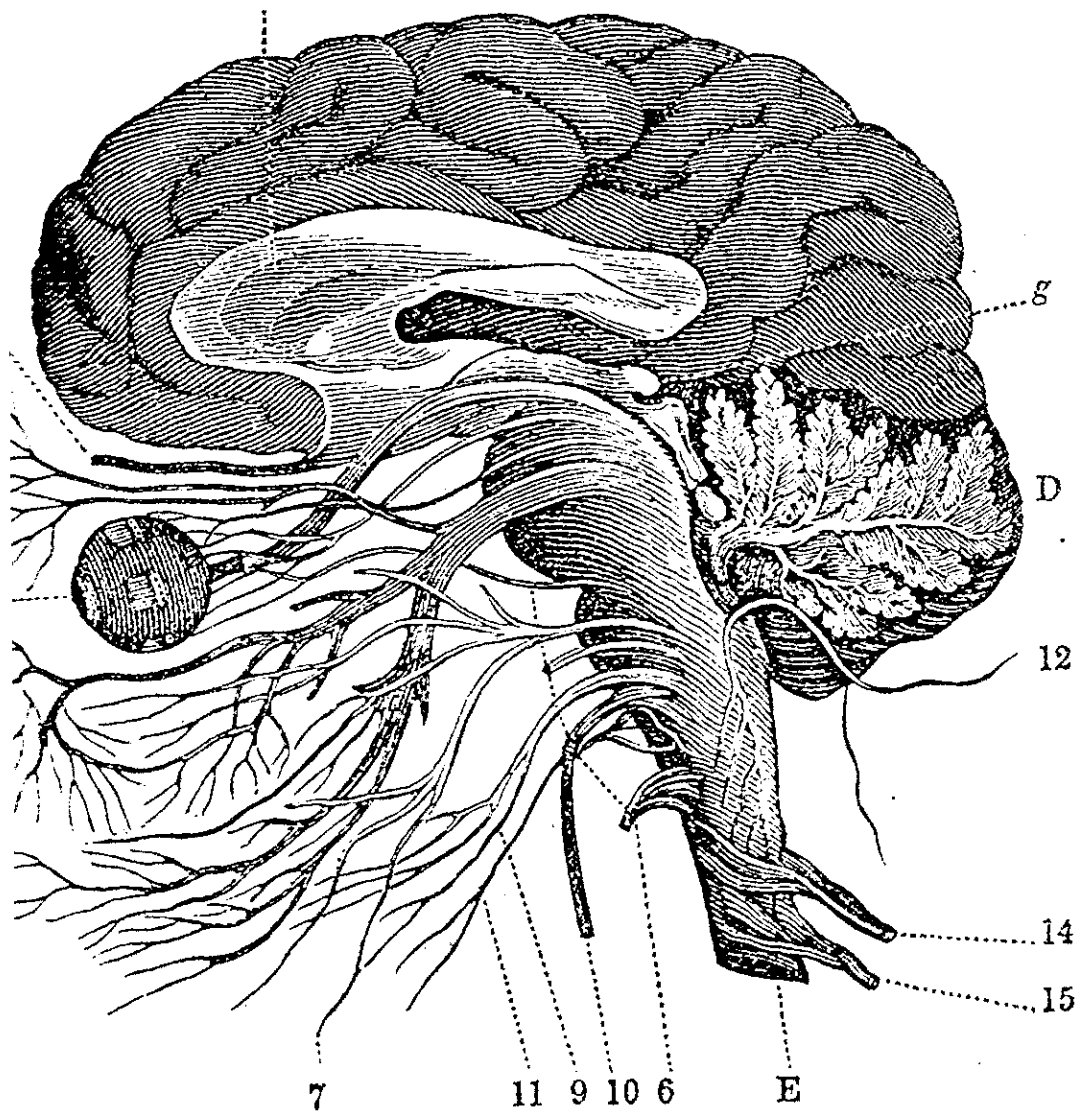


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Use of trans sodium crocetinate for sensitizing glioblastoma multiforme to radiation

Laboratory investigation

JASON SHEEHAN, M.D., PH.D., ADINA IONESCU, M.D., NADER POURATIAN, M.D., PH.D.,
D. KOJO HAMILTON, M.D., DAVID SCHLESINGER, PH.D., ROD J. OSKOUJIAN JR., M.D.,
AND CHARLES SANSUR, M.D.

