articles

molecular pharmaceutics

Loop 2 of *Ophiophagus hannah* Toxin b Binds with Neuronal Nicotinic Acetylcholine Receptors and Enhances Intracranial Drug Delivery

Changyou Zhan, Zhiqiang Yan, Cao Xie, and Weiyue Lu*

School of Pharmacy, Fudan University, Shanghai 201203, China

Received July 18, 2010; Revised Manuscript Received October 8, 2010; Accepted October 21, 2010

Abstract: Three-finger snake neurotoxins have been widely investigated for their high binding affinities with nicotinic acetylcholine receptors (nAChRs), which are widely expressed in the central nervous system including the blood−brain barrier and thus mediate intracranial drug delivery. The loop 2 segments of three-finger snake neurotoxins are considered as the binding domain with nAChRs, and thus, they may have the potential to enhance drug or drug delivery system intracranial transport. In the present work, binding of the synthetic peptides to the neuronal nAChRs was assessed by measuring their ability to inhibit the binding of ^{125}I-α-bungarotoxin to the receptor. The loop 2 segment of *Ophiophagus hannah* toxin b (KC2S) showed high binding affinity, and the competitive binding IC_{50} value was 32.51 nM. Furthermore, the brain targeting efficiency of KC2S had been investigated in vitro and in vivo. The specific uptake by brain capillary endothelial cells (BCECs) demonstrated that KC2S could be endocytosized after binding with nAChRs. In vivo, the qualitative and quantitative biodistribution results of fluorescent dyes (DiR or coumarin-6) indicated that KC2S modified poly(ethylene glycol)-poly(lactic acid) micelles (KC2S-PEG-PLA micelles) could enhance intracranial drug delivery. Furthermore, intravenous treatment with paclitaxel-encapsulated KC2S-PEG-PLA micelles (KC2S-PEG-PLA-PTX micelles) afforded robust inhibition of intracranial glioblastoma. The median survival time of KC2S-PEG-PLA-PTX-micelle-treated mice (47.5 days) was significantly longer than that of mice treated by mPEG-PLA-PTX micelles (41.5 days), Taxol (38.5 days), or saline (34 days). Compared with the short peptide derived from rabies virus glycoprotein (RVG29) that has been previously reported as an excellent brain targeting ligand, KC2S has a similar binding affinity with neuronal nAChRs but fewer amino acid residues. Thus, we concluded that the loop 2 segment of *Ophiophagus hannah* toxin b could bind with neuronal nAChRs and thus enhance intracranial drug delivery for the treatment of central nervous system diseases.

Keywords: Snake neurotoxin; KC2S; brain targeted; PEG-PLA micelle; drug delivery

1. Introduction

A key challenge in the development of drugs for the therapy of central nervous system (CNS) diseases is their transport across the blood−brain barrier (BBB).[1] The BBB consists of capillary endothelial cells that are closely sealed by tight junctions, which prevents paracellular molecule exchange between blood and brain interstitial fluid. Transport of substances destined for the brain parenchymal cells hence requires their uptake across the luminal (blood-facing) membrane into the endothelial cells, their transcellular transport, and their efflux across the abluminal (brain-facing) membrane into the interstitial fluid. The brain side of the capillary is almost completely covered by astrocyte foot processes; astrocytes and pericytes maintain and support, at least in part, the differentiation of the endothelial cell.[2]

* To whom correspondence should be addressed. Mailing address: School of Pharmacy, Fudan University, 826 Zhangheng Road, 815 Research Building, Shanghai 201203, China. Telephone/fax: +86-21-51980090. E-mail: wylu@shmu.edu.cn.

(1) Flemming, A. siRNA: brain delivery breakthrough. *Nat. Rev. Drug Discovery* **2007**, *6*, 2386–2386.

10.1021/mp100238j
Published on Web 10/21/2010

One of the most important strategies to implement brain targeted drug delivery is receptor mediated transport (RMT). Certain endogenous large-molecule neuropeptides such as insulin, transferrin, or leptin access the brain from blood via RMT across the BBB. This transport is mediated by specialized ligand-specific receptor systems, including the insulin receptor (IR) or the transferrin receptor (TfR), which are highly expressed on the capillary endothelium of the brain.[3]

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels expressed mainly in the nervous system and at the neuromuscular junction.[4] They are widely expressed in the brain, including the brain capillary endothelial cells (BCECs).[5] Therefore, nAChR mediated intracranial transport may be a promising strategy. Moreover, Kumar et al.[6] utilized RVG29 (a 29-residue peptide derived from rabies virus glycoprotein) to mediate transvascular delivery of siRNA to the central nervous system via nAChRs. The results indicated that RVG29 specifically bound to the nAChRs expressed on BCECs and provided a safe and noninvasive approach for delivery of siRNA and potentially other therapeutic molecules across the blood−brain barrier.

Neurotoxins from snake venoms are well-known for their binding with high affinity and selectivity to nicotinic acetylcholine receptors and are classified as short chain neurotoxins (e.g., erabutoxin-b (*Laticauda semifasciata*)) that have 60−62 residues and 4 conserved disulfide bonds and long chain neurotoxins (e.g., α-bungarotoxin (*Bungarus multicinctus*); α-cobratoxin (*Naja kaouthia*)) with 66−75 residues and 5 disulfide bonds.[7] They belong to a family of proteins called "three-finger toxins", which adopt a flat, leaflike shape formed by three adjacent loops that emerge from a small globular core.[8−10] The loop 2 segments of neurotoxins are considered to be the binding domain with nAChRs.[11] Thus, we hypothesized that the synthetic peptides derived from the loop 2 of neurotoxin have high binding affinities with nAChRs and thus overcome the BBB via nAChR mediated transport.

