Featured Article

Therapeutic Efficacy of DTI-015 using Diffusion Magnetic Resonance Imaging as an Early Surrogate Marker

Daniel E. Hall,1,2 Bradford A. Moffat,1,2 Jadranka Stojanovska,1,2 Timothy D. Johnson,5 Zhuolin Li,5 Daniel A. Hamstra,4 Alnawaz Rehemtulla,1,2,4 Thomas L. Chen evert,1,2 Julie Carter,6 Dennis Pietronigro,6 and Brian D. Ross1,2,3

1Center for Molecular Imaging and Departments of 2Radiology, 3Biological Chemistry, 4Radiation Oncology, and 5Biostatistics, University of Michigan, Ann Arbor, Michigan; and 6Direct Therapeutics Inc., Redwood City, California

ABSTRACT

To investigate diffusion weighted magnetic resonance imaging as a quantitative surrogate marker for evaluating the therapy-induced cellular changes in an orthotopic experimental glioma model, tumors were treated with direct intratumoral administration of DTI-015, a solution of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in 100% EtOH. Intracerebral 9L tumors were induced in Fischer 344 rats, and three treatment groups were established: DTI-015, EtOH, and sham. Two groups of rats received intratumoral injection of either 67 mg/mL BCNU in EtOH or EtOH alone at 50% of the tumor volume up to a maximum of 30 μl under stereotactic guidance. Diffusion magnetic resonance images were acquired before treatment and after treatment at 1, 24, 48, and 72 hours and then 3 times per week thereafter. Tumor cell viability was examined using multislice diffusion weighted magnetic resonance imaging with diffusion weighted transverse magnetic resonance images and histogram plots of each tumor quantified over time. Control animals (EtOH- or sham-treated animals) showed mean apparent diffusion coefficients (ADCs) that remained essentially unchanged over the experimental time course. In contrast, rats treated with DTI-015 showed a significant increase in ADC relative to the pretreatment within 24 hours, which further increased over time, followed by a significant therapeutic response as evidenced by subsequent tumor volume shrinkage, development of a cystic region, and enhanced animal survival. Finally, not only were ADC measurements predictive of differences between treatment groups, but they also yielded spatial and temporal data regarding the efficacy of treatment within individual treated animals that could be used to guide subsequent therapy.

INTRODUCTION

The treatment of glioblastoma multiforme remains an intractable problem that is evident in the constancy of a 1-year median survival time from initial diagnosis in these patients over the past several decades (1). In vivo animal models have been used for decades to evaluate the efficacy of new brain tumor therapeutic approaches (2). Numerous studies have revealed that systemic 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) is a semieffective treatment for the rat 9L brain tumor model (3). Intravenously administered BCNU has been a leading chemotherapeutic agent for the treatment of glioma patients for the past 30 years (4). However, the clinical outcome of patients treated with systemic BCNU has offered only modest increases in patient survival (5) and is limited by both acute toxic effects of bone marrow and the gastrointestinal tract mucosal cells, along with delayed toxicity in liver and lungs (4, 6–8). Efforts to improve the tumor to normal tissue ratio of administered drugs or agents have involved intra-arterial dosing (9–11), convection enhanced delivery (12), and incorporation into slow release polymeric implants (13, 14).

A recent development in BCNU delivery to solid tumors has been reported that utilizes the approach of solvent-facilitated perfusion (SFP). DTI-015 is a formulation of BCNU in a water-miscible organic solvent vehicle (100% EtOH) that has been shown to facilitate the movement of solubilized BCNU through both water and cell membranes (15–19). Direct administration of DTI-015 into the tumor site yields substantially higher concentrations of BCNU within the tumor tissue than is possible with systemic injection, carotid artery infusion, or intratumoral administration in an aqueous phase (16, 18, 19, 20). Furthermore, although BCNU administered via biodegradable wafers at the time of resection does offer the advantage of targeted chemotherapy, the movement of BCNU is limited by diffusion and has been demonstrated to only penetrate a few millimeters into the underlying tissue (21). In contrast, evidence suggests that SFP using DTI-015 drives the tumor penetration of BCNU over centimeter distances (18). Furthermore, DTI-015 has been shown to significantly increase life span in rats with intracerebral 9L tumors (15), and a phase I/II trial of recurrent malignant gliomas has provided evidence for its antitumoral activity in patients (17). In this clinical study, the median survival time for recurrent inoperable glioblastoma multiforme patients after...
In the current study, we used anatomic imaging to establish the coordinates and dosage of the intratumoral injection of DTI-015 into rat 9L gliomas and to follow the changes in tumor volumes over time after treatment. In addition, diffusion MRI was used to quantitate early therapy-induced changes in tissue structure over time and also to provide spatial information related to cell kill within the tumor mass. Overall, DTI-015 treatment of intracerebral 9L tumors provided excellent therapeutic benefit in all animals studied, and diffusion weighted imaging (DWI) yielded vital information regarding the spatial and temporal response to therapy in this animal model.

