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LOCAL DELIVERY OF THE TOPOISOMERASE I INHIBITOR CAMPTOTHECIN SODIUM PROLONGS SURVIVAL IN THE RAT INTRACRANIAL 9L GLIOSARCOMA MODEL

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Camptothecin, a naturally occurring inhibitor of the DNA-replicating enzyme topoisomerase I, demonstrated promising anti-tumor activity in pre-clinical testing; however, because of unexpected toxicity and low anti-tumor effects in the initial clinical trials, further testing was discontinued. We hypothesized that local controlled delivery of camptothecin sodium would achieve effective concentrations in brain tumors without the observed systemic side effects, thereby allowing this novel drug to be used to treat patients with malignant gliomas. To test this hypothesis, we evaluated the sensitivity of rat glioma lines and established human glioma lines to camptothecin *in vitro*. We found that the LD_{90} for the established rat and human lines was 0.3 to 1.4 μM after a 1 hr exposure and decreased to less than 0.1 μM after continuous exposure for 7 days. We loaded camptothecin into a controlled-release polymer (ethylene-vinyl acetate co-polymer; EVAc) and showed by high-pressure liquid chromatography that controlled release occurred over at least 21 days. We then tested camptothecin against 9L gliosarcoma, implanted into the brain of Fischer 344 rats. Five days after tumor implantation, animals were treated with camptothecin delivered either systemically or locally by release from EVAc. Local controlled delivery by the polymer significantly extended survival: 59% of the treated animals were long-term survivors (>120 days) compared to 0% of controls. Systemic administration did not extend survival compared to controls. We compared the efficacy of camptothecin delivered locally with a polymer to camptothecin injected directly into the tumor. Camptothecin increased survival only when delivered locally by polymer.

Camptothecin is a naturally occurring alkaloid isolated from *Camptotheca acuminata,* a tree indigenous to China (Potmesil, 1994; Slichenmyer *et al.,* 1993; Wall *et al.,* 1966). It exerts its pharmacological effects by inhibiting topoisomerase I, an enzyme intimately involved in DNA replication (Hsiang *et al.,* 1985). Camptothecin has been shown to have strong cytotoxic anti-tumor activity against a variety of experimental tumors *in vitro* (DeWys *et al.,* 1968; Drewinko *et al.,* 1974; Vendetti and Abott, 1967). Phase I and II clinical trials of camptothecin in patients with melanoma and advanced gastrointestinal carcinoma, however, showed unexpectedly severe systemic toxicity, with poor tumor responses, and clinical investigation was therefore halted (Gottlieb *et al.,* 1970; Moertel *et al.,* 1972; Muggia *et al.,* 1972).

Local delivery of a chemotherapeutic agent by controlled-release polymers is a new strategy with the potential to maximize the anti-tumor effect of a drug and reduce systemic toxicity (Brem, 1990; Brem *et al.,* 1991; Langer *et al.,* 1989). Local delivery of such agents is particularly well suited to the treatment of brain tumors, as 90% of malignant gliomas recur within 2 cm of the original tumor (Burger *et al.,* 1988; Hochberg and Pruitt, 1980). We hypothesized that local delivery of camptothecin would be an effective strategy to treat patients with malignant gliomas. This report suggests a possible role for the treatment of malignant gliomas with camptothecin and describes a new method for delivering this agent to tumors.

MATERIAL AND METHODS

Polymer preparation

Ethylene-vinyl acetate co-polymer (EVAc; 40% vinyl acetate by weight; Elvax 40P) was obtained from DuPont (Wilmington, DE). EVAc was washed in absolute ethyl alcohol to extract the inflammatory anti-oxidant butylhydroxytoluene, as previously described (Langer *et al.,* 1981). All studies were performed with the sodium salt formulation of camptothecin. The terms "camptothecin" and "camptothecin sodium" are used interchangeably in the text. Sodium camptothecin, obtained from the National Cancer Institute (Bethesda, MD), was incorporated into the polymer matrix by a modification of the procedure described by Rhine *et al.* (1980). Camptothecin and EVAc were combined to yield 20%, 40% or 50% loaded polymers by weight. Methylene chloride was added to the mixture to yield a 10% solution of EVAc and methylene chloride and agitated on a Vortex mixer until completely dissolved. The solution of camptothecin–EVAc–methylene chloride was then poured into a glass mold at −70°C. After 20 min, the solidified polymers were transferred to a −30°C freezer for 4 days. Polymers were then placed in a vacuum desiccator for 4 days at room temperature to facilitate evaporation of methylene chloride, after which they were stored at 4°C.

