Chemistry and Physics of Lipids 224 (2019) 104727

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

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

Ascorbic acid-modified brain-specific liposomes drug delivery system with "lock-in" function

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ARTICLE INFO

Keywords:
Ascorbic acid
Brain targeting
Thiamine disulfide
Synthesis
Liposome

ABSTRACT

In this study, a novel brain targeting ascorbic acid (AA) derivative with "lock-in" function was designed and synthesized as a liposome ligand to prepare novel liposomes to achieve the effective delivery of drug formulations to brain via glucose transporter 1 ($GLUT_1$) and the Na^+-dependent vitamin C transporter ($SVCT_2$). The liposome was prepared and characterized in terms of the particle size, zeta potential, encapsulation efficiency, release profile, stability, hemolysis and cell cytotoxicity. The preliminary evaluation *in vivo* demonstrated that the AA-thiamine disulfide system (TDS)-coated liposome had an improved targeting ability and significantly increased the brain concentration of docetaxel (DTX) as compared to the naked docetaxel, the non-coated and the AA-coated liposomes. The relative uptake efficiency and concentration efficiency were enhanced by 3.24- and 5.62-fold compared to that of the naked docetaxel, respectively. Both distribution data and pharmacokinetic parameters suggested that the ascorbic acid thiamine disulfide delivery system was a promising carrier to enhance central nervous system (CNS) drug's delivery ability into brain.

1. Introduction

Over the past few decades, with the rapid development of medical sciences, many diseases have gradually been overcome. But the treatment of central nervous system (CNS) diseases, such as Parkinson's disease, brain tumor and other neurodegenerative disorders (Chou et al., 2017; Fan et al., 2017; Tapeinos et al., 2017), are still one of the most dangerous threats to human health. So, the treatment for CNS diseases has become a pressing problem that needs to be solved. The greatest obstacle to deliver drugs into brain is the presence of the blood-brain barrier (BBB) between the blood circulation system and central nervous system (Cardenia et al., 2017; Singh et al., 2015). The BBB plays an important role in its further protecting action towards the brain microenvironment, the functions of BBB is not only a physical barrier but also a biochemical barrier. The BBB screens the biochemical, physicochemical, and structural features of solutes at its periphery, building a fence to selectively pass the desired molecules into the brain parenchyma.

In the treatment of brain cancers, to overcome the BBB and maintain a effective drug concentration, a high-dose of chemotherapeutic agent is always given, which in turn causes substantial adverse effects. In order to facilitate the brain entrance and tumor targeting abilities, many CNS delivery techniques have been proposed. Among the various approaches, the carrier-mediated transporter (CMT) system could be an promising strategy to facilitate the brain delivery of drugs, due to its high transport affinity between the transporter and substance. As a targeting carrier, liposome can be easily modified and exhibits many advantages, such as the non-toxicity, biocompatibility and biodegradability (Barenholz, 2001). In the past decades, many types of modified liposomes have been developed, for instance, the polyethylene glycol (PEG)-modified liposome (Jain et al., 2006) and immune liposome (Gao et al., 2011). Although liposomes modified by PEGs can prevent themselves from getting trapped by the reticuloendothelial system such as liver and spleen and thus exhibit good long-circulating character, they are barely brain targeting (Lasic, 1996). Immune liposome achieves targeting effect by combine monoclonal antibody or gene antibody with drug-loaded liposome, and the antibody can bind antigen or receptor of target cell surface.

Ascorbic acid (AA), also known as vitamin C, has an important role in many enzymatic reactions (Qiu et al., 2017). In recent studies, it was found that the concentration of AA in brain is the highest (Wu et al., 2013). The transportation mechanisms of AA across the barriers have been expressly illustrated. One is through the sugar transporter $GLUT_1$, which can transport dehydro-ascorbic acid (DHAA, the oxidized form of AA and can be reduced to AA in brain). The other way is through the transporter $SVCT_2$ which transports AA directly into brain (Pavan et al.,

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https://doi.org/10.1016/j.chemphyslip.2019.01.005
Received 12 December 2017; Received in revised form 18 October 2018; Accepted 16 January 2019
Available online 17 January 2019
0009-3084/

2008). It was also reported that the C2 − OH and C3 − OH are critical for the transportation of AA, while the C5 − OH and C6 − OH are not essential [Li et al., 2014]. Because of these reports, most researchers focused their attention on the preparation of C6-O-modified AA. In our previous works, we have designed and synthesized ibuprofen prodrugs coupled with AA. The results suggested that these prodrugs had good brain targeting ability (Qiu et al., 2017; Wu et al., 2013). Therefore, conjugating the potential CNS-active drugs with AA is also an effective strategy for brain targeting delivery of drugs.

We have been working on brain-targeting drugs for many years (Fan et al., 2011; Qiu et al., 2017; Wu et al., 2013), and are still exploring how to further improve the carrier's BBB permeation. However, the $GLUT_1$ and $SVCT_2$ were found to be bidirectional transporters that mediate the blood-to-brain and brain-to-blood transport of AA in either direction across the BBB (Portugal et al., 2009), owing to its expression on both the luminal and abluminal membranes of the endothelial cells of BBB. This indicated that the AA-modified liposome (AA-Lip) would be pumped out to the bloodstream after entering CNS, thus preventing the concentration of chemotherapeutic agent in brain. Therefore, to overcome the bidirectional delivery problem of $GLUT_1$ and $SVCT_2$, the TDS (Ishikura et al., 1995), with ability of "lock-in", was introduced and attached to the AA moiety at the C-6 position by ester bond. Once entered the BBB, TDS can be reduced by disulfide reductase and then ring-closed to be a thiazolium, which is lipophobic and cannot across the BBB via diffusion. Furthermore, the liposome with this system would be "locked" in the brain, releasing the chemotherapeutic agents sustainably (Fig. 1).

In this study, a new ligand, AA-TDS-Chol, with "lock-in" function, was designed and synthesized. DTX-loaded liposomes, non-coated, ligand AA-Chol coated and ligand AA-TDS-Chol coated, were prepared by the lipid film hydration-ultrasound method. After altering the behavior of the conjugate from bidirectional to unidirectional transport, the drug concentration would be enhanced in brain. The in vivo distribution and pharmacokinetics of DTX-AA-TDS-Lip in plasma and brain were assayed and compared with the naked docetaxel, DTX-Lip and DTX-AA-Lip.

