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New Therapeutic Approaches for Brainstem Tumors: A Comparison of Delivery Routes Using Nanoliposomal Irinotecan in an Animal Model

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Abstract

Despite the advances in imaging, surgery and radiotherapy, the majority of patients with brainstem gliomas die within 2 years after initial diagnosis. Factors that contribute to the dismal prognosis of these patients include the infiltrative nature and anatomic location in an eloquent area of the brain, which prevents total surgical resection and the presence of the blood-brain barrier (BBB), which reduces the distribution of systemically administered agents. The development of new therapeutic approaches which can circumvent the BBB is a potential path to improve outcomes for these children. Convection-enhanced delivery (CED) and intranasal delivery (IND) are strategies that permit direct drug delivery into the central nervous system (CNS) and are an alternative to intravenous injection (IV). We treated rats bearing human brainstem tumor xenografts with nanoliposomal irinotecan (CPT-11) using CED, IND, and IV. A single treatment of CED irinotecan had a similar effect on overall survival as multiple treatments by IV route. IND CPT-11 showed significantly increased survival of animals with brainstem tumors, and demonstrated the promise of this non-invasive approach of drug delivery bypassing the BBB when combined with nanoliposomal chemotherapy. Our results indicated that using CED and IND of nanoliposomal therapy increase likelihood of practical therapeutic approach for the treatment of brainstem gliomas.

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Introduction

Among brain tumors, brainstem tumors are particularly rare, occurring in only several hundred children in the United States each year and only in 1–2% of adults. Despite improvements in survival for hematologic and other solid tumors, the survival for children with brainstem gliomas remains poor with few patients remaining alive two years after diagnosis [1]. The factors that have limited treatment improvement include an infiltrative character in a non-resectable location, an aggressive pattern of growth, and finally, the inability to achieve high tissue drug concentrations due to an intact blood brain barrier (BBB) [2, 3]. A large number of Phase I studies conducted over the past several decades have failed to demonstrate any improvement in survival, or even any response, in the majority of these patients [1, 4]. Indeed, the only modality of therapy that prolongs survival, fractionated radiotherapy, has not changed in several decades. While surgical resection is not possible, biopsies and directed therapies are being explored. In particular, there is growing interest in direct delivery of chemotherapeutic agents.

During the last ten years, there have been improvements in the technology designed to directly delivery of therapeutic agents by convection-enhanced delivery (CED) into the central nervous system (CNS) [5]. CED relies upon continuous infusion with a positive pressure gradient to drive a therapeutic agent through a target volume. CED has shown promising results in animal models and is being evaluated in human clinical trials [6–8]. As expected, there are many variables that may affect the clinical efficacy of CED including volume of distribution, reflux, drug concentration, and delivery time [9, 10].

CED is well suited for the delivery of liposomes and particulate drug carriers which have the potential to provide a sustained level of drug and to reach cellular targets with improved specificity [11, 12]. The theoretical and observed consequence of liposomal delivery is markedly reduced CNS toxicity when compared to CED with free drug. Irinotecan, in particular, shows promise for the treatment of gliomas based on both *in vitro* and *in vivo* pre-clinical results with nanoliposomal irinotecan (nal-IRI) [6, 12], as well as demonstrated clinical free activity of free irinotecan [13]. Nal-IRI is a novel highly stabilized formulation of irinotecan that allows for slow and sustained release of the encapsulated irinotecan [14], which was recently approved for the treatment of gemcitabine refractory pancreatic cancer [15], but also has significant activity in treating brain tumors if delivered effectively. The limitations of CED, however, include the need for a surgical procedure, with its accompanying risks and complications, and the exacting technical requirements.

Intranasal delivery (IND) is a promising and practical noninvasive method for delivering therapeutic agents to the brain bypassing BBB using the unique anatomic connections of the olfactory and trigeminal nerves from the nasal mucosa to the CNS [16]. Intranasally administered drugs reach the CNS and/or CSF within minutes of administration by using an extracellular route through perineural and perivascular channels, without binding to any receptor or relying upon axonal transport. There are also the advantages of avoidance of hepatic first-pass elimination, thereby reducing systemic side effects and elimination of surgical risk. In brain tumor models, many anti-cancer agents such as methotrexate [17], 5-

fluorouracil [18], and raltitrexed [19], have been delivered successfully to the brain using IND.

In this study, our goal was to compare three delivery routes, intravenous injection (IV), CED, and IND, for nal-IRI in an orthotopic rodent brainstem tumor model.

