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Convection-enhanced delivery of Ls-TPT enables an effective, continuous, low-dose chemotherapy against malignant glioma xenograft model¹

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Treatment of malignant gliomas represents one of the most formidable challenges in oncology. The combination of surgery, radiation, and chemotherapy yields median survivals of less than one year. Here we demonstrate the use of a minimally invasive surgical technique, convection-enhanced delivery (CED), for local administration of a novel nanoparticle liposome containing topotecan. CED of this liposomal topotecan (Ls-TPT) resulted in extended brain tissue retention ($t_{1/2} = 1.5$ days), whereas free topotecan was rapidly cleared ($t_{1/2} = 0.1$ days) after CED. The favorable pharmacokinetic profile

of extended topotecan release for about seven days, along with biodistribution featuring perivascular accumulation of the nanoparticles, provided, in addition to the known topoisomerase I inhibition, an effective antiangiogenic therapy. In the rat intracranial U87MG tumor model, vascular targeting of Ls-TPT with CED was associated with reductions in laminin expression and vascular density compared to free topotecan or control treatments. A single CED treatment on day 7 showed that free topotecan conferred no survival benefit versus control. However, Ls-TPT produced a significant ($P = 0.0002$) survival

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relationship with Johnson & Johnson, Genentech, and Pfizer, and he is a coinvestigator on patents or patent applications with the University of California. Krystof S. Bankiewicz or UCSF is considering a patent application that may include data in this article.

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³Abbreviations used are as follows: CED, convection-enhanced delivery; DiI_{C18}, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine-perchlorate; GBM, glioblastoma multiforme; HBSS, Hanks' balanced salt solution; H&E, hematoxylin and eosin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; Ls-TPT, liposomal topotecan; TEA₈SOS, triethylammonium sucrose octasulfate.

benefit, with six of seven complete cures. Larger U87MG tumors, where CED of Ls-TPT on day 12 resulted in one of six cures, indicated the necessity to cover the entire tumor with the infused therapeutic agent. CED of Ls-TPT was also efficacious in the intracranial U251MG tumor model ($P = 0.0005$ versus control). We conclude that the combination of a novel nanoparticle Ls-TPT and CED administration was very effective in treating experimental brain tumors. *Neuro-Oncology* 8, 205-214, 2006 (Posted to *Neuro-Oncology* [serial online], Doc. D05-00015, May 24, 2006. URL www.dukeupress.edu/neuro-oncology; DOI: 10.1215/15228517-2006-001)

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The prognosis for patients with malignant gliomas has remained dismal, and the median survival for patients with glioblastomas is still less than one year after diagnosis (Gupta and Sarin, 2002). The standard of care for patients with high-grade malignant glioma is resection, followed by focal radiation therapy. The utility of chemotherapy, although accepted as one of the standard strategies for other malignancies, is still a matter of debate for these patients because malignant gliomas have largely been refractory to treatment with systemic chemotherapy (Stewart, 2002). The blood-brain barrier is partially responsible for this refractoriness (Groothuis, 2000). Even though it is largely disrupted within the tumor core, allowing most systemic chemotherapeutic agents to access the tumor, the barrier is mostly intact at the growing tumor margin. Thus, systemic chemotherapy may be unable to deliver tumorocidal drug concentrations to the tumor target site without incurring unacceptable systemic side effects. To overcome these difficulties, and from the clinical observations that 90% of malignant glioma recur within 2 cm of an original resection site, intratumoral chemotherapy has been proposed and holds promise (Walter et al., 1995). However, many local drug delivery techniques face the major problem of poor diffusion and distribution of infused drugs. In 1994, Bobo and colleagues (1994) introduced convection-enhanced delivery (CED)³ as a strategy to overcome this difficulty. CED is a direct infusion technique that takes advantage of bulk flow to enable the delivery of small and large molecules to a targeted site, offering an improved volume of distribution in comparison to simple diffusion. CED of therapeutic agents bypasses the blood-brain barrier, delivers a high concentration of therapeutic agents to the injection site, provides wider distribution of therapeutic agents within the target site, and minimizes systemic exposure, resulting in fewer systemic side effects. Since the introduction of this technique, CED has shown considerable promise for the treatment of brain tumors, with some protocols now in clinical trials (Kunwar, 2003; Vogelbaum, 2005).

Topotecan is a water-soluble camptothecin analog that binds and inhibits topoisomerase I, leading to DNA damage in tumors (Kohn et al., 2000). Topotecan has shown cytotoxicity toward a variety of tumor types, including ovarian cancer, small-cell lung cancer, and gli-

omas (Friedman et al., 1994; Houghton et al., 1995; ten Bokkel Huinink et al., 1997, 2004). However, while significant activity has been noted in treating brain tumors (Rapisarda et al., 2004), its clinical use has been limited by its systemic toxicity (Lesimple et al., 2003; Pipas et al., 2005). Delivery via CED of free topotecan to brain tumors is currently under investigation as a means to avoid systemic toxicity (Kaiser et al., 2000).

To improve the systemic pharmacological profile of camptothecin drugs, drug delivery systems such as liposome encapsulation have been actively pursued. Topotecan transforms from an active lactone configuration to an inactive carboxylate configuration while in the circulation system, an equilibrium that is in turn affected by binding to plasma proteins (Burke and Gao, 1994; Chourpa et al., 1998; Mi et al., 1995). Encapsulation in acidic liposomes can help maintain topotecan in the active lactone form (Burke and Gao, 1994) and thus may help improve its activity *in vivo*.

