Glialbloma is an all too common and aggressive type of intracranial tumor. The mainstays of treatment are cytoreductive surgery followed by radiotherapy and chemotherapy. The mainstays of treatment are cytoreductive surgery followed by radiotherapy and chemotherapy. Temozolomide is a frequently used chemotherapeutic agent for patients with high-grade gliomas. Areas within high-grade gliomas have been found to be hypoxic, and hypoxia may decrease the effectiveness of adjuvant therapy. In particular, the C6 line has demonstrated tumor hypoxia when used in animal models. 

Oxygen is known to be a powerful radiosensitizer of mammalian cells. It can increase the dose efficiency of ionizing radiation by a factor of 3. It may also help to improve the effectiveness of oral alkylating agents such as temozolomide. Given the number of intracranial tumors that have regions of relative hypoxia, the importance of raising partial brain oxygen concentrations to physiological or supraphysiological levels during therapeutic intervention cannot be overstated.

Trans-sodium crocetinate, a carotenoid compound, has been shown to increase the diffusion of oxygen in plasma and, hence, lead to improvements in oxygen availability within various types of tissues including the brain. The mechanism for increased oxygen diffusion is believed to be due to interactions between the hydrophobic TSC molecules and water. As such, it should occur in any aqueous solution. In vitro measurements confirm
that there is an approximate 30% increase in the diffusivity of oxygen through blood plasma.\textsuperscript{23,32}

Previously, TSC has been found to radiosensitize and increase oxygen diffusion into hypoxic C6 glioma cells in vivo.\textsuperscript{28,29} In the present study we examine the ability of TSC to potentiate the effectiveness of temozolomide and radiation for C6 gliomas in an in vivo model.

**Methods**

**Study Design and Animals**

A total of 24 Sprague-Dawley rats (Charles River Laboratories International, Inc.) were included in the study. Each animal weighed between 200 and 250 g. They were fed ad libitum, and a 12:12-hour light/dark cycle was maintained.

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 implantation, the animals were randomly assigned to 1 of 3 treatment groups: 1) temozolomide alone (8 rats); 2) temozolomide and radiation therapy (8 rats); or 3) temozolomide, radiation therapy, and TSC (8 rats). The timeline for intervention and follow-up assessments is depicted in Fig. 1.

**Cell Line**

The tumor cell line used was the C6 glioma cells from American Type Culture Collection. Although all animal models of high-grade glioma have their advantages and disadvantages, the C6 glioma model was used because of its previously demonstrated tumor hypoxia.\textsuperscript{2,3,17,20,29}

Cells were cultured for 4–5 passages in F-12 Kaighn 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 cm\(^2\) culture flasks. Cells were incubated at 37°C in 5% carbon dioxide, 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 with glucose to a final concentration 5 \(\times\) 10\(^5\)/5 μl. The cell density was determined by microscope stage micrometer. The time between cell removal 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/xylazine 60–80 mg/5–10 mg/kg. The rat’s head was fixed in a stereotactic frame (David Kopf Instruments). Using sterile technique, a small craniectomy was drilled (2 \(\times\) 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-μl volume of 5 \(\times\) 10\(^5\) cells was implanted at a rate of 1 μl/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.

**Magnetic Resonance Imaging**

Each rat’s brain was imaged 15, 30, 45, and 60 days after tumor implantation. We used Varian Medical System (4.7-T, 40-cm bore magnet, accessible diameter of 32.4 cm) at the University of Virginia Health System’s Small Animal Multi-Modality Imaging Core.

A Gd-enhanced, T1-weighted pulse sequence was used for imaging (TR/TE 400/20 msec, field of view 8 cm, matrix size 256 \(\times\) 192, slice thickness 3 mm). The pulse sequences allowed 30 slices per scan, which covered the entire brain. The average scan duration was 3 minutes. All imaging was performed 1 minute after an intraperitoneal injection of 1 ml (70 mg) of gadoteridol (Prohance, Bracco Diagnostics Inc.).