**MATERIALS AND METHODS**

**Cell Culture.** Rat 9L glioma cells, obtained from the Brain Tumor Research Center (University of California, San Francisco, CA), were grown as monolayers in minimal essential medium supplemented with 10% fetal calf serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin at 37°C in a 95%/5% air/CO\(_2\) atmosphere. Cells were harvested and resuspended for tumor implantation.

**Animal Model.** Intracerebral 9L tumors were induced in male Fischer 344 rats weighing between 125 and 150 g as described previously (3). Briefly, 9L cells (10\(^5\) cells per 5 \(\mu\)L) were implanted in the right forebrain at a depth of 2.5 mm through a 1-mm burr hole. The surgical field was cleaned with 70% EtOH, and the burr hole was filled with bone wax to prevent extracerebral extension of the tumor. There were no animals lost either at implantation or during treatment.

**Treatment.** Orthotropic tumors in Fischer 344 rats were grown to approximately 40 to 90 \(\mu\)L in volume, as determined by T2-weighted images. Tumor volumes were calculated and used for determination of intratumoral injection volumes of DTI-015 (67 mg BCNU/mL EtOH) or vehicle as described in the phase I/II clinical trial of DTI-015 in recurrent high-grade glioma (17). MRI anatomic scans were also used to define the injection coordinates for insertion of the needle at the center of the tumor mass. Three treatment groups were established: DTI-015 (\(n = 4\)), EtOH (\(n = 5\)), and sham (\(n = 4\)). The DTI-015 group of rats received intratumoral DTI-015 delivered at a volume of 50% of the tumor volume up to a maximum of 30 \(\mu\)L under stereotactic guidance. The EtOH group of rats received intratumoral injection of 100% EtOH, using the same 50% tumor volume criteria up to a maximum of 30 \(\mu\)L. The sham group received intratumoral placement of the needle as another control arm. All intratumoral injections were given with the needle tip positioned in the center of the tumor mass with an infusion flow rate of 7.5 \(\mu\)L/minute.

**Diffusion Magnetic Resonance Imaging.** Images were acquired pre- and posttreatment at 1, 24, 48, and 72 hours and then 3 times per week thereafter on a Varian Unity Inova imaging system equipped with a 7.0-Tesla, 18.3-cm horizontal bore magnet and a quadrature bird cage coil (USA Instruments, Aurora, OH). For MRI examination, rats were anesthetized with an isoflurane/air mixture and maintained at 37°C inside the magnet using a heated, thermostatted circulating water bath. A single-slice gradient-echo sequence was used to confirm proper animal positioning and to prescribe subsequent imaging. An isotropic, diffusion weighted sequence (22) was used with two
interleaved $b$-factors ($\Delta = 1,148 \text{s/mm}^2$) and the following acquisition parameters: TR/TE = 3500/60 milliseconds, $128 \times 128$ matrix, and a 3-cm field-of-view. Thirteen 1-mm–thick slices separated by a 0.5-mm gap were used to cover the whole rat brain. The $z$ gradient first moment was zeroed to reduce the dominant source of motion artifact. To further reduce motion artifact, a 32-point navigator echo was prepended to each phase-encode echo. The phase deviation of each navigator echo relative to their mean was subtracted from the respective image echoes before the phase-encode Fourier transform. Isotropic ADC maps were calculated for each image set, and ADC pixel value histograms were generated from tumor regions of interest combined across slices.

The tumor boundary was manually defined on each slice using a region-of-interest tool, and then integrated across slices to provide a volume estimate.

Statistical Methods. One-way analysis of variance was used to compare pretreatment volumes and ADC between the three groups. Survival differences among the three groups are compared using the log-rank statistic. After 29 to 30 days, any animal surviving was sacrificed, and this time was taken to be the censoring time.

Posttreatment volume changes and ADC changes are analyzed using a mixed effects longitudinal model in SAS Proc Mixed. The models fit are as follows: volume = group + time + $t^2$ + time $\times$ group + $t^3$ $\times$ group + error, and ADC = group + time + $t^2$ + time $\times$ group + $t^3$ $\times$ group + error.

