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Dual-Targeting Topotecan Liposomes Modified with Tamoxifen and Wheat Germ Agglutinin Significantly Improve Drug Transport across the Blood−Brain Barrier and Survival of Brain Tumor-Bearing Animals

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Abstract: Chemotherapy of brain tumors remains a big challenge owing to the low drug transport across the blood−brain barrier (BBB), multidrug resistance (MDR), and poor penetration into the tumor tissue. We developed a novel dual-targeting liposomal carrier that enabled drug to transport across the BBB and then target the brain tumor. In the dual-targeting liposomal carrier, tamoxifen (TAM) was incorporated into the lipid bilayer membrane of liposomes and wheat germ agglutinin (WGA) was conjugated to the liposomes' surface. Topotecan was then loaded into the above liposomes. In vitro, topotecan liposomes modified with TAM and WGA were applied to the glioma cells, BBB model, and avascular C6 glioma spheroids, respectively. In vivo, they were systemically administered via vein to brain C6 glioma-bearing rats. In view of the microtiter tetrazolium (MTT) results, topotecan liposomes modified with TAM and WGA exhibited a significant inhibitory effect compared to unmodified topotecan liposomes, suggesting that TAM plus WGA contributed strong drug delivery effects into the brain tumor cells after direct drug exposure. In the experiments of drug transport across the BBB model following drug exposure to tumor cells, topotecan liposomes modified with TAM and WGA exhibited the most robust dual-targeting effects: crossing the BBB and then targeting brain tumor cells. Similar strong activity was found in the reduction of C6 glioma tumor spheroid volume and in the apoptosis of the spheroids. In the brain tumor-bearing rats, the dual-targeting effects of topotecan liposomes modified with TAM and WGA could be evidently observed, resulting in a significant improvement in the overall survival of the brain tumor-bearing rats compared with free topotecan and topotecan liposomes. Moreover, results from an extended treatment group indicated that the survival could be further significantly enhanced, indicating that an extended chemotherapy with topotecan liposomes modified with TAM and WGA would be beneficial for treatment. The dual-targeting effects in vivo of topotecan liposomes modified with TAM and WGA could be related to an enhanced effect by TAM via inhibiting efflux of MDR proteins in the BBB and the brain tumor, and an enhanced effect by WGA via endocytosis in the BBB and in the brain tumor. In conclusion, topotecan liposomes modified with TAM and WGA significantly improve topotecan transport across the blood−brain barrier and the survival of brain tumor-bearing animals, showing dual-targeting effects. These findings would encourage further developments of noninvasive therapy for brain tumor.

Keywords: Topotecan liposomes modified with TAM and WGA; dual-targeting; BBB model; brain tumor model; survival

Introduction

Brain tumors remain a significant health problem worldwide. In several recent meta-analyses, albeit chemotherapy has shown a survival benefit to high-grade glioma patients, the results are still modest at best.[1] To be effective, a chemotherapy agent must be exposed to brain tumor cells at concentrations that can overcome intrinsic resistance mechanisms. However, this is difficult for most drugs, owing to the blood−brain barrier (BBB), which is formed by a network of closely sealed endothelial cells in the brain's capillaries. The BBB expresses a high level of proteins that pump foreign molecules away from the brain, while allowing others that are necessary to the function of the brain cells to cross the barrier. It is estimated that less than 1% of the circulation compounds are able to reach the vasculature of a CNS tumor via systemic administration.[2,3] Therefore, there is a need for a functional carrier that can transfer drugs across the BBB and then target the tumor. In this regard, modified liposomes could reach such functions due to their lipid membrane bilayer and functional advantages instigating from the multiple surfaces that can be used for conjugating functional ligands or monoclonal antibody[4] or drugs[5] and from the presence of an aqueous inner volume in which water-soluble drugs can be encapsulated.[6] We hypothesized that a dual-targeting carrier may act as drug transporting carrier which could cross the BBB and then target the brain tumor.

Topotecan is a water-soluble derivative of camptothecin, which is able to stabilize the DNA topoisomerase I complex thereby interfering with DNA replication and DNA repair. In preclinical studies, topotecan showed antitumor activity against xenograft models of primary CNS tumors, such as medulloblastoma and high-grade gliomas.[7,8] However, the results from clinical trials of topotecan for gliomas have been shown to be discouraging.[9,10] The poor response of primary CNS tumors to chemotherapy agents could be multifactorial, but inadequate delivery of active compounds to the tumor site in the brain is the major obstacle due to the fact that ATP-binding cassette (ABC) proteins lead to multidrug resistance in primary CNS malignancies.[11,12] Previous studies have demonstrated that topotecan is a substrate for P-glycoprotein (MDR1 protein; ABCB1),[13] MRP4 (ABCC4),[14] and breast cancer resistance protein (BCRP).[15,16] Overcoming MDR1, MRP4 and BCRP proteins is thus a strategy to improve CNS penetration and to deliver anticancer agents

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(1) Stewart, L. A. Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials. *Lancet* **2002**, *359*, 1011–1018.

(2) Newton, H. B. Advances in strategious to improve drug delivery to brain tumor. *Expert. Rev. Neurother.* **2006**, *6*, 1495–1509.

(3) Groothuis, D. R. The blood-brain and blood-tumor barriers: review of strategies for increasing drug delivery. *Neuro-Oncology* **2000**, *2*, 45–59.

(4) Bendas, G.; Rothe, U.; Scherphof, G. L.; Kamps, J. A. The influence of repeated injections on pharmacokinetics and biodistribution of different types of sterically stabilized immunoliposomes. *Biochim. Biophys. Acta* **2003**, *1609*, 63–70.

(5) Duncan, R. Polymer conjugates as anticancer nanomedicines. *Nat. Rev. Cancer* **2006**, *6*, 688–701.

(6) Arnold, R. D.; Mager, D. E.; Slack, J. E.; Straubinger, R. M. Effect of repetitive administration of Doxorubicin-containing liposomes on plasma pharmacokinetics and drug biodistribution in a rat brain tumor model. *Clin. Cancer Res.* **2005**, *11*, 8856–8865.

(7) Pawlik, C. A.; Houghton, P. J.; Stewart, C. F.; Cheshire, P. J.; Richmond, L. B.; Danks, M. K. Effective schedules of exposure of medulloblastoma and rhabdomyosarcoma xenografts to topotecan correlate with in vitro assays. *Clin. Cancer Res.* **1998**, *4*, 1995–2002.

(8) Zhuang, Y.; Fraga, C. H.; Hubbard, K. E.; Hagedorn, N.; Panetta, J. C.; Waters, C. M.; Stewart, C. F. Topotecan central nervous system penetration is altered by a tyrosine kinase inhibitor. *Cancer Res.* **2006**, *66*, 11305–11313.

(9) Bernier-Chastagner, V.; Grill, J.; Doz, F.; Bracard, S.; Gentet, J. C.; Marie-Cardine, A.; Luporsi, E.; Margueritte, G.; Lejars, O.; Laithier, V.; Mechinaud, F.; Millot, F.; Kalifa, C.; Chastagner, P. Topotecan as a radiosensitizer in the treatment of children with malignant diffuse brainstem gliomas: results of a French Society of Paediatric Oncology Phase II Study. *Cancer* **2005**, *104*, 2792–2797.

(10) Blaney, S. M.; Phillips, P. C.; Packer, R. J.; Heideman, R. L.; Berg, S. L.; Adamson, P. C.; Allen, J. C.; Sallan, S. E.; Jakacki, R. I.; Lange, B. J.; Reaman, G. H.; Horowitz, M. E.; Poplack, D. G.; Balis, F. M. Phase II evaluation of topotecan for pediatric central nervous system tumors. *Cancer* **1996**, *78*, 527–531.

(11) Löscher, W.; Potschka, H. Drug resistance in brain diseases and the role of drug efflux transporters. *Nat. Rev. Neurosci.* **2005**, *6*, 591–602.

(12) Friedman, H. S.; Kerby, T.; Fields, S.; Zilisch, J. E.; Graden, D.; McLendon, R. E.; Houghton, P. J.; Arbuck, S.; Cokgor, I.; Friedman, A. H. Topotecan treatment of adults with primary malignant glioma. *Cancer* **1999**, *85*, 1160–1165.

(13) Hendricks, C. B.; Rowinsky, E. K.; Grochow, L. B.; Donehower, R. C.; Kaufmann, S. H. Effect of P-glycoprotein expression on the accumulation and cytotoxicity of topotecan (SK&F 104864), a new camptothecin analogue. *Cancer Res.* **1992**, *52*, 2268–2278.

(14) Leggas, M.; Adachi, M.; Scheffer, G. L.; Sun, D.; Wielinga, P.; Du, G.; Mercer, K. E.; Zhuang, Y.; Panetta, J. C.; Johnston, B.; Scheper, R. J.; Stewart, C. F.; Schuetz, J. D. Mrp4 confers resistance to topotecan and protects the brain from chemotherapy. *Mol. Cell. Biol.* **2004**, *24*, 7612–7621.

(15) Maliepaard, M.; van Gastelen, M. A.; Tohgo, A.; Hausheer, F. H.; van Waardenburg, R. C.; de Jong, L. A.; Pluim, D.; Beijnen, J. H.; Schellens, J. H. Circumvention of breast cancer resistance protein (BCRP)-mediated resistance to camptothecins in vitro using non-substrate drugs or the BCRP inhibitor GF120918. *Clin. Cancer Res.* **2001**, *7*, 935–941.

(16) Maliepaard, M.; van Gastelen, M. A.; de Jong, L. A.; Pluim, D.; van Waardenburg, R. C.; Ruevekamp-Helmers, M. C.; Floot, B. G.; Schellens, J. H. Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line. *Cancer Res.* **1999**, *59*, 4559–4563.

due to these proteins' overexpressing in the BBB and also in brain tumors like gliomas.

