Trans sodium crocetinate has been shown to increase oxygen in the setting of hypoxia related to brain tumors and stroke. Reduction of hypoxia in malignant brain tumors could yield improvements in their response to radiosurgery, radiation therapy, or chemotherapy. In particular, cells surviving hypoxia can lead to increased migration, increased vascular signaling/recruitment, enhanced anaerobic glycolysis, and resistance to apoptosis. The Licox monitor (Integra) revealed a TSC-associated partial restoration of a hypoxic tumor to more normoxic conditions. However, such Licox probes had a limited sampling volume that may not have reflected changes throughout the entire tumor.

Although TSC is postulated to work through enhancement of oxygen diffusion across blood vessels, it is not known to what extent the administration of TSC results in a functional improvement in hypoxic tissue. If the parenchymal concentration of oxygen increases to a subfunctional degree, the consequences of TSC administration on hypoxic tissue may not be clinically meaningful. Positron emission tomography is a nuclear medicine technique that produces a 3D image of functional processes within the body; therefore, PET imaging has become a very important tool for functional imaging within the brain. In particular, Cu-ATSM–based PET imaging has been used to study functional changes in the oxygen levels of tissues.

Key Words • trans sodium crocetinate • glioma • hypoxia-inducing factor • Copper(II) diacetyl-di(N4-methylthiosemicarbazone) • rat • oncology

Abbreviations used in this paper: CA9 = carbonic anhydrase 9; Cu-ATSM = Copper(II) diacetyl-di(N4-methylthiosemicarbazone); HIF-1α = hypoxia-inducing factor–1α; PBS = phosphate-buffered saline; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TSC = trans sodium crocetinate.

This article contains some figures that are displayed in color online but in black and white in the print edition.
to have a region of hypoxia on direct measurements with parenchyma Licox oxygen probes and also by other techniques. However, it is not clear whether TSC’s enhancement of oxygen diffusion represents a functional change in the physiology (that is, cellular metabolism) of cells throughout the entire tumor. In this study, we use Cu-ATSM PET imaging to study the effect of TSC on a hypoxic brain tumor. We also study the expression of HIF-1α and CA9 in this same tumor model.

Methods

The C6 Glioma Technique

The C6 glioma cells were obtained from the American Type Culture Collection. Cells were cultured for 4–5 passages in Ham F-12 medium (GIBCO, Inc.) containing 15% horse serum, 2.5% fetal bovine serum, and 1% antibiotic-antimycotic solution. The medium was changed at least twice a week, and the cells were grown using 75-cm² culture flasks. Cells were incubated at 37°C in 5% CO₂, passed weekly by washing with PBS, detached in 10 ml of 0.5 mM EDTA, and subcultured at a ratio of 1:4.

For implantation, cells were washed in PBS, then detached in 10 ml of 0.5 mM EDTA. Cells were then pelletted by centrifugation (1500 G for 4 minutes) and resuspended in 10 ml PBS-glucose to a final concentration of 0.5 mM EDTA, and subcultured at a ratio of 1:4.

For implantation, cells were washed in PBS, then detached in 10 ml of 0.5 mM EDTA. Cells were then pelletized by centrifugation (1500 G for 4 minutes) and resuspended in 10 ml PBS-glucose to a final concentration of 10⁶ cells/10 μl. The cell density was determined by a microscope stage micrometer. The time between removal of the cells from incubation to implantation in the rat host was limited to 2 hours or less.

The Stereotactic Technique

The anesthetized rat was placed in a stereotactic head frame (David Kopf Instruments), and a small craniectomy (2 x 1 mm) was drilled, 3 mm from the midline and 1 mm anterior to the coronal suture. The dura mater was then opened. With a Hamilton syringe, a 2-μl volume of 10⁶ glioma cells was implanted with stereotactic guidance to a depth of 4 mm below the craniectomy, into the right frontotemporal region. The craniectomy was then sealed with bone wax, and the scalp was closed with suture.

The MR Imaging Technique

Two weeks after implantation of the C6 glioma cells in the rats, each animal was assessed using 3-T MR imaging at the University of Virginia’s Small Animal Multimodality Imaging Core. The T1-weighted MR images were obtained before and after addition of contrast material to determine if tumor was present.