2. Materials and methods

2.1. Materials

All liquid reagents were distilled before use. All unspecified reagents were from commercial resources. TLC was performed using precoated silica gel GF254 (0.2 mm), while column chromatography was performed using silica gel (100–200 mesh). The melting point was measured on a YRT-3 melting point apparatus (Shantou Keyi instrument & Equipment Co. Ltd, Shantou, China). Elemental analyses were performed by Atlantic Microlab (Atlanta, GA, USA). ^{1}H NMR spectra were taken on a Varian INOVA 400 (Varian, Palo Alto, CA, USA) using $CDCl_3$, d_6-DMSO and D_2O as solvent. Chemical shifts are expressed in δ (ppm), with tetramethylsilane (TMS) functioning as the internal reference, and coupling constants (J) were expressed in Hz. Mass spectra were recorded on an Agilent 1946B ESI-MS instrument (Agilent, Palo Alto, CA, USA). Docetaxel and diazepam were obtained from National Institute for Food and Drug Control. Soybean phospholipids (SPC) were purchased from Kelong Chemical. Cholesterol (Chol) was purchased from Bio Life Science& Technology Co., Ltd (Shanghai, People's Republic of China). Reversed-phase chromatography performed on C18 chromatographic analysis was carried out using the high performance liquid chromatography (HPLC) system (Alltech, Woodridge, IL) consisted of a RF-530 fluorescence detector (Shimadzu, Kyoto, Japan) and Allchorom plus data operator, respectively. A Diamonsil column (200 × 4.6 mm, 5 mm) was used. A LC-10 A liquid chromatographic system (Shimadzu) and a reverse-phase HPLC column (ODS-C18 column, 4.6 mm × 200 mm, 5 mm, SinoChrom, Dalian, P.R. China) were used.

2.2. Methods

2.2.1. Chemistry (The synthetic route of each compound was shown in Scheme 1 or Scheme 2)

*2.2.1.1. Synthesis of compound **3**.* To a solution of compound **2** (1.38 g, 2.39 mmol) in dichloromethane (40 mL) was added 4-methylmorpholine (0.36 g, 3.59 mmol) and isobutyl chloroformate (0.49 g, 3.59 mmol) at −15 °C. After 30 min, 4-methyl-5-thiazoleethanamine (0.41 g, 2.87 mmol) was added. Then the reaction mixture was stirred at room temperature for 24 h. The solution was washed with 1 M $NaHCO_3$ (10 mL). The organic layer was dried over anhydrous Na_2SO_4. After concentration, the crude product was purified by column-chromatography to obtain **3** (1.20 g, 71.9%) as an oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.67 (s, 3 H), 0.86 (d, 6H, J = 6.6 Hz), 0.91 (d, 3H, J = 6.6 Hz), 0.98 (s, 3 H), 0.67–2.35 (remaining cholesterol protons), 2.44 (s, 3 H), 3.03 (t, 2H, J = 6.6 Hz), 3.13–3.18 (m, 1 H), 3.52 (q, 2H, J = 6.6 Hz), 3.61–3.64 (m, 12 H), 3.98 (s, 2 H), 5.32 (d, 1H, J = 4.8 Hz), 7.28 (br, 1 H), 8.67 (s, 1 H). HRMS: (ESI+) calculated for $C_{41}H_{68}N_2O_5SNa$ $[M + Na]^+$ 724.0438, found 724.0440. Elemental Analysis: C, 70.24; H, 9.78; N, 4.00; S, 4.57, found C, 70.20; H, 9.85; N, 4.07; S, 4.60 ().

*2.2.1.2. Synthesis of compound **4**.* A solution of compound **3** (4.00 g, 5.70 mmol) and t-butyl bromoacetate (1.67 g, 8.56 mmol) in acetonitrile (50 mL) was heated to reflux and stirred overnight. After completion of the reaction, the mixture was concentrated under

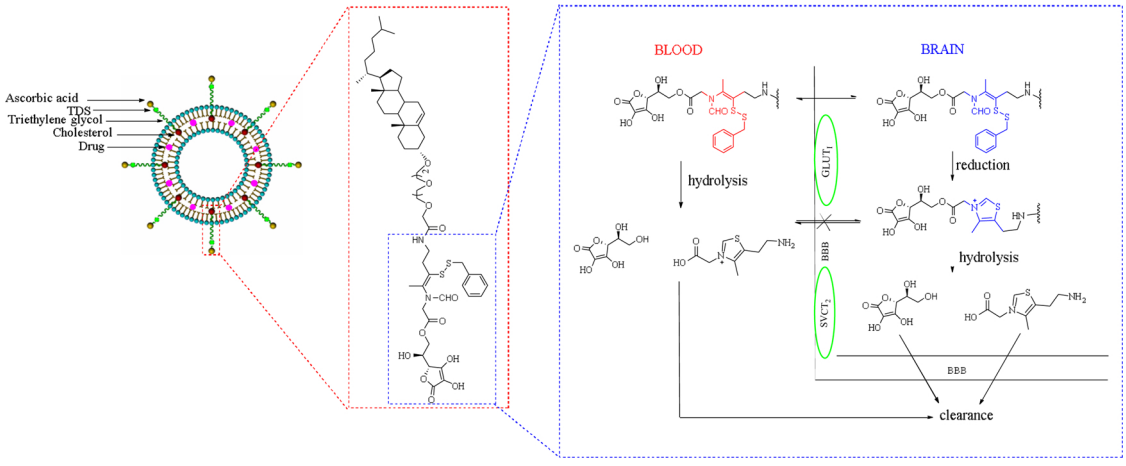
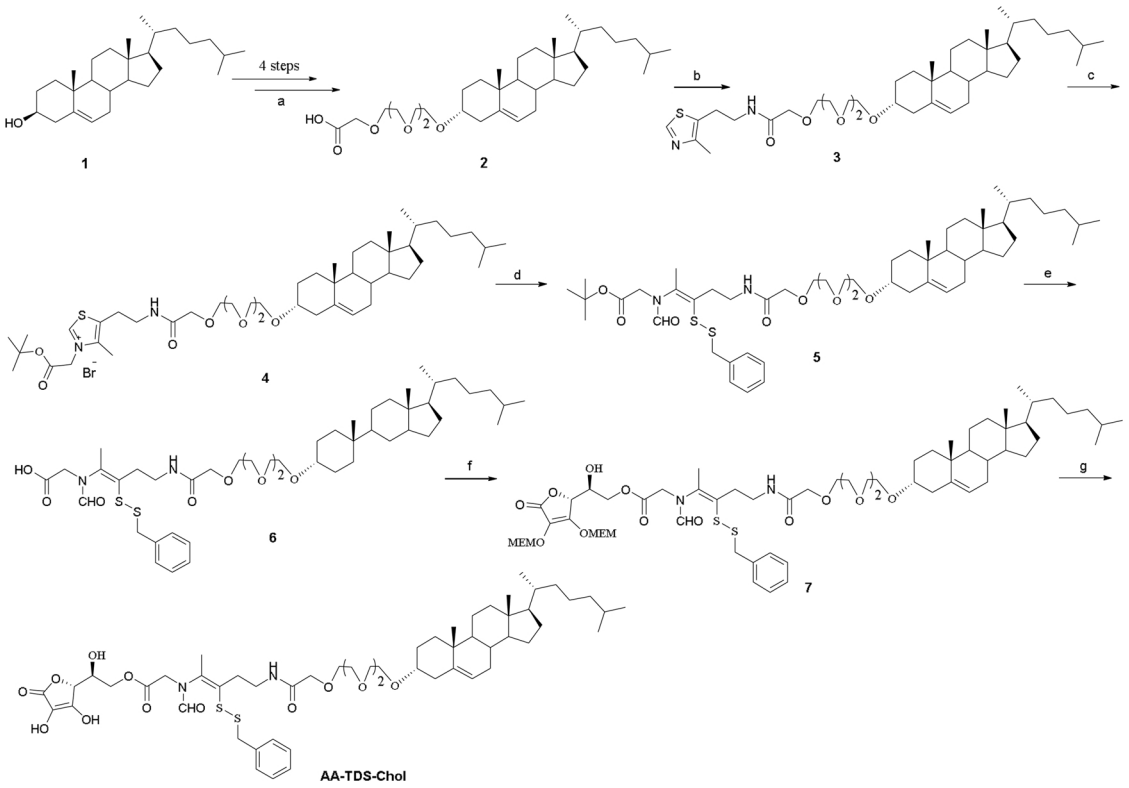


