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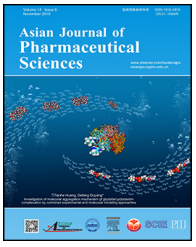

Review

The functions and applications of A7R in anti-angiogenic therapy, imaging and drug delivery systems

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ABSTRACT

Vascular endothelial growth factor receptor 2 (VEGFR-2) and neuropilin-1 (NRP-1) are two prominent antiangiogenic targets. They are highly expressed on vascular endothelial cells and some tumor cells. Therefore, targeting VEGFR-2 and NRP-1 may be a potential antiangiogenic and antitumor strategy. A7R, a peptide with sequence of Ala-Thr-Trp-Leu-Pro-Pro-Arg that was found by phage display of peptide libraries, can preferentially target VEGFR-2 and NRP-1 and destroy the binding between vascular endothelial growth factor 165 (VEGF165) and VEGFR-2 or NRP-1. This peptide is a new potent inhibitor of tumor angiogenesis and a targeting ligand for cancer therapy. This review describes the discovery, function and mechanism of the action of A7R, and further introduces the applications of A7R in antitumor angiogenic treatments, tumor angiogenesis imaging and targeted drug delivery systems. In this review, strategies to deliver different drugs by A7R-modified liposomes and nanoparticles are highlighted. A7R, a new dual targeting ligand of VEGFR-2 and NRP-1, is expected to have efficient therapeutic or targeting roles in tumor drug delivery.

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1. Introduction

In 1971, Folkman speculated that tumors could not grow beyond 1 mm in diameter without developing their own blood supply by angiogenesis [1]. Thus, angiogenesis was first proposed as a therapeutic target for tumors. Blood vessels in solid tumors are composed of endothelial cells and tumor cells. Research has revealed that angiogenesis is required for tumor cells to grow beyond a certain size, break away from an established solid tumor, enter blood vessels, implant, and initiate the growth of a secondary tumor at a distant site, i.e., tumor metastasis [2].

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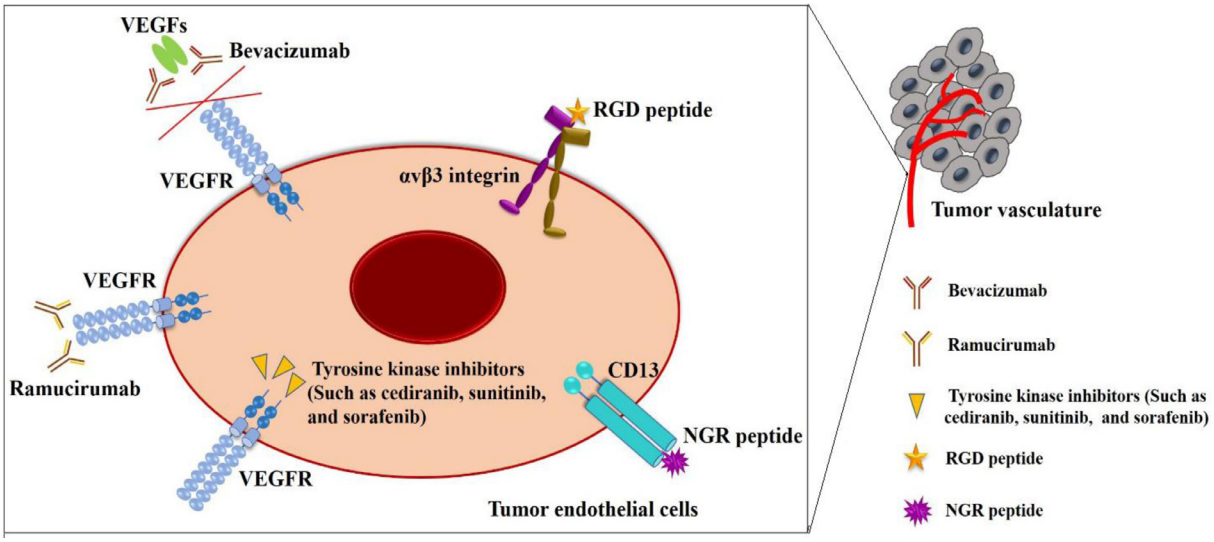

Fig. 1 – Schematic representation of other targeted strategies to tumor vasculature.

During the past decades, a series of strategies have emerged to target tumor endothelial cells, and many angiogenesis inhibitors have also been developed [3]. Tumor endothelial cells and cancer cells show abnormal surface expression of numerous molecular markers, such as endothelial cell growth factor receptors, integrins and cell surface proteoglycans, which differ from those expressed by normal cells [4]. Thus, targeting tumor cells and tumor vasculature is important for effective cancer treatment. During tumor growth, many proangiogenic factors are stimulated and upregulated. Vascular endothelial growth factors (VEGFs), which regulate angiogenesis through binding VEGF receptors (VEGFRs), are regarded as major regulators of tumor angiogenesis [5]. Thus, antiangiogenic strategies can be achieved through targeting VEGF or VEGFR (Fig. 1). Bevacizumab (a humanitzed monoclonal antibody) and aflibercept (a recombinant fusion protein) are the most popular antiangiogenesis drugs. These VEGF-targeted drugs can inhibit VEGF activity though binding to VEGFs [6]. Antiangiogenic therapies also involve targeting VEGFR. Ramucirumab can block signaling activation through binding VEGFR. In addition, cediranib, sunitinib, and sorafenib are tyrosine kinase inhibitors that suppress the kinase activity of VEGFR [7]. The above treatment methods have been clinically used for colorectal cancer, breast cancer and advanced gastric cancer. However, current antiangiogenic targeted therapies have many drawbacks [3]. For example, VEGF-targeted blockers are generally nonselective, show poor tissue penetration in normal vascular tissue and are always associated with acquired resistance and severe side effects, including hypertension, proteinuria and stroke. The high cost of the production and purification of monoclonal antibodies further limits their clinical applications [8].

To overcome the shortcomings of the above agents, scientists and clinicians have investigated peptides as therapeutics. Peptides with high specificity and low immune responses in hosts may overcome some of the limitations of other inhibitors and may be promising vascular-targeting diagnostic and therapeutic agents [9,10].

The selection of phage display libraries allows us to discover and acquire numerous peptides targeting tumor angiogenesis due to their high specificity and affinity for molecular markers and vascular receptors on the endothelial cell surface [11]. To date, the asparagine-glycine-arginine (NGR) and arginine-glycine-aspartic acid (RGD) peptides (Fig. 1) are the two most studied vascular-homing peptides screened from phage display libraries [12]. Aminopeptidase-N (CD13) is a zinc-dependent transmembrane ectopeptidase that is mainly overexpressed on tumor vascular cells and some tumor cells compared with normal blood vessels [13]. CD13 can promote the proliferation, invasion and migration of tumor endothelial cells. The NGR peptide and NGR-containing peptides can recognize and bind to CD13 expressed on the surface of endothelial cells of tumor blood vessels *via* the arginine and asparagine residues [14].

