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The aims of this study were to determine whether these MRI parameters measured in vivo in 6 glioma models at the same tumor size and prior treatment could be predictive of the tumor response to carmustin (BCNU) and could describe the tumor phenotype and microvasculature.

The six glioma models exhibited different characteristics in terms of morphology and vasculature, as well as different responses to BCNU treatment. The chemosensitivity in vivo was not related to the in vitro sensitivity. When measured prior treatment, neither ADC, nor BVf nor VSI alone or in combination was predictive of the in vivo response to BCNU treatment in these glioma models. Our results indicate that the vascular network of a tumor cannot be described by only one parameter such as BVf. VSI provided added value to BVf, which depends on both vessel density and size. The tumor phenotype was better described by combining BVf with VSI and BVf with ADC than by any of these parameters alone. This emphasizes the need for a multiparametric approach to characterize brain tumors, for which MRI is a promising imaging modality.
From: Chantal Rémy  
Tél. +33 (0)4 56 52 05 89  
+33 (0)7 56 52 05 99 (Secr.)  
Fax +33 (0)4 56 52 05 98  
Chantal.Remy@ujf-grenoble.fr

To: Neuro-Oncology  
1515 Holcombe Blvd  
Unit 234  
Houston, TX 77030  
USA

Notre réf : CR/CR 2009-09

Grenoble, the 21st of September 2009

Dear Dr Anderson

Please find enclosed a manuscript entitled: “Multiparametric MRI characterization of six glioma models – Link to carmustine treatment outcome”, which we wish to submit for publication in Neuro-Oncology as “Basic and translational investigations”. The subject category that best describes the manuscript is “Imaging”.

On behalf of all co-authors, I declare that:
- The manuscript has been seen and approved by all co-authors.
- There is no conflict of interest regarding this work.
- Neither the submitted paper nor any similar paper, in whole or in part, has been or will be submitted to or published in any other printed or digital publication.

Novel findings of the study:
- The microstructure and vasculature of 6 glioma models were characterized using in vivo MR imaging and histology. Among them, 3 ones were human gliomas xenografted to nude rats (not to nude mice).
- Despite the same tumor size, the 6 glioma models differed in terms of morphology and vasculature, as well as of response to carmustine (BCNU) treatment. It shows that attention must be paid to relate the results of studies conducted on a single model to the particular characteristics of that model.
- None of the measured MRI parameters measured at the same tumor size and prior to treatment could be predictive of the tumor response to BCNU. Combination of 2 parameters provided better characterization of the tumor phenotype, however. This study emphasizes the need of multiparametric imaging approaches for brain tumors characterization.

We might suggest a reviewer to whom the manuscript might be assigned:
Prof. Rolf F. Barth, M.D.
Professor of Pathology
The Ohio State University  
165 Hamilton Hall  
1645 Neil Ave.  
Columbus, Ohio 43210

Tel. (614) 292-2177  
Fax (614) 292-7072  
rolf.barth@osumc.edu

With best regards

Chantal Rémy
Neuro-Oncology

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Multiparametric MRI Characterization of Six Glioma Models - Link to Carmustine Treatment Outcome

Benjamin Lemasson, Olivier Duchamp, Emmanuel Barbier, Peggy Provent, Xavier Tizon, Nathalie Just, Marion Coquand-Gandit, Christoph Segebarth, Nicolas Guilbaud, Boudewijn van der Sanden, Philippe Genne and Chantal Rémy

Inserm, U836, 38042 Grenoble Cedex 9, France (B.L., E.B., M.G., C.S., B.vdS., C.R.)
Université Joseph Fourier, Institut des Neurosciences, BP 170, 38042 Grenoble Cedex 9, France (B.L., E.B., M.G., C.S., B.vdS., C.R.)
Oncodesign Biotechnology, 20 Rue Jean Mazen, B.P. 27 627, 21 076 Dijon Cedex, France (B.L., O.D., N.J., P.P, X.T., P.G.)
Institut de Recherche Pierre FABRE, Centre de Recherche en Oncologie Expérimentale, Parc Technologique du Canal - BP 94244 - 31432 Toulouse Cedex, France (N. G.)

