Choline-Derivate-Modified Nanoparticles for Brain-Targeting Gene Delivery

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The major obstacle for drug delivery to the central nervous system (CNS) is the blood–brain barrier (BBB), which protects the CNS from potentially harmful xenobiotics and endogenous molecules to ensure an optimal environment for brain function.[1] Despite this natural barricade, small molecules and macromolecules including peptides and proteins could be transported into the CNS to maintain its normal physiological function via the endogenous BBB transporters. There are three families of endogenous BBB transporters: carrier-mediated transporters (CMT), active efflux transporters (AET), and receptor-mediated transporters (RMT). The CMT and AET systems are mainly responsible for the transport of small molecules, while the RMT systems are responsible for endogenous large molecules.[2,3]

Drugs or drug-delivery systems can be modified with the substrates of these transporters to realize their accumulation in the CNS. The RMT mechanism has been mainly investigated and utilized for brain-targeting drug delivery.[2] For example, the ligands of transferrin receptor and insulin receptor have been used to modify polymers for constructing brain-targeting drug-delivery systems to transport small molecules, proteins, and gene drugs into the CNS.[4,5] A number of specific transporters are expressed on the brain capillary endothelial cells (BCECs), which are responsible for the endogenous and exogenous nutrient supplies for the brain. They possess inspiring features including high transport capacity, selectivity, and an adequate transfer rate.[6] Thus, a native BBB nutrient transporter could be utilized as an alternative strategy to enable polar, water-soluble drugs to cross the BBB via a CMT mechanism. Several successful applications have been reported. For example, the BBB large neutral amino acid transport system, the glucose transporter (GluT1), and the BBB choline transporter (BBB ChT) could be used to deliver D,L-2-amino-7-bis[(2-chloroethyl)amino]-1,2,3,4-tetrahydro-2-naphthoic acid (D,L-NAM), ketoprofen, and *N*-n-octylnicotinium iodide (NONI), respectively.[7,8] Despite these successful applications, the CMT mechanism remains an untapped resource for delivery systems. In this work, CMT mechanism was challenged to facilitate the brain-targeting drug-delivery system across the BBB.

Choline is a biochemical precursor of the neurotransmitter acetylcholine and other essential components of cell membrane phospholipids such as phosphatidylcholine. The high choline concentration in the brain demonstrates the amazing ability of the BBB ChT to transport choline across the BBB.[9,10] Furthermore, the low physiologic plasma concentration of choline (approximately 25% of the Michaelis–Menten constant, K_m) makes the BBB ChT free to transport choline derivates without interrupting the supply of CNS choline.[11] Therefore, BBB ChT is considered a potential target for active drug delivery into the CNS.

A quaternary ammonium group and a free hydroxyl group were suggested as the key requirements for BBB ChT substrates.[8] As a native BBB ChT substrate, choline was not suitable for further modification. Recent studies revealed that bis-quaternary ammonium compounds could also be applied as BBB ChT substrates with even higher affinity.[12,13] Thus, bis-quaternary ammonium compounds were challenged to modify dendrimers for constructing the brain-targeting drug-delivery system without affecting their BBB ChT affinity.

First, a series of novel bis-quaternary ammonium compounds with high BBB ChT affinity were designed. There were two major factors affecting the BBB ChT affinity of these compounds: i) the lipophilicity of the quaternary ammonium moieties and ii) the length and conformation rigidity of the linkers between two quaternary ammonium moieties.[13] Given these factors, high lipophilic isoquinoline (**4a**) and relatively low lipophilic 3-methylpyridine (**4b**) were chosen as quaternary ammonium moieties. (3,5-bis(3-bromopropoxy)phenyl)methanol (**3a**, 11 carbons) and (3,5-bis(4-bromobutoxy)phenyl)methanol (**3b**, 13 carbons) were selected as linkers (**Scheme 1**). High conformation rigidity of the benzene ring could result in higher BBB ChT affinity and benzylic hydroxyl could also provide a free reactive site for conjugation to a brain-targeting drug-delivery system. Based on the above considerations, four bis-quaternary ammonium compounds (**5**,**6**,**7**,**8**) have been crossover designed, synthesized (as shown in Scheme 1), and characterized by mass spectrometry (MS), ^{1}H NMR, and ^{13}C NMR (see Supporting Information). The characterization showed successful synthesis of compounds **5**, **6**, **7**, and **8**.

