Temozolomide Delivered by Intracerebral Microinfusion is Safe and Efficacious Against Malignant Gliomas in Rats

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ABSTRACT

Intracerebral microinfusion (ICM) is an innovative technique of delivering therapeutic agents throughout large portions of the brain that circumvents the blood-brain barrier, minimizes systemic toxicity, and provides a homogeneous distribution of the infused agent. Temozolomide is a novel methylating agent with proven efficacy against malignant gliomas (MGs) after systemic administration but with dose-limiting myelotoxicity. Because MGs rarely metastasize, systemic drug delivery is unnecessary. Therefore, we evaluated the efficacy and toxicity of ICM with temozolomide in an athymic rat model of human MGs. Treatment of rats by ICM with temozolomide 3 days after intracerebral challenge with D54 human MG xenograft increased median survival by 128% compared with rats treated by ICM with saline, by 113% compared with rats treated with i.p. saline, and by 100% compared with rats treated with i.p. temozolomide (P < 0.001). Delay of treatment until 9 days after tumor challenge still resulted in a 23% increase in median survival in rats treated by ICM of temozolomide compared with rats treated with i.p. temozolomide. In addition, overall, 21.7% of rats treated by ICM with temozolomide survived for >100 days without clinical or histological evidence of tumor. The dose of temozolomide delivered by ICM in this study was limited only by drug solubility, and no neurological or systemic toxicity could be attributed to ICM with temozolomide. Therefore, ICM of temozolomide may offer significant advantages in the treatment of MGs.

INTRODUCTION

Temozolomide (8-carbamoyl-3-methylimidazo [5,1-d]-1,2,3,5-tetrazin-4 (3H)-one) is a novel methylating agent with a mechanism of action similar to that of dacarbazine in that it requires conversion to the active DNA-methylating agent 5-(3-methyl-triazen-1-yl) imidazole-4-carboxamide. In contrast to dacarbazine, which requires metabolic dealkylation (a relatively inefficient process in humans compared with rodents) to form 5-(3-methyl-triazen-1-yl) imidazole-4-carboxamide, temozolomide undergoes spontaneous conversion under physiological conditions. Temozolomide has proven efficacy against MGs after systemic administration but has dose-limiting myelotoxicity (1, 2). Because MGs rarely metastasize, systemic chemotherapy is unnecessary, and direct intratumoral delivery of chemotherapy agents could increase drug efficacy by increasing drug delivery and reducing systemic toxicity. In addition, alkylating agents, like temozolomide, characteristically demonstrate a steep dose-response curve. This predicts that the dose escalation achievable by direct intratumoral administration may produce a marked increase in antitumor activity while minimizing extraneural exposure and toxicity.

ICM is an innovative technique of delivering therapeutic agents directly into brain parenchyma, which circumvents the blood-brain barrier and minimizes systemic toxicity. ICM is capable of achieving concentrations of therapeutic agents within the brain several orders of magnitude greater than that obtainable after systemic delivery (3–5) and provides a more homogeneous distribution of the therapeutic agent than that obtained after drug-impregnated polymer implants (6–8).

In this article, we demonstrate that temozolomide delivered by ICM is capable of increasing the median survival of athymic rats with well-established intracerebral MG xenografts in the absence of any neurological or systemic toxicity. Thus, ICM of temozolomide may offer significant advantages over systemic therapy in the treatment of MGs.

MATERIALS AND METHODS

Xenografts and Tumor Implantation. The well-characterized human MG xenograft D54-MG is the Duke University subline of A-172 (9). s.c. xenografts passed in athymic mice were excised, minced, and disassociated with 0.5% collagenase at room temperature in a trypsinization flask for 2 h. Viable cells were separated on a Ficoll density gradient, washed twice with DPBS, resuspended in 2.5% methylcellulose at a concentration of 1 × 10^7 cells/ml, and injected into the implanted guide cannula through the 33-gauge infusion cannula in a volume of 10 µl by using a 500-µl Hamilton gas-tight syringe and injector (Hamilton Co., Reno, NV).