Materials and Methods

Cell Culture

U-87 MG human glioblastoma cell line was obtained from the Department of Neurological Surgery Tissue Bank at the University of California, San Francisco (UCSF), and was propagated as exponentially growing monolayers in complete medium consisting of Eagle's minimal essential medium supplemented with 10% fetal bovine serum and non-essential amino acids. A standard U87 MG cell line is maintained at the UCSF Tissue Bank to provide for consistency among institutional investigators. GS2 cell line was obtained from Manfred Westphal, Department of Neurological Surgery, University Hospital Eppendorf, Hamburg, Germany, and maintained as a neurosphere culture, as previously described [20]. DNA fingerprints were obtained to confirm the identity of the cell lines. Cells from pediatric H3K27M diffuse midline gliomas were not used due to their unavailability at the time this study was conducted.

Cell Proliferation Assay

Tumor cells were cultured in the presence of 0, 0.0001, 0.001, 0.01, 0.1, 1, 10, 100, or 1000 nM SN38 (7-Ethyl-10-hydroxycamptothecin, Sigma-Aldrich) for 4 days. Proliferation effect was assessed by counting viable cells. Trypsinized cell suspensions were stained with trypan blue, and viable cells determined by hemocytometer counting. All *in vitro* assays and analyses were performed with mean and standard deviation (SD) values plotted from triplicate samples.

Animals

Six-week-old male athymic rats (rnu/rnu, homozygous) were purchased from the National Cancer Institute (Frederick, MD). Rats were housed in an animal facility and were maintained in a temperature-controlled and light-controlled environment with an alternating 12-hour light/dark cycle. All protocols were approved by the UCSF Institutional Animal Care and Use Committee.

Surgical Procedure for Implantation of Tumor Cells

Before injecting tumor cells into the brainstem, rats were anesthetized by intraperitoneal injection of 75 mg/kg of ketamine and 7.5 mg/kg of xylazine. Anesthetized rats were then positioned in a stereotactic device (David Kopf Instruments, Tujunga, CA) using ear bars. A burr hole was drilled through the skull 1.0 mm behind the lambda, and 9.6 mm deep from the inner surface of the skull. 1×10^5 tumor cells suspended in 1 μ l HBSS were injected slowly (over 1 minute) into the pontine tegmentum using a guide-screw system [20]. All procedures were carried out under sterile conditions.

***In vivo* BLI Monitoring**

GS2 cells were transduced with a lentiviral vector containing firefly luciferase (Fluc) under the control of the spleen focus forming virus (SFFV) promoter as previously described [20]. Briefly, lentiviral vectors were generated by transfection of 293T (human embryonal kidney) cells with plasmids encoding the vesicular stomatitis virus G envelope, gag-pol, and Fluc genes [21, 22]. Cells were screened for transfection efficiency by treatment with luciferin (D-luciferin potassium salt, 150mg/kg, Gold Biotechnology, St Louis, MO) *in vitro* and examination by a Xenogen IVIS Lumina System (Xenogen Corp., Alameda, CA).

In vivo BLI was performed with the Xenogen IVIS Lumina System coupled LivingImage software for data acquisition (Xenogen Corp.). Rodents were anesthetized with 75mg/kg of ketamine and 7.5mg/kg of xylazine and imaged 12 min after intraperitoneal injection of luciferin. Signal intensity was quantified within a region of interest over the head that was defined by the LivingImage software. To facilitate comparison of growth rates, each rat's luminescence readings were normalized against its own luminescence reading at the day before initiation of therapy, thereby allowing each rat to serve as its own control [20].

Liposomal Agent

Nanoliposomal irinotecan (nal-IRI) is a highly stabilized liposomal formulation containing nano-sized irinotecan crystals complexed with sucrose octasulfate in the liposome interior [14] and was generously provided by Merrimack Pharmaceuticals (Cambridge, MA). The preparation of nanoliposomal nal-IRI used in the experiments that follow had a particle size of 112.0 ± 11.6 nm, as determined by dynamic light scattering, and a drug-to-phospholipid (PL) ratio of 754 ± 21 g irinotecan/mol PL. N,NV-bis-octadecyl-4,4,4V,4V-tetramethylindacarbocyanin iodide [DiIC18(3); Molecular Probes, Inc., Eugene, OR] was included in the formulation at a ratio of 0.3 mol % of the total phospholipid for fluorescent labeling. The final concentration of the drug based on irinotecan content was 50 mg irinotecan/ml.