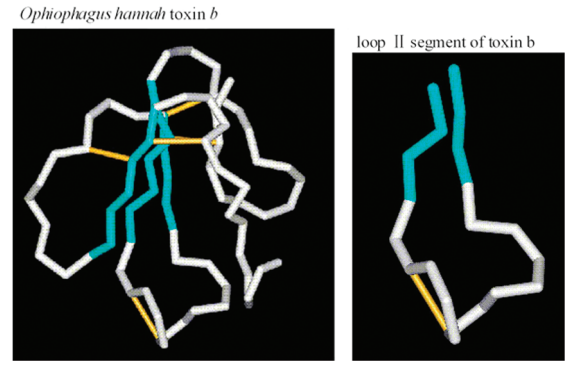

Figure 1. Solution NMR structure of *Ophiophagus hannah* toxin b, a long neurotoxin from the venom of the King Cobra (PDB code is 1TXA) and its loop 2 segment, KC2S. Yellow bonds corresponds to disulfide.

KC2S was derived from the loop 2 of *O. hannah* toxin b (long chain neurotoxin, as shown in Figure 1; PDB code is 1TXA),[12] and the amino acid sequence is shown in Figure 2. In the present work, we synthesized and evaluated the binding affinities of KC2S and RVG29 to neuronal nAChRs. The brain tropism of KC2S, which had high binding affinity with nAChRs, was further investigated in vitro and in vivo. Furthermore, we prepared KC2S modified poly(ethylene glycol)-poly(lactic acid) micelles (KC2S-PEG-PLA micelles), which could enhance encapsulated dye intracranial transport and demonstrated high brain targeting efficiency. The paclitaxel-encapsulated KC2S-PEG-PLA micelles (KC2S-PEG-PLA-PTX micelles) demonstrated effective anti-glioblastoma effect in vivo and significantly prolonged the survival time of intracranial glioblastoma model nude mice compared with mPEG-PLA-PTX micelles, Taxol, and saline. Therefore, we concluded that KC2S could bind with neuronal

(2) Nies, A. T. The role of membrane transporters in drug delivery to brain tumors. *Cancer Lett.* **2007**, *254*, 11–29.
(3) Pardridge, W. M. *Brain Drug Targeting: The Future of Brain Drug Development*; Cambridge University Press: Cambridge, UK, 2001.
(4) Person, A. M.; Bills, K. L.; Liu, H.; Botting, S. K.; Lindstrom, J.; Wells, G. B. Extracellular domain nicotine acetylcholine receptors formed by α_4 and β_2 subunits. *J. Biol. Chem.* **2005**, *280*, 39990–40002.
(5) Abbruscato, T. J.; Lopez, S. P.; Mark, K. S.; Hawkins, B. T.; Davis, T. P. Nicotine and Cotinine Modulate Cerebral Microvascular Permeability and Protein Expression of ZO-1 through Nicotinic Acetylcholine Receptors Expressed on Brain Endothelial Cells. *J. Pharm. Sci.* **2002**, *91*, 2525–2538.
(6) Kumar, P.; Wu, H.; McBride, J. L.; Jung, K. E.; Kim, M. H.; Davidson, B. L.; Lee, S. K.; Shankar, P.; Manjunath, N. Transvascular delivery of small interfering RNA to the central nervous system. *Nature* **2007**, *448*, 39–43.
(7) Nirthanan, S.; Charpantier, E.; Gopalakrishnakone, P.; Gwee, M. C. E.; Khoo, H. E.; Cheah, L. S.; Bertrand, D.; Kini, R. M. Candoxin, a novel toxin from *Bungarus candidus*, is a reversible antagonist of muscle ($\alpha\beta\gamma\delta$) but a poorly reversible antagonist of neuronal α7 nicotinic acetylcholine receptors. *J. Biol. Chem.* **2002**, *277*, 17811–17820.
(8) Tsernoglou, D.; Petsko, G. A.; Hudson, R. A. Structure and function of snake venom curarimimetic neurotoxins. *Mol. Pharmacol.* **1978**, *14*, 710–716.
(9) Basus, V. J.; Song, G.; Hawrot, E. NMR solution structure of an alpha-Bungarotoxin/nicotinic receptor peptide complex. *Biochemistry* **1993**, *32*, 12290–12298.
(10) Lou, X.; Liu, Q.; Tu, X.; Wang, J.; Teng, M.; Niu, L.; Schuller, D. J.; Huang, Q.; Hao, Q. The atomic resolution crystal structure of atratoxin determined by single wavelength anomalous diffraction phasing. *J. Biol. Chem.* **2004**, *279*, 39094–39104.
(11) Kumar, T. K. S.; Pandian, T. K.; Jayaraman, G.; Peng, H. J.; Yu, C. Understanding the structure, function and folding of cobra toxins. *Proc. Natl. Sci. Counc., Repub. China, Part A: Phys. Sci. Eng.* **1999**, *23*, 1–19.
(12) Lentz, T. L. Structure-function relationships of curaremimetic neurotoxin loop 2 and of a structurally similar segment of rabies virus glycoprotein in their interaction with the nicotinic acetylcholine receptor. *Biochemistry* **1991**, *30*, 10949–10957.

nAChRs and thus enhance intracranial drug delivery for the treatment of central nervous system diseases.

