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Original article

Design, synthesis and biological evaluation of multivalent glucosides with high affinity as ligands for brain targeting liposomes

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ABSTRACT

The new bifunctional cluster glucosides were designed and synthesized as liposome ligands for preparing novel liposome to achieve the effective delivery of drug formulations to brain by GLUT1. Docetaxel-loaded five liposomes were prepared successfully and tested in the animals. Results from the in vivo distribution study after i.v. administration of these five liposomes and blank-docetaxel indicated that the coupled liposomes **Lip-1**, **Lip-2**, **Lip-3**, **Lip-5** exhibited excellent transport ability across the BBB. In particular, they significantly increased the level of docetaxel in brain compared to blank-docetaxel and **Lip**. Among them, **Lip-5** showed higher brain concentration. Both pharmacokinetics and distribution study in mice confirmed that this novel brain targeting drug delivery system was a promising carrier to enhance brain delivery capacity for CNS drugs.

1. Introduction

In modern society, central nervous systems diseases, such as Parkinson's disease, brain tumor and other neurodegenerative disorders, have become one of the most dangerous threats to human health, due to the dramatic increase of their incidence rates, especially in younger people, and their low recovery rates. So, the treatment for CNS diseases has become a pressing problem that needs to be solved.

The greatest obstacle of drugs delivery into brain is the presence of blood–brain barrier (BBB) between blood circulation system and central nervous system [1]. The BBB functions as not only a physical barrier but also a biochemical barrier which plays an important role in its further protecting action towards the brain microenvironment [2]. Moreover, the BBB is often the rate-limiting factor in determining the permeation of therapeutic drugs into the brain [3], because the BBB is formed by brain vessel endothelial cells linked together by tight junctions [4]. Therefore, the rate of drugs crossing BBB is relevant to its physico-chemical characteristics such as molecular size, lipid solubility, charge, hydrogen bonding, and ionization profile [5].

In order to facilitate the delivery of drugs to the brain and achieve active targeting, many CNS delivery techniques have been adopted. Because of the high transport affinity and capacity between the substance being transported and components of the membrane, carrier-mediated transporter (CMT) system seems to be one of the most promising methods. The CMT system usually expresses on both the luminal and abluminal membranes of the brain capillary endothelium [6,7]. Among all the physiological transport systems for nutrients and endogenous compounds in CMT, glucose transporter 1 (GLUT1) expressed on the surface of brain capillary endothelial cells is considered as one of the most efficient transport systems [8,9]. GLUT1 can transport glucose, an active small molecule and an essential energy resource for brain function sustenance across the BBB into brain effectively. This unique character also makes itself a potential transporter of brain-targeting drugs [10–12]. To date, several different glycoconjugates serving as candidates for the treatment of CNS diseases have been reported, which could increase the permeability of BBB by carrier mediated transport GLUT1, such as dopamine [13,14] and 7-chlorokynurenic acid [15,16]. Recently, structure–activity relationship studies show that analogs with substituents at the C-6 position of glucose reflect a stronger affinity to GLUT1 than those substituted at other positions [17]. Consequently, based on the above studies, glucose could be an effective means to improve drugs permeability through the BBB. However, preparation of the macromolecular drugs and ionic drugs

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into prodrugs is not ideal, so we should pay more efforts to solve these drawbacks.

As the drug carrier, liposome has many advantages such as non-toxicity, biocompatibility and biodegradability [18]. The modified liposomes can carry hydrophilic, lipophilic and also amphoteric drug molecules to the diseased tissues or target organs [3,19,20]. Thus, the novel liposomal technology for brain targeting is attracting more and more attention of researchers. The modified liposome, in which the multiple surfaces and lipid membrane bilayer are prone to the connection of the monoclonal antibodies or functional ligands, showed efficient drug delivery ability [21]. Therefore, this strategy could be utilized in the design of glucosyl liposome ligands, which are able to cross BBB via GLUT1 as drug carriers in targeting delivery system.

Ligand contains glucose molecule could mediate liposomes through the BBB. Compared to the immune liposomes, which have good targeting properties but their use is restricted because of their own immunogenicity, expensive preparation process and complex operation extremely. Glucosyl modified liposomes showed potential application with brain targeting, high transfer efficiency, good in vivo cycling stability and easy preparation. Based on the previously mentioned ideas, we designed a series of ligands **23**, **24**, **25** and **26** with ester linkage between glucose and cholesterol derivatives (Fig. 1). Using post-insertion technique [22], the lipophilic steroidal portion was embedded into the liposome membrane, while exposing hydrophilic glucose residue outside the membrane for recognizing GLUT1 and connected with the ethylene glycols, which reduced the non-specific binding of proteins and other bioactive molecules [23]. Simultaneously, we assumed that the more the quantity of glucose residues exposed to the liposome surface, the stronger the affinity for GLUT1 showed.

Based on the assumption above, we focused on the quin-antennary glucosides ligand **26** with five glucose residues and one steroid unite, which linked glucose residue via ethylene glycols at C-6 position of glucose [17].This ligand, briefly, still maintained its two functional properties: the lipophilic steroid inset into lipid bilayer, while the five glucose residues were exposed on the surface of liposome for recognizing by GLUT1 and then transported across the BBB to fulfill the blood-to-brain delivery. In order to observe the effect of glucose residues exposed to the liposome surface, we also synthesized the single-antennary glucoside ligand **23**, the reported di-antennary glucosides ligand **24** and tri-antennary glucosides **25** ligand [24] as the reference.

Herein these four designed ligands were described in detail. The objective of our study was to propose a novel brain targeting delivery system mediated by GLUT1. Docetaxel, as a unique anticancer active drug, was used to model drug, which was stable and PH-independent. Furthermore, the brain biodistribution and pharmacokinetics of these liposomes in mice after i.v administration were discussed.

2. Chemistry

The synthetic route of di-antennary glucosides ligand **24** and the tri-antennary glucosides ligand **25** has already been reported in the previous article [24]. The synthetic route of **24** and **25** outlined in Schemes 1 and 2.

In order to successfully complete our synthetic work, glucosylated derivatives have been first synthesized as previously reported [24]. The synthetic route of glucosylated derivative **3** was demonstrated in Scheme 3. The hydroxyl of D-glucose **1** was full protected, then selectively deprotected at C-6 position to get reported compound **2** [25].Finally compound **2** coupled with succinic anhydride to give acid **3** by using DMAP as catalyst.