Integrins are heterodimeric membrane glycoproteins composed of noncovalently associated α- and β- subunits. Different subunit combinations comprise various types of heterodimers that determine the affinity of extracellular domains in integrins to diverse extracellular matrix (ECM) ligands (e.g., proteins, growth factors, immunoglobulin, cytokines) [15]. As cell adhesion receptors, these integrins regulate multiple intracellular signal transduction pathways by binding different ECM ligands. Among various integrins, $\alpha v\beta 3$ integrin, which is overexpressed in endothelial cells and tumor cells, is regarded as the strongest regulator of tumor angiogenesis [16]. Researchers have discovered that the RGD sequence exhibits a high affinity for the active sites present in the $\alpha v\beta 3$ integrin, and later, a wide variety of synthetic RGD-based peptides were designed as targeting ligands for the $\alpha v\beta 3$ integrin receptor [17]. The two peptides have been successfully used to deliver various antiangiogenic and antitumor drugs, including chemotherapeutic drugs, therapeutic proteins, cytokines,

nucleic acids and nanoparticles, for cancer therapy. In addition, after these molecules are coupled with different dyes or radiolabeled agents, they can be highly efficient imaging probes for molecular imaging studies in the diagnosis of various cancers or other angiogenic diseases [15]. In addition to NGR and RGD peptides, other vascular-homing peptides have been shown to target tumor angiogenesis. For example, the CAGALCY cyclic peptide exhibited a high-affinity interaction with the endothelium of the blood-brain barrier (BBB) [11]. The GX1 (CGNSNPKSC) peptide, a cyclic 9-mer peptide, displayed efficient targeting of the gastric tumor vasculature; the TCP-1 (CTPSPFSHC) peptide showed a high affinity for the blood vessels of orthotopic colorectal cancer; and SP5-52 (SVSVGMKPSPRP) showed selective recognition of tumor blood vessels, not normal vessels, and was used as a targeting ligand for the treatment of non-small cell lung carcinoma (NSCLC) [12]. Although these vasculature-homing peptides have been proven to be useful and potential targeting agents in tumor-targeted diagnosis and therapy, only preclinical studies and a few clinical trials have been conducted to date. Notably, the exact mechanisms underlying the interactions of the tumor vasculature with most vasculature-homing peptides, such as NGR, are undefined, and the receptors of some peptides have not yet been identified. Moreover, because some known molecular markers are present in both tumor vasculature and ordinary inflammatory vasculature, many peptides, such as NGR and RGD, have shown low selectivity to tumor vasculature under some specific inflammatory pathologic conditions. In addition, more studies should be carried out on the structural stability, toxicity, immunogenicity and biodistribution of these vasculature-homing peptides. Hence, development of new vasculature-homing peptides that have definite receptors and highly selective targets on the tumor vasculature should be carried out.

Vascular endothelial growth factor receptor 2 (VEGFR-2/KDR) and neuropilin-1 (NRP-1) are two important markers that contribute to the high proliferation and migration of endothelial cells [4]. Blocking VEGFR-2 or NRP-1 may be an effective therapeutic strategy for antitumor angiogenesis and drug delivery. ATWLPPR (A7R) was identified by a phage display peptide library [18] and was shown to simultaneously inhibit VEGF binding to VEGFR-2 and NRP-1 with high specificity and to decrease tumor angiogenesis and growth. Subsequently, scientists found that A7R could also induce endothelial cell and tumor cell apoptosis [19]; decrease vascular permeability, brain hemorrhage and BBB disruption [20]; and reduce early retinal damage by preserving vascular integrity [21]. Based on these results, A7R may become a new potent dual inhibitor of both VEGFR-2 and NRP-1 and a targeting peptide for tumor angiogenesis in cancer therapy.

To help understand and use A7R in disease treatment and diagnosis, in this review, we first introduce the discovery and mechanisms of action of A7R and then summarize the research progress in the applications of A7R and A7R-based strategies, including the use of A7R as an antiangiogenic and anticancer agent, radiolabeled A7R in imaging, and the grafting of A7R as a targeting ligand on the surface of nanocarriers.

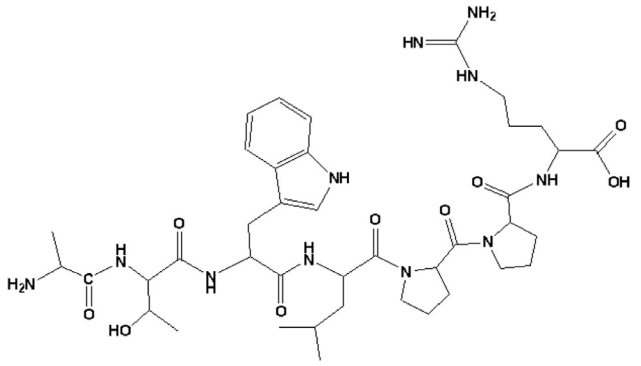

Fig. 2 – Structure of the A7R sequence.

Finally, we provide our opinions on the future research and development of A7R.

2. A7R peptide and its mechanism of action

Screening peptides from phage display peptide libraries is a powerful technique for identifying agonists or antagonists according to their ability to bind to the desired targets [22]. With the assistance of this technique, a new heptapeptide, ATWLPPR (A7R) (Fig. 2), with the ability to selectively inhibit human endothelial cell proliferation *in vitro*, was identified [18]. A7R has been extensively studied by researchers because is a specific ligand for VEGFR-2 and NRP-1. The identification of A7R and its mechanism of action were closely related to the regulatory roles of VEGFR-2 and NRP-1 in angiogenesis.

2.1. *The regulatory roles of VEGFR-2 and NRP-1 in angiogenesis*

The delicate balance between inducers (proangiogenic factors) and inhibitors (antiangiogenic factors) is critical for physiological homeostasis of tumor angiogenesis [23]. Triggered by signals such as metabolic stress (low pH, low oxygen pressure), mechanical stress, genetic mutations, etc., tumors often activate angiogenesis by shifting the balance towards proangiogenic conditions. For example (Fig. 3), hypoxia increases cellular hypoxia-inducible factor (HIF) transcription, leading to the upregulation of various proangiogenic factors, such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), angiopoietins, and VEGF [24]. Among these factors, the signaling pathway initiated by VEGFs and their receptors plays determinant roles in angiogenesis [25].

Among the VEGF family, VEGF165 (one isoform of VEGF-A) shows varied binding abilities with its receptors. In the tumor endothelium, VEGF165 interacts with VEGFR-2 and then induces capillary growth, which fulfills the increased tumor demand for oxygen and nutrients, contributing to tumor metastasis [26]. Studies have shown that VEGFR-2 may be the most important receptor in VEGF-induced endothelial cell mitogenesis and permeability [27]. NRP-1, a cell-surface glycoprotein that lacks intrinsic kinase sequences, was found to contribute to the high migration of endothelial cells [28]. As a type I