In these models, group is an indicator variable taking the three groups values (sham, EtOH, and DTI-015) and is the model intercept. Furthermore, each animal is given its own random intercept. Of primary interest in these models are the contrasts between the interaction terms. More specifically, the time $\times$ group and time$^2$ $\times$ group interactions that show changes in volume growth and ADC over time between the three different treatment arms.

RESULTS

Effects of DTI-015 on Tumor Water Diffusion Values using Magnetic Resonance Imaging. Magnetic resonance imaging was used to longitudinally follow and quantify the temporal effects of each intervention for all groups of animals. Serial T2-weighted magnetic resonance images were acquired and used to quantify tumor volumes and growth rates. ADC maps were then used to assess the water diffusion values for each group over time (Fig. 2A). These ADC maps reflect the spatial variations in water mobility throughout the tumor. High
signal intensity (bright regions) represents areas in which the cellularity has been significantly diminished due to therapeutic intervention (22). The distribution of ADC values for the entire three-dimensional tumor mass was also obtained from the multislice data sets and is displayed in a stacked histogram format (Fig. 2B). Stacked histograms provide the ability to assess overall changes in tumor ADC values over time. Because the area under the curve is proportional to tumor size, it also provides the ability to follow dynamic changes in the overall tumor volume.

Fig. 2A and B show data from a representative animal from each group over time. Sham tumor treatment, consisting of insertion of the needle into the center of the tumor mass without any injection, revealed no significant increase in tumor diffusion values until a small area of spontaneous necrosis that was unrelated spatially to the needle placement appeared 7 days later. The tumor growth rate also appeared unaffected in this sham-treated animal with a tumor doubling time of approximately 48 hours, which is consistent with previous reports (3). EtOH administration resulted in a small transient (1 day) increase in tumor ADC values after intratumoral dosage. This is reflected by a small regional increase in the signal intensity of the ADC maps for this animal shown in Fig. 2A, which quickly resolves by day 3 postadministration. A corresponding slight broadening of the ADC histogram for this animal was also observed (Fig. 2B) at 1 day after EtOH injection, which also resolved by day 3 along with a significant increase in overall tumor size. There was no significant increase in tumor doubling time when compared with the sham-treated animal, as witnessed by the rapidly increasing area under the curve for both sham- and EtOH-treated animals. In contrast, tumors treated with DTI-015 showed a significant increase in ADC values throughout the tumor mass beginning at 1 day after treatment (Fig. 2A), reflecting a massive kill of tumor cells (26). The increase in ADC values for the entire tumor mass for the DTI-015–treated animal is also displayed in Fig. 2B, which reveals a significant broadening of ADC values for this tumor along with a significant decrease in tumor volume over time, as witnessed by the flattening of the peak with a substantial decrease in the area under the curve.

Histologic sections taken from a slice approximately corresponding to the MRI scans of each animal from Fig. 2A are shown in Fig. 2C. The presence of a significant tumor burden for the sham- and EtOH-treated animals is easily observed along with minimal regions of necrosis. However, for the animal treated with DTI-015, the macroscopic analysis revealed a fluid-filled cystic region instead of a tumor mass, which is similar to previous findings (15). Further microscopic examination of this DTI-015–treated tumor demonstrated no viable tumor cells within the cystic region or within adjacent normal brain tissue.

**Effects of DTI-015 on Tumor Growth and ADC Values using Magnetic Resonance Imaging.** The data from Fig. 2 represent individual representative animals. To more carefully evaluate the effect of DTI-015 on intracerebral 9L tumors, volumes were quantified and compared with sham and EtOH groups, as shown in Fig. 3A. Administration of EtOH had a slight but insignificant effect on tumor growth rate over time. However, treatment of tumors with DTI-015 resulted in an inhibition of tumor growth within 2 days after treatment, with subsequent regression occurring over the next 20 days. Animals were sacrificed at the end of 30 days for histologic analysis, and only one out of the four DTI-015–treated animals had residual tumor regrowth.

Another approach for displaying quantitative tumor diffusion data is to calculate the ADC value for the entire tumor mass as a function of time for each animal and to sum these values for each group of animals and plot the mean percentage change in ADC values over time as shown in Fig. 3B. This figure reveals that in sham- or EtOH-treated animals, only minimal and transient changes in tumor diffusion values occurred in the first day after intervention. In contrast, tumors treated with DTI-015 revealed a 50% to 60% increase in their tumor ADC values as a result of this therapeutic intervention that persisted for >2 weeks after therapy.

