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ORIGINAL ARTICLE

Local delivery of temozolomide by biodegradable polymers is superior to oral administration in a rodent glioma model

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Abstract
Purpose Dose-limiting adverse effects of thrombocytopenia and leukopenia prevent augmentation of current temozolomide (TMZ) dosing protocols; therefore, we hypothesized that the direct intracranial delivery of TMZ would lead to improved efficacy in an animal model of malignant glioma in an animal model.
Methods Temozolomide was incorporated into biodegradable polymers and the active drug was released over 80 h. Intracranial toxicity was assessed in F344 rats and a maximally tolerated dose was not achieved.
Results In vivo drug biodistribution demonstrated that intracranial concentrations of TMZ increased threefold compared with orally delivered TMZ. In a rodent glioma model, animals treated with a single TMZ polymer (50% w/w) had a median survival of 28 days ($P < 0.001$ vs. controls, $P < 0.001$ vs. oral treatment), whereas animals treated with oral TMZ had a median survival of 22 days compared to control animals (median survival of 13 days). Animals treated with two TMZ polymers (50% w/w) had a median survival of 92 days ($P < 0.001$ vs. controls, $P < 0.001$ vs. oral treatment). The percentage of long-term survivors (LTS) for groups receiving intracranial TMZ ranged from 25 to 37.5%; there were no LTS with oral TMZ treatment. Animals treated with radiation therapy (XRT) and intracranial TMZ (median survival not reached, LTS = 87.5%) demonstrated improved survival compared to those with intracranial TMZ alone (median survival, 41 days; LTS = 37.5%), or oral TMZ and XRT (median survival, 43 days, LTS = 38.9%).
Conclusions The survival of tumor-bearing animals was improved with local delivery of TMZ compared with systemic administration. XRT in combination with intracranial TMZ did not cause additional toxicity and prolonged the survival even further.

Keywords Temozolomide · Local delivery · Polymer · 9L gliosarcoma

Abbreviations

TMZ	Temozolomide
GBM	Glioblastoma multiforme
pCPP:SA	Poly[bis(*p*-carboxyphenoxy propane) sebacic acid]

Introduction

Temozolomide (TMZ) is an imidazotetrazine second-generation alkylating agent. Temodar® is orally available and is used to treat patients with malignant glioma. As an alternative to the FDA-approved Gliadel® wafer with radiation therapy, radiation therapy with Temodar® has been approved for patients with Glioblastoma multiforme [1]. In these patients, Temodar® in combination with radiation therapy improved survival compared to patients with radiation alone, extending median survival by 2.5 months [2] at a dose of 150–200 mg/m^2. Higher doses of Temodar® were proscribed because of dose-limiting myelosuppression. Phase I trials have demonstrated

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that the maximally tolerated dose (MTD) of Temodar® is 200 mg/m$^{2.}$ Above this dose, severe leukopenia and thrombocytopoenia are evident. Even at these higher doses, however, only minimal adverse neurological effects have been encountered with Temodar® [3]. Consequently, we reasoned that direct intracranial delivery of TMZ by biodegradable polymers might also enhance its effectiveness without additional adverse effects.

We have previously shown that BCNU, incorporated into biodegradable polymers and implanted locally at the time of tumor resection, prolonged survival in patients with malignant gliomas [4–7]. Direct delivery to the brain increases intracranial drug concentrations while minimizing systemic toxicity. Therefore, we considered that delivery of TMZ directly to the tumor might further enhance the effectiveness of this important agent.

TMZ is an ideal candidate for direct local polymeric delivery to the brain, since it does not require hepatic activation and neurological toxicity has not been demonstrated. Under neutral and alkaline conditions, TMZ undergoes rapid hydrolysis with a half-life of 1.24 h [8]. Incorporation of TMZ into biodegradable polymers would theoretically prevent drug hydrolysis prior to release. Langer et al have previously shown that the hydrophobic components of the polyanhydride polymer improve drug stability and preserve biological activity [9–11].

We have developed a local delivery system for TMZ utilizing biodegradable polymers. We examined the safety and efficacy of this drug delivery system in a rodent glioma model. To mimic current clinical practice, we also examined the efficacy of locally delivered TMZ given with radiation therapy.