Wheat germ agglutinin (WGA) has previously been proven to be able to enhance the uptake of HIV-1 virus (gp120) without disrupting the BBB function; gp120 is likely to bind the same sugar moieties on the endothelial membrane surface as WGA does, and therefore gp120 is able to take advantage of adsorptive endocytosis induced by WGA.[17] Meanwhile, WGA seems to be a good candidate for drug carrier targeting the BBB due to its high affinity for the cerebral capillary endothelium compared with other lectins and its low cytotoxicity.[18] The propensity of malignant cells exhibits high lectin agglutination, thus leading to the development of lectins as tumor diagnostic tools. Some findings show that the surface conjugation of paclitaxel-loaded PLGA nanoparticles with WGA could potentiate the extent and selectivity of the anticancer activity.[19]Accordingly, WGA may have the potential to target the BBB and C6 glioma cells. P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) are drug efflux transporters. Both of them are located not only in endothelial cells of the BBB[20] but also in cancer cells.[21,22] Tamoxifen (TAM), an orally active selective estrogen receptor modulator (SERM) that is used in the treatment of breast cancer, has shown the ability to reverse multidrug resistance (MDR) protein in human and murine leukemic cells. Meanwhile it also showed a BCRP-reversing activity, and was found to enhance topotecan uptake in K562/BCRP cells.[23] Thus tamoxifen may potentially be useful for inhibiting the drug efflux transporters expressed on the brain endothelial cells and on tumor cells.[24]

The brain is a delicate organ that efficiently protects itself from harmful compounds and precisely regulates its microenvironment. Nonetheless, such mechanisms are also proved to be hurdles in drug development and chemotherapy of brain tumors. A well-characterized BBB model in vitro could provide a useful tool for studying mechanistic aspects of transport as well as biological and pathological processes related to the BBB.[25] Recently, various BBB models in vitro have been used to investigate permeability of drugs and delivery systems across the BBB in the early development stage.[26] These models keep many features of the BBB such as expression of endothelial cell markers, tight junction formation between the cells, expression of MRD transporters, and high transendothelial electrical resistance (TEER).[27] Consequently, the established BBB model in vitro could be included for assessment of new developments aimed at the chemotherapy of brain tumors.

Most brain tumors are solid tumors. The relative inability of certain drugs to penetrate deeply into solid tumor masses was the result of unfavorable interstitial pressure gradients and nonuniform blood supply. This is one of the postulated reasons explaining why tumor cells grown in monolayer culture are frequently much more sensitive intrinsically to certain cytotoxicity drugs than when grown as three-dimensional multicellular spheroids.[28] Tumor spheroids show a three-dimensional representation of avascular regions observed in many solid tumor tissues, and the avascular regions of solid tumors represent a chief barrier in achieving the effects of inhibiting the tumor growth. Therefore, distribution of drug in avascular tumor regions is a challenging task, and tumor spheroids could serve as a tool for optimizing delivery systems for chemotherapy research.

For the reason mentioned above, the government of China has funded some key grants against significant diseases during the national 11th five-year plan (year 2005−2010),

(17) Banks, W. A.; Kastin, A. J. Characterization of lectin-mediated brain uptake of HIV-1 GP120. *J. Neurosci. Res.* **1998**, *54*, 522–529.

(18) Fischer, D.; Kissel, T. Histochemical characterization of primary capillary endothelial cells from porcine brains using monoclonal antibodies and fluorescein isothiocyanate-labelled lectins: implications for drug delivery. *Eur. J. Pharm. Biopharm.* **2001**, *52*, 1–11.

(19) Mo, Y.; Lim, L. Y. Paclitaxel-loaded PLGA nanoparticles: potentiation of anticancer activity by surface conjugation with wheat germ agglutinin. *J. Controlled Release* **2005**, *108*, 244–262.

(20) Borst, P.; Evers, R.; Kool, M.; Wijnholds, J. A family of drug transporters: The multidrug resistance-associated proteins. *J. Natl. Cancer Inst.* **2000**, *92*, 1295–1302.

(21) Tóth, K.; Vaughan, M. M.; Peress, N. S.; Slocum, H. K.; Rustum, Y. M. MDR1 P-glycoprotein is expressed by endothelial cells of newly formed capillaries in human gliomas but is not expressed in the neovasculature of other primary tumors. *Am. J. Pathol.* **1996**, *149*, 853–858.

(22) Demeule, M.; Shedid, D.; Beaulieu, E.; Del Maestro, R. F.; Moghrabi, A.; Ghosn, P. B.; Moumdjian, R.; Berthelet, F.; Béliveau, R. Expression of multidrug-resistance P-glycoprotein (MDR1) in human brain tumors. *Int. J. Cancer* **2001**, *93*, 62–66.

(23) Sugimoto, Y.; Tsukahara, S.; Imai, Y.; Sugimoto, Y.; Ueda, K.; Tsuruo, T. Reversal of breast cancer resistance protein-mediated drug resistance by estrogen antagonists and agonists. *Mol. Cancer Ther.* **2003**, *2*, 105–112.

(24) Kayyali, R.; Marriott, C.; Wiseman, H. Tamoxifen decreases drug efflux from liposomes: relevance to its ability to reverse multidrug resistance in cancer cells. *FEBS. Lett.* **1994**, *344*, 221–224.

(25) Cecchelli, R.; Berezowski, V.; Lundquist, S.; Culot, M.; Renftel, M.; Dehouck, M. P.; Fenart, L. Modelling of the blood-brain barrier in drug discovery and development. *Nat. Rev. Drug Discovery* **2007**, *6*, 650–661.

(26) Dhanikula, R. S.; Argaw, A.; Bouchard, J. F.; Hildgen, P. Methotrexate Loaded Polyether-Copolyester Dendrimers for the Treatment of Gliomas: Enhanced Efficacy and Intratumoral Transport Capability. *Mol. Pharmaceutics* **2008**, *5*, 105–116.

(27) Gaillard, P. J.; Voorwinden, L. H.; Nielsen, J. L.; Ivanov, A.; Atsumi, R.; Engman, H.; Ringbom, C.; de Boer, A. G.; Breimer, D. D. Establishment and functional characterization of an in vitro model of the blood-brain barrier, comprising a co-culture of brain capillary endothelial cells and astrocytes. *Eur. J. Pharm. Sci.* **2001**, *12*, 215–222.

(28) Kobayashi, H.; Man, S.; Graham, C. H.; Kapitain, S. J.; Teicher, B. A.; Kerbel, R. S. Acquired multicellular-mediated resistance to alkylating agents in cancer. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3294–3298.

including this project for improving chemotherapy of brain tumors using nanodrug carriers. The objective of the present study was to assess the potential of dual-targeting topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA), where WGA was conjugated to liposomes for enhancing transport of topotecan across the BBB as well as for targeting the tumor, and TAM was encapsulated into the lipid bilayer membrane of the liposomes to overcome the drug efflux transporters which overexpress on both the BBB endothelial cells and brain tumor cells. The tumoricidal activities of these topotecan formulations were evaluated against C6 glioma cells and the avascular C6 glioma tumor spheroids, and then the antitumor effect was evaluated in C6 glioma-bearing rats.

Materials and Methods

Preparation of Dual-Targeting Topotecan Liposomes. Topotecan liposomes modified with both tamoxifen (TAM) and wheat germ agglutinin (WGA) were prepared as the dual-targeting drug carriers for transporting across the blood−brain barrier (BBB) and then targeting brain tumor. Other three types of topotecan liposomes were prepared consisting of topotecan liposomes, topotecan liposomes modified with TAM, and topotecan liposomes modified with WGA as controls. Formulation designs are depicted in Figure 1. (1) Topotecan liposomes: egg phosphatidylcholine (EPC), cholesterol and polyethylene glycol distearoylphosphosphatidylethanolamine (PEG2000-DSPE, NOF Corporation, Japan) (55/40/2.5, μmol/μmol) were dissolved in chloroform in a pear-shaped flask. The chloroform was removed by evaporation with a rotary vacuum evaporator, and the lipid film was then hydrated with 250 mM ammonium sulfate by sonication in water bath for 5 min, then blank liposomes were obtained, and successively extruded through polycarbonate membranes (Millipore, Bedford, MA) with pore size of 400 nm and 200 nm 2 times, respectively. After dialysis in the solution of Hepes buffered saline (HBS, 25 nM Hepes/150 nM NaCl) 4 times, blank liposomes were mixed with an appropriate volume of topotecan hydrochloride (Chengdu Furunde Enterprise, Co., Ltd. Sichuan, China) HBS buffer (EPC/cholesterol/PEG2000-DSPE/topotecan: 55/40/2.5/8, μmol/μmol), incubated at 40 °C water bath and intermittently shaken for 20 min.[29] (2) Topotecan liposomes modified with TAM: EPC, cholesterol, PEG2000-DSPE and TAM (Peking University Pharmaceuticals Co., Ltd. Beijing, China) (55/40/2.5/8, μmol/μmol) were dissolved in chloroform in a pear-shaped flask. The following procedures were the same as those of topotecan liposomes. (3) Topotecan liposomes modified with WGA: EPC, cholesterol, PEG2000-DSPE and 1,2-distearoyl-*sn*-glycero-3- phosphoethamolamine-*N*-[carboxy(polyethylene glycol) 2000] (COOH-PEG2000-DSPE, Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform in a pear-shaped flask. After the blank liposomes were prepared, appropriate amounts of *N*-hydroxysuccinimide (NHS, Beijing Sansheng Tengda Technology Co., Ltd., China) and 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDCI, Shanghai Medpep Co., Ltd., China) were added to the above liposome suspensions. After incubation at room temperature for 30 min, an appropriate amount of WGA (Shanghai BioSun Sci & Tech Co., Ltd., Shanghai, China) was added, followed by 10 h incubation at room temperature and then chromatography separation (Sephadex G-200, Beijing Biodee Biotechnology Co., Ltd. Beijing, China) to remove the unbound WGA. The following procedures were the same as those of topotecan liposomes. The final compositions of lipids and ligands were listed as follows: EPC:cholesterol:PEG2000-DSPE:COOH-PEG2000-DSPE:WGA = 55:40:2.5:1.25:0.035 (μmol/μmol). (4) Topotecan liposomes modified with TAM and WGA: EPC, cholesterol, PEG2000-DSPE, COOH-PEG2000-DSPE, and TAM were dissolved in chloroform in a pear-shaped flask. The following procedures were the same as those of topotecan liposomes modified with WGA. The final compositions of lipids and ligands were listed as follows: EPC:

