A d-Peptide Ligand of Nicotine Acetylcholine Receptors for Brain-Targeted Drug Delivery**

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Abstract: Lysosomes of brain capillary endothelial cells are implicated in nicotine acetylcholine receptor (nAChR)-mediated transcytosis and act as an enzymatic barrier for the transport of peptide ligands to the brain. A d-peptide ligand of nAChRs (termed DCDX), which binds to nAChRs with an IC_{50} value of 84.5 nM, was developed by retro–inverso isomerization. DCDX displayed exceptional stability in lysosomal homogenate and serum, and demonstrated significantly higher transcytosis efficiency in an in vitro blood–brain barrier monolayer compared with the parent L-peptide. When modified on liposomal surface, DCDX facilitated significant brain-targeted delivery of liposomes. As a result, brain-targeted delivery of DCDX modified liposomes enhanced therapeutic targeted delivery of liposomes. This study illustrates the importance of ligand stability in nAChRs-mediated transcytosis, and paves the way for developing stable brain-targeted entities.

The blood–brain barrier (BBB), which mainly consists of the endothelial cells that line cerebral microvessels, prevents drugs and drug-delivery systems from reaching the site of central nervous system (CNS) diseases.[1] Receptor-mediated transcytosis (RMT) has been exploited as an efficient pathway to circumvent the BBB. Diverse receptors that expressed on brain capillary endothelial cells, including nicotine acetylcholine receptors (nAChRs),[2] transferrin receptors (TfR),[3] and low-density lipoprotein receptor-related protein-1 (LRP-1),[4] can recognize ligands in blood circulation and facilitate specific transport to the brain.

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nAChRs are ligand-gated ion channels mainly expressed at neuromuscular junction of the CNS. Extensive expression of nAChRs on brain capillary endothelial cells and susceptibility to the inhibition by peptide neurotoxins and neurotropic viral proteins endow them with the ability to mediate peptide-based transvascular delivery of various therapeutic agents to the brain.[5] 1CDX (FKESWRARGTRIERG), a 16-residue peptide developed by structure-guided design, demonstrates submicromolar binding affinity to nAChRs and enables micelle-based brain-targeted drug delivery.[6] To assess the trafficking route of 1CDX upon nAChR-mediated transcytosis, intracellular distributions of endocytosed 1CDX was investigated. Fluorescein-labeled 1CDX was incubated with an in vitro BBB monolayer and intracellular fluorescence was detected 1 and 2 h after treatment. 1CDX displayed punctuate intracellular distribution (Supporting Information, Figure S1), and the most of punctuate compartments colocalized with lysosome, suggesting the involvement of lysosome in nAChRs-mediated transcytosis. 1CDX demonstrated high susceptibility to proteolytic degradation. The bulk of 1CDX disappeared after 15 min incubation with lysosomal homogenate (Supporting Information, Figure S2), indicating that at least a part of endocytosed 1CDX underwent intracellular degradation and the exocytosis was undermined.

We hypothesize that proteolytically stable ligands of nAChRs may possess higher brain targeting efficiency by overcoming the enzymatic barriers in brain capillary endothelial cells. DCDX, the retro–inverso isomer of 1CDX, was chemically synthesized and its randomly coiled conformation was validated with circular dichroism spectrometry (Supporting Information, Figure S3). To investigate whether or not DCDX is capable of interacting with neuronal nAChRs, we conducted a competition binding assay where different concentrations of peptide competed for receptors binding with radiolabeled 125I-α-bungarotoxin (125I-α-Bgt), a natural antagonist of neuronal nAChRs. Both 1CDX and 2CDX functioned as competitive antagonists in a dose-dependent manner (Supporting Information, Figure S4). 2CDX registered an IC_{50} value of 84.5 nM, which was approximately five times lower than that of 1CDX (441.6 nM). To better understand the binding of 2CDX to α7 neuronal nAChR, molecular modeling and docking were conducted on α7 nAChR with both 1CDX and 2CDX. The binding of both peptides to receptors was mainly dominated by electrostatic, cation–π, and hydrophobic interactions. 2CDX underwent great conformational changes to enable the side chains to occupy the binding pocket of α7 nAChR (Supporting Information, Figure S6).
Even though Arg9 in ⁴CDX and its counterpart DArg8 in ⁵CDX extended into the bottom of interfacial cleft and established a conserved cation-π interaction with Trp148⁶,⁷ in subunit A, the two peptides displayed different binding modes (Figure 1; Supporting Information, S7–S9). Specifically, ⁴Glu10 in ⁴CDX inserted deeply into the binding pocket and formed hydrogen bond with NH group in the backbone of Ser147 and van der Waals interactions with the residues Pro193, Tyr194, Ser147, and Trp148 in subunit A, while the corresponding residue Glu7 in ⁵CDX located outside the pocket and formed a hydrogen bond with Arg185 in subunit A. The side chain of ⁴Trp12 in ⁴CDX stretched deeply into the pocket and formed a hydrophobic interaction with Trp54, Leu36, and Leu37 in subunit B, while the counterpart Trp5 in ⁵CDX interacted with Tyr167 in subunit B but did not bind into this hydrophobic pocket. The flexible character of ⁴CDX made DArg5 form a hydrogen bond with Gln116 in subunit B, while the corresponding residue Arg12 in ⁵CDX formed an intramolecular hydrogen bond with Glu141 in ⁵CDX and a hydrogen bond with Glu188 in subunit A. The residue ⁴Ile4 in ⁴CDX formed van der Waals interaction with the residue Met159 in subunit B, which was not found in the interactions of ⁵CDX with θ7 nAChR.