Materials and methods

Polymer formulation

TMZ was incorporated into a polyanhydride CPP:SA polymer at concentrations of 10, 20 and 50% by methods described previously [12]. The polymers were then pressed into a disc shape weighing approximately 10 mg. For in vitro release kinetics and biodistribution studies, ^{3}H-TMZ (Moravek, Brea, CA) was added to the drug/polymer formulation.

In vitro release kinetics

Release kinetics were performed with ^{3}H-TMZ polymers (50% w/w). The polymers were placed in a 1-ml solution of PBS and stored at 37°C. The solution was removed and replaced at 1, 3.5, 10, 25, 48, 72, 96, 120 and 144 h. These samples were then added to GelReady scintillation fluid (Beckman Coulter, Inc., Fullerton, CA) and counted on a liquid scintillation counter (Beckman Coulter Inc., Fullerton, CA). The degree of drug release was calculated based on the radioactivity of the samples versus total radioactivity of the polymer.

Determination of maximum tolerated dose for oral TMZ and intracranial TMZ

In vivo dose-escalation toxicity studies for systemically delivered TMZ were performed so as to establish the MTD and to evaluate histological evidence of potential toxicity in Fisher 344 rats. Oral TMZ was given to animals at doses of 25, 50, 100 and 500 mg/kg/day (n = 3/group) daily for 5 days. These doses correlated with the human dosing regimen [13].

TMZ polymers were intracranially implanted at doses of 10, 20 or 50% (w/w); in addition, one group received two 50% polymers. Rats were anesthetized and prepared for intracranial implantation. After a midline scalp incision, the galea overlying the left cranium was swept laterally. With the aid of an operating microscope, a 3-mm burr-hole was made over the left parietal bone, with its center 3 mm lateral to the sagittal suture and 5 mm posterior to the coronal suture. A dural opening and then a cortical opening were made and the polymer was placed subdurally. The scalp incision was then closed with surgical staples. The animals were evaluated preoperatively and daily for 60 days to determine signs of neurological deficits.

Systemic toxicity and neurotoxicity were evaluated and appropriate tissue specimens were examined histologically. Complete blood counts were obtained periodically to determine possible systemic toxicity.

In vivo biodistribution

In vivo biodistribution was studied with ^{3}H-labeled TMZ (Moravek, Brea, CA). Polymer wafers containing 50% TMZ were prepared as described above, except that 60 mCi of ^{3}H-TMZ (specific activity Ci/mmol) was incorporated into the polymers. The mixture was then dried under a high-pressure vacuum and pressed into 10-mg discs. Oral TMZ was treated with 0.36 mCi/mg. All oral treatment of TMZ was delivered via gavage. The naïve animals were either implanted with the radiolabeled TMZ wafers or received a 5-day course of oral TMZ (50 mg/kg/day). At 4, 28, 76 or 172 h, the animals were euthanized, serum levels of TMZ and blood counts were examined and the brains were harvested. The polymers were removed, and the brain was divided into hemispheres and then cut into 2 mm sections, both ipsi-

laterally and contralaterally. Each section was then homogenized and examined for radioactivity to determine the drug concentration. Polymers removed from the parenchyma were also examined for remaining drug content. Intact temozolomide was not specifically measured, however, due to the positive results obtained in the efficacy studies, there was an indication that the drug was intact and bioactive upon release from the polymer.