Next, the synthetic pathway of single-antennary glucoside ligand **23** has been display in Scheme 4. The reaction of available **6** and glucosylated derivative **3** to achieve ester **16** used DCC as dehydrating agent for ester condensation. Then, debenzylation of ester **16** reacted under a mild hydrogen reduction condition with 10%Pd/C in a mixture of MeOH and CH_2Cl_2 to afford the desired product **23**.

The synthetic route used to prepared the quin-antennary glucosides ligand **26** has been illustrated in Scheme 5. The inositol **17** was chosen as the six-branch skeleton, under the conditions of 40% KOH, coupled with redistilled acrylonitrile to produce hexacyano compound **18** at a suitable temperature. The temperature should be controlled below 20 °C to reduce side reactions and increase yield. Hexacyano **18** was converted with H_2SO_4 and EtOH to get hexaester compound **19** in reflux, which was reduced by treating with $LiAlH_4$ using normal operation. The resulting hexol **20** in THF was coupled with cholesteryl carboxylate derivatives **12** at the same mole by dealing with condensing agent to give pentaol **21**, conventional acid as a catalyst used in this reaction is not ideal. For the purpose of the key intermediate **22**, large excess moles of glucosylated derivatives **3** were added in the reaction to ensure a good yield in the presence of DCC as dehydrating agent. Finally, debenzylation of **22** with 10%

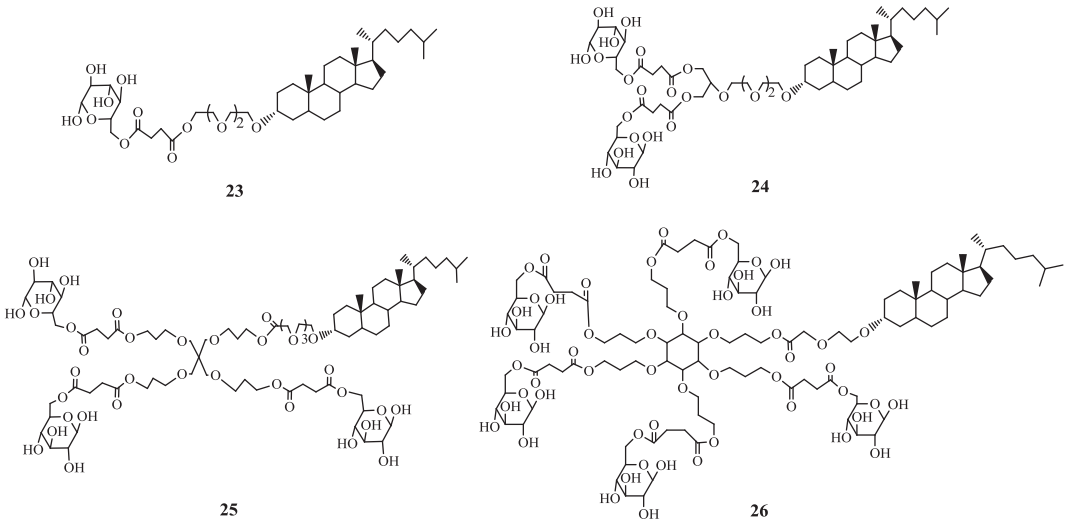
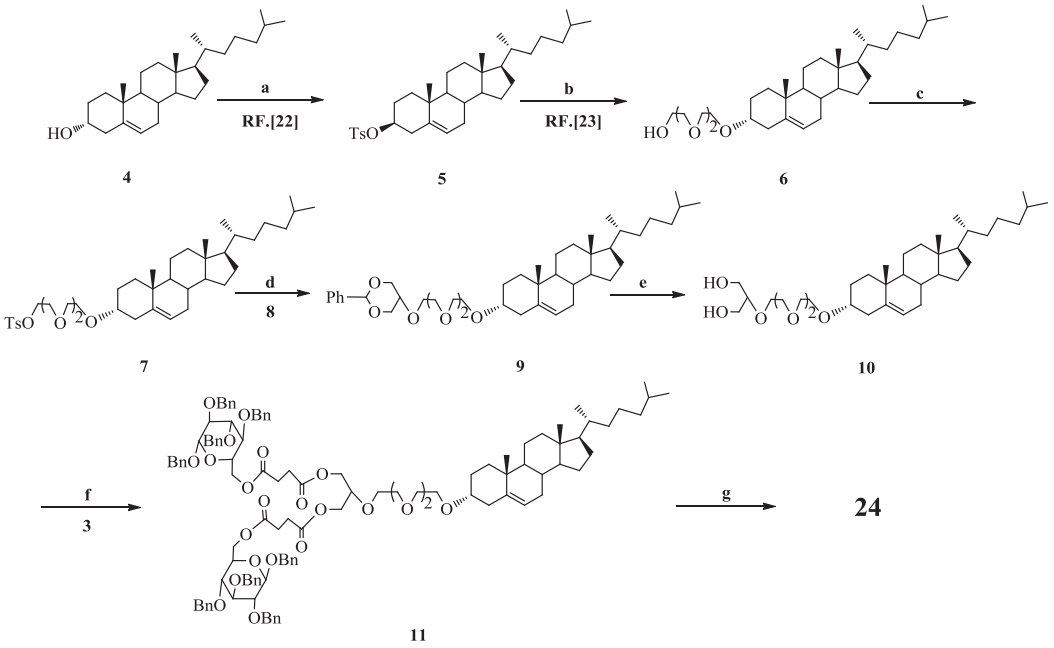


Fig. 1. The structure of multivalent glucosides ligand **23**, **24**, **25**, **26**.



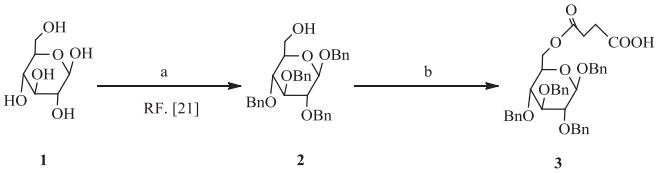
Scheme 1. Reagents and conditions: (a) TsCl, pyridine, 50 °C, 8 h; (b) $HOCH_2$ $(CH_2OCH_2)_2CH_2OH$, dioxane, reflux, 8 h; (c) TsCl, triethylamine, THF, rt, 36 h; (d) 2-phenyl-1,3-dioxan-5-ol **8**, NaH, THF, reflux, 8 h; (e) TsOH,CH_3OH, rt, overnight; (f) glucosylated derivatives **3** DCC, DMAP, rt, CH_2Cl_2, 20 h; (g) H_2, 10%Pd/C, CH_2Cl_2, CH_3OH, 12 h.

