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Effect of Interstitial and/or Systemic Delivery of Tirapazamine on the Radiosensitivity of Human Glioblastoma Multiforme in Nude Mice

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SUMMARY The purpose of this study was to investigate the feasibility and the efficacy of administering tirapazamine by a slow-releasing polymer disc that was implanted interstitially into a U251 (human glioblastoma multiforme) tumor grown in nude mice. Tumor-bearing animals, with a tumor nodule 0.8 cm³ in size, were distributed to groups receiving combinations of empty or drug-containing polymer implants in the tumor or contralateral leg, intraperitoneal (i.p.) drug, and/or irradiation. The drug (i.p.) alone (14 mg/kg ×6) or in combination with tumor drug implant (2 mg) did not significantly increase the tumor volume doubling time compared to that of control animals. Given with 12 Gy of irradiation in twice a day 2-Gy fractions, combined i.p. drug and tumor drug implant significantly delayed tumor growth compared to irradiation alone, which was not achieved with either drug treatment alone added to irradiation. Toxicity, as manifested by transient weight loss, was primarily seen in animals receiving radiation and i.p. tirapazamine. These results indicated that a slow-releasing tirapazamine disc can be produced and the addition of an interstitially implanted tirapazamine disc further increased the effectiveness of i.p. tirapazamine. *Radiat. Oncol. Invest. 6:63–70, 1998.*

Key words: tirapazamine (SR 4233); glioblastoma multiforme; hypoxic cell cytotoxin; implantable polymer disc

INTRODUCTION

Postoperative radiation therapy improves median survival of the patients with malignant glioma, and the addition of chemotherapy with nitrosoureas adds a modest gain for selected patients [1,2]. The benefit of radiotherapy, however, is limited by several factors. Although intrinsic radioresistance, rapid cellular proliferation, and normal tissue tolerance may contribute to the lack of therapeutic efficacy [3], tumor hypoxia is still one of the major factors causing radioresistance. Oxygen measurements document regional hypoxia in a high percentage of patients [4–6]. Thus, hypoxia may limit response to radiotherapy, as has been demonstrated for head and neck tumors [7], and a treatment that eradicates radioresistant hypoxic tumor cells should improve the efficacy of radiation therapy.

When given as multiple injections in conjunction with fractionated irradiation, tirapazamine (SR 4233), a bioreductive agent that preferentially kills hypoxic cells, increases tumor cell kill while sparing normal tissues in mouse SCCVII and other tumors [8,9]. Brown and colleagues have considered the possibility that tumor hypoxia may actually be therapeutically advantageous if fractionated radio-

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therapy were combined with a hypoxic cytotoxin such as tirapazamine [10,11].

It may be beneficial to deliver tirapazamine intratumorally in order to avoid systemic toxicity, maximize exposure directly to the tumor, and target hypoxic regions. Intratumoral implantation of a drug-containing disc made of synthetic biodegradable polyanhydride polymers allows sustained release of drugs, and provides an alternative, or adjunct, to systemic administration [12–14]. The benefit of such an approach includes predictable biodegradation, continuous release of drug, and absorption by the body after implantation, rendering removal unnecessary [12–14]. We have explored the feasibility and efficacy of administering tirapazamine by intratumoral implantation of a slow-releasing disc made of biodegradable polymers on human glioblastoma multiforme xenografts grown in nude mice.

METHODS AND MATERIALS

Tirapazamine Disc

Carboxyphenoxypropane monomer and prepolymer, sebacic acid (SA) prepolymer, and poly(bis(p-carboxyphenoxy)-propane (PCPP):SA) polymer were prepared by a melt polycondensation process (PCPP:SA ratio = 20:80). As previously described, the prepolymers were refluxed with acetic acid anhydride, recrystallized from dry toluene, immersed in dry petroleum ether to extract acetic acid and toluene, and placed under high vacuum to allow polymerization [12,15,16]. The acetic anhydride produced by the polymerization process was removed under vacuum. The polymer was allowed to solidify at room temperature and then was dissolved in methylene chloride with petroleum ether and hexane. The precipitate was washed three times with diethyl ether to remove residual acetic acid anhydride and was then dried under vacuum. To prepare drug-containing discs, 2 mg tirapazamine and 8 mg PCPP:SA polymer were combined in methylene chloride (10% wt/wt). The solvent was removed by desiccation under vacuum. The tirapazamine-containing polymers were then pressed into 10-mg discs measuring 3 mm in diameter and 1 mm in height.