Department of Neurological Surgery, University of Virginia Health System, Charlottesville, Virginia

Object. Adjuvant treatment with radiation (radiation therapy or radiosurgery) is a mainstay of treatment for patients harboring glioblastomas multiforme (GBM). Hypoxic regions within the tumor make cells less sensitive to radiation therapy. Trans sodium crocetinate (TSC) has been shown to increase oxygen diffusion in the brain and elevate the partial brain oxygen level. The goal of this study was to evaluate the radiosensitizing effects of TSC on GBM tumors.

Methods. A rat C6 glioma model was used, in which C6 glioma cells were stereotactically injected into the rat brain to create a tumor. Following creation of a right frontal tumor, animals were randomized into 1 of 4 groups: 1) TSC alone (animal treated with moderate-dose TSC only); 2) radiation (animals receiving 8 Gy of cranial radiation); 3) radiation and low-dose TSC (animals receiving 8 Gy of radiation and 50 µg/kg of TSC); or 4) radiation and moderate-dose TSC (animals receiving 8 Gy of radiation and 100 µg/kg of TSC). Animals were observed clinically for 60 days or until death. Magnetic resonance (MR) imaging was performed at 2-week intervals on each animal and quantitatively evaluated for tumor response. Immunohistochemical analysis was performed on all brain tumors. Survival differences were also evaluated using the Kaplan–Meier method.

Results. On MR imaging, a statistically significant reduction in tumor size was seen in the group receiving moderate-dose TSC and radiation treatment compared with the group receiving radiation treatment alone. The rate of tumor growth was significantly less for the combination of TSC and radiation treatment compared with either modality alone. Median survival times for the TSC-only and the radiation therapy-only groups were 15 and 30 days, respectively. The 60-day median survival times for the groups receiving a combination of either low- or moderate-dose TSC with radiation therapy were statistically improved compared with those for the other treatment groups.

Conclusions. Use of TSC improves the extent of GBM tumor regression following radiation therapy and enhances survival. Radiosensitization of hypoxic tumors through increased oxygen diffusion may have clinical utility in patients with GBM tumors but must be explored in a clinical trial. (DOI: 10.3171/JNS.2008.108.5.972)

KEY WORDS • diffusion • glioblastoma multiforme • oxygen • radiosensitization • trans sodium crocetinate

GLIOMASTOMA multiforme is an invasive and highly aggressive intracranial tumor. The mainstays of treatment for GBM are cytoreductive surgery followed by radiation therapy.³ Areas of hypoxia have been described in these tumors, and hypoxia may decrease the effectiveness of radiation therapy or radiosurgery.³⁰

Oxygen is known to be a powerful radiosensitizing agent for mammalian cells.^{9,34} It can increase the dose efficiency of ionizing radiation by a factor of 3. Considering the many intracranial tumors that have regions of relative hypoxia, the importance of raising partial brain oxygen concentrations to physiological or suprphysiological levels cannot be overstated.

Trans sodium crocetinate, a carotenoid compound, has been shown to increase the diffusion of oxygen in plasma and, hence, to lead to improvements in oxygen availability

to other tissues. The mechanism for this effect is believed to be due to interactions between the hydrophobic TSC molecules and water, and it should occur in any aqueous solution.³⁷ In vitro measurements confirm that there is an approximately 30% increase in the diffusivity of oxygen through blood plasma.^{27,37} Because TSC also increases the diffusivity of oxygen through other aqueous-based systems, it may also have an effect on transport through extravascular fluid as well; however, the major resistance of oxygen diffusion resides at the level of the plasma.^{35,38} It is also believed that TSC lowers this resistance by altering the intermolecular forces between water molecules.

Use of TSC has been shown to increase oxygen delivery to the brain parenchyma in rats.²⁷ A related compound, crocetin, enhanced the radiosensitivity of Walker-256 carcinoma in an animal model.⁴⁰ Because TSC is more soluble than crocetin and it is easier to deliver a dose that provides therapeutic benefit, TSC could be an exquisite radiosensitizer for brain tumors treated with fractionated radiation therapy or radiosurgery. This study examines the radiosensitizing effects of TSC in an in vivo GBM model.

Abbreviations used in this paper: GBM = glioblastoma multiforme; MR = magnetic resonance; PBS = phosphate buffered saline; TSC = trans sodium crocetinate.