To verify the design strategy, the BBB ChT affinityiesof the synthesized bis-quaternary ammonium compounds were compared by evaluation of their ability to inhibit the [^{3}H]-choline chloride uptake by BCECs (**Figure 1**a). The IC_{50} value (concentration

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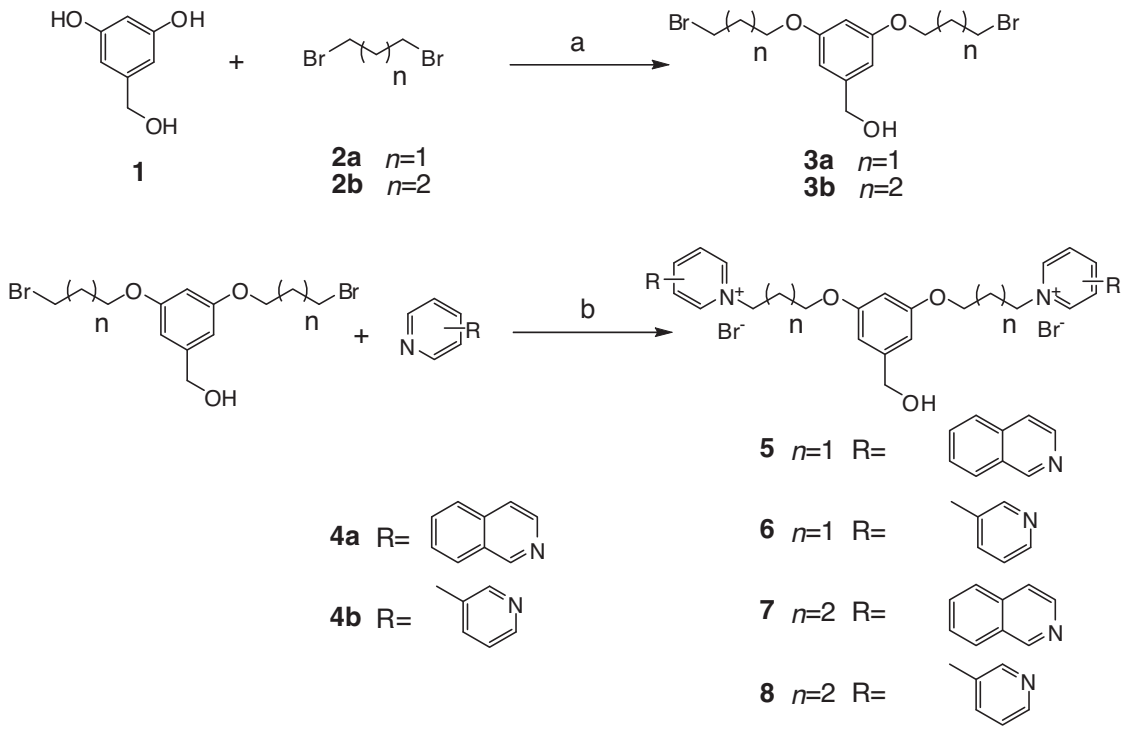

Scheme 1. Synthesis of bis-quaternary ammonium compounds **5-8**. Reagents and conditions: a) K_2CO_3, ACN, reflux 4 h and b) 65 °C, 1 h.

