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ORIGINAL ARTICLE

**Phillip B. Storm · Violette M. Renard
John L. Moriarity · Betty Tyler · Robb E. Wilentz
Henry Brem · Jon D. Weingart**

Systemic BCNU enhances the efficacy of local delivery of a topoisomerase I inhibitor against malignant glioma

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Abstract *Purpose*: To investigate the ability of systemically delivered BCNU to enhance the activity of either systemically delivered irinotecan (CPT-11) or locally delivered camptothecin from a biodegradable polymer for treatment of an intracranial 9L gliosarcoma. *Methods*: We used a single systemic dose of BCNU on treatment day 1 in combination with systemic doses of CPT-11 on treatment days 1–5 and 8–12 against an intracranial rat 9L gliosarcoma model implanted into female Fischer 344 rats. We also used the same systemic dose of BCNU given on treatment day 1, followed by a local dose of a 20% loaded camptothecin biodegradable polymer implanted on the same day. *Results*: Two doses of CPT-11 (10 and 60 mg/kg) were delivered systemically against intracranial 9L. Neither dose showed an increase in survival compared to controls ($P > 0.2$ for 10 mg/kg and $P = 0.17$ for 60 mg/kg). Systemic delivery of CPT-11 (10 mg/kg per day) in combination with systemic BCNU (15 mg/kg) did not show a significant effect on survival compared to systemic BCNU alone ($P > 0.2$), even at the maximally tolerated systemic dose of CPT-11 (60 mg/kg per day; $P = 0.06$). The combination of systemic BCNU (15 mg/kg) and intracranial delivery of camptothecin (20% loaded polymer), however, significantly extended survival compared to systemic BCNU alone ($P < 0.001$) and compared to intracranial delivery of camptothecin alone ($P = 0.01$). *Conclusions*: In a 9L gliosarcoma model, systemic delivery of CPT-11 showed no benefit in survival when delivered alone or in combination with systemic BCNU, because CPT-11 is unable to cross the blood–brain barrier in cytotoxic levels. When cytotoxic levels of a topoisomerase I inhibitor are delivered directly to the brain tumor via a biodegradable polymer, however, the systemic delivery of the alkylating agent BCNU significantly enhances the antitumor effects of camptothecin in a 9L gliosarcoma model.

Keywords Topoisomerase I inhibitor · BCNU · Brain tumor · Polymer delivery · Combination · Camptothecin · CPT-11 (Irinotecan)

P. B. Storm
Department of Neurological Surgery,
The Children's Hospital of Philadelphia
and University of Pennsylvania, Philadelphia,
PA 19104, USA

V. M. Renard · J. L. Moriarity · B. Tyler
H. Brem (✉) · J. D. Weingart
Hunterian Brain Tumor Laboratory,
Department of Neurological Surgery,
The Johns Hopkins University School of Medicine,
Baltimore, MD 21205, USA
E-mail: hbrem@jhmi.edu
Tel.: +1-410-6140477
Fax: +1-410-6140478

J. L. Moriarity
Mississippi Neurosurgical Spine Center,
1 Layfair Drive/Ste 120, Flowood,
MS 39232, USA

R. E. Wilentz
Department of Pathology,
Johns Hopkins University School of Medicine,
Baltimore, MD, USA

Introduction

In cancer patients, combination chemotherapy is used to increase therapeutic effects while limiting toxicity. The ideal combination is one that results in synergy against the tumor but has less than additive toxicity to normal tissues. Recent studies have shown that combining the potent topoisomerase I inhibitor irinotecan (CPT-11) with the alkylating agent 1,3-bis-(chloroethyl)-1-nitrosourea (BCNU) produces an antitumor effect that is greater than additive [1].