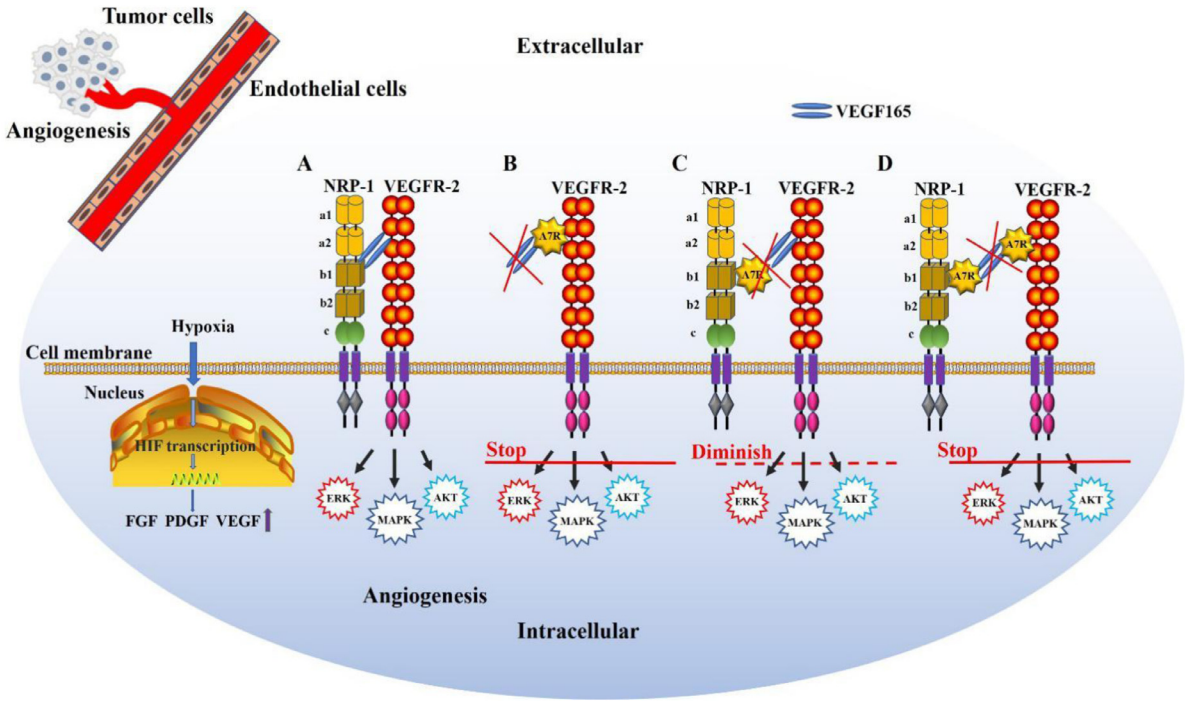

Fig. 3 – Roles of VEGFR-2 and NRP-1 in angiogenesis and the antiangiogenic mechanism of action of A7R. Hypoxia increases cellular hypoxia inducible factor (HIF) transcription, leading to the upregulation of FGF, PDGF, and VEGF expression in endothelial cells. (A) NRP-1 functioning as a coreceptor binds to VEGF165 and enhances VEGF165-VEGFR-2 intracellular trafficking, facilitating angiogenesis. (B) A7R can target and compete with VEGF165 to bind VEGFR-2 and suppress the downstream signal transduction of VEGFR-2. (C) A7R can bind to the b1 domain of NRP-1, inhibit VEGF165 binding to NRP-1 and diminish VEGF165-VEGFR-2 intracellular trafficking. (D) A7R can bind preferentially to both VEGFR-2 and NRP-1 simultaneously and suppress the signal transduction downstream of VEGFR-2, displaying the strongest antiangiogenic activity.

transmembrane receptor, NRP-1 was primarily identified as an axonal adhesion protein, a receptor for semaphorin 3A (SEMA3A) [29]. Researchers have proved that NRP-1 is a coreceptor that binds to VEGF165 and enhances the binding of VEGF165 to VEGFR-2 [30].

In addition to the highly conserved short cytoplasmic domain, NRP-1 possesses five discrete extracellular domains for ligand binding, two domains with homology to complement components C1r and C1s (CUB domains, a1 and a2), two coagulation factor V/VIII domains (CF V/VIII, b1 and b2), and one C-terminal MAM (meprin, A5, μ-phosphatase) domain (c) [30]. Thus, with a core conserved binding pocket formed by the b1 coagulation factor loops, NRP-1 binds to VEGF165, forming a VEGF165-NRP-1 complex [31]. This complex then enhances VEGF165-VEGFR-2 intracellular trafficking, activating receptor kinase activity that leads to receptor autophosphorylation. The phosphorylated receptors then recruit interacting proteins, such as Src homology 2 (SH2) domain-containing proteins, and induce the activation of signaling pathways that involve another second messenger, thereby activating the phosphatidylinositol 3-kinase/Akt pathway, which promotes endothelial cell survival; the Src-FAK pathway, which mediates endothelial cell migration and vascular permeability [26]; and the Raf-MEK-ERK pathway, which induces endothelial cell proliferation and network formation, facilitating angiogenesis (Fig. 3A) [32].

In addition to binding to VEGF, NRP-1 can also interact with platelet-derived growth factor (PDGF) and PDGF receptor (PDGFR), which promotes the proliferation of endothelial cells and blood vessel formation [29]. Furthermore, studies have shown that NRP-1 can regulate angiogenesis in a VEGFR-2-independent manner [33,34]. As an adhesion molecule, NRP-1 interacts with integrins and promotes endothelial cell adhesion to the ECM. Then, the cytoplasmic domain of NRP-1 forms a complex with the intracellular kinase ABL1 and promotes endothelial cell migration in response to integrin ligand-mediated angiogenesis [34].

Due to the close relationship of VEGF165, VEGFR-2 and NRP-1, interrupting the formation of the VEGF165/NRP-1/VEGFR-2 complex or specifically inhibiting VEGFR-2 or NRP-1 will inhibit tumor angiogenesis. Although A7R has been verified to interrupt the VEGF165/NRP-1/VEGFR-2 pathway and display antiangiogenic effects, different strategies for interrupting the pathway have been proposed (Fig. 3).

2.2. *The mechanism underlying A7R peptide function*

2.2.1. *Binding to VEGFR-2*

In 2000, Tournaire et al. found that A7R could completely abolish VEGF binding to VEGFR-2. The researchers used two different strategies to identify peptides blocking the binding between VEGF and VEGFR-2 [18]. First, they selected

Chinese hamster ovary (CHO) cells that expressed recombinant VEGFR-2 at the membrane surface to test the binding ability of different peptides to VEGFR-2. After screening, seven peptides (K1–K7) were chosen. However, these peptides showed low affinity to VEGFR-2 in ELISAs. Then, the researchers screened the peptides by an anti-VEGF antibody binding test. Another seven peptides (V1–V7) with no consensus motifs were selected, and all of them showed strong affinity for VEGFR-2. Among these peptides, V1 (ATWLPPR) and V6 (LPPNPTK), which both had an LPP motif, showed the best reactivity. Interestingly, only V1 (ATWLPPR) competed with VEGF for binding to VEGFR-2, inhibited human endothelial cell proliferation *in vitro* and abolished VEGF-induced angiogenesis *in vivo*. Subsequently, Perret et al. also proposed that this heptapeptide (A7R) might target VEGFR-2 since it could suppress [^{125}I]-VEGF binding to endothelial cells [35]. In a 2005 report, Barr et al. observed that 5-(6)-carboxyfluorescein-labeled A7R could interact with human umbilical vein endothelial cells (HUVECs) expressing VEGFR-2, as shown by confocal microscopy *in vitro* [19]. All these studies demonstrated that A7R could target VEGFR-2 and potentially inhibit tumor angiogenesis (Fig. 3B).

