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Controlled Release of Liposome-Encapsulated Temozolomide for Brain Tumor Treatment by Convection-Enhanced Delivery

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Abstract

Convection-enhanced delivery (CED) is a promising technique for the delivery of drugs directly into the central nervous system (CNS) and, more specifically, the brain. CED can increase drug concentration within a brain tumor, thereby improving the therapeutic efficacy and limiting the systemic toxicity of tumoricidal agents. In this study, we evaluated a drug-liposome construct *in vitro* and *in vivo* using U87 tumor-bearing nude mice. Dipalmitoylphosphatidylcholine (DPPC)-based liposomes were designed to deliver a lipophilic temozolomide (TMZ) formulation (LipoTMZ). The LipoTMZ displayed good release of TMZ *in vitro* over a suitable range of time and temperatures. Encapsulating the TMZ into liposomes enhanced its tumoricidal activity against U87MG human glioma cells. The LipoTMZ also displayed good release and distribution of TMZ when delivered intracerebrally to U87MG tumor-bearing mice by CED infusion. Histological examination revealed that CED did not damage normal brain tissue. Our data indicate that CED was an effective method to deliver LipoTMZ to U87MG tumor-bearing mice, significantly inhibiting tumor growth without evidence of systemic toxicity.

Keywords: convention-enhanced delivery, drug delivery, liposome, temozolomide, brain tumor

Introduction

Glioblastoma multiforme (GBM) is the most common malignant brain tumor, accounting for approximately 2% of all primary cancers in adults in the United States [1]. Patients typically have a poor prognosis despite current standard therapy with an overall medium survival time of 15 months [2]. Available chemotherapy to kill cancer cells via oral or intravenous routes provide a modest prolongation of survival [3, 4]. Despite strong evidence of their effectiveness in animal models or in cultured cells, drugs fail to differentiate GBM cancer cells from healthy cells and surrounding healthy tissue [5]. Another obstacle that limits the delivery of chemotherapeutic compounds into the residual disease is the functional blood-brain barrier (BBB), which prevents effective delivery of sufficient quantities of drugs to the brain [6, 7]. GBMs thus present unique challenges to local treatment of brain tumors. It is important to devise strategies to increase drug availability and to obtain the desired therapeutic responses in ways that do not rely on the circulatory system.

Convection-enhanced delivery (CED) uses a direct infusion technique that relies on bulk flow within interstitial brain tissue, allowing higher, more uniform drug concentrations at the infusion site [8, 9]. Fluid convection within the brain via application of an external pressure gradient provides much greater volumes of drug distribution than are achievable by diffusion [10]. Compared with traditional convection delivery, CED produces higher concentrations of therapeutic agents, longer infusion times, and larger distribution volumes at the infusion site, resulting in minimal systemic toxicity [11, 12]. Hence, the benefits of CED include both an enhanced ability to target tumors locally, as well as the ability to deliver agents to large volumes of brain tissue without penetrating the BBB [13, 14]. Studies have also shown that drug delivery through direct CED injection provides for intraparenchymal

concentrations that are 100-fold greater than via intranasal delivery, and 1000- to 10,000-fold greater than by intravenous delivery [15-**17**]. CED facilitates GBM treatment by decreasing systemic toxicity and increasing the maximum-tolerated dosages **[18-20]**.

CED is one of the most promising delivery systems for enhancing therapeutic agents and reducing systemic toxicity. Temozolomide (TMZ), an antineoplastic alkylating agent that can cross the BBB, is usually administered orally in clinical practice, and has shown efficacy for systemic chemotherapy for GBMs [21]. However, TMZ is not an ideal drug for CED due to its lipophilic properties. In addition, TMZ has a lifetime dosage limit because it produces severe systemic toxicity involving the liver, lungs, and blood [21-23]. Furthermore, although a variety of effective methods have been developed to improve the drug delivery, liposome-encapsulated delivery systems have proven advantageous for circumventing drug degradation, prolonging drug half-life, improving therapeutic efficacy, and reducing acute toxicity from the free drug [24-26]. We found that TMZ that has been encapsulated into liposomal nanoparticles maintains hydrophilic solubility and increases colloidal viscosity, thereby limiting the volume of distribution and reducing its systemic toxicity. Liposomes labeled with a fluorescent probe (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, [DiI]) allowed the visualization of locally administered drugs. The development of CED-dye-liposome-nanoparticles to monitor drug distribution and real-time imaging for immediate assessment of concentration efficiency may improve CED for application in GBM treatment.