The initial imaging session was analyzed to verify tumor implantation in all animals as well as to compute the size of the tumor. 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 NIH imaging software ImageJ (http://rsb.info.nih.gov/ij/).\textsuperscript{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. The 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 millimeters squared 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 Gd enhancement on the initial 2-week postoperative MR image.
Administration of TSC

The TSC was administered to the animals via tail vein injection starting on the 11th day after tumor implantation. It was delivered in bolus intravenous form for 5 consecutive days (that is, Days 11–15 after tumor implantation). The TSC-treated animals received 100 µg/kg TSC per injection. This dose is comparable to one previously given in survival studies and tumor oxygenation studies in which the same rat glioma model was used.13,18,27

Radiotherapy

Animals in 2 of the experimental groups received cranial radiation treatment. Radiotherapy was performed on the 5th day of TSC administration (that is, 15 days after tumor cell implantation), approximately 5–10 minutes after the injection was completed. This dosing of TSC and the delivery of radiotherapy within minutes of the 5th and final dose of TSC was chosen to take advantage of pharmacokinetics of TSC and the previously demonstrating oxygen-enhancing effects within the hypoxic C6 glioma.14,15,28,29

Each animal’s head received a single fraction of 8 Gy delivered using a modified linear accelerator (Stabilipan, 250 kVp, VDH 28085; Siemens USA); the animal’s body was shielded from radiation with a lead apron.

Temozolomide Administration

Temozolomide (Temodar, Scherling-Plough) was dissolved in a small amount of dimethylsulfoxide to a concentration of 150 mM.13,28

All animals received temozolomide. Temozolomide was administered at a dose of 350 mg/m²/day. The dose was selected based on work done by others in animal glioma models.13,18,27 Each animal was given 5 consecutive intraperitoneal doses of temozolomide on Days 11–15 after tumor implantation. In the cohort that received TSC and temozolomide, the animals received both agents on the same days and at the same times.

Follow-Up of the Animals

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 postprocedure care (Appendix 2 of the University of Virginia guidelines and policy for determination of humane endpoints for animals used in research: http://www.virginia.edu/vprgs/iacuc/policies.html).

All animals were observed until death or up to 60 days after tumor implantation. The observation period of 60 days was selected as it was more than 2 times longer than the reported mean survival duration of rats with untreated tumors.11,21,28 The animals were killed if they reached the threshold euthanasia score or at the 60-day time point if the animals remained clinically well.

Statistical Analysis

To evaluate for differences in tissue oxygenation among treatment groups, a commercial statistical package (StatView, SAS Institute) was used. Survival data were plotted using the Kaplan-Meier methodology. The log-rank test was used to determine differences in the survival distributions of the radiotherapy/TSC/temozolomide group versus the other groups. Differences in the tumor volume as detected on MR imaging were evaluated using the t-test. A p ≤ 0.05 was considered to be statistically significant.

Results

Clinical and Survival Responses

The rats did not exhibit overt evidence of neurological toxicity attributable to TSC administration. In fact, the overall neurological status of the TSC-treated animals in terms of weight, feeding, and gait was higher than that in those of the other treatment groups at the 30-day and later evaluation time points.

The mean survival (± SEM) for the various treatment groups was as follows: 1) temozolomide alone 23.2 ± 0.9 days; 2) temozolomide/radiotherapy 29.4 ± 4.4 days; and 3) temozolomide/radiotherapy/TSC 39.8 ± 6.0 days. Survival in the various groups is depicted in Fig. 2.

Those animals treated with TSC, temozolomide, and radiotherapy had a statistically significant improvement in survival compared with those in the temozolomide/radiotherapy group (p = 0.0084) and the temozolomide-alone group (p = 0.0045). There was no statistically significant difference in survival between the temozolomide-alone group and the temozolomide/radiotherapy group (p > 0.05). In the group treated with temozolomide/radiotherapy/TSC, 37.5% of the animals reached the full 60-day observation end point whereas only 1 other animal in the temozolomide and radiation therapy group survived this long. All other animals succumbed to the side effects of worsening mass effect from their intracranial tumor; the presence of a substantial tumor was confirmed on post-mortem histological analysis in each of these animals.