Fig. 1. Schematic diagram of liposomes and brain "lock-in" pathways.

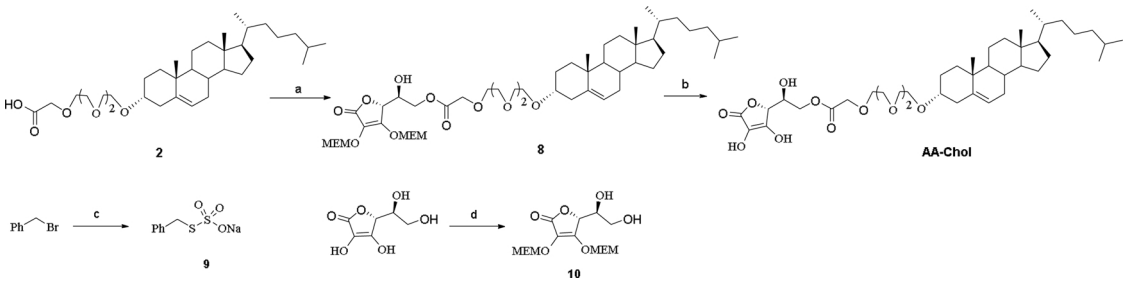


Scheme 1. The synthetic route of ligand AA-TDS-Chol. Reagents and conditions: (a) 1) TsCl, pyridine, 50 °C, 10 h; 2) $HOCH_2(CH_2OCH_2)_2CH_2OH$, dioxane, reflux, 8 h; 3) t-butyl bromoacetate, n-$Bu_4N^+HSO_4$–, 50% NaOH, toluene, r.t., overnight; 4) TsOH, tolunene, reflux, 4 h; (b) 4-methyl-5-thiazoleethanamine, IBCF, NMM, CH_2Cl_2, r.t., 2 h; (c) t-butyl bromoacetate, CH_3CN, 85 °C, 20 h; (d) S-benzyl solfothioate **9**, NaOH, ethyl acetate, 25 °C, 18 h; (e) $HClO_4$, CH_2Cl_2, r.t., 7 h; (f) **10**, DCC, DMAP, CH_2Cl_2, r.t., overnight; (g) 1% HCl − CH_3OH, 70 °C, 0.5 h.

vacuum. The residue was purified on a silica-gel chromatography column to get compound **4** (2.10, 41.2%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.67 (s, 3 H), 0.86 (d, 6H, J = 6.4 Hz), 0.91 (d, 3H, J = 6.4 Hz), 0.98 (s, 3 H), 1.55 (s, 9 H), 0.67–2.65 (remaining cholesterol protons), 2.45 (d, 3H, J = 10.4 Hz), 3.16–3.21 (m, 3 H), 3.59–3.74 (m, 14 H), 4.01 (s, 2 H), 5.33 (d, 1H, J = 4.4 Hz), 5.73 (s, 1 H), 5.91 (s, 1 H), 8.10 (br, 1 H). HRMS: (ESI+) calculated for $C_{47}H_{79}BrN_2O_7SNa$ [M + Na]$^+$ 919.0981, found 919.0980. Elemental Analysis: C, 62.99; H, 8.89; Br, 8.92; N, 3.13; S, 3.58, found C, 62.93; H, 8.82; Br, 8.97; N, 3.18; S, 3.50.

*2.2.1.3. Synthesis of compound **5**.* To a solution of compound **4** (1.80 g, 2.00 mmol) and sodium hydroxide (0.16 g, 4.00 mmol) in water (20 mL) was added compound **8** (0.90 g, 4.00 mmol) under argon protection. The reaction mixture was stirred for 1.5 h at room temperature. The resultant oily substance was extracted with ethyl acetate (20 mL) and the organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Purification of the crude product by column chromatography gave **5** (0.77 g, 40.0%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.67 (s, 3 H), 0.86 (d, 6H, J = 6.4 Hz), 0.91 (d, 3H, J = 6.6 Hz), 0.98 (s, 3 H), 1.46 (s, 9 H), 0.67–2.37 (remaining cholesterol protons), 1.98 (s, 3 H), 2.69 (t, 2H, J = 6.8 Hz), 3.14–3.19 (m, 1 H), 3.37 (q, 2H, J = 6.4 Hz), 3.61–3.66 (m, 12 H), 3.89 (s, 2 H), 3.94–4.01 (m, 4 H), 5.33 (d, 1H, J = 3.6 Hz), 7.29–7.33 (m, 5 H), 7.95 (s, 1 H). HRMS: (ESI+) calculated for $C_{54}H_{86}N_2O_8S_2Na$ [M + Na]$^+$ 978.3890, found 978.3893. Elemental Analysis: C, 67.89; H, 9.07; N, 2.93; S, 6.71, found C, 67.82; H, 9.01; N, 2.98; O, 13.45; S, 6.76.