The aim of this study was to develop a highly stable, more hydrophilic, and liposome-encapsulated TMZ (LipoTMZ) that can be delivered to brain tumors by

CED. We characterized the physical properties of the LipoTMZ system complex, and studied its physical release *in vitro* to understand and improve its therapeutic efficacy. We used fluorescence microscopy and an *in vivo* imaging system (IVIS) to examine the distribution of LipoTMZ and evaluate its *in vivo* distribution in normal brain and tumor-bearing mice. We also examined the biological toxicity of LipoTMZ and the corresponding therapeutic response using CED. CED of LipoTMZ was selectively toxic against human U87MG xenografts, and significantly prolonged the survival of tumor-bearing mice.

Materials and Methods

LipoTMZ development and characteristics

For liposome preparation, dipalmitoylphosphatidylcholine (DPPC), cholesterol (Chol), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG [2000]-amine; (Avanti Polar Lipids Inc., Alabaster, AL), and α-tocopherol in a 3:1:1:0.004 molar ratio were formulated using the film hydration method described previously [27, 28]. For histological study, we added the fluorescent membrane dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; ThermoFisher Scientific Inc., Grand Island, NY) at 0.1 mol% to the lipid solution. Liposomes were passively loaded with TMZ (Lotus Pharmaceuticals, Taipei, Taiwan) for therapeutic application. Briefly, 10 mg of TMZ with lipid mixture was dissolved in a volume ratio of 1:5 of methanol and chloroform and dried in a flask to produce a homogeneous lipid film. The film was hydrated with 1 mL ammonium sulfate (pH 4.0) at 42°C until it dispersed to form liposomes. The suspension was then extruded successively through 200-nm and 100-nm

polycarbonate filters using an Avanti Mini Extruder (Alabaster, AL). Then we removed the unencapsulated TMZ by passing the Liposome-TMZ over a spin column equilibrated with PBS (pH 7.0) to maintain the osmolality in biological media. We characterized the LipoTMZ by measuring the zeta potential and dynamic light-scattering (DLS) on a Nano-ZS90 particle analyzer (Malvern Instruments, Malvern, Worcestershire, UK). Each run required approximately three minutes, and we calculated the average of data obtained from 10 runs. The sample was prepared and imaged by transmission electron microscopy (TEM). We determined the concentration of TMZ in the liposomes with a HPLC system (Hitachi L-7200, Tokyo, Japan) by measuring the absorbance at 280 nm using a C_{18} column equipped with a UV detector. The sample was passed through the HPLC system using a mobile phase of 80% 0.1 M of phosphoric acid and 20% methanol at a flow rate of 1.0 mL/min. We then calculated the encapsulation efficiency as the fraction of the original TMZ incorporated into the LipoTMZ vesicles. Based on the amount of TMZ detected by HPLC of the LipoTMZ, approximately 87.0% of the original TMZ had been incorporated into the LipoTMZ recovered.

TMZ drug release and cell viability

Temperature effect: To evaluate the release of TMZ from the LipoTMZ, we incubated LipoTMZ solutions in PBS at pH 7.0 and maintained them in a shaking incubator at 4°C, 25°C or 37°C. We recovered the TMZ from the released media by centrifugation. This procedure was repeated for six incubation times of 0.5, 6, 12, 24, 48, and 72 h. The HPLC system enabled us to quantitatively determine the concentration of TMZ in the released media. Finally, we calculated the percentage of the drug released compared to the total drug in the liposomes using a unique incubation time for every sample. The release experiments were performed in

triplicate.

Intracellular localization of LipoTMZ: To visualize intracellular localization, LipoTMZ, 0.1 mol% DiI-liposome (LipoDiI), which served as the drug surrogate, was used only in localization experiments. U87MG human glioma cells from the American Type Culture Collection (ATCC, MD) (1×10^5 cells/μL) were seeded on 6-well plates and grown for 48 h. Five microliters of LipoDiI vesicles were added to the culture medium. Cells were incubated with LipoDiI for 24, 48, and 72 h. Then, the media were removed from all the wells, and the cells were washed three times with PBS to remove any unincorporated LipoDiI. To visualize the intracellular localization of DiI dye in U87MG cells, we used a Leica TCS SP8X confocal laser scanning microscope (40X objective) processing an argon laser with an excitation wavelength of 488 nm.