Tumor Response on MR Imaging

Brain MR images obtained in all living animals were evaluated at 15-day intervals after tumor implantation. All animals included in the study had demonstrable GBs on the 2-week postimplantation MR images. At 15 days, tumor sizes (mean ± SEM) in the groups were as follows: 1) temozolomide alone 15.4 ± 1.4 mm²; 2) temozolomide/radiotherapy 17.6 ± 1.3 mm²; and 3) temozolomide/radiotherapy/TSC 16.6 ± 0.91 mm². There was no difference in tumor size among groups just prior to initiation of treatment.

At 30 days after tumor implantation and 15 days after initiation of treatment (Fig. 3), the size (mean ± SEM) of the tumors in all surviving animals was as follows: 1) temozolomide alone 42.1 ± 2.7 mm²; 2) temozolomide/radiotherapy 35.8 ± 5.1 mm²; and 3) temozolomide/radiotherapy/TSC 18.9 ± 6.6 mm². The group treated with TSC in addition to temozolomide and radiotherapy demonstrated statistically significant smaller tumors than either the temozolomide-alone group (p = 0.047, t-test) or temozolomide/radiotherapy group (p = 0.004, t-test).

At 45 and 60 days after tumor implantation, all of the TSC-treated animals had no demonstrable tumor despite the use of thin-slice postcontrast MR imaging (Fig. 4).
Radiosensitization in a glioblastoma

Discussion

High-grade gliomas are well known to have regions of tissue hypoxia. In fact, the C6 glioma cell in vivo model has been shown to exhibit tumor hypoxia typical of a high-grade glioma.\textsuperscript{17,20,29} Hypoxia is believed to contribute to the resistance of gliomas to radiosurgery, radiation therapy, and chemotherapeutic agents such as temozolomide.\textsuperscript{31} It can also stimulate angiogenesis through vascular endothelial growth factor upregulation and lead to further glioma cell invasion.\textsuperscript{10,35} The development of effective therapies for CNS malignancies such as GBs will likely require a better understanding of tumor hypoxia and hypoxia-induced gene expression.\textsuperscript{19}

Normoxia rather than hyperoxia appears to be a reasonable goal in the clinical arena for patients with brain tumors.\textsuperscript{10} Normal brain tissue is very well perfused and, as such, well oxygenated.\textsuperscript{9} Quantitative MR imaging of human brain perfusion reveals that gray matter is perfused at rate of 93 ± 16 ml/100 g/min, white matter at a rate of 38 ± 10 ml/100 g/min, and whole brain at a rate of 52 ± 8 ml/100 g/min.\textsuperscript{25} This translates to a typical tissue oxygenation in the brain of > 25 mm Hg.\textsuperscript{33}

In the setting of normoxic brain tissue, TSC has not been shown to appreciably alter partial tissue oxygenation. However, in the context of hypoxia associated with a high-grade glioma, a single intravenous bolus of TSC temporarily has been demonstrated to elevate tissue oxygenation above its baseline hypoxic state, as measured directly by intraparenchymal Licox oxygen probes.\textsuperscript{29} Although the exact pharmacokinetics of TSC, its optimal frequency and dosing, and the full functional consequences of improved oxygen diffusion of a brain tumor are still being fully defined, delivery of adjuvant treatment to a more normoxic tumor may lessen resistance typically found in lesions that are hypoxic.