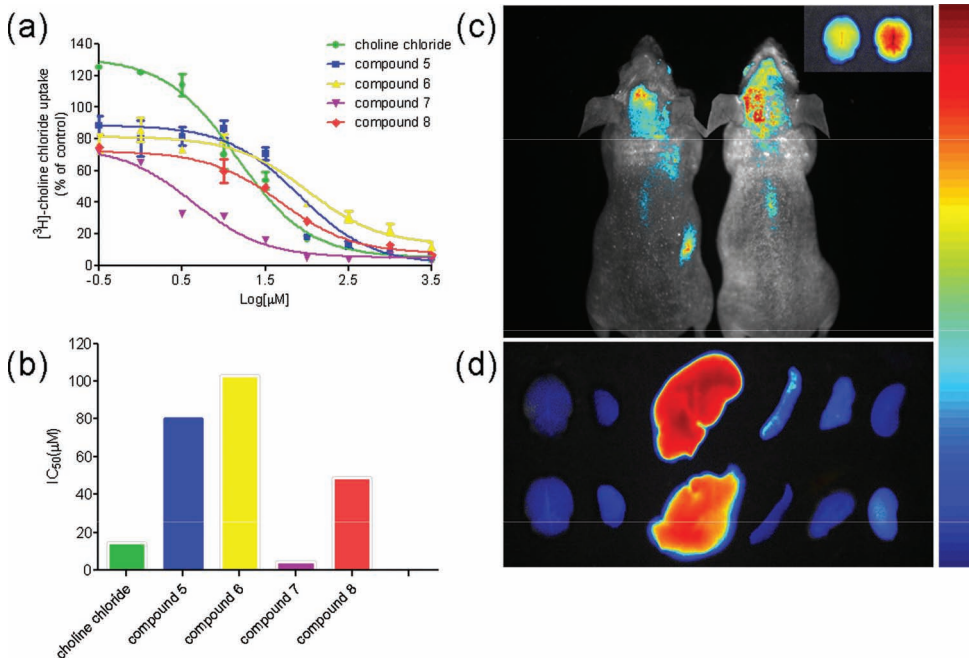

Figure 1. a) Dose–response relationship for the inhibition of [^{3}H]-choline chloride uptake by various compounds. Uptake of [^{3}H]-choline chloride (10 nM) into BCECs was measured with a 10-min incubation in the presence of NaCl at pH 7.4 either in the absence (control) or presence of assigned concentrations of various compounds. Data are expressed as mean ± standard deviation (S.D.) (number of samples, n = 4). b) Structure–activity relationship indicated by IC_{50} values of various compounds. c) In vivo imaging of mice administrated with 50 μM **10**-BODIPY (right) and free BODIPY of equal fluorescence (left). d) In vitro imaging of the main organs of mice administered with 50 μM **10**-BODIPY (low panel) and free BODIPY of equal fluorescence (upper panel). Images were taken 2 h after administration. Intensity of the signal: dark red is the strongest and dark blue is the weakest, as shown by the bar.

necessary for 50% inhibition) was calculated by One site-Fit logIC_{50} (an equation in GraphPad Prism 5). For compound **5**, **6**, **7**, and **8** and choline chloride, the IC_{50} values were 80.25 μM, 102.9 μM, 4.265 μM, 49.08 μM, and 14.50 μM, respectively. Comparison of compound **5** with **6** and **7** with **8** (Figure 1B) indicated that compounds with isoquinoline as quaternary ammonium moieties possessed higher affinity. The cationic binding site of BBB ChT may locate inside a hydrophobic pocket, thus higher lipophilicity of moieties could gain higher affinity. Comparison of compound **5** with **7** and **6** with **8** (Figure 1b) suggested that the optimal length of linker was 13 carbons. It may attribute to the potential binding sites of quaternary ammonium moieties with BBB ChT: i) one BBB ChT has several cationic binding sites and two quaternary ammonium cations bind to two of them and ii) one BBB ChT has only one cationic binding site and two quaternary ammonium cations bind to two nearby BBB ChTs. Independent of the binding arrangement, the results verified that a distance of 13 carbons was more suitable. Comparison of the BBB ChT affinity of the synthesized compounds revealed a simple structure–activity relationship. The bis-quaternary ammonium compound with isoquinoline as the quaternary ammonium moieties, which were connected by a high conformation rigidity linker with 13 carbons (compound **7**), had the highest BBB ChT affinity. Among the four synthesized compounds, compound **7** had the lowest IC_{50} value (4.265 μM), which was even lower than that of choline chloride (14.50 μM). This indicated that compound **7** could compete efficiently with endogenous choline in binding to BBB ChT in vivo. Thus compound **7**, as a novel BBB ChT substrate, was further conjugated to dendrimers for constructing the brain-targeting drug-delivery system.

