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Dual-targeting for brain-specific liposomes drug delivery system: Synthesis and preliminary evaluation

Yao Peng[a], Yi Zhao[a], Yang Chen[a], Zhongzhen Yang[a], Li Zhang[a], Wenjiao Xiao[a], Jincheng Yang[b], Li Guo[a,*], Yong Wu[a,*]

[a] Key Laboratory of Drug Targeting and Drug Delivery System of Education Ministry, Department of Medicinal Chemistry, West China School of Pharmacy, Sichuan University, Chengdu 610041, PR China
[b] Department of Pharmacy, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, PR China

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ABSTRACT

The treatment of glioma has become a great challenge because of the existence of brain barrier (BB). In order to develop an efficient brain targeting drug delivery system to greatly improve the brain permeability of anti-cancer drugs, a novel brain-targeted glucose-vitamin C (Glu-Vc) derivative was designed and synthesized as liposome ligand for preparing liposome to effectively deliver paclitaxel (PTX). The liposome was prepared and its particle size, zeta potential, encapsulation efficiency, release profile, stability, hemolysis and cytotoxicity were also characterized. What's more, the cellular uptake of CFPE-labeled Glu-Vc-Lip on $GLUT_1$- and $SVCT_2$-overexpressed C6 cells was 4.79-, 1.95-, 4.00- and 1.53-fold higher than that of Lip, Glu-Lip, Vc-Lip and Glu + Vc-Lip. Also, the Glu-Vc modified liposomes showed superior targeting ability *in vivo* evaluation compared with naked paclitaxel, non-coated, singly-modified and co-modified by physical blending liposomes. The relative uptake efficiency was enhanced by 7.53 fold to that of naked paclitaxel, while the concentration efficiency was up to 7.89 times. What's more, the Glu-Vc modified liposomes also displayed the maximum accumulation of DiD-loaded liposomes at tumor sites with the strongest fluorescence in the brain *in vivo* imaging. Our results suggest that chemical modification of liposomes with warheads of glucose and vitamin C represents a promising and efficient strategy for the development of brain-specific liposomes drug delivery system by utilizing the endogenous transportation mechanism of the warheads.

1. Introduction

With the rapid development of medical science, many diseases have been conquered over the past few decades. While, the central nervous system (CNS) diseases, such as brain tumor, have become one of the most dangerous threats to human health,[1,2] due to dramatic increase of brain diseases and their lower recovery. So, the treatment of brain tumor needs to be solved urgently. However, the two physiological barriers that separate the brain from its blood supply, would significantly decrease the accumulation of anti-cancer drug (including paclitaxel) in the brain and lead to an extremely limited distribution in CNS.[3]

One is the blood-brain barrier (BBB) and the other is the blood-cerebrospinal fluid barrier (BCSFB).[4–10] The barriers control the entry and exit of endogenous and exogenous compounds. Generally, the capability of molecules to cross the barriers by passive diffusion is related to their molecular weight, lipid solubility, charge, hydrogen bonding, ionization profile and physicochemical characteristics.[11,12] As a result, over 98% of small molecule drugs and all the macromolecular drugs could not reach the therapeutic concentration in the brain. Although the vascular-corrected brain concentration could be enhanced by increasing the dose of drug in treatments, it could cause serious side effects and severer toxicity meanwhile. Therefore, there is a huge amount of demand of, not only for paclitaxel (PTX) but in general, strategies that can effectively deliver drugs into the brain for the treatment of brain tumor.[13–15]

Because of the high transport affinity between the transporter and substance, carrier-mediated transporter (CMT) system has become one of the most promising methods to facilitate the delivery of drugs into brain.[16,17] There are plenty of physiological transport systems for nutrients and endogenous compounds, for example, glucose transporter 1 ($GLUT_1$), sodium-dependent vitamin C transporter 2 ($SVCT_2$), large neutral amino acid transporter 1 (LAT_1) and monocarboxylic acid transporter 1 (MCT_1).[18–20]

* Corresponding authors.
E-mail addresses: guoli@scu.edu.cn (L. Guo), wyong@scu.edu.cn (Y. Wu).

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$GLUT_1$ and $SVCT_2$ over-expressed on the surface of brain capillary endothelial cells and choroid plexus epithelium cells respectively, are considered as the most efficient transportation systems.[7,12]

It is well known that the enormous and sustained energy demand of the brain is provided almost exclusively by $GLUT_1$-transported glucose (Glu). It is estimated that transport value of $GLUT_1$ is 15–3000 fold more than other transporters. Liposomes modified by glucose have been proposed as a strategy to improve their brain uptake. Moreover, it has been widely reported that C-6 position glycosylation is an effective way to heighten the accumulation of drugs in brain.[21–23] Our previous studies have also shown that the liposomes modified with glucose could be transported into brain specifically and subsequently release the drugs, hence increasing the concentration in the brain.[16,19] These evidences all suggested that glucose could be used as a good carrier for brain targeting drugs.

In addition to glucose, it was also widely reported that the vitamin C (Vc) derivatives have a superior brain targeting efficiency. Studies have indicated that there is a highest concentration of Vc in the brain, which is 10 fold more than that in other organs.[24] In general, Vc is transported into the brain mainly through two different ways. One is the sugar transporter $GLUT_1$, which can transport dehydro-vitamin C (DHVC, the oxidized form of Vc and can be reduced to Vc in brain). The other way is the transporter $SVCT_2$ which transports Vc into cerebrospinal fluid (CSF) at the choroid plexus, from which Vc can be further diffused to brain extracellular fluid (ECF), and then taken up into the brain cells from ECF. What's more, it was reported that the hydroxyl groups of enediol lactone in C2 and C3 are vital reaction sites for Vc in the redox process, while the C5- and C6-hydroxyl groups of Vc are not critical for its transportation.[25] Therefore, the modification of Vc is always conducted at C5 and C6 sites. Given all the evidences, it is also a promising method to utilize Vc and its transporters to improve the brain targeting ability.