Pd/C in THF and MeOH gave the last desired compound **26**. All the title compounds and important intermedium were characterized by their respective IR, ^1H NMR, ^{13}C NMR and MS.

3. Results and discussion

3.1. Preparation of liposomes

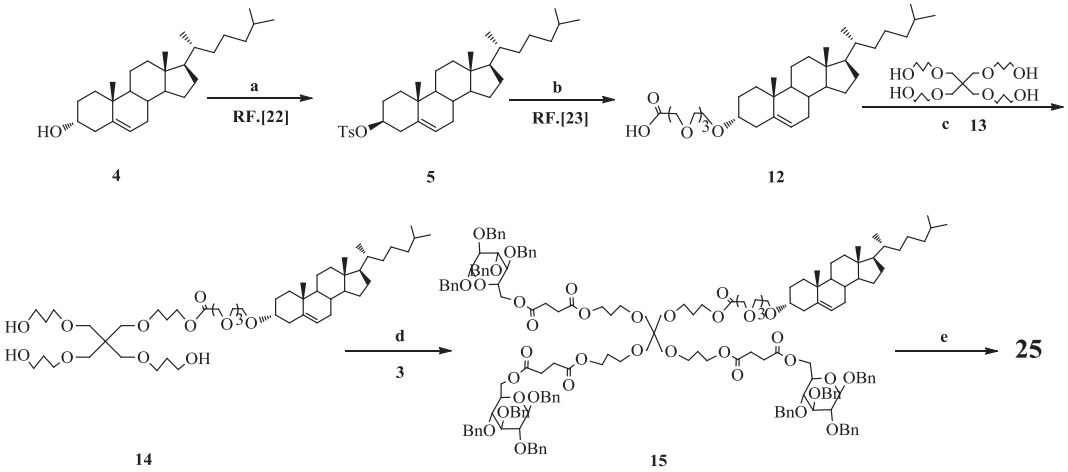
Glucosides-modified liposomes (**Lip-1**, **Lip-2**, **Lip-3**, **Lip-5**) and unmodified liposome **Lip** loaded with docetaxel were prepared by the lipid film hydration-ultrasound method with various modified ligands (**23**, **24**, **25**, **26**) or without ligand. Briefly, lipid materials (phospholipid and cholesterol in a molar ratio of 2:1) and ligand, docetaxel were dissolved in chloroform, and then chloroform was removed by vacuum evaporation and stored *in vacuo* overnight to remove the solvent completely. The lipid film was then incubated in



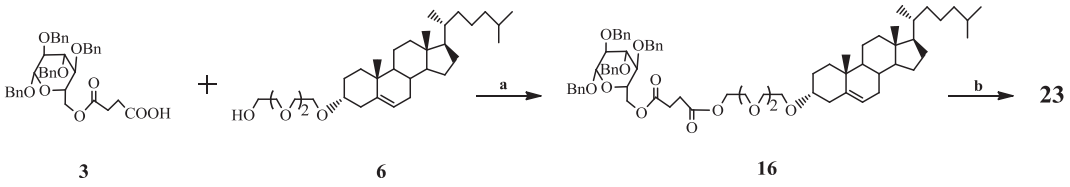
Scheme 3. Reagents and conditions: (a) i. NaH, BnBr, DMF, rt, 24 h; ii. HOAc–Ac_2O (5:1), $ZnCl_2$, 90 min; iii. NaOMe–MeOH, 5 h (b) succinic anhydride, DMAP, CH_2Cl_2, rt, overnight.

PBS rehydration solution (PH = 6.5, 0.02 M) for 90 min at 37 °C with constant mixing. Followed, it was further intermittently sonicated by a probe sonicator at 200 W for 100 s. The final concentration of docetaxel was 0.2 mg/mL.

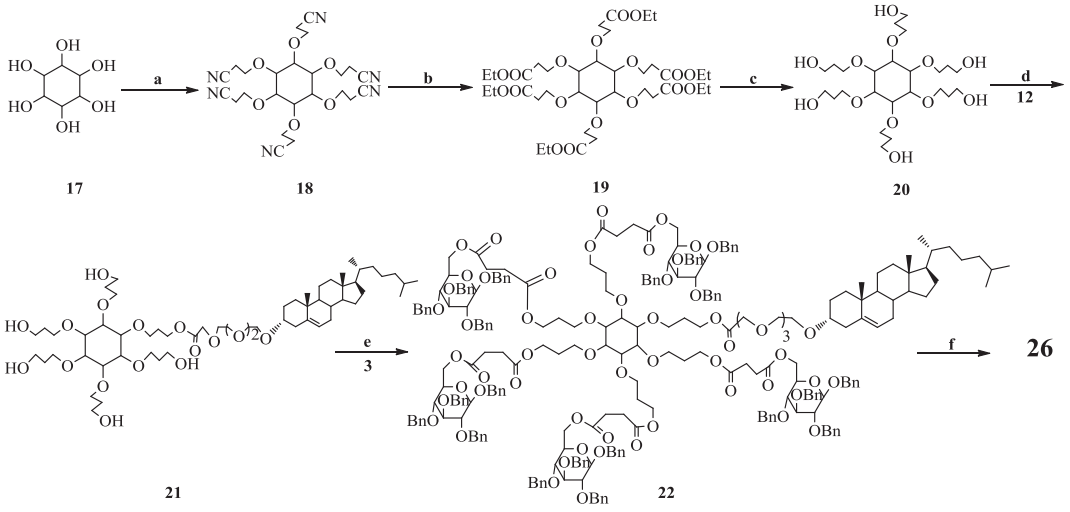
Conventional liposomes were evaluated as the control in this study. Liposome suspensions were diluted with PBS rehydration



Scheme 2. Reagents and conditions: (a) TsCl, pyridine, 50 °C, 8 h; (b) i. $HOCH_2$ $(CH_2OCH_2)_2CH_2OH$, dioxane, reflux, 8 h; ii. *t*-butyl bromoacetate, NaH, THF, reflux, 8 h; iii. TsOH, toluene, reflux, 2 h; (c) pentaerythritol derivatives **13**, DCC, DMAP, rt, CH_2Cl_2, overnight; (d) glucosylated derivatives 3, DCC, DMAP, rt, CH_2Cl_2, 20 h; (e) H_2, 10%Pd/C, THF, CH_3OH, 14 h.



Scheme 4. Reagents and conditions: (a) i. DCC, DMAP, rt, CH_2Cl_2, overnight; (b) H_2, 10%Pd/C, THF, CH_3OH, 10 h.