Visualization of drug distribution during and after CED is crucial in a clinical setting, where the objective is to cover the tumor mass completely to maximize therapeutic effect. We have established a noninvasive method for detection of gadolinium-loaded liposomes in rodent brain tumors after CED that uses MRI (Mamot et al., 2004; Saito et al., 2004). These studies have led to development of a method for real-time monitoring of liposomal agents with gadolinium-loaded liposomes in primate brain (Krauze et al., 2005a; Saito et al., 2005), which can be used to optimize CED administration of these nanoparticle agents.

In this study, we report the efficacy of a novel, highly stable, nanoparticle-liposome-encapsulated topotecan delivered by CED to brain tumors. This locally infused liposomal topotecan (Ls-TPT) exerted strong antiangiogenic activity and demonstrated an excellent selective toxicity against human glioblastoma xenografts, significantly prolonging the survival of tumor-bearing rats.

Materials and Methods

Drugs and Liposomes

Topotecan and Ls-TPT were gifts of Hermes Bioscience, Inc. (South San Francisco, Calif.). Fluorescent liposomes were composed of a 3:2 (mol/mol) phospholipid-cholesterol mixture. 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, Ala.) was used as the phospholipid component, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(methoxy[polyethylene glycol]-2000) (Avanti Polar Lipids) was incorporated at 10% (mol/mol) of the total phospholipid content. Cholesterol was obtained from Calbiochem (San Diego, Calif.). Liposomes were labeled with membrane-bound 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-perchlorate (DiI_{C18}; Sigma, St. Louis, Mo.). The dye composition was 1% (mol/mol) of the total phospholipids. Liposomes were prepared by lipid-film hydration in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline

(pH 6.5). Each sample was hydrated by six successive cycles of freezing (-80°C) and thawing (60°C). Unilamellar liposomes were formed by extrusion with use of a 10-ml-capacity extruder (Northern Lipids, Vancouver, B.C., Canada). Liposomes were sized by extrusion through polycarbonate membranes of an approximate pore size of $0.1\ \mu\text{m}$, which resulted in liposomes that averaged $112.5 \pm 18.6\ \text{nm}$, determined by dynamic light scattering using an N4 Plus particle size analyzer (Beckman Coulter, Fullerton, Calif.).

Topotecan-loaded liposomes were prepared with a modified, remote-loading, ion-gradient and intraliposomal drug-stabilization method. Briefly, after the lipids were dried in chloroform-methanol (9:1, v/v), they were dried by rotary evaporation and subsequently under vacuum for 2 h. The lipids were then resuspended in 1 ml of ethanol at 60°C . An aqueous solution of triethylammonium sucrose octasulfate (TEA_8SOS , 0.65 M) was prepared by cation-exchange chromatography as previously described (Drummond et al., 2005) and was also heated to 60°C . The TEA_8SOS solution was then injected rapidly into the ethanolic lipid solution to form liposomes. The liposomes were sized by extrusion through polycarbonate filters of average size $0.1\ \mu\text{m}$, which resulted in an average size of 105–122 nm. Unencapsulated TEA_8SOS was removed by Sepharose CL-4B (GE Healthcare, Chalfont St. Giles, U.K.) size-exclusion chromatography, eluting with HEPES-buffered dextrose (5 mM HEPES and 5% dextrose, pH 6.5). Topotecan was added at a ratio of 350 g TPT/mol phospholipid, and loading was initiated by adjusting the pH to 6.5 and incubating the mixture for 30 min at 60°C , followed by quenching on ice for 15 min. Unencapsulated TPT was removed by Sephadex G-75 (GE Healthcare) gel filtration chromatography, eluting with HEPES-buffered saline (5 mM HEPES and 145 mM NaCl, pH 6.5); the drug concentration and phospholipid concentration of the purified solution were determined, spectrophotometrically at 375 nm after dissolution in acidic methanol and by a standard phosphate assay (Bartlett, 1959), respectively. The loading efficiency was always greater than 95% for the preparations used in these studies.

Cell Lines and Animals

Established human glioblastoma multiforme (GBM) cell lines U87MG and U251MG were obtained from the Brain Tumor Research Center Tissue Bank at the University of California, San Francisco. Cells were maintained as monolayers in a complete medium consisting of Eagle's minimal essential medium supplemented with 10% fetal calf serum, nonessential amino acids, 0.1 mg/ml of streptomycin sulfate, and 100 U/ml of penicillin G. Cells were cultured at 37°C in a humidified atmosphere consisting of 95% air and 5% CO_2 . Congenitally athymic, male, nude rats (rnu/rnu, homozygous) were purchased from the National Cancer Institute (Bethesda, Md.) and were housed under aseptic conditions, which included filtered air and sterilized food, water, bedding, and cages. Animals weighed approximately 250 g at the initiation of the studies. All protocols used in the animal

studies were approved by the University of California, San Francisco, Institutional Animal Care and Use Committee.

Intracranial Tumor Implantation

Cells were harvested by trypsinization, washed once with Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS), and resuspended in HBSS for implantation. A cell suspension containing 5×10^5 cells/ $10\ \mu\text{l}$ HBSS was used for implantation into the striatum of rat brains. Under deep isoflurane anesthesia, rats were placed in a small-animal stereotactic frame (David Kopf Instruments, Tujunga, Calif.). A sagittal incision was made through the skin to expose the cranium, and a small dental drill was used to make a burr hole in the skull 0.5 mm anterior and 3 mm lateral to the bregma. A $5\text{-}\mu\text{l}$ cell suspension was injected at a depth of 4.5 mm from the brain surface. After 2 min, another $5\ \mu\text{l}$ was injected at a depth of 4 mm. After a final interval of 2 min, the needle was removed, and the wound was sutured.

