p-Hydroxybenzoic acid (*p*-HA) modified polymeric micelles for brain-targeted docetaxel delivery

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Chemotherapies for brain diseases have been hampered due to the inability of transport of drug across the blood-brain barrier (BBB). In order to overcome the barrier, *p*-hydroxybenzoic acid (*p*-HA), a small molecule of benzamide analogue, was used as a ligand for brain-targeted drug delivery. The *p*-HA was conjugated to PEG-DSPE to form *p*-HA-PEG-DSPE. Docetaxel-loaded polymeric micelles were prepared by a thin-film hydration method using methoxy-poly(ethylene glycol)-distearoylphosphatidylethanolamine (mPEG₂₀₀₀-DSPE) as a carrier and the *p*-HA-PEG-DSPE as a brain targeted material. The prepared micelles showed spherical with a mean diameter of (18±3) nm. Encapsulation efficiency and drug loading were (83.49±1.3)%, (7.7±1.2)% for unmodified micelles and (80.65±1.6)%, (7.47±1.8)% for *p*-HA-modified micelles, respectively. *In vitro* cellular uptake experiments showed that the *p*-HA-modified micelles increased BCECs cellular uptake by 1.2 times compared to the unmodified micelles. *Ex vivo* near-infrared fluorescence imaging showed that brain uptake of the *p*-HA-modified micelles was 1.3–1.8 times higher than that of the unmodified micelles. *In vitro* cytotoxicity assay against glioblastoma cell U87 MG showed that inhibition rate of the *p*-HA-modified micelles increased by 1.2 times compared to that of the unmodified micelles and 1.7 times compared to that of DTX. Survival time of nude mice bearing intracranial glioblastoma showed that the lifetime of saline group, Taxotere group, mPEG-DSPE/DTX micelles group and *p*-HA-PEG-DSPE/DTX micelles group was 22, 27, 32 and 45.8 d, representively, which indicated that anti-glioblastoma activity of DTX could be significantly enhanced by the *p*-HA-modified polymeric micelles. These results demonstrated that the *p*-HA-modified micelles could be a promising brain-targeted drug delivery system for hydrophobic drugs against glioblastoma.

***p*-hydroxybenzoic acid (*p*-HA), brain-targeting, polymeric micelles, docetaxel, glioblastoma**

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Blood-brain barrier (BBB) is a physical dynamic barrier between brain and blood, which serves as a protection barrier to effectively prevent certain foreign substances from entering the brain blood circulation. Due to poor permeability of the BBB, nearly 100% of large molecules and 98% of small molecules are forbidden access to central nervous system (CNS). Consequently, it hampers treatment and diagnosis of CNS diseases such as Alzheimer's diseases, brain tumors, and Parkinson's diseases [1–4].

To deliver therapeutic agents across the BBB, mechanisms for transport of endogenous molecules into the brain have been employed such as receptor-mediated endocytosis, adsorptive mediated transcytosis, transporter uptake and membrane permeation of lipophilic molecules [5,6]. Receptor-mediated drug delivery systems represent very promising non-invasive strategy for active targeted drug delivery to the site of brain tumors [7–9]. Various receptors and transporters are present on the luminal endothelial plasma membranes, including the transferrin receptor [10], the insulin receptor [11], endothelial growth factor receptor

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[12], low-density lipoprotein receptor [13] and glucose transporter [14] providing binding targets for drug delivery systems.

Benzamide analogue is a kind of small molecules used for the treatment of central nervous diseases. Among them, tiapride could be used for the treatment of behavioral disturbances and non-cognitive symptoms in children agitation; sultopride has strong antipsychotic effects [15,16]. Benzamide analogues were found to have high affinity with dopamine receptors that are prominent in most parts of CNS. D1 and D2 dopamine receptors could be found in nigrostriatal pathway comprising the neurons of the substantia nigra and neostriatum [17]. In recent years, a considerable amount of research has been directed towards synthesis of substituted benzamides as ligands for visualization of dopamine D2 receptor binding in the brain by positron emission tomography [18].

Of the nanoparticle drug delivery systems, PEG-lipid micelles have been extensively used as vehicles for hydrophobic drugs due to the high stability, controlled drug release, and low toxicity [19–24]. In the present study, a novel brain-targeted nanoparticle drug delivery system based on a small molecule ligand (*p*-Hydroxybenzoic Acid, *p*-HA) was developed [25]. Few studies have reported on PEG-DSPE micelles modified with *p*-HA. In this study, *p*-HA-modified micelles was first established, then *in vitro* and *in vivo* targeting effect of the micelles was investigated. The anti-glioblastoma efficacy of DTX-loaded micelles was studied on intracranial glioblastoma bearing nude mice.