Release kinetics

EVAc polymers loaded with camptothecin were placed in 3.0 ml of 0.9% NaCl in a 37°C incubator. The solution was removed at various time points and replaced with fresh 0.9% NaCl, thus maintaining the concentration of camptothecin in the release medium at infinite sink conditions. The amount of camptothecin released into the solution was measured by high-pressure liquid chromatography (HPLC) as described below. The cumulative dose released was determined by combining the release values at each time point.

HPLC method for measuring camptothecin

Quantitative analysis was performed on a Beckman (Palo Alto, CA) chromatographic system equipped with a 507 Autosampler, 126AA solvent module, 166 Detector, and a System Gold data system. The column was a reverse-phase microBondpak C18 Waters (Milford, MA) column (particle size 10 μm, 3.9 × 300 mm), which was protected by an Uptight Precolumn (Upchurch Scientific, Oak Harbor, WA). The HPLC system was eluted isocratically with methanol:water (63:37; v/v) at room temperature. The flow rate of the mobile phase was 1.0 ml/min and samples were measured at a wavelength of 254 nm. A standard curve was constructed by plotting peak area against concentration. The assay has been

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found to be highly accurate and reproducible, with a coefficient of determination = 0.998.

In vitro *studies*

Tumor cell lines for studies in vitro. In 1985, 9L gliosarcoma cells were obtained from Dr. M. Barker, San Francisco, CA. F98 glioma cells were provided by Dr. J. Goodman, Columbus, OH. Human glioma cell lines U87 and U373 were provided by Dr. O.M. Colvin, Baltimore, MD.

Clonogenic assay

The sensitivity of each glioma cell line was tested in a clonogenic assay. The medium used was minimum essential medium (Gibco BRL, Grand Island, NY) with 10% FBS, 0.5% L-glutamine, penicillin (base; 80.5 units/ml) and streptomycin (80.5 μg/ml). At confluence, the cells were trypsinized and plated at 400 cells per 60 mm well. After 24 hr, fresh medium containing camptothecin at various concentrations was added. For brief exposure experiments, camptothecin was removed and replaced with fresh medium after 1 hr; for continuous exposure, medium was left in place for 7 days. At 7 days, all plates were fixed and stained with Coomassie brilliant blue (BioRad, Richmond, CA). Colonies containing more than 50 cells were identified and counted. Survival was calculated as the number of colonies formed by the treated cells relative to the number of colonies formed by the untreated cells. Tests were performed in triplicate. Survival curves derived from the mean of 3 measurements were plotted as a function of camptothecin concentration. LD_{90} was extrapolated from these sensitivity curves.

In vivo *efficacy studies*

Animals. Male Fischer 344 rats weighing 200–250 g were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The animals were kept in standard animal facilities and given free access to Certified Rodent Chow 5002 (Ralston Purina, St. Louis, MO) and to Baltimore City water.

9L intracranial model

9L gliosarcoma was maintained in the flanks of male Fischer 344 rats. For intracranial implantation, the tumor was surgically excised from the carrier animal and cut into 1 × 2 × 2 mm pieces. The pieces were kept in sterile 0.9% NaCl on ice during the implantation procedure. Rats were anesthetized with an intra-peritoneal injection of 3–5 ml/kg of a stock solution containing ketamine hydrochloride 25 mg/ml, xylazine 2.5 mg/ml, and 14.25% ethyl alcohol in 0.9% NaCl. The surgical site was shaved and prepared with 70% ethyl alcohol and Prepodyne solution. After a mid-line incision, a 3 mm burr hole centered 5 mm posterior to the coronal suture and 3 mm lateral to the sagittal suture was made. The dura was opened and the cortex and white matter were resected using gentle suction until the brain stem was visualized. The surgical site was irrigated until clear with sterile 0.9% NaCl. A single tumor piece was placed in the depths of the cortical resection. The skin was closed with surgical staples.