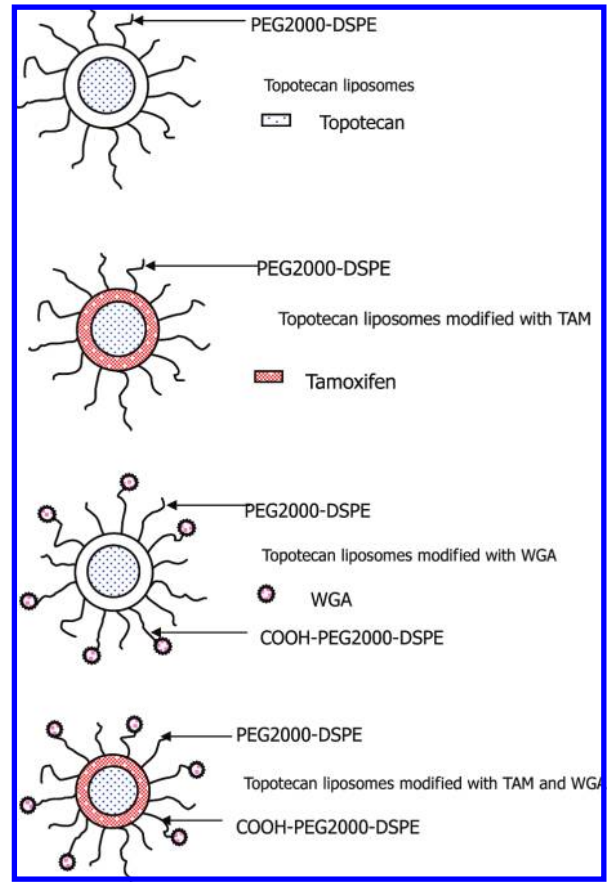

Figure 1. Schematic representations of four types of liposomes, including topotecan liposomes, topotecan liposomes modified with tamoxifen (TAM), topotecan liposomes modified with wheat germ agglutinin (WGA), and topotecan liposomes modified with TAM and WGA.

(29) Liu, Y.; Lu, W. L.; Guo, J.; Du, J.; Li, T.; Wu, J. W.; Wang, G. L.; Wang, J. C.; Zhang, X.; Zhang, Q. A potential target associated with both cancer and cancer stem cells: A combination therapy for eradication of breast cancer using vinorelbine stealthy liposomes plus parthenolide stealthy liposomes. *J. Controlled Release* **2008**, *129*, 18–25.

cholesterol:PEG2000-DSPE:COOH-PEG2000-DSPE:TAM:WGA = 55:40:2.5:1.25:8:0.035 (μmol/μmol).

Measurements. Topotecan in various liposomal formulations was measured using fluorospectrophotometry as reported previously.[30,31] The excitation and emission wavelengths were set at 381 and 531 nm, respectively.

Tamoxifen was measured with HPLC system equipped with ODS column (Kromasil, 250 × 4.6 mm), and the flow rate was 1.0 mL/min. The mobile phase consisted of methanol, water and diethylamine (90:10:0.1, v/v). The detected wavelength was set at 254 nm.

Wheat germ agglutinin was measured with Bradford protein assay by following the kit instruction (Beijing Saichi Shengwu Keji Co., Ltd., Beijing, China).

Mean particle size, polydisperisty index (PDI) and zeta potential of liposomes were measured using Zetasizer 3000HSA (Malvern Instruments Ltd., U.K.). The topotecan leakage ratio (%) of these liposomes was also measured in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, PBS, pH 7.4) at 4 °C, room temperature, and 37 °C, respectively or in 20% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM) at 37 °C.

Cell Culture. Three kinds of cell lines were applied including murine C6 glioma cells, murine brain microvascular endothelial cells, and rat astrocytes. Murine C6 glioma cells (Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China) were routinely grown in Dulbecco's modified Eagle's medium (DMEM, high glucose, Tianrun Shanda Biotech Co., Ltd., Beijing, China) supplemented by 10% heated-inactivated fetal bovine serum (FBS), antibiotics (penicillin 100 U/mL, and streptomycin 100 μg/mL (all from GIBCO-BRL Life Technologies, Beijing local agent, China).

Brain microvascular endothelial cells (BMVECs, Institute of Clinical Sciences, China−Japan Friendship Hospital, Beijing, China) were grown in endothelial cell culture medium (ECCM) [DMEM, 20% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, 100 μg/mL endothelial cell growth factor (ECGF), 20 μg/mL heparin, and 40 μU/mL insulin].

Rat astrocytes (RAs, Beijing Yuhengfeng Biotech Co., Ltd., Beijing, China). The RAs were grown in astrocyte medium (AM) [500 mL basal medium (Cat. No. 1081), 10 mL of fetal bovine serum (FBS, Cat. No. 0010), 5 mL of astrocyte growth supplement (AGS, Cat. No. 1852), and 5 mL of penicillin/streptomycin solution (P/S, Cat. No. 0503)]. In the logarithmic phase of growth, cells were used for cytotoxicity assay.

Cytotoxicity. Microtiter tetrazolium (MTT) assay was performed according to a standard MTT-based colorimetric assay.[29] Briefly, murine C6 cells were seeded onto 96-well plates at a density of 1×10^4 cells per well. Twenty-four hours later, fresh medium containing serial concentrations of various drug formulations, including unloaded liposomes modified with TAM, unloaded liposomes modified with TAM and WGA, free topotecan hydrochloride, topotecan liposomes, topotecan liposomes modified with tamoxifen (TAM), topotecan liposomes modified with wheat germ agglutinin (WGA), and topotecan liposomes modified with TAM and WGA. Topotecan in all samples was given at a concentration of 0.25 μM to 20.0 μM. Cells incubated in medium without any drug were used as blank controls. After drug treatment, cells were incubated for 43 h, and then 20 μL/well 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (5 mg/mL) was added. The plates were incubated for an additional 5 h. The cells were lysed using 100 μL of dimethylsulfoxide (DMSO) solution, and placed overnight in the incubator at 37 °C. The absorbance values of the lysed cells were read on a microplate reader (BIO-RAD model 680, Bio-Rad Laboratories, Inc. Shanghai, China) at the wavelength of 490 nm. The survival percentages were calculated using the following formula: survival % = [(A_{490} for the treated cells)/(A_{490} for the control cells)] × 100, where A_{490} is the absorbance value.

BBB Model. BMVECs and RAs were cocultured in a "contact through feet" model.[32−34] In a 12-well cell culture insert with 3 μM diameter microporous polyethylene terephtalate (PET) membrane (Millipore, Corporation, Billerica, MA), which were coated by 2% gelation D-Hank's solution, RAs were seeded on the backside at a density of 5×10^5 cells/mL by placing the insert upside down. After RAs attached firmly on the backside of insert membrane at 30 min, the membrane was turned over and placed in 12-well culture plate. Astrocyte medium was added and changed every other day. At day 5, BMVECs were seeded on the upper side at a concentration of 7.5×10^4 cells per insert, the BMVECs' culture medium was added and changed every other day. At day 8, transendothelial electrical resistance (TEER) values of the BBB were measured with the TEER

(30) Li, X.; Ruan, G. R.; Lu, W. L.; Hong, H. Y.; Liang, G. W.; Zhang, Y. T.; Liu, Y.; Long, C.; Ma, X.; Yuan, L.; Wang, J. C.; Zhang, X.; Zhang, Q. A novel stealth liposomal topotecan with amlodipine: apoptotic effect is associated with deletion of intracellular Ca2+ by amlodipine thus leading to an enhanced antitumor activity in leukemia. *J. Controlled Release* **2006**, *112*, 186–198.

(31) Zhang, Y. T.; Lu, W. L.; Li, T.; Liang, G. W.; Sun, J. B.; Guo, J.; Men, Y.; Du, J. A Continued Study on the Stealth Liposomal Topotecan Plus Amlodipine: In Vitro and In Vivo Characterization in Non-Resistant Solid Tumors. *J. Health Sci.* **2008**, *54*, 450–463.

(32) Cecchelli, R.; Dehouck, B.; Descamps, L.; Fenart, L.; Buée-Scherrer, V. V.; Duhem, C.; Lundquist, S.; Rentfel, M.; Torpier, G.; Dehouck, M. P. In vitro model for evaluating drug transport across the blood-brain barrier. *Adv. Drug Delivery Rev.* **1999**, *36*, 165–178.

(33) Boveri, M.; Berezowski, V.; Price, A.; Slupek, S.; Lenfant, A. M.; Benaud, C.; Hartung, T.; Cecchelli, R.; Prieto, P.; Dehouck, M. P. Induction of blood-brain barrier properties in cultured brain capillary endothelial cells: Comparison between primary glial cells and C6 cell line. *Glia* **2005**, *51*, 187–198.