Intra-nasal Delivery

To administer the drugs through the nasal cavity, animals were anesthetized with 2–2.5% isoflurane and placed in an anesthesia chamber. Six microliter (μ l) drops of soluble form of therapeutic agents were administered with a small pipette every 2 min into alternate sides of the nasal cavity for a total of 22 min (a total volume of 66 μ l) and 3.3 mg irinotecan. This method of administration results in consistent deposition in the olfactory epithelium without respiratory distress [23]. Following IND, the animals remained in a supine position for 15 min in order for absorption to occur through the nasal mucosa.

Convection-enhanced Delivery

CED was performed by micro-infusion of liposomal agents as previously described [24]. Briefly, the infusion system consisted of a fused-silica needle cannula with a 1-mm stepped design continuous with a fused silica tube (Polymicro Technologies, Phoenix, AZ) leading to a 24 gage needle that protruded from the silica guide base. A one mL syringe with silica cannula was loaded with liposomal agents and mounted onto a micro-infusion pump

(BeeHive, Bioanalytical Systems, West Lafayette, IN). The syringe with silica cannula was mounted onto a stereotactic holder and guided to targeted region of the brains through a puncture hole made in the skull for tumor cells implantation. The liposomal agent was infused following ascending rates to achieve the 20 μL total infusion volume: 0.1 $\mu\text{L}/\text{min}$ (5 minutes) + 0.2 $\mu\text{L}/\text{min}$ (5 minutes) + 0.5 $\mu\text{L}/\text{min}$ (5 minutes) + 0.8 $\mu\text{L}/\text{min}$ (20 minutes). For the 50 mg/mL nal-IRI this translated to a total irinotecan dose of 1 mg per rat. The cannula was removed 2 min following completion of infusion.

Statistical Analysis

The Kaplan–Meier estimator and Prism software were used to generate and analyze survival plots. Differences between survival plots were calculated using a log-rank test. For all other comparisons, a 2-tailed unpaired *t*-test was used (GraphPad Software).

RESULTS

Toxicity of Liposomal Irinotecan by CED and IND

In advance of conducting experiments to evaluate toxicity of nal-IRI *in vivo*, we examined *in vitro* response to the active metabolite of irinotecan, SN38, using U-87 MG and GS2 cell lines (Figure 1A). Cell proliferation assay results showed a 50% reduction in U-87 MG and GS2 cell number at 2.42 ± 0.13 and 0.65 ± 0.15 nM of SN38. In order to determine whether the liposomal formulation of irinotecan (nal-IRI) had any overt toxicity by either CED or IND, we treated naïve rats with single CED at dose of 0.25 mg (2 rats), 0.5 mg (3 rats), and 1.0 mg of nal-IRI (3 rats), 20 μL of PBS was used in the control group (4 rats). There was no effect on body weight of the animals that received nal-IRI by CED (Figure 1B), and no animals developed symptoms attributable to either the surgical procedure or drug delivery. Similarly, animals received varying total dose of nal-IRI by IND. We administered a maximum solubility dose (MSD) of nal-IRI (3.25 mg in 65 μL PBS) in 3 rats, 0.1 of MSD (0.325 mg in 65 μL PBS) in 3 rats, and 0.01 of MSD (0.0325 mg in 65 μL PBS) in 3 rats, through the nasal cavity, daily for 10 days. 65 μL of PBS was delivered intranasally in 2 rats as control (Figure 1C; shaded area represents duration of treatment). There was no effect on body weight and no animals experienced symptoms.

Distribution of Liposomes by CED and IND

In order to qualitatively confirm the distribution of DiI labeled liposomes (DiI-LS), DiI-LS was infused into the brainstem of naïve rats by CED. Two naïve rats receiving DiI-LS were euthanized with transcardial perfusion at 3 h following CED (Figure 2). Sequential sections were taken of the brainstem and *ex vivo* fluorescent imaging was performed. *Ex vivo* fluorescent image was detected in the infusion site over the ipsilateral pons (Figure 2A). The fluorescent microscope images showed diffuse distribution in the pons that indicates parenchymal penetration of liposomes (Figure 2B).

Following intranasal delivery of DiI nal-IRI, animals were euthanized and the brains isolated for subsequent analysis with fluorescent microscopy (Figure 3). There was clear evidence of fluorescent signal within the olfactory bulb, frontal cortex, hypothalamus, cerebellum and

medulla (Figure 3A). In tumor-bearing animals (Figure 3B), the fluorescent signal mainly clustered at the tumor/brain interface (Figure 3C).