2. Materials and Methods

2.1. Materials. Boc-protected amino acid derivatives were from Peptide Institute (Osaka, Japan). *O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate (HBTU) was purchased from American Bioanalytical Co. (Natick, MA). Diisopropylethylamine (DIEA) and Boc-Gly-PAM resin were both supplied by Fluka. Methoxyl poly(ethylene glycol) (mPEG−OH, M_n = 2.0 kDa) was purchased from Sigma, and maleimide-poly(ethylene glycol) (mal-PEG−OH, M_n = 3.5 kDa) was obtained from JenKem technology Co. Ltd. (Beijing, China). Bovine serum albumin (BSA), biotin, coumarin-6, and streptavidin-FITC (STA-FITC) were all supplied by Sigma-Aldrich. Near infrared dye DiR was from Invitrogen. α-Bungarotoxin (α-Bgt) was purchased from Sigma, and ^{125}I radiolabeled α-bungarotoxin (^{125}I-α-Bgt) was obtained from Perkin-Elmer Co. Paclitaxel was kindly gifted by Prof. Hao Wang (Shanghai Institute of Pharmaceutical Industry, China). Taxol was from Haikou Pharmaceutical Factory Co. Ltd. (Haikou, China).

Brain capillary endothelium cells (BCECs) were kindly provided by Prof. X. G. Jiang (School of pharmacy, Fudan University). BCECs were cultured in special Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). HeLa cells were cultured in RMPI 1640 supplemented with 10% bovine calf serum (BCS, Gibco). U87 glioblastoma cells were obtained from Shanghai Institute of Cell Biology. The cells were cultured in special Dulbecco's modified Eagle medium (Gibco) supplemented with 10% FBS.

Wister rats and KM mice were purchased from the Department of Experimental Animals, Fudan University, and maintained under standard housing conditions; male Balb/c nude mice of 4−6 weeks age were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. and maintained under SPF conditions. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University.

2.2. Synthesis and Characterization of Peptides. *2.2.1. Solid Phase Peptide Synthesis (SPPS).* The chain assembly of KC2S and RVG29 (as shown in Figure 2) was performed via SPPS using active ester chemistry to couple Boc-protected amino acid to the deprotected resin. The Boc protecting group was removed via 100% trifluoroactic acid (TFA), and dimethylformamide (DMF) was used as both the coupling and flow wash solvent throughout the cycle. The progress of the assembly was monitored by ninhydrin monitoring.

Biotinylated KC2S was synthesized by coupling biotin with $N_α$-Boc-deprotected peptide resin and routinely doubly coupled. Prior to HF cleavage, the $N_α$-Boc group was removed using TFA to prevent side reaction in subsequent steps, and then the formyl side chain protecting group on tryptophan was removed using a solution of piperidine (20%) in DMF.

The disulfide in KC2S was synthesized via a 20% dimethyl sulfoxide (DMS)O water solution and overnight. The crude peptides were examined by RP-HPLC (Agilent 1100 series) and then purified by preparative HPLC (Waters, 600E). The molecular weights were confirmed by ESI-MS.

2.2.2. Competition Assay of Synthetic Peptides with Neuronal nAChRs. The neuronal nAChRs was prepared as previously described.[13] Wister rats were killed by cervical dislocation and the hippocampus was quickly separated. It was homogenized in 10 times volume of Tris-HCl buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.5% BSA, 1 mM PMSF, 3 μg/mL protease inhibitor, 0.1% NaN_3, 0.32 M sucrose, pH 7.4) at 15 000 rpm for 30 s and repeated five times, prior to centrifugation (1000*g*, 4 °C, 10 min). The supernatant was recentrifugated at 39 000*g* for 10 min. The pellet was resuspended in 10 times volume of Tris-HCl buffer, and centrifugation was repeated three times. At last, the protein was determined via Fotin protein assay methods and stored at −80 °C before use.

Binding of the synthetic peptides with the neuronal nAChRs was determined by measuring their ability to compete binding of ^{125}I-α-Bgt to the receptor. In briefly, the microtiter tubes were incubated with 50 μg of neuronal nAChR membrane protein in 100 μL of Tris-HCl buffer at 37 °C. Six replicates were included for each condition. Competition experiments were performed by adding 20 μL of KC2S or RVG29 with different concentrations. To the nonspecific binding tubes was added 50 μL of α-Bgt with a final concentration of 10 μM. All the tubes were preincubated for 50 min prior to addition of 30 μL of ^{125}I-α-Bgt (with the final concentration of 2 nM), and the final volume was supplemented to 200 μL with Tris-HCl buffer. After incubation at 37 °C for another 2 h, the nonbound ^{125}I-α-Bgt was removed by washing with cold phosphate buffer saline for 10 times (2 mL each time) under vacuum filtration on 49 glass fiber filter membranes. The membranes were oven-dried and placed in scintillation vial with the addition of 1 mL of scintillation fluid. The samples were then counted by using a γ counter.

2.2.3. Cellular Uptake of KC2S. Biotinylated KC2S (Bio-KC2S) with the concentration of 5 μM and an amount of STA-FITC (one unit of STA-FITC can bind with 1 μg biotin) were dissolved in PBS. Then an equal volume of Bio-KC2S was dripped into STA-FITC and vortexed for 30 min. The conjugation (KC2S-STA-FITC) was formed and stored away from light under 4 °C. BCECs and HeLa cells were seeded at a density of 2×10^5 cells/well in 6-well plates (Corning Incorporated, Corning, NY), incubated for 24 h, and checked

(13) Davies, A. R. L.; Hardick, D. J.; Blagbrough, I. S.; Potter, B. V. L.; Wolstenholme, A. J.; Wonnacott, S. Characterisation of the binding of [^{3}H] methyllycaconitine: a new radioligand for labeling α7-type neuronal nicotinic acetylcholine receptors. *Neuropharmacology* **1999**, *38*, 679–690.

under the microscope for confluency and morphology. Before uptake, all the cells were incubated with 1% BSA solution to inhibit protein nonspecific binding. BCECs and HeLa cells were incubated with 800 μL KC2S-STA-FITC in a peptide concentration of 2.5 μM for 2 h. The cells were washed three times with PBS and visualized under an IX2-RFACA fluorescent microscope (Olympus, Osaka, Japan). For quantitative analysis, BCECs and HeLa cells treated as above were trypsinized and centrifuged at 1600 rpm for 10 min to obtain a cell pellet, which was subsequently washed with PBS three times and resuspended in PBS to analyze via flow cytometer.