CPT-11 is a water-soluble camptothecin analog that is converted into the active metabolite SN-38 in the liver. SN-38 stabilizes the covalent bond formed between topoisomerase I and DNA during replication and causes apoptotic cell death [2]. BCNU is an alkylating agent that covalently binds one alkyl group to a strand of DNA,

with formation of DNA interstrand crosslink. Studies have shown that CPT-11 in combination with BCNU is more effective than either compound alone for the treatment of human malignant glioma-derived xenografts in the flanks of athymic nude mice [3]. This combination is most effective when a single systemic dose of BCNU is given on treatment day 1, followed by systemic doses of CPT-11 on days 1–5 and 8–12 [3]. Because of the exciting results obtained by this BCNU/CPT-11 combination in the mouse flank model, a phase I clinical trial is currently underway for adults with recurrent glioma.

A major obstacle in treating malignant glioma with systemic chemotherapy is the blood–brain barrier. Many studies have indicated that CPT-11 is a powerful antineoplastic agent when administered alone and in combination with other agents in the treatment of lung, pancreatic, gastrointestinal, and ovarian tumors [4–9]. Unfortunately, there are also several reports that suggest that CPT-11 (and its active metabolite SN-38) and other water-soluble camptothecin analogs cross the blood–brain barrier poorly, which severely limits their efficacy for treating malignant glioma [10]. Therefore we investigated the efficacy of systemic delivery of this promising drug combination in our intracranial 9L gliosarcoma model. We showed, however, that the systemic delivery of BCNU and CPT-11 does not significantly extend survival, but it does show a trend toward statistical significance. This finding coupled with many exciting results when an alkylating agent is combined with a topoisomerase I inhibitor led us to investigate this combination further. Since CPT-11 must be metabolized in the liver to the active SN-38 form, it is not a candidate for local delivery. We have shown, however, that local delivery of the topoisomerase I inhibitor, camptothecin-sodium, from a biodegradable polymer is safe, and significantly extends survival [11]. Therefore, we investigated the combination of systemic delivery of BCNU with local delivery of camptothecin-sodium and showed that systemic BCNU significantly enhances the efficacy of locally delivered camptothecin.

Materials and methods

Animals

Female Fischer 344 rats weighing 150–200 g were obtained from Charles River and housed in the animal storage facility at the Johns Hopkins University School of Medicine. The animals were maintained on Purina Certified Rodent Chow with free access to Baltimore City water. The rats were kept in cages with three or four animals per cage and cared for by members of the Department of Animal Services at the Johns Hopkins University School of Medicine. The animals were checked daily by the investigators, by members of the Department of Animal Services, and by veterinarians for changes in weight, grooming and behavior. Animals showing signs of neurological deficits were killed.

Anesthesia

Rats were anesthetized with an intraperitoneal (i.p.) injection of 2–4 ml/kg of a stock solution containing ketamine hydrochloride (25 mg/ml), xylazine (2.5 mg/ml), and 14.25% ethyl alcohol in normal saline. Rats were killed with an intracardiac injection of 0.3 ml of Euthanasia-6 Solution CII (Veterinary Laboratories, Lenexa, Kan.) after being anesthetized as described above.

Chemotherapy drugs

CPT-11 was generously provided by Pharmacia & Upjohn (Global Distribution Center, Kalamazoo, Mich.). For systemic administration, the clinical formulation (Camptosar) was used, but we refer to this solution as CPT-11 in the text. Camptosar was formulated by combining irinotecan hydrochloride (CPT-11) 20 mg/ml, sorbitol 45 mg/ml, and lactic acid 0.9 mg/ml, and adjusting to pH 3–3.8. Dilutions were made in a 5% dextrose solution. Sorbitol and lactic acid were purchased from Sigma Chemical Co. (St Louis, Mo.). BCNU (Bristol-Myers Squibb) was purchased from the oncology pharmacy at the Johns Hopkins Hospital (Baltimore, Md.); BCNU solutions were made in 0.9% saline. The National Cancer Institute (Bethesda, Md.) generously provided the camptothecin sodium. Because camptothecin sodium is the water-soluble form of camptothecin, and it exists in an equilibrium with camptothecin [12], we refer to camptothecin sodium as camptothecin from this point forward.