To investigate the efficiency of nAChRs-mediated transcytosis, in vitro BBB monolayer was incubated with 50 μM fluorescein-labeled ⁴CDX and ⁵CDX at 37°C. Solutions collected from the lower compartment at different time points were analyzed by reverse-phase HPLC coupled with a fluorescence detector. As shown in Figure 2A, the percentage of ⁴CDX across the BBB displayed a linear increase in a time-dependent fashion. After 2 h, 7.9% ⁴CDX traversed the in vitro BBB monolayer. However, ⁵CDX displayed significantly lower efficiency at all tested time points in comparison to ⁴CDX. When incubated at 4°C, both peptides displayed minimum transcytosis efficiency over the BBB (<1.0%), which suggests energy consumption and possible participation of receptor mediation during transcytosis.⁹ Considering that both ⁴CDX and ⁵CDX displayed high binding affinities to nAChRs, we studied potential participation of nAChRs during transcytosis. The BBB monolayer was pre-incubated with 150 μM α-Bgt for one hour to block nAChRs at the luminal side, followed by incubation with fluorescein labeled ⁴CDX and ⁵CDX. It was found that transcytosis of ⁴CDX and ⁵CDX was dramatically undermined with α-Bgt pretreatment, confirming that the transcytosis of ⁴CDX and ⁵CDX was mediated by nAChRs.

Liposome-based vehicle is a class of versatile nanocarriers for targeting delivery of various therapeutic agents.⁶ Herein, we conjugated ⁴CDX and ⁵CDX on the surface of PEGylated liposomes to investigate the efficiency of nAChRs-mediated drug delivery to the brain. Rhodamine B-labeled liposomes were placed at the luminal side of in vitro BBB monolayer and fluorescence intensities at the abluminal side after different incubation periods were detected by fluorescence spectrophotometer. Liposomes were fluorescently labeled by inserting rhodamine B-conjugated 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine (rhodamine B-DPPE), excluding the possibility that free dye was released from liposomes and crossed the in vitro BBB monolayer. As shown in Figure 2B, ⁴CDX and ⁵CDX modification boosted liposome transcytosis. Among those, ⁴CDX modification assumed the highest transcytosis efficiency of liposomes, which was consistent with the results of peptide transcytosis efficiency.

The intracellular distribution of the endocytosed ⁴CDX in the in vitro BBB monolayer demonstrated the similar pathway to that of ⁴CDX (Figure 3). Most of the peptide colocalized with lysosome. We also labeled early and late endosomes by using anti-EEA1 and anti-M6PR antibodies, respectively. Colocalization of CDX peptides with late endosome was evident and increased upon chase from 2 h to 12 h (Supporting Information, Figure S10). Both ⁴CDX- and ⁵CDX-liposomes exhibited obvious colocalization with lysosome of brain capillary endothelial cells (Figure S11), while plain liposomes displayed very low endocytosis efficiency (data not shown here). nAChRs-mediated endocytosis was previously revealed in C2C12 myocytes,⁹ Binding of α-Bgt
mediated transcytosis is unknown to date. Both DCX and dynamin. However, the intracellular pathway of nAChRs-compartment, while in the absence of clathrin, caveolin, or dynamin, the participation of lysosomal compartments may at least partially explain the significantly higher transcytosis efficiency of DCX displayed in the BBB model than that of LCDX. In previous reports, lower-affinity antibody of TfR at therapeutic concentration (saturating concentration) demonstrated higher brain uptake capacity owing to the quick dissociation of antibody after receptor-mediated transcytosis than did higher-affinity antibody. Herein, a high concentration of fluorescein-labeled peptides (50 μM) was incubated with the in vitro BBB monolayer to meet the detection limitation of analytic HPLC. The saturating concentration of peptides may lead to ligands binding to nAChRs on the luminal side of BBB regardless of affinities.

