Biomaterials 32 (2011) 4943–4950

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Targeting the brain with PEG–PLGA nanoparticles modified with phage-displayed peptides

Jingwei Li [a,b], Liang Feng [a,b], Li Fan [a,b], Yuan Zha [a,b], Liangran Guo [a,b], Qizhi Zhang [a,b], Jun Chen [a,b], Zhiqing Pang [a,b], Yuchen Wang [a,b], Xinguo Jiang [a,b,*], Victor C. Yang [c,e,**], Longping Wen [d,***]

[a] Department of Pharmaceutics, School of Pharmacy, Fudan University, 826 Zhangheng Rd., Shanghai 201203, People's Republic of China
[b] Key Laboratory of Smart Drug Delivery, Ministry of Education & PLA, 826 Zhangheng Rd., Shanghai 201203, People's Republic of China
[c] Department of Pharmaceutical Science, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109, USA
[d] Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science & Technology of China, Hefei, Anhui 230027, People's Republic of China
[e] School of Pharmacy, Tianjin Medical University & Tianjin Key Laboratory for Modern Drug Delivery and High Efficiency, Tianjin 300070, People's Republic of China

ARTICLE INFO

Article history:
Received 16 January 2011
Accepted 12 March 2011
Available online 5 April 2011

Keywords:
Phage display
Blood-brain barrier (BBB)
Brain delivery
Biodegradable nanoparticle

ABSTRACT

The relative impermeability of the blood-brain barrier (BBB) results from tight junctions and efflux transport systems limits drug delivery to the central nervous system (CNS), and thus severely restricts the therapy of many central nervous system diseases. In order to enhance the brain-specific drug delivery, we employed a 12-mer phage display peptide library to isolate peptides that could target the drug delivery system to the brain. A 12-amino-acid-peptide (denoted as Pep TGN) which was displayed by bacteriophage Clone 12-2 was finally selected by rounds of *in vivo* screening. Pep TGN was covalently conjugated onto the surface of poly (ethyleneglycol)-poly (lactic-co-glycolic acid) (PEG–PLGA) based nanoparticles (NPs). The cellular uptake of Pep TGN decorated nanoparticles was significantly higher than that of unmodified nanoparticles when incubated with bEnd.3 cells. Enhanced brain accumulation efficiency together with lower accumulation in liver and spleen was observed in the nude mice intravenously injected with Pep TGN conjugated nanoparticles compared with those injected with plain nanoparticles, showing powerful brain selectivity of Pep TGN. Coumarin 6 was used as a fluorescent probe for the evaluation of brain delivery properties. The brain Drug Targeting Index (DTI) of coumarin 6 incorporated in targeted nanoparticles was significantly higher than that of coumarin 6 incorporated in plain nanoparticles. In conclusion, the Pep TGN is a motif never been reported before and Pep TGN modified nanoparticles showed great potential in targeted drug delivery across the blood brain barrier.

1. Introduction

Brain diseases such as brain tumors, Alzheimer's disease and Parkinson's disease have caused heavy burden on the affected individual and the society in terms of disability, loss of productivity, premature mortality and health costs. Due to the blood-brain barrier (BBB) which performs as a formidable obstacle, 98% of small-molecule drugs and 100% of large-molecule drugs, including peptides, recombinant proteins, monoclonal antibodies, genes and short interfering RNAs cannot cross the blood-brain barrier.

BBB is formed by endothelial tight junctions and plays an effective way to protect the brain from harmful and toxic substances while hampering the systemic delivery of therapeutically important drugs from the blood into the brain [1,2]. The preferable characteristics of drugs to traverse the BBB are: <400 Da and lipophilic, non-substrate of the efflux system such as P-glycoprotein. However, very few drugs could meet these demands simultaneously to cross the BBB and reach their action targets within the brain parenchyma [3,4]. Proteins and gene drugs are restricted to enter the CNS from systemic circulation due to their hydrophilicities, protein bound properties and large molecular weights. Therefore, to reach the brain, most substances must cross the BBB through interaction with specific transporters and/or receptors expressed at the luminal (blood) side of the endothelial cells [5].

* Corresponding author. Department of Pharmaceutics, School of Pharmacy, Fudan University, 826 Zhangheng Rd., Shanghai 201203, People's Republic of China. Tel.: +86 21 51980067; fax: +86 21 51980069.
** Corresponding author. Department of Pharmaceutical Science, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109, USA. Tel.: +1 734 764 4273; fax: +1 734 763 9772.
*** Corresponding author. Tel.: +86 551 3600051; fax: +86 551 3601443.
E-mail addresses: xgjiang@shmu.edu.cn (X. Jiang), vcyang@umich.edu (V.C. Yang), lpwen@ustc.edu.cn (L. Wen).