(34) Abbott, N. J.; Rönnbäck, L.; Hansson, E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat. Rev. Neurosci.* **2006**, *7*, 41–53.

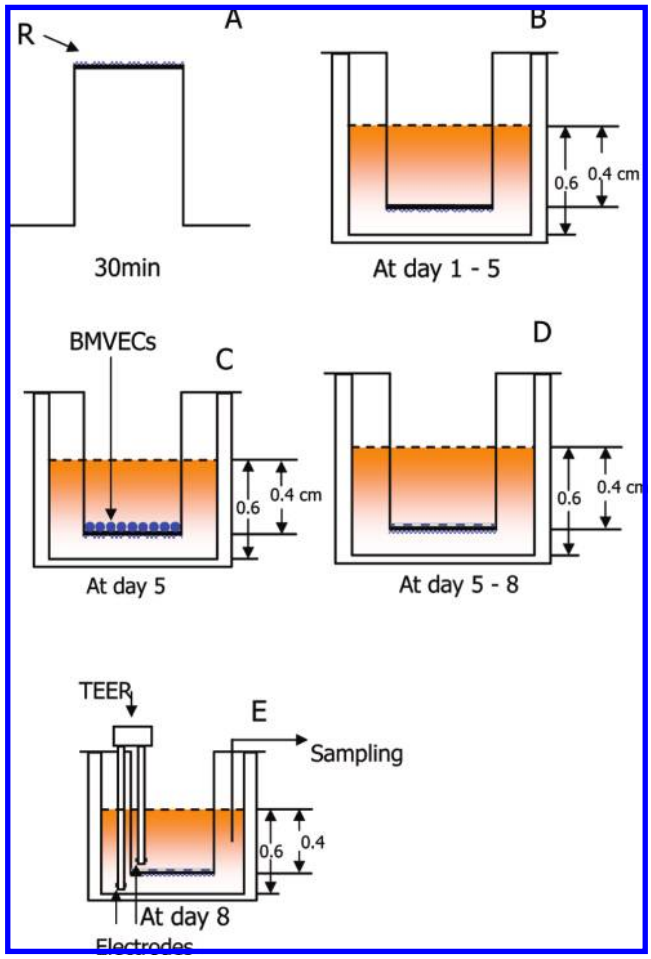

Figure 2. Schematic drawings of the blood−brain barrier (BBB) model in vitro. Rat astrocytes (RAs) were seeded on the backside at a density of 5×10^5 cells/mL by placing the insert upside down (A). After RAs attached firmly on the backside of insert membrane at 30 min, the membrane was turned over and placed in a 12-well culture plate. Astrocyte medium was added and changed every other day (B). At day 5, brain microvascular endothelial cells (BMVECs) were seeded on the upper side at a concentration of 7.5×10^4 cells per insert (C), and the BMVECs' culture medium was added and changed every other day (D). At day 8, transendothelial electrical resistance (TEER) values of the BBB were measured with the TEER instrument (E).

instrument (Word Precision Instruments, Inc. Sarasota, FL). When the TEER values reached $\geq 250\ \Omega\ cm^2$, the BBB model was included for experiments, as shown in Figure 2.

Transport across the BBB. Formulations were applied to BBB models in vitro, including free topotecan, topotecan liposomes, topotecan liposomes modified with tamoxifen (TAM), topotecan liposomes modified with wheat germ agglutinin (WGA), and topotecan liposomes modified with TAM and WGA, respectively. Final topotecan concentration added to the insert for all formulations was at 25.0 μM. The effects of different topotecan formulations on BBB integrity were monitored by measuring transendothelial electrical resistance (TEER) values during the experiment. A volume

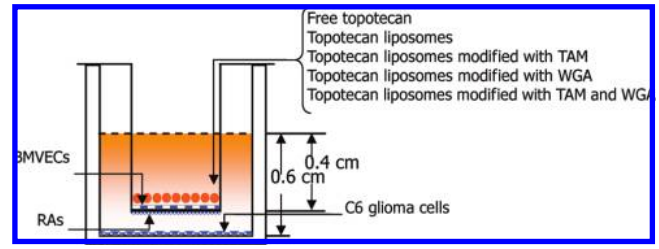

Figure 3. Schematic drawing for the dual-targeting effects in vitro of topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA): crossing the blood−brain barrier (BBB) model and then targeting murine C6 glioma cells.

of 100 μL of sample was taken from the receiver chamber at a given interval time ranging from 15 min to 2 h, as shown in Figure 2E, and the receiver chamber was replaced by 100 μL of fresh culture medium immediately.

For competition assay, free tamoxifen (TAM, 25.0 μM) and free wheat germ agglutinin (WGA 0.1 μM) were added into the inserts in advance, respectively. After 30 min, topotecan liposomes modified TAM, topotecan liposomes modified with WGA, and topotecan liposomes modified with TAM and WGA were added at a topotecan concentration of 25.0 μM, respectively. A volume of 100 μL of sample was taken from the receiver chamber at a given interval time ranging 15 min to 2 h, and then 100 μL of fresh culture medium was added to the chamber immediately.

Dual-Targeting Effects. The BBB model in vitro was established as above, and the inserts were then transferred to another 12-well culture plate where C6 glioma cells had been cultured for 1 day. Formulations were applied to BBB models in vitro, including Hepes buffer (pH 7.4, as a blank control), free topotecan, topotecan liposomes, topotecan liposomes modified with tamoxifen (TAM), topotecan liposomes modified with wheat germ agglutinin (WGA), and topotecan liposomes modified with TAM and WGA, respectively. Final topotecan concentration added to the insert for all formulations was at 25.0 μM, respectively, as shown in Figure 3. After 2 h incubation, the inserts were moved away, and C6 glioma cells were further incubated for 43 h. The following procedures were the same with MTT assay.

Effects on Avascular Murine C6 Glioma Spheroids. Avascular C6 glioma spheroids in vitro were made using a lipid overlay system, as reported previously.[26,35,36] Briefly, agarose solution (2% w/v) was prepared in serum free DMEM culture medium by heating at 80 °C for 30 min and then sterilized in autoclave. Each well of 24-well plates was coated with a thin layer of this sterilized solution. Tumor

(35) Goldbrunner, R. H.; Bernstein, J. J.; Plate, K. H.; Vince, G. H.; Roosen, K.; Tonn, J. C. Vascularization of human glioma spheroids implanted into rat cortex is conferred by two distinct mechanisms. *J. Neurosci. Res.* **1999**, *55*, 486–495.

(36) Kostarelos, K.; Emfietzoglou, D.; Papakostas, A.; Yang, W. H.; Ballangrud, A.; Sgouros, G. Binding and interstitial penetration of liposomes within avascular tumor spheroids. *Int. J. Cancer* **2004**, *112*, 713–721.

cells at a density of 1×10^5 cells/mL were seeded into each well. Then, plates were gently agitated for 5 min. Tumor spheroids were allowed to grow for 4 days, and culture medium was changed every 2 days.

For evaluating the inhibition of tumor spheroids, at day 5 murine C6 glioma spheroids were treated with Hepes buffer (pH 7.4, as a blank control), free topotecan, topotecan liposomes, and topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA), respectively. Final topotecan concentration added to the plate for all formulations was at 10.0 μM. Growth inhibition[37] was monitored by measuring the size of tumor spheroids under an inverted microscope (Chongqing Optical & Electrical Instrument, Co., Ltd., Chongqing, China). The major (d_{max}) and minor (d_{min}) diameters of each spheroid were determined, and spheroid volume was calculated by using the following formula: $V = (\pi \times d_{max} \times d_{min})/6$. The C6 glioma spheroid volume change ratio was calculated with the formula $R = (V_{dayi}/V_{day5}) \times 100\%$, where the V_{dayi} is the C6 glioma spheroid volume at the ith day (day 6, 7, 8, 9, 10) after applying drug, and V_{day5} is the C6 glioma spheroid volume prior to treatment.

For observing the morphology of spheroids, at day 5 murine C6 glioma spheroids were treated with Hepes buffer (pH 7.4, as a blank control), free topotecan, topotecan liposomes, and topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA), respectively. Topotecan concentration was at 10.0 μM. At day 8, the spheroids were fixed by 2.5% glutaraldehyde for 60 min, rinsed three times in 0.1 M phosphate buffered saline, then dehydrated and embedded. These tumor spheroid specimens were viewed with a scanning electron microscope (SEM, JSM-5600 LV, JEOL, Japan) at instrumental magnification of 500× and 5000×, respectively.

For investigating apoptosis in the spheroids, at day 5 tumor spheroids were treated with free topotecan, topotecan liposomes, and topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA). Topotecan concentration was at 10.0 μM. In addition, murine C6 glioma cells grown in monolayer culture and tumor spheroids incubated with Hepes buffer (pH 7.4) were used as blank controls. At day 6 they were washed twice with Hepes buffer, and disintegrated by extruding through a cell strainer. A volume of 200 μL of binding buffer was added to resuspend the collected cells. An aliquot of 10 μL of Annexin V-FITC and 5 μL of propidium iodide (Beijing Biosea Biotechnology Co., Ltd., Beijing, China) were added. After incubation in the dark at room temperature for 15 min, apoptosis was measured by flow cytometry.

Effects on the Survival of Brain Tumor-Bearing Animals. Male Sprague−Dawley rats weighing 200 ± 10% g were obtained from Experimental Animal Center of Peking University, and maintained on a light/dark cycle. Rats were acclimatized for 7 days prior to experiment, and were allowed free access to standard chow and water. Temperature and relative humidity were maintained at 25 °C and 50%, respectively. All care and handling of animals were performed with the approval of Institutional Authority for Laboratory Animal Care of Peking University.