Treatment

Animals were treated with camptothecin delivered either systemically, locally by a polymer, or locally by direct intratumoral injection. Intra-peritoneal injections of camptothecin were given on days 5, 6, 7 and 8 after tumor implantation at doses of 3, 5, 10, 20 and 40 mg/kg/day (n = 5 animals/group). For local delivery by polymer, camptothecin was incorporated into EVAc at a dose of 50% by weight. Polymer cylinders measuring 1 × 3 mm were fashioned with a number 11 scalpel and placed under UV light for sterilization for 1–2 hr prior to implantation. The camptothecin polymers used were the same for all experiments. The mean weight of the implanted polymer varied slightly for the different experiments due to the fashioning process. On average, each polymer disc weighed approximately 9.0 mg; therefore, each polymer contained approximately 4.5 mg of camptothecin. Polymers were placed directly into the tumor through the original burr hole 5 days after tumor implantation. Control animals and those receiving systemic camptothecin received blank intra-tumoral EVAc polymers of similar size and weight on day 5.

For direct injection, camptothecin was dissolved in sterile 0.9% NaCl to yield a 100 mg/ml solution. Five days after tumor implantation, animals were placed into a stereotactic frame and camptothecin was injected at a depth of 3.5 mm from the surface. Following injection the burr hole was covered with bone wax to prevent efflux of the camptothecin. Doses injected were 5 mg in 50 μl and 2.5 mg in 25 μl. The 5 mg dose is equivalent to the total amount of camptothecin contained in a single polymer. Control animals were injected with similar volumes of sterile 0.9% NaCl. Animals were assessed daily for signs of toxicity, especially neurological and behavioral changes. Deaths were quantified daily. At the time of death, the brain was removed and placed in 10% formalin for at least 1 week. Brains were prepared for hematoxylin and eosin (H and E) staining. Sections through the tumor were stained to verify the presence of tumor.

Statistics

For the animal efficacy studies, survival was plotted on a Kaplan-Meier survival curve and statistical significance was determined by the Kruskal-Wallis non-parametric analysis of variance followed by the non-parametric analogue of the Newman-Keuls multiple comparison test.

RESULTS

Release kinetics

EVAc polymers were prepared with loadings of 20%, 40% and 50% camptothecin by weight. Release is reported as a cumulative percentage of the total drug loaded into the polymer. Thus, for a polymer weighing 10 mg, the total drug load would be 2, 4 and 5 mg for the 20, 40 and 50% loaded polymers, respectively. As shown in Figure 1, with 50% loading, release was rapid over the first 4 days, where approximately 43% of the drug load had been released. After the initial burst, the release rate per day was approximately 0.1% (or 5.0 μg), which continued to 3 weeks. A slower release rate was achieved with the 20 and 40% loadings; compared to the 50% polymer, less camptothecin was released at each time

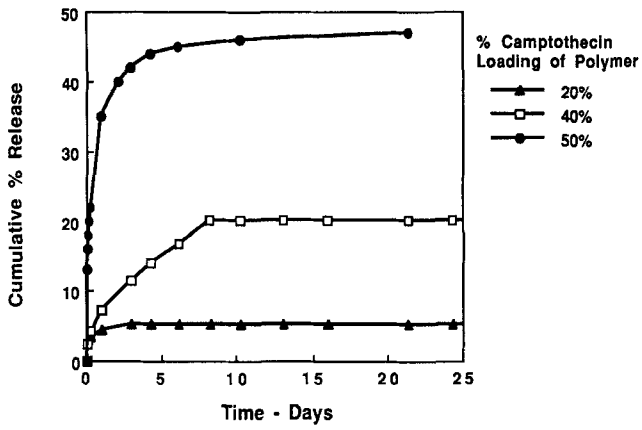

FIGURE 1 – Release kinetics of camptothecin from EVAc polymer *in vitro*. Polymers weighing 10 mg were formulated with 20% (▲), 40% (❑) and 50% (●) camptothecin by weight. Each point represents the mean of 3 measurements.

point. We selected the 50% loaded polymer for evaluation of efficacy *in vivo* based on its release profile.