In order to gain more efficient conjugation to dendrimers, the benzylic hydroxyl of compound **7** was converted to benzylic thiol. Compound **10** was the sulfhydrylation form of compound **7** (Scheme S1, Supporting Information) and its brain-targeting efficiency was investigated in vivo by labeling with a fluorescent agent, BODIPY (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, sulfosuccinimidyl ester, sodium salt). Compound **10**-BODIPY and free BODIPY of equal fluorescence were injected via the caudal vein of nude mice. As shown in Figure 1c, the fluorescence in the brain of the mice treated with compound **10**-BODIPY was significantly stronger than that of the mice treated with free BODIPY. In vitro imaging results showed that the accumulation of compound **10**-BODIPY in the brain and kidneys increased (Figure 1d). Compound **10**-BODIPY was more hydrophilic for the two cationic moieties and led to faster excretion, thus it had more accumulation in the kidneys. Traditionally it was thought that molecules with more lipophilicity could cross the BBB more easily. It was amazing that the decreased lipophilicity of compound **10**-BODIPY did not result in less, but instead more, accumulation in brain. This was due to the high BBB ChT affinity of compound **10**. Here the BBB ChT acted as a Trojan horse to increase the accumulation of compound **10**-BODIPY in brain. This indicated the possibility that BBB ChT could also mediate the brain accumulation of a drug-delivery system modified with compound **10**.

Next, compound **10** was conjugated to the drug-delivery system and the brain-targeting efficiency was evaluated in vitro and in vivo. Dendrigraft poly-L-lysines (DGLs, generation 3 with 123 amino groups per molecule) have been utilized for gene delivery to brain.[14,15] Compound **10** was conjugated to the surface of the DGL via a-malemidyl-u-*N*-hydroxysuccinimidyl polyethyleneglycol (NHS-PEG-MAL) and further constructed the gene delivery system. Details of the synthesis and characterization of DGL-PEG/pDNA (plasmid DNA) and DGL-PEG-**10**/pDNA nanoparticles (NPs) are shown in Figure S1 (Supporting Information).

To determine whether BBB ChT could mediate the brain accumulation of a gene-delivery system, the cellular uptake mechanism of NPs with YOYO-1-labeled (Invitrogen, Y3601) pDNA was investigated. The cellular uptake of DGL-PEG-**10**/pDNA (**Figure 2**b) was much more than that of DGL-PEG/pDNA (Figure 2a). Excess choline chloride could inhibit the uptake of DGL-PEG-**10**/pDNA (Figure 2c). The uptake of DGL-PEG-**10**/pDNA at 4 °C significantly decreased (Figure 2f). These indicated that the uptake showed energy-dependent and BBB-ChT-involving character. The uptake mechanism for the NPs is endocytosis. There are two main kinds of endocytosis on the BBB: i) receptor-mediated endocytosis (RME), in which a