***In Vivo* Efficacy of Liposomal Irinotecan by IV, CED, and IND**

We then examined the *in vivo* efficacy of nal-IRI using three delivery routes: IV, CED, and IND. IV injections were three times of 30 mg/kg of nal-IRI (3 rats) and free irinotecan (3 rats) once a week for three weeks. The IV control group comprised of 3 rats receiving IV PBS. We administered nal-IRI by CED once at 0 mg (3 rats), 0.01 mg (4 rats), 0.1 mg (4 rats), and 1.0 mg (4 rats). Intranasal nal-IRI was administered MSD dose at 3.25 mg/day (6 rats) as well as empty LS (5 rats) for 15 days as total 48.7 mg of irinotecan.

Following three IV doses of nal-IRI, there was a significant increase in survival compared to rats receiving empty liposome controls (empty-LS) - IV control (median survival of IV control 52 d vs. IV-nal-IRI 61 d, significant $p = 0.02$, Figure 4A). In contrast, there was no difference in survival between control and IV free irinotecan. This was consistent with previous reports utilizing this treatment approach [12].

We then compared the effects of local delivery with CED or IND using the same tumor model. CED of nal-IRI showed dose dependent inhibition of the growth of GS2 brainstem tumor and significant increased survival with median survival of 51 days for 0.01 mg ($p = 0.03$), 54 days for 0.1 mg ($p = 0.01$), and 60.5 days for 1.0 mg ($p = 0.01$) of nal-IRI when compared to CED of empty-LS with median survival of 47 days (Figure 4B). Fifteen-days treatment of IND of nal-IRI also inhibited brainstem tumor growth and increased survival with median survival of 61.5 days for nal-IRI ($p = 0.02$) compared to IND of empty-LS (median survival of 58 days) (Figure 4C).

DISCUSSION

The development of new strategies to deliver therapeutic agents across the BBB is a priority if the outcomes associated with malignant brain tumors are to improve. Local delivery strategies are being explored for the treatment of brainstem gliomas because of the specific therapeutic challenges regarding their site of origin, infiltrative nature, and presence of an intact BBB. Based on our previous experience with the development of brainstem tumor models [20, 25, 26, 27], we decided to evaluate the efficacy of three delivery strategies that may have clinical relevance.

CED relies on positive pressure infusion of a therapeutic agent into the brain using a MR-guided surgical approach. A previous phase III clinical trial examining the use of an IL-13 antagonist in adult patients with glioblastoma did not demonstrate efficacy but a number of technical limitations were noted [28]. In addition to CED, additional technical refinements can improve the duration of therapeutic agents in the target tissue. Liposomal carriers are used to package drugs that then allow sustained release. A highly stable nanoliposomal formulation of irinotecan (nal-IRI) has shown greater brain and tumor retention for a prolonged period than free irinotecan and effective anti-tumor activity in intracerebral glioblastoma xenografts, when administered by CED [6]. A clinical trial currently underway uses liposome encapsulated irinotecan delivered by CED for patients with recurrent GBM

(NCT02022644). This study delivers a variable volume of agent to a defined tumor volume using a MR-guided approach. There is interest in using a similar strategy for children with diffuse midline gliomas with histone H3K27M mutation, especially as the same formulation of liposomal nal-IRI can be adapted for use in children. In order to examine the feasibility of using liposomal nal-IRI in the brainstem, we used an orthotopic xenograft brainstem tumor model to assess the efficacy of nal-IRI delivered by the IV route, IND route, or by CED.

Our results demonstrate that naïve non-tumor bearing animals tolerated our local delivery strategies. Increasing the total drug delivered by CED four-fold did not result in any noticeable animal toxicity. Similarly, treatment by IND over a hundred-fold dose range did not cause any toxicity. Histological examination of the nasal mucosa was not performed as prior studies did not observe any congestion, edema, epithelial sloughing, necrosis or hemorrhage of the nasal mucosa with administration of plain liposomes in human volunteers [29] or calcitonin loaded liposomes in rats [30]. In the CED, reflux of infused nal-IRI to the upper parenchymal brain was observed (Figure 2A). The difficulties associated with accurate brainstem targeting by CED may be due to the high cellular density in the pons. Because of technical limitations regarding survival surgery and repeated treatments, we used only one CED treatment in our experimental model. This single treatment had a qualitatively similar effect on improvement in survival as compared to multiple treatments through either the IV or IND route. The survival benefit was significant but modest with nal-IRI treatment using each delivery method. This could be related to limited conversion rate of irinotecan to SN38 in the brain tumor due to low activity of carboxyl esterase. In addition, the duration of treatment was short for all treatment groups. Our expectation is that greater survival benefits will be observed with repeated treatment and prolonged delivery.