Polymer preparation

P(CPP/SA)(20:80) polymers containing camptothecin were synthesized according to the method of Domb and Langer [13]. Camptothecin (20% by weight) was mixed with p(CPP/SA)(20:80), and dissolved in methylene chloride to yield a 10% solution (w/v). The solvent was then evaporated by placing the mixture in a vacuum desiccator for 72 h, which yielded a dry powder. Camptothecin polymer discs (10 mg final weight) were prepared by compression molding as described by Domb and Langer [13].

Intracranial model

The 9L gliosarcoma was maintained in the flanks of female Fischer 344 rats. The tumor was initially obtained in 1985 from the Brain Tumor Research Center, University of California at San Francisco, and was passed every 2–3 weeks. For intracranial implantation, the carrier animal was antiseptically prepared with 70% ethanol and Prepodyne solution, and the tumor was surgically excised. The tumor was then cut into 1-mm^3

pieces that were placed in sterile 0.9% NaCl and kept on ice during the implantation procedure.

Toxicity

We used 40 female Fischer 344 rats. They were divided into groups of eight and treated with escalating doses (20, 40, 60, 80, 100 mg/kg) of i.p. CPT-11 on days 1–5 and 8–12 to determine the maximum tolerated dose. The rats were examined twice daily for signs of weight loss, seizures, paralysis, loose stools, abnormal gait, and poor grooming. On the day of death, or after 200 days, we removed the brains, lungs, hearts, kidneys, livers, and gastrointestinal tracts from the animals, fixed them in formalin, and stained them with hematoxylin and eosin (H&E).

Efficacy studies

We used our 9L model for efficacy studies because the model is highly reproducible, and the survival curves from preclinical testing of polymer delivery of BCNU in this 9L model closely paralleled the survival curves of polymer delivery of BCNU in phase III clinical trials [14–16]. The efficacy of systemic administration of CPT-11 alone and in combination with systemic BCNU was first tested against the intracranial 9L tumor model. The efficacy of systemic administration of BCNU in combination with local delivery of camptothecin was then tested in the same tumor model. Fischer 344 rats were anesthetized as described above, and the surgical site was prepared by shaving and applying 70% EtOH and Prepodyne solution. A midline incision was made on the posterior aspect of the animal's head, and the coronal suture was identified. Using a ruler, a point 5 mm posterior to the coronal suture and 3 mm lateral to the sagittal suture on the left parietal bone was identified. A dental drill fitted with a 2-mm cutting burr was used to make a 3-mm burr hole. The dura was opened with a cruciate incision, and the underlying cortex and white matter were gently aspirated using a sterile glass micropipette attached to wall suction. The cortex was aspirated until the superior aspect of the brainstem was exposed. After achieving hemostasis, a 1-mm^3 tumor piece was placed into the defect. The wound was closed with surgical clips.

Five days after tumor implantation (treatment day 1), the animals were randomized into groups, reanesthetized, and the wounds were reopened under sterile conditions. The investigators confirmed tumor in each animal by visual inspection of the implantation site upon reoperation on day 5. Every animal receiving a tumor piece had viable tumor at the time of polymer implantation. After the presence of tumor was confirmed, each animal was implanted with a 10-mg p(CPP/SA)(20:80) disc containing no drug (control group and groups receiving only systemic drug). Control animals also received i.p. injections of carrier solutions without drug. Animals receiving systemic CPT-11 were given drug on treatment days 1–5 and 8–12. Animals receiving systemic BCNU were given a single dose on day 1. Animals receiving both CPT-11 and BCNU systemically were given BCNU on day 1 and CPT-11 on days 1–5 and 8–12.

Animals receiving intracranial implantation of 10-mg p(CPP/SA) discs loaded with 20% camptothecin alone or in combination with systemic BCNU received both the polymers and the systemic dose of BCNU on treatment day 1. The BCNU dose was given first and followed 5 h later with the systemic or intracranial topoisomerase I inhibitor dose. All animals alive on day 120 were considered long-term survivors and were killed. The efficacy experiments were terminated on day 120 because in our well-established 9L gliosarcoma model no animals surviving for 120 days have demonstrated gross or histological evidence of tumor. The organs (brain, liver, lung, kidney, heart, and gastrointestinal tract) of the long-term survivors were fixed in formalin, sectioned and stained with H&E, and examined by a pathologist for gross and microscopic evidence of toxicity and/or tumor.