Scheme 5. Reagents and conditions: (a) acrylonitrile, 40%KOH, 10 °C, overnight; (b) H_2SO_4, EtOH, reflux, 30 h; (c) $LiAlH_4$, anhydrous THF, 6 h; (d) cholesteryl carboxylate derivatives **12**, DCC, DMAP, rt, CH_2Cl_2, 20 h; (e) glucosylated derivatives 3, DCC, DMAP, rt, CH_2Cl_2, 20 h; (f) H_2, 10%Pd/C, THF, CH_3OH, 14 h.

solution, and the average diameters, polydispersity indices (PDI) and zeta-potentials of **Lip**, **Lip-1**, **Lip-2**, **Lip-3**, **Lip-5** were determined.

3.2. Characterization of modified liposomes of different ligands

For the five types of liposomes, encapsulation efficiencies of docetaxel were all greater than or nearly 80%, respectively. Briefly, the lipid materials (Soybean phospholipids and cholesterol) and ligand were in a molar ratio of 8:4:1. The average particle sizes of all liposomes were close to or less than 120 nm, with the value of PDI less than 0.3, fully complied with the conditions. The composition and characterization of all liposomes were shown in Table 1.

3.3. In vivo studies

3.3.1. Methodology evaluation

The chromatograms of blank mouse plasma and brain tissues showed no peaks at the retention time of docetaxel (12 min approximately) and the internal standard diazepam (8 min approximately). A good baseline separation of docetaxel, diazepam, and other major components from mouse plasma and brain tissue samples was achieved under this chromatographic condition.

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

	Molar ratio of lipids and ligand	Size (nm)	PDI	Encapsulating efficiency (EE, %)	Zeta (mv)
Lip	2:1:0	121.6 ± 5.6	0.219 ± 0.024	93.54 ± 2.41	−64.1 ± 9.2
Lip-1	8:4:1	118.3 ± 8.4	0.264 ± 0.019	84.79 ± 3.46	−44.8 ± 1.6
Lip-2	8:4:1	108.4 ± 6.1	0.245 ± 0.022	82.83 ± 5.37	−32.6 ± 3.5
Lip-3	8:4:1	105.5 ± 7.4	0.232 ± 0.027	80.21 ± 7.36	−29.5 ± 2.2
Lip-5	8:4:1	115.1 ± 8.8	0.277 ± 0.034	78.89 ± 9.45	−27.4 ± 3.6

3.3.2. Pharmacokinetics in plasma and brain of each liposome

For in vivo study, **Lip**, **Lip-1**, **Lip-2**, **Lip-3**, **Lip-5** and docetaxel original drug were injected through caudal vein of the mice with a single dose equivalent to 5 mg/kg body weight of docetaxel. At 30 min, 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.

In order to demonstrate the in vivo behavior of the different liposomes, the plasma pharmacokinetics of docetaxel and various liposomes were assessed (Fig. 2). Pharmacokinetics parameters of docetaxel in mice were reported in Table 2 after i.v. administration of each liposome (**Lip**, **Lip-1**, **Lip-2**, **Lip-3**, **Lip-5**) and docetaxel.

The result showed that the area under the concentration–time profile (AUC_{0-t}) of docetaxel in the five types of liposomes was much larger than that of naked docetaxel within 24 h. Free docetaxel of liposomes **Lip**, **Lip-1**, **Lip-2**, **Lip-3**, **Lip-5** and docetaxel presented with an area ratio of 1.14, 1.85, 2.24, 2.72 and 2.53, and the maximum concentration for free docetaxel was 1.12, 2.52, 3.92, 4.71 and 5.42 times that of the five types of docetaxel liposomes. These data indicated that the liposomes showed certain stability which increased the chance to be transported across BBB. The mean residence times (MRT) of docetaxel in plasma after i.v. administration of **Lip**, **Lip-1**, **Lip-2**, **Lip-3**, **Lip-5** were 1.12, 1.04, 1.07, 1.04 and 1.01 times that of docetaxel, respectively, the MRT was the same as the blank-docetaxel or a slight increase. The half lives ($t_{1/2}$) and MRT of docetaxel by these liposomes indicated that the liposomes can prolong the circulation time of docetaxel, by delaying its

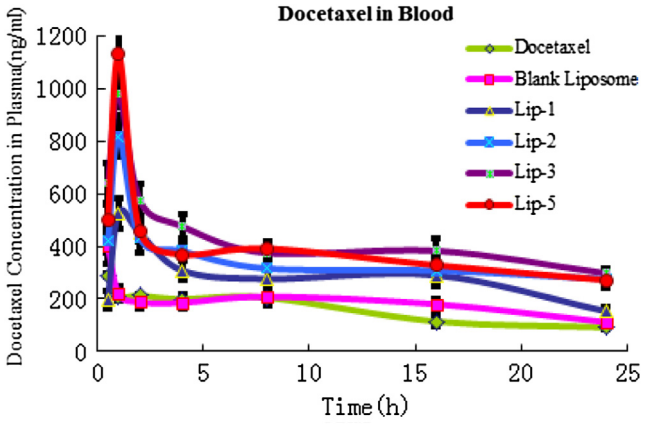


Fig. 2. The concentration curve of docetaxel in plasma versus time after i.v. injection of **Lip**, **Lip-1**, **Lip-2**, **Lip-3**, **Lip-5** and Docetaxel in mice. Data represent the mean ± SD ($n = 3$).

metabolic rate in vivo. So it is obvious that all the liposomes can keep the certain concentration of docetaxel in plasma, which might increase the chance for the drug to be delivered across BBB.

3.3.3. *Distribution in brain of different liposomes in mice*

For further evaluation of the possibility of the glucose-mediated liposome's being transported across BBB, the distributions in brain of **Lip**, **Lip-1**, **Lip-2**, **Lip-3**, **Lip-5** and docetaxel were determined. The distribution of docetaxel was measured at 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, and 24 h after i.v. injection. The concentrations of docetaxel in brain versus time curves were displayed in Fig. 3. After i.v. administration of **Lip**, **Lip-1**, **Lip-2**, **Lip-3**, **Lip-5** and docetaxel, the pharmacokinetic parameters, RE and CE, of docetaxel in brain were reported in Table 3.