Ex vivo cytotoxicity assay: To study the *in vitro* response of these cells to LipoTMZ, four experimental groups were evaluated consisting of the control, liposome-only, and free TMZ (500, 1000, 2000, 4000, and 8000 μmoles of TMZ in dimethyl sulfoxide [DMSO]) groups, and the LipoTMZ (TMZ at 500, 1000, 2000, 4000, and 8000 μmoles) group. We evaluated cell proliferation 24, 48 and 72 h prior to the addition of 0.25 mg/mL MTT-tetrazolium salts (Sigma-Aldrich Corp., St. Louis, MO) in PBS. Viability of the U87MG cells was calculated relative to the control cells at 24, 48 and 72 h. Following incubation with the MTT at 37°C for 2 h, we discarded the cell culture media and added DMSO to dissolve the dye. After shaking the mixture gently, we measured the absorbance at 450 nm using a spectrophotometer. All assays were performed in triplicate.

Convection-enhanced delivery (CED) system

CED procedures were performed using an infusion cannula composed of fused silica tubing with a 0.5-mm stepped-tip needle, as described previously [13, 29]. The

cannula was connected to a Hamilton syringe (Hamilton, Reno, NV) and attached to a micro-infusion pump (Bioanalytical Systems, Lafayette, IN). The syringe with the silica cannula was placed onto a stereotaxic head frame and then lowered into the brain_via a bun hole in the skull. The test agents were administered by CED, at an infusion rate at 1 μL/min until a volume of 5 μL or 10 μL of drug had been delivered. Afterwards, the syringe was gradually withdrawn over another two minutes.

Brain-tumor animal model

To study the effects of the CED-LipoTMZ system on biodistribution and therapeutic efficacy in normal and tumorous brain models, we used six-week-old male Nu/Nu mice, each weighing approximately 25 g, for all experiments. For the tumor model, U87MG human glioma cells (1×10^5 cells/μL) were collected, and 5 μL of tumor cell suspension was injected into each mouse's left striatum (with coordinates of 2.0 mm left and 1 mm anterior to bregma and 3 mm in depth). To enable *in vivo* imaging, we used U87MG human cells that were modified by lentiviral infection to achieve stable expression of firefly luciferase, as previously described [13]. Ten days after inoculation of the tumor cells, we selected animals with tumor growth confirmed using the *in vivo* imaging system (IVIS) Spectrum (Caliper, Hopkington, MA) for the experiments. All experiments met criteria outlined by the Institutional Animal Care and Use Committee of Chang Gung University (CGU-IACUC), and the mice were cared for according to the guidelines in *The Handbook of the Laboratory Animal Center*, Chang Gung University. The design of the experiments served to minimize the animals' suffering.

Brain biodistribution study

We used LipoDiI as a drug surrogate instead of visualizing the biodistribution of LipoTMZ in the normal brain parenchyma and in the U87MG tumor model for

drug delivery to the desired sites. Clearance of the fluorescence signal generated by LipoDiI in the left hemisphere when the drug surrogate was infused into the intact brain via CED was analyzed. The images were acquired after CED infusions of LipoDiI (5 μL/site), to analyze the signal intensity after treatment in order to determine the drug distribution of DiI-dye in the brain tissues. After IVIS imaging for three days, the mice were euthanized and all tissues were collected and then blocked in Tissue-Tek OCT Compound (Sakura Finetek Inc., CA) for later use. The entire fresh frozen brain and liver samples were sectioned into 15-μm thick sections in an axial direction and the DiI signal was imaged using a fluorescence microscope (TissueFAXS Plus, Tissue Gnostics, Austria). The tissue sections were stained with hematoxylin and eosin (H&E) for light microscopy. Five mice were used for each experiment.

Assessment of liposomal toxicity

To evaluate the toxicity of LipoTMZ on normal brain, we performed *in vivo* experiments with three experimental groups consisting of mice that received saline-only, liposomes only, and LipoTMZ (TMZ at 8.7 mg/mL) via CED infusion. The weight of mice in each group was monitored before the CED infusion and twice a week for a four-week period after treatment. Three days after infusion of 5 μL of the test agent into the brain by CED, three mice from each group were euthanized and their brains and livers tissues were removed and processed for histologic examination.