Materials and Methods

Study Design and Experimental Animals

A total of 31 female Sprague-Dawley rats (Charles River Laboratories, Inc.) were included in the study. Each animal weighed between 200 and 220 g, was fed ad libitum, and was maintained on a 12-hour light/dark cycle. All aspects of this research were performed according to the National Institutes of Health animal experimentation guidelines and approved by the University of Virginia's Animal Care and Utilization Committee.

After tumor cell implantation, animals were randomly assigned to 1 of 4 groups: 1) moderate dose of TSC only; 2) radiation therapy only; 3) radiation therapy and low-dose TSC; or 4) radiation therapy and moderate-dose TSC. There were 4 animals in the moderate dose of TSC only group; the radiation therapy-only group consisted of 8 animals; the radiation therapy and low-dose TSC group consisted of 10 animals; and the radiation therapy and moderate-dose TSC group contained 9 animals.

In 3 of the 4 experimental groups, 5 doses of TSC (1 dose/day) were administered intravenously with the first dose delivered 5 days after tumor implantation. The radiation therapy was performed on the 5th day of TSC administration (10 days after tumor implantation). All animals were observed until death or for as long as 60 days after tumor implantation. The observation period of 60 days was selected because it was more than 2 times longer than the reported mean survival time of rats with untreated tumors.^{14,19,26,31,32}

Cell Line

The tumor cell line used was the C6 glioma cell line from American Type Culture Collection. Cells were cultured for 4–5 passages in F-12 Kaighn's nutrient mixture medium (Gibco) supplemented with 15% horse serum, 2.5% fetal bovine serum, and 1% antibiotic-antimycotic solution. The medium was changed twice per week, and the cells were grown in 75 cm² culture flasks. Cells were incubated at 37°C in 5% CO₂, passaged weekly by washing with PBS, detached in 10 ml of 0.5 mM ethylenediamine tetraacetic acid, and subcultured at a ratio of 1:10.

Cells for implantation were obtained by trypsinization from subconfluent cultures, washed twice with PBS, and resuspended in 10 mM PBS-glucose to a final concentration 5×10^5 μ l. The cell density was determined by a microscope stage micrometer. The time between removal of the cells from culture to implantation in the rat host was < 90 minutes.

Stereotactic Tumor Implantation Technique

Prior to the operative procedure, all animals were anesthetized by intraperitoneal injection of a mixture of ketamine (60–80 mg)/xylazine (5–10 mg/kg). The head was fixed in a stereotactic frame (David Kopf Instruments). Using sterile techniques, a small craniectomy was drilled (2 × 1 mm) at 3 mm from the midline and 1 mm anterior to the coronal suture. The dura mater was then pierced with a small-gauge needle. A 5- μ l volume of 5×10^5 cells was implanted at a rate of 1 μ l/minute to a depth of 4 mm below the craniectomy into the right frontotemporal region. The needle was left in place for 2 minutes before being removed. The craniectomy was then sealed with bone wax, and the scalp was closed with an interrupted nylon suture.

Administration of TSC

The TSC used in the study was provided as a gift by Diffusion Pharmaceuticals. In the low- and moderate-dose TSC groups, TSC was administered to the animals by tail vein injection starting on the 5th day after tumor implantation. The TSC was delivered in bolus intravenous form for 5 consecutive days (Days 6 through 10 after tumor implantation). Animals in the low-dose TSC group received 50 μ g/kg of TSC per injection. The moderate-dose TSC animals were treated with 100 μ g/kg of TSC per injection.

Radiation Therapy

Animals in 3 of the experimental groups received cranial radia-

tion. Radiation therapy was performed on the 5th day of TSC administration (postoperative Day 10), 45 minutes after the injection was completed. Each animal's head received a single fraction of 8 Gy using a modified linear accelerator (Stabilpan, 250 kVp; Siemens USA); the animal's body was shielded from radiation with a lead apron.

Magnetic Resonance Imaging

Each rat's brain was imaged every 2 weeks using a Varian MR imaging system (4.7 Tesla, 40 cm bore magnet, accessible diameter of 32.4 cm; Varian Medical Systems) in the University of Virginia's Small Animal Multimodality Imaging Core.

A gadolinium-enhanced, T1-weighted pulse sequence was used for imaging (TR/TE = 400/20 msec, number of excitations = 2, field of view = 8 cm, matrix size = 256 × 192, slice thickness = 3 mm). The pulse sequences allowed 30 slices per scan, which covered the entire brain. The average scan time was 3 minutes. All imaging was performed 1 minute after an intraperitoneal injection of 1 ml (70 mg) of gadoteridol (Bracco Diagnostics, Inc.). The initial imaging session was analyzed to verify tumor implantation in all animals. Subsequent sessions were used to compute the maximum cross-sectional area of the tumor as a function of time.