1 Materials and methods

1.1 Materials

DIEA (Diisopropylethylamine) were supplied by Fluka (Milwaukee, USA). Docetaxel was kindly provided by Prof. Y. P. Li from Shanghai Institute of Materia Medica (SIMM). EDC·HCl (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) were purchased from GL Biochem Ltd (Shanghai, China). mPEG₂₀₀₀-DSPE (methoxy-poly(ethylene glycol)-distearoylphosphatidylethanolamine) was purchased from Lipoid GmbH (Ludwigshafen, Germany). NH₂-PEG-DSPE was purchased from Laysan Bio Co. (USA). DiR (1,1'-dioctadecyl-3,3',3'-tetramethyl indotricarbocyanine iodide) was from Invitrogen (USA). DMEM medium and FBS (fetal bovine serum) were purchased from Gibco Co. (USA). Methanol, acetonitrile, and other HPLC grade reagents were obtained from Fisher Scientific (USA). All the chemicals were analytic reagent grade.

1.2 Cell lines and animals

Brain capillary endothelial cells (BCECs) were kindly provided by Prof. X. G. Jiang (School of Pharmacy, Fudan University). U87 MG cell line was obtained from the Insti-

tute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. Both cells were cultured in DMEM medium, supplemented with 10% FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin sulfate. All the cells were cultured in incubators maintained at 37°C with 5% CO₂ under fully humidified conditions. All the experiments were performed on cells (passages from 5 to 30) in the logarithmic phase of growth.

BALB/c nude mice (male, 4–5 weeks, weighing 18–20 g) were supplied by Department of Experimental Animals, Fudan University (Shanghai, China). All the animal experiments were carried out in accordance with guidelines evaluated and approved by the Ethics Committee of Fudan University.

1.3 Synthesis and characterization of *p*-HA-PEG-DSPE

p-HA (27.6 mg) was dissolved in anhydrous DMF (3 mL) together with NHS and EDC·HCl. Then 20 μL of DIEA were added and incubated for 3 h. The resulting mixture was centrifuged and the supernatant was added dropwise into a solution of NH₂-PEG-DSPE in anhydrous DMF (72 mg/mL). The solution was stirred overnight at room temperature and precipitated using cold diethyl ether for three times. The precipitation was then vacuum-dried for 2 h. The resulting *p*-HA-PEG-DSPE was purified using a dialysis method (MWCO 3.5 kD, Millipore) against distilled water. Finally the *p*-HA-PEG-DSPE solution was lyophilized and stored at –20°C. The structure of the conjugates was characterized via ¹H-NMR spectroscopy using CDCl₃ as a solvent at 25°C.

1.4 Preparation and characterization of *p*-HA-PEG-DSPE micelles

(1) Preparation of micelles. Micelles loaded with coumarin 6, DiR or DTX were prepared using the thin-film hydration and extrusion method [26]. A mixture of mPEG-DSPE/*p*-HA-PEG-DSPE (95:5 by molar) with coumarin 6, DiR or DTX in acetonitrile was rotary evaporated to form a thin film. Then the film was vacuum desiccated overnight. To form spontaneous self-assembly of the micelles, the dried polymeric film was dissolved in 10 mmol/L HEPES-buffered saline (HBS, pH 7.4) and incubated at a constant temperature of 37°C under stirring for 0.5 h. The obtained micelle was filtrated against 0.22 μm filter membrane (millipore) to remove unencapsulated DTX. To purify the coumarin 6 and DiR-loaded micelles, size exclusive chromatography was carried on a G-50 gel column using HBS as the mobile phase.

The particle size and distribution were determined via dynamic light scattering (DLS) analysis using Malvern Zetasizer Nano ZS (Malvern, UK) at 25°C. Each test was measured in triplicate. The morphology of the micelles was

studied using Transmission Electron Microscope (TEM).

(2) Drug-loading capacity, encapsulation efficiency and *in vitro* release. To value the encapsulation efficiency (E.E.) and loading capacity (L.C.), the DTX-loaded micelle was diluted in acetonitrile and the concentration of DTX was measured via HPLC (mobile phase: acetonitrile/water (65/35, v/v), λ : 230 nm) [27]. E.E. was defined as the ratio between actual DTX amount determined and theoretic feeding DTX amount. L.C. was defined as the ratio between DTX amount determined and the weight of lyophilized micelles.