Statistical analysis

Survival was plotted on Kaplan-Meier survival curves and statistical significance was determined by a nonparametric Kruskal–Wallis analysis of variance followed by a nonparametric Wilcoxon Rank Sum Test. The endpoint for the Kaplan-Meier curve was death. Animals demonstrating weight loss, abnormal grooming, gait disturbances or seizures were killed and treated as deaths. Values were considered significant at an alpha level of 0.05.

Results

Efficacy of systemic CPT-11 and systemic BCNU

The Kaplan-Meier survival curve (Fig. 1) showed that systemic delivery of CPT-11 (10 mg/kg per day on days 1–5 and 8–12) had no effect against intracranially implanted 9L gliosarcoma when administered alone or in combination with systemic BCNU. The median survivals (Table 1, experiment 1) were 16 days for controls, 15 days for the CPT-11 group, 31 days for the BCNU group, and 23 days for the BCNU and CPT-11 combination. After controlling for the effect of BCNU, the systemic CPT-11 and systemic BCNU combination did not extend survival compared to BCNU alone ($P > 0.2$).

The doses for the animals in Fig. 1 were based on experiments that showed a significant reduction in tumor volume in the flanks of athymic mice [3]. Because we used Fischer 344 rats instead of athymic mice, we next determined the maximum tolerated systemic dose in

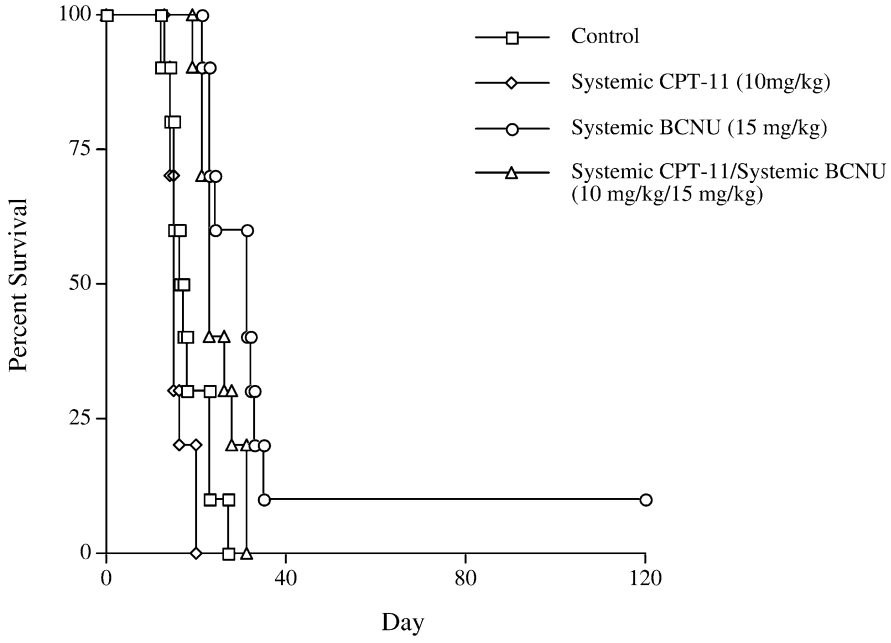

Fig. 1 Kaplan-Meier survival curve for experiment 1 (Table 1). Fischer 344 rats received intracranial 9L gliosarcoma on day 0; on day 5 (treatment day 1) they received i.p. injections of BCNU, CPT-11 or both BCNU and CPT-11. The rats received CPT-11 on treatment days 1–5 and 8–12. Controls received i.p. injections of the carrier solutions

female Fischer 344 rats, thus establishing that the lack of response was not a result of underdosing. Table 2 shows that all of the animals in the 100-mg/kg group were dead by day 5. The animals in the 80-mg/kg group survived long enough to receive all ten injections. All of the animals in this group had loose stools, weight loss, and developed severe abdominal irritation at the injection site. Four animals in this group had died by the end of the 3rd week of the experiment. Those that survived had begun to gain weight by week 4 and no longer had loose stools. All eight of the 60-mg/kg group survived and tolerated the dosing well, apart from development of moderate abdominal irritation at the injection site. The rats in the 40-mg/kg and 20-mg/kg groups did not show any signs of toxicity or abdominal irritation.