Convection-Enhanced Delivery

A sagittal incision was made in the skin to expose the cranium, and with a small dental drill, a burr hole was made in the skull 0.5 mm anterior and 3 mm lateral to the bregma (for CED into tumor-bearing rats, the same burr hole made at the time of tumor implantation was used). Infusions were performed at a depth of 4.5 mm from the brain surface by using the CED method described in our previous studies (Mamot et al., 2004; Saito et al., 2004). Briefly, an infusion cannula connected to a Hamilton syringe (Hamilton, Reno, Nev.) was attached to a rate-controllable microinfusion pump (Bioanalytical Systems, Lafayette, Ind.). Slow-infusion CED was performed with an ascending schedule of infusion rates to achieve a total volume of $20\ \mu\text{l}$ ($0.2\ \mu\text{l}/\text{min}$ for 15 min, $0.5\ \mu\text{l}/\text{min}$ for 10 min, and $0.8\ \mu\text{l}/\text{min}$ for 15 min). After infusion, the cannula was removed and the wound sutured.

Detection of Fluorescently Labeled Liposomes

Ten days after tumor implantation, four rats received CED of fluorescent liposomes labeled with DiIC₁₈(3)-DS at a phospholipid concentration of 0.5 mM. All four rats were euthanized immediately after the infusion. The brains were harvested, freshly frozen in ice-cold isopentane, and cut into serial coronal sections ($25\ \mu\text{m}$) with a cryostat. The fluorescent signal generated by DiIC₁₈(3)-DS was visualized with a fluorescence microscope by excitation at 540/25 nm with a band-pass filter and with a long-pass filter at 565 nm for emission. A charge-coupled device camera with a fixed aperture was used to capture the image.

Evaluation of Toxicity

The CED of Ls-TPT (5 mg/ml [$n = 4$] or 0.5 mg/ml [$n = 4$]) was performed with normal Sprague-Dawley rats. Rats

were observed for the development of any neurological symptoms and weight loss. On day 21 after drug administration, the brains were harvested and fixed with 10% formalin. Paraffin sections (5 μ m) were cut and stained with hematoxylin and eosin (H&E).

Quantification of Topotecan After CED into Rat Brains

Rats were sacrificed at the prescribed times and the brains removed after perfusion with PBS. The brains were then frozen and kept at -80°C until analysis. The hemisphere receiving drug was homogenized by mortar and pestle under liquid nitrogen. Blank tissue homogenates were spiked with TPT to determine the efficiency of extraction. Homogenates of 50% tissue/water (100 μ l) were extracted with 400 μ l of methanol/0.1 M phosphoric acid (80:20) by vortexing for 10 s. The extraction mixture was kept at -80°C for 2 h and then centrifuged at 13,000 rpm for 15 min. The supernatant was transferred to a high-performance liquid chromatography (HPLC) autosampler vial that was maintained at 4°C . A 50- μ l injection volume was used for each sample/standard. Separation was conducted on a C-18 reverse-phase silica column (Supelco, Bellefonte, Pa.; C-18 column, 250 mm $\times$ 4-mm i.d., particle size of 5 μ m) with a C-18 guard column. A mobile phase, consisting of 3% triethylammonium acetate pH 5.5/acetonitrile (85:15), was used for isocratic elution, and TPT was detected by absorbance at 375 nm. TPT tissue spike extraction recovery was determined to be 101%.

Visualization of the Tumor Vasculature

To visualize blood vessels in tumors, immunohistochemical staining with laminin antibody (1:200 dilution; Sigma) was performed. Brains were fixed with 10% formalin, frozen with ice-cold isopentane, and cut into 40- μ m sections. Floating sections were incubated with blocking solution (1% normal goat serum and 0.01% Triton X-100 in PBS) to reduce nonspecific background staining. Primary antibody was applied overnight at room temperature. On the following day, rhodamine-conjugated antirabbit serum (1:100 in blocking solution; Jackson ImmunoResearch Laboratories, West Grove, Pa.) was applied at room temperature for 1 h. After washing with PBS, sections were mounted onto microscope slides, covered with mounting medium (Gel/Mount; Biomedex, Foster City, Calif.), and sealed with coverslips. Cells were observed by phase contrast and fluorescence microscopy with a TRITC (tetramethylrhodamine isothiocyanate) filter (no. 31002; Chroma Technology Corp., Rockingham, Vt.), and a charge-coupled device camera with a fixed aperture was used to capture the images.

Western Blots

Tumors were harvested from the brain of rats infused by CED with fluorescent liposomes, free topotecan, or Ls-TPT. These tumors were homogenized, and protein was extracted in cell lysis buffer (Cell Signaling Tech-

nology, Beverly, Mass.). Equal amounts of protein were separated electrophoretically on a 7.5% sodium dodecyl sulfate-polyacrylamide gel and blotted onto polyvinylidene fluoride membrane (Bio-Rad, Hercules, Calif.). The polyvinylidene fluoride membrane was incubated in blocking buffer TBS (tris-buffered saline)/casein blocker; Bio-Rad), followed by anti-laminin β -1 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.; 1:200, 4°C overnight), anti-phospho-Akt antibody (Cell Signaling Technology; 1:500, 4°C overnight), and anti-beta actin antibody (Sigma Bioscience; 1:1000, room temperature, 1 h). The blot was visualized with a colorimetric detection kit (Opti-4CN detection kit; Bio-Rad).

