Integrins	RGD modified and doxorubicin-loaded selenium nanoparticles	RGD	HUVECs and MCF-7 breast cancer cells	Selectively binding to tumor vessel and achieving anti-angiogenesis.	Fu et al., 2016
	c(RGDfk) mediated nanoparticle encapsulatedVEGFR2-siRNA	C(RGDfk)	HUVECs and A549 lung cancer cells	Inducing effective gene silence in vitro and inhibiting tumor growth in vivo	Liu et al., 2014
Neuropilin-1	Peptide modified liposome loaded with DOX	ATWLPPR peptide	B16-F1 murine melanoma cells	Increased tumor targeting and suppressed tumor growth	Herringson and Altin, 2011
gC1qR	LyP-1 conjugated to PEGylated liposomes loaded with or DOX nanoformulations	Lyp-1 M2pep	SPC-A1 lung adenocarcinoma cells Mouse BALB/c	Enhanced inhibition effect on tumor cells in vitro and lymphatic metastatic tumors in vivo Immunomodulating	Yan et al., 2012 Conde et

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M2-like TAM	composed of (RNAi)-peptide biohybrid	macrophage J774.2 cells	TAMs in tumor microenvironment and efficaciously eradicating tumor cells	al., 2015

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Figures:

Figure. 1.

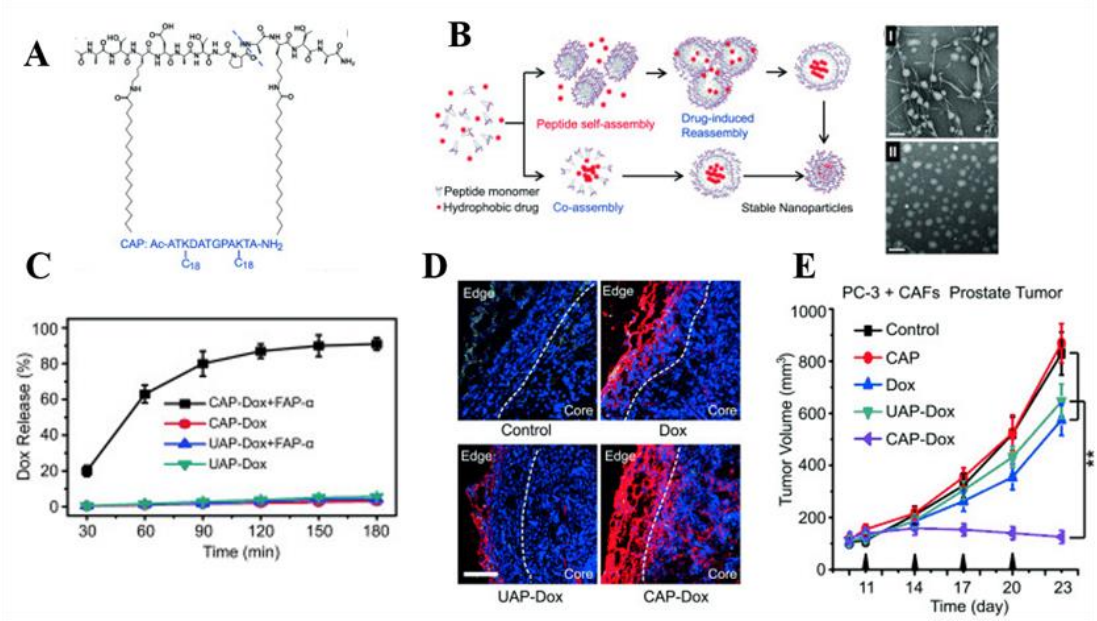

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Figure. 2.