2.3. Preparation of Peptide Modified PEG-PLA Micelles. *2.3.1. Synthesis of KC2S-PEG-PLA.* mPEG-PLA and mal-PEG-PLA were synthesized as described in a previous report.[14] KC2S modified PEG-PLA copolymer was prepared via addition between thiol of KC2S and maleimide of mal-PEG-PLA. To obtain the disulfide in KC2S, we synthesized KC(Acm)$_2$-Cys and the sequence was YTKTWC(Acm)-DGFC(Acm)SSRGKRIDLGC. The thiol groups in the first two cysteines were protected by the acetamidomethyl residue. A total of 100 mg of mal-PEG-PLA was dissolved in 5 mL of acetonitrile, rotary evaporated to form a thin film at 37 °C, and then hydrated with 3 mL PBS (pH 8.0, 0.2M). A total of 50 mg of KC(Acm)$_2$-Cys was added during 8 h, and the reaction was kept stirring overnight under nitrogen atmosphere. The product was purified via a Sephadex G-50 column and lyophlized. To obtain the disulfide bond of KC2S, 80 mg of the lyophilized product was further formed micelle in 30 mL of citric acid solution (0.2M) and 3 mL of hydrochloric acid (1 M) was added. Then 3 mL of iodine methanol solution (5 mM) was added immediately. The reaction was stirred under room temperature for 30 min and interrupted by adding vitamin C to reduce the excessive iodine. The product was further purified via a Sephadex G-50 column. The obtained KC2S-PEG-PLA was lyophilized for further use. mPEG-PLA, mal-PEG-PLA, and KC2S-PEG-PLA were characterized via ^{1}H NMR.

2.3.2. Preparation of Paclitaxel Encapsulated KC2S-PEG-PLA Micelles. Paclitaxel-encapsulated micelles were prepared as described in the previous report.[13] Amounts of 20 mg of mPEG-PLA and 10 mg of paclitaxel were codissolved in 3 mL acetonitrile, rotary evaporated to form thin film at 37 °C, and hydrated with 3 mL of physiological saline. The obtained micelles were filtrated against a 0.22 μm filter membrane (Millipore) to remove unencapsulated paclitaxel. For KC2S-PEG-PLA-PTX micelles, a similar procedure was taken except that KC2S-PEG-PLA (5 wt %) was mixed with mPEG-PLA. The diameters were detected via a dynamic light scattering instrument (Nicomp 380 DLS), and the results demonstrated that both of mPEG-PLA-PTX and KC2S-PEG-PLA-PTX micelles were about 30 nm in size with narrow polydispersity.

2.4. Brain Targeted Biodistribution. DiR or coumarin-6-encapsulated mPEG-PLA and KC2S-PEG-PLA micelles were prepared via film-hydration methods. In brief, 20 mg of mPEG-PLA (or containing 5 wt % KC2S-PEG-PLA) and 15 μg of DiR or coumarin-6 were codissolved in 3 mL of acetonitrile and rotary evaporated to form thin films at 37 °C, and then 3 mL of physiological saline was added for hydration. The obtained micelles were purified with a CL4B gel column. The obtained fluorescent labeled micelles were stored away from light for further use.

2.4.1. Intravital Near-Infrared (NIR) Imaging. A total of 100 μL of DiR-encapsulated KC2S-PEG-PLA micelle or mPEG-PLA micelle was injected into the caudal vein of Balb/c nude mice. After 2, 6, 12, and 24 h, the fluorescent images were detected using an in vivo image system (FX Pro, Kodak). The brain biodistributions were analyzed via the fluorescent intensities.

2.4.2. Quantitative Studies of Coumarin-6-Encapsulated KC2S-PEG-PLA and mPEG-PLA Micelles in Vivo. Forty-eight mice were randomly divided into two groups, receiving coumarin-6-encapsulated KC2S-PEG-PLA and mPEG-PLA micelles via the caudal veins (micelles dose 40 mg/kg). At 0.083, 0.25, 0.5, 1, 2, 4, 8, and 12 h following i.v. injection, the blood sample was collected. The mice were anesthetized and sacrificed after heart perfusion. The brain tissues were harvested and then subjected to n-hexane extraction for HPLC analysis with coumarin-7 as internal standard.[15] Concentration data were dose-normalized and plotted as drug concentration−time curves in the blood and brain. The pharmacokinetic parameters were determined by using WinNonlin 5.2 (Pharsight Corp., CA).

2.5. Anti-Glioblastoma Study of KC2S-PEG-PLA-PTX. To explore the anti-glioblastoma effect of KC2S-PEG-PLA-PTX micelles, we investigated the survival time of intracranial U87 glioblastoma bearing mice. U87 cells (5×10^5 cells suspended in 5 μL of PBS) were implanted into the right striatum (1.8 mm lateral, 0.6 mm anterior to the bregma, and 3 mm of depth) of male Balb/c nude mice by using a stereotactic fixation device with mouse adaptor. The mice were randomly divided into four groups ($n = 6$) and treated with 100 μL of KC2S-PEG-PLA-PTX or mPEG-PLA-PTX micelles, Taxol (all of 10 mg/kg paclitaxel to body weight), or physiological saline at 7, 12, 17, and 22 days after implantation. The survival time was recorded.