*2.2.1.4. Synthesis of compound **6**.* To a solution of compound **5** (3.00 g, 3.14 mmol) in CH_2Cl_2 was added $HClO_4$ (0.5 mL), and the reaction was stirred for 7 h at room temperature. The mixture was concentrated under reduced pressure and purified by column chromatography to yield compound **6** (1.81 g, 64.2%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.67 (s, 3 H), 0.86 (d, 6H, J = 6.8 Hz), 0.91 (d, 3H, J = 6.4 Hz), 0.99 (s, 3 H), 0.67–2.37 (remaining cholesterol protons), 1.91 (s, 3 H), 2.74 (t, 2H, J = 6.0 Hz), 3.16–3.21 (m, 1 H), 3.42–3.44 (m, 2 H), 3.62–3.67 (m, 12 H), 3.88–3.95 (m, 4 H), 4.22 (s, 2 H), 5.34 (d, 1H, J = 4.0 Hz), 6.96 (t, 2H, J = 6.0 Hz), 7.27–7.31 (m, 5 H), 8.05 (s, 1 H). HRMS: (ESI+)



Scheme 2. The synthetic route of ligand AA-Chol. Reagents and conditions: (a) **10**, DCC, DMAP, CH_2Cl_2, r.t., overnight; (b) 1% HCl − CH_3OH, 70 °C, 0.5 h; (c) C_2H_5OH/H_2O, reflux,14 h; (d) MEMCl, DIPEA, CH_2Cl_2, r.t., 0.5 h.

calculated for $C_{50}H_{78}N_2O_8S_2Na$ $[M + Na]^+$ 922.2827, found 922.2825. Elemental Analysis: C, 66.78; H, 8.74; N, 3.12; S, 7.13, found C, 66.70; H, 8.79; N, 3.17; S, 7.20.

*2.2.1.5. Synthesis of compound **7**.* To a solution of compound 6 (1.80 g, 2.00 mmol) in 30 mL dichloromethane was added DCC (0.62 g, 3.00 mmol) and cat. DMAP, then the mixture was stirred at room temperature for 30 min. Compound 10 (0.70 g, 2.00 mmol) in CH_2Cl_2 (10 mL) was added to the above reaction mixture. After stirring at room temperature overnight, the reaction was terminated and then filtered. The filtrate was concentrated, and the residue was purified by flash chromatography to yield 7 (1.51 g, 61.0%) as a yellow oil. 1H NMR (400 MHz, CDCl3): δ 0.67 (s, 3 H), 0.86 (d, 6H, J = 6.4 Hz), 0.91 (d, 3H, J = 6.4 Hz), 0.98 (s, 3 H), 0.67–2.36 (remaining cholesterol protons), 1.99 (s, 3 H), 2.59–2.70 (m, 2 H), 3.14–3.19 (m, 1 H), 3.37–3.39 (m, 6 H), 3.56–3.66 (m, 18 H), 3.81–3.90 (m, 6 H), 3.95 (s, 2 H), 4.08 (s, 2 H), 4.18 (t, 1H, J = 6.0 Hz), 4.28–4.32 (m, 1 H), 4.39–4.44 (m, 1 H), 4.72 (s, 1 H), 5.25 (s, 2 H), 5.33 (d, 1H, J = 4.8 Hz), 5.39 (d, 1H, J = 6.4 Hz), 5.73 (d, 1H, J = 6.0 Hz), 7.29–7.31 (m, 5 H), 7.97 (s, 1 H). HRMS: (ESI+) calculated for $C_{64}H_{100}N_2O_{17}S_2Na$ $[M + Na]^+$ 1256.6018, found 1256.6015. Elemental Analysis: C, 62.31; H, 8.17; N, 2.27; S, 5.20, found C, 62.37; H, 8.11; N, 2.20; S, 5.26.

2.2.1.6. Synthesis of ligand AA-TDS-Chol. The compound **7** (0.10 g, 0.08 mmol) was dissolve in 10 mL THF, then 1% HCl in CH_3OH (5 mL) was added. The reaction was stirred at 70 °C for 0.5 h, and concentrated under vacuum. The residue was purified on a silica-gel chromatography column to get desired ligand AA-TDS-Chol (0.07, 82.3%) as a yellow oil. 1H NMR (400 MHz, $CDCl_3$): δ 0.67 (s, 3 H), 0.86 (d, 6H, J = 6.4 Hz), 0.91 (d, 3H, J = 6.4 Hz), 0.98 (s, 3 H), 0.67–2.37(remaining cholesterol protons), 1.97 (s, 3 H), 2.68–2.73 (m, 2 H), 3.17–3.22 (m, 1 H), 3.42 (d, 2H, J = 6.4 Hz), 3.63–3.66 (m, 12 H), 3.87 (s, 2H), 3.99 (s, 2 H), 4.09 (s, 2 H), 4.05–4.37 (m, 3 H), 4.71 (s, 1 H), 5.33 (d, 1H, J = 3.2 Hz), 7.24–7.32 (m, 5 H), 7.98 (s, 1 H). HRMS: (ESI+) calculated for $C_{56}H_{84}N_2O_{13}S_2Na$ $[M + Na]^+$ 1080.3915, found 1080.3917. Elemental Analysis: C, 63.61; H, 8.01; N, 2.65; S, 6.06, found C, 63.67; H, 8.05; N, 2.60; S, 6.01.

*2.2.1.7. Synthesis of compound **8**.* To a solution of compound **3** (1.44 g, 2.50 mmol) in 50 mL dichloromethane was added DCC (0.52 g, 2.70 mmol) and cat. DMAP, then the mixture was stirred at room temperature for 30 min. Compound **10** (0.97 g, 2.75 mmol) in CH_2Cl_2 (10 mL) was added to the above reaction mixture. After stirring at room temperature overnight, the reaction was terminated and then filtered. The filtrate was concentrated under reduce pressure, and the residue was purified by flash chromatography to yield **8** (1.60 g, 70.3%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.67 (s, 3 H), 0.87 (d, 6H, J = 6.4 Hz), 0.91 (d, 3H, J = 6.6 Hz), 0.99 (s, 3 H), 0.67–2.38 (remaining cholesterol protons), 3.14–3.21 (m, 1 H), 3.37 (d, 6H, J = 2.8 Hz), 3.55–3.59 (m, 4 H), 3.62 (s, 4 H), 3.66 (s, 4 H), 3.68–3.75 (m, 4 H), 3.84–3.90 (m, 4 H), 4.15–4.28 (m, 1 H), 4.21 (s, 2 H), 4.26–4.30 (m, 1 H), 4.40–4.45 (m, 1 H), 4.71 (d, 1H, J = 0.8 Hz), 5.25 (s, 2 H), 5.30–5.34 (m, 1 H), 5.37 (d, 1H, J = 6.4 Hz), 5.76 (d, 1H, J = 6.4 Hz). HRMS: (ESI+) calculated for $C_{49}H_{82}O_{15}Na$ $[M + Na]^+$ 934.1561, found 934.1563. Elemental Analysis: C, 64.59; H, 9.07, found C, 64.52; H, 9.13.