Release Kinetics of Tirapazamine Disc In Vitro

Tirapazamine discs were incubated for known intervals in 1 ml of 0.1 M phosphate-buffered saline (PBS) at 37°C. The PBS was periodically removed and replaced with fresh saline. Tirapazamine concentrations released into PBS were measured by spectrophotometry (Beckman model DU-65, Beckman Instruments, Fullerton, CA) at a wavelength of 515 nm. The percentage of loaded tirapazamine that accumulated in the supernatant was plotted with respect to time.

U251 Tumor Model

The human GBM cell line (U251) used in this study was obtained from the DCT Tumor Repository, National Cancer Institute, Frederick, MD. Cells were cultured in Dulbecco's MEM/Ham's F-12 nutrient mixture with 10% fetal bovine serum (GIBCO, Gaithersburg, MD) and antibiotics (penicillin and streptomycin) in 12 × 80 mm plastic culture dishes or T-25 culture flasks. Cells were incubated (37°C) in a mixture of 5% carbon dioxide and 95% air. Media were changed twice weekly and cells were passaged at confluence with 0.5% trypsin. Six-week-old male Balb/c athymic nude mice received subcutaneous injections of 4 or 5×10^6 U251 cells in the left posterior extremity. Tumors were visible after 6 to 8 days. Tumor sizes ranged from 0.5–1.2 cm^3 at the start of treatment. Mice were assigned to treatment groups so that each group had a mean tumor volume of approximately 0.8 cm^3. Based on our previous experience on the variability in tumor growth response of this tumor system [17], 5 animals were assigned to groups comparing tirapazamine treatments and fewer to the control groups. Mice were sacrificed after tumors reached at least twice their original tumor volume.

Systemic Administration of Tirapazamine

The drug was supplied by Sanofi Winthrop, Collegeville, PA. Tirapazamine was dissolved in physiological saline at a concentration of 0.71 mg/ml, and intraperitoneal (i.p.) injections were given in a volume of 0.02 ml/g body weight (0.08 mmol/kg) either daily ×6 or twice a day for 3 days. When combined with radiation treatments, i.p. injections were given 5–20 min prior to 2-Gy irradiation.

Implantation of Tirapazamine Discs

For surgical implantation of tirapazamine discs, animals were anesthetized with methoxyflurane, a skin incision was made next to the xenograft, and an empty disc or tirapazamine disc was inserted in the center of the tumor via a single 3–4-mm incision. The incision was closed using wound clips. Contralateral flank discs were placed subcutaneously by the same technique.

In Vivo Irradiation

A Mark I irradiator (Model 68, J.L. Shepherd and Assoc., San Fernando, CA) was used for in vivo

irradiation. The dose to the xenograft was calibrated by TLD dosimetry and confirmed with radiochromic dye medium (Gafchromic) mounted in polystyrene mouse phantoms [18]. The tumor-bearing flank was selectively irradiated, with shielding of the mouse trunk and head. In the experiment with i.p. injections alone, 2 Gy was given daily for 6 fractions; for the experiment with polymer implants, 2 Gy was given twice daily for 6 fractions starting 1 day after polymer implant.

Tumor Volumetrics

Tumor length (L), width (W), and height (H) were measured twice weekly and tumor volumes were calculated as pi/6 × L × W × H. The logarithm of the ratio of this product (V) to the initial product (V0) for each animal was calculated and the mean logarithm of this ratio for each treatment group was plotted against time. The time of regrowth to twice the original tumor volume (V/V0 = 2; log V/V0 = 0.3) for each animal and treatment group was calculated from the plot of log V/V0 vs. time. Mean tumor volume doubling time (VDT) was used as the endpoint to determine the effect of a given treatment arm on U251 xenograft tumors. Each animal was weighed bi-weekly to assess possible toxicity to treatment.

Statistical Evaluation

Data show the mean ± standard error of the mean (SEM). Outcomes among experimental groups were compared by the two-tailed Student's *t*-test [19].

RESULTS

In Vitro Release Kinetics

Figure 1 shows the percentage of the drug released from discs containing 20% (2 mg) tirapazamine. The rate of release of the drug was highest initially. The percentage of the drug released was 8% after 1 day and 11% after 3 days with a continuing steady-state release thereafter. These release data are predictive of but not identical to in vivo kinetics [20].