Murine C6 glioma cells were inoculated into the brain of rats using a stereotaxic instrument (RWD Life Science Co., Ltd., Shenzhen, China). A sagittal incision was made through the skin to expose the cranium. Approximate 1×10^6 cells/10 μL was stereotaxically implanted into the right forebrain of each rat by using the following coordinates: 1.0 mm anterior and 3.0 mm lateral from the bregma, and at a depth of 5.0 mm from the brain surface.[38,39] At days 8, 10, and 12 since inoculation, physiological saline (as a blank control), unloaded liposomes modified with tamoxifen (TAM), unloaded liposomes modified with TAM and wheat germ agglutinin (WGA), free topotecan, topotecan liposomes, and topotecan liposomes modified with TAM and WGA were administered via tail vein at a dose of 5 mg topotecan/kg or at a dose of tamoxifen was 4 mg/kg, respectively. Besides the above one-week treatment, an extended treatment group was administered with topotecan liposomes modified with TAM and WGA at the same dose at day 8, 10, 12, 15, 17, and 19, respectively. The survival of rats was monitored.

Statistics. Data are presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine significance among groups, after which post hoc tests with the Bonferroni correction were used for comparison between individual groups. Survival data were presented as Kaplan−Meier plots and analyzed with a log−rank test. A value of $P < 0.05$ was considered to be significant.

Results

Preparation and Characterization of the Liposomes. In the four types of liposomes, topotecan encapsulation efficiencies were ≥85%, respectively. In the topotecan liposomes modified with TAM, and the topotecan liposomes modified with TAM and WGA, tamoxifen (TAM) encapsulation efficiency was ≥90.0%, respectively. In the topotecan liposomes modified with WGA, and the topotecan liposomes modified with TAM and WGA, the content of wheat germ agglutinin (WGA) was 35 μg per mg liposomes. The average particle sizes of all liposomes were approximately in the range of 100−110 nm. Particle size of

(37) Ballangrud, A. M.; Yang, W. H.; Dnistrian, A.; Lampen, N. M.; Sgouros, G. Growth and Characterization of LNCaP Prostate Cancer Cell Spheroids. *Clin. Cancer Res.* **1999**, *5*, 3171s–3176s.

(38) Lu, W.; Sun, Q.; Wan, J.; She, Z.; Jiang, X. G. Cationic Albumin-Conjugated Pegylated Nanoparticles Allow Gene Delivery into Brain Tumors via Intravenous Administration. *Cancer Res.* **2006**, *66*, 11878–11887.

(39) Reddy, G. R.; Bhojani, M. S.; McConville, P.; Moody, J.; Moffat, B. A.; Hall, D. E.; Kim, G.; Koo, Y. E.; Woolliscroft, M. J.; Sugai, J. V.; Johnson, T. D.; Philbert, M. A.; Kopelman, R.; Rehemtulla, A.; Ross, B. D. Vascular Targeted Nanoparticles for Imaging and Treatment of Brain Tumors. *Clin. Cancer Res.* **2006**, *12*, 6677–6686.

Table 1. Characterization of Topotecan Liposomes Modified with Tamoxifen (TAM) and Wheat Germ Agglutinin (WGA)

	mean size (nm)	polydispersity index	zeta potential (mV)
topotecan liposomes	103.7 ± 0.3	0.20 ± 0.02	−0.6 ± 0.5
topotecan liposomes modified with TAM	107.3 ± 1.5	0.20 ± 0.02	0.6 ± 0.2
topotecan liposomes modified with WGA	107.5 ± 0.9	0.18 ± 0.01	−3.4 ± 0.5
topotecan liposomes modified with TAM and WGA	110.0 ± 4.0	0.22 ± 0.01	−3.1 ± 2.0

the liposomes modified with TAM, WGA, or both together was slightly bigger than that of the unmodified. The charge values were close to a neutral state with slightly negative charges distributed around the liposomes vesicles, as shown in Table 1.

Topotecan leakage ratios from these four types of liposomes incubated in PBS (pH 7.4) were <1% at 4 °C for 48 h (Figure 4A), <12% at room temperature for 48 h (Figure 4B), <16% at 37 °C for 24 h and <30% at 37 °C for 48 h (Figure 4C). The ratios of the liposomes incubated serum were <13% at 37 °C for 24 h, and approximately 50% at 37 °C for 48 h (Figure 4D).

Cytotoxicity. After direct exposure, effect of topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA) on the proliferation of murine C6 glioma cells is depicted in Figure 5. MTT assay showed that topotecan liposomes modified with TAM and WGA at various concentrations exhibited a strong inhibition to the proliferation of tumor cells. Significant inhibitory effects were also observed after applying various controls, including free topotecan, topotecan liposomes modified with TAM, and topotecan liposomes modified with WGA excluding topotecan liposomes. Among the four types of liposomes, topotecan liposomes modified with TAM and WGA exhibited the best inhibitory effect, and topotecan liposomes did a weak action. Under direct exposure condition, free topotecan showed the strongest inhibitory effect. As TAM may potentially affect the survival of C6 glioma cells, the unloaded liposomes modified TAM and unloaded liposomes modified with TAM and WGA were included as controls, respectively. Results showed that these two controls had less effect on the survival of the C6 glioma cells (<15% inhibitory ratio) at a lower concentration of TAM (0.25 μM to 5 μM). With the concentration of TAM increasing (10 μM to 20 μM), both controls exhibited inhibitory effects up to 35%.

BBB Model and Drug Transport across the BBB. The BBB model was constructed according to Dr. Jiang's establishment in 2005[40] by coculturing BMVECs and RAs. By judging transendothelial electrical resistance (TEER)

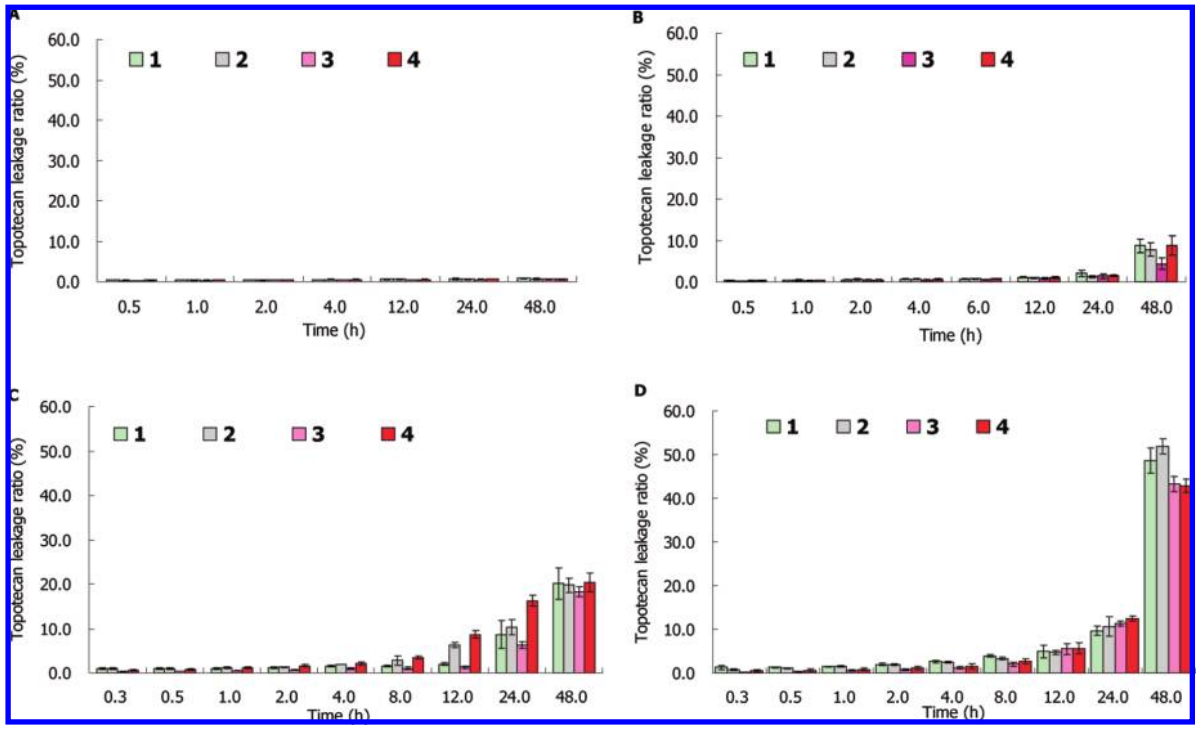

Figure 4. Topotecan leakage ratios (%) from topotecan liposomes, topotecan liposomes modified with tamoxifen (TAM), topotecan liposomes modified with wheat germ agglutinin (WGA), and topotecan liposomes modified with TAM and WGA. The ratios were measured in PBS (pH 7.4) at 4 °C (A), in PBS (pH 7.4) at room temperature (B), in PBS (pH 7.4) at 37 °C (C), and 20% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM) at 37 °C (D). Key: 1, topotecan liposomes; 2, topotecan liposomes modified with TAM; 3, topotecan liposomes modified with WGA; 4, topotecan liposomes modified with TAM and WGA.