Recently, some studies have explored the possibility of using Glu or Vc as carriers to promote the permeation across the BB into brain. But single-modification with Glu or Vc has limited targeting efficiency with 2–4 fold increase.[16,18,19] In our previous work, we designed and synthesized dual-brain targeting ibuprofen prodrugs, which had much better efficiency *in vitro* and *in vivo* than the parent drug and the single-modified prodrugs.[11] In this study, we will explore the possibility to develop a brain-targeting liposomes drug delivery system which uses glucose and vitamin C as the mediators to improve the brain permeability. We aim to construct a dual-targeting moiety Glu-Vc-Chol as liposomes ligand to delivery PTX into brain effectively. What's more, the Glu-Vc modified, non-coated, singly-modified and co-modified by physical blending liposomes (Glu + Vc-Lip) were prepared by the lipid film hydration-ultrasound method, and the characteristics and brain target abilities were conducted *in vitro* and *in vivo*.

2. Results and discussion

2.1. Synthesis of liposomes ligands

The synthetic route of ligand Glu-Vc-Chol was illustrated in Scheme 1. Firstly, we synthesized the glycosylated derivative **5** and the ascorbic acid intermediate **9**. Briefly, glucose **1** was totally etherified with chlorotrimethylsilane (TMSCl) and hexamethyl disilazane in pyridine to give penta-O-trimethylsilyl-glucopyranose **2**, which was treated by the mixture of acetone, acetic acid and methanol to deprotect the C-6 TMS group selectively to obtain compound **3**. Then, the linker 4-(benzyloxy)-4-oxobutanoic acid was connected with intermediate **3** to generate compound **4**. After deprotecting the benzyl group from **4**, glucose-TMS derivative **5** was obtained.[12] The synthesis of intermediate **9** started from the available material Vc. Treatment of **6** with acetyl chloride in acetone following our earlier method afforded 5,6-O,O-isopropylidene protected Vc ketol **7**. Benzylation of the C-2 and C-3 hydroxy groups of the ketol **7** was accomplished using K_2CO_3 and benzyl bromide in acetone to provide **8**. Deblocking of the 5,6-O,O-protected derivative of **8** with HCl in CH_3CN solution gave 2,3-O,O-dibenzyl Vc intermediate **9**.[7] What's more, cholesterol **10** underwent five steps to generate compound **15**,[13] which was then conjugated with glycosylated derivative **5** in the presence of DCC and DMAP to give compound **16**. Finally, compound **16** was readily underwent a deprotection reaction to get compound **17** when subjected the condition of trifluoroacetic acid in dichloromethane, then the 2,3-O-di-benzyl groups of **17** was removed under hydrogen catalyzed by Pd/C to provide the target ligand Glu-Vc-Chol.

The synthetic route of ligand Vc-Chol was illustrated in Scheme 2. Briefly, the 2,3-O-di-benzyl groups of **15** was removed under hydrogen catalyzed by Pd/C to provide the target ligand Vc-Chol. All the title compounds and important intermediates were characterized by their respective ^{1}H NMR and MS.

2.2. Preparation and characterization of liposomes

One of the requirement for liposomes to penetrate BBB is that they must have proper sizes and uniform distribution. The particle sizes and zeta potentials of different liposomes in this study were listed in Table 1. The encapsulation efficiencies (EE%) of paclitaxel in the each type of liposomes were all greater than 82%. The average particle sizes of all liposomes were less than 110 nm, and the values of polymer dispersity index (PDI) were close to 0.19. What's more, the transmission electron microscopy (TEM) of PTX-Glu-Vc-Lip showed that the liposomes exhibited uniform spherical in shape (Fig. 1). To our knowledge, the particle size and zeta potential of liposomes were crucial to *in vivo* study.[20]

2.3. In vitro drug release study

PTX release properties were evaluated in PBS containing 0.1% Tween 80. As shown in Fig. 2, for free PTX, the release exhibited a rapid property, with over 80% of the drug released into the media within 12 h incubation. On the other hand, the PTX-loaded liposomes exhibited the sustained release behaviors, with the cumulative PTX released from liposomes was less than 60% after 48 h incubation. No significant difference on release properties was observed among PTX-Lip, PTX-Glu-Lip, PTX-Vc-Lip, PTX-Glu-Vc-Lip and PTX-Glu + Vc-Lip, and none of these PTX-loaded liposomes displayed burst initial release patterns.

2.4. In vitro stability of liposomes in serum

It is important for liposomes to have superior stability in biological conditions, which is closely related to 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 able to prevent the interaction between liposomes and serum protein, which was important for the long blood half-life *in vivo*.

2.5. Hemolysis assays

Hemocompatibility is a key point for *in vivo* applications of liposomes. As shown in Fig. 4, hemolysis assay of ligand-modified liposomes demonstrated that all the liposomes did not significantly increase the hemocompatibility (less than 10%) even the concentration of phospholipids up to 400 nmoles, which indicated that the lipid material was almost no-toxic.

2.6. Cytotoxicity

The cytotoxicity of different liposomes on C6 cells was evaluated