The PET Imaging Technique

For the PET experiments, those animals in which a tumor was confirmed on MR imaging were randomly assigned to either the TSC or saline infusion group (6 animals each). The PET imaging studies were performed at the University of Virginia’s Small Animal Multimodality Imaging Core. Colleagues in the Department of Radiology provided a copper nuclide specific for PET imaging to assess oxygenation. Copper(II) diacetyl-di(N4-methylthiosemicarbazone) has been shown to demonstrate significant selectivity for hypoxic tissues in vivo; this relates to a reduction-oxidation trapping mechanism. The Cu-ATSM preferentially accumulates in hypoxic cells and delineates hypoxic areas within tumors. The mechanism of Cu-ATSM involves a reduction of Cu(II) to Cu(I), followed by a loss of the radiometal from the complex. The Cu-ATSM accumulates in tumor masses with active but hypoxic tumor cells. It has been shown to be effective in both rodent studies and in clinical studies of cancer in humans.

Each animal was injected with 1 mCi of Cu-ATSM prior to imaging. Each animal then underwent 1.5 hours of PET imaging and 0.5 hours of CT imaging. The PET imaging was continued throughout the intravenous delivery of 100 μg/kg TSC or an equal volume of saline. All animals were breathing room air (approximately 21% fraction of inspired oxygen).

The degree of uptake of the Cu-ATSM was computed in the hypoxic brain tumor of each animal. The PET ⁶⁴Cu-ATSM images demonstrated activity concentration (μCi/cm³). Image analysis was performed using AMIDE software (http://amide.sourceforge.net / Accessed June 1, 2011). Regions of interest corresponding to the glioma were contoured, and the volume of hypoxic tissue was computed for each animal by integration of the contoured areas on each slice. Hypoxia was defined as a Cu-ATSM uptake at least 2 times greater than normally perfused brain tissue.

The relative hypoxic tumor volume was computed by dividing the volume of hypoxic brain tumor on PET imaging by the total tumor volume. This accounted for slight variations in tumor size between animals. The relative hypoxic tumor volume was computed for each animal in the TSC and saline infusion groups.

Immunoblot Analysis

Tumors were implanted and confirmed on MR imaging in 5 animals. Animals were infused with 100 μg/kg TSC (3 rats) or an equivalent volume of saline (2 rats). Tumor tissue was then harvested at 1 day after infusion of TSC or saline. The 3 TSC- and 2 saline-treated animals were killed. Tumor was dissected from the surrounding brain by using microsurgical techniques. From each animal, 100 mg of brain tumor tissue was minced into very small pieces by using a clean razor blade.

Brain tumor tissues were lysed with RIPA (radioimmunoprecipitation assay) buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA) containing protease inhibitor cocktail (Sigma Chemical Co.) and phosphatase inhibitor cocktail (Sigma). Protein content was measured with BCA Protein Assay Reagent (Pierce). The samples were diluted with 2× Laemmlı lysis buffer (2.4 M glycerol; 0.14 M Tris, pH 6.8; 0.21 M SDS; 0.3 M bromophenol blue) containing 1.28 M β-mercaptoethanol, and equal quantities of protein were loaded on 10% SDS-polyacrylamide gels. Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk in PBS-Tween 20 (0.1% vol/vol) for 1 hour. The
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membrane was incubated overnight at 4°C with appropriate antibodies raised against HIF-1α (anti–HIF-1α 1:500, Novus Biologicals), CA9 (anti-CA9 1:2000, Novus Biologicals), and β-actin (1:5000, MP Biomedicals). Horse-radish peroxidase–conjugated antirabbit or antimouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL Kit, Amersham). To ensure equal protein loading, each membrane was stripped and reprobed with anti–actin antibody to normalize for differences in protein loading.

The Western blot assay was performed with 5 tumor samples, looking at expression of CA9 (anti-CA9 1:2000, Novus Biologicals); HIF-1α (anti–HIF-1α 1:500, Novus Biologicals); and β-actin (1:5000, MP Biomedicals). Horse-radish peroxidase–conjugated antirabbit or antimouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL Kit, Amersham). To ensure equal protein loading, each membrane was stripped and reprobed with anti–actin antibody to normalize for differences in protein loading.