2.2.1.8. Synthesis of ligand AA-Chol. The prepared compound **8** (0.90 g, 0.99 mmol) was dissolve in 10 mL THF, then 1% HCl in CH_3OH (5 mL) was added. The reaction was stirred at 70 °C for 0.5 h, and concentrated under vacuum. The residue was purified on a silica-gel chromatography column to get desired ligand AA-Chol (0.59, 81.6%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.65 (d, 3H, J = 12.4 Hz), 0.86 (d, 6H, J = 8.4 Hz), 0.89 (d, 3H, J = 7.2 Hz), 0.98 (s, 3 H), 0.60–37 (remaining cholesterol protons), 2.63 (s, 1 H), 3.18–3.32 (m, 2 H), 3.65–3.79 (m, 12 H), 4.13–4.40 (m, 5 H), 4.80 (d, 1H, J = 2.4 Hz), 5.39 (br, 1 H). HRMS: (ESI+) calculated for $C_{41}H_{66}O_{11}Na$ $[M + Na]^+$ 757.9459, found 757.9458. Elemental Analysis: C, 67.00; H, 9.05, found C, 67.04; H, 9.01.

2.2.2. Preparation and characterization of liposomes

Liposomes were prepared by thin film hydration method. Lipid compositions of the prepared liposomes were as follows: (1) conventional liposomes (Lip), SPC/cholesterol/ (molar ratio = 62: 33); (2) Ligand AA-Chol modified liposomes (AA-Lip), SPC/cholesterol/ligand AA-Chol (molar ratio = 62: 33: 5); (3) Ligand AA-TDS-Chol modified liposomes (AA-TDS-Lip), SPC/cholesterol/Ligand AA-TDS-Chol (molar ratio = 62: 33: 5). All lipid materials were dissolved in the mixture solvent chloroform/methanol (v/v = 2:1), and then the organic solvent was removed by rotary evaporation to form a lipid film. After kept in vacuum overnight, the obtained film was hydrated in PBS (pH 7.4) for 0.5 h at 20 °C. Then it was further intermittently sonicated by a probe sonicator at 80 W for 80 s to form liposomes. DTX-loaded liposomes were prepared with DTX added to the lipid organic solution prior to the solvent evaporation. The entrapment efficiency (EE) and drug loading efficiency (DL) of DTX was determined by HPLC. The mean size and zeta potential of Lip, AA-Lip and AA-TDS-Lip were detected by Malvern Zeta sizer Nano ZS90 (Malvern Instruments Ltd., UK).

2.2.3. In vitro drug release study

In vitro DTX release study was performed using dialysis method. Each DTX-loaded liposomes (0.4 mL) or free DTX were placed into dialysis tubes (MWCO = 8000–14000 Da) and tightly sealed. Then the dialysis tubes were placed into 40 mL PBS containing 0.1% (v/v) Tween 80 and incubated under 37 °C for 48 h with gentle oscillating at 45 rpm. At predetermined time points (0 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h), 0.1 mL release medium was taken out and replaced with equal volume of fresh release medium. Then the samples were diluted with acetonitrile and the concentrations of DTX were determined at the wavelength of 225 nm by HPLC.

2.2.4. In vitro stability of liposomes in serum

Turbidity variations were measured to demonstrate the serum stability of liposomes in the presence of fetal bovine serum (FBS). Briefly, liposomes were mixed with equal volume of FBS under 37 °C with moderate shaking at 45 rpm. The transmittance of the mixture was measured at predetermined time points (0 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h) at 750 nm by a microplate reader (Thermo Scientific Varioskan Flash, USA).

2.2.5. Hemolysis assays

To evaluate the safety of ligands-modified liposomes during body circulation, hemolysis assay was performed. Fresh mouse blood was collected in heparin sodium-containing tubes. The red blood cells (RBCs) were separated and collected by centrifugation at 5×10^3 rpm for 5 min and washed several times with PBS until the supernatant became colorless. After the last wash, the RBCs were diluted with PBS to a concentration of 2% (v/v). Various concentrations of liposomes were incubated with equal volume of 2% RBCs solutions for 1 h at 37 °C with gentle shaking, followed by centrifugation at 1×10^4 rpm for 10 min. Absorbance of hemoglobin was measured using a microplate reader (Thermo Scientific Varioskan Flash) at 540 nm. The values for 0% and 100% hemolysis were determined by incubating erythrocytes with PBS or 1% (v/v) Triton X-100. The hemolysis percentage was calculated using the following equation:

$$Thepercenthemolysis = \frac{ASample - ANegative}{APostive - ANegative} \times 100$$

where A is the absorbance of hemoglobin.

2.2.6. Cytotoxicity assay

Murine glioma cells (C6) and murine brain endothelial cells

(bEnd.3) were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL streptomycin and 100 U/mL penicillin at 37 °C in a 5% CO_2 humidified environment incubator (Thermo Scientific, USA), human cervical.

The cytotoxicity of DTX-loaded liposomes was measured with MTT assay. Generally, the cells were seeded in a 96-well tissue culture plate at a density of 5×10^3 cells/well and cultured for 24 h at 37 °C. DTX-loaded liposomes and free DTX were diluted to predetermined concentrations with PBS, and added into each well for 24 h incubation. The final concentrations of DTX were in the range of 0.1–20 μg/ml. Blank liposomes and the solvent of free DTX (DMSO) were added at the same concentration of DTX-loaded liposomes as well. Then, 20 μL MTT solutions (5.0 mg/mL) was added to the medium and incubated for another 4 h at 37 °C. After removal of the culture medium, the reduced MTT dye was solubi1ized by DMSO (150 μL) and the absorbance was read at 490 nm wavelength on an automatic microplate spectrophotometer. Cell viability (%) was calculated as the following equation: A_{test} / $A_{control}$ × 100%, where A_{test} and $A_{control}$ represented the absorbance of treated cells and control cells, respectively.

2.2.7. Biodistribution studies in vivo

2.2.7.1. Test animals. Adult Kunming mice weighing 20–22 g were obtained from the animal center of Sichuan University. The animals were left for two days to acclimatize to animal room conditions and were maintained on standard pellet diet and water ad libitum. Food was withdrawn on the day before the experiment, but free access to water was allowed. Since the experiment could be completed within 24 h, there was no significant change in the mice's' body weight during the experiment. All animals received human care, and the study protocols complied with the guidelines of Sichuan University animal ethical experimentation committee. Throughout the experiments, the animals were handled according to the requirements of the National Act on the use of experimental animals (People's Republic of China).