All imaging measurements were performed by at least 2 of the investigators using the National Institutes of Health imaging software ImageJ.¹ The area of tumor on a given image slice was considered to be the area of enhancing tissue at the operative site as seen on coronal images. Maximal cross-sectional area was defined as the total number of pixels on the image slice with the largest number of enhancing pixels, converted to units of mm² based on the known in-plane resolution of the images.

Animals were excluded from the analysis if there was no discernible evidence of tumor as indicated by the presence of a circumscribed lesion with gadolinium enhancement on the initial 2-week postoperative MR image. Rejection of tumors over time should be a function of the model (animal type and cell line used) and occur in all groups, thereby not resulting in a therapeutic bias for one group versus another.

Clinical Follow-Up

Animals were examined daily for alertness, feeding ability, external appearance, changes in body weight, focal motor deficits, gait disturbance, and responses to contact. Clinical end points for animals used in research scoring systems were followed as published by the University of Virginia postprocedural care. The animals were killed if they reached the threshold euthanasia score, or at the 60-day time point if the animals remained clinically well.

Histological Tissue Preparation

All animals were killed under anesthesia by exsanguination and were perfused with formalin. Each brain was removed and placed in a buffered 10% formalin solution prior to sectioning. Tissue blocks were paraffin embedded and sectioned at a thickness of 7 μ m in the coronal plane through the level of the tumor. Hematoxylin and eosin staining was used for each section. Tumor size and the presence of tumor were assessed.

Light microscopy was used to determine the presence of tumor and evaluate tumor size. Consecutive slices of each sample were examined to determine tumor size, which was defined as the maximum cross-sectional area. The software package ImagePro (Media Cybernetics) was used for quantitative analysis.

Statistical Methods

Statistical analysis was performed by Dr. Mark Conaway, Director of the Division of Biostatistics and Epidemiology at the University of Virginia. To compare tumor size on MR images and histological features between treatment groups, a commercial statistical package (SPSS version 15.0, SPSS, Inc.) was used. A probability value of ≤ 0.05 was considered statistically significant.

Survival curves were created using Kaplan-Meier survival charts. Animal survival was computed from the day of tumor implantation until the day of euthanasia or the 60-day end point. Differences in

survival curves were analyzed using the log-rank test. The F test was used to assess for differences in tumor growth across groups. Statistical samples were computed by analyzing the "slope" as defined by the natural log (ln) of the histological area of the tumor plus 1, divided by the survival time in days of the particular animal (that is, for each animal, the tumor slope was the \ln [histological area + 1]/survival time in days).

The MR imaging tumor data were plotted on a natural log scale and fitted to a quadratic model. Statistical differences in the MR imaging data between groups were also assessed. Such analysis takes into account the trends in tumor growth even in animals that have a shortened survival.

Results

Tumor Size on MR Imaging

The maximal tumor cross-sectional areas were computed on the basis of MR imaging (Fig. 1). These tumor sizes are depicted as a function of time and treatment group in Fig. 2. Overall tumor sizes increased in all groups from 2 to 4 weeks after tumor implantation. No animals treated with TSC alone were alive longer than 4 weeks. There was no statistically significant difference in tumor size between the radiation therapy-only and TSC-only treatment animals at 2 weeks postimplantation; only 1 TSC-only animal reached the 4-week time point, preventing a valid statistical comparison at that interval. Wide standard error bars at the 6-week time point in the radiation therapy alone group (Fig. 2) are indicative of the fact that few animals in this group survived beyond 30 days.

The natural log of the tumor size as depicted on MR imaging for the radiation alone, radiation and low-dose TSC, and radiation and moderate-dose TSC groups is depicted in Fig. 3. Because no TSC-only animals had MR imaging data beyond 30 days, that group could not be included in this particular statistical analysis. A significant reduction in tumor size detected by MR imaging was observed in the radiation therapy and moderate-dose TSC group as compared with the radiation therapy-only group ($p = 0.018$).

Animal Survival

A Kaplan-Meier survival curve of the treatment groups is depicted in Fig. 4. The C6 glioma tumor was fatal to all animals treated with TSC alone and also to the vast majority (75%) of animals treated with radiation therapy alone, but 70% of the animals from the low-dose TSC and radiation therapy group and 67% of the animals in the moderate-dose TSC and radiation therapy group reached the study's 60-day end point.