Running title
MRI characterization of glioma vasculature

Corresponding author
Chantal Rémy
Grenoble Institut des Neurosciences - U836
Université Joseph Fourier - Site Santé
BP 170
38042 Grenoble Cedex 9
France
Telephone: 33 (0)4 56 52 05 89
Fax: 33 (0)4 56 52 05 98
e-mail: Chantal.Remy@ujf-grenoble.fr
Abstract

Commonly used diagnostic factors have been shown to be essential but not sufficient to predict response to chemotherapy. Besides genetic alterations, the chemosensitivity of gliomas may be influenced by tumor microstructure and functional properties of the microvasculature, which can be assessed non-invasively using water apparent diffusion coefficient (ADC), blood volume (BVf) and vessel size (VSI) MRI.

The aims of this study were to determine whether these MRI parameters measured in vivo in 6 glioma models at the same tumor size and prior treatment could be predictive of the tumor response to Carmustine (BCNU) and could describe the tumor phenotype and microvasculature.

The six glioma models exhibited different characteristics in terms of morphology and vasculature, as well as different responses to BCNU treatment. The chemosensitivity in vivo was not related to the in vitro sensitivity. When measured prior treatment, neither ADC, nor BVf nor VSI alone or in combination was predictive of the in vivo response to BCNU treatment in these glioma models. Our results indicate that the vascular network of a tumor cannot be described by only one parameter such as BVf. VSI provided added value to BVf, which depends on both vessel density and size. The tumor phenotype was better described by combining BVf with VSI and BVf with ADC than by any of these parameters alone. This emphasizes the need for a multiparametric approach to characterize brain tumors, for which MRI is a promising imaging modality.

Key-words

MRI, microvasculature, glioma, angiogenesis, chemosensitivity
Cancer incidence is progressing worldwide with a high impact on mortality and healthcare costs. Current WHO’s estimations suggest that cancer-associated mortality will increase by 62% by 2030, requiring in the occidental countries, alone, hundreds of billions of dollars. Healthcare systems will need to cover directly 40% of these costs to take in charge patients and their treatments. On the other hand, medical cures in oncology are not efficient enough, with a mean positive response rate below 20%. New targeted therapies have to enhance the efficacy of treatments; nevertheless selection of responsive patients will be a requirement in the near future.

The development of biomarkers will lead to the dynamic of personalized medicine. They help fill the unsatisfied needs in oncology for cancer diagnosis, therapeutic strategy, patient selection, prediction of therapeutic response, and monitoring of the antitumoral treatment efficacy.¹

Commonly used diagnostic and prognostic factors, such as clinical, radiological and histological investigations, do not always predict tumor sensitivity effectively.²,³ In the case of gliomas, histology of the original tumor has been shown to be essential but not sufficient to predict response to chemotherapy.⁴ Besides, there is now growing evidence that some molecular and genetic alterations are relevant predictors of chemo sensitivity in gliomas, for example loss of chromosome 1p/19q in oligodendrogliomas and expression of O-6-methylguanine-DNA methyltransferase (MGMT) or epidermal growth factor receptor (EGFR) in glioblastomas.⁴,⁵ However the assessment of these biomarkers require biopsies of the brain tissue, which are obviously invasive, and sometimes result in injury or death of the patient. In addition, the biopsied tissue does not always represent the most relevant part of the tumor because only a small fragment of a heterogeneous tumor is sampled. Quantitative biomarkers of tumor physiology derived from in vivo imaging overcome these limitations and
have recently shown promise for prognosis, treatment outcome prediction and patient follow-up.6

Besides genetic alterations, the chemosensitivity of tumors may also be influenced by the presence of edema and hypoxic areas inside the tumor and by the uptake and diffusion properties of the chemotherapeutic agent within tumor tissue.5 Previous histological studies applied to several glioma models have suggested that structural and functional specificities of microvasculature might influence the chemosensitivity of these tumors.7 These microenvironmental properties can be assessed non-invasively using Magnetic Resonance Imaging (MRI).

First, diffusion-weighted MRI is a valuable tool to characterize tissue microstructure non-invasively on the basis of water diffusion properties. Measurement of the Apparent Diffusion Coefficient (ADC) of water has been shown to bring information on cell death and cell density.8 ADC values before treatment onset have been found predictive of therapy outcome for patients with rectal carcinoma,2,3 and in animal models with colon and mammary carcinomas,9,10 and gliosarcomas.11

Second, several studies using Dynamic Contrast-Enhanced (DCE) MRI have suggested that an index of perfusion, combining information about permeability and blood volume/flow, measured before treatment could be predictive of therapy success or failure in patients with rectal carcinoma,2,12-14 uterine cervical carcinoma,15 and with malignant glioma.16