Antitumor Efficacy of Ls-TPT in U87 and U251 Xenografts

Thirty-three rats that received U87MG tumor cell implants were randomly divided into five groups: for the day 7 CED, the control group ($n = 7$), the free TPT group ($n = 7$), and the Ls-TPT group ($n = 7$), and for the day 12 CED, the control group ($n = 6$) and the liposomal TPT group ($n = 6$). Fourteen rats that received U251MG tumor cells implants were also randomly divided into two groups: the control group ($n = 7$) and the Ls-TPT group ($n = 7$).

Statistical Analysis

Results for the survival studies are expressed as a Kaplan-Meier plot. Survival between the treatment groups was compared with a log-rank test.

Results

Detection of Fluorescent Liposomes After CED

Ten days after tumor implantation, CED of liposomal DiI₁₈(3)-DS (20 μ l) was performed. Detection by fluorescence microscopy revealed that liposomes covered almost the entire U87MG intracranial tumor xenograft (Fig. 1A). Observation at higher magnification ($\times 200$) of these sections revealed fluorescent liposomes at the intercellular gap region in the tumor (Fig. 1B). In addition, accumulation of infused liposomes at the distribution margin in the perivascular or periaxonal space was observed (Fig. 1C).

Evaluation of Local Brain Toxicity After CED of Ls-TPT

The local tissue toxicity of Ls-TPT was evaluated in normal rat brain. Since we have already demonstrated that the tissue distribution of infused liposomes has a linear correlation with infused volume, and that concentration of liposomes has little impact on volume of distribution (Saito et al., 2005), the same infusion volume (20 μ l) with different drug concentrations was used to test for toxicity. Previous studies demonstrating the effect of free topotecan infused by CED into the brain tumor xeno-

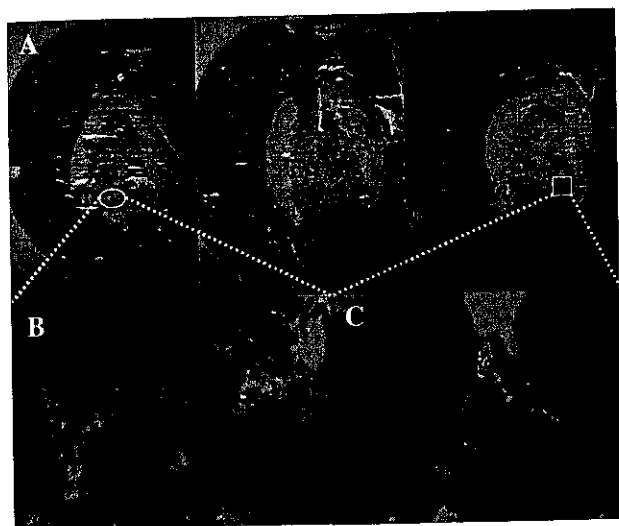


Fig. 1. CED of fluorescent liposomes into U87MG intracranial xenografts. Four rats received CED of liposomal DiI_{C18}(3)-DS 10 days after tumor implantation. Rats were euthanized immediately after the infusions, and their brains were freshly frozen, sectioned on a cryostat, and subjected to histological examination. After determining the distribution of liposomes by fluorescence microscopy, the same sections were stained with H&E. Fluorescence images were overlapped with H&E images. A. Serial sections 25 μ m thick, collected at 1-mm intervals (magnification $\times 12.5$). B. Higher magnification ($\times 200$) of the circled region is shown within the volume of distribution. C. Boxed region at the tumor boundary. Similar findings were observed in all four rats.

graft model used up to 160 μ g/kg/day for two days (Kaiser et al., 2000), which translates to 80 μ g per animal if a 250-g rat is used. We therefore tested for toxicity of Ls-TPT at a concentration of 5 mg/ml (20- μ l infusion = 100 μ g Ls-TPT per infusion). This concentration turned out to be highly toxic, and two of four rats died within seven days. Examination of the two rats that survived for seven days revealed extensive brain damage at necropsy (Fig. 2A). However, at an Ls-TPT concentration of 0.5 mg/ml (20- μ l infusion = 10 μ g Ls-TPT per infusion), rats demonstrated no symptoms of toxicity after infusion and were euthanized three weeks after infusion. Histological examination revealed no signs of toxicity at the infusion site or surrounding brain tissue (Fig. 2B).

Prolonged Retention of Topotecan by Liposomal Encapsulation

Either Ls-TPT or free topotecan was infused at 0.5 mg/ml into healthy Sprague-Dawley rat brains by CED to study drug clearance. Analysis of brain tissue revealed a dramatic improvement in brain retention of Ls-TPT as compared to that of free topotecan (Fig. 3). Tissue half-life of Ls-TPT was 1.5 days versus 0.1 days for free topotecan. The liposomal formulation extends the residence time of topotecan to the degree that it can be detected for as long as seven days after CED, whereas