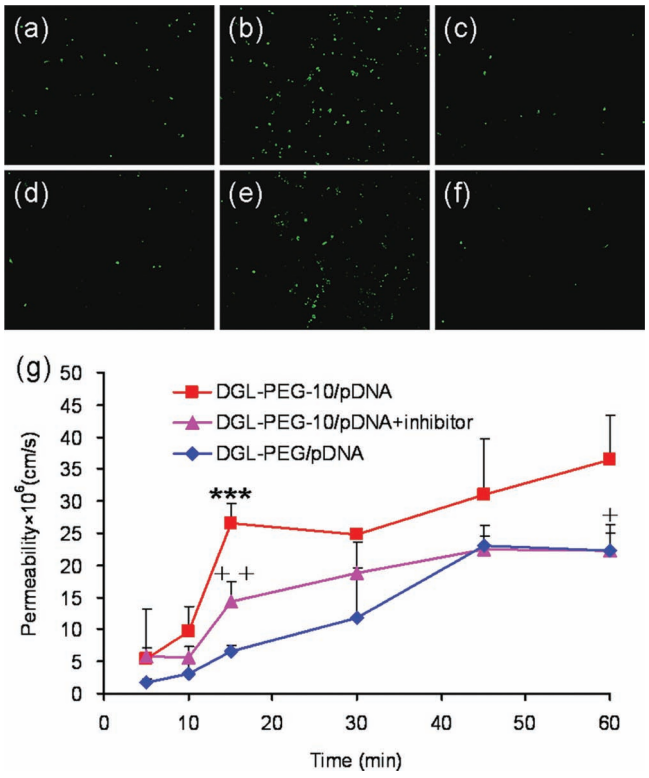

Figure 2. Cellular uptake of DGL-PEG/pDNA (a), DGL-PEG-**10**/pDNA (b) with different inhibitors (c–e) were examined by fluorescent microscopy after a 30-min incubation at 37 °C. BCECs were treated with different inhibitors including choline chloride (c), filipin complex (d), phenylarsine (e). The cellular uptake of DGL-PEG-**10**/pDNA was also carried out at 4 °C (f). Green: YOYO-1 labeled pDNA. Original magnification: ×100. g) Results of transport studies of NPs across the BCECs monolayer. Data is shown as P_{app} at assigned time points. Significance: $+p < 0.05$; $++p < 0.01$, represents DGL-PEG-**10**/pDNA vs. DGL-PEG-**10**/pDNA+inhibitor, while $***p < 0.001$, represents DGL-PEG-**10**/pDNA vs. DGL-PEG/pDNA. Results are expressed as mean ± S.D. ($n = 4$).

ligand-modified system binds to its cognate receptor specifically to elicit cellular internalization and can be inhibited by phenylarsine oxide and ii) adsorptive endocytosis (AE), in which the drug-delivery system will bind to the cellular surface through non-specific mechanisms, such as electrostatic interactions, and can be blocked by a filipin complex.[1] It was reported that the uptake of Angiopep (a high-affinity ligand of low-density lipoprotein-receptor-related protein) modified NPs could significantly be inhibited by phenylarsine oxide.[16] The modification of Angiopep gave the NPs RME character. The uptake of NPs decreased with addition of the filipin complex (Figure 2d) but not phenylarsine oxide (Figure 2e), thus showing that the main pathway of endocytosis of was AE not RME. The surface amino groups were slightly cationic and this contributed to the AE pathway. However, unlike ordinary AE, the modification of compound **10** increased the capture possibility of NPs by the cell membrane. Subsequently, the transport efficiency across the BCECs monolayer was evaluated. As shown in Figure 2g, the apparent permeability (P_{app}) of DGL-PEG-**10**/pDNA was much higher than that of DGL-PEG/pDNA, especially in the first 15 min (DGL-PEG-**10**/pDNA, 26.47 cm s^{-1}; DGL-PEG/pDNA, 6.61 cm s^{-1}). The higher permeability was probably due to the transporting character of BBB-ChT. When adding the excess choline chloride as inhibitor, P_{app} decreased due to the competition with compound **10**. The incorporation of compound **10** could lead to higher permeability across the BCECs monolayer.

Inspired by the in vitro results, in vivo brain-targeting efficiency of the NPs was investigated 2 h after injection of the NPs. The accumulation of ethidium monoazide bromide (EMA) labled pDNA in the brain treated with DGL-PEG-**10**/pDNA was more than that treated with DGL-PEG/pDNA (**Figure 3**a). Then, distribution of gene expression in mouse brain was detected. For DGL-PEG/pEGFP, green fluorescent protein (GFP) expression was found mainly in fourth ventricle (Figure 3g) and little was found in other regions (Figure 3c–f). It was reported that exogenous gene expression could be observed in limited regions around the cerebral ventricles due to their relatively weak BBB.[17] DGL-PEG/pEGFP had high GFP expression in fourth ventricle. It could be hypothesized that NPs modified with compound **10** were likely to accumulate in high-choline-demanding regions, thus resulting in more GFP expression in these regions. For DGL-PEG-**10**/pEGFP, GFP expression was mainly found in cortical layer (Figure 3h), caudate putamen (Figure 3i), and fourth ventricle (Figure 3l). The cortical layer and caudate putamen are rich in neurons and have high

