Journal of Controlled Release 192 (2014) 141–147

Contents lists available at ScienceDirect

Journal of Controlled Release

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

A high-affinity peptide for nicotinic acetylcholine receptor-α1 and its potential use in pulmonary drug delivery

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ARTICLE INFO

Article history:
Received 24 January 2014
Accepted 6 July 2014
Available online 12 July 2014

Keywords:
Aptides
Nicotinic acetylcholine receptors
Pulmonary delivery
Phage display

ABSTRACT

In pulmonary drug delivery, the ability of an affinity molecule to bind to lung epithelium may prolong retention of therapeutic molecules within the lung and consequently yield higher overall bioavailability. To this end, we screened a library of structurally constrained peptides ('aptides') using phage-display technology and identified a high-affinity aptide for the mouse nicotinic acetylcholine receptor-α1 (nAChR-α1). The isolated aptide ($APT_{nAChR-\alpha1}$) bound to its target protein with high affinity (K_d = 47 nM). Alexa 488-labeled $APT_{nAChR-\alpha1}$ showed preferential binding to nAChR-α1-positive mouse lung epithelial cells and mouse muscle cells. Furthermore, the aptide exhibited substantial binding in nAChR-α1-positive tissue sections of muscle, trachea and lung, but not in liver, kidney or spleen tissues, which are nAChR-α1-negative. In an *in vivo* experiment, a high-intensity fluorescence signal was observed in the entire lung up to 50 h after tracheal injection of Cy5.5-$APT_{nAChR-\alpha1}$, whereas most of the fluorescence signal from a Cy5.5-labeled scrambled peptide washed out within 20 h after injection. Taken together, these results indicate that the high-affinity peptide for nAChR-α1 identified here bound tightly to lung epithelium and thus exhibited a long residence time in this tissue, suggesting that the peptide could be used for pulmonary delivery of active pharmaceuticals.

1. Introduction

Pulmonary delivery has been widely used for numerous applications, including delivery of a variety of drugs ranging from small molecules to proteins and genes, as well as for vaccination purposes, among others [1–6]. Lung-targeted drug delivery via the respiratory tract has been considered an attractive solution for local (lung) as well as systemic therapy for a number of reasons. First, compared with other non-injection routes (i.e., oral, buccal, transdermal, and nasal), the lung offers the most ready access to the bloodstream for large-sized molecules; even IgG (>150 kDa) can be absorbed into the bloodstream through the pulmonary route [7,8]. Second, it is easy to achieve high bioavailability because metabolic enzymes, which are abundant in the liver and gastrointestinal tract encountered in oral delivery, are scarce in the luminal space of the lung [9,10]; thus, lung-delivered drugs or biomolecules remain intact longer in lung than in other tissues. Third, rapid entry into the systemic circulation is facilitated by the large surface area for absorption in the lung (~70–100 m^2 for human alveoli) and a very thin barrier between the pulmonary lumen and blood capillaries (~0.1–0.2 μm in humans) [11–13]. Fourth, devices and delivery protocols for inhalation or tracheal instillation are already well established [14]. Despite such advantages, however, there are several obstacles encountered in pulmonary drug delivery. A major hurdle is natural cleansing mechanisms of the lung, including coughing, swallowing, mucociliary clearance, and phagocytosis by alveolar macrophages [15,16]. In fact, a considerable fraction of delivered molecules is removed by these mechanisms. Thus, a method for overcoming such rapid clearance and achieving maximal drug absorption is much in demand. Absorption time in the lung is mainly affected by molecular weight (M_w) and solubility; for example, high-M_w molecules, such as proteins and lipophilic molecules, require much longer residence time in the lung for effective absorption than in low-M_w and hydrophilic molecules. In fact, many chemical drugs have been efficiently delivered to the lung using inhalation devices, whereas only a few therapeutic proteins or peptides, including insulin [4,17], human growth hormone [18], GM-CSF (granulocyte/monocyte-colony stimulating factor) [19], interferon (IFN)-γ [20] and erythropoietin [21], have shown potential for pulmonary delivery. A plausible approach for the pulmonary delivery of high-M_w molecules would be the use of a ligand capable of tightly binding lung epithelia via specific interactions, thereby prolonging residence time and enhancing absorption. Nicotinic acetylcholine receptors (nAChRs) are present in lung epithelia. Among nAChRs, nAChR-α1, a subunit of nAChRs, is reported to be present in the bronchial epithelium of lung [22]. Therefore, an affinity molecule capable of tightly binding to nAChR-α1 and conjugating to drugs of interest could be utilized for successful pulmonary delivery of high-M_w drugs.