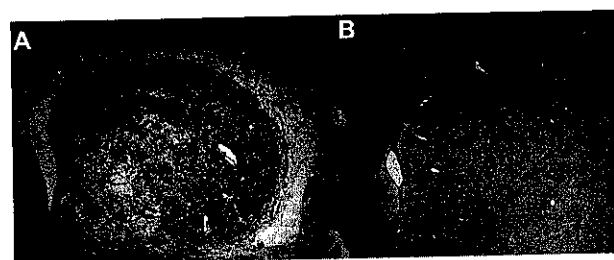


Fig. 2. Histological evaluation of Ls-TPT after CED infusion into intact striatum of 20 μ l. Ls-TPT was administered to normal Sprague-Dawley rats. A. All four rats receiving Ls-TPT at a concentration of 5 mg/ml were euthanized within seven days because of pathological symptoms. B. Four rats receiving 0.5 mg/ml Ls-TPT remained healthy for the duration of the study and were euthanized three weeks after infusion for histological examination (termination of toxicity study). The brains were harvested and fixed with 10% formalin, and paraffin sections (5 μ m) were obtained and stained with H&E.

free TPT is completely cleared within one day. The longer residence time suggests that the Ls-TPT formulation remains stable during CED. After infusion, Ls-TPT presumably acts as a reservoir for the slow release of drug and thereby facilitates prolonged exposure of tumor tissue to the drug.

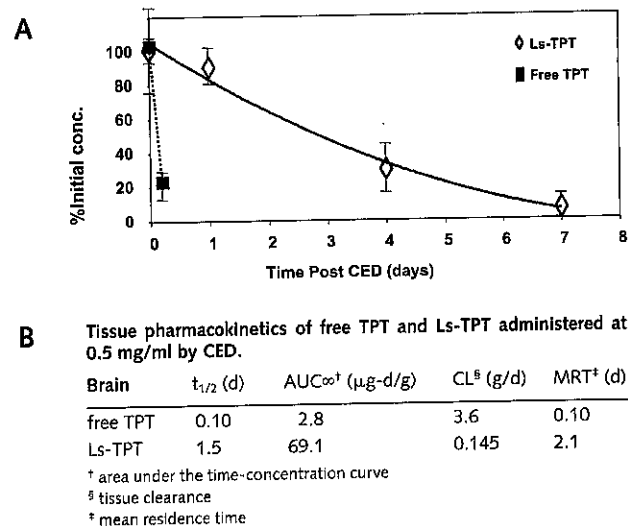


Fig. 3. Drug retention of liposomal topotecan and free topotecan and the pharmacokinetics after CED. Either Ls-TPT or free TPT was infused into intact striatum of Sprague-Dawley rats by CED at an equivalent dose of 0.5 mg/ml (10 μ g) and a 20- μ l volume of infusion. Rats were euthanized, and their brains were harvested at the indicated time. Topotecan content was determined by HPLC. A. Topotecan values are reported as percent of initial concentration (y-axis) and plotted against the number of days after CED (x-axis). B. Calculations of tissue pharmacokinetics for free TPT and Ls-TPT were based on HPLC data.

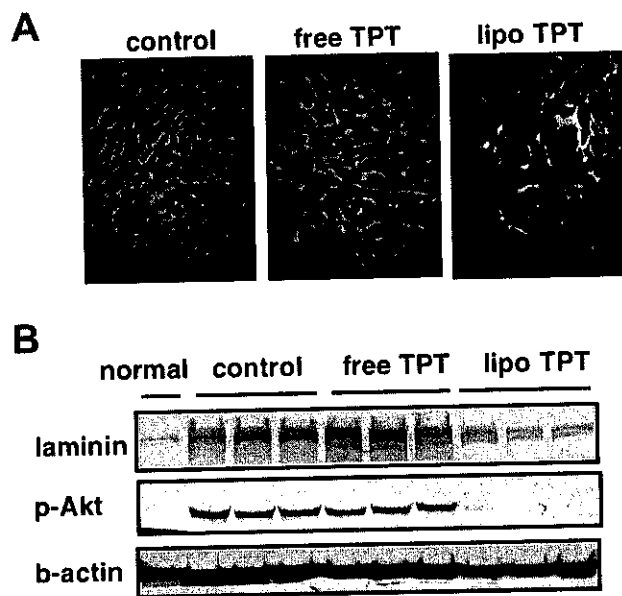


Fig. 4. Tumor vascular densities of the treated groups evaluated by immunohistological detection and Western blot detection of laminin. Seven days after tumor implantation, rats were randomly divided into three groups ($n = 6$ for each group) and treated with CED infusion of liposomal DiI_{C18} (control), free topotecan (0.5 mg/ml), or Ls-TPT (0.5 mg/ml topotecan). Seven days after treatment, rats were euthanized and subjected to (A) immunohistochemical detection of laminin ($n = 3$ for each group) or (B) Western blot quantification of laminin ($n = 3$).

Antiangiogenic Effects Detected After Local Application of Ls-TPT

To evaluate the antiangiogenic effect of this strategy, 18 rats were implanted intracranially with U87MG tumor cells. Ten days after tumor implantation, these rats were randomly divided into three groups (six rats per group): a control group that received no treatment, a free-topotecan group that received CED of free topotecan (0.5 mg/ml), and an Ls-TPT group that received CED of Ls-TPT (0.5 mg/ml). These rats were euthanized seven days after treatment, and the brains of three rats in each group were processed for immunohistochemical detection of blood vessels with antilaminin antibody (Fig. 4A). The tumor mass was removed from the brains of the other three rats in each group, and tumor protein was analyzed by Western blot to quantify blood vessels with antilaminin antibody. Immunohistochemical staining for laminin showed a marked decrease in blood vessels in the Ls-TPT group, whereas control and free-topotecan groups showed a similar high density (Fig. 4B). All three brains of rats treated with Ls-TPT and evaluated by Western blot demonstrated diminished expression of laminin in agreement with immunohistochemical data (Fig. 4B). Phosphorylation of Akt was also evaluated by Western blot, since topotecan has been demonstrated to inhibit the phosphorylation of Akt (Nakashio et al., 2000). Diminished levels of phosphorylated Akt were found in the Ls-TPT group, but not in the control or the free-topotecan groups (Fig. 4B).