In brain, it is obvious that all of the liposomes could be delivered to the brain following i.v. administration. At different time interval, the concentration of docetaxel released from coupled liposomes was much higher than that from uncoupled liposomes and blank-docetaxel during 24 h. The AUC_{0-t} and C_{max} of docetaxel in brain after i.v. administration of liposomes were fairly higher than that after the injection of blank-docetaxel, and it is worth noting that it showed an increasing trend with the increase of the number of glucose residues. The relative uptake efficiencies (REs) were enhanced to 1.80, 2.63, 4.53, 5.70 and 6.09 times that of naked docetaxel for liposomes **Lip**, **Lip-1**, **Lip-2**, **Lip-3**, **Lip-5** respectively. The concentration efficiencies (CEs) were also enhanced to 1.94, 4.11, 5.32, 8.19 and 9.38 times that of docetaxel. So these data further proved our conjecture that all the ligand-modified liposomes can deliver docetaxel across the BBB, and the pharmacokinetic parameters suggested that the brain targeted abilities of these designed liposomes had a descending trend as **Lip-5** > **Lip-3** > **Lip-2** > **Lip-1** > **Lip**. The results indicated that **Lip** also delivered docetaxel across the BBB, but to a significantly lower extent than other liposomes, which may be the reason that **Lip** was not the ligand-modified liposome, but rather rely on the nature of the liposome itself. The higher REs and CEs of modified liposomes demonstrated that exposing glucose residues outside the membrane of coupled liposomes could recognize GLUT1. Moreover, the more the quantity of glucose residues exposed to the liposome surface, the stronger the affinity for GLUT1, which means the stronger ability to transport docetaxel into CNS. Therefore, the **Lip-5** showed the best affinity. Details above indicated that more docetaxel were distributed into brain.

4. Conclusion

For developing a brain-specific drug liposomal carrier, in the present work, we designed and successfully synthesized a serial of multivalent glucosides with high affinity as ligands for brain targeting liposomes. According to the in vivo study, after i.v. administration of the coupled liposomes **Lip-1**, **Lip-2**, **Lip-3**, **Lip-5**, the results showed that the concentrations of docetaxel in brain at different time points were significantly higher than that of uncoupled **Lip** and naked-docetaxel with the same dose. The coupled liposomes exhibited excellent transport ability across the BBB, among which the biodistribution data had indicated higher brain concentration and AUC_{0-t} of Lip-5 than those of other three ones. **Lip-5** seemed to be easier to be transported into CNS, which was attributed to the larger quantity of glucose residues. So, Lip-5 was a potent brain targeting liposome ligand which could enhance liposome's ability of delivering drug into brain.

Generally, this work presented a way that the synthesis of liposome ligand may be used as potential means to improve the therapeutic efficiency in brain diseases.

5. Experimental protocols

5.1. *Chemistry*

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). IR spectra were obtained on a Perkin Elmer983 (Perkin Elmer, Norwalk, CT, USA). Elemental analyses were performed by Atlantic Microlab (Atlanta, GA, USA). ^{1}H NMR spectra were taken on a Varian INOVA400 (Varian, Palo Alto, CA, USA) using $CDCl_3$, *d*-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

Table 2
Pharmacokinetic parameters of docetaxel in blood after administration of docetaxel and liposomes ($n = 3$).

Compounds	$AUC_{(0-t)}$ (ng/ml h)	MRT(h)	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/ml)
Docetaxel	3800.578 ± 173.40	9.664 ± 0.17	16.24	4	208.633 ± 13.33
Lip	4320.478 ± 93.19	10.862 ± 0.44*	20.134	3.333	233.133 ± 22.18***
Lip-1	7044.894 ± 68.78**	10.043 ± 0.70	19.891	1	526.725 ± 57.54***
Lip-2	8509.774 ± 502.60**	10.310 ± 0.11	30.166	1	817.575 ± 78.33***
Lip-3	10322.43 ± 555.59**	10.076 ± 0.21	26.347	1	983.175 ± 56.57***
Lip-5	9625.14 ± 625.06**	9.751 ± 0.28	35.987	1	1129.95 ± 93.40***

*$P < 0.05$ versus Docetaxel.
**$P < 0.01$ versus Docetaxel.
***$P < 0.001$ versus Docetaxel.

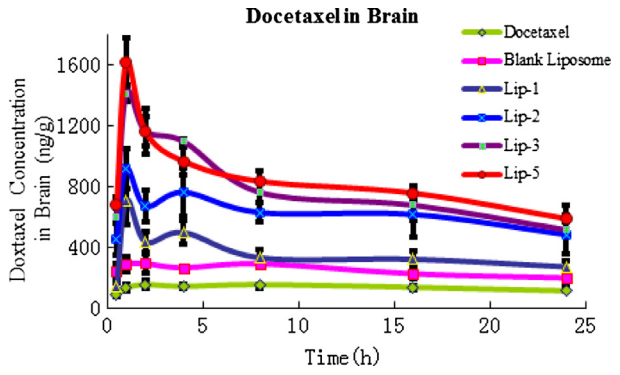


Fig. 3. The concentration curve of docetaxel in brain versus time after i.v. injection of **Lip**, **Lip-1**, **Lip-2**, **Lip-3**, **Lip-5** and Docetaxel in mice. Data represent the mean ± SD ($n = 3$).

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 (CHOLE) was purchased from Bio Life Science& Technology Co., Ltd (Shanghai, People's Republic of China).

*5.1.1. Synthesis of di-antennary glucosides compound **24** [24]*

The known cholesteryl tosylate **5** [26] and tosyl chloride was refluxed for 8 h with triethylene glycol in dry dioxane to get alcohol **6** [27]. Alcohol **6** was converted with tosyl chloride to give tosylate **7** in triethylamine and THF. Tosylate **7** reacted with 2-phenyl-1,3-dioxan-5-ol **8**, which was prepared by the reaction of glycerol with benzaldehyde according to the literature procedure [28], in the presence of NaH in refluxing THF to afford the 1,3-dioxane compound **9**. Then, under the conditions of TsOH, compound **9** was deprotected to achieve 1,3-diol compound **10**. Condensation of intermediate **10** and glucosylated derivative **3** in the presence of DCC as dehydrating agent gave diester **11**. Finally, debenzylation of diester **11** was achieved by catalyzed with 10%Pd/C under hydrogen in MeOH and CH_2Cl_2 to afford the desired product **24**.

*5.1.2. Synthesis of tri-antennary glucosides compound **25** [24]*

The cholesteryl carboxylate derivative **12** was prepared from cholesterol **4** via 3 steps as described in the literature [27]. Tetrol **13** [29] was coupled with cholesteryl carboxylate derivative 12 by dealing with DCC/DMAP in CH_2Cl_2 to get triol **14**. Similarly, as the same method to achieve **24** from **10**, we readily got the second desired product **25** from triol **14**.

