

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

Xiaoli Wei, Changyou Zhan,* Qing Shen, Wei Fu, Cao Xie, Jie Gao, Chunmei Peng, Ping Zheng, and Weiyue Lu*

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 IC₅₀ 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 efficiency of encapsulated doxorubicin for glioblastoma. This study illustrates the importance of ligand stability in nAChR-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.

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] ^LCDX (FKESWREARGTRIERG), 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 ^LCDX upon nAChR-mediated transcytosis, intracellular distributions of endocytosed ^LCDX was investigated. Fluorescein-labeled ^LCDX was incubated with an in vitro BBB monolayer and intracellular fluorescence was detected 1 and 2 h after treatment. ^LCDX 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. ^LCDX demonstrated high susceptibility to proteolytic degradation. The bulk of ^LCDX disappeared after 15 min incubation with lysosomal homogenate (Supporting Information, Figure S2), indicating that at least a part of endocytosed ^LCDX 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 ^LCDX, 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 ^DCDX and ^LCDX functioned as competitive antagonists in a dose-dependent manner (Supporting Information, Figure S4). ^DCDX registered an IC₅₀ value of 84.5 nM, which was approximately five times lower than that of ^LCDX (441.6 nM). To better understand the binding of ^DCDX to $\alpha 7$ neuronal nAChR, molecular modeling and docking were conducted on $\alpha 7$ nAChR with both ^LCDX and ^DCDX. The binding of both peptides to receptors was mainly dominated by electrostatic, cation- π , and hydrophobic interactions. ^DCDX underwent great conformational changes to enable the side chains to occupy the binding pocket of $\alpha 7$ nAChR (Supporting Information, Figure S6).

[*] X. Wei, Dr. C. Zhan, Q. Shen, Prof. W. Fu, C. Xie, J. Gao, C. Peng, Prof. W. Lu
Department of Pharmaceutics, School of Pharmacy
Fudan University & Key Laboratory of Smart Drug Delivery
(Fudan University), Ministry of Education
Shanghai, 201203 (P.R. China)
E-mail: zcy439@hotmail.com
wylu@shmu.edu.cn

X. Wei, Prof. P. Zheng, Prof. W. Lu
State Key Laboratory of Medical Neurobiology
Fudan University, Shanghai, 200032 (P.R. China)
Prof. W. Lu
State Key Laboratory of Molecular Engineering of Polymers
Fudan University, Shanghai, 200433 (P.R. China)

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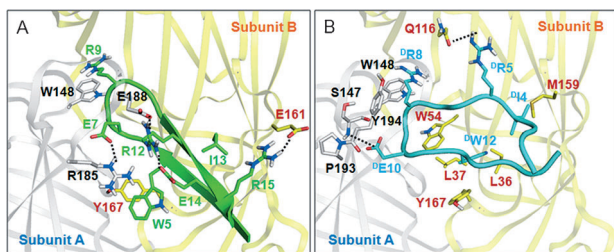


Figure 1. Binding mode of ¹CDX (A, green) and ²CDX (B, aqua) with $\alpha 7$ nAChR. The subunit A of the receptor is shown in white and subunit B in yellow. Residues involved in binding are represented with sticks, and hydrogen bonds are denoted by black dashed lines.