Intracranial glioma model

The 9L gliosarcoma was maintained in the flanks of Fisher 344 (Harlan Sprague Dawley, Indianapolis, IN) rats. For intracranial implantation, the 9L gliosarcoma tumor was surgically excised from the carrier animal, cut into 1-mm^3 pieces and placed in sterile 0.9% NaCl on ice. For intracranial implantation of the 9L glioma, 167 female Fischer 344 rats, weighing 150–200 g, were anesthetized with an intraperitoneal injection of 3–5 ml/kg of a stock solution containing ketamine hydrochloride 25 mg/ml (Ketlar; Parke-Davis Corporation Morris Plains, NJ), xylazine 2.5 mg/ml (Rompun; Mobay Corp., Shawnee, Kansas) and 14.25% ethyl alcohol in 0.9% NaCl. All surgical procedures were carried out using sterile surgical techniques. The head was prepared with alcohol and prepodyne solution, and a midline scalp incision was made, exposing the sagittal and coronal sutures. A small burr hole was made with an electric drill and 2 mm round cutting burr, centered 3 mm lateral to the sagittal suture, avoiding the sagittal sinus, and 5 mm posterior to the coronal suture. Forceps were used to lift off the remaining bone. A dural opening and then a cortical opening were made. With gentle suction, a small area of cortex and white matter was resected. Once hemostasis was achieved, a single tumor piece (1 mm^3) was placed in the depths of the cortical resection. The skin was then closed with surgical staples.

Efficacy of locally delivered TMZ

To determine the effectiveness of locally delivered TMZ, tumor-bearing animals were treated with either TMZ polymers (50% w/w) on Day 5 (Fig. 1b), or by gavage daily for 5 days (Fig. 1a) (days 5–9) with 50 mg/kg of oral TMZ. The overall survival was compared to that of the controls. The euthanized animals were examined to confirm the presence of tumor.

Efficacy of locally delivered TMZ with radiation therapy

To determine the effectiveness of local delivery of TMZ with polymers and radiation therapy, tumor-bearing animals underwent implantation of TMZ polymers (50% w/w), followed by external beam single-dose radiation treatment by using a ^{137}Cs laboratory irradiator (Mark I Irradiator, Model 68) at a dose of 20 Gy. The animals were anesthetized, placed at a fixed distance from the radiation source and shielded with a square primary collimator (7 cm × 7 cm) and a circular secondary collimator (1 cm diameter) centered over the tumor implantation site. Survival was compared to that of controls, animals treated with TMZ polymer without radiation therapy and animals treated with oral TMZ and concomitant XRT.

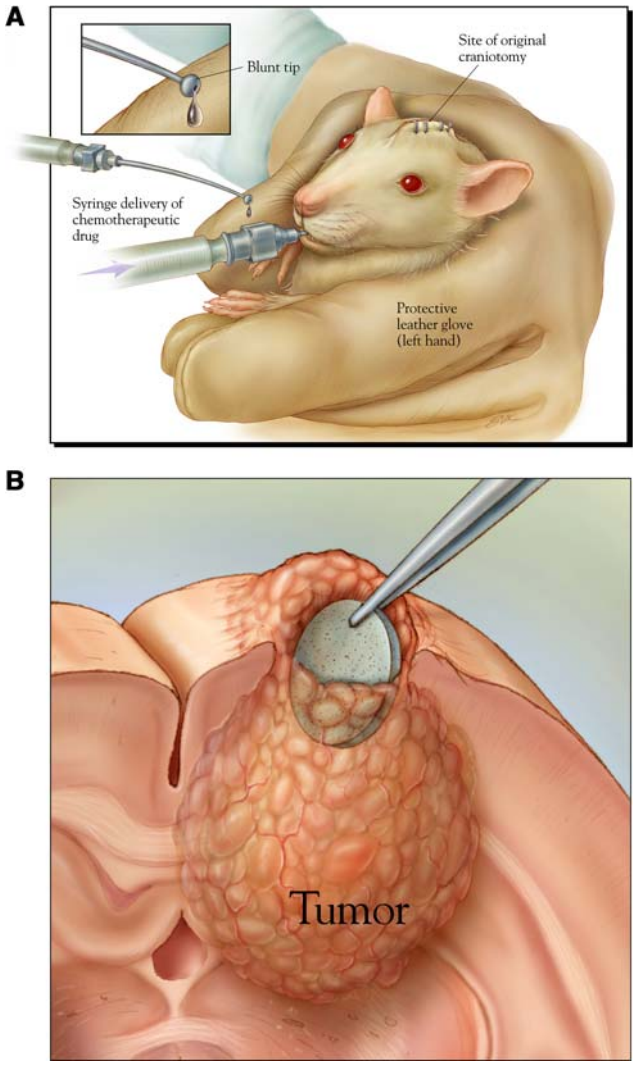

Fig. 1 **a** Artist's illustration of F344 rat previously intracranially implanted with 9L gliosarcoma, receiving oral TMZ by gavage. **b** Artist's illustration of polymer wafer being implanted into an established 9L glioma in the rodent brain

Animal care

All animals were housed in standard facilities and given free access to food and water. They were treated

in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee. At Day 120, the study was terminated and the surviving rats were deemed long-term survivors (LTS). They were then euthanized and tissue specimens were collected in formalin after perfusion.