*5.1.3. Synthesis of glucosylated derivatives (**3**)*

The prepared known compound **2** [25] (2.56 g, 4.75 mmol) was dissolved in CH_2Cl_2 (50 ml), followed by adding catalytic amount of DMAP and Succinic Anhydride (0.523 g, 5.23 mmol) to the system. The reaction mixture was stirred overnight at room temperature. The solution was then concentrated under vacuum, the resulting syrup was purified by the column chromatography with petroleum ether–acetone as eluent to get a colorless semi-solid. (65%) ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm):7.27–7.39 (m, 20H + $CHCl_3$, Ar–**H**), 4.86 (d, 2H, CH_2Ar, $J = 10.8$ Hz), 4.79 (d, 2H, CH_2Ar, $J = 10.8$ Hz), 4.72 (d, 2H, CH_2Ar, $J = 10.8$ Hz), 4.57 (d, 2H, CH_2Ar, $J = 10.8$ Hz), 4.52 (d, 1H, H-1, $J = 7.6$ Hz), 4.37–4.40 (m, 1H, H-6), 4.29–4.34 (m, 1H, H-2), 4.22–4.26 (m, 2H, H-6′, H-5), 3.54–3.57 (m, 2H, H-4, H-3), 2.63–2.65 (m, 4H, COC**H**$_2$C**H**$_2$CO) MS (m/z): 663.3 ([M + Na]$^+$); $[\alpha]_D^{20}$ +35° ($c = 1.0$, $CHCl_3$).

*5.1.4. Synthesis of glucose-cholesterol derivatives (**16**)*

A solution of compound **3** (1.57 g, 2.46 mmol) in THF, DCC(610.5 mg, 2.95 mmol), and catalytic amount of DMAP were added in reaction bottle. The reaction was activated for half an hour, followed by the addition of alcohol **6** (1.27 g, 2.46 mmol) in THF. The solution was stirred at room temperature overnight, then the white precipitate was filtered off and THF was evaporated under reduced pressure, the crude oil was purified on a silica-gel chromatography column to yield colorless oil **16**. (70%) IR (KBr): ν 3025, 2974, 2887, 1748, 1592, 1242, 1112 cm^{-1} ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 7.27–7.39 (m, 20H + $CHCl_3$, Ar–**H**), 5.34 (m, 1H, chol H-6), 4.86 (d, 2H, CH_2Ar, $J = 10.8$ Hz), 4.79 (d, 2H, CH_2Ar, $J = 10.8$ Hz), 4.72 (d, 2H, CH_2Ar, $J = 10.8$ Hz), 4.57 (d, 2H, CH_2Ar, $J = 10.8$ Hz), 4.52 (d, 1H, H-1, $J = 7.6$ Hz), 4.37–4.40 (m, 1H, H-6), 4.29–4.34 (m, 1H, H-2), 4.22–4.26 (m, 2H, H-6′, H-5), 3.62–3.68 (m, 12H, OC**H**$_2$C**H**$_2$O), 3.54–3.57 (m, 2H, H-4, H-3), 3.18 (m, 1H, chol H-3), 2.63–2.65 (m, 4H, COC**H**$_2$C**H**$_2$CO), 2.37–0.66 (remaining chol protons) with 0.67 (s, 3H, CH_3-18), 0.86 (d, 6H, $J = 6.8$ Hz, CH_3-26, CH_3-27), 0.91 (d, 3H, $J = 6.8$ Hz, CH_3-21), 0.99 (s, 3H, CH_3-19); MS (m/z): 1163.6 ([M + Na]$^+$). Anal. Calcd for $C_{71}H_{96}O_{12}$ C, 74.70; H, 8.48. Found: C, 74.66; H, 8.52.

*5.1.5. Synthesis of single-antennary glucosides compound **23***

The prepared compound **16** (1.8 g, 1.58 mmol) was dissolve in the mixture of methanol and THF, then 10%Pd over activated carbon (180 mg) was added. The reaction was agitated at room temperature under H_2 for 10 h, the reaction was filtered and the filtrate was concentrated under vacuum. The residue was purified on a silica-gel chromatography column, using dichloromethane–methanol as eluent, to get desired ligand **23** as semi-solid. (76%) IR (KBr): ν 3476, 2960, 2892, 1746, 1262, 1086 cm^{-1} ^{1}H NMR (400 MHz, DMSO, δ ppm): 4.54 (d, 1H, H-1, $J = 6.4$ Hz), 4.32–4.30 (m, 1H, H-6), 4.29–4.34 (m, 1H, H-2), 4.27–4.28 (m, 2H, H-6′, H-5), 3.72–3.78 (m, 12H, OC**H**$_2$C**H**$_2$O), 3.64–3.67 (m, 2H, H-4, H-3), 2.93 (m, 1H, chol H-3), 2.55–2.56 (m, 4H, COC**H**$_2$C**H**$_2$CO), 2.37–0.66 (remaining chol protons) with 0.62 (s, 3H, CH_3-18), 0.83 (d, 6H, $J = 6.8$ Hz, CH_3-26, CH_3-27), 0.89 (d, 3H, $J = 6.8$ Hz, CH_3-21), 0.97 (s, 3H, CH_3-19); ^{13}C NMR (CD_3OD-d_6, ppm): 173.2 (1C, **C**OCH$_2$), 171.6 (1C, **C**OCH$_2$), 93.4 (1C, C-1), 82.3 (1C, chol C-3), 75.2 (1C), 74.2 (1C), 72.3 (1C), 71.4 (1C), 70.8 (1C), 70.13 (1C), 69.5 (1C), 68.4 (1C), 67.2 (1C), 64.5 (1C), 63.4 (1C, C-

Table 3
Pharmacokinetic parameters of docetaxel in brain after administration of docetaxel and liposomes ($n = 3$).

Compounds	$AUC_{(0-t)}$ (ng/g h)	MRT (h)	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/g)	Re	CE
Docetaxel	3365.934 ± 125.15	11.437 ± 0.15	98.108	4	171.9 ± 10.27	–	–
Lip	6045.388 ± 358.51*	11.026 ± 0.39	35.675	1.667	334.369 ± 29.48*	1.79605	1.94514
Lip-1	8839.033 ± 322.14**	10.321 ± 0.47	29.219	1	707.696 ± 162.14	2.62603	4.11691
Lip-2	15260.34 ± 868.95**	10.939 ± 0.68	23.123	1	914.424 ± 129.23*	4.53376	5.31951
Lip-3	19180.276 ± 1288.42**	9.914 ± 0.37	18.407	1	1408.03 ± 153.05**	5.69835	8.19098
Lip-5	20526.546 ± 1020.61***	10.226 ± 0.50	24.439	1	1613.023 ± 163.79*	6.09832	9.3835

*$P < 0.05$ versus Docetaxel.
**$P < 0.01$ versus Docetaxel.
***$P < 0.001$ versus Docetaxel.