The primary limitation of this model is the use of U87 MG and GS2 GBM cells which show significant difference in genetics, epigenetics, and *in vivo* growth in compared to pediatric diffuse midline glioma cells with histone H3K27M mutation. At the time that these experiments were planned and conducted, reliable pediatric diffuse midline glioma models were not available. Nevertheless, the use of these adult GBM cells is to compare new drug delivery strategies in appropriate anatomic xenograft models for brainstem glioma which do grow in the pons with a reasonable time period, and that the growth of these tumors can be monitored accurately with bioluminescent imaging [20]. We subsequently successfully developed cell lines derived from a pediatric diffuse midline glioma with histone H3K27M mutation [25, 26]. The delivery methods utilized in this model system can be easily adapted to examine a xenograft model derived from pediatric diffuse midline glioma cells with histone H3K27M mutation. An additional technical limitation of this study is that the therapeutic agent (nal-IRI) cannot be directly visualized using non-invasive techniques. However, our goal with this study was to obtain pre-clinical data using the same agent which is currently being evaluated in human clinical trials (NCT03086616) [31]. In existing and planned human studies, gadolinium is being used as a surrogate marker for drug distribution during CED. Finally, liposomes allow the potential for simultaneous packaging of markers such as gadolinium which can be monitored the distribution of liposomal therapeutics with CED by MRI in rodents [32], non-human primates [33, 34] and in human [31, 35].

The results demonstrate that there is pre-clinical evidence in support of the use of nal-IRI via CED for the treatment of brainstem tumors. There are clearly key differences between a rodent model and the human clinical situation. While the entire tumor volume in rodents can be likely covered with a small volume of infusate, in humans, the target volume in the pons may be as large as 20–30 cc's. There will be clear risks associated with direct delivery to the brainstem that will need to be incorporated in the clinical trial design. There are additional variables such as total infusate volume, infusion rate, and multiple infusions that will need to be examined directly in human studies.

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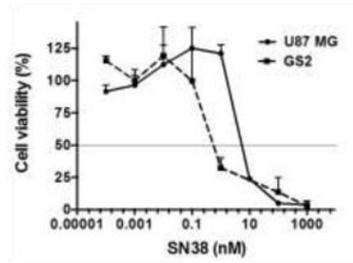
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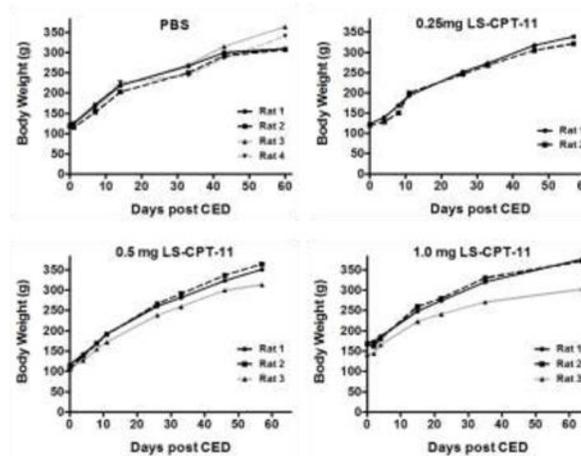
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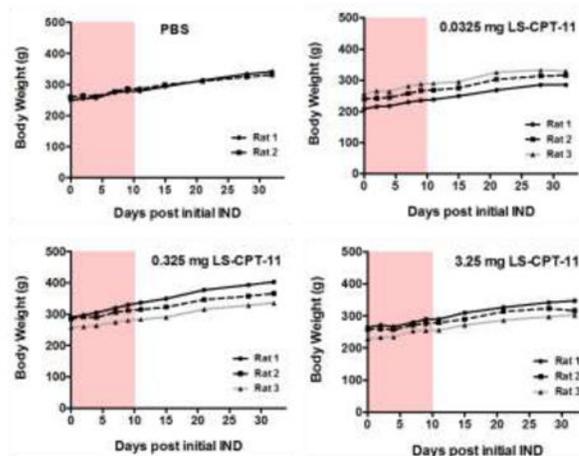
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**Figure 1.**

Effect of active metabolite of CPT-11, SN38, on human glioma cell proliferation and toxicity of nanoliposomal CPT-11 (LS-CPT11) by CED and IND in rats. **A.** Graph showing proliferation response of human glioma cells to increasing concentration of SN38. Values shown are the average (mean \pm SD) from triplicate samples for each incubation condition. SN38 shows valuable, but consistently anti-proliferative effect at concentrations of 0.1–100 nM. **B.** Graphs showing direct injection of either PBS or nanoliposomal CPT-11 by CED demonstrates no obvious toxicity. A maximum volume of 20 μ l of infusate volume was used.

C. IND with nanoliposomal CPT-11 over a hundred-fold dose range for 10 consecutive days (pink area) did not result in any appreciable toxicity.