Systemic Administration of Tirapazamine

As shown in Table 1 and Figure 2, drug alone had no effect on mean VDT. For animal xenografts receiving saline (control) or daily tirapazamine alone, tumors had a mean VDT of 10 and 12 days, respectively ($P = 0.6$). Fractionated radiation plus saline injections produced a significant growth delay (VDT = 22 days, $P = 0.012$) and this radiation effect was further enhanced with the addition of i.p. injections of tirapazamine (VDT = 35 days, $P = 0.012$).

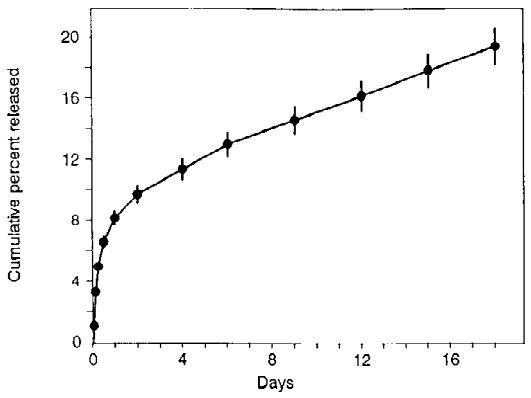

Fig. 1. Cumulative percentage release of the drug from tirapazamine discs as a function of time. Values are the mean for three discs; error bars are plus or minus the standard errors.

Table 1. Response of U251 Human Glioma to I.P. Administration of Tirapazamine With or Without Radiation*

		Assigned treatment		
Group	No. of animals	Radiation, 2 Gy (daily × 6)	Tirapazamine (i.p.; daily × 6)	VDT (days)[a]
1	3	No	No	10 ± 0
2	4	No	Yes	12 ± 0.6
3	5	Yes	No	22 ± 2.5
4	5	Yes	Yes	35 ± 2.5

*Student's *t*-test: Effect of irradiation: group 1 vs. 3, $P = 0.012$; 2 vs. 4, $P < 0.001$. Effect of i.p. tirapazamine: group 1 vs. 2, $P = 0.6$; 3 vs. 4, $P = 0.012$.
[a]Mean xenograft tumor volume doubling time ± SEM.

Intratumoral Implantation of Tirapazamine Discs

Radiation and/or i.p. treatments began 1 day following implantation of the discs. Irradiation was given twice per day to take advantage of the initial burst of tirapazamine released from the disc. In order to distinguish a systemic effect of the drug released from a tirapazamine disc from a true local effect, multiple groups were designed, including a group with tirapazamine discs placed in the opposite flank. Table 2 and Figure 3 show the assigned treatment groups, mean VDT of each group, and *P* values for various comparisons.

The combination of intratumoral tirapazamine disc plus i.p. tirapazamine added to irradiation (group 7) produced a large growth delay (16.5 days, $P < 0.001$) as compared to irradiation alone. This was much larger than the growth delay resulting from intratumoral tirapazamine disc implant plus i.p. tirapazamine without radiation (group 2)

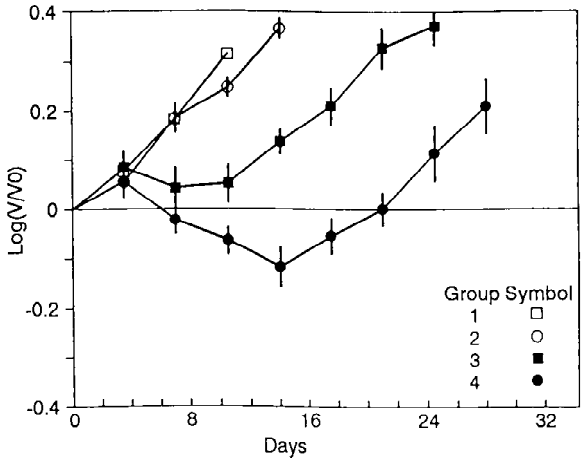

Fig. 2. Response of U251 human glioma to i.p. administration of tirapazamine with or without radiation. Mean $\log_{10}$ tumor volume/initial tumor volume was plotted as a function of time from the beginning of treatment for each group. Table 1 lists the treatments for each group. Error bars are plus or minus the standard errors. Data are shown for each group up to the time at which the first animal in the group was sacrificed.