0142-9612/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2011.03.031

To solve the problem of drug delivery across the BBB, quite a few CNS delivery strategies have been developed [6,7], among which the most promising approach is the receptor-mediated transport (RMT) [8]. By coupling drug-loaded vehicles with ligands which specifically recognize receptors on the BBB, the RMT strategy combines the advantages of brain targeting, high incorporation capacity, reduction of side effects, and circumvention of the multidrug efflux system [9]. Some receptors are highly expressed on the endothelial cells forming the BBB, such as the insulin receptor, transferrin receptor, low-density lipoprotein receptor (LDLR) and its related protein, and others [10]. There have been many reports about RMT strategy employing transferrin, lactoferrin, OX26 or angiopeps as the specific ligands to enable nanocarriers crossing the BBB [11–14]. Research is still on-going to identify new receptors and their respective ligands.

Therefore, identification of brain-specific markers is critical to the improved therapy of brain diseases. Finding such brain-specific targets may help drugs get into brain more specifically through targeted delivery, thus providing higher therapeutic efficiency while simultaneously decreasing systemic toxicity. To achieve this goal we chose a powerful tool called phage display technique for brain-specific ligand identification in our study. We chose a powerful tool called phage display technique for brain specific ligand identification in our study. Phage display is a well-developed technique used to obtain peptide sequences that interact with a particular molecule [15]. It has been used for the selection of peptides which can bind to defined proteins, cultured cells and even inorganic materials [16–18].

Since the in vivo phage display was first introduced by Pasqualini in 1996 [19], this technique has been expanded and has provided tissue-specific peptides as targeting moieties for tumors and organs. The three-amino-acid sequence Arg–Gly–Asp (RGD) is one of the most successful targeting ligands for tumor vascular endothelial cells screened by phage display technique [20]. The *in vivo* screening method was also conducted to search for tissue-homing peptides [19,21,22]. Wan XM applied a C7C phage display library intranasally to rats and recovered phage from the brain tissue and finally gained a peptide sequence (ACTTPHAWLCG) that can bypass the BBB through the nasal-to-brain passage [23]. Rooy et al. selected two 15 amino acid-peptides (GLA and GYR) that can bind to the murine brain in an *in situ* brain perfusion model [24].

The aim of our study is to identify peptides that could traverse the BBB from the system circulation and to construct a drug delivery system that can target the brain using the identified peptides. In our study, a random 12-mer peptide library displayed on the surface of filamentous phage M13 was screened for peptides that could lead nanocarriers to traverse BBB into the CNS. A longer circulation time was arranged according to the brain/blood ratios of phage particles. Poly (ethyleneglycol) - poly (lactide-co-glycolic acid) nanoparticles (PEG–PLGA NP) were prepared by the emulsion/evaporation method, and then the ligands screened from phage display were attached covalently to the surface of PEG–PLGA NP. The physicochemical characteristics of the nanoparticles were investigated. The brain-targeting efficiency of this system was evaluated *in vitro* and *in vivo* using coumarin 6 as a probe. The *in vitro* cytotoxicity of the nanoparticle system was investigated by CCK-8 assay.

2. Materials and methods

2.1. Materials and animals

Ph.D.-12™ phage display library Kit was purchased from New England Biolabs (Beverly, MA, USA). *Escherichia. coli* ER2738 (an F^+ strain) was used for M13 phage propagation and was cultured on Luria–Bertani agar or broth at 37 °C. Mouse anti-M13 monoclonal antibody was obtained from GE Healthcare(Piscataway, NJ, USA); Cy3 labeled goat anti-mouse IgG, 5-bromo-4-chloro-3-indolyl-beta-$_D$-galactopyranoside (Xgal), coumarin 6 and coumarin 7 were all purchased from Sigma–Aldrich (St. Louis, MO, USA); 4,6-diamidino-2-phenylindole (DAPI) was purchased from Molecular Probes (Eugene, OR, USA); Isopropyl-beta-$_D$-thiogalastoside (IPTG) was purchased from Merck (Germany); Optimal Cutting Temperature-compound 'Tissue-Tek' (O.C.T. compound) was from Miles Laboraories Inc. (USA). Methoxy-polyethyleneglycol Poly (lactic-co-glycolic acid) (MePEG3000-PLGA40000 (25:75)) copolymer, Maleimide-polyethyleneglycol Poly (lactic-co-glycolic acid) (Mal-PEG3400-PLGA40000 (25:75)) copolymer were prepared by University of Electronic Science and Technology of China. Plastic cell culture dishes, plates and flasks were obtained from Corning Incorporation (Lowell, MA); Dulbecco's Modified Eagle Medium (DMEM) (high glucose) cell culture medium and fetal bovine serum (FBS) were from Gibco (Carlsbad, CA); Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories (Japan). Deionized water (Millipore, Bedford, MA) was used through the entire study.