Statistical Analysis

To evaluate for differences in PET-defined hypoxic tissue between treatment groups, a commercially available statistical package (StatView, SAS Institute) was used. A p value ≤ 0.05 was defined as statistically significant.

Results

Clinical Outcomes

All animals included in the study had demonstrable tumor on postcontrast MR imaging and Cu-ATSM PET studies. However, at the time of MR and PET imaging, no animals demonstrated signs or symptoms of tumor mass effect (for example, weakness, poor grooming, > 20% weight loss). No adverse clinical effects were seen as a result of TSC or saline infusion in these animals.

Tumor Compared with Contralateral Brain Tissue on PET Imaging

The PET imaging studies demonstrated decreased uptake of Cu-ATSM within the C6 glioma compared with the contralateral cerebral hemisphere. All tumors demonstrated regions of hypoxia at the time of initial PET imaging. The mean relative uptake value of the tumor was 3900 (range 2203–6836). The mean relative uptake value of the contralateral brain tissue was 1017 (range 488–2304). Thus, the tumor tissue demonstrated a relative hypoxia 3.8 times greater than normal brain tissue (p = 0.000002, t-test).

Differences Between Tumor Tissue in TSC- and Saline-Infused Groups on PET Imaging

After infusion, the median volume of hypoxic tumor in the TSC group was 136 mm³ (Fig. 1 left), and the median volume of hypoxic tumor in the saline group was 192 mm³ (Fig. 1 right). The mean relative hypoxic tumor volume for the saline group (6 rats) was 1.01 ± 0.063 (mean ± SEM). The range for the saline group was 0.86–1.14. For the TSC-infused group (6 rats), the mean relative hypoxic tumor volume was 0.69 ± 0.062 (mean ± SEM). The range for the TSC group was 0.46–0.85. Treatment with TSC reduced the relative volume of hypoxic tumor by 31% (Fig. 2). This difference was statistically significant (p = 0.002, t-test).

Expression of CA9 and HIF-1α

Immunoblot revealed that all tumor specimens showed protein expression for CA9 and HIF-1α (Fig. 3). However, there was no consistent difference in the expression of CA9 and HIF-1α between animals at 24 hours after infusion of either TSC or saline.

Discussion

Trans sodium crocetinate has previously been shown to reduce hypoxia in gliomas and in stroke.10,20 It has also been shown to have promising therapeutic effects in a recent clinical trial (ClinicalTrials.gov Identifier NCT00725881) for reducing the effects of peripheral vascular disease.3 The addition of TSC appears to improve oxygenation in hypoxic tissue by facilitating oxygen diffusion in plasma.22
Fig. 3. Western blots showing the effect of TSC in a rat C6 glioma model. The C6 glioma cells (CCL-107 cell line, American Type Culture Collection) were implanted into 1 hemisphere of 5 rats (mean mass 200 g). Animals were treated with TSC (100 μg/kg). Equal amounts of protein (20 μg) from tissue lysates were separated by SDS-PAGE and immunoblotted with anti–HIF-1α and anti-CA9 antibodies.

Positron emission tomography imaging most frequently uses a glucose analog, FDG, to study a given tissue’s metabolic activity based on regional glucose uptake. The use of FDG-PET imaging has been proposed as a means of identifying tissues less responsive to radiation, and of targeting these tissues with an additional boost to cancerous lesions. However, other radionuclides have been introduced to study specific aspects of cellular activity. Copper-64 is one such radioactive nuclide that has proven useful for PET imaging; Cu-ATSM is preferentially taken up by hypoxic compared with normoxic cells. Copper-64 emits positrons in the cells that take up the tracer. Thus, within physiological systems such as the brain, it can provide real-time images of the oxygenation status of tissues in vivo.

In the current study, all tumors demonstrated a statistically significant uptake of Cu-ATSM compared with brain tissue in the contralateral cerebral hemisphere. Uptake of Cu-ATSM was 3.6 times greater on average in the tumor than in normal brain tissue. This underscores the significant hypoxia within the C6 glioma model. When animals were infused with TSC compared with saline, the relative hypoxia was lessened by approximately 31%. It is also conceivable that Cu-ATSM PET imaging could be used to provide targeting information for boost radiation therapy or radiosurgery to high-grade, hypoxic tumors such as glioblastoma. This type of approach with Cu-ATSM targeting has been shown to work for image-guided radiation therapy. It is important to validate this neuroimaging technique and the effect of TSC in other animal glioma models.