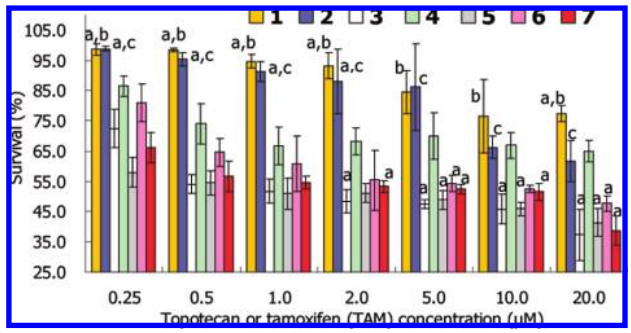

Figure 5. Effect of topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA) on the proliferation of murine C6 glioma cells. Free topotecan, topotecan liposomes, topotecan liposomes modified with TAM, topotecan liposomes modified with WGA, unloaded liposomes modified with TAM, and unloaded liposomes modified with TAM and WGA were included for comparisons. Cells were incubated for 43 h at the indicated concentration ranging 0.25 μM to 20.0 μM, and the viability was measured using the MTT assay. Key: 1, unloaded liposomes modified with TAM; 2, unloaded liposomes modified with TAM and WGA; 3, free topotecan; 4, topotecan liposomes; 5, topotecan liposomes modified with TAM; 6, topotecan liposomes modified with WGA; 7, topotecan liposomes modified with TAM and WGA; a, $P < 0.05$, versus topotecan liposomes; b, $P < 0.05$, unloaded liposomes modified with TAM versus topotecan liposomes modified with TAM; c, $P < 0.05$, unloaded liposomes modified with TAM and WGA versus topotecan liposomes modified with TAM and WGA.

primarily ($\geq$250 Ω cm^2), they were employed to evaluate the transport of various topotecan formulations across the BBB model in vitro. No obvious reduction in the TEER values was seen during the study, indicating that transport of drug did not disrupt the BBB barrier properties.

Results showed that the rank of transport ratio (%) across the BBB was topotecan liposomes modified with TAM and WGA > topotecan liposomes modified with TAM > topotecan liposomes modified with WGA $\geq$ topotecan liposomes > free topotecan at 120 min, displaying that the dual modifications with TAM and WGA showed a significant drug transport across the BBB, as illustrated in Figure 6. In addition, the drug transport across the BBB was in a time-dependent manner.

In competition assay, when free tamoxifen (TAM) was added in advance, the transfer ratios across the BBB of topotecan liposomes modified with TAM, and topotecan liposomes modified with TAM and WGA were significantly increased. When free WGA was added in advance, the transfer ratios across the BBB of topotecan liposomes modified with WGA, and topotecan liposomes modified with TAM and WGA were evidently decreased, as illustrated in Figure 6.

Dual-Targeting Effects. Dual-targeting effects, which exhibited crossing the BBB model and targeting murine C6 glioma cells, of topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA), are depicted in Figure 7. The results showed that topotecan liposomes modified with TAM and WGA exhibited the highest inhibitory effect to the C6 glioma cells than other topotecan formulations according to the survival rates following applying (65.8% for topotecan liposomes modified with TAM and WGA; 75.6% for topotecan liposomes modified with WGA; 76.1% for topotecan liposomes modified with TAM; 86.5% for topotecan liposomes; 88.0% for free topotecan), and these results were consistent with those seen in drug transport across the BBB.

Effects on Avascular Murine C6 Glioma Spheroids. The effect of topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA) on the growth of murine C6 glioma spheroids is shown in Figure 8. It was observed that the tumor spheroids had no changes in size and volume in the absence of topotecan. Free topotecan, topotecan liposomes, and topotecan liposomes modified with TAM and WGA were able to significantly inhibit the growth of tumor spheroids. By comparing with the spheroid volume prior to treatment at day 5, the spheroid volume change ratios at day 6 to day 10 were in the range of 97.9−56.6%, 84.9−59.3%, and 65.2−45.9% after applying a dose at day 5 of free topotecan, topotecan liposomes, and topotecan liposomes modified with TAM and WGA, respectively.

The apoptotic percentages of tumor spheroids at day 5 following 12 h drug treatment were 33.3%, 33.9%, and 64.1% after applying a dose at day 5 followed by a 12 h treatment with free topotecan, topotecan liposomes, and topotecan liposomes modified with TAM and WGA, respectively.

SEM observations showed that the murine C6 glioma cells became round spheroids in a diameter range of 150−250 μm at day 5, as shown in Figure 9A1,A2. After 3 days further incubation with free topotecan, the cell membrane microvilli disappeared, cells became swollen, and their surface became smooth (Figure 9B1,B2 versus Figure 9C1,C2). The same phenomenon happened when tumor spheroids were incubated with topotecan liposomes, while the cell leakage and cell membrane lysis occurred (Figure 9D1,D2). When tumor spheroids were incubated with topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA), the marginal of murine C6 glioma cells became disintegrated and shrunken (Figure 9E1,E2).

Effects on the Survival of Brain Tumor-Bearing Animals. Effects of topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA) on brain tumor-bearing animal survival are presented in Figure 10. After one-week treatment, the median survival time of rats treated with topotecan liposomes modified with TAM

(40) Lu, W.; Tan, Y. Z.; Hu, K. L.; Jiang, X. G. Cationic albumin conjugated pegylated nanoparticle with its transcytosis ability and little toxicity against blood-brain barrier. *Int. J. Pharm.* **2005**, *295*, 247–260.

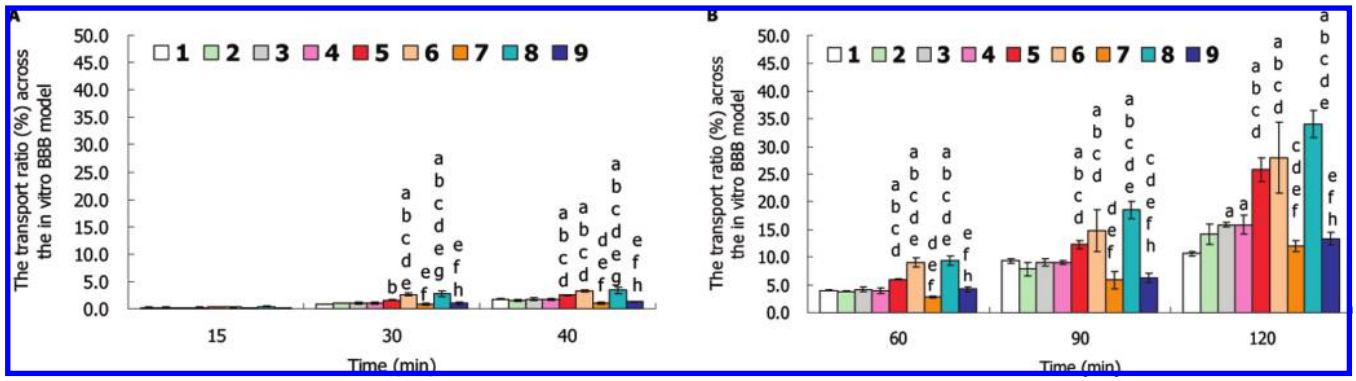

Figure 6. The transport ratio (%) across the in vitro BBB model of topotecan liposomes modified with TAM and WGA. Free topotecan, topotecan liposomes, topotecan liposomes modified with TAM, topotecan liposomes modified with WGA and the competition assay were included for comparisons. In the competition assay, the BBB model was preconditioned with TAM (25.0 μM) or WGA (0.1 μM) for 30 min, and followed by applying topotecan liposomes modified with TAM, topotecan liposomes modified with WGA, and topotecan liposomes modified with TAM and WGA. Final topotecan concentration for all was at 25.0 μM, respectively. The experiment was performed for 120 min. A: time range 15−40 min. B: time range 60−120 min. Key: 1, free topotecan; 2, topotecan liposomes; 3, topotecan liposomes modified with TAM; 4, topotecan liposomes modified with WGA; 5, topotecan liposomes modified with TAM and WGA; 6, preconditioned with TAM and applying topotecan liposomes modified with TAM; 7, preconditioned with WGA and applying topotecan liposomes modified with WGA; 8, preconditioned with TAM and applying topotecan liposomes modified with TAM and WGA; 9, preconditioned with WGA and applying topotecan liposomes modified with TAM and WGA; a, $P < 0.05$, versus free topotecan; b, $P < 0.05$, versus topotecan liposomes; c, $P < 0.05$, versus topotecan liposomes modified with TAM; d, $P < 0.05$, versus topotecan liposomes modified with WGA; e, $P < 0.05$, versus topotecan liposomes modified with TAM and WGA; f, $P < 0.05$, versus preconditioned with TAM and applying topotecan liposomes modified with TAM; g, $P < 0.05$, versus preconditioned with WGA and applying topotecan liposomes modified with WGA; h, $P < 0.05$, versus preconditioned with TAM and applying topotecan liposomes modified with TAM and WGA.

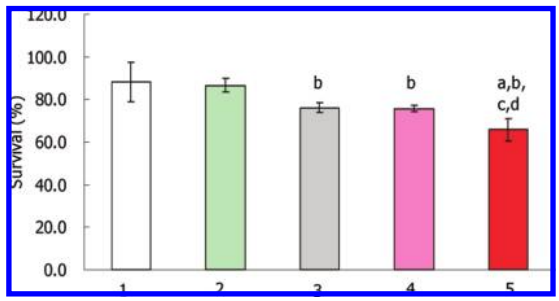

Figure 7. Dual-targeting effects of topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA): crossing the BBB model in vitro and then targeting murine C6 glioma cells. Free topotecan, topotecan liposomes, topotecan liposomes modified with TAM, topotecan liposomes modified with WGA were included for comparisons. Final topotecan concentration added for all was at 25.0 μM. Key: 1, free topotecan; 2, topotecan liposomes; 3, topotecan liposomes modified with TAM; 4, topotecan liposomes modified with WGA; 5, topotecan liposomes modified with TAM and WGA; a, $P < 0.05$, versus free topotecan; b, $P < 0.05$, versus topotecan liposomes; c, $P < 0.05$, versus topotecan liposomes modified with TAM; d, $P < 0.05$, versus topotecan liposomes modified with WGA.