In vitro *sensitivity studies*

The results of the exposure of gliomas to camptothecin in cell culture are presented in Table I. The experiment was designed to assess the sensitivity of gliomas *in vitro* to a brief (1 hr) exposure and a continuous (7 day) exposure. When exposed for 1 hr, the calculated LD_{90} ranged from 1.4 μM for 9L, F98 and U87 to 0.3 μM for U373. For all cell lines, continuous exposure for 7 days decreased the LD_{90} by up to 10-fold. For the rat and established human cell lines, the LD_{90} after continuous exposure was approximately 0.1 μM or less.

Efficacy studies

We evaluated camptothecin for its potential to prolong survival in the rat 9L intracranial model when administered either systemically or by local polymer-mediated delivery. Table II shows the survival data for the different treatment groups. Camptothecin, delivered by the polymer, significantly extended survival compared to controls ($p < 0.001$). Systemic delivery of camptothecin did not increase survival relative to controls. Rather, at the highest dose tested, 40 mg/kg/day for 4 days, the animals died before the controls, although this result was not statistically significant. Kaplan-Meier survival curves comparing systemic delivery with polymer delivery of camptothecin are shown in Figure 2.

The results of this experiment showed a benefit of local camptothecin in prolonging survival in the 9L intracranial gliosarcoma model. To evaluate further the role of the polymer, we compared direct intratumoral injection of camptothecin with polymer delivery. Survival data are presented in Table III. Camptothecin delivered by the polymer significantly increased median survival compared to controls and to the direct injection treatment group. Direct injection of camptothecin did not increase survival relative to controls.

There were no signs of neurological or behavioral abnormalities noted in any of the animals to suggest toxicity. All animals had tumor present grossly in the brain at the time of death. Presence of intra-cranial tumor was confirmed microscopically on H and E staining.

TABLE I – *IN VITRO* SENSITIVITY OF GLIOMAS TO CAMPTOTHECIN SODIUM

Cell line[1]	LD_{90} 1 hr exposure (μM)	LD_{90} continuous exposure (μM)
F98	1.40	0.10
9L	1.40	0.10
U87	1.20	0.10
U373	0.30	0.026

[1]F98 and 9L are rat glioma lines. U87 and U373 are established human glioma lines. All were tested for sensitivity to camptothecin in a clonogenic assay. Tests were performed in triplicate. Survival curves were derived from the mean of 3 measurements. LD90 for each cell line was extrapolated from survival curves.

DISCUSSION

We have shown that camptothecin can be effectively utilized by local delivery with a controlled-release polymer to prolong survival in rats implanted intracranially with 9L gliosarcoma. Our results suggest that local controlled drug delivery may allow the clinical use of highly effective anti-neoplastic agents that could not be utilized systemically because of their toxicity and narrow therapeutic window.

Camptothecin

Topoisomerase inhibitors are a class of anti-cancer drugs with a novel mechanism of action. The main target of camptothecin is the DNA-replicating enzyme topoisomerase I, resulting in inhibition of DNA and RNA synthesis (Hsiang and Liu, 1988; Li *et al.,* 1972). When tested *in vivo* against the L1210 and the rat Walker 256 carcinosarcoma, camptothecin was found to have significant anti-tumor activity (DeWys *et al.,* 1968; Drewinko *et al.,* 1974; Venditti and Abott, 1967). Consequently, camptothecin was tested in Phase I and II studies in patients with advanced gastrointestinal cancer and melanoma (Gottlieb and Luce, 1972; Moertel *et al.,* 1972; Muggia *et al.,* 1972). However, the initial clinical trials were halted because very few tumor responses were observed and significant systemic toxicity occurred. Several problems were later identified to explain the clinical results (Potmesil, 1994; Slichenmyer *et al.,* 1993; Slichenmyer and Von Hoff, 1990). First, laboratory studies confirmed the importance of the lactone structure for camptothecin cytotoxicity. Because of the insolubility of the lactone structure, clinical trials were carried out with the sodium salt, which is in equilibrium with the lactone but is less active. Additionally, pharmacological studies revealed that the water soluble sodium salt formulation was strongly protein-bound. These properties suggested a problem with bioavailability. Thus, the failure of the initial clinical trials was related to the inability to deliver active drug to the tumor.