Prolonged Survival of U87MG or U251MG Intracranial Brain Tumor Xenograft Models with CED of Ls-TPT

A pilot study was initiated to evaluate the antitumor effect of Ls-TPT. Six athymic rats received U87MG tumor cell implantation and were randomly divided into a control group ($n = 3$) or Ls-TPT treatment group ($n = 3$). Ten days after tumor implantation, the latter group received CED of Ls-TPT (0.5 mg/ml, 20 μ l), whereas the control group received fluorescent liposomal DiI_{C18}. Nine days after infusion, rats were euthanized, and their brains were subjected to histological examination. Whereas all control rats developed large U87MG tumors (Fig. 5A), two rats from the treatment group revealed only tissue fibrosis (Fig. 5B, black arrowheads), and one rat from the same group revealed fibrosis tissue (Fig. 5C, black arrowhead) with a small, remaining viable tumor (Fig. 5C, white arrowhead). The brain tissue surrounding the fibrotic tissue was intact, which shows the excellent selective activity of this treatment against tumor tissue (Fig. 5B and C).

Survival studies were performed using two well-established intracranial glioblastoma tumor xenograft models: U87MG and U251MG (Fig. 6). Twenty-one athymic rats received an implantation of U87MG tumor cells into their right striatum, and the rats were divided randomly into three groups. Seven days (almost one third of the life span of control animal) after tumor implantation, the first group ($n = 7$) received fluorescent liposomal DiI_{C18} as a control, the second group ($n = 7$) received CED of free topotecan (0.5 mg/ml, 20 μ l), and the third group ($n = 7$) received CED of Ls-TPT (0.5 mg/ml topotecan, 20 μ l) (Fig. 6A). Around days 17–26, all control rats and free-topotecan rats developed neurological symptoms due to large intracranial tumors confirmed as such by histology. Although free topotecan demonstrated no survival benefits at this low dose ($P = 0.78$), Ls-TPT at the same drug dose exerted a strong antitumor effect, and six out of seven rats sur-

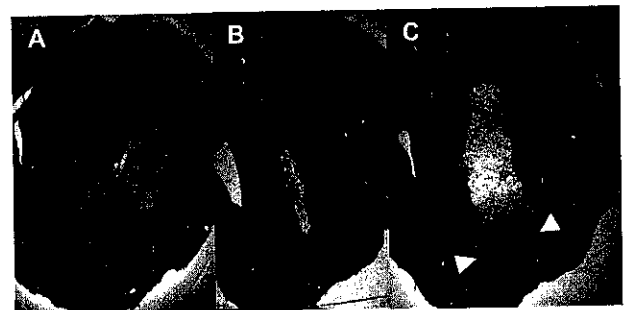


Fig. 5. Tumoricidal effects of Ls-TPT on a U87 xenograft model. A. Representative U87 xenograft size on day 19 after implantation in rodent striatum after CED with fluorescent liposomes on day 10 (black arrows point at tumor). B. U87MG tumor eradication after CED with Ls-TPT on day 10 (black arrows point at fibrotic tissue). C. Fibrotic cavity (black arrows) containing a small remnant of U87MG tumor (white arrows) after CED with Ls-TPT on day 10 after U87MG implantation in rodent striatum.