Gross examination of the rats at the time of death did not reveal hemorrhaging or an obvious cause of death in the 80-mg/kg group. Two of the eight animals in the 100-mg/kg per day group had evidence of pulmonary hemorrhage. Likewise, microscopic examination of the organs following H&E staining did not reveal a cause of death. Only non-specific changes, including vascular congestion, were seen. No histological evidence of hepatotoxicity, nephrotoxicity, or intestinal toxicity was identified.

The efficacy experiment was repeated with a systemic dose of 60 mg/kg per day CPT-11 per rat. We also added a fifth group that received 30 mg/kg BCNU (double the initial dose). We were concerned that if the BCNU and CPT-11 combination prolonged survival, it would be impossible to conclude a synergistic or even additive effect if we did not increase the doses of the groups receiving a single drug. That is, would we be demonstrating a specific effect of the combination, or just showing that a higher dose was more effective than a lower dose? The CPT-11 dose of 60 mg/kg per day could not be doubled because of its toxicity.

Even at the maximum tolerated dose of CPT-11 (60 mg/kg per day) survival was not prolonged when CPT-11 was administered alone or in combination with BCNU (Fig. 2). Further, doubling of the BCNU dose (30 mg/kg) failed to offer a benefit over the original dose

Table 1 Efficacy of combination therapy of systemically delivered BCNU and CPT-11

Experiment	Treatment	No. of rats	Median survival (days)	Long-term survivors	*P*-value[a]	*P*-value[b]
1	Control	10	16	0		
	CPT-11 (10 mg/kg/day)	10	15	0	>0.2	
	BCNU (15 mg/kg)	10	31	2	0.002	
	CPT-11 (10 mg/kg/day) and BCNU (15 mg/kg)	10	23	0	0.02	$>0.2^{\#}$
2	Control	10	19	0		
	CPT-11 (60 mg/kg/day)	10	25	0	0.17	
	BCNU (15 mg/kg)	9	31	0	0.001	
	BCNU (30 mg/kg)	10	40	0	<0.001	$0.13^{\#\#}$
	CPT-11 (60 mg/kg/day) and BCNU (15 mg/kg)	11	43	2	<0.001	$0.06^{\#\#\#}$

[a]Results of nonparametric Wilcoxon Rank Sum Test
[b]Results of nonparametric Wilcoxon Rank Sum Test comparing the CPT-11/BCNU combination to BCNU alone in experiment 1 (#) and in Experiment 2 (###). ## Result of single dose of BCNU compared to a double dose of BCNU

Table 2 Toxicity study of systemically delivered CPT-11 in rats. Eight rats were used in each group

CPT-11 dose (mg/kg/day)[a]	Percent survival
20	100
40	100
60	100
80	50
100	0[b]

[a]CPT-11 was administered as an intraperitoneal injection on days 1–5 and 8–12
[b]All of the animals had died by day 5

of BCNU (15 mg/kg). The median survivals (Table 1, experiment 2) were 19 days for controls, 23 days for CPT-11 (60 mg/kg) alone, 31 days for BCNU (15 mg/kg) alone, 43 days for the CPT-11 (60 mg/kg)/BCNU (15 mg/kg) combination, and 40 days for the doubled BCNU dose (30 mg/kg). Again, the CPT-11/BCNU combination significantly extended survival compared to controls ($P < 0.001$), but when the effect of BCNU alone was controlled for, the combination was no better than BCNU alone ($P = 0.06$).