Even though Arg9 in ¹CDX and its counterpart ²Arg8 in ²CDX extended into the bottom of interfacial cleft and established a conserved cation– π interaction with Trp148^[6,7] in subunit A, the two peptides displayed different binding modes (Figure 1; Supporting Information, S7–S9). Specifically, ²Glu10 in ²CDX 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 ¹CDX located outside the pocket and formed a hydrogen bond with Arg185 in subunit A. The side chain of ²Trp12 in ²CDX stretched deeply into the pocket and formed a hydrophobic interaction with Trp54, Leu36, and Leu37 in subunit B, while the counterpart Trp5 in ¹CDX interacted with Tyr167 in subunit B but did not bind into this hydrophobic pocket. The flexible character of ²CDX made ²Arg5 form a hydrogen bond with Gln116 in subunit B, while the corresponding residue Arg12 in ¹CDX formed an intramolecular hydrogen bond with Glu14 in ¹CDX and a hydrogen bond with Glu188 in subunit A. The residue ²Ile4 in ²CDX formed van der Waals interaction with the residue Met159 in subunit B, which was not found in the interactions of ¹CDX with $\alpha 7$ nAChR. However, Arg15 in ¹CDX formed a hydrogen bond with Glu161 in subunit B, which was not found in the interaction of ²CDX with $\alpha 7$ nAChR. As a whole, ²CDX displayed more potent interaction than did ¹CDX. Importantly, the binding affinities of the two peptides calculated for $\alpha 7$ nAChR by X-score were in good agreement with experimentally determined data (Supporting Information, Table S1), thus validating the design of ²CDX as functional D-peptide antagonist of $\alpha 7$ nAChR.

To investigate the efficiency of nAChRs-mediated transcytosis, in vitro BBB monolayer was incubated with 50 μ M fluorescein-labeled ²CDX and ¹CDX 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 ²CDX across the BBB displayed a linear increase in a time-dependent fashion. After 2 h, 7.9% ²CDX traversed the in vitro BBB monolayer. However, ¹CDX displayed significantly lower efficiency at all tested time points in comparison to ²CDX. When incubated at 4°C, both peptides displayed minimum transcytosis efficiency over the BBB (<1.0%), which suggests energy consumption and possible participation

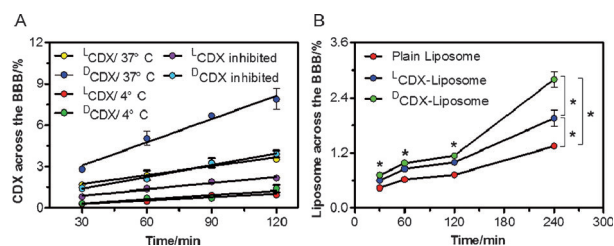


Figure 2. Transcytosis efficiency of ²CDX, ¹CDX, and peptide-modified liposomes in the in vitro BBB monolayer. A) Fluorescein-labeled ²CDX or ¹CDX (50 μ M) was incubated at the luminal side of primary rat brain capillary endothelial cells monolayer at 4°C and 37°C, or with pre-incubation with 150 μ M α -Bgt for one hour at 37°C. At different time points, intact fluorescein labeled peptide at the abluminal side was analyzed by RP-HPLC coupled with a fluorescence detector (mean $\pm$ SD, $n = 3$). B) Transcytosis efficiency of ²CDX-liposomes, ¹CDX-liposomes, and plain liposomes at 37°C. Mean $\pm$ SD, $n = 3$, * $p < 0.001$, ²CDX-liposome vs. plain liposome at 30, 60, and 120 min.

of receptor mediation during transcytosis.^[8] Considering that both ²CDX and ¹CDX displayed high binding affinities to nAChRs, we studied potential participation of nAChRs during transcytosis. The BBB monolayer was pre-incubated with 150 μ M α -Bgt for one hour to block nAChRs at the luminal side, followed by incubation with fluorescein labeled ²CDX and ¹CDX. It was found that transcytosis of ²CDX and ¹CDX was dramatically undermined with α -Bgt pretreatment, confirming that the transcytosis of ²CDX and ¹CDX was mediated by nAChRs.

Liposome-based vehicle is a class of versatile nanocarriers for targeting delivery of various therapeutic agents.^[9] Herein, we conjugated ²CDX and ¹CDX 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-glycero-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, ²CDX and ¹CDX modification boosted liposome transcytosis. Among those, ²CDX modification assumed the highest transcytosis efficiency of liposomes, which was consistent with the results of peptide transcytosis efficiency.