Trans-sodium crocetinate appears to alter the hydrogen bonding patterns in serum and thereby cause an increase in the rate of oxygen diffusion across capillary membranes.\textsuperscript{14–16} In the setting of hypoxia, the effect of increased oxygen diffusion seems to be accentuated. Trans-sodium crocetinate has been shown to increase the diffusion of glucose and oxygen through water by 25–30%.\textsuperscript{32} This effect is likely achieved through induction of order in the surrounding water structure through increased hydrogen bonding of water molecules.\textsuperscript{32} In the setting of hemorrhagic shock, TSC has been shown to reduce lactate levels presumably through increased oxygen diffu-

![Fig. 2. Kaplan-Meier plot depicting survival of animals in the various treatment groups as a function of days after C6 tumor cell implantation. Animals treated with the combination of TSC, temozolomide (Temodar), and radiotherapy (RT) had a statistically significant survival advantage over those in the other groups (p < 0.05).](image)

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![Fig. 3. At 30 days after tumor implantation, coronal postcontrast MR images depict C6 gliomas in an animal treated with temozolomide alone (A), an animal treated with temozolomide/radiotherapy (B), and an animal treated with temozolomide/radiotherapy/TSC (C).](image)
sivity.14,30 Also, in those animals receiving supplemental oxygen, TSC was found to increase oxygen delivery to baseline normoxic brain tissue.

In previous work by our group, TSC was seen to increase the sensitivity of the C6 glioma to radiation, but TSC was not found to have an effect on either survival or tumor control alone.28 Thus, TSC itself has no intrinsic value as a tumoricidal agent. For TSC to prove useful in the neurooncology arena, it will have to enhance the benefits of standard adjuvant treatment for gliomas—this currently being radiation therapy and temozolomide. The current study indicates that TSC potentiates the radiological and survival responses of GBs to the standard therapy of temozolomide and radiation. Tumors in the TSC group treated with TSC were statistically smaller than those in the other groups after just 2 weeks posttreatment. Those animals treated with TSC and surviving to 6 weeks after tumor implantation had no discernible tumor on high-resolution, postcontrast MR imaging studies. Trans-sodium crocetinate resulted in improved survival in this animal model over standard therapy (that is, radiation therapy and temozolomide) alone.

In the setting of tissue hypoxia within a GB, TSC has been shown to temporarily reverse hypoxia. By intervening during this period, the beneficial effects of radiation therapy and alkylating chemotherapeutic agents such as temozolomide are improved.31 In particular, the ability to reduce or eliminate tumor hypoxia may provide a therapeutic window to intercede with adjuvant therapies. In addition, an agent that changes hypoxic conditions in gliomas could alter expression of such agents as hypoxia-inducible factors (for example, carbonic anhydrase, Hif1A, Hif2a, and Hif3a). However, not all gliomas show significant hypoxia, and TSC and other such agents may be of maximal value in patients harboring hypoxic gliomas.20,24 Nevertheless, significant expression of hypoxia-related tissue expression has been shown to be a poor prognostic factor, and improvement in tumor hypoxia even if small in magnitude or short in duration could potentially have great impact in this poor-outcome subgroup of patients with glioma.22

Further study of the intracranial potential of this agent seems warranted to shed light on its potential utility in the clinical arena and its limitations in the heterogeneous group of high-grade gliomas with wide ranging oxygen characteristics.20,24 Also, this study is limited with regard to the fact that only one rat brain tumor model was used to obtain these results. The C6 model is a hypoxic tumor and serves as a generally reliable in vivo model. However, unintended immunological response has been noted in its use.23 Additional preclinical testing of TSC will be required and likely will necessitate the use of additional animal models.

Conclusions

Intratumoral hypoxia is a common finding in the setting of high-grade gliomas and has been shown to diminish the effectiveness of chemotherapy and radiation. Trans-sodium crocetinate has been previously shown to temporarily increase the tissue oxygenation in hypoxic C6 gliomas. In conjunction with temozolomide and radiation therapy, TSC potentiated the radiological response and clinical survival in this GB preclinical model. Additional testing of TSC appears warranted to explore potential therapeutic advantages afforded by a compound that reduces hypoxia in brain tumors.

Disclosure

The TSC was provided as a gift by Diffusion Pharmaceuticals (Charlottesville, VA), and the temozolomide was provided as a gift by Schering-Plough Research Institute division (Kenilworth, NJ). 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.

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