Statistical analysis

For all efficacy studies, death was the primary endpoint. The distribution of the intervals until death was determined by the method of Kaplan and Meier. Efficacy and biosdistribution studies underwent two non-parametric statistical analyses, the Mann–Whitney *U* test and the Kruskal–Wallis test. Statview 4.51 (Abacus Concepts Inc., Berkeley ,CA) software was used for statistical analyses.

Results

Incorporation of TMZ into pCPP:SA polymers and release kinetics

TMZ was readily incorporated into CPP:SA polymers at loading doses of 10, 20 and 50% (w/w) by using the solvent method. We found that 50% TMZ polymers released 60% of the drug after 72 h, with a total release of 70% over 144 h (Fig. 2).

Determination of MTD for oral TMZ

For Fisher rats, the MTD for oral TMZ was determined to be 50 mg/kg, qd, for 5 days. Oral TMZ was found to be toxic at doses of 100 and 500 mg/kg, with median survival in these groups less than 15 days. Doses up to 50 mg/kg were well tolerated without evidence of significant clinical or histological toxicity.

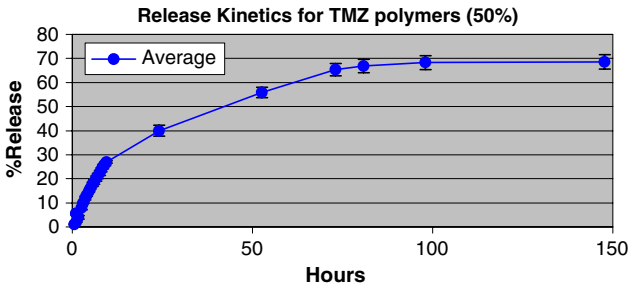

Fig. 2 Average in vitro release kinetic profile of the poly(carboxyphenoxy)propane and sebacic acid polymer (measured in triplicate). Each polymer weighed 10 mg and was loaded with 50% temozolomide by weight

Animals treated with 50 mg/kg of oral TMZ showed transient leukopenia and thrombocytopenia at 72 h, which resolved after 7 days. This dose was used for all subsequent experiments with oral TMZ.

Determination of MTD for intracranial TMZ

The MTD for intracranial delivery of TMZ was not reached. The polymers were loaded up to the maximal loading dose of 50% and rats that were implanted with TMZ polymers up to this loading dose did not display any neurological or systemic toxicity. Weight gain was similar to that in a group of animals receiving polymers without drug. Similarly, rats receiving the maximal number of two 50%-loaded TMZ polymer wafers (equaling 20 mg) that would fit into the rodent skull did not demonstrate any toxicity. Moreover, animals treated with intracranial TMZ did not show any evidence of leukopenia or thrombocytopenia, as was seen with animals treated with oral TMZ.

Intracranial and systemic biodistribution of local TMZ

Intracranial concentrations of TMZ in animals treated with intracranial ^{3}H-TMZ polymers (50% w/w) reached a maximum of 224 ng of TMZ/mg brain tissue within 2 mm of the polymer implant at 4 h after polymer implantation. After 28 h, this concentration decreased to 163 ng/mg; at 76 h, it was 62 ng/mg; and at 172 h, it was 17 ng/mg. The contralateral hemisphere of these intracranially implanted animals had concentrations ranging from 18 ng/mg at 4 h to a maximum of 31.3 ng/mg at 28 h, followed by a decrease to 11 ng/mg at 172 h. In comparison, the mean concentration of TMZ within the brain of animals treated with oral ^{3}H-TMZ was only 36 ng/mg at 4 h and 73 ng/mg at 28 h.