2.2.7.2. HPLC analysis. The waters liquid chromatographic system employed was an LC-10 A liquid chromatographic system (Shimadzu Japan). The analysis was carried out on a SinoChrom ODS-C18 column (200 mm × 4.6 mm, 5 mm), thermostated at 25 °C. The solution of acetonitrile-0.1% phosphoric acid solution (55:45) was used as the mobile phase at a flow rate of 1.0 mL/min and the UV detector was set to monitor the signal at 230 nm corresponding to the maximum absorbance for the docetaxel.

2.2.7.3. Sample preparation. Blood was collected from the eye socket of mouse into a tube containing heparin, and centrifuged at 5000 rpm for 5 min. The supernatant was collected as plasma sample. The organs were removed and flushed with water for three times to remove the blood remained and then the brains were roll over on the filter paper carefully to remove the main vessel. All the tissues were homogenized with triple amount of water. An aliquot of 10 μL of internal standard (diazepam) was added into 200 μL plasma or 200 μL organ homogenate, and extracted with 1 mL ether. The mixture was vortexed for 5 min, and centrifuged at 10,000 rpm for 5 min. The supernatant was transferred to another centrifuge tube, and dried under air stream at room temperature. The dry residue was reconstituted with 80 μL of methanol. The solution was centrifuged at 12,000 rpm for 10 min, and then 20 μL of the supernatant was injected into the HPLC system for analysis. All centrifugation processes were carried out at 4 °C.

2.2.7.4. Body distribution study. Twenty-one mice were randomly assigned to 4 groups for each liposome or docetaxel. DTX-Lip, DTX-AA-Lip, DTX-AA-TDS-Lip and docetaxel were given to the mice via the tail vein and each was equivalent to the administration dose of docetaxel of 5 mg/kg. At 0.5 h, 1 h, 2 h, 4 h, 8 h, 16 h and 24 h after injection, blood sample was collected from the eye socket of mice after anesthesia with 0.4% chloral hydrate, and placed in heparin tubes. The organs were removed after excretion of blood by cardiac perfusion with saline, and washed twice with water. The organs were weighed and prepared as described earlier. The concentration the docetaxel was analyzed by HPLC.

2.2.8. Statistical analysis

The area under the concentration-time profile (AUC_{0-t}), maximal concentration (C_{max}), and mean residence times (MRT) were calculated by Data and max Statistics (DAS 2.0, Shanghai, China). Statistical evaluation was performed using analysis of variance followed by t-test. A value of $p < 0.05$ was considered significant. The relative uptake efficiency (RE) and concentration efficiency (CE) were calculated to evaluate the brain targeting property of liposome. The value of RE and CE were defined as follows:

$$RE = (AUC_{0-t})s / (AUC_{0-t})c$$

$$CE = (C_{max})s / (C_{max})c,$$

Where S and C represented sample (the liposomes) and control (docetaxel), respectively.

3. Results and discussion

3.1. Chemistry

The synthetic route of ligand AA-TDS-Chol, has been illustrated in Scheme 1. The starting materials are the commercially available AA and cholesterol. Briefly, cholesterol **1**, the starting material, underwent four steps to generate compound **2** (Qu et al., 2014), which was then conjugated with 4-methyl-5-thiazoleethanamine in the presence of isobutyl chloroformate (IBCF) and N-methyl morpholine (NMM) to give compound **3**. Subsequently, the intermediate **3** reacted with tert-butyl bromoacetate in acetonitrile to yield tert-butyl ester **4**, which was then reacted with **9** under alkaline conditions to obtain the ring-opened compound **5**. After deprotected the tert-butyl group, compound **6** was generated. The small steric hindrance 6-hydroxy group of AA compound **10** coupled with **6** by using dicyclohexyl carbodiimide (DCC) as dehydrating agent for ester condensation to get ester **7**. Finally, under the conditions of 1% HCl − CH_3OH, we removed the 2,3-hydroxy protecting group to reached anticipated ligand AA-TDS-Chol. All the title compounds and important intermedium were characterized by their respective ^{1}H NMR and MS.

Scheme 2 showed the synthetic route of ligand AA-Chol. Briefly, condensation of compound **2** and **10** in the presence of DCC and 4-dimethylamiopyridine (DMAP) gave ester **8**, which was then deprotected to afford the desired product AA-Chol. The synthesis of intermediates **9** and **10** was also exhibited in Scheme 2 according to our previous reported procedures (Fan et al., 2011), which will not be repeated here.

3.2. Preparation and characterization of liposomes

A proper size and uniform distribution of nanoparticles were required for both BBB permeation and brain tumor targeting. The particle sizes and zeta potentials of different liposomes in this study were listed in Table 1. For the three types of liposomes, EE of docetaxel were greater than 82%, and DL of docetaxel were close to 4%, respectively. The average particle sizes of all liposomes were close to or less than 120 nm, and the values of PDI were close to 0.2, fully complied with the conditions. To our knowledge, the particle size and zeta potential of liposomes were crucial to *in vivo* bio-distribution (Shi et al., 2015).

3.3. In vitro drug release study

DTX release properties were evaluated in PBS containing 0.1% Tween 80. As shown in Fig. 2, free DTX exhibited a rapid release, with

Table 1
The composition and characterization of different docetaxel-loaded liposomes (n = 3).

Liposomes	Size(nm)	PDI	EE (%)	DL (%)	Zeta potential (mV)
DTX-Lip	110.6 ± 3.6	0.205 ± 0.024	92.87 ± 2.41	4.23 ± 0.14	−14.1 ± 1.2
DTX-AA-Lip	116.5 ± 3.2	0.235 ± 0.017	86.54 ± 3.38	3.96 ± 0.20	−16.7 ± 2.3
DTX-AA-TDS-Lip	123.7 ± 6.9	0.247 ± 0.029	82.16 ± 6.45	3.78 ± 0.25	−10.4 ± 0.7

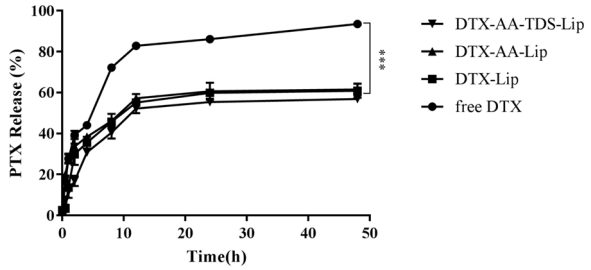


Fig. 2. The DTX release profiles of free DTX, DTX-Lip, DTX-AA-Lip and DTX-AA-TDS-Lip in PBS (pH 7.4) containing 0.1% Tween 80 over 48 h (n = 3, mean ± SD). The DTX-loaded liposome groups was compared with the free DTX group. *** $p < 0.001$, compared with the free DTX group.

over 80% of the drug released into the media within 12 h incubation. On the other hand, DTX-loaded liposomes achieved sustained release behaviors that the cumulative DTX release of drug loaded liposomes was less than 60% after 48 h incubation in PBS. No significant difference on release properties was observed among DTX-Lip, DTX-AA-Lip and DTX-AA-TDS-Lip, and none of these DTX-loaded liposomes displayed burst initial release patterns.

