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A stabilized peptide ligand for multifunctional glioma targeted drug delivery

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ABSTRACT

Peptide ligands consisting of L-amino acids are subject to proteolysis *in vivo*. When modified on the surface of nanocarriers, those peptide ligands would readily degrade and the targeting efficacy is significantly attenuated. It has received increasing scrutiny to design stable peptide ligands for targeted drug delivery. Here, we present the design of a stable peptide ligand by the formation of a head-to-tail amide bond as an example. Even though the linear L-peptide A7R (termed ^{L}A7R) can bind specifically to vascular endothelial growth factor receptor 2 (VEGFR2) and neuropilin-1 (NRP-1) that are overexpressed on glioma cells, neovasculature and glioma vasculogenic mimicry (VM), the tumor-homing capacity of ^{L}A7R is greatly impaired *in vivo* due to proteolysis (*e.g.* in the serum). A cyclic A7R (cA7R) peptide was identified by computer-aided peptide design and synthesized with high yield by combining solid phase peptide synthesis and native chemical ligation. The binding of cA7R to both receptors was theoretically and experimentally assessed. In our simulated model hydrophobic and ionic interactions dominated the binding of ^{L}A7R to receptors. It is very interesting that cA7R adopting a different structure from ^{L}A7R retained high binding affinities to receptors without affecting the hydrophobic and ionic interactions. After head-to-tail cyclization by the formation of an amide bond, cA7R exhibited exceptional stability in mouse serum. Either cA7R or ^{L}A7R was conjugated on the surface of doxorubicin (DOX) loaded liposomes (cA7R-LS/DOX or ^{L}A7R-LS/DOX). The results of *in vitro* cellular assays indicated that cA7R-LS/DOX not only displayed stronger anti-proliferative effect against glioma cells, but also demonstrated to be more efficient in destruction of VM and HUVEC tubes in comparison to ^{L}A7R-LS/DOX and plain liposomes (LS/DOX, without peptide conjugation). cA7R conjugation could achieve significantly higher accumulation of liposomes in glioma than did ^{L}A7R conjugation, which in turn, cA7R-LS/DOX could substantially suppress subcutaneous tumor growth when compared with other DOX formulations (free DOX, LS/DOX and ^{L}A7R-LS/DOX). The designed cyclic A7R exhibited the capability of targeting glioma cells, neovasculature and VM simultaneously *in vivo*. Considering the ease of synthesis, high binding affinity to receptors and increased stability of cA7R peptide in the present study, the design of head-to-tail cyclized peptides by the formation of amide bond based on computer-aided peptide design presents an alternative method to identify proteolytically stable peptide ligands.

1. Introduction

In recent decades, the design of actively targeted drug delivery systems has been emerging as a successful strategy to improve the drug accumulation in the tumor and/or tumor-related tissues [1]. When decorated on the surface of nanocarriers, targeting ligands would play pivotal roles in directing tumor-targeted accumulation of payloads. Among the various targeting ligands, tumor-homing peptides have attracted extensive attention due to their unique advantages in design, synthesis, and modification, and always high binding affinity and specificity to cognate receptors. A plurality of peptides identified by phage display screening have been widely exploited as tumor-homing ligands [2–5]. A phage display selected L-peptide ^{L}A7R (ATWLPPR) [6] exhibited specific binding to vascular endothelial growth factor receptor 2 (VEGFR2) and neuropilin-1 (NRP-1), which are overexpressed in glioma, neovasculature and vasculogenic mimicry (VM) [6–10].

However, the *in vivo* applications of linear L-peptides are limited mainly owing to their poor biological stability. They are subject to proteolysis in the blood after systemic administration, which may devastatingly undermine the targeting efficiency *in vivo*. Therefore, it becomes

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prevalent to design proteolytically stable peptide ligands for targeted drug delivery. A variety of methods have been utilized to increase the *in vivo* stability of peptides, including the design of retro-all-D or retro-inverso peptide analogues, head-to-tail cyclization, terminal amino acid modification as well as the introduction of non-natural amino acids [11]. We and others have designed a series of retro-inverso isomers of L-peptide ligands to overcome the enzymatic barriers *in vivo* [12–17]. However, retro-inverso isomerization has its limitation, especially in the molecular mimicry of biologically active peptides containing α-helical structure [18,19]. Moreover, the safety of D-peptides and D-amino acids remains elusive due to their potential antigenicity and immunogenicity [20].

Head-to-tail cyclization by the formation of amide bond provides an alternative method to stabilize peptide ligands. However, the design is very challenging since simple head-to-tail cyclization would change the peptide conformation and undermine the bioactivity. In addition, the synthesis of head-to-tail cyclized peptides is always of low yield. Aided by computer-aided peptide design, we identified a cyclic A7R peptide (cA7R, CATWLPPR, head-to-tail cyclization *via* an amide bond) that could specifically bind VEGFR2 and NRP-1, which are known to be overexpressed in glioma, neovasculature and VM. cA7R was synthesized with high yield using native chemical ligation (NCL), which was developed by the Kent laboratory (Scheme 1) [21]. The binding affinity of cA7R was assessed by surface plasmon resonance, and its targeting properties were investigated *in vitro* and *in vivo*. cA7R was conjugated on the surface of DOX loaded liposomes as a targeting ligand and the anti-glioma effect of various DOX loaded liposomal formulations was studied in subcutaneous glioma-bearing nude mice.