Received 2/25/00; revised 7/12/00; accepted 7/13/00.

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1 Supported by Grants CA11898 and NS20023 from the NIH, a grant from the American Association of Neurological Surgeons, and by a NIH Neuro-Oncology Research Fellowship.

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3 The abbreviations used are: MG, malignant glioma; DPBS, Dulbecco’s PBS; ICM, intracerebral microinfusion.
Temozolomide Preparations. Standard formulation temozolomide (M, 194) was provided by W. Robert Bishop (Schering-Plow Research Institute, Kenilworth, NJ). Saturated dosing solutions (16 mM) of this formulation were prepared by dissolving the solid drug in sterile DPBS to a final concentration of 3.1 mg/ml with 45 s of sonication at room temperature and neutral pH. The microcrystalline formulation of temozolomide was provided by Sparta Pharmaceuticals, Inc. (Horsham, PA) and prepared by using the phospholipid excipients 1,2-dilauroyl-sn-glycero-3-phosphocholine and 1,2-dimyristoyl-sn-glycero-3-phosphocholine. Saturated dosing solutions (42.5 mM) of this microcrystalline formulation were prepared by dissolving the sterile microcrystalline drug into sterile water to a final concentration of 8.25 mg/ml at room temperature at neutral pH in a sonicating water bath at 37°C for 5 min.

Athymic Rat Cannula Implantation. Athymic male rats were maintained in the Duke University Cancer Center Isolation Facility according to institutional policy. Rats were anesthetized by i.p. injection of a mixture of ketamine (55 mg/ml) and xylazine (9 mg/ml) at a dose of 1 ml/kg and placed into a stereotactic frame (Kopf Instruments, Tujunga, CA). A 26-gauge intracranial guide cannula (Plastics One Inc., Roanoke, VA) was placed 1 mm anterior to bregma and 3 mm to the right of midline into the caudate nucleus 5 mm below the dura and was permanently secured to the calvaria by cranioplastic cement (Plastics One). The cannula system was closed by a cannula stopcock, and the incision was stapled closed. The rats were allowed to recover for a minimum of 7 days, and only rats showing normal weight and neurological function and no evidence of infection were randomized by a random numbers table into experiments.

Xenograft Growth Characteristics. To ascertain xenograft growth characteristics, we performed pathological and histological examinations on rat brains sectioned at the level of the cannula after fixing them in 10% neutral buffered formalin 3, 5, 7, 8, and 9 days after tumor challenge (n = 3 rats/day).

ICM. ICM was performed using an Alzet osmotic pump (ALZA Corp., Palo Alto, CA) implanted s.c. that had been primed for 8–10 h at 37°C in sterile normal saline. The Alzet pump was connected by polyethylene tubing (Becton Dickinson, Sparks, MD) to a 33-gauge infusion cannula, which fit securely in the intracranial guide cannula. Rats underwent a total infusion of 200 μl at 8 μl/h temozolomide, 0.5% Evan’s blue dye, or DPBS for 25 h. Systemic administration was initiated by implantation of the same Alzet pump i.p. for the same time period. Both pumps were explanted on completion of the infusions.

Assessment of Toxicity and Efficacy. Toxicity was monitored by daily weights, daily neurological examinations consisting of stepping and placing reflex and 60-degree incline ramp climbing ability, and histological examination of the brain and systemic organs. Efficacy was assessed by comparing the median survival time between treated and control groups.

Statistical Analysis. Survival estimates and median survivals were determined by using the method of Kaplan and Meier (10). Survival data were compared by using the nonparametric Wilcoxon’s rank-sum test.

RESULTS

Tumor Growth. On histological examination, tumor was consistently evident microscopically three days after tumor challenge (Fig. 1A). Nine days after tumor challenge, tumor was consistently evident macroscopically at gross autopsy (Fig. 1B). These macroscopic tumors were comparable in size, by volume extrapolation, to human gliomas ~4 cm in diameter.