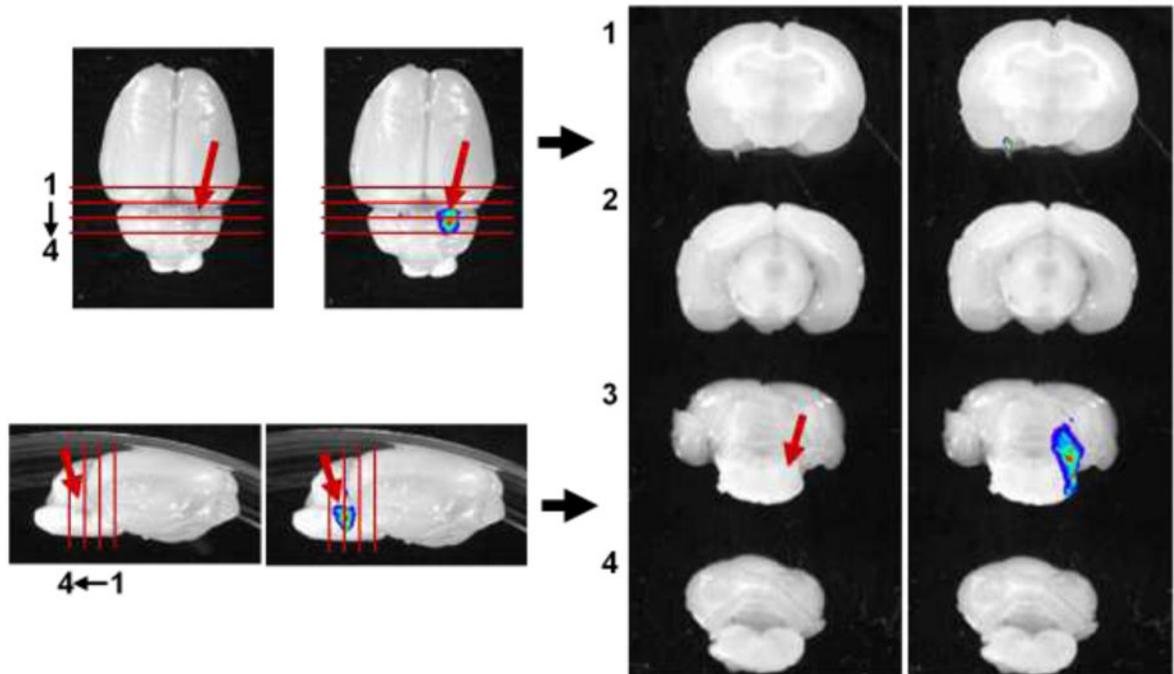
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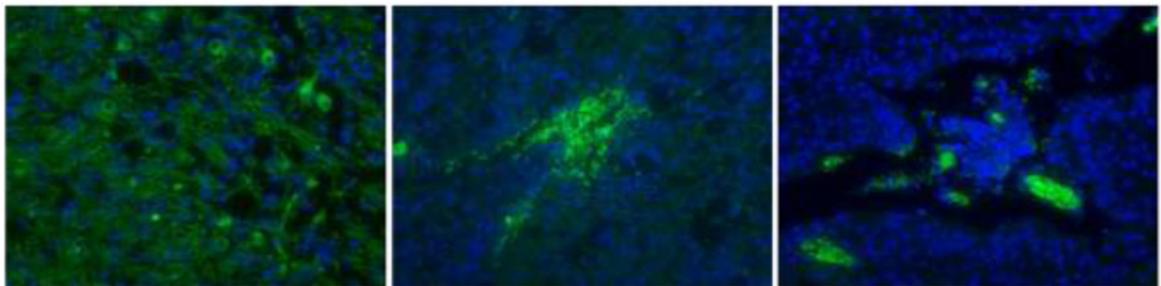


Figure 2.

Ex vivo distribution of fluorescent liposomes using CED in the rat brainstem. **A.** *Ex vivo* image demonstrates relatively efficient distribution at the target site (narrows) and for a short distance along injection track. **B.** Fluorescence microscope indicates diffuse distribution of DiI liposomes in the pons. DNA staining is by DAPI.