3.4. In vitro stability of liposomes in serum

The stability of liposomes under biological conditions is an important parameter governing the activity of the associated therapeutic agent. Transmittance of different liposomes were monitored in the presence of 50% FBS. As shown in Fig. 3, the transmittance of the liposomes were above 90% and hardly changed after 48 h incubation with 50% FBS. This stability study of liposomes indicated that the liposomes were enough to prevent the interaction between liposomes and serum protein, which was important to achieve a long blood half-life *in vivo*.

3.5. Hemolysis assays

Hemocompatibility is a key point for in vivo applications of liposomes. As shown in Fig. 4, hemolysis assay of ligand-modified

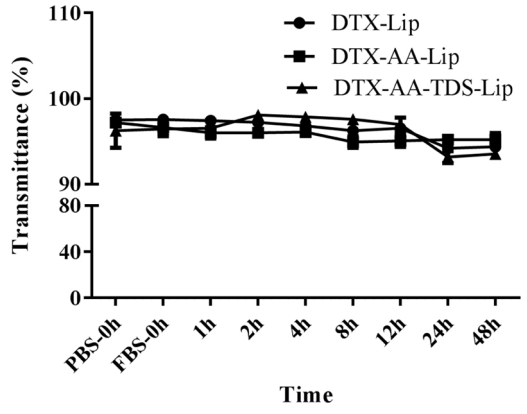


Fig. 3. The variations of transmittance of different modified liposomes in 50% FBS (n = 3, mean ± SD).

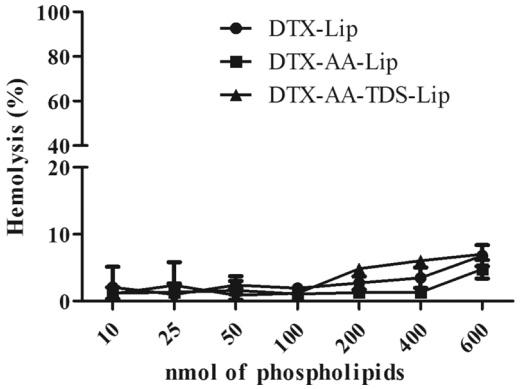


Fig. 4. Hemolysis percentage of different liposomes. Values are represented as mean ± SD (n = 3).

liposomes demonstrated that all the liposomes did not show any significant increase in the hemoglobin release up to 600 nmoles of phospholipids. DTX-AA-TDS-Lip did not display concentration-dependent increase in hemolysis as well and less than 10% hemolysis was always regarded as non-toxic (Shi et al., 2015).

3.6. Cytotoxicity

The cytotoxicity of different liposomes on C6 cells was evaluated using MTT assay. As shown in Fig. 5A, the DTX-loaded liposomes showed higher cell viability than the free DTX, because free drugs could be transported into the cells directly without a drug release process. On the other hand, the cytotoxicity of blank liposomes was also measured, and all these three kinds of blank liposomes exhibited no significant cytotoxicity (Fig. 5B). Therefore our liposomal drug delivery systems were safe and nonvenomous to be further used in vivo.

3.7. Distribution study in plasma and brain

For *in vivo* study, DTX-Lip, DTX-AA-Lip, DTX-AA-TDS-Lip and docetaxel original drug were injected through caudal vein of the mice with a single dose equivalent to 10 mg/kg body weight of docetaxel. At 0.5 h, 1 h, 2 h, 4 h, 8 h, 16 h and 24 h after injection, blood sample was collected from the eye socket of mice, and placed in heparin tubes. Then blood and brain were collected to analyze the concentration of docetaxel at different intervals by HPLC method.

The plasma DTX concentration-time profiles were shown in Fig. 6 and the pharmacokinetic parameters of DTX from different formulations were summarized in Table 2. The result showed that the area under the concentration-time profile (AUC_{0-t}) of docetaxel in the three types of liposomes was much higher than that of the naked docetaxel within 24 h. Free docetaxel of liposomes DTX-Lip, DTX-AA-Lip, DTX-AA-TDS-Lip and docetaxel presented with an area ratio of 1.08, 1.41, and 1.51, and the max concentration (C_{max}) for free docetaxel was 1.04, 1.68 and 2.36 times that of the three types of docetaxel liposomes. These data indicated that the liposomes showed certain stability which would increase the chance to be transported across BBB.

To further evaluate the possibility of the AA-mediated liposomes being transported across BBB, all of animals had been perfused before the tissues were removed. the distribution in brain of DTX-loaded

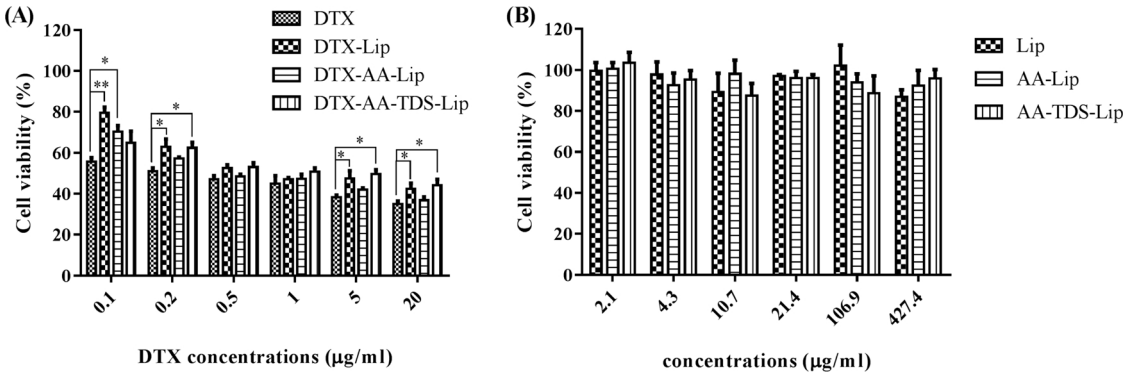


Fig. 5. (A) The cytotoxicity study of DTX-loaded liposomes and free DTX on C6 cells. Free DTX-treated cells were used as a control group compared with DTX-loaded liposome-treated cells. (B) The cytotoxicity study of blank liposomes on C6 cells. Horizontal coordinates indicate lipid concentrations of blank liposomes. Values are represented as mean ± SD (n = 3). The DTX-loaded liposome groups were compared with the free DTX group (A). * $p < 0.05$, ** $p < 0.01$, compared with the control group.