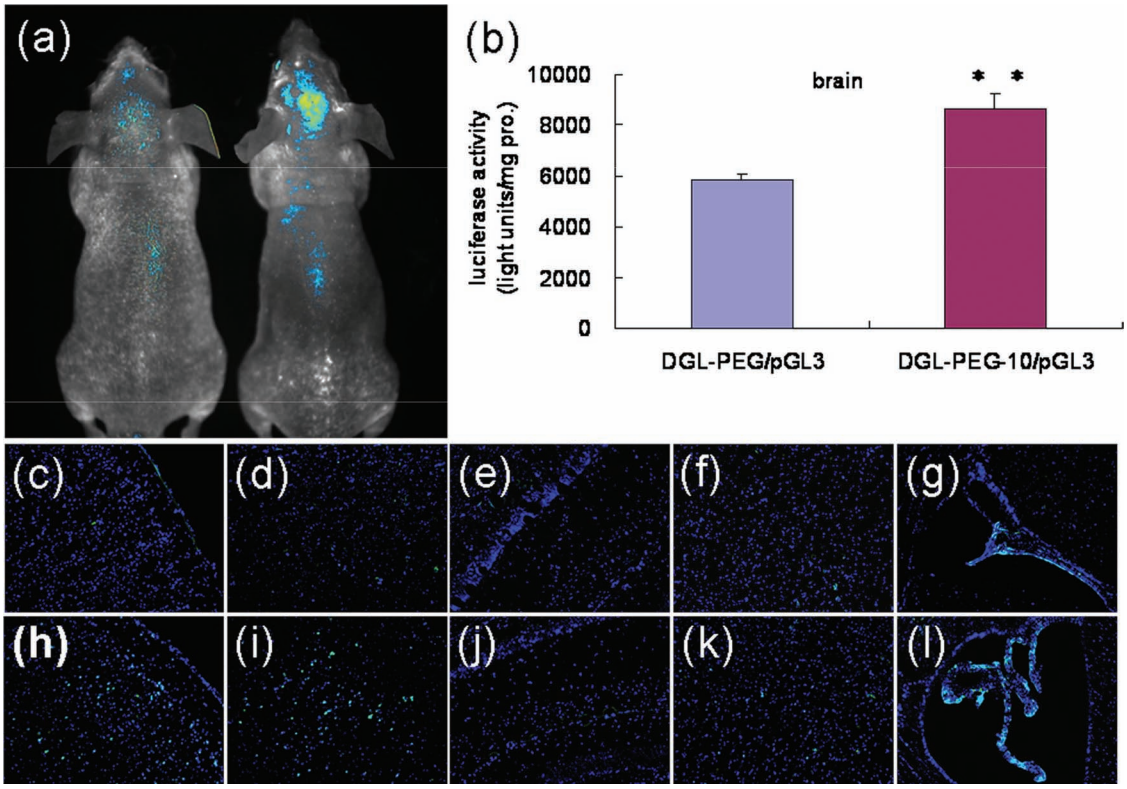

Figure 3. a) In vivo imaging of mice administrated with DGL-PEG/pDNA (left) or DGL-PEG-**10**/pDNA (right). Images were taken 2 h after administration. b) Luciferase expression 48 h after intravenous administration of DGL-PEG/pGL3 and DGL-PEG-**10**/pGL3 into Balb/c mice at a dose of 50 μg DNA per mouse. Luciferase expression is plotted as light units per mg protein. $^{**}p < 0.01$ represents DGL-PEG-**10**/pGL3 vs. DGL-PEG/pGL3. Data are expressed as mean ± S.D. ($n = 4$). c–l) Distribution of gene expression in brains of mice treated with DGL-PEG/pEGFP (c–g) or DGL-PEG-**10**/pEGFP (h–l) 48 h after intravenous administration. Frozen sections (thickness of 20 μm) of cortical layer (c,h), caudate putamen (d,i), hippocampus (e, j), and substantia nigra (f,k), and fourth ventricle (g,l) were examined by fluorescent microscopy. The sections were stained with 300 nM DAPI for 10 min at room temperature. Green: GFP. Blue: cell nuclei. Original magnification: ×100.