Third, information on microvasculature can also be obtained using susceptibility contrast imaging, based on dynamic or steady-state techniques. In the clinic, monitoring the first pass of an i.v. injected bolus of small gadolinium-based contrast agents has been used since many years.17 However broadening of the arterial input function and/or alteration of blood brain barrier permeability might affect the accuracy of the measured parameters.18 More recently, steady-state susceptibility contrast imaging methods, based on transverse relaxation time
measurement before and after injection of an intravascular iron-based contrast agent, have been developed. Both dynamic and steady–state susceptibility MRI approaches allowed estimation of blood volume fraction (BVf) and information on microvessel radii distribution in the form of a vessel size index (VSI, µm).\(^\text{18-21}\) Only one study showed that susceptibility contrast imaging and diffusion MRI before treatment onset might be helpful in predicting chemosensitivity of a patient with a malignant oligodendroglioma.\(^\text{22}\)

The aim of this study was twofold (i) to characterize \textit{in vivo}, with MRI, tumor microstructure and microvasculature of six orthotopic glioma models in rats, at about the same stage of tumor development and (ii) to assess the potential value of ADC, BVf and VSI, alone or in combination, to differentiate tumor from normal tissue, to discriminate the 6 models and to predict tumor response before the onset of chemotherapy. For this purpose, 3 rat glioma models (GV1A1, 9L and C6) and 3 human xenograft models (CGL3, CGL9 and U-87 MG) which differed with regards to their chemosensitivity to BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea, an alkylating agent) were subjected to diffusion, BVf and VSI MRI, when mean tumor volumes reached 75 mm\(^3\).
Materials and methods

Cell lines

All cell lines except CGL3 and CGL9 were purchased from the American Type Culture Collection (Manassas, VA). Tumor cells were used between the 5th and 15th in vitro passage.

Rat glioma cell lines

- The GV1A1 cell line was established from a mixed glioma induced by N-ethyl-N-nitrosourea in a BD-IX rat.23
- The 9L cell line derived from a gliosarcoma induced by treatment with N-methylnitrosourea (MNU) of Fischer rats.24
- The C6 cell line derived from an astrocytoma induced by treatment with MNU of Wistar rats.25

Human glioma cell lines

- The CGL3 and CGL9 cell lines were generated in Oncodesign by mechanic dissociation of tumor fragments collected in Nude mice bearing subcutaneously implanted human glioblastomas, TG-8 and SNB-19 respectively (obtained from Dr MF Poupon, Curie Institute, Paris, France).
- U-87 MG cells derived from a human malignant glioblastoma / astrocytoma grade III tumor.26

Tumor cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine at 37°C in a humidified atmosphere (5% CO₂, 95% air).

In vitro studies

All cell lines were screened for their intrinsic sensitivity to a 96 hours BCNU treatment (Carmustine, Sigma-Aldrich St. Louis, MO, USA). Cells were seeded in 96-well plates (5000 cells/well, 180 μl of medium/well) and incubated at 37°C for 24 h in drug-free culture
medium. Tumor cells were then incubated for 96 h at 37°C with 9 concentrations (ranging from 6.25 to 1600 µM in 1/2 dilution steps) of BCNU. As negative control, cells were treated with corresponding vehicle alone. At the end of treatment, the cytotoxic activity of BCNU was evaluated using an MTT assay according to manufacturer’s instructions. The optical density was measured at 570 nm using an automated microplate reader (Victor 1420, Perkin Elmer, Waltham, MA, USA). Experiment was repeated three times and performed in quadruplicate for each cell line and BCNU concentration.

Dose-response curves were plotted using XLFit3 software (IDBS, United Kingdom). The index of cytotoxicity for 50% inhibition of cell proliferation (IC₅₀) values was calculated from semi-log curves using XLFit3 software.

Animal tumor models

Animal experiments were performed according to the European ethical guidelines for animal experimentation, and the English guidelines for welfare of animals in experimental neoplasia. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Pharmacy and Medicine University (Dijon, France).