(3.5 days, $P = 0.15$) compared to intratumoral empty disc implant (group 1). Group 7 also had a significant growth delay when compared to i.p. tirapazamine alone plus irradiation (group 6) (11 days, $P = 0.016$) or intratumoral tirapazamine disc implant alone plus irradiation (group 5) (12.8 days, $P = 0.004$). Thus, both the intratumoral tirapazamine disc implant and i.p. tirapazamine injection appear to be contributing to the effectiveness of the combination with irradiation.

Growth delays produced by intratumoral tirapazamine disc or imp. tirapazamine injection when combined with irradiation did not differ significantly. The growth delay for intratumoral implantation of tirapazamine disc plus irradiation (group 5) compared to intratumoral implantation of empty disc plus irradiation (group 3) was 3.7 days ($P = 0.24$), and compared to intratumoral empty disc implant plus contralateral tirapazamine disc implant with irradiation was 6.6 days ($P = 0.055$). For intratumoral empty disc implant plus i.p. tirapazamine injection with irradiation (group 6) compared to intratumoral empty disc implant plus irradiation, the delay was 5.5 days ($P = 0.15$). Of note, the addition of i.p. tirapazamine to irradiation appeared to be less effective than in the previous experiment. Several factors might contribute to these differences, including the different irradiation schedule (twice daily rather than daily), which might not allow enough time for rehypoxiation between fractions (see Discussion) and the effect of surgical procedure for disc implantation, which may cause tumor swelling and affect uptake of systemic drug.

Toxicity

Figure 3 also shows the minimum animal weights post-treatment expressed as a fraction of the pretreatment weight for each group in Table 2. Transient weight loss occurred in all treatment groups as compared to group 1, which only received intratumoral implantation of an empty disc. The results indicate that irradiation or the combination of i.p. tirapazamine and intratumoral implantation of tirapazamine disc produced systemic toxicity and that for the latter, toxicity appeared to be primarily associated with the i.p. treatments. The weight losses were transient, with all groups exceeding their pretreatment weights before they were sacrificed.

DISCUSSION

Tirapazamine (also designated SR 4233, WIN 59075, or 1,2,4-benzotriazine 1,4-di-oxide) is a bioreductive agent, which, by undergoing an enzymatic one-electron reduction, forms cytotoxic free radicals that cause DNA strand breaks and cell death; hypoxia elevates the level of these radicals due to the slower rate of ''back-oxidation'' to the parent compound [21]. In vitro studies show that tirapazamine is 40–150 times more toxic to cells exposed to drug under hypoxic than under oxygenated conditions [22,23].

Evidence supporting the existence and significance of tumor hypoxia is strong. Thomlinson and Gray reported that histological specimens of human lung cancers contain zones of necrosis, consistent with an estimated oxygen diffusion distance of 150 μm from capillaries, and beyond that distance tumor cells were hypoxic and necrotic [24]. Gatenby et al. [7] used CT-guided needle electrodes to measure oxygen distribution in 31 neck masses in patients with head and neck squamous cell carcinoma. They found a near zero oxygen concentration in some tumors and this was associated with poor outcome after RT. Hockel et al. measured the intratumoral oxygen concentration with needle electrodes in 33 patients who had carcinoma of the uterine cervix treated with radiotherapy ± chemotherapy. Their results showed a significantly lower survival and recurrence-free survival for patients with a median oxygen concentration of ⩽10 mm Hg compared to those with a median oxygen concentration >10 mm Hg [25].

Nitroimidazole hypoxic cell sensitizers have been studied in clinical trials for the past 20 years, and with few exceptions, the results are either negative or inconclusive [26–28]. The factors that limit

Table 2. Response of U251 Human Glioma to Intratumoral Implantation of a Slow-Releasing Tirapazamine Disc ± I.P. Administration of Tirapazamine*

Group	Number of animals	Assigned treatment			VDT[b]
		Irradiation, 2 Gy (bid × 3 days)	Tirapazamine disc implant[a]	I.p. Tirapazamine (i.p.; bid × 3 days)	
1	4	No	Tumor, empty	No	7 ± 1.96
2	4	No	Tumor, drug	Yes	10.5 ± .87
3	4	Yes	Tumor, empty	No	19.3 ± .76
4	5	Yes	Contralateral flank, drug and tumor, empty	No	16.4 ± 1.57
5	5	Yes	Tumor, drug	No	23.0 ± 2.49
6	5	Yes	Tumor, empty	Yes	24.8 ± 3.0
7	5	Yes	Tumor, drug	Yes	35.8 ± 2.0