The median survival time for the TSC-only group was 15 days. For the radiation therapy only group, the median survival time was 30 days. The median survival times for the low- and moderate-dose TSC with radiation therapy groups were each 60 days. Using the log-rank analysis, the survival times of the groups with low- and moderate-dose TSC with radiation therapy were statistically improved compared with either the radiation therapy-only or TSC-only groups ($p = 0.001$).

Histological Results

All tumors showed histological evidence of intratumoral necrosis consistent with regions of hypoxia within this model of malignant GBM (Fig. 5). As previously noted,



FIG. 1. Contrast-enhanced coronal MR images of representative animals 4 weeks after tumor implantation. *Upper*: Image from an animal in the radiation therapy-only group. Note the marked tumor size in the right frontal lobe (*white arrows*). *Lower*: Image from an animal in the radiation therapy and moderate-dose TSC group. The tumor (*white arrow*) in this animal is markedly smaller than the one in the animal treated with only radiation (*upper*).

tumor growth was computed as a function of the natural log of the tumor area on histological assessment + 1 divided by the survival time in days. The values for this tumor growth (the slope) are depicted in Fig. 6 for each treatment group. The tumor growth slopes were as follows (mean \pm standard error of the mean): radiation therapy only = 0.40 ± 0.10 ; TSC only = 0.69 ± 0.15 ; radiation therapy and low-dose TSC = 0.20 ± 0.1 ; and radiation therapy and moderate-dose TSC = 0.14 ± 0.07 . The tumor growth slopes were significantly lower in the combined treatment groups than in either radiation therapy-only or TSC-only groups ($p = 0.024$).

Discussion

In cancer and other malignant intracranial processes, failure of local tumor control is the cause in 40–60% of deaths and occurs in 60–80% of patients at the time of death.³⁹ Regions of hypoxia within malignant tumors such as GBMs have been well described.^{2,30} Cells in such regions will invariably show diminished response to radiation therapy or radiosurgery. Optimally, more radiation or repeated radiation could be delivered to inactivate tumor cells in

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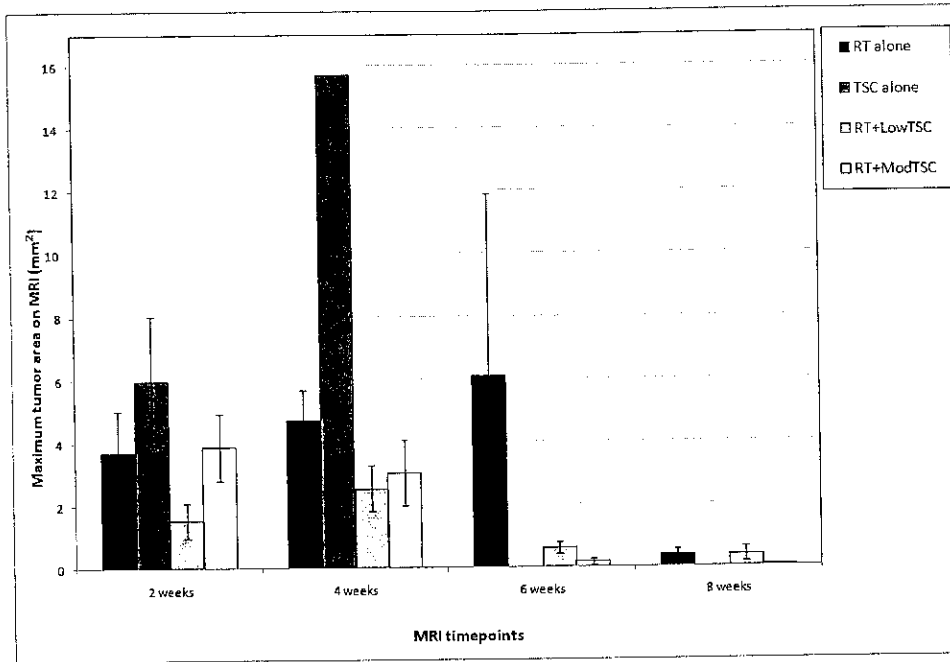


FIG. 2. Bar graph showing mean size (in mm²) of the tumors (with standard error bars) as noted on MR imaging in each of the 4 treatment groups according to 4 different time points. No animals in the TSC-only group survived longer than 4 weeks. The long standard error bar at Week 6 in the radiation therapy (RT) alone group indicates that few animals in this group survived more than 30 days. Mod = moderate.