Efficacy analysis for LipoTMZ

To evaluate intracerebral tumor growth, we used an IVIS Spectrum system to monitor the tumor-bearing mice. Before imaging, D-luciferin (3 mg/mouse) was injected intraperitoneally. Eight minutes after injection, luminescent signals from the tumor were obtained by IVIS. We conducted imaging twice a week to assess the

efficacy and the dose-response to CED of LipoTMZ in animals with brain tumor xenografts. We anaesthetized mice with vaporized isoflurane, dividing them into six experimental groups, consisting of the control mice, mice that had received 10 μL of free TMZ administered orally (TMZ dissolved in DMSO at a concentration of 8.7 mg/mL), once per day for three days, and mice that received CED of liposome-only or LipoTMZ (8.7 mg TMZ/mL) at an infusion rate of 1 μL/min with 5 μL volume once on day 10 after tumor inoculation; 5 μL given twice on days 10 and 17; and 10 μL volume once on day 10. At the end of the study period, all mouse tissues were collected and then blocked in Tissue-Tek OCT Compound (Sakura Finetek Inc., CA) for later study.

Statistical analysis

The results are expressed as mean ± SD. We used SPSS version 16.0 statistical software (SPSS Inc., Chicago, IL) for data management and analysis. We used ANOVA combined with post-hoc testing to identify statistical differences. We plotted survival curves according to the Kaplan-Meier method. A *P*-value ≤ 0.05 was considered to indicative of a significant difference.

Results

Characterization of liposomal temozolomide (LipoTMZ)

To formulate liposomes containing temozolomide (LipoTMZ) for better drug delivery in a physiological environment, TMZ was loaded into liposomes using the film hydration technique [27, 28]. After encapsulating the TMZ with liposomes and removing the unincorporated TMZ by ion exchange chromatography, we calculated the amount of encapsulated TMZ from the quantity of TMZ in the resulting LipoTMZ. The zeta potential was approximately neutral after encapsulation (Fig. 1A). The size distribution of liposomes and LipoTMZ with average diameters was approximately 120 nm and 160 nm, respectively (Fig. 1B). A TEM image of LipoTMZ illustrates the formulation of LipoTMZ in the presence of sphere-shaped particles (Fig. 1C). The HPLC calibration curve demonstrated the relationship between absorbance intensity and TMZ concentration, which we calculated as the weight percentage of TMZ entrapped in the liposomes (Fig. 1D). The LipoTMZ had a high entrapment efficiency of 87%, and a load of approximately 8.7 mg TMZ per mL of LipoTMZ suspension.

Ex vivo release of LipoTMZ and cell viability

To study the effect of temperature on LipoTMZ release *ex vivo*, we examined the TMZ release profile of LipoTMZ at 4°C, 25°C, and 37°C. The release of TMZ over 72 h was very slow at 4°C and 25°C, and increased quite obviously once the temperature changed to 37°C, with nearly 80% released, compared to only 15% released at 25°C and only 5% released at 4°C (Fig. 2A). These data suggested that the LipoTMZ was stably maintained in its physical state at low temperatures, but could respond quickly to changes in physiological conditions. These results indicate that drug release increased with increases in both time and temperature.

To test the cellular uptake and subcellular distribution of the drug and its release, the liposomes were labeled with a fluorescent dye, DiI, and incubated with U87MG cells for 24, 48, and 72 h. The images showed that the LipoDiI bound the cell surfaces and was also internalized into the nucleus over time (Fig. 2B). These results revealed that liposomes can encapsulate TMZ to carry drugs to cancer cells, and that the cellular uptake and nuclear distribution was suitable for application in drug delivery.

To evaluate the *in vitro* antitumor effects of LipoTMZ for anticipated therapeutic applications, we exposed U87MG human glioma tumor cells to either liposomes alone, free-TMZ, or LipoTMZ. The control group received no liposomes, no TMZ, and no LipoTMZ. The TMZ concentrations of the free TMZ group and the LipoTMZ group ranged from 500 μmoles to 8000 μmoles in the cell suspension. We measured cell viability for all experimental groups after 24, 48, and 72 h after treatment with the corresponding agents. LipoTMZ was more potent than free TMZ, indicated by the considerably lower IC_{50} for LipoTMZ (approximately 4000 μmoles after incubation for 24 h), lower than that for free TMZ (Fig. 2C). At a concentration of 4000 μmoles, LipoTMZ had significantly enhanced cytotoxicity against U87MG cells compared to the control, liposome-only, and free TMZ-treated cell groups ($P<0.05$) after 24, 48, and 72 h (Fig. 2D). The liposome exhibited no apparent cytotoxicity in tumor cells, whereas free TMZ and LipoTMZ resulted in higher cytotoxicity against U87MG cells. The cytotoxicity of free TMZ and LipoTMZ rapidly declined, with 67% and 43% cell viability after 24 h, respectively; 66% and 38% cell viability after 48 h, respectively; and 8% and 35% cell viability after 72 h, respectively. Although free TMZ resulted in similar cytotoxicity when compared to LipoTMZ, the difference was not statistically significant after 48 h. In contrast, LipoTMZ still demonstrated relatively high cytotoxicity against tumor cells after 24 h. These results indicate that LipoTMZ