Adult male nude mice (16–20 g) and ICR mice (18–22 g) were obtained from the Sino-British Sippr/BK Lab. Animals were maintained at 22 ± 2 °C on a 12 h light-dark cycle with access to food and water ad libitum. The animal experiments were carried out in accordance with the protocols evaluated and approved by the ethical committee of Fudan University.

2.2. Screening of phage libraries in vivo

To obtain brain-homing phage-displayed peptides, the appropriate time to recover phages from brain is determined according to the brain/blood ratio. The brain/blood ratio was calculated by dividing the phage titer (in TU/g tissue, TU is transducing unit) in the brain at a given time by the phage titer in blood (in TU/ml) at that time. Mice were injected in the tail vein with 10^{11} plaque forming units (pfu) of Ph.D.-12™ Phage Display Library in 100 μL TBS. The phages were allowed to circulate in the mice for 15, 30 min and 1, 2, 4, 8, 12, 16, 20, 24, 30 and 36 h. The mice were then sacrificed by cervical dislocation. Blood was collected and the brain was withdrawn and weighed.

A total of 4 rounds of screening were performed with adult male ICR mice (18–22 g). For the first round, ICR mice (n = 3) were injected intravenously (i.v.) with 10^{11} pfu of Ph.D.-12™ Phage Display Library in 100 μL TBS. Phages were allowed to circulate for a period of time prior to recovery from brain. Then, the mice were perfused through the heart with 500 mL of sterile normal saline (containing 1% heparin). The brain tissue was withdrawn, weighed and homogenized under a bacteria-free environment. The homogenate was mixed together with rapidly growing *E. coli* (ER2738 host strain, New England Biolabs Inc.). Phages in the homogenate were amplified by *E. coli* ER2738 infection and subsequently pooled for the next round of biopanning (Txt S1). From round2 to round3, BSA was added in to TBS (round 2: 0.5% BSA; round3: 1% BSA; round4: without BSA). Other procedures were repeated as described in round 1.

20 bacteriophage clones from the last round were randomly picked up and were subjected to DNA sequencing (ABI3730). The peptide-encoding nucleotide sequences were determined with −96 g III primer (5′-^{HO}CCC TCA TAG TTA GCG TAA CG-3′) included in Ph.D.-12™ phage display library.

2.3. Immunohistochemistry

Clone 12-2 (displayed the Pep TGN) and native M13 phage were given to two groups of ICR mice (n = 3) via tail vein respectively (10^{11}pfu in 100 μL TBS). Mice were sacrificed 1 h later. Cerebrum samples were fixed in 4% paraformaldehyde, followed by dehydration in 15% sucrose for 24 h and 30% sucrose till deposition. Samples were frozen quickly in O.C.T. compound. Immunofluorescent detection of phages was performed by using anti-M13 monoclonal antibody and a secondary Cy3 conjugated anti-mouse IgG (Txt S1). Distribution of Clone 12-2 and native M13 phage in cerebrum was observed.

2.4. Peptide synthesis

All peptides were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd using standard solid-phase FMOC method and purified to >95% by high-performance liquid chromatography (HPLC). All peptides were verified using a mass spectrometer (lcms-2010a, Shimadzu, Japan).

2.5. Peptide competition

The competitive inhibitions of Clone 12-2 homing to brain were detected by the addition of synthetic Pep TGN. Clone 12-2 phages were mixed with increasing concentrations of synthetic Pep TGN respectively and then injected into mouse tail vein. The competitive inhibitory effect by added Pep TGN was quantified by evaluating phage titers.

2.6. Preparation of NP and TGN-NP

PEG–PLGA nanoparticles were prepared using the emulsion/solvent evaporation method. The ratio of MePEG-PLGA and Maleimide-PEG-PLGA is 9:1(weight). Coumarin-6-loaded nanoparticles were prepared with the same procedure except that 0.1% of coumarin 6 was added to the dichloromethane solution before