2.2.2. *Binding to NRP-1*

In 2004, Perret labeled A7R with ^{99m}Tc to explore whether it could target NRP-1 or NRP-2. Interestingly, the researchers found that ^{99m}Tc-labeled A7R could bind NRP-1 but not NRP-2. Incubation of ^{99m}Tc-labeled A7R with recombinant NRP-1 protein resulted in a visual monophasic binding curve [36]. Subsequently, Starzec et al. proved that A7R could inhibit the binding of VEGF165 to recombinant NRP-1 protein and NRP-1-expressing MDA-MB-231 cells but could not inhibit the binding between VEGF165 and recombinant VEGFR-2 protein or VEGFR-2-expressing porcine aortic endothelial (PAE) cells. The researchers also found that A7R could form a specific complex with recombinant NRP-1 by affinity crosslinking experiments (Fig. 3C) [37].

In 2007, the above mentioned laboratory performed a structural characterization of A7R. According to their research, the interface between NRP-1 and VEGF165 is predominantly stabilized by a network of hydrogen bonds and salt bridges based on analysis of the complex crystal structure of NRP-1-VEGF165 [38]. VEGF165 contributes two separate principal regions to the heterodimeric interactions: the exon 8 region, including the C-terminal arginine, is essential for the high affinity of VEGF165 for NRP-1; and the electronegative residues of the exon 7 region form additional interactions with the L1 loop of NRP-1. In addition, the C-terminal tail (residues 160–165, CDKPRR) of VEGF165 is the binding site for NRP-1 [39,40]

Based on the NRP-1 structure (PDB ID: 2ORZ), a molecular dynamics (MD) simulation of the NRP-1-A7R complex was performed [38]. The complex structure showed that the hydrogen bonds between the terminal amino acid Arg7 of A7R and NRP-1 played a key role in stabilization of the complex, which was the same as the structural basis for the interaction between VEGF165 and NRP-1 mentioned before. The guanidinium group of Arg7 of A7R (donor) formed hydrogen bonds with the side-chain carboxylic group of Asp320 in NRP-1 (acceptor). The hydroxyl groups of Ser346, Thr349 and Tyr353 (donor) formed hydrogen bonds with the C-terminal carboxylic group of Arg7 in A7R (acceptor). Interestingly, the C-terminal tails of both VEGF165 and A7R possess a CendR sequence (C-terminal arginine), which was confirmed to be essential for the interaction with the b1 domain of NRP-1. The cell binding and internalization abilities were lost when the C-terminal arginine was mutated to alanine, thus confirming the presumed hydrogen bonding network between Arg7 and NRP-1 [41]. In addition, based on alanine scanning (mutation of each amino acid to alanine) or amino acid deletion, the C-terminal LPPR sequence is important for A7R activity; mutation of both Pro5 and Pro6 to alanine in A7R significantly reduced the inhibitory effect. Thus, A7R contains a CendR sequence targeting NRP-1: the C-terminal arginine is optimally accommodated at the binding pocket in the b1 domain of NRP-1 by a hydrogen bond network, and the two internal proline residues in the LXXR motif may be responsible for stabilization of the NRP-1-peptide complex. In 2009, Tuttle further studied the dynamic behavior of A7R through a hybrid quantum mechanical/molecular mechanical (QM/MM) method, which may contribute to revealing its conformation [42].

In summary, A7R may mimic the C-terminal tail of VEGF165, simulate the interaction with the b1 domain of NRP-1 and thus generate steric hindrance between NRP-1 and VEGF165. A7R competes with VEGF165 for binding to NRP-1, which can disrupt the VEGFR-2-NRP-1 complex, thereby interrupting VEGFR-2-mediated angiogenesis through NRP-1. These results provide useful information for future studies aimed at designing new peptide inhibitors of angiogenesis.

2.2.3. *Binding to VEGFR-2 and NRP-1*

Although some recent studies showed that A7R targeted NRP-1 but not VEGFR-2, Ying et al. [43, 44]. proved that this peptide specifically bound to both NRP-1 and VEGFR-2 (Fig. 3D). Molecular docking and surface plasmon resonance (SPR) analyses were conducted to explore the interactions of A7R with its receptors.

In the molecular docking study, A7R was shown to be deeply docked at two domains of VEGFR-2, among which the Arg could form ionic interactions with Asp1054 of VEGFR-2, while the indole fragment of Trp could form hydrophobic interactions with Phe1045, Phe916, Ala864 and Leu1033 of VEGFR-2. In addition, the Leu and Pro of A7R could permeate into the binding pocket through hydrophobic interactions. The binding mode between A7R and NRP-1 showed that A7R localized in the head of the b1 domain of NRP-1, and ionic interactions between Arg of A7R and Asp320 of NRP-1, as well as the Pro and Leu residues of the peptide and the Tyr297 and Trp301 residues of NRP-1, were observed and were consistent with previous mechanistic research [44]. In the SPR assay, A7R displayed a similar high binding affinity to VEGFR-2 and NRP-1 with an equilibrium dissociation constant (KD) of 9.29 and 6.62 nM, respectively [43]. In conclusion, all these results confirmed the binding affinity of A7R to both VEGFR-2 and NRP-1.

Based on the above findings, although A7R was originally thought to target VEGFR-2 and display antiangiogenic activity, the hypothesis that A7R directly targets NRP-1 and thus blocks the binding between VEGF165 and NRP-1 has the most support. Nevertheless, the latest studies by Ying et al. showed that this peptide might exhibit specific dual binding to VEGFR-2 and NRP-1, suggesting that the antiangiogenic effects of A7R

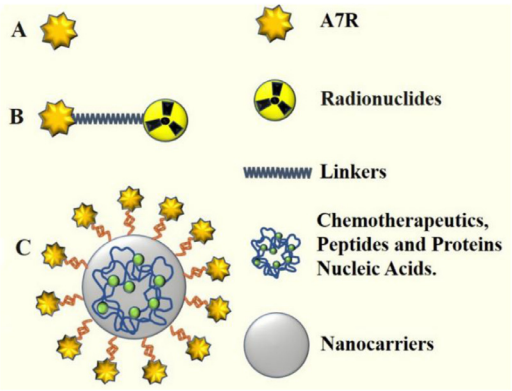

Fig. 4 – The applications of A7R. (A) A7R was used as an antiangiogenic and anticancer agent. (B) Radiolabeled A7R. The A7R peptide is covalently conjugated to radionuclides. (C) A7R peptides or peptidomimetics are grafted at the nanocarrier surface (liposomes, nanoparticles, etc.). These nanocarriers are loaded with various drugs, such as anticancer therapeutic drugs, peptides, proteins, or nucleic acids.

may be caused by blocking both VEGFR-2 and NRP-1 instead of only one receptor. Certainly, the underlying mechanisms need to be elucidated, which will be indispensable for applications of this peptide.

3. The applications of A7R

Although the mechanism underlying A7R targeting of VEGFR-2 or NRP-1 is still controversial, applications of A7R based on its inhibitory effects on tumor angiogenesis have been extensively examined in recent years. A7R has several advantages for scientific research and practical applications. First, owing to its ability to target VEGFR-2 and NRP-1, which are highly expressed on tumor neovasculature, A7R can be preferentially recognized and taken up by the endothelial system. Second, modified A7R (N-to-C cyclization or the retro-inverso isomer of A7R) showed enhanced proteolytic and thermal stability against proteolytic degradation and retained comparable binding affinities to the receptors (VEGFR-2 and NRP-1), and A7R conjugates could show increased accumulation in tumor tissue. Third, A7R, which consists of only seven amino acids and is much smaller than monoclonal antibodies, has a lower risk of immunoreactivity than therapeutic antibodies and is easy to produce. Based on the merits of A7R mentioned above, we primarily examined three applications of A7R: A7R used as an antiangiogenic and anticancer agent, radiolabeled A7R for tumor imaging and diagnostics, and A7R-mediated targeted delivery systems (Fig. 4).