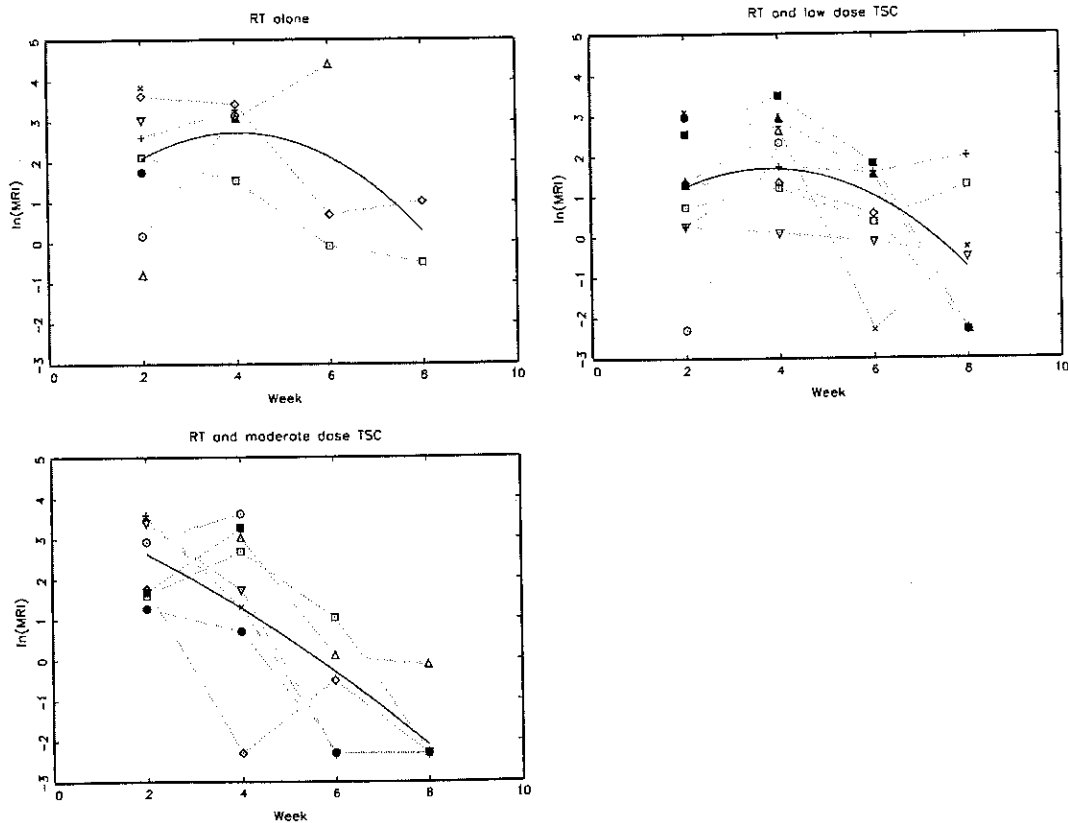


FIG. 3. Graphs of the natural log (ln) of the MR imaging tumor size as a function of week after tumor implantation in all animals. Using a quadratic model, a statistically significant difference was found between the RT alone group and the RT and moderate-dose TSC group ($p = 0.018$). Symbols represent specific animals at specific time points.

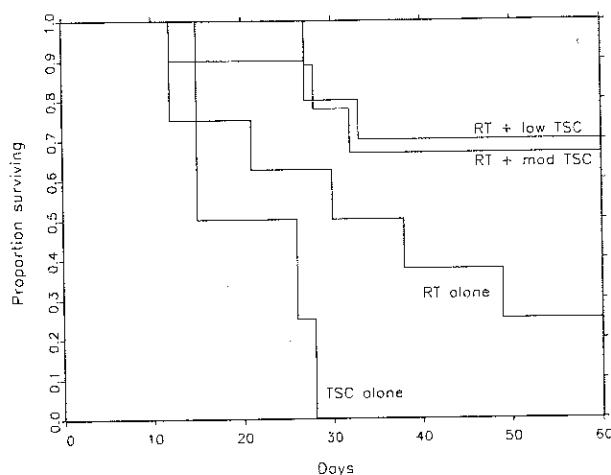


FIG. 4. Line graph of the Kaplan-Meier survival plot illustrating the proportion of surviving animals per group as a function of days after tumor implantation.