BDIX, Fisher F344 and Wistar male rats (200–250 g; Charles River, L’Arbresle, France) were used as host for GV1A1 (n = 13), 9L (n = 14) and C6 (n = 14) syngenic glioma models respectively, and male RH-rnu/rnu Nude rats (180-220g, Harlan Sprague Dawley, Indianapolis, USA) were used for the three human xenogenic tumor models CGL3 (n = 11), CGL9 (n = 17) and U-87 MG (n = 15). Nude rats were prepared for xenograft by whole-body irradiation with a ⁶⁰Co γ-source (7 Gy), 24-48 h prior to human tumor cell inoculation. Animals were anesthetized with an intraperitoneal injection of Ketamine at 75 mg/kg (Ketamine500®, Centravet, France) and Xylazine at 5 mg/kg (Rompun®, Centravet, France) in 0.9% NaCl solution and immobilized in a stereotactic frame (David Kopf Instrument, Germany). A suspension containing 10⁵ cells in 5 µl serum-free RPMI1640 medium was
inoculated in the right caudate nucleus by stereotactic injection through a 1-mm burr hole (2.5 mm lateral to the bregma and 4.5 mm in depth from the dura). The surgical field was cleaned with 70% ethanol. The injection was performed slowly over 15 minutes, and the needle was withdrawn over another 5 minutes. The burr hole was filled with bone wax to prevent extracerebral extension of the tumor. The day of tumor cell implantation was considered as D0.

**In vivo studies**

For each tumor type, tumor-bearing rats were randomized into 3 groups when the mean tumor volume (measured on MRI T2-weighted images) reached 75 mm³. A first group (n=4 to 7) was used for MRI parameters evaluation and tumor collection for histological analysis. MRI imaging was performed on randomization day and tumors collected just after imaging. A second group (n=3 to 5) was treated i.v. with BCNU at 10 mg/kg (two administrations separated by a 14-day rest period), the first treatment being performed on randomization day. A third group (n=3 to 5) was used as control (i.e. no BCNU treatment). Control and BCNU-treated rats were monitored for survival, behavior and body weights. Rats were euthanized when any clinical sign appeared or when body weight had decreased of more than 20% from randomization day.

The number of rats in each group and days of BCNU administration are reported in Table 1. The number of rats in MRI groups and randomization days are indicated in Figure 1.
MRI experiments

All the MRI experiments were performed on a horizontal 2.35 Tesla (40 cm diameter) magnet equipped with actively shielded gradient coils (Magnex Scientific Ltd., Oxford, UK) and interfaced to a SMIS console (SMIS Ltd, Guildford, UK).

The rats were anesthetized with 5% isoflurane for induction, 1-1.5% for maintenance in 70% air / 30% oxygen. Throughout the experiment, the rat body temperature was monitored by a rectal probe and maintained at a physiological level (37°C±1°C) by a heating pad placed under the abdomen. The tail vein of the rat was equipped with a heparinized NaCl-containing catheter. The rat was placed in a plastic stereotactic head-holder and introduced inside the magnet.

After preliminary adjustments, 15 coronal spin-echo T₂-weighted (T₂w) MR images (TR/TE = 2000/80 ms; voxel size = 234x468x1000 μm) were acquired for anatomical imaging and tumor volume measurement. To map water ADC, 1 reference image and 3 diffusion-weighted spin-echo images with diffusion gradients applied in X, Y, and Z directions were acquired successively (b value = 500 s/mm², TR/TE = 2000/80 ms; 15 coronal slices; voxel size = 234x468x1000 μm). To map BVf and VSI, a multi gradient echo and spin echo sequence (TR = 6000 ms, 6 gradient echoes with TE = [9.3 – 17.6 – 25.9 – 34.2 – 42.5] ms, 1 spin-echo with TE = 100 ms, voxel size = 234x468x1000 μm) was performed just before and 4 minutes after injection of an intravascular contrast agent (Sinerem®, Guerbet, Roissy, France; Combidex®, AMAG Pharmaceuticals Inc, MA, USA, 200 μmol Fe/kg) in the tail vein. Sinerem® is a dextran-coated iron-based contrast agent of about 20 nm in diameter.

Histology

At the end of the MRI examination, animals were euthanized 1 minute after intravenous injection of a 0.3 ml Hoechst 33342 saline solution (11 mg/ml, Sigma-Aldrich, Inc.). Hoechst
33342 was used for the detection of perfused vasculature and characterization of blood-brain barrier (BBB) integrity. Brains were quickly removed (within 3 min after the rat’s death), frozen in liquid nitrogen then stored at -80°C to prevent the Hoechst from diffusing further into the tissue. For each rat, two adjacent coronal slices (10 µm thick) were cut at mid-tumor (where the tumor section was the largest).

To analyze the morphology of the tumor, the first section was stained with haematoxylin/eosin (HE) and observed by light microscopy using a Nikon Eclipse E600 microscope equipped with an Olympus ColorView video camera and an image analysis system (analySIS, Olympus Soft Imaging System, Münster, Germany).