demand for choline. High-affinity choline transporter-immunoreactive cell bodies were demonstrated in these regions.[18] As a result, compound-**10**-modified NPs accumulated more in these regions and had higher GFP expression. To examine the transfection efficiencies quantitatively, the expression of pGL3 (luciferase reporter vector)-control vector in brain was measured (Figure 3b). The brain gene expression of DGL-PEG-**10**/pGL3 was $8.63 \pm 0.61 \times 10^3$ light units per mg protein, which is 1.48-fold higher than that of DGL-PEG/pGL3 ($5.85 \pm 0.26 \times 10^3$ light units per mg protein). The significant level (p) is smaller than 0.01.

In summary, a series of novel bis-quaternary ammonium compounds with high BBB-ChT affinity have been synthesized. Although BBB ChT has not been crystallized, the structure–activity relationships shown here could inspire design of BBB ChT substrates with higher affinity. The sulfhydrylation form of the novel compound **7**, i.e., compound **10**, was utilized for the construction of the brain-targeting gene-delivery system. And DGL-PEG-**10**/pDNA NPs demonstrated higher uptake efficiency in vitro and higher gene expression in vivo. The BBB ChT-mediated brain-targeting strategy was first employed in a drug-delivery system and proved to be an encouraging way to overcome the BBB.

Experimental Section

Synthesis of Compounds **5**–**8**: Compounds **3a** and **3b** were prepared by dissolving 3,5-dihydroxy-benzyl alcohol (**1**, 5 mmol) in acetonitrile, adding 1,3-dibromopropane (**2a**, 30 mmol) or 1,4-dibromobutane (**2b**, 30 mmol), and K_2CO_3 (30 mmol), then stirring the the solution under reflux for 4 h. The completion of the reactions was monitored by thin-layer chromatography (TLC). After filtration and removal of the solvent via rotary evaporation, the crude products were purified by flash chromatography on silica (1:4 ethyl acetate/petroleum ether eluent) and identified by ^{1}H NMR. Compounds **5**–**8** were prepared by reacting an excess of isoquinoline (**4a**) or 3-methylpyridine (**4b**) with compound **3a** or **3b** for 1 h at 65 °C in the absence of solvent. The resulting solids were collected by filtration and purified by recrystallisation in ethanol.

Cell Line and Animals: BCECs were kindly provided by Prof. J. N. Lou (the Clinical Medicine Research Institute of the China–Japan Friendship Hospital). Balb/c nude mice (male, age 4–5 weeks, 20–25 g) were maintained under standard housing conditions. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University.

Inhibition of [^{3}H]-Choline Chloride Uptake: Incubation buffer (0.25 mL) containing 10 nmol [^{3}H]-choline chloride dissolved in Hanks buffer with different compounds at designated concentrations was added to each well and incubated at 37 °C for 10 min. The unlabeled choline chloride served as a comparison. Incubation buffer without inhibitions was the total uptake as a control. The uptake was stopped by aspirating the incubation buffer and washing the cells three times with ice-cold Hanks buffer. 2 N NaOH (0.2 mL) was added in each well to lyse the cells and neutralized with 4 N HCl (0.1 mL). Radioactivity was determined by adding scintillant liquid (2 mL) to the solubilized cell solution (0.1 mL) and counting it in a liquid scintillation counter (LS 6000SE, Beckman, USA).

Synthesis and Identify of DGL Derivatives and Derivative/DNA NPs and Other Bioassay Parts: Experimental details for in vivo imaging of compound 10-BODIPY, synthesis and identify of DGL derivatives and derivative/DNA NPs, the cellular uptake mechanism of NPs, transport studies of NPs across the BCECs monolayer, in vivo imaging of NPs, the distribution of gene expression in the mouse brain, and in vivo gene quantitative expression are described in the the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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