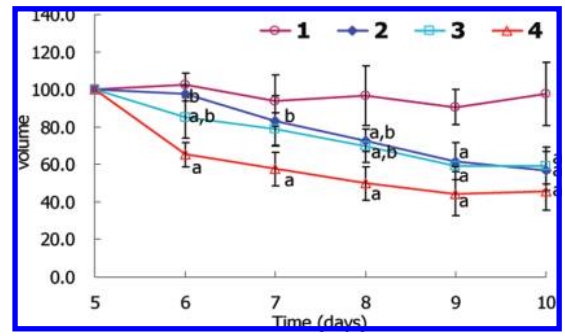

Figure 8. Effect of topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA) on the growth of murine C6 glioma spheroids. Hepes buffer (pH 7.4, as a blank control), free topotecan, and topotecan liposomes were included for comparisons. Final topotecan concentration for all was at 10.0 μM. Key: 1, Hepes buffer; 2, free topotecan 3, topotecan liposomes; 4, topotecan liposomes modified with TAM and WGA; a, $P < 0.05$, versus Hepes buffer (pH 7.4, as a blank control); b, $P < 0.05$, versus topotecan liposomes modified with TAM and WGA.

and WGA (26 days) was significantly longer than that of rats treated with saline (15 days, $P = 0.003$), free topotecan (19 days, $P = 0.002$) and topotecan liposomes (20 days, $P = 0.002$). There were also statistical differences in the survival time between the group treated with physiological saline and the group treated with free topotecan ($P = 0.003$) or between the group treated with physiological saline and the group treated with topotecan liposomes ($P = 0.003$). The extended treatment of topotecan liposomes modified with TAM and WGA for two weeks could prolong the median

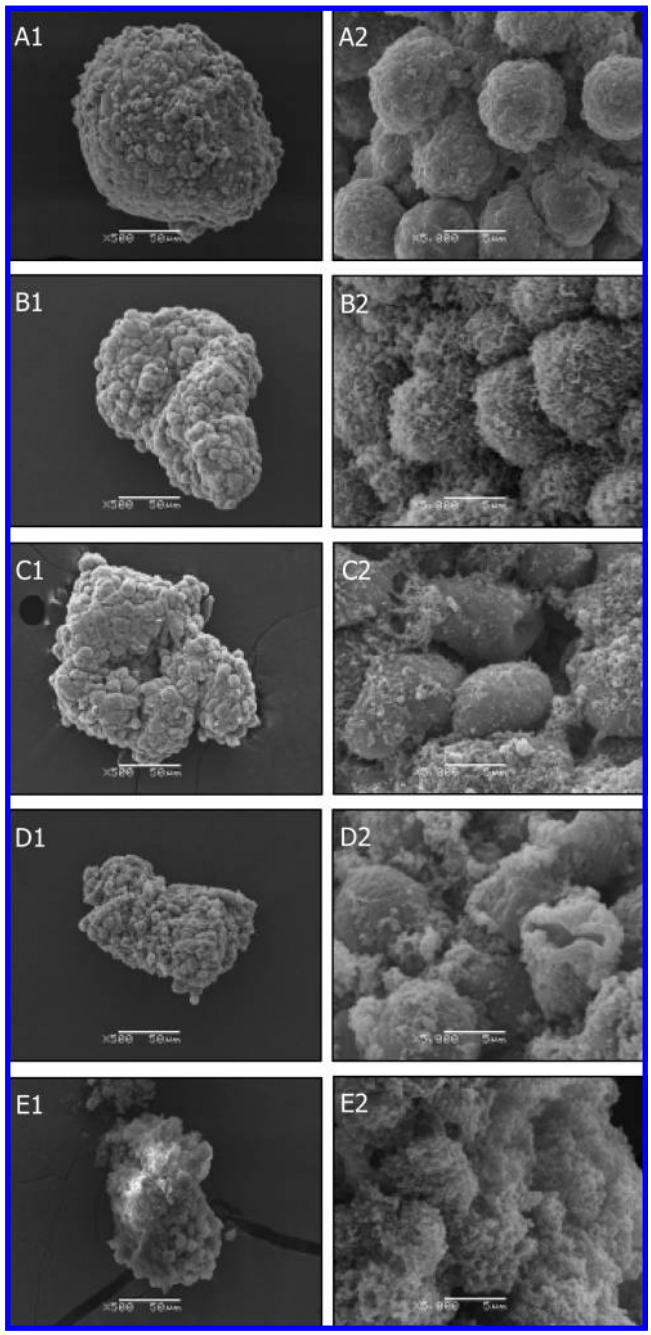

Figure 9. Scanning electronic microscope (SEM) photographs of murine C6 glioma spheroids. Key: A1 and A2, spheroids at day 5; B1 and B2, spheroids at day 8 after applying Hepes buffer (pH 7.4) at day 5; C1 and C2, spheroids at day 8 after applying free topotecan at day 5; D1 and D2, spheroids at day 8 after applying topotecan liposomes at day 5; E1 and E2, spheroids at day 8 after applying topotecan liposomes modified with TAM and WGA at day 5. Final topotecan for all was at 10.0 μM.

survival time to 31 days, and there was significant difference between one-week treatment and two-week treatment ($P = 0.011$).

As TAM may potentially affect the survival of C6 glioma cells, the unloaded liposomes modified with TAM and the

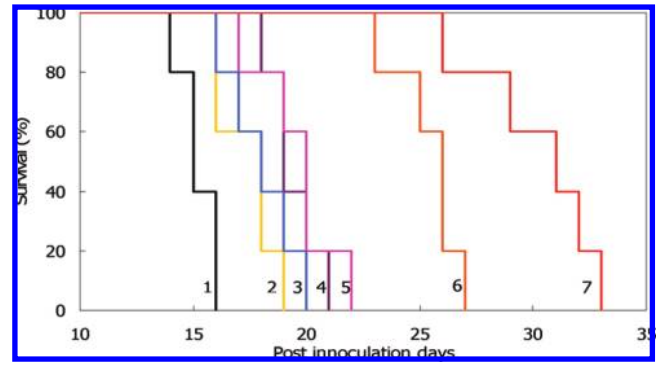

Figure 10. Kaplan−Meier survival curves of Sprague−Dawley rats inoculated with murine C6 glioma cells using a stereotaxic instrument. At days 8, 10, 12 (one-week treatment) after inoculation, physiological saline (as a blank control), unloaded liposomes modified with tamoxifen (TAM), unloaded liposomes modified with TAM and wheat germ agglutinin (WGA), free topotecan, topotecan liposomes, and topotecan liposomes modified with TAM and WGA were administered via tail vein, respectively. Besides the above one-week treatment groups, an extended treatment group with the same dose of topotecan liposomes modified with TAM and WGA at days 8, 10, 12, 15, 17, and 19 (two-week treatment) was conducted for survival monitoring. The dose for topotecan was 5 mg/kg, and the dose for tamoxifen was 4 mg/kg. Key: 1, physiological saline; 2, unloaded liposomes modified with TAM; 3, unloaded liposomes modified with TAM and WGA; 4, free topotecan; 5, topotecan liposomes; 6, topotecan liposomes modified with TAM and WGA (one-week treatment); 7, topotecan liposomes modified with TAM and WGA (two-week treatment).

unloaded liposomes modified with TAM and WGA were included as controls, respectively. Results showed that the median survival times of rats both given as the unloaded liposomes modified TAM and given as the unloaded liposomes modified with TAM and WGA were 18 days. The median survival days of these two controls are longer than that of rats treated with saline ($p = 0.018$) but significantly shorter than that of rats treated with topotecan liposomes modified with WGA and TAM (one-week treatment, $p = 0.002$; two-week treatment, $p = 0.002$), respectively. There are no differences among these two controls, free topotecan and the topotecan liposomes.

Discussion

The blood−brain barrier (BBB) is a metabolic cellular structure in the central nervous system (CNS), which restricts the passage of various small molecular chemical agents and micro-objects between the bloodstream and the neutral tissues; overcoming the difficulty of delivering anticancer agents to brain tumors presents a major challenge. However, BBB allows the passage of endogenous substances essential to metabolic function such as insulin, a large molecule. This suggests that a suitable carrier could help in the transport of

drug across the BBB. Mechanisms for drug targeting in the brain tumors involve two aspects: going "through" the BBB, and specifically looking for the tumor behind the BBB. In the present study, we proposed a novel type of dual-targeting nanoliposomal carriers with the purposes of delivering anticancer agent through the BBB and then targeting the inside brain tumor cells.

To construct the dual-targeting nanocarriers, PEGylated liposomes loaded with topotecan were included for consideration as our previous studies showed that such carriers were more stable in both physiochemical and biological conditions, which were associated with sterical stability and with the bulky polyethylene glycol (PEG) headgroup which inhibits the rapid uptake of reticuloendothelial system (RES). Furthermore, the weak acidic microenvironment in the liposomes benefited for the lactone species by suppressing hydrolysis.[30]

Tamoxifen was incorporated into the liposome membrane acting as a potential ligand for inhibiting the efflux of BBB protein transporters. In addition, the surface of brain tumor also highly expresses protein transporters like MDR1 protein, MRP and BCRP; tamoxifen (TAM) therefore would be able to assist in delivering topotecan into brain tumor cells. Wheat germ agglutinin (WGA) has shown the feature of targeting tumor cells based on the mechanism of receptor-mediated endocytosis;[19,41] it may help to transfer topotecan liposomes across the BBB and then target the "behind" brain tumor, therefore being included as another dual-targeting ligand.