*Student's *t*-test: Effect of irradiation: group 1 vs. 3, $P = 0.001$; 2 vs. 7, $P < 0.001$. Effect of i.p. tirapazamine: group 3 vs. 6, $P = 0.15$; 5 vs. 7, $P = 0.004$. Effect of tirapazamine disc: group 3 vs. 5, $P = 0.24$; 6 vs. 7, $P = 0.016$; 4 vs. 5, $P = 0.055$. Effect of i.p. tirapazamine plus tirapazamine disc: group 1 vs. 2, $P = 0.15$; 3 vs. 7, $P < 0.001$.

[a]Site of implant, drug = tirapazamine.

[b]Mean xenograft tumor volume doubling time ± SEM.

the effectiveness of hypoxic cell sensitizers include inherently small enhancement ratios, limited dosing due to systemic toxicities, the fact that only a fraction of patients contain tumor hypoxia, and that reoxygenation occurs during fractionated RT [26–28].

Several observations lead us to believe that patients with GBM represent an appropriate group for combined tirapazamine and radiation therapy. Tumor hypoxia in GBM has been demonstrated by polarographic oxygen electrode measurements and by positron emission tomography with [F-18]fluoromisonidazole [5,6]. Rampling et al. also reported the presence of bioreductive enzymes for activation of tirapazamine [5].

In the studies reported here, i.p. injected tirapazamine alone had no effect on the mean VDT. This is not surprising, since any (chronically) hypoxic cells killed by tirapazamine would have died eventually; such hypoxia is only a factor because such cells can become reoxygenated after radiation treatment. However, tirapazamine had a significant effect when combined with irradiation. The mean VDT was increased from 12 days for fractionated irradiation (2 Gy × 6) to 25 days for fractionated irradiation plus the drug. This result is consistent with reports by Brown and Lemmon [8] and by Dorie and Brown [29], in that a significant enhancement of fractionated radiation was produced by tirapazamine in different mouse tumors [8,29].

A study by Kim and Brown using mouse squamous cell SCCVII tumors has demonstrated that within 1 hour of injecting tirapazamine, the hypoxic fraction fell to 0.57% (about 7% of pretreatment levels) and returned to pretreatment levels 3 to 5 h later [30]. This rehypoxiation phenomenon led us to explore the feasibility and the efficacy of administering tirapazamine by controlled release polymer implants. Brem et al. first reported the safety and efficacy of using BCNU-containing polymer implants for treating patients with recurrent gliomas [31]. They also demonstrated that this approach was safe for treating newly diagnosed malignant glioma [32]. Implantable slow-releasing biodegradable disc-containing chemotherapeutic agents (carboplatin, BCNU, camptothecin, cytoxan, and taxol) have been successfully produced, and the safety and efficacy were established in animal models [33–36].

The technology of making biodegradable polymer discs is readily available [14–16,33,36]. We experienced no difficulties in producing tirapazamine-containing discs. Intratumoral implantation of a tirapazamine disc has the advantage of maintaining a local antitumor effect while avoiding systemic toxicity. But there could be even more reasons. Recently Lin and Ho have reported that tirapazamine forms a complex with copper and the complex exhibits 2- to >10-fold greater cytotoxicity than metal-free tirapazamine [37]. Copper tends to accumulate more in tumor than in normal tissues. The distribution ratio of copper between nucleus and cytoplasm is greater for tumor than for normal cells [38]. These could lead to a synergistic effect when tirapazamine was administered intratumorally.

In this study, a polymer disc containing 2 mg of tirapazamine steadily released the drug, in vitro, for up to several weeks. Incorporation of drug into tumor-implanted disks resulted in longer, but not