enhanced cytotoxicity compared to the free TMZ, confirming the slow release of TMZ from the LipoTMZ, as well as a time-dependent cytotoxicity.

Direct visualization of drug distribution via the CED system

To visualize the distribution of the liposome-encapsulated drugs in the brain directly, LipoDiI was reconstituted with the IVIS contrast agent and used as drug surrogate for monitoring LipoTMZ distribution. LipoDiI (5 μL of 0.1 mol%) was infused into the parenchyma of normal mouse brains via CED. We measured the distribution of liposomal carriers using histological detection of fluorescence generated and IVIS signals from DiI dye. The signals from infused DiI-liposome were clearly observed *in vivo* and in brain tissues after a three-day period (Fig. 3A). The brain and liver sections also were examined after 3 days of infusion (Figs. 3B and 3C). The results of the fluorescence analysis showed that the LipoDiI had been cleared, with residual LipoDiI remaining only within the CED brain site and surrounding tissue area. Interestingly, no signs of accumulation or deposition of DiI-liposomes in the liver tissue were observed. H & E stained sections of brain and liver showed no evidence of pathologic effects attributable to infusion (Figs. 3B′ and 3C′). Both histologic and fluorescence analysis confirm the improved stability and diffusive distribution of drug carriers into targeted infusions without concomitant systemic toxicity. These data also indicated that CED did not produce hematologic toxicity, while effectively targeting the cancer cells.

Toxicity evaluation

To evaluate the toxicity of LipoTMZ, mice received 5-μL CED infusions of saline, liposome alone, or LipoTMZ (TMZ at 8.7 mg/mL) into their left hemispheres. Histopathologic examination revealed no obvious difference in the brain tissue sections of the three groups of mice (Fig. 4). These results suggest that there was no

additional tissue damage from LipoTMZ treatment over a three-day observation period. In addition, the mice appeared healthy and maintained their body weight, indicating an absence of neurological toxicity during CED infusion over the four-week observation period.

Therapeutic effects in a brain-tumor model

To further visualize the effects of the CED-LipoTMZ system in brain tumors, LipoDiI was used as a drug surrogate for monitoring LipoTMZ distribution *in vivo*. Tumor-bearing mice were euthanized 1 h after infusion of 5-μL of LipoDiI. Histological evaluation of the U87MG brain-tumor sections using HE staining and fluorescence microscopic analysis showed extensive peritumoral extravasation throughout the majority of the tumor (Figs. 5A and 5B). To evaluate the therapeutic response to tumor treatment using the CED-LipoTMZ system, the tumor-bearing mice were infused with liposomes alone, 5 μL and 10 μL of LipoTMZ (8.7 mg/ml TMZ), or 5 μL of LipoTMZ with an additional 5 μL administered seven days after the initial infusion. For the TMZ group, the tumor-bearing mice received a single, 10 μL oral dose of TMZ dissolved in DMSO at a concentration of 8.7 mg/mL. Representative tumor sizes are shown as regions-of-interest (ROI) ratios. The control group developed large U87MG brain tumors in the treated hemisphere, whereas tumor-bearing mice that received either TMZ (orally) or LipoTMZ (by CED), developed significantly smaller brain tumors compared to the controls (Fig. 5C). Interestingly, two 5-μL doses of CED-LipoTMZ effectively inhibited tumor growth, and also resulted in a significantly improved tumor-growth-ratio reduction. Overall, LipoTMZ via CED enhanced therapeutic efficacy. In particular, low doses of TMZ achieved higher therapeutic responses with the CED delivery of LipoTMZ. We also analyzed animal survival plotting the results using the Kaplan-Meier method (Fig.