Peptides represent a class of affinity molecules that are considered appropriate candidate targeting ligands because they are usually

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http://dx.doi.org/10.1016/j.jconrel.2014.07.006
0168-3659/

biocompatible with low toxicity, can be easily synthesized, and can be chemically conjugated with drugs in a site-specific manner. We recently reported the development of a platform technology enabling screening and identification of high-affinity peptides, termed 'aptides' [23]. Phage display using an aptide library has allowed us to obtain high-affinity peptides for various biological targets. Here we report the identification of an aptide specific for nAChR-α1 ($APT_{nAChR\text{-}\alpha1}$) in phage-display screens. We further investigated its potential for use in pulmonary drug delivery, testing the hypothesis that a nAChR-α1-specific peptide would tightly bind the receptor in the lung epithelium, resulting in prolonged residence time and enhanced absorption.

2. Materials and methods

2.1. Materials

Peptides were custom-synthesized by AnyGen Inc. (Gwangju, South Korea). Alexa Fluor 488-C5 maleimide and cell culture media, including RPMI-1640, high-glucose Dulbecco's modified Eagle medium (DMEM) and Opti-MEM reduced serum medium, were purchased from Gibco-Life Technologies (Grand Island, NY). Cy5.5-maleimide was purchased from Lumiprobe Co. (Hallandale Beach, FL). CrljOri:CD1(ICR) mice were purchased from Orient-Bio Inc. (Seoul, South Korea). Rompun (Bayer Co., Germany) and Zoletil-50 (Virbac, France) were used as anesthetics. Rabbit anti-mouse nAChR-α1 IgG, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, and HRP-conjugated anti-M13 monoclonal IgG were purchased from Abfrontier Inc. (Seoul, South Korea), Santa Cruz Biotechnology Inc. (Dallas, TX), and GE Healthcare (Piscataway, NJ), respectively.

2.2. Cell culture

C2C12 (mouse myoblast), MLE12 (mouse lung epithelium), and HEK293 (human embryonic kidney) cell lines were grown in DMEM (11995; Gibco-Life Sciences). CHO-K1 (Chinese hamster ovary) and Neuro-2a (mouse neural crest-derived) cell lines were grown in RPMI-1640 medium. All media were supplemented with 10% (v/v) fetal bovine serum, 100 IU/mL penicillin and 100 IU/mL streptomycin. Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. Biopanning with an aptide phage library

A peptide fragment of nAChR-α1 corresponding to Ser193-His224 (SGEWVIKEARGWKHWVFYSCCPTTPYLDITYH), designated $P_{nAChR\text{-}\alpha1}$, was chosen as a target for aptide screening. An N-terminal biotin-labeled target peptide (biotin-$P_{nAChR\text{-}\alpha1}$) was immobilized on a streptavidin-coated 96-well plate for 15 min. Unbound biotin-$P_{nAChR\text{-}\alpha1}$ was removed by washing with phosphate-buffered saline (PBS), and wells were blocked by incubating with PBS containing 2% bovine serum albumin (BSA) for 2 h at room temperature. BSA blocking was also performed on plates containing wells coated with streptavidin only, which were used for counter-selection. After washing with PBS to remove BSA, plates were loaded with a freshly prepared aptide phage library (M13 virus; 10^{11} plaque-forming units [p.f.u.]) by incubating for 2 h at 25 °C. Unbound phages were removed by washing with PBST (PBS, 0.1% Tween-20); plates were washed three times after the 1st biopanning round, and 6, 9, 12 and 20 times after the 2nd, 3rd, 4th and 5th biopanning rounds, respectively. Bound phages were eluted by adding 50 μL of 0.2 M glycine-HCl (pH 2.2) to each well and incubating for 20 min, after which the eluent was immediately neutralized by adding 1/5 elution volume of 2 M Tris–HCl (pH 9.2). Phages with an affinity for streptavidin or BSA were removed by loading neutralized phage eluent onto streptavidin/BSA-coated wells. The non-bound phages collected in this final step were subjected to the following amplification process. Phages were mixed with 1 mL of log-phase *Escherichia coli* ER2738 strain (New England Biolabs Ltd., Hitchin, UK) and cultured in a shaking incubator for 1 h at 37 °C, followed by treatment with EX12 helper phages (*E. coli*:helper phage ratio, 1:20) for an additional 1 h. Superinfected *E. coli* was inoculated into 40 mL fresh LB liquid medium containing ampicillin (50 μg/mL) and kanamycin (25 μg/mL), and then cultured in a shaking incubator at 37 °C overnight. Amplified phages were prepared from the supernatant of culture medium using a polyethylene glycol (PEG6000) precipitation method. Purified phages were re-suspended in PBS/2% BSA and used for the next round of biopanning. The output/input ratio of phage p.f.u. was monitored, with a sharp increase in the ratio indicating successful progression of biopanning.