As a bioadhesion agent, the stability of wheat germ agglutinin (WGA) is very important. A previous report showed that, after preincubation of WGA with abnormally high amounts of pepsin, trypsin, pancreatin, and elastase, WGA did not cause any degradation products, and its cell-binding characteristics were fully retained.[42] The covalent attachment of WGA to the distal end of a PEGylated lipid resulted in a stable liposomal carrier with high binding specificity. These properties above were essential prerequisites for the development of long circulating and site specific drug delivery systems.[43] During the whole experimental period, the particle sizes of these four types of liposomes were monitored frequently and no macroscopic aggregation was observed.

MTT assay demonstrated that topotecan resulted in obviously inhibitory effects to C6 glioma cells following direct exposure, thus proving the anticancer effects on such brain tumors. In addition, there was significant difference in the inhibitory effect between topotecan liposomes and topotecan liposomes modified with wheat germ agglutinin (WGA). This could be explained by the high affinity of WGA to C6 glioma cells, while common PEGylated liposomes would hinder the contact of topotecan with tumor cells in vitro. Such a phenomenon was also found in other reports.[44] Topotecan liposomes modified with tamoxifen (TAM) exhibited a stronger toxicity to C6 glioma cells than topotecan liposomes modified with WGA, demonstrating that tamoxifen could inhibit MDR1 and BCRP proteins highly expressed on the C6 glioma cells, leading to more topotecan stays in the cells. More significantly, topotecan liposomes modified with TAM and WGA exhibited the strongest inhibitory effects, and showed a topotecan-concentration dependent manner, suggesting that TAM plus WGA contributes to a stronger drug delivering effect into C6 glioma cells.

To build a BBB in vitro model and to closely mimic the situation in vivo, brain microvascular endothelial cells (BMVECs) were cultured on the upper side of the insert and rat astrocytes (RAs) on the backside. This model had been characterized by displaying BBB characteristics and linking the barrier junction with its transendothelial electrical resistance (TEER) value.[32,45] Drug transport across the BBB model showed that the transport of topotecan liposomes modified with tamoxifen (TAM), wheat germ agglutinin (WGA), or both was evidently higher than that of topotecan liposomes, demonstrating that TAM and WGA contributed to the transfer of topotecan across the BBB.

From the results of competition assay, the BBB preconditioned with free tamoxifen led to a significant increase in the liposomes crossing the BBB for topotecan liposomes modified with TAM or for topotecan liposomes modified with TAM and WGA, further proving that tamoxifen could reverse the function of efflux pump proteins on the brain endothelial cells. As tamoxifen is a potential anticancer agent, the TEER values of the in vitro BBB were monitored during the whole experiment (data not shown). The TEER values were not obviously changed before or after applying tamoxifen, suggesting that the in vitro BBB membrane was not altered. However, results showed that there was no inhibitory competition effect on the transport of topotecan liposomes modified with TAM or topotecan liposomes modified with TAM and WGA after applying tamoxifen in advance. In contrast, when the BBB was preconditioned with free wheat germ agglutinin (WGA), a significant reduction in the liposomes crossing the BBB was observed for topotecan

(41) Mo, Y.; Lim, L. Y. Mechanistic study of the uptake of wheat germ agglutinin-conjugated PLGA nanoparticles by A549 cells. *J. Pharm. Sci.* **2004**, *93*, 20–28.

(42) Gabor, F.; Wirth, M.; Jurkovich, B.; Haberl, I.; Theyer, G.; Walcher, G.; Hamilton, G. Lectin-mediated bioadhesion: Proteolytic stability and binding-characteristics of wheat germ agglutinin and Solanum tuberosum lectin on Caco-2, HT-29 and human colonocytes. *J. Controlled Release* **1997**, *49*, 27–37.

(43) Bakowsky, H.; Richter, T.; Kneuer, C.; Hoekstra, D.; Rothe, U.; Bendas, G.; Ehrhardt, C.; Bakowsky, U. Adhesion characteristics and stability assessment of lectin-modified liposomes for site-specific drug delivery. *Biochim. Biophys. Acta* **2008**, *1778*, 242–249.

(44) Xiong, X. B.; Huang, Y.; Lu, W. L.; Zhang, X.; Zhang, H.; Nagai, T.; Zhang, Q. Enhanced intracellular delivery and improved antitumor efficacy of doxorubicin by sterically stabilized liposomes modified with a synthetic RGD mimetic. *J. Controlled Release* **2005**, *107*, 262–275.

(45) Perrière, N.; Yousif, S.; Cazaubon, S.; Chaverot, N.; Bourasset, F.; Cisternino, S.; Declèves, X.; Hori, S.; Terasaki, T.; Deli, M.; Scherrmann, J. M.; Temsamani, J.; Roux, F.; Couraud, P. O. A functional in vitro model of rat blood-brain barrier for molecular analysis of efflux transporters. *Brain. Res.* **2007**, *1150*, 1–13.

liposomes modified with WGA or for topotecan liposomes modified with TAM and WGA. This phenomenon was caused by the saturation of the sugar moieties by WGA. Such saturation led to a decreased adsorptive endocytosis,[17] and thus resulted in a reduction in the transport of the liposomes modified with WGA across the BBB.

To understand the dual-targeting effect in vitro, topotecan liposomes modified with TAM and WGA were passed through the BBB model on the insert first and then reached the tumor cells in the culture plate tank. The inhibitory effect on the C6 cells further proved the results obtained from drug transport across the BBB. Albeit free topotecan showed a similar transporting effect across the BBB to topotecan loaded liposomes, its inhibitory effect on tumor cells was significantly less than that from topotecan liposomes modified with TAM, topotecan liposomes modified with WGA, and topotecan liposomes modified with TAM and WGA, respectively. Among these, topotecan liposomes modified with TAM and WGA exhibited the most robust dual-targeting effects: crossing the BBB and then targeting brain tumor cells.

To demonstrate the penetrating ability of drug into solid brain tumor masses, three-dimensional murine C6 glioma spheroids were studied. Results indicated that topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA) displayed the most significant inhibitory effect on the growth of spheroids compared to blank control. In addition, free topotecan and topotecan liposomes also exhibited evident inhibitory effect. Apoptosis analysis of tumor spheroids followed by applying drugs showed a similar trend that topotecan liposomes modified with TAM and WGA had the most significant apoptosis effect on spheroids. Through SEM observations, the ability of inhibitory effects on three-dimensional glioma spheroids could be intuitively seen following applying topotecan liposomes modified with TAM and WGA, suggesting that they have the potential to kill brain tumor after crossing the BBB.

In the brain tumor-bearing rats, the dual-targeting effects of topotecan liposomes modified with TAM and WGA could be evidently observed as this combination leads to a significantly improved chemotherapy in the overall survival of the brain tumor-bearing rats, compared with free topotecan or with topotecan liposomes. Results from an extended treatment group indicated that the survival could be further significantly enhanced, indicating that an extended chemotherapy with topotecan liposomes modified with TAM and WGA would be beneficial for the treatment. The dual-targeting effects in vivo of topotecan liposomes modified with TAM and WGA could be relevant to the following aspects: an enhanced effect with tamoxifen via inhibiting efflux of MDR1/BCRP protein effluxes in the BBB and the brain tumor; an enhanced effect with wheat germ agglutinin via adsorptive endocytosis in the BBB[17] and receptor-mediated endocytosis.[19,41] In addition, the encapsulation of topotecan in acidic medium in the liposomes could also contribute to the improved chemotherapy because of an increase in active form of topotecan (lactone species), as described above. Results from the controls administered as the unloaded liposomes modified with TAM and the unloaded liposomes modified with TAM and WGA indicate that the unloaded liposomes but containing tamoxifen slightly contributed to the inhibitory effect to the brain tumor in animals. Tamoxifen has been proven to be a potent inhibitor to human[46] and murine glioma proliferations in vitro,[47] and is able to penetrate the brain−blood barrier.[48−50] Nevertheless, in view of the overall survival curves in the present study, the inhibitory effect of tamoxifen to brain tumor takes a secondary status compared with those of topotecan formulations.

In conclusion, topotecan liposomes modified with tamoxifen (TAM) and wheat germ agglutinin (WGA) significantly improve the transport of topotecan across the blood−brain barrier and survival of brain tumor-bearing animals, showing a dual-targeting effect. These findings would encourage further developments by noninvasive therapy of brain tumors.

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(46) Pollack, I. F.; Randall, M. S.; Kristofik, M. P.; Kelly, R. H.; Selker, R. G.; Vertosick, F. T. Effect of tamoxifen on DNA synthesis and proliferation of human malignant glioma lines in vitro. *Cancer Res.* **1990**, *50*, 7134–7138.

(47) Kim, Y. J.; Lee, C. J.; Lee, U.; Yoo, Y. M. Tamoxifen-induced cell death and expression of neurotrophic factors in cultured C6 glioma cells. *J. Neurooncol.* **2005**, *71*, 121–125.

(48) Mastronardi, L.; Guiducci, A.; Puzzilli, F.; Ruggeri, A. l. Relationship between Ki-67 labeling index and survival in high-grade glioma patients treated after surgery with tamoxifen. *J. Neurosurg. Sci.* **1999**, *43*, 263–270.

(49) Döhler, K. D.; Coquelin, A.; Davis, F.; Hines, M.; Shryne, J. E.; Sickmöller, P. M.; Jarzab, B.; Gorski, R. A. Pre- and postnatal influence of an estrogen antagonist and an androgen antagonist on differentiation of the sexually dimorphic nucleus of the preoptic area in male and female rats. *Neuroendocrinology* **1986**, *42*, 443–448.

(50) Ferretti, C.; Blengio, M.; Ghi, P.; Racca, S.; Genazzani, E.; Portaleone, P. Tamoxifen counteracts estradiol induced effects on striatal and hypophyseal dopamine receptors. *Life Sci.* **1988**, *42*, 2457–2465.