**Animal Survival.** Overall, there was no significant difference in the pretreatment tumor volumes among the three groups of animals (P = 0.22). The DTI-015–treated group lived significantly longer than the sham and EtOH groups (P = 0.007 and P = 0.005, respectively), as shown in the Kaplan-Meier survival curve displayed in Fig. 4. Additionally, no statistically significant difference in survival between the sham and EtOH groups was found (P = 0.06). All DTI-015–treated animals survived for the entire experimental period, except for one animal that died of non-cancer–related death 2 days before the...
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Diffusion Magnetic Resonance Imaging as a Method to Assess Therapeutic Heterogeneity. To better understand why one of the DTI-015–treated tumors exhibited regrowth of the tumor mass, a careful retrospective examination of the ADC maps from the single DTI-015 animal that demonstrated recurrence was undertaken. Tumor growth was analyzed over time by taking a single slice through the tumor mass and overlaying the ADC values (in color) on the T2-weighted anatomic images as shown in Fig. 5A. This initial MRI revealed that the tumor developed with an elongated shape with a small lobe toward the ventral midline. After treatment, the major central portion of the tumor mass revealed significantly elevated mean ADC values, between 1.7 and 2.1 ADC units (yellow and red) at 3 to 6 days after treatment, as shown in Fig. 5B. However, the previously identified ventral tumor lobe demonstrated significantly smaller increases in tumor diffusion values, with a high value between 1.4 and 1.5 ADC units. From subsequent images, it is apparent that the tumor regrowth occurred from the small tumor lobe corresponding to the region that had a significantly reduced maximal diffusion change relative to the main tumor mass. The large tumor that recurred exhibited a shift of ADC back toward that witnessed before treatment (Fig. 5B, blue), suggestive of viable growing tumor, except for a slight rise near the end of the study coincident with the development of an area of central necrosis. This is consistent with our previous studies of BCNU therapy in the 9L model, where an ADC value of ≥1.7 was associated with complete loss of viable tumor cells on histologic analysis, whereas smaller ADC values were associated with subsequent tumor regrowth (22, 26).

DISCUSSION

This study was undertaken to evaluate the utility of MRI-guided direct intratumoral injection of DTI-015 in a rodent gliosarcoma model. In addition, DWI to monitor the early cellular changes in response to therapy was also used to determine whether it could predict not only differences between treatment groups but also heterogeneity within individual animals. Direct tumor-targeted therapies such as that used here are of great need in the treatment of primary brain tumors. The standard treatment for patients with high-grade malignant gliomas at this time is maximal surgical resection followed by external beam radiation therapy with or without adjuvant chemotherapy (1). Despite aggressive local treatment with surgery and radiation therapy, the majority of patients have recurrence of disease within the site of the initial tumor bed (30). Furthermore, the outcome for these patients remains dismal, with a median survival of less than 1 year from the time of diagnosis.

Systemic chemotherapy has been demonstrated to offer only minimal improvement in time to progression and overall survival with a recent meta-analysis of the randomized trials of adjuvant systemic chemotherapy in high-grade glioma, revealing only an absolute 6% increase in survival at 1 year and a 2-month increase in overall median survival from 12 to 14 months (5). Given the predominant local recurrence of high-grade gliomas with rare intra- or extracerebral spread, there was initial enthusiasm for targeted chemotherapy using carotid artery infusion (11), implanted biodegradable wafers (13), or direct injection using convection enhanced delivery with aqueous solutions (12). In theory, these would offer an enhanced tumor to systemic ratio of the chemotherapeutic agents and may provide both increased efficacy and decreased normal tissue toxicity.

Unfortunately, these treatments, to date, have been unable to provide significant advances over systemic chemotherapy. Intracarotid administration of BCNU only resulted in a 4-fold increase in tumor to systemic ratio for BCNU (31) and was associated with a high risk of neurologic complications or unilateral blindness (9, 10). Direct intratumoral injection of BCNU in an aqueous solvent has been demonstrated to result only in limited spread of the drug from the injected site and no significant improvement in therapeutic index when compared with systemic administration (20). Moreover, the solubility of BCNU in aqueous solution is very small versus EtOH. In addition, the volume of aqueous solution (10% EtOH) that can be injected into the tumor is also smaller as compared with absolute EtOH due to differences in tissue resistance to the different solvent systems. In fact, a recent report used a direct infusion of BCNU in buffer at a rate of 0.3 μL/minute for 2.2 hours for a total delivered volume of 40 μL (32). The concentration of BCNU in the aqueous formulation was 66 μg/mL versus our current study, which had a concentration of 67,000 μg/mL. Treatment of 9L tumors using SFP of DTI-015 provided cures in four of five animals with no central nervous system or systemic toxicity.
whereas the previous study using BCNU dissolved in buffer yielded no cures. Biodegradable BCNU containing wafers did produce a minimal 2.3-month increase in median survival over resection and radiotherapy alone in a recent randomized trial, but this is similar in nature to that seen when BCNU is administered systemically (5, 14). The limited response of the BCNU wafers may be due to the fact that there is minimal depth of penetration of BCNU from these wafers into the surrounding tissue (21), which most certainly is inadequate, given the infiltrative nature of high-grade diffuse gliomas (1).