2.4. Phage ELISAs for the selection of high-specificity aptide 'hits'

Phages with high affinity for $P_{nAChR\text{-}\alpha1}$ were identified by conducting phage ELISAs (enzyme-linked immunosorbent assays) using rescued phage clones. *E. coli* ER2738 infected with eluted phages from the 5th biopanning round was spread on a solid LB-ampicillin plate and incubated at 37 °C overnight. Forty *E. coli* colonies were selected randomly and inoculated into 1.5 mL centrifuge tubes containing 1 mL LB-ampicillin liquid media and EX12 helper phage. Superinfected *E. coli* was cultured in a shaking incubator at 37 °C for 48 h. After pelleting cells by centrifugation, the supernatant containing each rescued phage clone was used for ELISAs. The entire ELISA procedure was as follows. First, 10 μg of streptavidin, BSA, and streptavidin-$P_{nAChR\text{-}\alpha1}$ in PBS were loaded into wells of a 96-well plate and incubated for 2 h. For the preparation of streptavidin-$P_{nAChR\text{-}\alpha1}$-coated wells, 10 μg of streptavidin in PBS was added initially and incubated for 2 h; then, wells were briefly washed and a 5-μM $P_{nAChR\text{-}\alpha1}$ solution was loaded. After washing with PBS to remove unbound proteins, wells were blocked by incubating for 2 h at 25 °C with 2% skim milk in PBS. Blocking solution was then removed by washing three times with PBS, after which a phage/skim milk solution, prepared by mixing 500 μL of phage-containing supernatant with 500 μL 2% skim milk/PBS and rotating at 4 °C for at least 15 min, was loaded into the test-protein-coated 96-well plate and incubated at 25 °C for 2 h. The plate was then washed three times with 0.1% PBST and incubated for 2 h with HRP-conjugated anti-M13 antibody in 2% skim milk/PBS. After washing each well four times with 0.1% PBST, TMB substrate (BD Biosciences, Franklin Lakes, NJ) was added and color intensity was measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). Fold-change in color intensity was calculated (streptavidin-$P_{nAChR\text{-}\alpha1}$ relative to the control proteins, streptavidin and BSA), and phage clones showing more than a 2.5-fold change were selected and the corresponding phagemid DNA was sequenced. Selected phages were further examined for specificity with ELISAs using the procedures described above for phage ELISA experiments. Selected phage clones were first prepared from phagemid-containing *E. coli* and used for ELISAs. Six proteins–streptavidin-$P_{nAChR\text{-}\alpha1}$, streptavidin, BSA, MBP, myoglobin, thrombin and visfatin–were used as test proteins in ELISAs (in triplicate). Ultimately, the phage clone showing the best specificity was selected for further study.

2.5. Estimation of affinity of $APT_{nAChR\text{-}\alpha1}$

The sequence of the selected candidate aptide, $APT_{nAChR\text{-}\alpha1}$, was deduced to be *N′*-EASFWLGSWTWENGKWTWKGKGTLNR-*C′* and was subsequently custom-synthesized by AnyGen Inc. (Gwangju, South Korea). A BIAcore X instrument (GE Healthcare) was used for affinity measurements, as described by the manufacturer. Briefly, 100 μL of 1 mM biotinylated $P_{nAChR\text{-}\alpha1}$ was flowed into the first channel of Sensor Chip SA (GE Healthcare) for 15 min. Streptavidin bound in the second channel was used as control. Thereafter, 100, 200 and 300 nM $APT_{nAChR\text{-}\alpha1}$, respectively, were applied to the sensor chip. Surface plasmon resonance (SPR) sensorgrams were generated and kinetic parameters, including affinity (K_d) of the interaction, were calculated using BIA evaluation 3.1 software.