The intracellular distribution of the endocytosed ²CDX in the in vitro BBB monolayer demonstrated the similar pathway to that of ¹CDX (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 ²CDX- and ¹CDX-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.^[10] Binding of α -Bgt

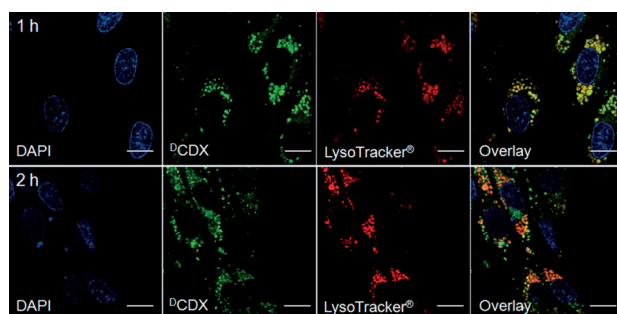


Figure 3. Colocalization of endocytosed D CDX and lysosome in the in vitro BBB monolayer. Fluorescein-labelled D CDX ($50\ \mu\text{M}$) was incubated with in vitro BBB monolayer at 37°C for 1 and 2 h, followed by lysosome staining. After fixation with formaldehyde, cells were subject to DAPI staining. Transwell membrane was detached and imaged by confocal laser scanning microscope. Scale bar = $10\ \mu\text{m}$.

induces the internalization of cell surface nAChRs to late endosomes. This internalization occurs by sequestration of nAChR- α -Bgt complex in narrow, tubular, surface-connected compartments, while in the absence of clathrin, caveolin, or dynamin. However, the intracellular pathway of nAChRs-mediated transcytosis is unknown to date. Both D CDX and L CDX entered the brain capillary endothelial cells in the in vitro BBB monolayer and at least partially distributed in lysosomal compartments.

Different pathways have been proposed to depict the intracellular pathway of receptor-mediated transcytosis.^[11] It is believed that lactoferrin^[12] and low-density lipoprotein^[13] bypass the lysosomal compartments by receptor mediation and are exocytosed without degradation; however, anti-TfR antibody (OX-26) was partially distributed in lysosome.^[14] By virtue of exceptional stability of D CDX in lysosome homogenates, the participation of lysosomal compartments may at least partially explain the significantly higher transcytosis efficiency of D CDX displayed in the BBB model than that of L CDX. Lysosomal compartments may be quickly saturated with D CDX owing to full resistance of the D-peptide to proteolysis, resulting in higher efficiency of exocytotic transport than that of L CDX. 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.^[15] Herein, a high concentration of fluorescein-labeled peptides ($50\ \mu\text{M}$) 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 D CDX and L CDX 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 D CDX modification on the liposomal surface induced the highest brain distribution, validating high brain-targeting efficiency of D CDX

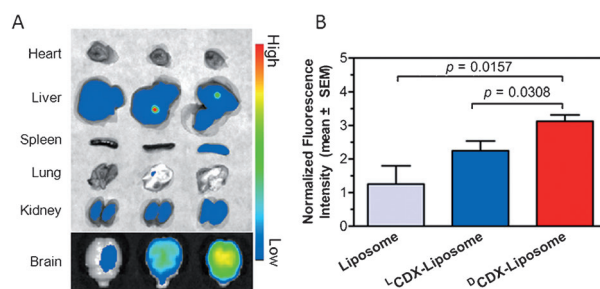


Figure 4. In vivo biodistribution of rhodamine B-labeled D CDX-liposomes, L CDX-liposomes, and plain liposomes. A) Ex vivo imaging of dissected tissues including brains and other organs of mice 8 h after injection. Organs from left to right are from mice administrated with plain liposomes, L CDX-liposomes, and D CDX-liposomes. B) Normalized fluorescence intensity of brain in each group. Mean $\pm$ SD, $n = 3$.

in vivo. L CDX 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). D CDX-liposomes displayed higher distributions in cortex, hippocampus, and ventricle in comparison to L CDX-liposomes and plain liposomes.