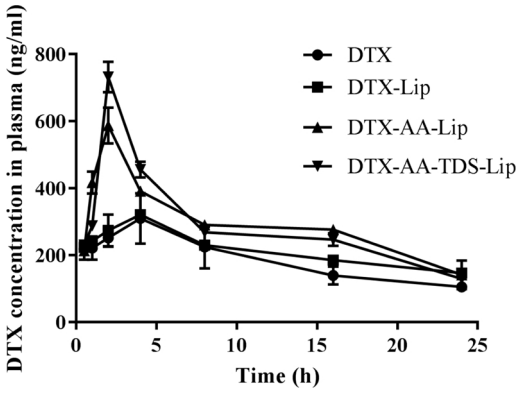


Fig. 6. The concentration curve of docetaxel versus time in plasma after i.v. injection of docetaxel and DTX-loaded liposomes in mice (n = 3, mean ± SD).

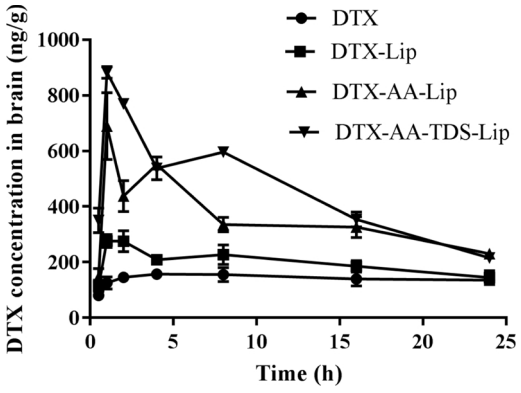


Fig. 7. The concentration curve of docetaxel versus time in brain after i.v. injection of docetaxel and DTX-loaded liposomes in mice (n = 3, mean ± SD).

liposomes and docetaxel was determined. The concentrations of docetaxel in brain versus time curves were displayed in Fig. 7 and the pharmacokinetic parameters were listed in Table 3.

It is obvious that the three types of liposomes could be delivered to the brain following i.v. administration. At different time interval, the concentration of docetaxel released from liposomes was much higher than that from docetaxel original drug during 24 h. The $AUC_{0\text{-}t}$ and C_{max} of docetaxel in brain after i.v. administration of liposomes were fairly higher than that of the free docetaxel, and it is worth noting that it showed an increasing trend with the introduction of the thiamine disulfide system (TDS). The relative uptake efficiencies (REs) of DTX-Lip, DTX-AA-Lip and DTX-AA-TDS-Lip were enhanced by 1.40, 2.51 and 3.24 times of the naked docetaxel, respectively. The concentration efficiencies (CEs) were also enhanced by 1.75, 4.38 and 5.62 times than that of the docetaxel. So these data further proved our conjecture that liposomes with ligands having the "lock-in" function can better deliver and keep docetaxel in the CNS. Furthermore, the pharmacokinetic parameters suggested that the brain targeting ability of this designed liposome is better than DTX-Lip and DTX-AA-Lip. Moreover, the higher REs and CEs of the modified liposomes demonstrated that exposing AA residues outside the membrane of coupled liposomes could recognize $GLUT_1$ and $SVCT_2$. Furthermore, the TDS system could be reduced and ring-closed to be a thiazolium in the BBB. Thus, the liposome with this system was "locked" in the brain and then it can serve a sustained releaser of the chemotherapeutic agent. Moreover, the higher docetaxel concentration of DTX-AA-TDS-Lip shown in brain also confirmed the bidirectional transport characteristics of $GLUT_1$ and $SVCT_2$ as well as the effectiveness of the TDS system.

4. Conclusion

Generally, to overcome the bidirectional delivery problem of $GLUT_1$ and $SVCT_2$, the TDS, with the ability of "lock-in", was introduced, and a new AA-TDS-Chol coated liposome was designed and prepared in this work. The experimental results confirmed the hypothesis that liposome coated with AA-TDS-Chol was a promising brain targeting drug delivery strategy given its higher transendothelial ability, higher brain concentration, increased $AUC_{0\text{–}t}$ and better RE values in brain compared to

Table 2
Pharmacokinetic parameters of docetaxel in blood after administration of docetaxel and liposomes (n = 3, mean ± SD). The DTX-loaded liposome groups was compared with the free DTX group. * $p < 0.05$, ** $p < 0.01$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the free DTX group.

	$AUC_{(0\text{-}t)}$ (ng/ml·h)	MRT (h)	T_{max} (h)	C_{max} (ng/ml)
Docetaxel	4528.17 ± 136.23	9.61 ± 0.16	4	310.23 ± 14.24
DTX-Lip	5178.44 ± 87.35**	10.41 ± 0.33	4	321.58 ± 23.65
DTX-AA-Lip	7030.39 ± 70.14***	9.94 ± 0.65	2	587.39 ± 76.41**
DTX-AA-TDS-Lip	7153.67 ± 298.43***	9.10 ± 0.23	2	732.57 ± 88.37**

Table 3
Pharmacokinetic parameters of docetaxel in brain after administration of docetaxel and liposomes (n = 3, mean ± SD). The DTX-loaded liposome groups was compared with the free DTX group. * $p < 0.05$, ** $p < 0.01$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the free DTX group.

	$AUC_{(0-t)}$ (ng/g·h)	MRT (h)	T_{max} (h)	C_{max} (ng/g)	RE	CE
Docetaxel	3368.42 ± 125.36	11.67 ± 0.14	4	157.23 ± 10.05	–	–
DTX-Lip	4727.96 ± 356.77**	11.01 ± 0.46	2	275.61 ± 26.98**	1.40	1.75
DTX-AA-Lip	8449.74 ± 389.05***	10.46 ± 0.12	1	689.35 ± 67.88***	2.51	4.38
DTX-AA-TDS-Lip	10906.65 ± 578.16***	9.54 ± 0.21	1	883.25 ± 123.34***	3.24	5.62

the naked docetaxel, non-coated and AA-Chol coated liposomes. Furthermore, the results indicated that the ascorbic acid and thiamine disulfide moiety (AA-TDS) could act as a vector, transporting the liposome across the BBB and then sustainably releasing drug in CNS. Given its efficiency and ease of synthesis, this approach may be applied in design of other brain targeting drug delivery systems.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (No. 81573286, No. 81773577 & No. 21472130).

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