To study in vivo brain targeting efficiency, we compared brain distributions of 1CDX and 1CDX-modified liposomes with plain liposomes. All of the liposomes were fluorescently labeled with rhodamine B-DPPE. Each labeled liposomal formulation was injected via a tail vein, and organs were harvested and subject to ex vivo fluorescent imaging 8 h after injection (Figure 4). It was evident that DCX modification on the liposomal surface induced the highest brain distribution, validating high brain-targeting efficiency of DCX in vivo. 1CDX modification gave rise to higher fluorescence intensity in the brain in comparison to plain liposomes. The brains were sectioned and slices were examined with confocal laser scanning microscope for closer observation of in vivo liposome distribution (Supporting Information, Figure S12).

In vivo biodistribution of rhodamine B-labeled 1CDX-liposomes, 1CDX-liposomes, and plain liposomes. A) Ex vivo imaging of dissected tissues including brains and other organs of mice 8 h after injection. Organs from left to right are from mice administrated with plain liposomes, 1CDX-liposomes, and 1CDX-liposomes. B) Normalized fluorescence intensity of brain in each group. Mean ± SD, n = 3.

To evaluate the potential therapeutic value of 1CDX-decorated liposomes for CNS diseases, we studied the therapeutic efficacy of liposomal formulations encapsulating doxorubicin (DOX) in a xenograft nude mouse model of human glioblastoma multiforme (GBM). Five groups of nude mice (n = 10) bearing intracranial U87 cells were intravenously injected with saline, free DOX, DOX-loaded plain liposomes, DOX-loaded 1CDX-liposomes, and DOX-loaded 1CDX-liposomes. As shown in Figure 5, in the absence of CDX peptides, treatments with free or liposome-formulated doxorubicin at a dose of 2 mg per kg body weight (at 6, 9, 12, and 15 days post-tumor implantation) did little in improving mouse survival, registering a median survival of 26.5 days (p = 0.105) and 27 days (p = 0.092) versus 24 days for the saline-treated group. Both 1CDX (33.5 days, p < 0.005) and 1CDX
(28 days, \( p < 0.05 \)) decorating significantly prolonged the average survival time of nude mice. In comparison to plain liposomes containing DOX, \( ^{125}\text{CDX} \) modification significantly lengthened the survival of model mice (\( p < 0.005 \)). \( ^{125}\text{CDX} \) was superior to \( ^{125}\text{CDX} \) by extending additional 5.5 days (140% of the prolongation resulted from the treatment of \( ^{125}\text{CDX} \)-modified liposomes encapsulating DOX) of median survival time of intracranial GBM-bearing nude mice, in which BBB is the main obstacle to efficient drug delivery.[16]

Along with enzymatic barriers in brain capillary endothelial cells, blood plasma is another barrier to inactivate \( ^{125}\text{CDX} \) and undermine the interaction with nAChRs on the BBB. It was evident that \( ^{125}\text{CDX} \) demonstrated fast degradation and most intact peptide disappeared after 4 h incubation with 50% fresh rat serum. In conspicuous contrast, \( ^{125}\text{CDX} \) displayed nearly no degradation under the same condition (Figure 6). Preincubation with rat serum also undermined the cellular uptake of \( ^{125}\text{CDX} \)-liposomes by brain capillary endothelial cells. Fluorescein loaded liposomes were incubated with cells for 4 h and intracellular fluorescence was tracked using confocal laser scanning microscope and flow cytometry. Both \( ^{125}\text{CDX} \) and \( ^{125}\text{CDX} \) modified liposomes were substantially taken up. After incubation with 50% rat serum, cellular uptake of preincubated \( ^{125}\text{CDX} \)-modified liposomes exhibited no perceptible difference with that of non-treated \( ^{125}\text{CDX} \)-liposomes, whereas preincubation with rat serum significantly impaired the cellular uptake of \( ^{125}\text{CDX} \)-modified liposomes, indicating that \( ^{125}\text{CDX} \) peptide ligand modified liposomes are able to maintain fully targeting ability in blood circulation.

In the present study, a \( ^{125}\text{CDX} \) peptide ligand of nAChRs was experimentally and computationally validated, and the brain targeting efficacy was evaluated in vitro and in vivo. When modified on the surface of PEGylated liposomes, \( ^{125}\text{CDX} \) efficiently inspired brain-targeted delivery of the encapsulated payloads. In vivo studies verified the potential value of \( ^{125}\text{CDX} \) in improving therapeutic efficacy of existing anticancer drugs such as doxorubicin in the treatment of glioblastoma. As the promising of \( ^{125}\text{CDX} \) peptide shown in brain-targeted drug delivery, the present study paved the way for designing proteolytically stable ligands of nAChRs for the treatment of CNS diseases.

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![Figure 6. Stability of CDX peptides in 50% rat serum at 37 °C and targeting ability of CDX peptides modified liposomes with brain capillary endothelial cells. A) Degradation of CDX peptide in 50% rat serum determined with HPLC. B) Brain capillary endothelial cells uptake of CDX peptides modified liposomes with and without preincubation with rat serum. C) Quantitative cellular uptake by using flow cytometer. Scale bar = 10 μm; *** \( p < 0.005 \), ** \( p < 0.001 \).](image-url)