3.1. *A7R as an antiangiogenic and anticancer agent*

3.1.1. *Inhibiting tumor and ocular angiogenesis*

Tournaire et al. discovered that A7R could completely abolish VEGF binding to VEGFR-2 expressed by CHO cells [18]. *In vitro*, the proliferation of bovine pulmonary endothelial cells was reduced by 60% by A7R, and the mitogenic activity of HUVECs was inhibited by A7R in a dose-dependent manner. *In vivo*, A7R could significantly suppress neovascularization stimulated by VEGF in a rabbit corneal model. Rodrigues et al. found that A7R (200 μM) efficiently suppressed the angiogenic response stimulated by VEGF in a chorioallantoic membrane (CAM) assay and capillary formation of HUVECs plated on Matrigel [45].

Anna et al. also confirmed that A7R diminished VEGF-induced proliferation of HUVECs in a dose-dependent manner with an IC50 of 450 μM. In HUVECs cocultured with human fibroblasts, tubule length and the density of branching junctions formed by endothelial cells were decreased by A7R. *In vivo*, the intratumor vessel density decreased by 22% compared to that of the control group after A7R treatment in a nude mouse model harboring MDA-MB-231 breast cancer cell-derived xenografts expressing NRP-1 [37]. In summary, A7R can be used as a potent therapeutic agent to treat angiogenic diseases.

3.1.2. *Reducing vascular permeability*

Upon activation by VEGF, VEGFR-2, which is highly expressed on retinal and cerebral endothelial cells, adopts an activated form and stimulates downstream pathways, such as the Src-FAK pathway. The signal transduction induced by VEGFR-2 weakens endothelial tight junctions and increases vascular permeability [46]. Moreover, this signal transduction induced by VEGFR-2 is strengthened by NRP-1 binding to VEGF165 [33]. Different studies have proven that A7R can reduce vascular permeability in diseases characterized by BBB and blood-retinal barrier (BRB) disruption [20,21].

Vascular permeability dysfunction is an indispensable factor in BBB disruption [47]. CD8 T cells that have a close relationship with central nervous system (CNS) vascular permeability can promote BBB disruption [48]. In 2012, Suidan's group built a murine model of CD8 T cell-initiated BBB disruption [20]. By blocking VEGF signal transduction with the NRP-1 inhibitor A7R, researchers reduced vascular permeability, brain hemorrhage and mortality in this murine model. The VEGFR-2 expression level was also decreased in this BBB disruption model. This research demonstrated that A7R effectively suppressed NRP-1 to protect against neuroinflammatory diseases, including BBB dysfunction and brain hemorrhage.

Diabetic retinopathy (DR) is a cause of blindness, and BRB disruption is a prominent sign of DR [49]. In 2015, Wang et al. [21] evaluated the effects of A7R on the early stages of DR. The researchers established an experimental diabetic mouse model and treated it with A7R at a dosage of 400 μg/kg once daily for one week. The results showed that A7R not only noticeably decreased diabetes-induced leucocyte attachment, oxidative stress, and the expression of inflammation-associated proteins but also prevented occludin degradation and extravasation of albumin. Occludin proteins play irreplaceable roles in tight junctions formed by vascular endothelial cells in the BRB. VEGF autocrine signaling and occludin degradation in DR can be induced by glucose conditions [50]. Li et al. found that A7R (100 μM) significantly prevented occludin downregulation under high-glucose conditions in rhesus monkey retinal fovea vascular endothelial (RF/6A) cells, which suggested that A7R could reduce vascular permeability

in DR [51]. All these results indicated that A7R may prevent the early retinal damage induced by diabetes and preserve vascular integrity. Furthermore, this study proposes a new strategy to treat DR with A7R.

3.1.3. *Inducing apoptosis of endothelial cells*

In addition to inducing the proliferation and differentiation of endothelial cells, Barr et al. studied the effects of A7R on the apoptosis of endothelial cells. Confocal microscopy showed that A7R labeled with 5-(6)-carboxyfluorescein bound to HUVECs expressing functional VEGFR-2. Treatment with A7R resulted in a significant increase in apoptosis of the HUVECs. Therefore, A7R may be an effective apoptosis-inducing peptide in antiangiogenic strategies [19].

3.1.4. *Inhibiting tumor growth and angiogenesis with A7R-containing peptides*

As described above, A7R has been used as an independent antitumor angiogenic agent. In 2010, Wu et al. connected A7R to NLLMAAS via a flexible linker, Ala-Ala, and obtained the novel peptide ATWLPPRAANLLMAAS [52]. AANLLMAAS was screened from a phage display peptide library and suppressed the binding of angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) to endothelial cell-specific receptor tyrosine kinase (Tie-2) and further inhibit angiogenesis [53]. The researchers tested the novel peptide on sarcoma S180- and hepatoma H22-bearing BALB/c nude mouse models. After subcutaneous injection for 7 days, the conjugated peptide showed potent antitumor activity in reducing tumor weight, volume, and microvessel density (MD) without significant side effects on normal tissue compared with A7R and AANLLMAAS. Thus, these results showed that the conjugated peptide could be an effective inhibitor of tumor growth and angiogenesis.

3.2. *Tumor imaging and diagnosis with radiolabeled A7R*

Radiolabeled receptor-binding peptides have been extensively investigated as potential molecular imaging probes and diagnostic agents [54,55]. Due to the affinity of A7R to NRP-1 and VEGFR-2 overexpressed in the tumor vasculature, A7R can be used for tumor angiogenesis imaging.

The peptide A7R was labeled with ^{99m}Tc (a benzoyl mercaptoacetyl group was added to the peptide *N*-terminus in order to allow the formation of an SNNN tetradentate ^{99m}Tc complex), and a stable radiolabeled peptide was obtained. *In vitro* ^{99m}Tc-labeled A7R showed high binding to recombinant NRP-1. After intravenous injection, high-level radioactive biodistribution of ^{99m}Tc-labeled A7R was observed in the digestive tract of mice and rats rather than in the tumor region. The rapid elimination and low tumor accumulation of this molecule *in vivo* may result in low tumor uptake and rapid dissociation from tumors, which indicates that improved labeling methods are needed to obtain better imaging results [36].

Integrins are essential cell adhesion receptors and play important roles in mediating adhesive events, tumor angiogenesis and metastasis [56]. $\alpha_v\beta_3$ integrins are some of the most important integrins because they facilitate endothelial cell migration. High expression of these molecules on tumor cells is stimulated by proangiogenic factors. The RGD peptide, a cell adhesion motif, shows high affinity for $\alpha_v\beta_3$ integrins. Given that both NRP-1 and $\alpha_v\beta_3$ integrins are overexpressed in gliomas, Wu et al. [57] and Ma et al. [58] synthesized a conjugated RGD-ATWLPPR peptide that was labeled with fluorine-18 (^{18}F-RGD-A7R) and evaluated the receptor-binding properties and tumor-targeting efficacy. Cell uptake experiments *in vitro* showed that ^{18}F-RGD-A7R had a higher cell uptake rate than ^{18}F-RGD or ^{18}F-A7R at the 2 h time point, and the uptake of ^{18}F-RGD-A7R was completely inhibited in the presence of both RGD and A7R. In static micro-PET/CT scans of the U87MG glioma cell-derived xenograft model *in vivo*, conspicuous accumulation of ^{18}F-RGD-A7R was observed in the tumor compared with normal organs and blood. All these results demonstrated that the ^{18}F-labeled RGD-A7R peptide exhibited improved *in vitro* and *in vivo* pharmacokinetics and superior imaging quality [57,58].