2.6. Statistical Analysis. The IC_{50} values were calculated by nonlinear regression analysis with the GraphPad Prism 5.0 version program. Statistical significance of differences in AUC and C_{max} were tested using the two-tail Student's t test. Survival data were presented using Kaplan−Meier plots and analyzed using a log-rank test. $p < 0.05$ was considered significant.

(14) Zhan, C.; Gu, B.; Xie, C.; Li, J.; Liu, Y.; Lu, W. Cyclic RGD Conjugated Poly(ethylene glycol)-*co*-poly(lactic acid) Micelle Enhances Paclitaxel Anti-Glioblastoma Effect. *J. Controlled Release* **2010**, *143*, 136–142.

(15) Hu, K.; Li, J.; Shen, Y.; Lu, W.; Gao, X.; Zhang, Q.; Jiang, X. Lactoferrin-conjugated PEG-PLA nanoparticles with improved brain delivery: *In vitro* and *in vivo* evaluations. *J. Controlled Release* **2009**, *134*, 55–61.

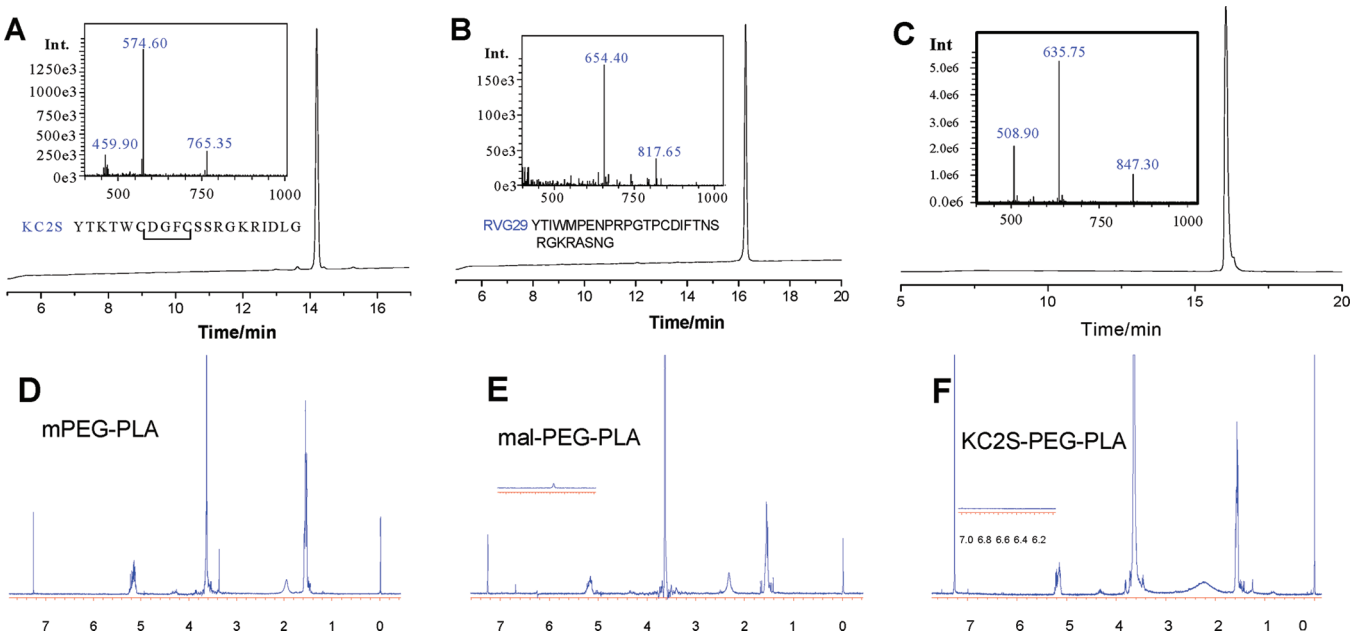

Figure 2. HPLC/ESI-MS spectra of KC2S (A), RVG29 (B), and KC(Acm)$_2$-Cys (C). (D−F) ^{1}H NMR spectra of (D) mPEG-PLA (M_n 4.3 kDa), (E) mal-PEG-PLA (M_n 5.9 kDa), and (F) KC2S-PEG-PLA.

3. Results

3.1. Synthesis of Peptides and KC2S-PEG-PLA. KC2S, RVG29, and KC(Acm)$_2$-cys were synthesized via SPPS, and the HPLC/ESI-MS results are shown in Figure 2A−C. The chain assembly of peptides proceeded without major difficulty. The crude products were purified on a preparative C18 RP-HPLC column (Symmetry, C18, 7 μm, 300 Å) using a 10−45% acetonitrile containing 0.1% TFA linear gradient at 10 mL/min over 60 min. ESI-MS spectra indicated that the molecular weights of KC2S, RVG29, and KC(Acm)$_2$-Cys were consistent with theoretical data (from http://www.expasy.ch/tools/peptide-mass.html).

mPEG-PLA (4.3 kDa) and mal-PEG-PLA (5.9 kDa) were synthesized as described in the previous report.[14] Next, KC(Acm)$_2$-cys was conjugated with mal-PEG-PLA and further oxidized by iodine. The excessive KC2S-Cys and the salts were removed by using a Sephadex G-50 column. The absence of the characteristic resonance of maleimide at 6.7 ppm (as shown in Figure 2F) in ^{1}H NMR spectra also indicated the conjugating reaction.