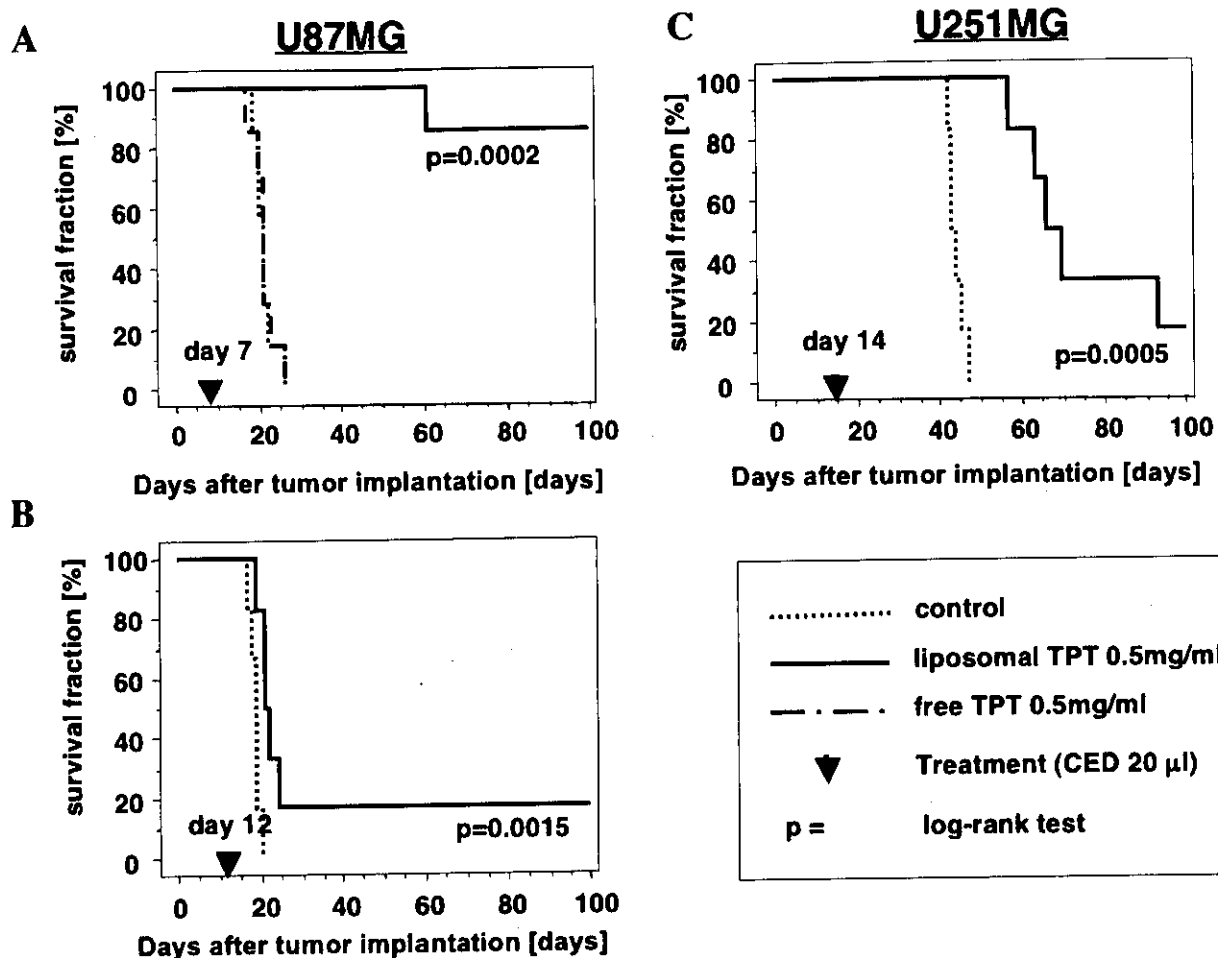


Fig. 6. Survival studies in U87MG intracranial xenograft model or U251MG intracranial xenograft tumor model. A. Effects of free topotecan or Ls-TPT administered with CED were evaluated in a U87MG tumor model with CED infusions (20 μ l) of fluorescent liposomes (n = 7, dotted line), free topotecan (0.5 mg/ml, n = 7, dashed line), or Ls-TPT (0.5 mg/ml topotecan, n = 7, solid line) performed seven days after tumor implantation (one third of average life span). B. Effect of Ls-TPT on larger tumors was assessed in the same U87MG model. Fluorescent liposome (n = 6, dotted line) or Ls-TPT (0.5 mg/ml topotecan, n = 6, solid line) was infused by CED 12 days after tumor implantation. C. Efficacy of Ls-TPT was also evaluated in the U251MG intracranial xenograft tumor model with either CED infusion of fluorescent liposomes (n = 6, dotted line) or Ls-TPT (0.5 mg/ml topotecan, n = 6, solid line) 14 days after tumor implantation (one third of average life span).

vived until the study termination point of 100 days with no histological evidence of a tumor ($P = 0.0002$, Fig. 6A). Since the impact on survival of free topotecan is low at this dose (0.5 mg/ml, 20 μ l), we focused on the efficacy of Ls-TPT in our following studies.

To evaluate the treatment of larger tumors, the U87MG intracranial model was again developed in 12 rats. However, in this experiment, CED was performed 12 days after tumor implantation (Fig. 6B). Treatment groups were administered either a 20- μ l infusion of control liposomes (n = 6) or 0.5 mg/ml of Ls-TPT (n = 6). Previous experiments showed that a U87 xenograft tumor at day 12 is not entirely covered by 20 μ l liposomes (M.T.K., unpublished data). Although significant survival benefit was observed in this study ($P = 0.0015$, Fig. 6B), the response is clearly inferior to that in the treatment of smaller tumors, which are completely

encompassed by the infused therapeutic agent. An intracranial tumor model with U251MG tumor cells was also developed with 12 athymic rats. Fourteen days (almost one third of the life span of this tumor model) after tumor implantation, six rats received fluorescent control liposomes, and the other six rats received Ls-TPT (0.5 mg/ml topotecan, 20 μ l). Again, Ls-TPT showed significant survival benefit in the U251MG tumor model ($P = 0.0005$, Fig. 6C) demonstrating that the efficacy of Ls-TPT is not restricted to a single tumor model.

Discussion

We have previously reported that CED infusion of liposome constructs in rodent and primate brains, as well as in a rodent intracranial brain tumor model, is feasible

and represents a new approach for brain tumor therapy (Saito et al., 2005). To achieve robust distribution within the tumor area, nanoparticles with lower affinity to surrounding tissue were shown to be the most effective carriers (Saito et al., 2006). Neutral liposomes, when infused locally into brain tumors by CED, distribute within the intercellular space and enable wide coverage of tumor and surrounding brain tissues. At the border of the distribution area, the lower interstitial pressure of perivascular and periaxonal spaces (Saito et al., 2006) results in accumulation of liposomes in these tracts. We have also previously demonstrated liposomal accumulation around blood vessels at 48 h after injection by CED into subcutaneous mouse tumor, and we have proposed the possibility of targeting blood vessels with liposomal drugs as a therapeutic strategy (Mamot et al., 2004). This accumulation may result from the existence of perivascular interstitial fluid drainage pathways in brains where nanoparticle and macromolecule drainage from the brain might occur (Browder et al., 2000; Weller et al., 1992). The present study showed that CED of Ls-TPT results in perivascular accumulation and consequent disruption of tumor vessels. These observations suggest a possible antiangiogenic mechanism for CED of liposomes loaded with a chemotherapeutic cargo.