ICM. To examine the distribution characteristics of ICM in our model, we infused 200 μl of 0.5% Evan’s blue dye at 8 μl/h for 25 h. The rats were then killed, and their brains were fixed in 10% neutral buffered formalin. Sections through the brains were made at 3-mm intervals and photographed. The dye was most concentrated around the infusion cannula at the site of tumor implantation and throughout the neighboring caudate nucleus (Fig. 2A). However, after ICM, the dye also crossed to the contralateral hemisphere via the corpus callosum and tracked along white matter tracts (Fig. 2B). Dye was also identified along the cerebral convexities, within the lateral, third, and fourth ventricles and within the cortical subarachnoid space (Fig. 2C).

Toxicity. To define any inherent intracerebral or systemic toxicity of ICM of temozolomide, athymic rats without tumors were infused with 200 μl of the maximal concentrations of the standard formulation of temozolomide (16 mM; n = 10),
the microcrystalline formulation of temozolomide (42.5 mM; 
\( n = 10 \)), or DPBS (\( n = 10 \)) as described above. No weight loss of >10% or neurological deficits were seen during an observation period of 6 weeks after ICM. Furthermore, histological evaluation of the brain at the level of the infusion cannula (\( n = 10 \) rats/group) demonstrated only focal gliosis surrounding the cannula tract, likely resulting from the implantation of the intracranial guide cannula in all treatment groups including control groups treated by ICM with DPBS alone. No evidence of hemorrhage, necrosis, edema, or demyelination was identified in the brain after treatment with either formulation of temozolomide.

Detailed histological examination of the spleen, kidney, lung, heart, and sternum bone marrow by an observer blinded to the treatment group failed to reveal any significant abnormalities in rats treated with ICM of the standard or the microcrystalline formulation of temozolomide (Table 1). Mild hepatic centrilobular edema consistent with reversible hepatic toxicity was identified in 20% (2 of 10) of rats receiving standard formulation temozolomide and in 10% (1 of 10) of rats receiving microcrystalline temozolomide but was also seen in 10% (1 of 10) of control rats that received ICM of DPBS. One of 10 rats that received ICM of the standard formulation of temozolomide was found to have a mononuclear infiltrate in the liver that could be attributed to a low-grade systemic subclinical infection based on transient weight loss. No myelosuppression was noted in any animal.

**Efficacy.** To determine whether ICM of temozolomide offered an advantage over systemic therapy with temozolomide in the context of microscopic disease, athymic rats were treated with temozolomide 3 days after intracerebral challenge with D54-MG xenograft. Treatment consisted of 200 \( \mu l \) of i.p. DPBS, i.p. maximally concentrated (16 mM) standard formulation temozolomide, or ICM of an equal dose of standard formulation temozolomide. Each solution was delivered at a rate of 8 \( \mu l/h \) over 25 h. The median survival of rats treated i.p. with DPBS (\( n = 7 \)) was 15 days (Fig. 3A). Rats receiving standard formulation temozolomide i.p. (\( n = 9 \)) did have an increase in median survival to 16 days, but this was not significantly different from the rats treated i.p. with DPBS (\( P > 0.087 \)). In contrast, ICM of the standard temozolomide formulation (\( n = 8 \)) increased median survival to 32 days (\( P < 0.001 \)), resulting in a 100% increase in median survival compared with i.p. treatment with standard formulation temozolomide. In addition, the microcrystalline formulation of temozolomide (42.5 mM; 
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whereas all rats treated with DPBS or temozolomide i.p. succumbed to tumor, with ICM of standard formulation temozolomide, 25% (two of eight) of rats survived for 100 days without clinical or histological evidence of tumor.