Serum concentrations of TMZ in the systemically treated group at 4, 28, 76 and 172 h were 0.06, 0.089, 0.111 and 0.077 ng/ml, respectively. By contrast, serum concentrations of TMZ in the locally treated group were substantially reduced at the same time points: 0.016, 0.042, 0.065 and 0.04 ng/ml, respectively (Fig. 3). This difference in drug concentrations between local and systemic administration of TMZ was found to be statistically significant at all time points tested. Since systemic toxicity has been cited with orally delivered TMZ, this decrease in serum concentration using locally delivered TMZ may be beneficial.

In vivo efficacy of locally delivered TMZ

Intracranial delivery of TMZ via polymers consistently improved the survival of tumor-bearing animals

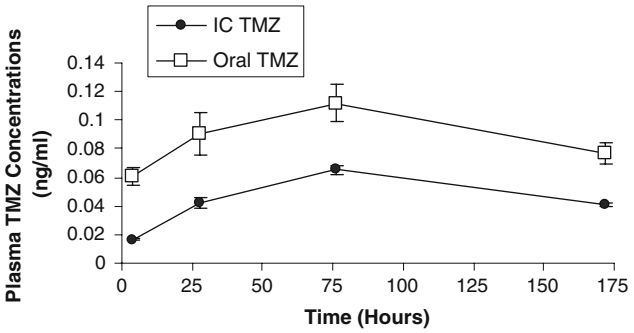

Fig. 3 Serum TMZ levels following intracranial (*IC*) and oral delivery of TMZ. The animals were either implanted with radiolabeled TMZ wafers ($n = 3$) or received a 5-day course of radiolabeled oral TMZ ($n = 3$; 50 mg/kg/day). After killing the animals, serum concentrations were measured and the levels of TMZ were found to be substantially reduced in the locally treated group as compared to the orally treated animals

compared to that of both control animals and animals receiving only oral TMZ, with more than 25% of the animals treated with locally delivered TMZ showing no evidence of tumor burden at the end of the study (Table 1 and Fig. 4). The median survival of animals receiving a single 50% TMZ polymer was 28 days, compared with 22.5 days for animals receiving a 5-day course of oral TMZ ($P < 0.0001$ 58% improvement in prolongation of survival), and 13 days for control ($P < 0.0001$). Survival was further increased with the implantation of two 50% TMZ polymers with a median survival of 92 days ($P < 0.0001$ vs. controls, $P < 0.0001$ vs. oral TMZ, a sevenfold improvement in prolongation of survival). Long-term survival (LTS) was not observed with oral TMZ. In contrast, animals receiving either one or two 50% intracranial TMZ polymers had long-term survival rates of 25 and 37.5%, respectively.

In vivo efficacy of TMZ with radiation therapy

Locally delivered TMZ with radiation therapy significantly improved survival compared to radiation therapy or locally delivered TMZ alone (Table 1 and Fig. 5). All treatment groups had significantly improved survival as compared to controls (Table 1); median survival was as follows: control, 14 days; oral TMZ, 22.5 days; radiation therapy, 24 days; local TMZ, 41 days; combination oral TMZ and radiation therapy, 43 days; combination local TMZ and radiation therapy, >120 days. There were no long-term survivors for those animals receiving oral TMZ. The long-term survival rate was 7.1% for XRT alone, 37.5% for locally delivered TMZ, 38.9% for oral TMZ with XRT and 87.5% for locally delivered TMZ with XRT.

Discussion

Alkylating agents such as TMZ have a clear dose–response for many glioma cell lines in vitro [14–16]. Thus, a more substantial prolongation of survival might be expected if higher dosages of TMZ could be administered over a substantial amount of time. Clinical dose escalation studies, however, demonstrated severe hematologic toxicity, including leukopenia and thrombocytopenia at higher systemic dosages [17–20]. Neurological toxicity, interestingly, has not been

Table 1 Treatment of malignant glioma with locally delivered TMZ with and without radiation therapy