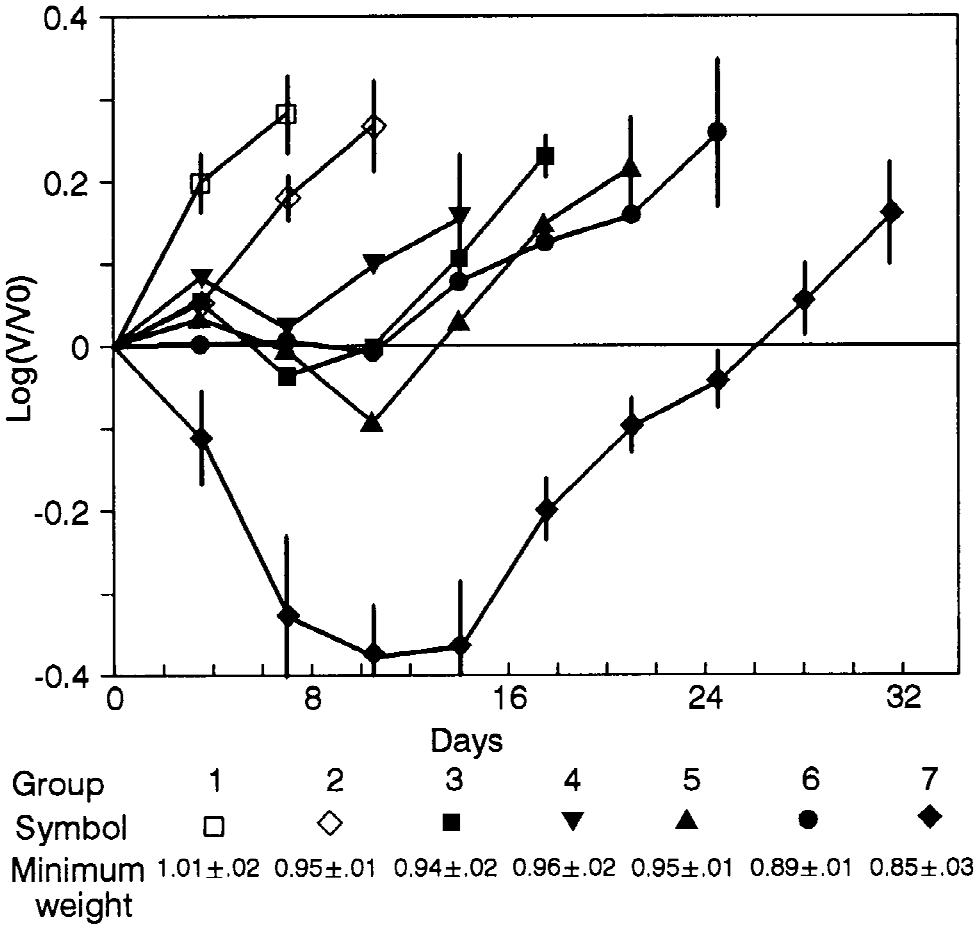

Fig. 3. Response of U251 human glioma to intratumoral implantation of a slow-releasing tirapazamine disc in various combinations with i.p. tirapazamine and radiation. Mean $\log_{10}$ tumor volume/initial tumor volume was plotted as a function of time from the beginning of treatment for each group. Table 2 lists the treatments for each group. Error bars are plus or minus the standard errors. Error bars overlapped for groups 3 through 6; therefore, for clarity, error bars were omitted except for the last point for each of these groups. Data are shown for each group up to the time at which the first animal in the group was sacrificed. Also shown for each treatment group is the minimum post-treatment weight expressed as the fraction of the pretreatment weight.

statistically significant, delays compared to empty tumor disks with no radiation ($P = 0.15$) or with radiation ($P = 0.24$) or with drug with drug disc on the contralateral leg with radiation ($P = 0.055$); a significant effect was observed ($P = 0.016$) when tumor drug disc was added to i.p. drug plus radiation. Our data do not allow us to say whether the contribution of the tumor drug disc in the last case was necessarily from released drug diffusing through the tumor rather than a drug whose prolonged systematic release complimented the ''pulses'' of drug from the i.p. injections.

Injections (i.p.) had a smaller and less significant effect in combination with radiation in the second experiment than in the first. A possible explanation is that the time between fractions (approximately 6 hr) for the twice-a-day fractions in the second experiment did not allow enough time for as much rehypoxiation (and thus resensitization to tirapazamine) as the once-a-day fractions used in the first experiment.

Our experimental results indicate a slow-releasing tirapazamine disc can be produced. Intratumoral implantation of a tirapazamine disc enhances the antitumor effect of fractionated irradiation on a human GBM xenograft. The treatment is well tolerated by animals. We suggest this combined approach should be considered in the design of clinical trials for patients with malignant gliomas.

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