regions of hypoxia. After a standard course of radiation therapy for a GBM, surviving cells tend to repopulate the tumor.¹² In fact, during a typical radiotherapy course of 60 Gy in 2 Gy per fraction for GBMs, 25–30% of the dose is required just to sterilize newly formed tumor cells arising during the treatment period.⁴¹ Unfortunately, because of inherent limitations of the brain and the severity of radiation toxicity, neither fractionated doses of > 60 Gy nor repeat fractionated radiation therapy are advisable for patients with GBMs.⁵ Processes that enhance tumor cell death for a given dose of radiation (sensitization) could lead to therapeutic improvement.

With radiosurgery or radiation therapy, tumor cell death results in part from direct ionization within the target tissue. Free radical generation within the treatment volume by exposure to ionizing radiation can be enhanced by oxidation or reduction.⁷ Thus, the addition of oxygen to the tumor can be lethal to tumor cells. Oxygen is an exquisite radiosensitizing agent. Hyperbaric oxygen treatment is one such way to increase brain tissue oxygenation, and some work has shown efficacy of hyperbaric therapy for high-grade gliomas.¹⁸ Other approaches attempted include transfusions to increase the red blood cell count, utilizing perfluorocarbons that carry oxygen, administering erythropoietin, and breathing carbogen (hyperoxic gas) combined with nicotinamide.^{4,10,13,20–22} Because oxygen modifies the effectiveness of a particular radiation dose, the agent should be administered close to the time of radiation or radiosurgery delivery.⁸

Much research and many clinical trials have been performed on chemical agents that mimic the radiosensitizing effects of oxygen. Some of the electron-affinic agents that have been studied include metronidazole, misonidazole, etanidazole, and nimorazole. A Phase III glioma trial using misonidazole showed no significant benefit.²⁵ In another GBM clinical trial, the overall survival rate in the group receiving nicotinamide during radiotherapy was similar to those receiving radiotherapy alone.²⁴ Efavoxiral (RSR-13) acts to shift the dissociation curve to the left; Phase II and III trials indicate that subsets of patients with brain metastases may benefit from its radiosensitizing effect.^{23,33}



FIG. 5. Photomicrograph of a coronal cross-section through the tumor in an animal treated with only radiation therapy. The right frontal lobe is shown with the tumor demonstrating areas of necrosis and cellular hyperproliferation. H & E, original magnification $\times 10$.

Elevating the concentration of oxygen to regions of a brain tumor in patients receiving radiation therapy can also be accomplished by increasing the rate of oxygen transport from the blood to the tumor. Use of TSC and its parent compound, crocetin have been shown to increase oxygen in blood plasma in vitro and in vivo.^{16,27,37,38,40} Trans sodium crocetin has been shown to increase the diffusion coefficient or diffusivity of oxygen in plasma. It appears to do so through a novel physiochemical change in which TSC creates a water solvation shell that lies just 4 to 5 Å away from the TSC molecule.³⁷ This increased chemical structure leads to a decreased density and allows for increased diffusion of solutes (such as oxygen) through a medium.^{15,37} In a Walker-256 carcinoma model of a tumor implanted into a rat thigh, crocetin was found to sensitize the tumor cells to radiation therapy.⁴⁰

In this study, moderate-dose TSC when combined with radiation improved the animal survival rate, radiological response, and histologically confirmed decrease in tumor growth rate of a GBM. Low-dose TSC and radiation improved animal survival and decreased the tumor growth rate but did not achieve statistical significance with regard to radiological response. This difference may be a function of the limited sample size. The ability of TSC to improve tissue oxygenation in the setting of a hypoxic tumor receiving single-dose radiation may have important clinical applications for the treatment of tumors. Nevertheless, further investigation of whether TSC alone can decrease tumor growth due to improved oxygenation will need to be performed. In the radiosurgery or radiation therapy settings,