Group	Median survival (days)	Long-term survivors (%)	*P*-values
Expt. 1: Oral delivery compared to local delivery of TMZ			
Control ($n = 19$)	13 (11–18)	0	
50 mg/kg oral TMZ ($n = 18$)	22.5 (16–37)	0	<0.0001 vs. controls
50% TMZ polymer ($n = 16$)	28 (13–120)	25	<0.0001 vs. controls <0.0015 vs. Oral TMZ
2 × 50% TMZ polymer ($n = 8$)	92 (23–120)	37.5	<0.0001 vs. controls <0.0001 vs. oral TMZ
Expt. 2: Oral and local TMZ delivery with/without radiation therapy			
Control ($n = 23$)	14 (12–20)	0	
50 mg/kg oral TMZ ($n = 18$)	22.5 (17–33)	0	<0.0001 vs. controls
XRT (20Gy) ($n = 21$)	24 (18–120)	7.1	<0.0001 vs. controls <0.01612 vs. Oral
50 mg/kg Oral TMZ + XRT ($n = 18$)	43 (36–120)	38.9	<0.0001 vs. Oral TMZ <0.0033 vs. XRT
50% TMZ polymer ($n = 8$)	41 (15–120)	37.5	<0.0001 vs. controls <0.1863 vs. XRT
50% TMZ polymer + XRT ($n = 8$)	Median not reached (55–120)	87.5	<0.0005 vs. XRT <0.0277 vs. Local TMZ

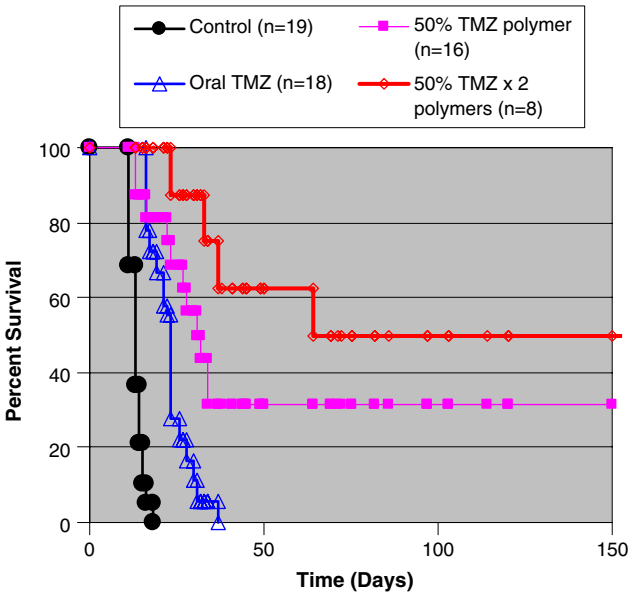

Fig. 4 Survival of F344 rats after treatment with oral or locally delivered TMZ. The animals underwent intracranial implantation of 9L gliosarcoma. Five days following tumor implant, the animals that received no treatment ($n = 19$) had a median survival of 13 days. Those animals that received oral TMZ treatment by gavage ($n = 18$) had a median survival of 22.5 days ($P < 0.0001$ vs. controls). Animals that received one 50% TMZ polymer ($n = 16$) had a median survival of 28 days with 25% of the animals deemed long-term survivors ($P < 0.0001$ vs. controls; $P < 0.0015$ vs. oral TMZ). Animals that received two 50% TMZ polymers ($n = 8$) had a median survival of 92 days with 37.5% of the animals deemed long-term survivors ($P < 0.0001$ vs. controls; $P < 0.0001$ vs. oral TMZ)