Efficacy of local camptothecin and systemic BCNU

We chose a 20% loaded camptothecin polymer because this dose modestly extends survival [11], and any enhancement by systemic BCNU would be observed. The animals in the combination group received an i.p. injection of BCNU (15 mg/kg), and 5 h later a 20% loaded camptothecin polymer. The groups getting single therapy received their respective treatments at the same time as the combination group. Since doubling the dose of BCNU to 30 mg/kg did not extend survival compared to the BCNU 15 mg/kg dose (see Fig. 2), that group was omitted. The Kaplan-Meier curves are shown in Fig. 3. All four groups had significantly extended survival compared to controls (Table 3). The combination group of a 20% camptothecin polymer and systemic BCNU significantly extended survival compared to the control group ($P < 0.001$), the systemic BCNU group ($P < 0.001$), and the 20% camptothecin polymer group ($P = 0.01$). Furthermore, the combination group of intracranial camptothecin and systemic BCNU had 70% (7/10) long-term survivors. The study was terminated on day 120. Thus, unlike the experiments where systemic delivery of the topoisomerase I inhibitor, CPT-11, failed to affect survival, the efficacy of local delivery of the topoisomerase I inhibitor, camptothecin, was significantly enhanced by systemic delivery of BCNU.

At the time of death, each rat brain was inspected for gross tumor. All of the animals that died spontaneously had gross evidence of tumor. The long-term survivors were killed on day 120; none of them had macroscopic or microscopic evidence of tumor cells.

Discussion

The blood–brain barrier prevents potentially therapeutic agents from effectively treating malignant gliomas. Several chemotherapeutic agents that are extremely potent against gliomas in vitro are ineffective in vivo because dose-limiting systemic toxicity occurs before cytotoxic levels of the drug cross the blood–brain barrier and reach the brain tumor. The alkylating agent BCNU is lipophilic and is able to cross the blood–brain barrier, but it has shown limited success because the high systemic doses required for treating the tumor cause unacceptable side effects. Combination chemotherapy and local delivery have been used to overcome the

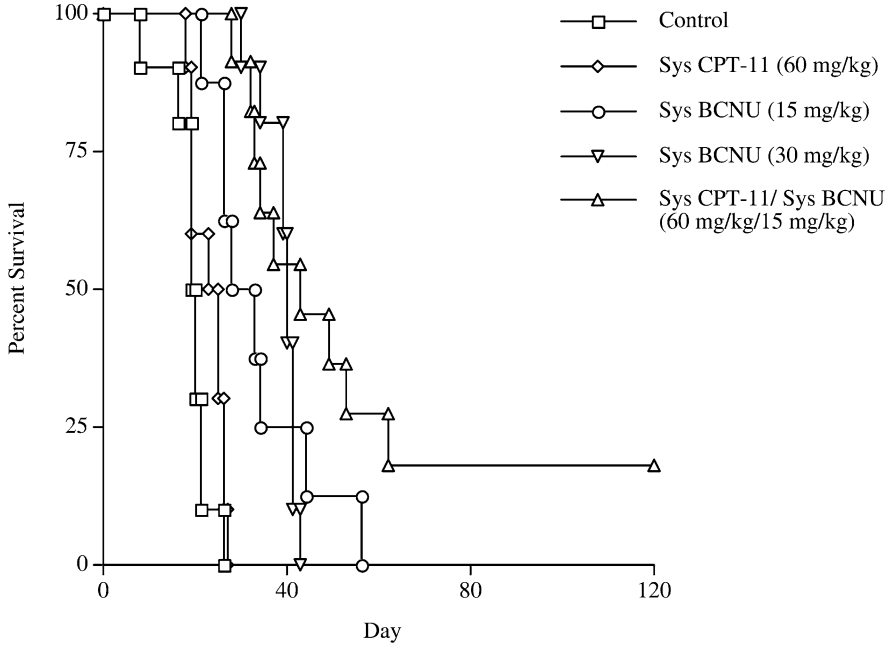

Fig. 2 Kaplan-Meier survival curve for high-dose CPT-11, experiment 2 (Table 1). Fischer 344 rats received intracranial 9L gliosarcoma on day 0; on day 5 (treatment day 1) they received i.p. injections of BCNU, high-dose CPT-11 or both BCNU and high-dose CPT-11. The rats received high-dose CPT-11 treatment days 1–5 and 8–12. Controls received i.p. injections of the carrier solutions