Expression of HIF-1α and CA9 stimulates a resistance of cells to hypoxic conditions and, in turn, makes cells more able to withstand adjuvant treatments such as radiosurgery. The expression of HIF-1 has correlated with radiation resistance of C6 xenografts. In the current research, we observed HIF-1α and CA9 expression in the C6 tumors. This is consistent with the previous findings that the C6 glioma is a hypoxic tumor model. At 24 hours postinfusion, there were no consistent differences between TSC- and control-treated animals. Evaluation of additional time points and varying administration (for example, amounts and frequencies) of TSC should be studied to determine its optimum effects in terms of hypoxic protein expression in glioblastoma. In particular, as opposed to the single TSC dose given in the current experiments, 5 doses of TSC were required to provide a survival advantage in this glioblastoma model. An additional amount or duration of TSC administration may be required to alter expression of certain hypoxic proteins.

Hypoxia in gliomas represents a significant therapeutic challenge. Tumor hypoxia in high-grade gliomas has been widely described, and it may be responsible for stem cell–induced glioma growth and tumor progression. Improvements in intratumoral oxygenation have led to therapeutic gains related to radiation therapy and chemotherapy.

Novel approaches to reduce tumor hypoxia have been tried. For instance, erythropoietin was shown to improve the response to radiation of GBM (glioblastoma multiforme) Nan1 and U87 cells xenografted to nude mice. Other clinical trials and animal studies in which recombinant human erythropoietin was used have had a mixture of responses, including some with significant clinical improvement. More recently, by having animals breathe carbogen, partial oxygen concentration in both tumor and contralateral brain tissue increased in an F98 glioma model. Interceding with hypofractionated radiation therapy (9.3 Gy in 4 fractions) in animals treated with carbogen led to a significant decrease in glioma tumor growth.

In a similar fashion, TSC infusion at or around the time of radiation delivery could be used to lessen intratumoral hypoxia and increase the therapeutic gains of radiation therapy or radiosurgery. Also, for those chemotherapeutic agents in which tumor hypoxia may lessen clinical efficacy, TSC may serve to improve the therapeutic response too. The optimal dose and timing of delivery to capitalize on TSC’s apparent lessening of glioma hypoxia during adjuvant therapy remains to be determined. Further investigation is underway with additional PET imaging studies to evaluate the effects of TSC given concurrently with Temodar. Determining the functional response of TSC with Temodar will be important, because Temodar is not given concurrently with standard and hypofractionated radiation therapy for high-grade gliomas.

Conclusions

The C6 gliomas consistently demonstrated significant intratumoral hypoxia compared with normal brain tissue. Based on Cu-ATSM PET imaging findings, TSC was noted to lessen intratumoral hypoxia by 31%. The reduction in tumor hypoxia, if achieved at the time of tumor irradiation, may afford a therapeutic gain. Further pre-
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clinical studies will need to be performed to determine the optimal dose and timing of TSC infusion as well as to characterize interactions between TSC and other pharmacological agents (for example, Temodar, Dilantin, and corticosteroids).

Disclosure

The TSC was provided as a gift from Diffusion Pharmaceuticals (Charlottesville, Virginia). The research was funded by a grant to the University of Virginia from Diffusion Pharmaceuticals. The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Sheehan. Acquisition of data: all authors. Analysis and interpretation of data: Sheehan, Popp, Lee, Park. Drafting the article: Sheehan, Lee, Park. Critically revising the article: Popp, Monteleth, Park.

Acknowledgments

The authors appreciate the assistance of Drs. Dongfeng Pan and Stuart Berr in the University of Virginia Department of Radiology; they assisted with the MR and PET imaging as well as final preparation of the Cu-ATSM.

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Manuscript submitted November 20, 2010. Accepted May 17, 2011. Please include this information when citing this paper: published online June 17, 2011; DOI: 10.3171/2011.5.JNS101954.

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