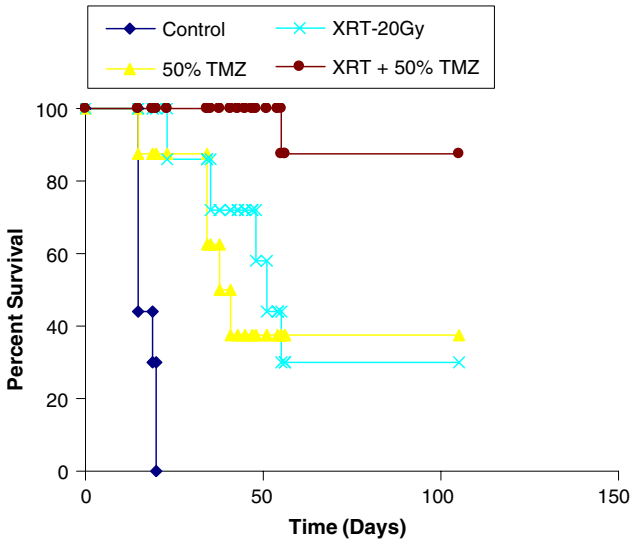

Fig. 5 Survival of F344 rats after treatment with oral or locally delivered TMZ and radiation therapy. The animals underwent intracranial implantation of 9L gliosarcoma. Five days following tumor implant, the animals that received no treatment ($n = 23$) had a median survival of 14 days. Animals that received one 50% TMZ polymer had a median survival of 41 days with 37.5% deemed long-term survivors ($P < 0.0001$ vs. control). Animals undergoing radiation therapy (XRT) (20 Gy) had a median survival of 24 days ($P < 0.0001$ vs. controls). Animals who received a combination of 50% TMZ polymer and XRT did not reach median survival and 87.5% were deemed long-term survivors ($P < 0.0005$ vs. XRT alone; $P < 0.0277$ vs. 50% TMZ polymer alone)

observed as a dose-limiting factor for TMZ [2, 13]. Thus, if intracranial concentrations of TMZ could be increased without increasing systemic exposure, the anti-glioma properties of TMZ could be augmented, and the survival benefit conferred by oral TMZ could be improved.

Consequently, we hypothesized that direct intracranial delivery of TMZ could improve intracranial and intratumoral drug concentrations without a concomitant increase in systemic exposure. Local delivery of TMZ would also lead to improved efficacy in animal glioma models and potentially offer a new therapeutic strategy for treatment of gliomas.

We developed local delivery for TMZ with a biodegradable polymer. TMZ was readily incorporated into CPP:SA polymers, and this formulation released the bioactive drug for several days. In this experimental model, animals treated with the maximal dose of local TMZ that we could insert did not have any overt neurological or systemic toxicity. Based on our experience with BCNU, we would predict that a dose limiting level exists; however, with the constraints of the size of the rodent skull, we did not find one [21]. Local TMZ treatment increased intracranial TMZ concentrations near the site of the tumor compared to the maximally tolerated oral dose of TMZ, while minimizing systemic exposure to TMZ. Furthermore, animals treated with local TMZ did not show any evidence of hematological toxicity that was seen with orally delivered TMZ.

In our experimental brain tumor model, local delivery of TMZ significantly prolonged survival when compared to that in animals treated with oral TMZ and in control animals. More than 25% of animals treated with locally delivered TMZ showed no evidence of tumor burden at the end of the study. Conversely, no animals treated with oral TMZ survived the length of the study. Biodistribution studies demonstrated that the intracranial concentration of TMZ was enhanced as compared to systemic delivery. We attribute this improvement in efficacy to the higher concentration of TMZ.

All patients with malignant gliomas are treated with some form of radiation therapy [22, 23]. Therefore, it is important to determine the interaction of any novel strategies with radiation therapy. We demonstrated that our new strategy of local TMZ delivery with concurrent XRT was safe. We also showed that the

addition of XRT to locally delivered TMZ improved survival significantly as compared to either treatment alone. Median survival was not reached for animals treated with local TMZ and XRT; and seven of eight animals survived through the duration of the study. This striking synergy suggests that this novel system should be studied for the treatment of brain tumors.

Future studies will determine the effectiveness of this TMZ local delivery system when combined with systemic chemotherapy, other local delivery strategies [24] and TMZ resistance modifiers such as O6-benzylguanine [25].

Conclusions

In summary, we have established that it is safe and effective to deliver TMZ in a locally releasing polymer delivery system. In our experimental rodent glioma model, local delivery appears to have advantages over systemic administration. We also showed that locally delivered TMZ with concomitant radiation therapy significantly prolongs survival as compared to either treatment administered alone. Future studies are needed to determine whether combinations of systemic and locally delivered agents may further improve their benefit. Local delivery of TMZ from biodegradable polymer is a potentially new therapy for the treatment of brain tumors.

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