3.2. Competitive Binding to Neuronal nAChRs. The snake venom toxin α-bungarotoxin (α-Bgt) could specifically bind to the nAChRs.[16] We assessed the binding of KC2S and RVG29 with neuronal nAChRs by testing their ability to inhibit the binding of ^{125}I-α-Bgt with the receptor (as shown in Figure 3). IC_{50} values of KC2S and RVG29 were determined as 32.51 and 27.65 nM, respectively. Previous

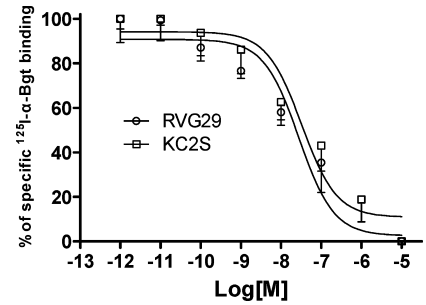

Figure 3. Inhibition of ^{125}I-α-Bgt binding to neuronal nAChRs by KC2S and RVG29.

research[5,17] had demonstrated that nAChRs were widely expressed in the brain, including brain capillary endothelial cells, which are the main constituent of the brain−blood barrier. Since RVG29 had proven the efficacy of intracranial transport via nAChRs, we hypothesized that the loop 2 of snake neurotoxins also had the potential of brain targeting for their high binding affinities with neuronal nAChRs.

The results demonstrated that KC2S and RVG29 had similar binding affinity with neuronal nAChRs. However, KC2S has much advantage compared with RVG29. For example, KC2S has fewer residues than RVG29, making it more convenient to be prepared.

3.3. BCEC Cellular Uptake. BCEC specific uptake of KC2S was evaluated, and the qualitative and quantitative results are shown in Figure 4. HeLa cell uptake was also performed as control. After 2 h incubation, KC2S-STA-FITC

(16) Leonard, S.; Bertrand, D. Neuronal nicotinic receptors: from structure to function. *Nicotine Tob. Res.* **2001**, *3*, 203–223.

(17) Broadwell, R. D.; Balin, B. J.; Salcman, M.; Kaplan, R. S. Blood-Brain barrier? Yes or No. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 7352–7356.

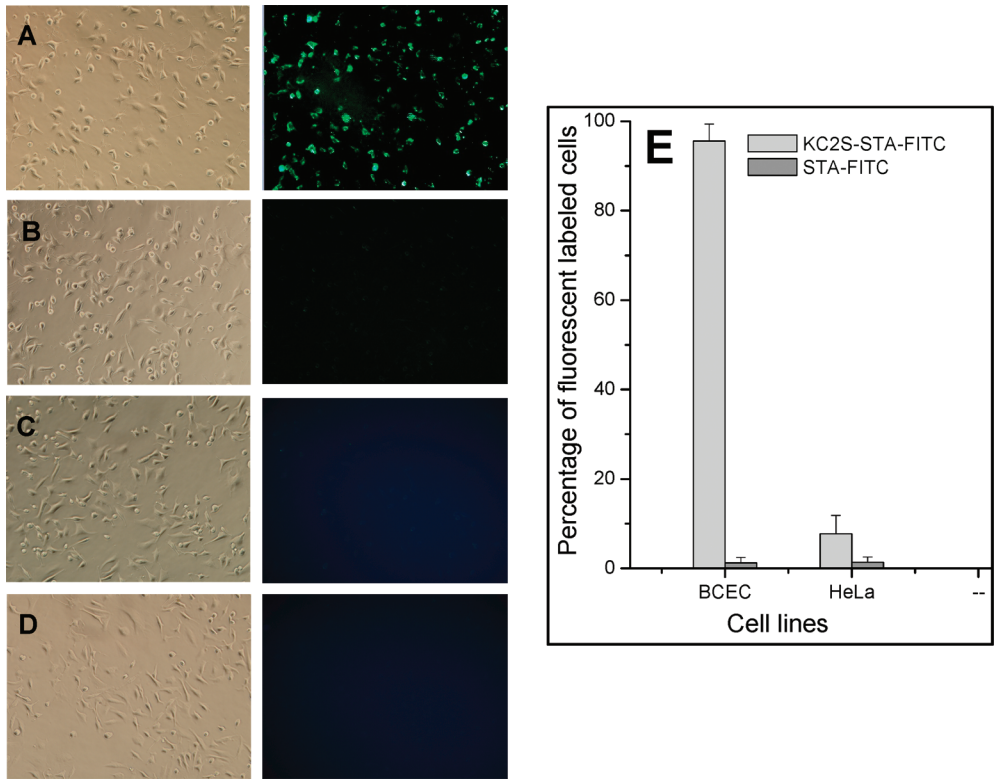

Figure 4. BCEC (A, B) and HeLa cell (C, D) uptake of KC2S-STA-FITC and STA-FITC (negative control, B and D). (E) % FITC-positive cells in flow cytometry.

was specifically taken up by BCECs; on the contrary, there was much weaker fluorescence after incubation with HeLa cells. The FACS results as shown in Figure 4E also demonstrated the BCEC specific uptake. It had been confirmed that for nAChRs expressed on brain endothelial cells via previous reports[5,17] in our study it was found that KC2S could enhance endocytosis by the BBB composing cell, which indicated its potential for brain targeting.

3.4. Brain Targeted Biodistribution. *3.4.1. Intravital NIR Optical Imaging.* To visualize the brain target property of KC2S-PEG-PLA micelles, we prepared DiR-encapsulated KC2S-PEG-PLA and mPEG-PLA micelles and images were acquired at 2, 6, 12, and 24 h. As shown in Figure 5, compared with mPEG-PLA micelles, the obvious accumulation of NIR fluorescence in the brain indicated that KC2S-PEG-PLA micelles could effectively mediate the encapsulated drug, overcoming the BBB.