To exclude a confounding effect of the ICM alone, athymic rats were treated 3 days after intracerebral challenge with D54-MG by ICM with DPBS, maximally concentrated standard formulation temozolomide, or maximally concentrated microcrystalline formulation temozolomide as described above. In this experiment, the median survival of rats (n = 7) treated by ICM with DPBS was 18 days (Fig. 3B). In contrast, ICM with standard formulation temozolomide (16 mM; n = 7), or ICM microcrystalline temozolomide (42.5 mM; n = 8) infused for 25 h at a rate of 8 μl/h 3 days after tumor challenge. Median survival times were 18, 41, and 37 days, respectively. ICM of standard formulation and microcrystalline formulation temozolomide significantly increased median survival compared with ICM of DPBS (P < 0.001). There was no significant difference in the median survival resulting from ICM with the two different formulations of temozolomide (P = 0.347).

Because there was no significant difference of in vivo efficacy between the two formulations delivered by ICM on macroscopic tumors, we therefore evaluated whether treatment of macroscopic tumor by ICM with the more soluble microcrystalline formulation temozolomide could also be efficacious. The median survival of rats treated with i.p. microcrystalline temozolomide (n = 10) in this context was 17.5 days. In contrast, although therapy was delayed to a time point midway between tumor challenge and death, treatment by ICM with microcrystalline temozolomide (n = 8) increased median survival 23% to 21.5 days compared with rats treated i.p. with temozolomide (P < 0.001; Fig. 4). Again, 25% (two of eight) of rats treated by ICM with temozolomide survived for >100 days without clinical or histological evidence of tumor.

Fig. 3 ICM of temozolomide is efficacious against microscopic human MG xenografts. Infusion cannulas were implanted in the caudate nuclei of athymic rat brains, and rats were challenged with 1 × 10^5 cells obtained from dissociated D54-MG xenograft. A, treatment consisted of i.p. DPBS (n = 7), i.p. temozolomide (16 mM; n = 9), or ICM of temozolomide (16 mM; n = 8) infused for 25 h at a rate of 8 μl/h 3 days after tumor challenge. Median survival times were 15, 16, and 32 days, respectively. ICM of temozolomide significantly increased median survival compared with i.p. DPBS or i.p. temozolomide (P < 0.001). ICM of temozolomide did not significantly increase median survival compared with i.p. DPBS (P > 0.087). B, treatment consisted of ICM DPBS (n = 7), ICM standard formulation temozolomide (16 mM; n = 7), or ICM microcrystalline temozolomide (42.5 mM; n = 8) infused for 25 h at a rate of 8 μl/h 3 days after tumor challenge. Median survival times were 18, 41, and 37 days, respectively. ICM of standard formulation and microcrystalline formulation temozolomide significantly increased median survival compared with ICM of DPBS (P < 0.001). There was no significant difference in the median survival resulting from ICM with the two different formulations of temozolomide (P = 0.347).
DISCUSSION

MGs are almost always uniformly lethal, despite aggressive surgical resection and incapacitating radiation therapy. Chemotherapy has been limited by systemic drug-induced toxicity and by the restrictions the capillary barrier of the cerebral vasculature places on drug delivery. Because MGs are locally aggressive neoplasms that rarely metastasize, systemic delivery of chemotherapy may be unnecessary; therefore, techniques to deliver therapeutic agents directly to the brain have been investigated. Whereas direct implantation of carmustine-impregnated polymers has reduced systemic toxicity, the increase in efficacy with this approach has been modest (11, 12). The limitations of such an approach may be explained by the steep concentration gradient that radiates from the point of drug delivery (6–8), which may result in subtherapeutic drug concentrations at the advancing tumor border. In contrast, ICM results in a more homogeneous distribution of drug (3, 4). As evidenced by the dye distribution and therapeutic efficacy data reported here, ICM is apparently capable of completely encompassing relatively large experimental tumors with therapeutic concentrations of drug. The macroscopic tumors treated in the last set of experiments are comparable, by a volumetric extrapolation, to human gliomas ~4 cm in diameter. Failure of ICM of temozolomide to fully eradicate all of the macroscopic tumors may be related to the development of drug resistance in the larger tumors; inhomogeneous delivery of the drug at a microscopic level, allowing sufficient cells to escape cytotoxic levels of temozolomide; or macroscopic mismatches between tumor growth and drug distribution using this technique. Some of these same factors may also explain the complete resistance of intracranial tumors to an equivalent dose of temozolomide delivered i.p., despite demonstrations by previous studies that D54 is sensitive to temozolomide in other model systems (2).