One of the factors limiting the clinical use of camptothecin as a treatment for gliomas is CNS penetration. Because of the

TABLE II – EFFICACY OF CAMPTOTHECIN SODIUM AGAINST INTRACRANIAL 9L GLIOSARCOMA IN FISCHER 344 RATS: SYSTEMIC TREATMENT *VS.* LOCAL POLYMER DELIVERY

Treatment group	Number	Method of drug delivery	Dose	Median survival: days (range)	*p* value[1]
Control	20	*	Blank intratumoral EVAc	19 (13–31)	—
Systemic campto 20	5	i.p./*	20 mg/kg/day camptothecin × 4 days	23 (21–29)	NS[2]
Systemic campto 40	5	i.p./*	40 mg/kg/day camptothecin × 4 days	11 (11–28)	NS
Campto polymer	17	Intra-tumoral EVAc polymer	4.4 mg camptothecin[3]	>120 (39–120)	< 0.001

*Denotes treatment groups that received blank intra-tumoral discs of EVAc.–[1]Results of non-parametric Newman-Keuls test comparing treatment groups to control.–[2]NS, not significant.–[3]Dose is based on 50% load, average polymer weight = 8.8 ± 0.5 mg.

high degree of protein binding (above 97%), very little drug is available for diffusion into the CNS. Furthermore, camptothecin was not detectable in the cerebrospinal fluid (CSF) of patients in the initial clinical studies (Gottlieb *et al.,* 1970). Local delivery of camptothecin to brain tumors provides active drug directly at the tumor site across the blood–brain barrier.

Local drug delivery

Langer and Folkman (1976) reported the development of EVAc as a local delivery system for proteins and other macromolecules. Drug loaded into the non-biodegradable polymer matrix is released by diffusion through the polymer's micropore lattice. Drug release from the polymer occurs in a sustained and predictable fashion. The release profile can be tailored to provide a rapid or a slower release rate by altering the percent drug loading. The clinical use of controlled-release polymers for local drug delivery has expanded considerably. Current clinical applications include drug delivery for contraception, insulin therapy, glaucoma treatment, asthma therapy, prevention of dental caries and cancer chemotherapy (Langer and Wise, 1986). By avoiding the systemic toxicity associated with standard chemotherapy, the use of local drug delivery has the potential to expand the spectrum of drugs available for clinical use in the treatment of patients with malignant brain tumors.

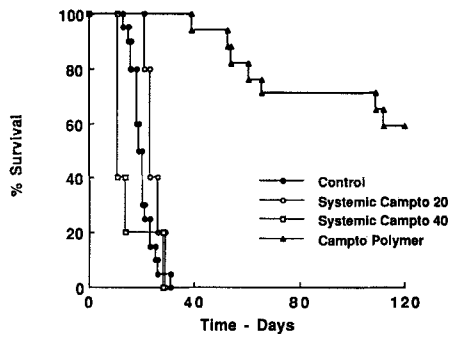

FIGURE 2 – Kaplan-Meier survival curves comparing systemic delivery of camptothecin with local delivery from EVAc polymer. Rats received an intra-cranial 9L gliosarcoma implant on day 0 and treatment was initiated on day 5. Control animals and those treated with i.p. camptothecin received a 9.0 mg EVAc polymer implant with no loaded drug. Systemic camptothecin (campto) at 20 or 40 mg/kg/day was administered i.p. over 4 days, beginning on day 5. The campto polymer group received an intra-tumoral implant of EVAc containing 4.4 mg of camptothecin. Dosing was based on polymers weighing 8.8 ± 0.5 mg, loaded with 50% camptothecin by weight.