SFP utilizes a water-miscible organic solvent that more easily moves through both water and cellular membranes to deliver the solubilized anticancer drug throughout the tumor. DTI-015 has been demonstrated to offer a 164-fold increase in BCNU-produced adducts within experimental rodent fibrosarcomas compared with systemic administration of the drug (16), and increased levels of DNA adducts were also identified in humans even up to 2 cm from the site of injection (18). Furthermore, despite this high local concentration, there was limited spread of DTI-015 into normal brain parenchyma (19). In addition, the increased levels of BCNU within the tumor after direct intratumoral administration of DTI-015 have translated into substantial responses in preclinical models (15, 20).

A previous report on the use of DTI-015 in a rodent gliosarcoma model, similar to the one used here, demonstrated a 400% increase in animal survival and a 44% long-term cure rate (15). One limitation of that study was that the injections were performed in an unguided manner through the burr hole used for tumor cell inoculation. In fact, this blind administration of DTI-015 may actually have underestimated the efficacy of the treatment because it was demonstrated that some tumors were largely missed by the treatment and resulted in minimal efficacy, unchecked tumor growth, and early demise. Indeed, histologic analysis revealed three main groups of animals: a first group in which only the top of the tumor was treated; a second group in which the center of the tumor was treated, but there were residual cells at the margins; and a final group in which no viable tumor cells were evident, and the tumor mass was replaced with a cyst. It is likely that only this final group had a direct injection within the tumor mass and experienced the full benefit of the therapy. This heterogeneity of response is seen in the survival curve, which has at least three different slopes (likely corresponding to partial tumor injection and partial response), with animals succumbing to disease before a plateau phase with long-term survival (corresponding to direct tumor injection and complete response; ref. 15).

Through the use of image-guided therapy, we were able to assure that the injection occurred within the center of the tumor in three-dimensional space. Because changes in tumor cellularity after treatment have been previously shown to correlate with changes in cell density/membrane integrity (26), we investigated the use of quantitating spatial changes...
in ADC values for assessing tumor response to DTI-015. In addition, by using DWI to follow the cellular changes in response to therapy, we, for the first time, document the rapid response of the implanted brain tumors to this therapy. As early as 24 hours after injection, there were statistically significant differences in the ADC values between the treated animals and both control groups that preceded volumetric differences by several days. The rise in ADC was uniform across the tumor in most animals, except for the one documented “near miss” due to a ventral tumor lobe outside the main tumor mass. The peak rise in ADC was similar to that seen previously using a much higher and more toxic systemic injection of BCNU (26). However, in that study, despite reaching a similar peak ADC value, it was maintained only for approximately 6 days, with a subsequent fall of ADC with an eventual return to the pretreatment values by 3 weeks after treatment, which coincided with tumor regrowth. Not surprisingly, in that study BCNU was only minimally effective when administered systemically, with no long-term survival. This is in contrast to the present study, in which ADC rose dramatically over the first week after treatment and was maintained at this elevated level for at least an additional 2 weeks. Because ADC represents the diffusibility of water, there is an upper limit to this value as one approaches a completely acellular environment; therefore, as treatments approach this value, it is not surprising that different treatments achieve similar peaks, but the duration of a sustained response is predictive of both increased cytotoxicity and durability of response (26). Furthermore, the elevation in ADC was not only predictive of improved efficacy between treated groups but also was predictive of heterogenous responses within individual animals as witnessed by tumor regrowth from a small ventral tumor lobe that exhibited only relatively mild changes in ADC after treatment. The spatial and temporal nature of DWI could potentially be used to offer retreatment to areas that were initially missed or were minimally responsive to therapy.

In conclusion, using image-guided intratumoral delivery of DTI-015 followed by close monitoring with DWI offers further evidence for the efficacy of DTI-015 in high-grade gliomas. In addition, the use of noninvasive imaging to follow cellular changes to therapy offers an exciting means to more rapidly and accurately predict clinical responses to DTI-015 and to direct other therapies within individual tumors. More importantly, this study provides further evidence for the utility of DWI for the evaluation of treatment response in brain tumors; this utility is not limited to preclinical models but can also be directly translated into clinical practice to facilitate the design and evaluation of further randomized trials using DTI-015 or other interventions for brain tumors (26–28).

REFERENCES


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