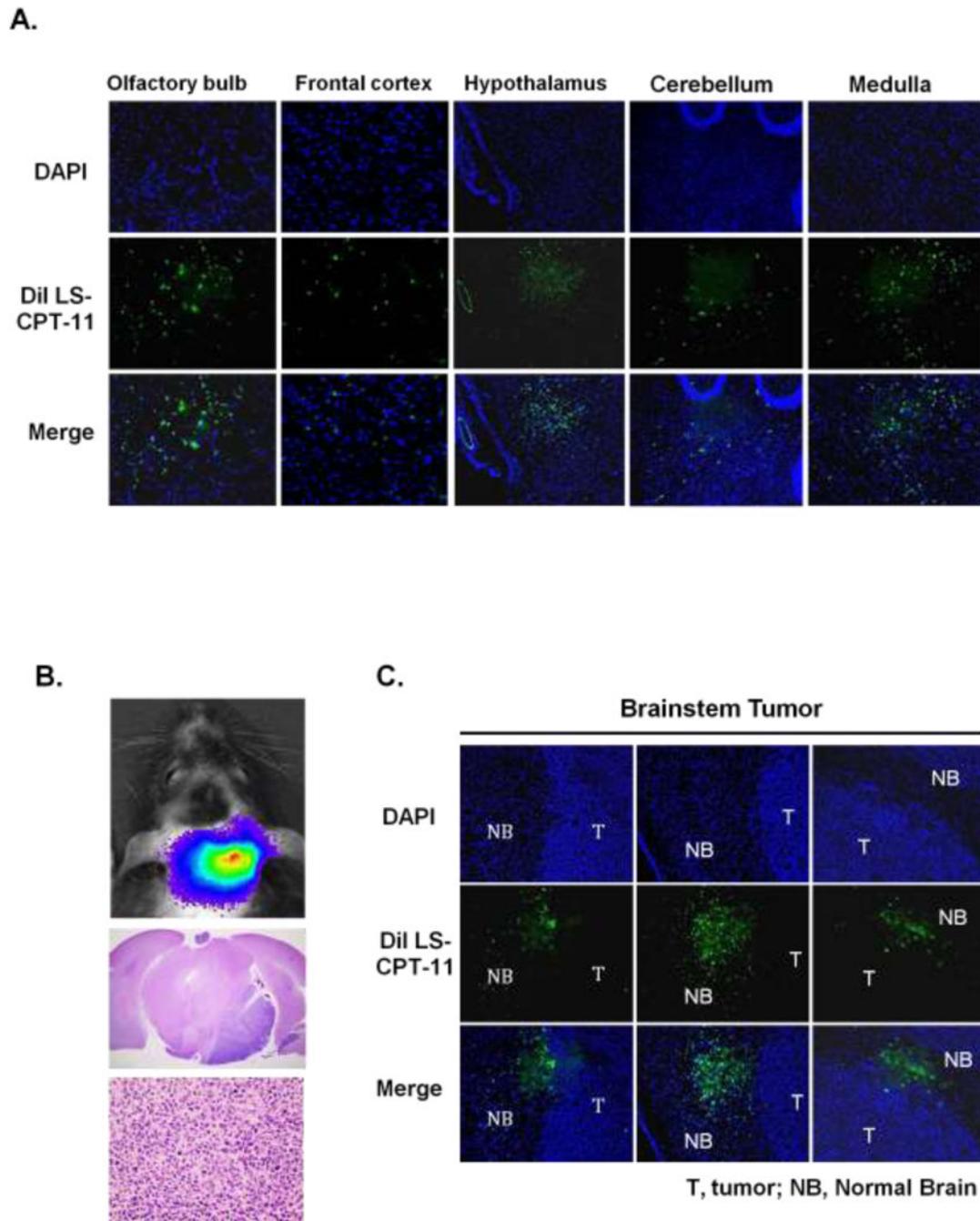


Figure 3.

Distribution of fluorescently labeled liposomal CPT-11 by IND in normal brain and brainstem tumor. **A.** Fluorescent signals were detected from olfactory bulb throughout the different brain regions. **B.** 1×10^5 luciferase-modified GS2 glioblastoma cells were injected into brainstem in athymic rats using an implantable guide-screw system. Bioluminescence imaging (BLI) shows a corresponding signal from the brainstem tumor (*upper*). Histologic analysis reveals GS2 tumor growth in the pons (*middle: 2 × magnification, lower: 40 ×*

magnification). C. Fluorescent labeled liposomal CPT-11 accumulated in the brainstem 6 hours following IND.