In vitro release profiles of the DTX-loaded micelles were performed in 20 mL of PBS containing 0.5% of Tween-80. Release samples analyzed via HPLC.

1.5 Targeting ability study of *p*-HA-PEG-DSPE micelles

(1) Cellular uptake of *p*-HA-PEG-DSPE micelles. Cellular internalization of coumarin 6-loaded micelles were studied. BCECs cells were seeded into 12-well plates, and each micelle formulation with the same coumarin 6 concentration of 25 ng/mL was added and incubated at 37°C for 1 h. After incubation, the medium was removed and the cells were washed three times with PBS, then visualized using a fluorescent microscope. For quantitative analysis, BCECs cells were trypsinized and centrifuged (1000 r/min, 5 min), washed, resuspended in PBS and analyzed using a flow cytometer (FACSaria, BD, USA).

(2) *In vivo* distribution of *p*-HA-PEG-DSPE micelles. Potential brain-targeting effect of *p*-HA-PEG-DSPE micelles was evaluated in normal nude mice [25]. Following *i.v.* administration, time-dependent biodistribution of the DiR-labeled micelles was observed at 1, 2, 4, 8, 12 and 24 h, respectively. The mice were then sacrificed to excise major organs that were photographed *ex vivo*.

1.6 Pharmacodynamic study of *p*-HA-PEG-DSPE micelles

(1) *In vitro* cytotoxicity assay. *In vitro* cytotoxicity was determined using the MTT assay [28]. U87 MG cells were seeded to 96-well plates in 200 μ L of medium to obtain a

concentration of 3×10^3 cells per well, and then the plates were incubated for 24 h. The medium in each well was refreshed with 200 μ L of the *p*-HA-PEG-DSPE/DTX micelles, mPEG-PEG-DSPE/DTX micelles and Taxotere[®] respectively and incubated at 37°C for 72 h. The concentration of DTX ranged from 0.625 to 102.4 μ mol/L. Experiments were repeated for at least three times. Percent cell viability was calculated on the basis of optical density values of sample wells versus reference wells (PowerWave XS, Bio-TEK, USA).

(2) Anti-glioblastoma study of *p*-HA-PEG-DSPE/DTX micelles. Therapeutic efficacy of DTX-loaded micelles was investigated in a xenograft mouse model bearing human U87 multiforme glioblastoma [29]. The intracranial U87MG bearing nude mice were randomly divided into four groups ($n=9$) and treated with the *p*-HA-PEG-DSPE/DTX micelles, unmodified mPEG-PEG-DSPE/DTX micelles, Taxotere[®] and saline (dose: 8 mg/kg of body weight at 6, 9, 12, and 15 d post-tumor implantation). The survival times were recorded.

2 Result and discussion

2.1 Characterization of *p*-HA-PEG-DSPE

The conjugation of *p*-HA to PEG-DSPE was confirmed from the ¹H-NMR spectrum. Results showed that the protons of *p*-HA at 6.8–7.8 ppm. The chemical shifts at 3.2–4.0 ppm and 0.8–1.5 represent the PEG (–O–CH₂–CH₂–O–) and the DSPE, respectively. The ¹H-NMR spectrum results indicated the successful synthesis of *p*-HA-PEG-DSPE.

2.2 Characterization of the *p*-HA-PEG-DSPE micelles

As shown in Figure 1, the *p*-HA-PEG-DSPE/DTX micelles, the mPEG-DSPE/DTX micelles and blank micelles showed spherical morphology with mean diameters around 18 nm.

The micelles with or without *p*-HA modification showed similar particle sizes, polydispersity indexes as well as drug leakage rates, which indicated that the incorporation of *p*-HA-PEG-DSPE into micelles had no influence on the physical properties of the PEG-DSPE micelle system.

Encapsulation efficiency and drug loading were $(83.49 \pm$

Figure 1 Transmission electron microscope photographs of the micelles ($\times 80000$). (a) mPEG-PEG-DSPE/DTX micelles; (b) *p*-HA-PEG-DSPE/DTX micelles; (c) mPEG-PEG-DSPE micelles (blank).

1.3)%, (7.7±1.2)% for unmodified micelles and (80.65±1.6)%, (7.47±1.8)% for *p*-HA-modified micelles, respectively.