Continuous low-level chemotherapy, known as *metronomic dosing*, is an attractive strategy for targeting cancer blood vessels (Browder et al., 2000; Hanahan et al., 2000; Kerbel and Kamen, 2004). In contrast to conventional chemotherapeutic regimens that use maximum tolerated doses of cytotoxic agents, followed by periods of rest to enable normal tissue to recover, metronomic dosing delivers a lower, uninterrupted dose that continuously targets the genetically stable, newly developing endothelial cells. Many studies demonstrate improved efficacy by continuous low-dose scheduling compared to conventional dosing (Bocci et al., 2003; Klement et al., 2000, 2002; Man et al., 2002). The *in vitro* experiments of Bocci and colleagues (2002) demonstrated a difference between tumor cells and proliferating endothelial cells in sensitivity to various chemotherapeutic drugs, and they proved that continuous low-dose exposure to certain kinds of drugs predominantly suppresses the growth of endothelial cells (Bocci et al., 2002). Several groups also have reported that very low (e.g., nanomolar) concentrations of certain drugs such as paclitaxel (Belotti et al., 1996), topotecan, camptothecin (Clements et al., 1999; O'Leary et al., 1999), or vinblastine (Vacca et al., 1999) can significantly block the growth of endothelial cells, but not necessarily tumor cells *in vitro*. One significant advantage of stably encapsulated liposomal cytotoxic drugs over corresponding unencapsulated agents, when infused systemically, is extended drug half-life (Drummond et al., 1999). Similarly, prolongation of liposome drug half-life was observed in our study after CED to the CNS. Thus, we hypothesized that, after local delivery, liposomes slowly leak the encapsulated drug to the interstitium, enabling a continuous low dose of chemotherapy, which prolongs exposure of the targeted tissue to the drug.

We evaluated the effects of Ls-TPT against intracranial glioblastoma xenograft in the rat. The choice

of drug was based on the fact that topotecan displays preferential antiangiogenic effects at low concentrations (Clements et al., 1999; O'Leary et al., 1999) and that systemic topotecan is now under clinical investigation for treating human, malignant gliomas (Gross et al., 2005; Lesimple et al., 2003). At doses not toxic to normal brain tissue, Ls-TPT exerted strong antiangiogenic activity in the U87MG intracranial xenograft model. Moreover, free topotecan at the same dose as Ls-TPT demonstrated no efficacy, which was likely due in part to the rapid clearance observed in our tissue-retention study. Thus, once dispensed locally, liposomes work as a drug source for effective continuous low-dose chemotherapy, slowly releasing the encapsulated drugs to surrounding tissues. This delay is also suggestive of a slow release of encapsulated compound from liposomes. Stable encapsulation of drugs is an important criterion for effective intravenous drug delivery via liposomes, as well (Drummond et al., 1999). We have recently developed a novel intraliposomal drug stabilization technology for preparing highly stable formulations of potent anticancer drugs (Drummond et al., 2005, and unpublished experiments). This stability appears to be at least partially responsible for the prolonged retention in brain tissue, as well.

The mechanism responsible for inhibition of angiogenesis by topotecan is not clearly understood. However, Nakashio et al. (2002) have reported that topotecan inhibits phosphorylation of Akt and that this may provide antiangiogenic activity. In our study, this apparent inhibition of Akt phosphorylation was observed to parallel the decrease in laminin expression. In addition, similar to results in other reports demonstrating the efficacy of antiangiogenic regimens, CED of Ls-TPT demonstrated an excellent selective toxicity against tumor tissues (Fig. 5). CED of Ls-TPT inhibited growth or completely eradicated orthotopic U87MG or U251MG xenografts, whereas free drug exerted almost no effect with the dose of 10 μ g used in this study. We believe that the limited efficacy of Ls-TPT in day 12 treatment in the U87MG xenograft model (Fig. 6B) is caused by two problems: (1) incomplete tumor coverage by the therapeutic agent after CED and (2) inadequate release of TPT from liposomes at this late stage of tumor progression. The ability to image in real time the distribution of liposomes within the brain (Saito et al., 2004, 2005) provides a means for verifying optimal tumor coverage of liposomal therapeutics with a coinjected surrogate liposomal marker.

To expand our promising results into a clinical trial, several key points need to be discussed. To date, CED of chemotherapeutics is used in clinical trials only after surgical tumor resection to perfuse the resection margins and therefore should prevent regrowth of malignancy. We believe also that very large inoperable brain tumors may be amenable to liposomal CED. Because the human CNS is large, this application would require implantation of several catheters into the targeted area to achieve complete coverage of malignant tissue. The slow release of Ls-TPT and the antiangiogenic effect of Ls-TPT in combination with CED have the potential to improve outcomes in brain tumor patients.

In conclusion, we have shown that Ls-TPT in combination with CED displayed excellent selective toxicity for tumor tissue relative to normal tissue, an important requirement when applying CED to clinical treatment of malignant brain tumors. In addition to the tumoricidal effects of topoisomerase I inhibition by TPT, the peri-

vascular accumulation and the favorable pharmacokinetic profile of Ls-TPT provide an antiangiogenic effect. Because of its high selectivity and superior efficacy in the context of CED, Ls-TPT may be an excellent candidate for future clinical development.

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