2. Materials and methods

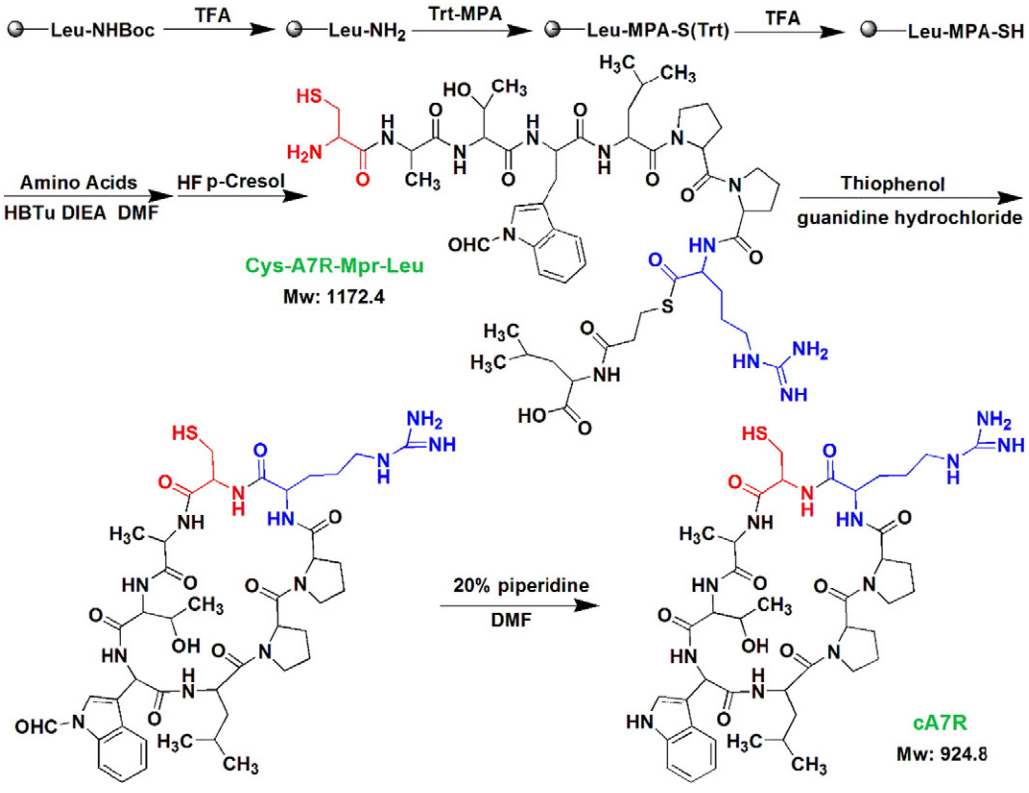
2.1. Materials

Fluorescein-5-maleimide was purchased from Fanbo Biochemicals (Beijing, China). DAPI was obtained from Roche (Basel, Switzerland). Near infrared dye DiR was from Invitrogen (Grand Island, NY). 5-carboxyfluorescein (FAM) was obtained from Sigma (St. Louis, MO). Cholesterol was purchased from Sinopharm Chemical Reagent Co. LTD. (Shanghai, China). HSPC (Hydrogenated soy phosphatidylcholine) and $mPEG_{2000}$-DSPE were from Lipoid GmbH (Ludwigshafen, Germany). mal-PEG_{3400}-DSPE was from Laysan Bio Co·(Arab, AL). Growth factor-reduced Matrigel matrix was supplied by BD Biosciences (San Diego, *CA*, USA). Rabbit anti-CD31 antibody was purchased from Abcam (USA). Dylight 647-conjugated donkey anti-rabbit antibody was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA, USA). Mouse serum was from Guangzhou Jianlun Biotechnology Co. (Guangzhou, China). Anticancer drug doxorubicin hydrochloride (DOX) was purchased from Haizheng Co. (Zhejiang, China).

HUVECs (human umbilical vein endothelial cells) and U87 (human glioblastoma cells) were from Shanghai Institute of Cell Biology. Both cells were cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% FBS (Gibco) in a humidified atmosphere containing 5% CO_2 at 37 °C. Male BALB/c nude mice of 4–6 weeks age were supplied by Shanghai SLAC laboratory animal Co. LTD (Shanghai, China) and housed under SPF conditions.

2.2. Synthesis of A7R peptides

Linear peptide ^{L}A7R was synthesized by solid phase peptide synthesis using active ester chemistry to couple Boc-protected amino acid to the deprotected resin. The crude peptide was purified to homogeneity, and the purity and molecular weight were ascertained by HPLC and ESI-MS, respectively. To synthesize cA7R, the linear peptide Cys-A7R-Mpr-Leu was designed and synthesized and purified for native chemical ligation. Briefly, Cys-A7R-Mpr-Leu was dissolved in Guanidine·HCl (6 M, pH was adjusted by 0.2 M phosphate buffer to 7.0) at a concentration of 1 mg/mL. Thiophenol (0.5‰, v/v) was added into the ligation buffer and the mixture was vortexed overnight. Followed that, 20% (v/v) piperidine was added into the reaction to remove the aldehyde group of the



Scheme 1. Synthesis of cA7R by combing solid phase peptide synthesis and native chemical ligation (black bead represents resin for the solid phase peptide synthesis).