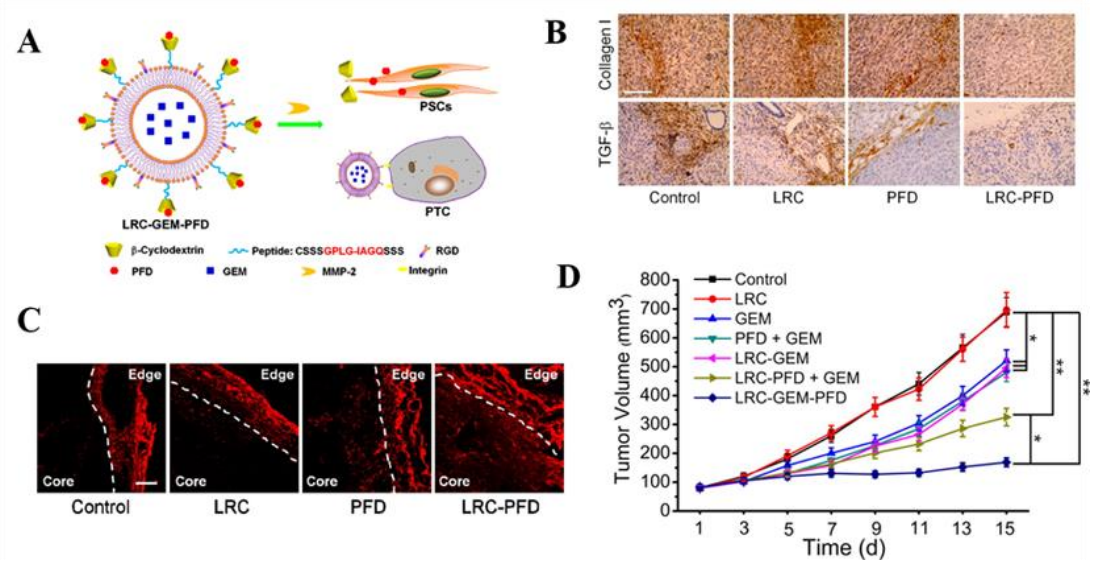

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Figure. 3.

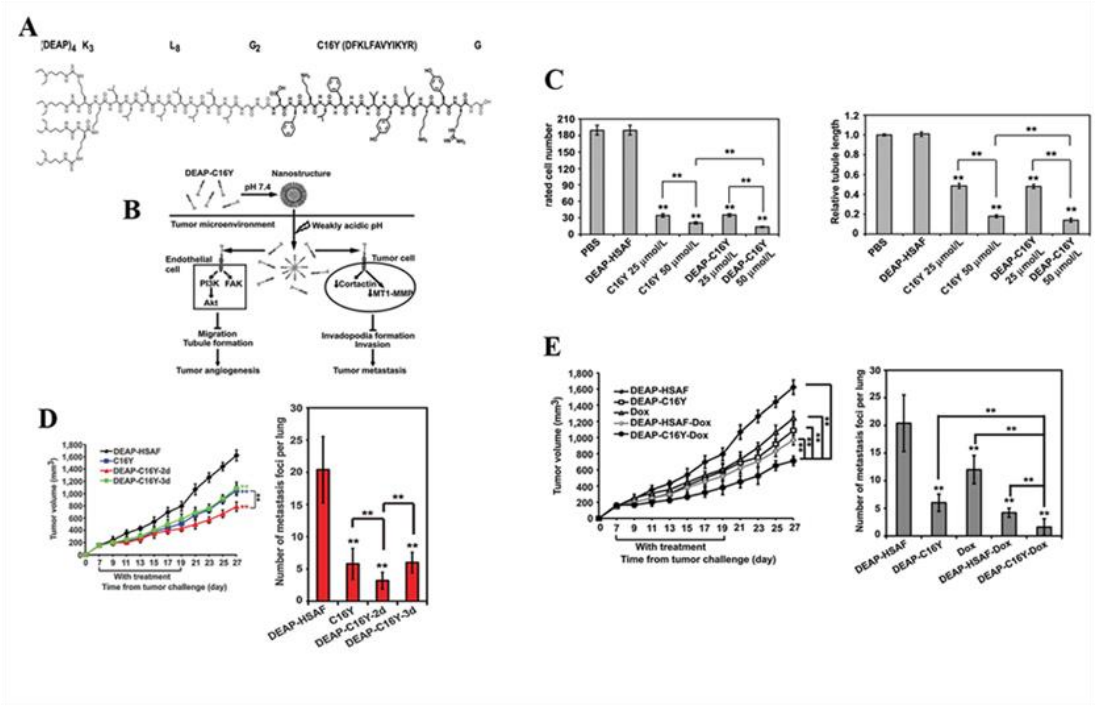

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Figure. 4.