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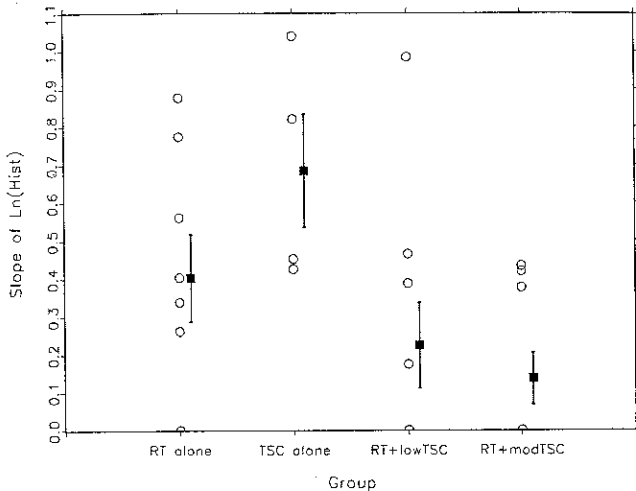


FIG. 6. Scatterplot showing a comparison of tumor growth in all 4 groups. The slope of the tumor growth was computed for each animal as the natural log \ln (histological tumor area + 1) divided by the survival time in days. The tumor growth in the combined TSC and radiation groups was significantly less than in the groups receiving either treatment modality alone ($p < 0.05$). Solid line shows mean (square) and standard error.

one could conceive of administering an agent to enhance oxygen diffusion. Using equivalent current doses of radiation and a radiosensitizing agent may improve tumor response rates. Moreover, using a radiosensitizing agent and lowering the dose may achieve results comparable to those found with higher doses but reduce the side effect profile.

Radiosensitizing and radioenhancing agents are designed to increase the toxicity of radiation to cancerous tissues but spare or cause less damage to adjacent normal parenchyma. Besides hypoxia-based radiosensitizers, various types of other sensitizing agents have also been tried for treating GBMs. Unfortunately, although results from preclinical studies often show significant promise, the efficacy of such agents in a human trial have generally been modest at best.^{6,11,12,38,29,36,42-44} In the European Organization for Research and Treatment of Cancer (EORTC) Phase III study, the best studied of these agents, temozolomide, showed survival benefits when it was combined with radiation therapy.⁴⁴ Estramustine, an estradiol-based antimicrotubule effector, did show a positive trend for improved response of Grade III gliomas when combined with conventional radiotherapy.¹⁷

Using tumor tissue, immunohistochemical assessments of tumor hypoxia can be inferred from the molecular expression of vascular endothelial growth factor, hypoxia-induced factor 1 α , CD-31, Ki 67, and carbonic anhydrase IX. Magnetic resonance imaging, electron paramagnetic resonance, MR spectroscopy, blood oxygenation-level dependent imaging, and Eppendorf microelectrode measurements of the partial pressure of oxygen can also be used to directly or indirectly yield information regarding tumor hypoxia.³⁹ Ideally, the oxygenation state of each tumor could be measured, and those with significant hypoxic regions could be treated with a safe and effective oxygen radiosensitizer. Fortunately, in good laboratory practices of animal testing of TSC reported to the US Food and Drug Administration,

there were no adverse events observed in rats with doses as high as 50 mg/kg; the doses demonstrating radiosensitization in this study were 0.05 mg/kg and 0.1 mg/kg (Dr. John Gainer, personal communications).

Although the results of TSC in this GBM animal model look promising, additional preclinical testing must be performed. The importance of developing radiosensitizing agents for intracranial tumors cannot be understated, however, as the limits of clinical advances related to optimum radiation dose, fractionation (single, hypofractionation, or conventional fractionation schemes), and image-guided delivery are realized.

Conclusions

Intratumoral hypoxia lessens the effectiveness of radiosurgery and radiation therapy. Use of TSC with radiation treatment resulted in greater tumor shrinkage and enhanced animal survival than radiation therapy only or TSC only. Tumor reduction probably occurs through improvements in tumor oxygenation as a result of TSC's enhancement of diffusion. Further testing of TSC must be performed to better define the clinical safety and potential therapeutic effectiveness of this agent for intracranial tumors undergoing radiation therapy or radiosurgery.

Disclosure

The TSC used in the study was provided by Diffusion Pharmaceuticals. Also, a portion of this research was funded by a grant from Diffusion. Diffusion did not devise the scientific methodology and had no role in the data analysis or preparation of the manuscript.

Acknowledgments

Dr. Mark Conaway, Director of the Division of Biostatistics and Epidemiology at the University of Virginia, performed the statistical analysis of the data. Dr. Beatriz Lopes, Professor of Pathology at the University of Virginia, provided guidance regarding the glioma immunohistochemical analysis.

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Address correspondence to: Jason Sheehan, M.D., Ph.D., Box 800-212, University of Virginia Health System, Charlottesville, Virginia 22908. email: jps2f@virginia.edu.