The design and development of effective anti-tumor agents for treatment of patients with malignant neoplasms of the CNS have been influenced by 2 major factors: (*i*) the blood–brain barrier provides an anatomic obstruction, limiting access of drugs to these tumors, and (*ii*) the systemic doses required to achieve control of tumor growth frequently result in unacceptable systemic toxicity. Currently, the most effective chemotherapeutic agent for treating patients with malignant gliomas is 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Nevertheless, despite high systemic doses, tumor response remains poor and systemic toxicity is significant (Kornblith and Walker, 1988). Local drug delivery of a chemotherapeutic agent could obviate these problems. By achieving high concentrations of a drug directly at the tumor site and avoiding systemic toxicity, polymer-mediated drug delivery provides an innovative means of treating these malignant neoplasms.

We previously demonstrated that polymer-mediated delivery of BCNU is superior to systemic delivery in extending survival of animals with intracranial 9L gliosarcoma (Tamargo *et al.,* 1993). The higher sustained drug concentrations achieved with local polymer-mediated delivery significantly increased median survival. Indeed, 17–42% of the animals treated with local BCNU from the polymer survived > 120 days, while there were no long-term survivors in animals receiving systemic chemotherapy. These experimental studies were the basis of Phase I clinical trials of polymer delivery of BCNU in patients with recurrent malignant glioma (Brem *et al.,* 1991). In a Phase III trial involving 222 patients randomized between placebo polymer and BCNU polymer, there was a significant prolongation of survival in the treated group (Brem *et al.,* 1995). To date, 325 brain tumor patients have been evaluated with this treatment approach. These trials indicate that the technique is safe and effective.

We have shown that other chemotherapeutic agents, such as taxol (Walter *et al.,* 1994), 4-hydroperoxycyclophosphamide (Judy *et al.,* 1995) and carboplatin (Brem *et al.,* 1994; Domb *et al.,* 1991), also extend survival in animal models of gliomas when delivered locally *via* polymer. Additionally, the 9L glioma was treated effectively by a combination of a systemic chemotherapeutic agent, BCNU, and a polymer-delivered

TABLE III – EFFICACY OF CAMPTOTHECIN SODIUM AGAINST INTRACRANIAL 9L GLIOSARCOMA IN FISCHER 344 RATS: DIRECT INTRA-TUMORAL INJECTION *VS.* LOCAL POLYMER DELIVERY

Treatment group	Number	Method of drug delivery	Dose	Median survival: days (range)	*p* value[1]
Control injection	7	Intra-tumoral injection	0.9% NaCl	18 (15–25)	—
Control polymer	7	Blank intra-tumoral EVAc	—	19 (15–23)	NS[2]
Campto polymer	10	Intra-tumoral EVAc-campto polymer	4.75 mg camptothecin[3]	50 (35–76)	< 0.001
Campto injection	10	Intra-tumoral injection	2.5 mg camptothecin	18 (15–27)	NS
Campto injection	10	Intra-tumoral injection	5.0 mg camptothecin	19 (16–25)	NS

[1]Results of non-parametric Newman-Keuls test comparing treatment groups to control saline injection.–[2]NS, not significant.–[3]Dose is based on 50% load, average polymer weight = 9.5 ± 1.0 mg.

anti-angiogenic agent, minocycline, providing a new approach for the treatment of brain tumors (Weingart *et al.,* 1995).

This report, showing that gliomas are sensitive to camptothecin at nanomolar concentration *in vitro,* adds another potentially effective agent for treating malignant gliomas. Camptothecin can be readily incorporated into, and released from, a controlled-release polymer, making it suitable for local delivery. In the rat intracranial 9L gliosarcoma model, polymer-mediated delivery of camptothecin was a highly effective means of treatment, resulting in a significant increase in survival. Local delivery of camptothecin by polymer proved to be superior to local intra-tumoral injection of the drug. Our results highlight the therapeutic potential of polymer-mediated delivery of drugs and suggest that with further study camptothecin may be an effective treatment for patients with malignant gliomas when delivered locally by polymer.

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