Ultrasound molecular imaging has been widely used in early disease detection and disease progression monitoring [59]. In 2015, Zhang et al. conjugated A7R onto the surface of lipid microbubbles (A7R-MBs) to evaluate the molecular imaging of tumor angiogenesis in a breast cancer model. *In vitro*, the MBs modified with 1 mol% A7R bound to NRP-1-expressing primary prostate carcinoma-1 (PPC-1) cells at a 17.6 times higher rate than non-modified MBs. *In vivo*, A7R-MBs were successfully used to image NRP-1 content on angiogenic vessels [60]. Thus, NRP-1-targeting A7R-MBs offer a new strategy for molecular imaging of tumor angiogenesis.

3.3. *A7R-mediated targeted delivery systems*

A key factor in the successful treatment of a disease is to deliver effective therapeutic drugs at an optimal dosage and continuous concentration to the diseased tissues and organs without affecting the physiological function of normal tissue [61]. Drug delivery systems (DDS) are devices that can deliver chemotherapeutic drugs, immunomodulatory drugs and diagnostic radiopharmaceuticals to certain tissues of the body. Traditional DDS have many disadvantages, such as low targeting, poor therapeutic effects, inefficient delivery and issues of drug resistance [62]. Currently, numerous novel DDS have been focused on targeted drug delivery [63]. The number of targeted delivery strategies utilizing various nano-, micro- or macroscale DDS for cancer treatment and diagnosis has been increasing. These systems exhibit efficient therapeutic effects due to their excellent properties, such as deeper tumor penetration, superior specificity, reduced multidrug resistance and enhanced cellular uptake [64].

In tumor chemotherapy, multiple novel materials, such as multifunctional polymeric micelles [65], stimuli-responsive nanocarriers [66], and nanoparticle-assembled thermosensitive hydrogels [67], have been shown to not only improve the drug targeting distribution, accumulation and retention efficiency in tumor tissues but also reduce the side effects of chemotherapeutic agents (e.g., doxorubicin (DOX)) to normal tissues. Immunotherapy is an effective treatment method for cancer [68]. A series of engineered micro- and nanomaterials can be used as carriers to deliver vaccines, immune cells or immunomodulators to specific cells or tissues or to stimulate the host to produce durable anticancer immune responses directly through their own inherent properties

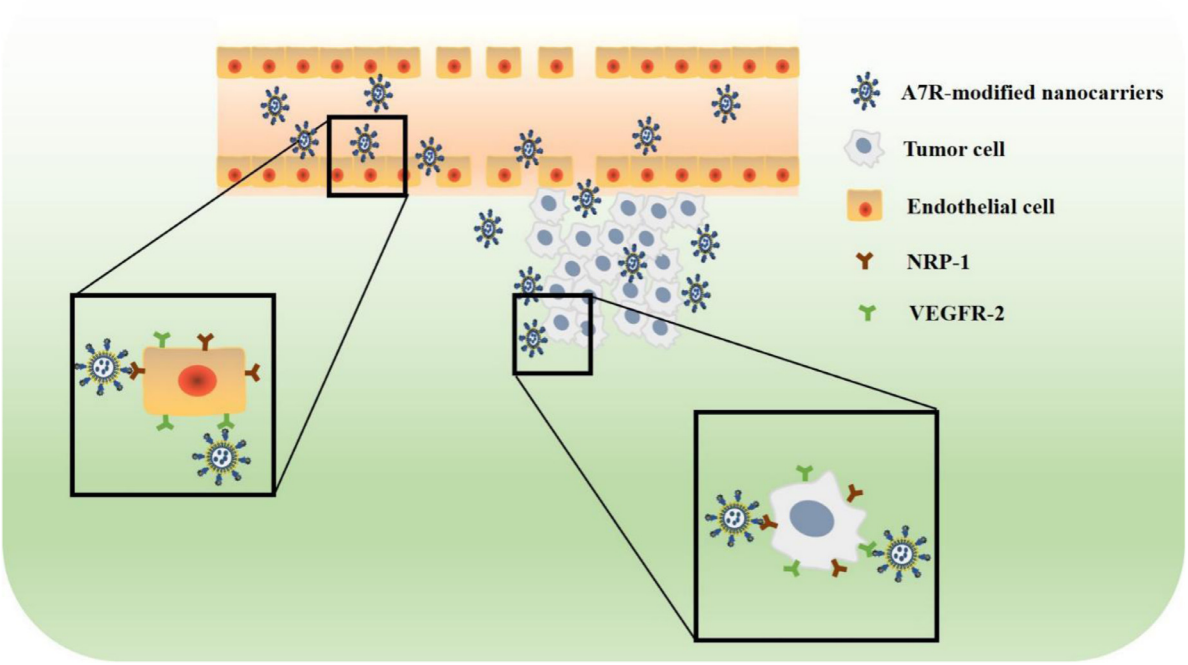

Fig. 5 – Schematic representation of the active targeting mechanisms of A7R-modified nanocarriers. A7R can guide nanocarriers to bind endothelial cells and tumor cells that express NRP-1 or VEGFR-2.

[69]. For example, the applications of mesoporous silica rods (MSRs), nanofibrous hydrogels, nanoscale colloids and other vehicles promote the development of DC-based antitumor immunotherapy [70,71]. Significant breakthroughs in the imaging and diagnosis of tumors, which take advantage of diverse delivery systems, have been reported [72]. Targeted nanoscale imaging molecules or radiolabeled nanocarriers such as gold nanoparticles (AuNPs) [73], Raman scattering nanoparticles (MPR) [74] and ^{19}F-MRI nanoprobes [75], which can be monitored by different imaging techniques such as magnetic resonance imaging (MRI), photoacoustic imaging (PAI), computed tomography (CT) and single photon emission computed tomography (SPECT), are helpful for the detection and measurement of biological and cellular events, the visualization of drug release and accumulation, and tumor diagnosis [76,77].

In recent years, active targeted nanocarriers modified with targeting ligands (such as antibodies, peptides, glycoproteins and carbohydrates) have become a research hotspot [78]. Among the diverse targeting ligands, targeting peptides have emerged as one of the most promising non-immunogenic methods to target tumors and cells. Liposomes or nanoparticles that can be grafted with targeting peptides (such as A7R) have several advantages, such as "passive targeting" to tumors due to their appropriate size (20–400 nm), prolonged blood circulation time, and "active targeting" to neovascular endothelial cells and tumor cells that overexpress specific receptors, for instance, NRP-1 and VEGFR-2 [79]. Fig. 5 shows the active targeting mechanisms of A7R-modified nanocarriers. A7R can guide nanocarriers to these cells through its targeting characteristics. Therefore, the applications of A7R-modified liposomes and nanoparticles in cancer therapy will be introduced in this section.