To evaluate the potential therapeutic value of D CDX-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 L CDX-liposomes, and DOX-loaded D CDX-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 D CDX (33.5 days, $p < 0.005$) and L CDX

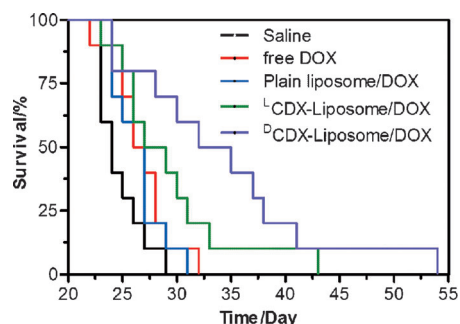


Figure 5. Kaplan–Meier survival curves of nude mice bearing intracranial U87 glioblastoma. Mice ($n = 10$) that received four doses (at 6, 9, 12, and 15 days after glioblastoma implantation) of D CDX (median survival time = 33.5 days) or L CDX-modified liposomes (median survival time = 28 days) encapsulating DOX survived significantly longer than the control groups that received saline (median survival time = 24 days).

(28 days, $p < 0.05$) decorating significantly prolonged the average survival time of nude mice. In comparison to plain liposomes containing DOX, D CDX modification significantly lengthened the survival of model mice ($p < 0.005$). D CDX was superior to L CDX by extending additional 5.5 days (140 % of the prolongation resulted from the treatment of L 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 L-peptide ligand and undermine the interaction with nAChRs on the BBB. It was evident that L CDX demonstrated fast degradation and most intact peptide disappeared after 4 h incubation with 50 % fresh rat serum. In conspicuous contrast, D CDX displayed nearly no degradation under the same condition (Figure 6). Preincubation with rat serum also

and in vivo. When modified on the surface of PEGylated liposomes, D CDX efficiently inspired brain-targeted delivery of the encapsulated payloads. In vivo studies verified the potential value of D CDX in improving therapeutic efficacy of existing anticancer drugs such as doxorubicin in the treatment of glioblastoma. As the promising of D-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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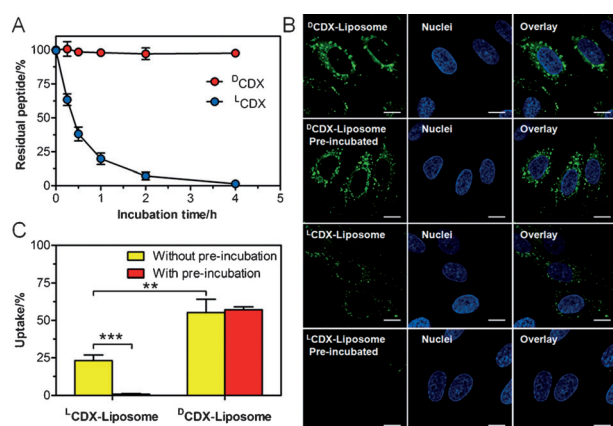


Figure 6. Stability of CDX peptides in 50% rat serum at 37°C and targeting ability of CDX peptides modified liposomes with brain capillary endothelial cells. A) Degradation of CDX peptide in 50% rat serum determined with HPLC. B) Brain capillary endothelial cells uptake of CDX peptides modified liposomes with and without pre-incubation with rat serum. C) Quantitative cellular uptake by using flow cytometer. Scale bar = 10 μ m; ** $p < 0.005$, *** $p < 0.001$.

undermined the cellular uptake of L 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 D CDX and L CDX modified liposomes were substantially taken up. After incubation with 50 % rat serum, cellular uptake of pre-incubated D CDX modified liposomes exhibited no perceptible difference with that of non-treated D CDX-liposomes, whereas pre-incubation with rat serum significantly impaired the cellular uptake of L CDX-modified liposomes, indicating that D-peptide ligand modified liposomes are able to maintain fully targeting ability in blood circulation.

In the present study, a D-peptide ligand of nAChRs was developed to overcome enzymatic barriers in the BBB as well as in circulating blood. The function of D CDX as an antagonist of nAChRs was experimentally and computationally validated, and the brain targeting efficacy was evaluated in vitro

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