Recent clinical studies have demonstrated that ICM is capable of distributing consistently high concentrations of even very large therapeutic constructs radiating several centimeters from a single source (13), suggesting that drug distribution is not a limiting factor of this technique. An advantage of ICM for intracerebral tumors is that drug distribution appears to be directed preferentially along common sites of tumor dissemination such as the white matter tracts including the corpus callosum. This was evident from our studies, but it has also been demonstrated by others (3, 4).

Perhaps the most significant advantage of ICM for the delivery of chemotherapy, however, is that it bypasses almost all organs with rapidly dividing cell populations. This is important because these organs are usually responsible for the dose-limiting toxicity of chemotherapeutic agents. Because the brain is relatively senescent with regard to cell division, ICM of most chemotherapeutic agents may be predicted to be relatively nontoxic and may provide for significant dose escalation relative to that achievable after systemic delivery. This was certainly the case in the experiments presented here, where no dose-limiting toxicity for ICM of temozolomide could be practically identified. On the other hand, the neurological effects of ICM may become evident only with more sophisticated neuropsychological testing. Still, the dramatic efficacy of ICM of temozolomide in the complete absence of neurological and systemic toxicity is encouraging and warrants further investigation.

Preclinical studies have demonstrated that temozolomide has significant dose-related antiproliferative activity in vitro at concentrations ranging from ~0.0001 to ~0.3 mM (14, 15). Similarly, clinical studies have shown that plasma levels of temozolomide peak at ~0.05 mM after systemic administration and that plasma concentrations ranging from 0.0001–0.01 mM are maintained for about 12 h (16). These data suggest that a very narrow therapeutic window exists for the treatment of primary or metastatic intracerebral tumors with systemic administration of temozolomide. In our experiments, 200 µl of temozolomide were administered at a concentration of 16 or 42.5 mM for the standard and microcrystalline formulations, respectively, without toxicity. Thus, even with use of poorly soluble standard formulation temozolomide, 0.62 mg of temozolomide was safely delivered intracerebrally in the rat. A corresponding human dose (corrected for body surface area) would be ~17.7 mg. If 10% of this total dose were used as a starting dose for an ICM clinical trial, where volumes as large as 40 ml have routinely been used to completely encompass a tumor, the initial infused

Fig. 4 ICM of temozolomide is efficacious against macroscopic human MG xenografts. Infusion cannulas were implanted in the caudate nuclei of athymic rat brains, and rats were challenged with 1 × 10⁷ cells obtained from dissociated D54-MG xenograft. Treatment consisted of i.p. microcrystalline temozolomide (42.5 mM; n = 10) or ICM of microcrystalline temozolomide (42.5 mM; n = 8) infused for 25 h at a rate of 8 µl/h 9 days after tumor challenge. Median survival times were 17.5 and 21.5 days, respectively. ICM of temozolomide significantly increased median survival compared with i.p. temozolomide in these well-established tumors (P < 0.001).
drug concentration would be 0.228 mM. Our preclinical studies indicate that this would be a very safe concentration for ICM with temozolomide, would match well the drug concentrations effective in vitro, and almost certainly would greatly exceed the peak drug levels obtained after toxicity-limited systemic delivery. These studies suggest that ICM of temozolomide may have a very favorable therapeutic window and should be evaluated in a Phase I clinical trial. In a recent clinical trial, ICM of an immunotoxin construct produced significant tumor responses without systemic toxicity in patients with malignant brain tumors refractory to conventional therapy (13). This study and our own suggest that ICM is a technique that should be explored for the delivery of a variety of therapeutic agents targeted to the brain that have previously been limited by systemic toxicity or poor penetration into the central nervous system.

ACKNOWLEDGMENTS

We acknowledge the expert technical assistance provided by and Tracy Chewing, Eddie Hanson, Ali Ibrahimiyi, and Steve Keir and the editorial assistance of Janet Parsons.

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