3.3.1. *A7R-modified liposomes*

Janssen demonstrated that coupling of the A7R peptide to the surface of liposomes increased the affinity of liposomes for HUVECs through flow cytometry-based fluorescence-activated cell sorting (FACS) analysis and confocal laser scanning microscopy (CLSM) analysis *in vitro* [80]. In 2015, Cao et al. designed paclitaxel liposomes modified with A7R-cysteine peptide (A7RC) on the surface (A7RC-LS/TAX). *In vitro*, the targeting and uptake efficiency of A7RC-LS/TAX in MDA-MB-231 cells with high NRP-1 expression were significantly enhanced, and the antiangiogenic effect on NRP-1-expressing HUVECs was strengthened at the same time. *In vivo*, 4 h after injection, the fluorescence intensity in local tumor tissue was stronger in the DiR-labeled A7RC-LS/TAX group than in the group of nude mice bearing MCF-7 and MDA-MB-231-derived tumors [81].

The BBB and blood-brain tumor barrier (BBTB) are major obstacles when treating malignant glioma with therapeutic drugs [82,83]. To overcome these physiological barriers, researchers have investigated targeting peptide-modified liposomes. Considering the poor biological stability of linear A7R peptide, in 2016, Lu's group designed a retro-inverso A7R peptide/^{D}A7R peptide (comprised of D-amino acids in the reverse sequence) [43] and a cyclic A7R peptide (head-to-tail joining via an amide bond) [44]. Molecular docking and SPR analyses demonstrated that ^{D}A7R and cyclic A7R displayed similar binding affinities to VEGFR-2 and NRP-1 compared with the linear A7R peptide (^{L}A7R). Both the BTB/U87 tumor spheroid coculture model imitating the blood tumor barriers *in vitro* and nude mice bearing subcutaneous U87 xenograft tumors *in vivo* indicated that ^{D}A7R-liposomes and cyclic A7R-liposomes showed stronger targeting to and accumulation in

Table 1 – Examples of recent studies on A7R-modified liposomes delivering different therapeutic agents.

Carrier	Therapeutic agent	Compound	Experimental model	Results	Refs.
	—	A7R-LS	HUVEC (endothelial cells)	A7R-PEG-liposome showed increased affinity for HUVEC *in vitro*.	[60]
	Paclitaxel	A7RC-LS/TAX	Breast cancer cell lines	Angiogenesis and tumor growth were inhibited simultaneously.	[61]
Liposomes (LS)	Doxorubicin	cA7R-LS/DOX	U87 xenograft tumors/glioma	Subcutaneous tumor growth was suppressed.	[41]
	Doxorubicin	^{D}CDX/ ^{D}A7R-LS/DOX	U87 xenograft tumors	Tumor growth and angiogenesis was inhibited.	[64]
	Doxorubicin and vincristine	T7/^{D}A7R-LS/DOX and VCR	Glioma-bearing mice model	Higher glioma localization was displayed than that of single ligand-modified liposomes or free drug.	[66]
	GFP gene	C16-A7R-LS/GFP	MDA-MB-231 cells	The expression of GFP reporter genes was enhanced.	[71]
Immuno-nano-Liposomes (INLS)	PEDF	A7R-INLS/PEDF	CNV in the rat model	The inhibitory effects of PEDF on CNV was strengthened and side effects was reduced.	[70]

tumors than ^{L}A7R-liposomes. Furthermore, DOX liposomes modified with cyclic A7R and ^{D}A7R peptide (cA7R-LS/DOX and ^{D}A7R-LS/DOX) showed improved inhibitory effects on HUVECs, HUVEC 3D tubes *in vitro* and a subcutaneous tumor model *in vivo*.

To increase the ability of liposomes to cross the BBB/BBTB and target malignant glioma, researchers have developed multiple ligand-modified liposomes. Ying et al. designed a ^{D}A7R and ^{D}CDX (a D-peptide ligand binding nicotine acetylcholine receptors) dual modified liposome. This new liposome showed improved penetration into HUVECs, brain capillary endothelial cells (BCECs), U87 cells and tumor spheroids *in vitro*. After they were loaded with DOX, ^{D}A7R/^{D}CDX-modified liposomes (^{D}A7R/^{D}CDX-LS) showed improved inhibitory effects on tube formation of HUVECs and U87 cells *in vitro* and antitumor effects in intracranial U87 glioma-bearing nude mice *in vivo* [84].

The T7 peptide (HAIYPRH), which has a high affinity for transferrin receptor (TfR) overexpressed in glioma cells and endothelial cells, was recently identified from a phage display library [85]. To overcome the BBB and BTB as well as single chemical drug resistance, Zhang et al. developed a T7 and ^{D}A7R peptide-modified liposome coloaded with DOX and vincristine (T7/^{D}A7R-LS/DOX and VCR) [86]. T7/^{D}A7R-LS/DOX and VCR showed high penetration efficiency in a coculture model of bEnd.3/C6 cells *in vitro* (simulating BBB and BTB models). Similarly, the accumulation of T7/^{D}A7R-LS/DOX and VCR in the mouse brain with intracranial C6 glioma was higher than that of T7-LS/DOX and VCR or ^{D}A7R-LS/DOX and VCR. The glioma diameter and volume were clearly reduced after the administration of T7/^{D}A7R-LS/DOX and VCR, suggesting its prominent therapeutic efficacy in glioma.

In addition to chemical drugs, therapeutic proteins and nucleic acids can be carried by liposomes to enhance antitumor activity and reduce systemic side effects [87,88]. Pigment epithelium derived factor (PEDF), which can directly induce the apoptosis of endothelial cells in neovascularization, is regarded as a promising drug for choroidal neovascularization (CNV) [89]. However, lack of an appropriate delivery method prevents the clinical use of PEDF. Li et al. designed PEDF-loaded immuno-nanoliposomes modified with A7R (A7R-INLS/PEDF). The researchers found that A7R-INLS/PEDF could specifically bind to endothelial cells of CNV, largely decrease the CNV area and increase the effects of PEDF in a rat model [90]. Thus, this new delivery system may provide us a new strategy for treating CNV. Slimani's laboratory synthesized a new C16-A7R peptide through an amide reaction between a palmitoyl fatty chain and A7R and then conjugated it to liposomes. The C16-A7R-modified liposomes loaded with green fluorescent protein (C16-A7R-LS/GFP) were well aggregated in MDA-MB-231 human breast cancer cells overexpressing NRP-1 and strengthened the expression of GFP in the above cells [91]. Examples of different A7R-modified liposomes that have been shown to improve the target efficiency of different drugs are summarized in Table 1.

3.3.2. *A7R-modified nanoparticles*

Photodynamic therapy (PDT) has been an effective strategy to treat cancer. PDT targeting neovasculature in tumor tissue is a potential strategy for treating cancer [92]. However, inadequate water solubility, insufficient pharmacokinetics and poor tumor selectivity of photosensitizers (PSs) limit their development and application. The design of PSs covalently attached to a tumor-targeting moiety or encapsulated within nanoparticles is currently a research hotspot. These tumor-targeting moieties consist of monosaccharides [93], low-density lipoprotein [94], antibodies [95], peptides [96] and so on. Among them, peptides are receiving increasing interest in the field of PDT.

A7R-modified 5-(4-carboxyphenyl)−10,15,20-triphenylchlorin (TPC, which is a kind of PS) via a spacer 6-aminohexanoic acid, Ahx (A7R-Ahx-TPC), which binds exclusively to NRP-1, is a much more potent PS than TPC [97].

Table 2 – Examples of recent studies on A7R-modified nanoparticles.