3.4.2. Quantitative Analysis of Brain Uptake in Vivo. We prepared coumarin-6-encapsulated KC2S-PEG-PLA and mPEG-PLA micelles to evaluate the brain uptake profiles. As shown in Table 1, the AUC and C_{max} of coumarin-6 in the brain of KC2S-PEG-PLA micelles were about 1.98- and 2.60-fold compared with those of mPEG-PLA micelles, respectively. The results indicated that KC2S modified micelles did increase the accumulation of encapsulated coumarin-6 in the brain, which might result from the fact that the KC2S induced active transport of drug delivery system through the BBB.

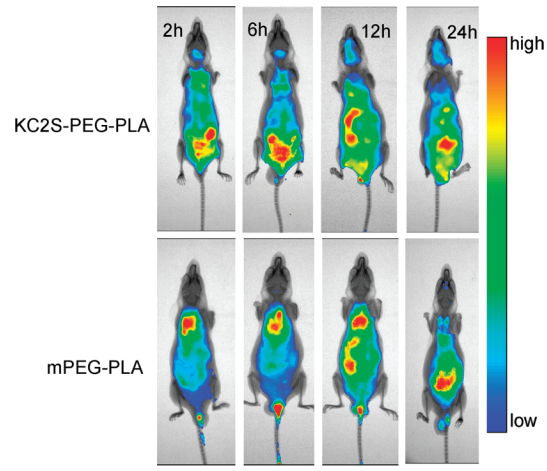

Figure 5. Near-infrared fluorescent imaging of DiR-encapsulated KC2S-PEG-PLA or mPEG-PLA micelles on Balb/c nude mice. The micelles were injected via the tail vein, and the images were collected at 2, 6, 12, and 24 h post-administration.

Figure 6 shows that the concentration of coumarin-6 in systemic circulation by KC2S-PEG-PLA micelles was not significantly different from that by mPEG-PLA micelles, which suggested the tested amount of KC2S on the surface of mPEG-PLA micelle did not impair the long-circulation characteristic of PEG.

3.5. Anti-Glioblastoma Effect in Vivo. The anti-glioblastoma effect of KC2S-PEG-PLA-PTX micelles was demonstrated by the survival time of intracranial U87

Table 1. Comparison of Pharmacokinetic Parameters (Coumarin-6) in Blood and Brain after i.v. Injection of Coumarin-6-Encapsulated mPEG-PLA or KC2S-PEG-PLA Micelles in Mice

	blood		brain	
PK parameters	mPEG-PLA	KC2S-PEG-PLA	mPEG-PLA	KC2S-PEG-PLA
K (h^{-1})	0.987	0.994	0.905	0.966
T_{max} (h)	0.083	0.083	0.5	0.25
$T_{1/2}$ (h)	0.702	0.697	0.766	0.717
C_{max} (ng/mL)	51.60 ± 9.48	51.40 ± 12.98	8.76 ± 3.11	22.79 ± 6.58[a]
$AUC_{(0-t)}$ (ng/mL·h)	39.436 ± 1.22	41.594 ± 9.45	17.41 ± 3.39	34.42 ± 2.63[a]

[a] $p < 0.05$ difference from mPEG-PLA group.

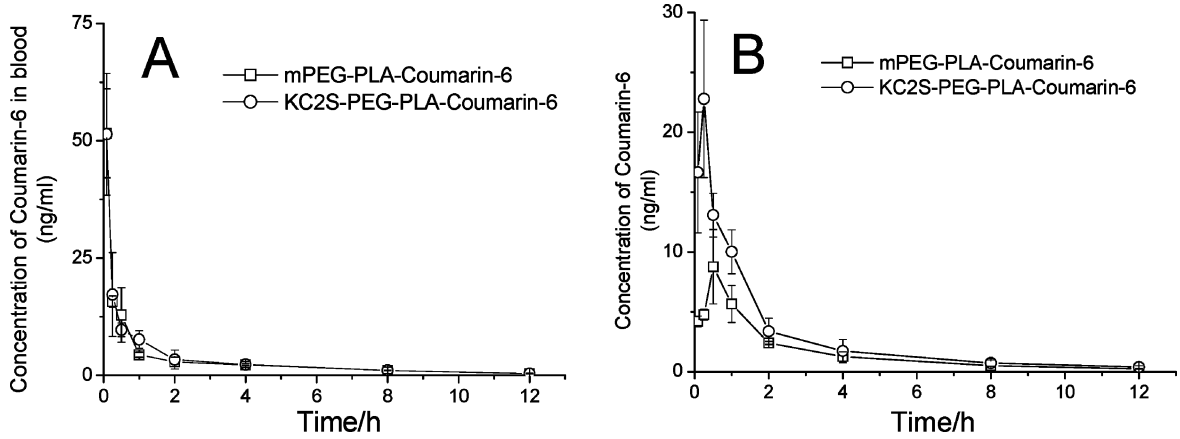

Figure 6. Biodistribution of coumarin-6-encapsulated in KC2S-PEG-PLA or mPEG-PLA micelles in blood (A) and brain (B) during 12 h ($n = 3$).