6), 29.8 (1C, **C**H_2CO), 29.5 (1C, **C**H_2CO), 12.7–56.4 (remaining chol protons) 56.4 (1C), 55.8 (1C), 55.2 (1C), 42.5 (1C), 41.7 (1C), 40.3 (1C), 38.8 (1C), 37.9 (1C), 37.3 (1C), 37.1 (1C), 35.3 (1C), 33.2 (1C), 33.1 (1C), 30.2 (1C), 28.4 (1C), 27.9 (1C), 25.3 (1C), 25.1 (1C), 23.1 (1C), 22.6 (1C), 22.3 (1C), 21.2 (1C), 19.8 (1C), 13.3 (1C), 12.8 (1C); MS (*m*/*z*): 802.5 ([M + Na]$^+$). Anal. Calcd for $C_{43}H_{74}O_{12}$ C, 65.96; H, 9.52. Found: C, 65.92; H, 9.57.

*5.1.6. Synthesis of hexacyano compound **18***

The inositol **17** (1.8 g, 0.01 mol) was dissolved in 1.2 ml 40%KOH solution, then redistilled acrylonitrile was added dropwise in an ice bath. After the dropwise addition, the reaction was controlled the temperature at 10 °C, overnight. TLC monitoring of the reaction is completed, adding dilute HCl adjust the reaction to neutral. Then, the resultant was extracted with DCM (30 ml) and the organic layer was dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. Purification of the crude product by recrystallization form MeOH to reach white solid. (83%) Mp: 124–126 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 2.64 (m, 12H, CH_2CN), 3.2–3.7 (m, 6H, CHO), 4.02 (m, 12H, CH_2O). MS (*m*/*z*): 490.22 ([M + 1]$^+$).

*5.1.7. Synthesis of hexaester compound **19***

In an ice bath, H_2SO_4 (5 ml) was adding dropwise into ethanol (15 ml), the mixture was stirred enough. Then in the solution of H_2SO_4–EtOH was added the hexacyano compound **18**, the reaction was heated to 90 °C and maintained for 30 h. The ethanol was removed under vacuum, then the residue was partitioned between ethyl acetate and water (1:1, v/v). The aqueous layer was the extracted with ethyl acetate, the combined organic layer was washed by 5%Na_2CO_3, then dried with anhydrous Na_2SO_4, and concentrated. The residue was purified on a silica gel chromatography column, using petroleum ether–acetone as eluent, to get compound **19** as colorless oil. (65%) ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 1.24–1.26 (m, 18H, $COOCH_2C\mathbf{H}_3$), 2.54–2.56 (m, 12H, CH_2CO), 3.42–3.92 (m, C 6H + 12H, **H**O**C**$\mathbf{H}_2$), 4.12 (m, 12H, $COOCH_2$). MS (*m*/*z*): 781.42 ([M + 1]$^+$).

*5.1.8. Synthesis of hexol compound **20***

To a suspensions of $LiAlH_4$ (0.414 g, 10.89 mmol) in dry THF at 0 °C, the prepared Hexaester in THF (1.0 g, 1.281 mmol) was added dropwise into the reaction. The reaction mixture was stirred at 25 °C for 20 h. Then, in ice bath the $LiAlH_4$ was destroyed by sequentially added H_2O, 15%NaOH, H_2O(1:1:3) and stirred for 30 min, the reaction was filtered and the filtrate was evaporated under vacuum. The residue was purified on a silica gel chromatography column, using dichloromethane–methanol as eluent, to get compound **20** as colorless oil. (73%) ^{1}H NMR (400 MHz, DMSO, δ ppm): 1.86 (m, 12H, CH_2), 3.31–3.89 (m, 18H, C**H**O**C**$\mathbf{H}_2$; 12H, CH_2O). MS (*m*/*z*): 529.31 ([M + 1]$^+$).

*5.1.9. Synthesis of pentaol compound **21***

A solution of cholesteryl carboxylate derivatives **12** (4.5 g, 7.8 mmol) in THF, DCC (2.1 g, 10.14 mmol), and catalytic amount of DMAP were added in reaction bottle. The reaction was activated for half an hour, followed by the addition of the prepared hexol compound **20** (4.12 g, 7.8 mmol) in THF. The solution was stirred overnight at room temperature, then the white precipitate was filtered off and THF was evaporated under reduced pressure, the crude oil was purified on a silica-gel chromatography column to yield product colorless oil **21**. (54%) ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 5.31 (m, 1H, chol H-6), 4.25 (m, 2H, CH_2OCO), 4.15 (s, 2H, $OCOCH_2O$), 3.82–3.97 (m, 6H, CHO), 3.71–3.78 (m, 12H, O**C**$\mathbf{H}_2$-C$\mathbf{H}_2$O), 3.44–3.71 (m, 12H, C**H**O**C**$\mathbf{H}_2$; 10H, CH_2OH), 3.16 (m, 1H, chol H-3), 1.82–1.86 (m, 12H, CH_2), 2.37–0.66 (remaining chol protons) with 0.65 (s, 3H, CH_3-18), 0.84 (d, 6H, J = 6.8 Hz, CH_3-26, CH_3-27), 0.89 (d, 3H, J = 6.8 Hz, CH_3-21), 0.97 (s, 3H, CH_3-19). MS (*m*/*z*): 1109.74 ([M + Na]$^+$).