Drug leakage rates at pH 7.4 and pH 5.0 in 96 h are shown in Figure 2. Both of the *p*-HA-PEG-DSPE/DTX micelles and mPEG-PEG-DSPE/DTX micelles exhibited a sustained-release profile in that the total DTX release from micelles was about 40% in the initial 24 h in PBS (pH 7.4), 50% in PBS (pH 7.4) and 74% in PBS (pH 5.0) for 96 h, respectively (Figure 2(a)). At the same time, there was no obvious difference between *p*-HA-DTX micelle and mPEG-DTX micelle (Figure 2(b)).

Figure 2 *In vitro* drug release profiles of DTX-loaded micelles in pH 5.0 and 7.4 PBS at 37°C (a) ($n=3$, mean±SD); *in vitro* drug release profiles of DTX-loaded micelles from 7.4 PBS at 37°C (b) ($n=3$, mean±SD).

Figure 3 Cellular internalization of free C6 and the C6-loaded micelles. Panels above: micrographs taken in the phase-contrast mode, panels below: the same field viewed in the fluorescence mode. (a)–(c) Free C6; (d)–(f) mPEG-PEG-DSPE/C6; (g)–(i) *p*-HA-PEG-DSPE/C6.

results indicated that *p*-HA could enhance micelles uptake by BCECs cells *in vitro*.

(2) *In vivo* distribution of *p*-HA-PEG-DSPE/DiR micelles. *p*-HA-PEG-DSPE/DiR micelles and mPEG-DSPE/DiR micelles were injected via caudal vein in normal nude mice. Visible fluorescence accumulation appeared much stronger in the brains of *p*-HA-PEG-DSPE/DiR micelle group, compared with the normal micelle group through the entire studies (Figure 4). *Ex vivo* fluorescent image and semi quantitative of excised organs further confirmed that fluorescence accumulation in the brains treated with the *p*-HA-PEG-DSPE/DiR micelles were 1.3–1.8 times higher compared with the normal micelle group, except for the highest fluorescence in liver which had high macrophage uptake nature. Compared with the normal micelle group, fluorescence signal in the brains of the *p*-HA-PEG-DSPE micelle group was remarkably stronger at any time post-injection ranged from 2 to 12 h (Figure 5).

(3) *In vitro* cytotoxicity studies. *In vitro* cytotoxicity of DTX-loaded micelles was evaluated on U87 MG cells via MTT assay. Growth inhibition effect against U87 MG cells was observed in both groups. The IC₅₀ values for Taxotere, the mPEG-DSPE/DTX micelles and the *p*-HA-PEG-DSPE/DTX micelles were 177.8, 125.9, and 104.7 nmol/L, respectively. The IC₅₀ of the DTX-loaded micelles were lower than Taxotere®, indicating that the micelles were more efficient to mediate the cytotoxic effect of DTX than Taxotere

Figure 4 *In vivo* non-invasive NIR fluorescence images of the *p*-HA-PEG-DSPE/DiR (rats at the right side) and the mPEG-DSPE/DiR (rats at the left side) after intravenous administration.

to U87 MG tumor cells.

(4) Anti-glioblastoma studies of the *p*-HA-PEG-DSPE/DTX micelles. Figure 6 represents Kaplan-Meier survival curves of the DTX-loaded formulations. The median survival time of the *p*-HA-PEG-DSPE/DTX micelle group, the

Figure 5 *Ex vivo* imaging and semi-quantitative analysis of dissected organs at different time points. (A) mPEG-DSPE/DiR micelles; (B) *p*-HA-PEG-DSPE/DiR micelles.

Figure 6 Kaplan-Meier survival curves of mice bearing intracranial U87 glioblastoma.

mPEG-DSPE/DTX micelle group, Taxotere® group and saline group were 45.8, 32, 27 and 22 d, respectively. These results demonstrated that the survival of the *p*-HA-PEG-DSPE/DTX micelle group was significantly longer than those of other groups ($P < 0.05$). These survival data suggested that *p*-HA could mediate BBB transport of polymeric micelles, thus enhancing therapeutic efficacy of chemotherapy drugs against brain tumors.

3 Conclusions

The *p*-HA-modified micelles exhibited strong enhancement on the uptake against the BBB both *in vitro* and *in vivo*. All data indicated that this novel small molecular (*p*-HA) can be used in the anti-tumor drug delivery system which targeting to the brain, and, *p*-HA-conjugated micelles could have a potential value of clinical application.

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