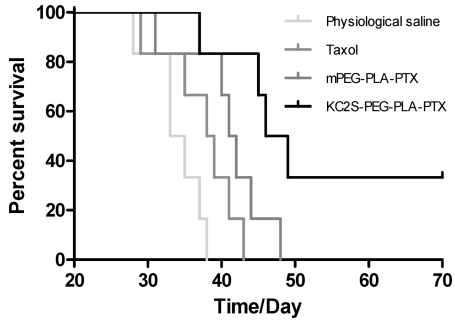

Figure 7. Kaplan−Meier survival curves of nude mice bearing intracranial U87 glioblastoma. Mice that received four doses of KC2S-PEG-PLA-PTX micelles survived significantly longer than mice that received i.v. administrations of mPEG-PLA-PTX micelles ($P < 0.05$, log-rank analysis), Taxol ($P < 0.01$), or saline ($P < 0.005$).

glioblastoma-bearing mice. As shown in Figure 7, the median survival time of the KC2S-PEG-PLA-PTX micelle group, mPEG-PLA-PTX micelle group, Taxol group, and saline group were 47.5, 41.5, 38.5, and 34 days, respectively. Compared to physiological saline, KC2S-PEG-PLA-PTX micelles ($P < 0.005$, log-rank analysis) and mPEG-PLA-PTX micelles ($P < 0.01$) significantly prolonged the survival time, in sharp contrast with Taxol which resulted in no significant prolongation ($P > 0.05$). Such superiority of the micelle formulations in vivo compared to Taxol may be attributed to the sustained release profiles and prolonged blood circulation time caused by micelles.[14] Moreover, the tumor neovasculature has not formed and the glioblastoma cells make use of the normal brain vessels at the early stage. With the aggravation of the glioblastoma, the cells can be roughly separated into an angiogenic component and an invasive or migratory component.[18] The infiltrating tumor around the edge makes use of the existing brain vasculature with a largely intact BBB. Thus, the BBB should still exist which hampers drug or delivery system brain transport. In our study, we began intervention with KC2S-PEG-PLA-PTX micelles 7 days after tumor implantation and repeated the process four times. KC2S-PEG-PLA micelles enhanced the encapsulated paclitaxel brain transport and thus improved the anti-glioblastoma effect in the intracranial GBM model.

4. Conclusion and Discussion

nAChRs are widely expressed in the central nervous system and are varied in different cell types and neurons located in different parts of the nervous system. The α7 receptor, which accounts for the majority of the α-Bgt-binding sites in the nervous system, are widely distributed and are present at a high concentration in the hippocampus[19] as well as the brain capillary endothelial cells.[20] Actually, nAChRs had been successfully utilized to mediate brain targeted gene therapy.[5,21]

(18) Verhoeff, J. J. C.; van Tellingen, O.; Claes, A.; Stalpers, L. J.; van Linde, M. E.; Richel, D. J.; Leenders, W. P.; van Furth, W. R. Concerns about anti-angiogenic treatment in patients with glioblastoma multiforme. *BMC Cancer* **2009**, *9*, 444.

It is well-known that snake neurotoxins can bind with high affinity and selectivity to nAChRs. They are classified as short chain neurotoxins that have 60−62 residues with four conserved disulfide bonds and long chain neurotoxins with 66−75 residues with five disulfide bonds.[6] The loop 2 of snake neurotoxin had been proven to be the main binding region for nAChRs.[22] The previous report[23] had investigated the model of the complex between snake neurotoxin and the α7 nicotinic receptor. It demonstrated that the loop 2 of the neurotoxin plugged into the loop C of α7 nAChRs with high binding affinities.

In the present work, the loop 2 segment of *Ophiophagus hannah* toxin b was synthesized and its binding affinity with the neuronal nAChRs was also investigated. Actually, our competitive binding assay demonstrated that the segment derived from the loop 2 of toxin b had high binding affinity with neuronal nAChRs. Furthermore, KC2S was investigated for its brain targeting efficiency. It could mediate the streptavidin conjugation specific endocytosis by the BBB composing cell and enhance PEG-PLA micelles to overcome the BBB. Thus, we concluded that KC2S had high binding affinity with neuronal nAChRs and thus had the potential to accelerate drug delivery system brain transport. Our study also indicated the efficiency of nAChRs mediated brain transport and the potential of short peptides derived from loop 2 of three-finger snake neurotoxins as brain targeting ligands.

Acknowledgment. This work was supported by National Basic Research Program of China (973 Program) 2007CB935800, the "Key new drug creation program" 2009ZX09310-006, Shanghai Key Technologies R&D Program (No. 0652 nm011 and 074319117), and creative foundation of Fudan University.

MP100238J

(19) Kalamida, D.; Poulas, K.; Avramopoulou, V.; Fostieri, E.; Lagoumintzis, G.; Lazaridis, K.; Sideri, A.; Zouridakis, M.; Tzartos, S. J. Muscle and neuronal nicotinic acetylcholine receptors structure, function and pathogenicity. *FEBS J.* **2007**, *274*, 3799–3845.

(20) Gotti, C.; Clementi, F. Neuronal nicotinic receptors: from structure to pathology. *Prog. Neurobiol.* **2004**, *74*, 363–396.

(21) Liu, Y.; Huang, R. Q.; Han, L.; Ke, W. L.; Shao, K.; Ye, L.; Lou, J.; Jiang, C. Brain-targeting gene delivery and cellular internalization mechanisms for modified rabies virus glycoprotein RVG29 nanoparticles. *Biomaterals* **2009**, *30*, 4195–4202.

(22) Kuo, K. W.; Chang, L. S.; Chang, C. C. The structural loop 2 of cobrotoxin is the main binding region for nAChRs and epitope in the region is conformation-dependent. *J. Biochem.* **1995**, *117*, 438–442.

(23) Fruchart-Gaillard, C.; Gilquin, B.; Antil-Delbeke, S.; Novère, N. L.; Tamiya, T.; Corringer, P. J.; Changeux, J. P.; Ménez, A.; Servent, D. Experimentally based model of a complex between a snake toxin and the α7 nicotinic receptor. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 3216–3221.