*5.1.10. Synthesis of key intermediate compound **22***

To a solution of compound **3** (4.7 g, 7.36 mmol) in CH_2Cl_2, DCC (1.83 g, 8.83 mmol) and catalytic amount of DMAP were added. The reaction mixture was stirring for half an hour, followed by addition of the solution Pentaol **21** (1.0 g, 0.92 mmol) in CH_2Cl_2 dropwise. Then the reaction mixture was kept stirring at room temperature for 20 h. The generated white precipitate was filtered off and CH_2Cl_2 was evaporated under reduced pressure to get the crude oil, which was further purified on a silica-gel chromatography column to give colorless oil. (65%) IR (KBr, cm^{-1}): ν_{max} 3061, 2970, 2885, 1741, 1594, 1262, 1115; ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.27–7.42 (m, 80H + $CHCl_3$, Ar–**H**), 5.30 (m, 1H, chol H-6), 4.97 (d, 5H, CH_2Ar, J = 10.8 Hz), 4.94 (d, 5H, CH_2Ar, J = 10.8 Hz), 4.87 (d, 5H, CH_2Ar, J = 10.8 Hz), 4.78 (d, 5H, CH_2Ar, J = 10.8 Hz), 4.72 (d, 5H, CH_2Ar, J = 10.8 Hz), 4.67 (d, 5H, CH_2Ar, J = 10.6 Hz), 4.64 (d, 5H, CH_2Ar, J = 10.8 Hz), 4.58 (d, 5H, CH_2Ar, J = 10.6 Hz), 4.52 (d, 5H, H-1, J = 7.6 Hz), 4.26–4.31 (m, 5H, H-6), 4.14–4.22 (m, 10H, H-2, H-5), 4.13 (m, 2H, CH_2OCO), 4.11–4.12 (m, 2H, $OCOCH_2O$), 3.67–3.75 (m, 12H, O**C**$\mathbf{H}_2$C$\mathbf{H}_2$O), 3.62–3.65 (m, 18H, C**H**O**C**$\mathbf{H}_2$), 3.58–3.62 (m, 10H, CH_2OCO), 3.45–3.57 (m, 15H, H-3, H-4, H-6′), 3.17 (m, 1H, chol H-3), 2.59–2.62 (m, 20H, CO**C**$\mathbf{H}_2$C$\mathbf{H}_2$CO), 1.84–1.86 (m, 12H, CH_2), 2.37–0.66 (remaining chol protons) with 0.68 (s, 3H, CH_3-18), 0.88 (d, 6H, J = 6.8 Hz, CH_3-26, CH_3-27), 0.91 (d, 3H, J = 6.8 Hz, CH_3-21), 1.01 (s, 3H, CH_3-19); MS (*m*/*z*): 4221.1 [M + Na]$^+$; Anal. Calcd for $C_{249}H_{296}O_{57}$: C, 71.19, H, 7.12; Found: C, 71.24, H, 7.15.

*5.1.11. Synthesis of the quin-antennary glucosides compound **26***

The prepared compound **22** (2.7 g, 0.968 mmol) was dissolve in the mixture of methanol and THF Then 10%Pd over activated carbon (270 mg) was added. The reaction was agitated under H_2 for 14 h, the reaction was filtered and the filtrate was evaporated under vacuum. The residue was purified on a silica gel chromatography column, using dichloromethane–methanol as eluent, to get desired ligand **26** as semi-solid. (70%) ^{1}H NMR (400 MHz, DMSO, δ ppm): ν_{max} 3454, 2973, 2896, 1752, 1251, 1098; ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 4.56 (d, 5H, H-1, J = 7.2 Hz), 4.26–4.31 (m, 5H, H-6), 4.30–4.32 (m, 5H, H-6), 4.26–4.28 (m, 5H, H-2), 4.24 (m, 2H, CH_2OCO), 4.14–4.16 (m, 2H, $OCOCH_2O$), 4.07–4.09 (m, 5H, H-5), 3.67–3.79 (m, 12H, O**C**$\mathbf{H}_2$C$\mathbf{H}_2$O), 3.63–3.67 (m, 18H, C**H**O**C**$\mathbf{H}_2$), 3.53–3.58 (m, 10H, CH_2OCO), 3.42–3.57 (m, 15H, H-3, H-4, H-6′), 3.07 (m, 1H, chol H-3), 2.52–2.54 (m, 20H, CO**C**$\mathbf{H}_2$C$\mathbf{H}_2$CO), 1.78–1.81 (m, 12H, CH_2), 2.37–0.62 (remaining chol protons) with 0.62 (s, 3H, CH_3-18), 0.84 (d, 6H, J = 6.8 Hz, CH_3-26, CH_3-27), 0.88 (d, 3H, J = 6.8 Hz, CH_3-21), 0.94 (s, 3H, CH_3-19); ^{13}C NMR (CD_3OD-d_6, ppm): 175.2 (5C, **C**OCH_2), 174.7 (5C, **C**OCH_2), 172.7 (1C, O**C**OCH_2), 95.1 (5C, C-1), 82.8 (1C, chol C-3), 79.2 (5C, CHO), 78.5 (1C), 77.6 (5C), 76.5 (5C), 74.9 (5C), 74.2 (1C), 73.7 (5C), 72.4 (1C), 71.3 (1C), 70.8 (5C), 70.2 (1C), 69.5 (1C), 69.3 (5C), 68.7 (1C), 68.4 (1C), 64.8 (1C), 63.8 (1C), 63.5 (5C, C-6), 29.8 (5C, **C**H_2CO), 29.6 (5C, **C**H_2CO), 29.2 (5C, CH_2**C**HCH_2), 29.0 (1C), 12.6–58.2 (remaining chol protons) 58.2 (1C), 57.9 (1C), 57.6 (1C), 46.6 (1C), 43.5 (1C), 41.1 (1C), 40.1 (1C), 38.5 (1C), 37.9 (1C), 37.4 (1C), 37.2 (1C), 36.8 (1C), 33.2 (1C), 33.1 (1C), 30.3 (1C), 28.7 (1C), 28.1 (1C), 25.3 (1C), 24.9 (1C), 23.3 (1C), 23.0 (1C), 22.2 (1C), 20.1 (1C), 19.5 (1C), 12.4 (1C), 12.6 (1C); MS (*m*/*z*): 2422.1 [M + Na]$^+$; Anal. Calcd for $C_{109}H_{178}O_{57}$: C, 54.54, H, 7.47; Found: C, 54.48, H, 7.52.

5.2. Biodistribution studies in vivo

5.2.1. 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 mices' 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).

5.2.2. HPLC analysis of liposomes

The waters liquid chromatographic system employed was an LC-10A 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.

5.2.3. 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 animals were killed by cervical dislocation, and the organs were removed and flushed with water for three times to remove the blood remained [30] 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.

5.2.4. Body distribution study

Twenty-one mice were randomly assigned to 7 groups for each liposome or docetaxel. **Lip**, **Lip-1**, **Lip-2**, **Lip-3**, **Lip-5** and docetaxel were given to the mice via the tail vein, each was equivalent to the administration dose of docetaxel of 5 mg/kg. At 30 min, 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. After exsanguinations, the mice were killed by cervical dislocation and the organ were removed and washed twice with water. The organs were weighed and prepared as described earlier. The concentration the docetaxel was analyzed by HPLC.

5.3. 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 (DAS2.0, 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.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.10.007.

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