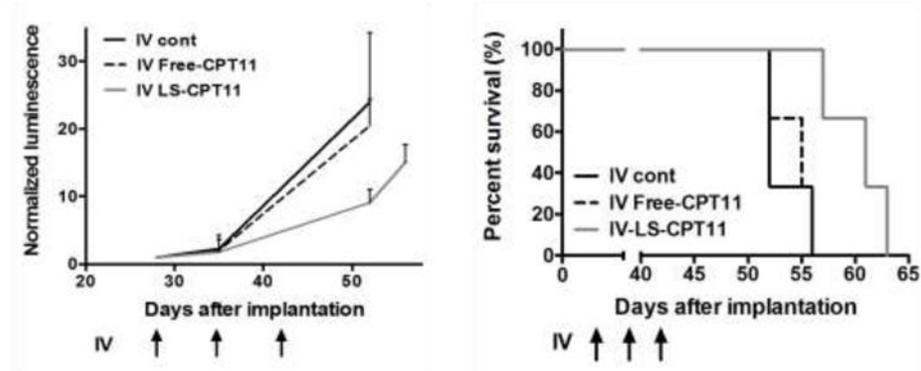
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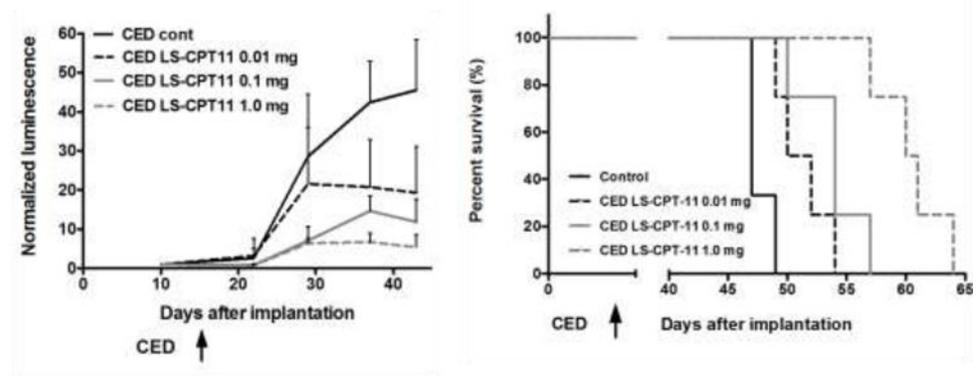
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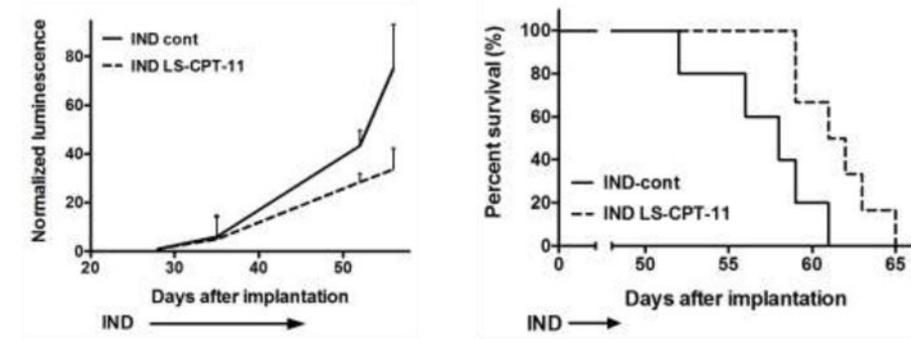
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**Figure 4.**

A comparison of survival between animals with brainstem tumors treated by IV, IND and CED routes. **A.** Rats with brainstem tumor were treated with vehicle (PBS), free-CPT11 or liposomal CPT-11 (LS-CPT11) by IV at daily dose of 30mg/kg once a week for three weeks. Bioluminescence values were normalized against bioluminescence values obtained at the beginning of therapy. Growth curve for brainstem tumor shows more growth delay of rats received IV LS-CPT11 in compared to the animals received vehicle control and free-CPT11 (*left*). Corresponding survival plots for each treatment (*right*). Statistical analysis was

performed using a log-rank test (IV LS-CPT11: $p = 0.0224$). **B.** Rats were treated with LS-CPT11 by CED once at 0, 0.01, 0.1, and 1.0 mg. Growth curve for brainstem tumor shows dose dependent inhibition of the brainstem tumor growth by CED of LS-CPT11 (*left*). Every dose of LS-CPT11 significantly prolonged animal survival in compared to the animal treated with CED of empty LS (*right*, 0.01 mg: $p = 0.0304$, 0.1 mg: $p = 0.001$, 1.0 mg: $p = 0.001$). **C.** Rats received IND of LS-CPT11 at daily dose of 3.25 mg for 15 days as total dose of 48.7 mg. IND of LS-CPT11 inhibits growth of brainstem tumor (*left*). IND of LS-CPT11 significantly prolonged animal survival in compared to the animals received IND of empty-LS (*right*, $p = 0.022$).