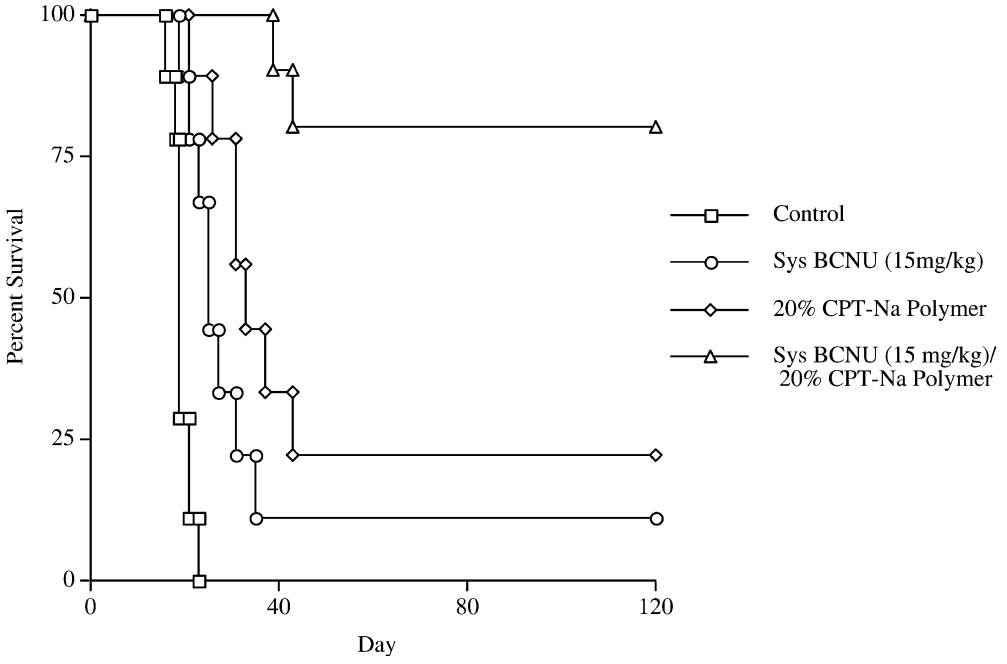

Fig. 3 Kaplan-Meier survival curve for the enhancement of local delivery of camptothecin with systemic BCNU, experiment 3 (Table 3). Fischer 344 rats received intracranial 9L gliosarcoma on day 0; on day 5 (treatment day 1) the animals received an intratumoral implant of a 10-mg p(CPP/SA) disc. The control animals and those receiving the i.p. injection of BCNU also received discs with no drug. The other two groups received discs loaded with 20% camptothecin alone or in combination with an i.p. injection of BCNU

obstacles imposed by the blood–brain barrier. In the present study we were unable to demonstrate a benefit when systemic CPT-11 was combined with systemic BCNU. When we combined local delivery of camptothecin from a biodegradable polymer with systemic delivery of BCNU, however, we showed a significant prolongation in survival.

The combination of CPT-11, a topoisomerase I inhibitor, with BCNU, an alkylating agent, has shown promise in treating malignant glioma both in vitro and in a mouse flank xenograft model [1, 3]. These drugs have different mechanisms of action and different dose-limiting toxicities, making them good choices to use in combination. CPT-11 showed promising results in preclinical testing, but results of a phase II trial in patients with recurrent malignant glioma were disappointing [17]. It is possible that doses of CPT-11 were reduced in these patients because their antiepileptics (dilantin and tegretol) caused enhanced clearance of SN-38 (the active metabolite of CPT-11) [3]. Moreover, systemic administration of CPT-11 and SN-38 results in limited penetration into brain and CSF [10].