5D). We determined overall median survival, setting tumor inoculation day as day 0; and performed a log-rank statistical analysis of the Kaplan-Meier plot. Tumor-bearing mice treated with CED-LipoTMZ at a volume of 5 μL (0.0435 mg/mouse) died by day 31.5, with a median survival time of 28.5 days. There was no significant improvement in overall median survival when compared to the control group (median survival, 25 days). Animals that received CED-LipoTMZ at a volume of 10 μL (0.087 mg/mouse) all died by day 48, with a median survival of 37.5 days. Although the administration of the 10-μL dose of CED-LipoTMZ did significantly prolong animal survival (median survival, 35 days), oral TMZ at a dose of 0.087 mg/mouse/day for three days did not significantly improve animal survival (median survival, 28.5 days). In contrast to the 10-μL dose of CED-LipoTMZ, the 5-μL dose of CED-LipoTMZ with the additional 5-μL administration increased survival in all animals to greater than 70 days (median survival, 50 days), while treatment resulted in two of the ten animals (20%) in the CED-LipoTMZ treatment group surviving beyond day 70.

Discussion

In this study, we successfully encapsulated TMZ into liposomes (denoted as "LipoTMZ"), which effectively released into U87MG human tumor cells killing many more cells compared to tumor cells exposed to liposomes only, or to free TMZ. The combination of TMZ and CED allowed the drug carriers located at the infusion sites to distribute a low, yet sufficient quantity of the TMZ without causing systemic toxicity in U87MG tumor-bearing mice. With CED, bioavailability of TMZ was greater than via the oral route. In addition, the therapeutic response to tumor treatment using the CED-LipoTMZ formulation enhanced therapeutic efficacy when 5 μL were administered, followed by an additional 5 μL administered seven days after the initial

infusion.

Conventional liposomes containing zwitterionic phospholipids and cholesterol produce stable constructs and exhibit several potential therapeutic advantages over corresponding lipophilic agents. Among these advantages are highly stable formulations that maintain the constancy of TMZ levels (with high entrapment efficiency of approximately 87%). We encapsulated TMZ into liposomes, which effectively prevented the drug from degradation and changed a lipophilic drug into a hydrophilic one. The DPPC-based liposome-TMZ displayed a fast-release kinetic profile, showing approximately 50%, 63%, and 80% of TMZ release after 24 h, 48 h, and 72 h, respectively, at 37°C. We concluded that DPPC/PEG(2000)-amine, a lipid formulation with a positive surface charge, could reliably interact with TMZ to form a stable bilayer structure and contributed to high drug-entrapping efficiency. In addition, the DPPC phase transition temperature is approximately 40°C, therefore, the temperature responses indicate drug release may be augmented around the liposome by disruption of the lipid physical state of the liposomal colloidal system. This effect could account for the effect of temperature or the LipoTMZ solution on both cellular uptake and subcellular distribution of TMZ, and was sufficient to enhance cellular transfer of the drug surrogate or drug carrier. The internalization of DiI-labeled liposomes, visualized using fluorescence microscopy indicate that these DPPC-based liposome TMZ complexes could interact with the brain tumor cells. The cytotoxicity induced by LipoTMZ was substantially higher ($P<0.05$) compared with that induced by free TMZ drug.

CED is a promising method that directly delivers drugs to CNS tumors. Entrapment of lipophilic TMZ in liposomal phospholipids, which were released effectively into U87MG human tumor cells, killed many more tumor cells than was

the case with tumor cells exposed either to liposomes only or to free TMZ. We developed LipoTMZ to enhance the efficacy of chemotherapy and reduce its side effects by constructing vesicles that would sequester the drug and then release it into the cytosol only in desired locations targeted by CED. Our data show that a CED-LipoTMZ system can extensively distribute the drug-carrying liposomes in the tumors in a way that enhances tumoricidal activity and inhibits tumor growth response. Immunohistochemical analysis revealed that, when infused into brain tumors via the CED, LipoTMZ was no more toxic to CNS cells compared to systemic administration.