Carrier	Therapeutic agent	Compound	Experimental model	Results	Refs.
Hybrid Silica Nanoparticle	Chlorin (photosensitizer)	A7R-HS-NP/TPC	Glioma-bearing rat model	A positive magnetic resonance imaging (MRI) contrast and intratumoral retention were increased greatly.	[78-80]
Superparamagnetic iron oxide-based nanoparticles	$Ni_{0.5}Zn_{0.5}Fe_2O_4$	A7R-SPIONs/Fe	HUVEC (endothelial cells)	The cell viability of HUVEC was reduced.	[84]
Nanoparticles	Paclitaxel	A7R-CGKRKR-NP/PTX	U87MG glioblastoma	Apoptosis induction and anti-proliferative activity were enhanced.	[87]

In addition, A7R-Ahx-TPC can target both tumor endothelial cells and tumor cells themselves, which overexpress NRP-1, efficiently potentiating the effects of PDT *in vivo* [97–99]. Reem et al. found that the conjugation of verteporfin as a PS to A7R not only retained its spectral and photosensitizing properties but also efficiently targeted CNV [100]. However, biodistribution and stability tests in glioma-bearing mice *in vivo* showed that although A7R-Ahx-TPC accumulated at the tumor site, significant degradation was observed 2 h after injection [97,98], which indicated that A7R-Ahx-TPC was weakly preserved.

In 2017, Benachour et al. designed a novel A7R-conjugated hybrid silica nanoparticle loaded with TPC (A7R-HS-NP/TPC). A competitive binding assay with VEGF165 showed that A7R-HS-NP/TPC bound recombinant NRP-1 protein in a concentration-dependent manner (EC50=56.6 μM) and displaced 50% of VEGF165 binding to NRP-1. *In vivo*, positive magnetic resonance imaging (MRI) contrast and intratumoral retention were substantially increased after intravenous injection of A7R-HS-NP/TPC in glioma-bearing rats [101]. These results showed that TPC loaded in A7R-modified nanoparticles had better retention and stability than A7R-conjugated TPC, providing us with a new strategy to use targeting peptide-modified nanoparticles for PDT.

Superparamagnetic iron oxide-based nanoparticles (SPIONs), with the help of an external magnetic field, are promising carriers as targeted drug delivery vehicles [102]. Cell cytotoxicity studies showed that SPIONs modified with A7R (A7R/SPIONs) significantly reduced the viability of HUVECs compared with SPIONs and A7R. Further *in vitro* and *in vivo* studies are required to confirm the internalization mechanism of these SPIONs functionalized with A7R [103].

Treating glioma is difficult not only because of the existence of the BBB but also because of the complicated tumor microenvironment [104]. Numerous biomarkers [such as heparan sulfate proteoglycan (HSPG) in the ECM of the tumor microenvironment] are potential targets [105]. Utilizing the targeting ability of the CGKRK peptide to HSPG overexpressed in the tumor microenvironment and the A7R peptide to NRP-1 overexpressed on tumor endothelial cells and glioma cells, Hu et al. developed a new nanoparticle loaded with paclitaxel and modified by the dual-targeting A7R peptide and the CGKRK peptide (A7R-CGKRKR-NP/PTX). The cellular association of A7R-CGKRKR-NP on the HUVECs and U87 cells was enhanced compared with that of CGKRK-NP and A7R-NP. A7R-CGKRKR-NP enhanced the apoptotic and antiproliferative activity of paclitaxel on U87MG cells. An *in vivo* U87MG-bearing mouse experiment showed that A7R-CGKRKR-NP increased the accumulation of paclitaxel at the glioma site and its therapeutic effect on glioma [106]. Examples of different A7R-modified nanoparticles are summarized in Table 2.

4. Conclusions

Endothelial cells and tumor cells can be targeted due to their expression of VEGFR-2 and NRP-1. The overexpression of these two receptors is accompanied by tumor angiogenesis and progression. A7R was initially confirmed to inhibit VEGF-induced angiogenesis by targeting VEGFR-2. However, most studies showed that A7R generates steric hindrance between VEGF165 and NRP-1 and then interrupts VEGFR-2-mediated angiogenesis. However, the latest molecular docking studies confirmed that A7R has binding affinity for both VEGFR-2 and NRP-1: the leucine and proline of A7R can permeate the binding pocket through hydrophobic interactions with VEGFR-2, while the C-terminal arginine (CendR) simulates the C-terminal tail of VEGF165 and interacts with the b1 domain of NRP-1. However, the exact molecular mechanism of A7R binding to VEGFR-2 and NRP-1 and whether or how it competes with VEGF165 to inhibit VEGFR-2 and NRP-1 need further confirmation. This finding suggests that we can conduct structural optimizations of A7R and use computational docking technology to design more specific and active new peptides based on the unique structure of A7R to target NRP-1 and VEGFR-2. Compared with other vasculature-homing peptides, A7R has unique advantages in antiangiogenic diagnosis and therapy. First, A7R can perform dual targeting through simultaneous binding to VEGFR-2 and NRP-1, which leads to higher selectivity for tumor vasculature compared to other single-target peptides. Second, unlike RGD or NGR, as the targeting ligand of tumor vasculature-targeted DDS, A7R can not only facilitate drug delivery effectively by interacting with VEGFR-2 and NRP-1 but also exert a synergistic antiangiogenic effect with the carried therapeutic drugs. Third, due to its unique C-terminal (C-end rule, arginine at the C-terminus) structure, A7R can increase vascular permeation and penetrate deep into

tumor tissues through binding to NRP-1 on endothelial cells, thereby overcoming the weak tumor tissue-penetrating properties of some existing vasculature-homing peptides. However, the IC50 of A7R on HUVECs is not ideal (450 μM), and this peptide is easily degraded in circulation. Furthermore, A7R cannot kill tumor cells, and once the treatment ceases, the incidence of tumor recurrence is high. These shortcomings may be the main reasons for restricting the applications of A7R as a direct antagonist in the antiangiogenic field. Thus, optimal antiangiogenic therapy may require complex solutions by combining A7R with other therapeutic strategies (surgery, chemotherapy and radiation therapy) should be studied in more detail in the future.

The use of radiolabeled A7R as an imaging molecule has some problems, such as rapid elimination and low tumor accumulation. After conjugating RGD or grafting A7R onto the surface of lipid MBs, researchers observed obvious accumulation in tumor vasculature. This finding indicated that a combination with other targeted peptides or selection of better imaging carriers may improve the imaging and diagnostic efficiency of tumor angiogenesis. However, because of the high targeting abilities of A7R to VEGFR-2 and NRP-1, the applications of A7R-modified nanocarriers in DDS have been extensively researched. As shown above, A7R has been used to deliver chemotherapeutic drugs to the brain for the treatment of malignant glioma. A series of studies indicated that A7R-modified liposomes and nanoparticles were superior to free drugs in treating malignant glioma. However, the poor stability of A7R should not be ignored. To avoid proteolytic degradation and prolong blood circulation, researchers may need to design more stable structures of A7R. Due to the current mature modification methods, such as cyclization, retro-inverso isomerization and partial amino acid removal and replacement, the development of effective drug delivery methods with A7R is possible. Accordingly, despite some drawbacks, as a short non-immunogenic peptide with good bioavailability, A7R will certainly be an effective means of targeting therapy in the future.

Conflicts of interest

The authors state no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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