When we investigated systemic administration of CPT-11 alone and in combination with BCNU in our intracranial rat 9L gliosarcoma model, we found that the addition of CPT-11 had no benefit compared to systemic BCNU alone (Table 1, Fig. 1, 2). The dose of 10 mg/kg per day of CPT-11 on days 1–5 and 8–12 in the first experiment was based on a prior study showing that this dosing regimen was the most effective in athymic mice with human xenografts implanted in the flank [3]. The failure of systemic delivery of CPT-11 to prolong survival alone or in combination with systemic delivery of BCNU in an intracranial model, could have been due to underdosing of the Fischer 344 rats because of a potentially increased clearance of CPT-11 in rats compared to athymic mice. A study by Bogaards et al. has shown that the rate of P450 drug metabolism varies not only between species, but also between drugs, suggesting that simple conversion tables used to predict drug dosing between species based on body surface area may not be accurate [18]. Therefore, we conducted a dose escalation study to determine the highest tolerated systemic dose of CPT-11 in rats (Table 2). Despite increasing the dose by a factor of 6, there still was not a statistically significant difference between the CPT-11 group and controls ($P = 0.17$) and the CPT-11/BCNU combination and BCNU alone groups ($P = 0.06$) (Fig. 2, Table 1). This suggests that SN-38 (the active metabolite of CPT-11) does not cross the blood–brain barrier in sufficient amounts to inhibit topoisomerase I.

To solve the problem of poor blood–brain barrier penetration of camptothecins, we investigated the efficacy

Table 3 Enhancement of intracranial camptothecin implants by systemic BCNU

Treatment	No. of rats	Median survival (days)	Long-term survivors	*P*-value[a]	*P*-value[b]
Control	9	19	0		
BCNU (15 mg/kg)	9	25	1	0.003	
Intracranial CPT (20%)	9	33	2	< 0.001	
Intracranial CPT (20%) + BCNU (15 mg/kg)	10	120[c]	7	< 0.001	< 0.001, 0.01

[a]Results of nonparametric Wilcoxon Rank Sum Test
[b]Results of nonparametric Wilcoxon Rank Sum Test comparing the camptothecin and BCNU combination to BCNU alone and camptothecin alone
[c]The experiment was terminated on day 120

of a locally delivered topoisomerase I inhibitor in combination with systemically delivered BCNU in an intracranial tumor model. CPT-11 is not a candidate for local delivery because it requires hepatic conversion to its active metabolite SN-38. We have previously shown, however, that local delivery of camptothecin from a biodegradable polymer significantly extends survival in an intracranial 9L glioma model [11]. Thus, by directly implanting a polymer loaded with camptothecin, we can bypass the blood–brain barrier, continuously expose the tumor to a topoisomerase I inhibitor, and enhance its effect by adding a systemic dose of BCNU. In contrast to the systemic CPT-11/BCNU combination, local delivery of camptothecin was significantly enhanced by systemic delivery of BCNU (Fig. 3, Table 3).

The mechanism of action for the enhancement of cytotoxicity is not established. A possible explanation is that the inhibition of topoisomerase I by camptothecin prevents the cells from correcting DNA mispairing caused by BCNU. It is also possible that camptothecin inhibits phosphatidylinositol (3) kinase, thereby lowering the threshold for BCNU-induced apoptosis. Nakashio et al. have demonstrated that the camptothecin analog, topotecan, inhibits phosphatidylinositol (3) kinase [19]. This kinase and its downstream phospholipid products are involved in suppression of apoptosis and implicated in oncogenesis [20–22].

This study established that intracranial delivery of camptothecin is enhanced by systemic delivery of BCNU. The results are consistent with previous studies that have shown a significant increase in cytotoxicity against gliomas when a topoisomerase I inhibitor is combined with an alkylating agent both in vitro and in models with gliomas being implanted extracranially, thereby lacking a blood–brain barrier. On the basis of this study we would predict a lack of response in glioma patients receiving systemic CPT-11 alone or in combination with systemic BCNU. By contrast using biodegradable polymers to bypass the blood–brain barrier and directly deliver a topoisomerase I inhibitor to brain tumor cells in rats, we were able to utilize this potent combination to extend survival significantly. Because of these encouraging results we are beginning to investigate local delivery of both BCNU and camptothecin from biodegradable polymers to further augment efficacy and eliminate systemic delivery.

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