We used LipoDiI as a drug surrogate and imaging contrast reagent in this study to explore the feasibility of the CED-LipoTMZ system for localized delivery in normal mouse parenchyma, and to monitor drug distribution and toxicity. No systemic toxicity nor further brain tissue damage was detected during the three-days after CED infusion of LipoTMZ comparing to CED of saline. **In contrast, Huo et al. [34] used liposomal cisplatin as a therapeutic agent to show that CED-liposomal cisplatin could be highly neurotoxic in the normal brain tissues for the following 10 or 14 days after infusion. Instead of temozolomide, cisplatin is known to produce significant toxicity to both CNS and PNS, in a cumulative and dose-dependent manner [35, 36]. Thus, toxicity examination for CED of candidate liposomal chemotherapeutics in normal brain is worth to perform and could help us to select not only effective but also safe drugs for brain tumor treatment.** The LipoDiI used in this study may be advantageous for *in vivo* use. Our results clearly show that DPPC-based liposomes can be locally delivered by CED, and that the half-life of TMZ in tissue can be extended with reduced toxicity using drug encapsulation techniques.

Tumor-bearing mice treated with CED-LipoTMZ had significantly reduced tumor growth and improved survival times compared to the control groups. Since TMZ is an alkylating agent that targets proliferative cells, and tumor cells do not undergo cell division in a concerted manner, repeated administration of TMZ may kill the tumor cells that have delayed proliferation and further inhibit tumor growth. CED of nanodrugs into tumor tissue is dependent on the concentration difference between the infusion site and the tumor tissues, as well as the bulk flow between tumors and normal tissues [30, 31]. The concentration of drugs in the vicinity of drug receptors can help determine their therapeutic efficacy and toxicity. At high concentrations, effects plateau so that further increases in drug concentration do not produce greater effects [32, 33]. In this study, the plateauing phenomenon might have been due to a high load of 8.7 mg TMZ per mL of LipoTMZ suspension. The cumulative rate of the external pressure gradient elevation to augment the therapy through an additional 5 μL-dose of infusion could enhance drug concentrations in tumors when compared to a single injection.

Despite the advantages of CED-LipoTMZ described here, other practical obstacles need to be solved before the method can be applied for clinical management of brain tumors. An appropriate imaging system is required to monitor and confirm drug distribution. In addition, nanodrugs with both chemotherapeutic and image-contrast functions may be required to monitor the therapeutic drug and its toxic effects in treated regions during and after CED-nanodrug infusion. The efficacy and safety of treatment observed in the animal brain tumor model suggests that the CED-LipoTMZ system is a potential alternative treatment modality for human glioma patients and warrants further development

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Figure Legends

Fig. 1 Characterization of the liposome nanoparticles used in this study: (A) the zeta potential of LipoTMZ; (B) the particle size distribution of liposome (blue) and LipoTMZ (red) measured by means of dynamic light scattering; (C) TEM image of LipoTMZ; D) TMZ encapsulation efficiency determined by HPLC.

Fig. 2 TMZ release from LipoTMZ. (A) TMZ release from LipoTMZ when incubated at 4°C, 25°C, and 37°C and over different incubation periods. (B) Confocal microscopic images of U87MG tumor cells after uptake of DiI-labeled liposomes (LipoDiI) at different time intervals. (C) Cell viability after different treatment conditions: control, liposome-only, free-TMZ, and LipoTMZ (graded drug concentration). (D) Cell viability measured at 24, 48, and 72 h for the indicated drug concentrations. Results are expressed as the mean ± SD ($n \geq 3$, for each treatment). $^{*}P<0.05$.

Fig. 3 (A) *In vivo* imaging system (IVIS) images of DiI-labeled liposomes (LipoDiI) distribution in normal brain tissue using convection-enhanced delivery (CED) 3 days after infusion. (B) & (C) The retention of LipoDiI in brain- and liver-tissue sections from a sacrificed mouse 3 days after infusion using fluorescence imaging to detect the fluorescence generated from DiI dye. The image shows the distribution of the drug surrogate LipoDiI, which served as the drug surrogate, in cross sections of tissue surrounding the brain tissue. (B', C') HE staining of the brain and liver tissue sections. The bioluminescence signal (p/sec/cm^2/sr) is represented as a measure of signal intensity after dye infusion. Images were obtained at 20X magnification.

Fig. 4 Toxicity from CED treatment with saline, liposome alone, and LipoTMZ infusion, as shown in three representative sections of brains after three days. The body weights of mice in each group were monitored twice a week for a four-week period after treatment.

Fig. 5 (A) Retention of LipoTMZ-labeled DiI dye was monitored using fluorescence imaging to detect the fluorescence generated from DiI dye. The image shows the distribution of LipoTMZ in cross sections of tissue surrounding the brain glioma 1 h after infusion. (B) HE staining of the brain-tumor tissue section. (C) Brain tumor growth inhibition under different treatment conditions and percentage of animals surviving after different treatment conditions.