The second section was placed on the fluorescence Nikon Eclipse E600 microscope (346 nm excitation, 460 nm emission) to observe the perfused vessels (Hoechst). Then the section was fixed in 4% buffered paraformaldehyde and the unspecific sites were saturated in PBS-Tween 0.01%-BSA 3% for 1 h at room temperature. The section was incubated overnight at 4°C with the primary antibody: 1/100 dilution of a goat polyclonal antibody against collagen IV (Southern Biotechnology Associates, Birmingham, AL, USA). This step was followed by incubation with the secondary fluorescent antibody for 2 h at room temperature: 1/100 dilution of a donkey antibody directed against goat IgG and linked to Alexa Fluor 546 (Molecular Probes, Eugene, OR, USA) in PBS with 0.5% BSA. Immunohistological staining of collagen IV, marker of the vessel basal lamina, allowed the detection of all vessels (perfused and nonperfused). They were visualized using a filter with excitation at 556 nm and emission at 573 nm.

Mosaic images were reconstructed for HE staining and for each fluorophore, after scanning the required number of fields of view to cover the entire tumor (X10 magnification, pixel size = 0.76 µm) using a digital image analysis and an automated scanning stage.30
Data analysis

Determination of tumor volume: Tumor volumes were obtained by manually delineating the tumor on T₂-weighted MR images from adjacent slices, counting the voxels within the tumor boundaries, and scaling with the voxel volume.

MRI parameter maps: All maps were computed using a program developed in-house within the Matlab 7 environment (The MathWorks, Inc., Natick, NA). ADC was computed as the mean of the ADCs observed in the three principal directions of the gradient system. Changes in transverse relaxation rates (ΔR₂* and ΔR₂) due to Sinerem® were obtained, respectively, from gradient echo and spin echo signals acquired before and after injection of Sinerem® as described by Tropres et al.²¹ BVf maps were computed from ΔR₂* maps and the increase in blood to tissue susceptibility value (Δχ=0.57 ppm).²¹ VSI MRI maps were computed from ΔR₂* and ΔR₂ maps, Δχ and mean ADC values determined from tumor and contralateral regions of interest (ROI) in each tumor model.

ADC, BVf and VSI MRI measurements: Tumor ROIs were obtained by manually delineating the tumor on the 3 T₂w images containing the largest tumor area. Contralateral ROIs were drawn in the contralateral striatum on the same slices. Each ROI was transferred on ADC, BVf and VSI MRI maps. Then, within each ROI and each map, voxels for which the analysis could not be performed were identified (e.g. voxels with non converging, with negative values and values outside the range of validity of the method – ADC > 3500 µm².s⁻¹; BVf > 17%, VSI MRI > 50 µm). Those voxels were excluded from the analysis.

Quantitative histological analysis: The analysis was performed on microscopic fields (between 3 and 6) on the section where the tumor was the largest. Collagen IV images were binarized (threshold manually defined) and vascular parameters (mean vessel density, fractional vascular surface (VS), distribution of vessel radii) were obtained using the
To allow comparison between MR and histological data, VSI$_{histo}$ was derived as described in Tropres et al.$^{29}$

**Antitumor efficacy evaluation of BCNU treatment:** The day of rat death or the day of euthanasia for any ethical criteria was monitored. The efficacy of BCNU treatment was evaluated by calculation of the increase life span (ILS). ILS% was determined as:

\[
\left(\frac{\text{MST}_{\text{control}} - \text{MST}_{\text{BCNU}}}{\text{MST}_{\text{control}}}\right) \times 100
\]

where MST is the median survival time.

**Statistics**

Paired student t-tests were used for tumor versus contralateral comparison. An independent t-test was used for comparison between gliomas. A p<0.05 was considered significant. All statistics were performed with SPSS (SPSS Inc, Chicago, Ill, USA).
Results

**MRI and histological characteristics of each tumor model**

At the time of analysis, tumor volumes were around 75 mm$^3$, which allowed comparing the different models at a similar stage of development. Due to different growth kinetics between tumors, this corresponded to different delays after tumor cell inoculation (D9-11 for GV1A1 and 9L, between days D17 and D23 for C6, U-87 MG and CGL9, D49 for CGL3).

Figure 1 shows examples of T$_2$w morphological images, ADC, BVf and VSI$_{MRI}$ maps from a representative rat for each glioma model. Figure 2 and Table 2 present the ADC, BVf and VSI$_{MRI}$ values of the different tumors and their contralateral counterpart. Similarly the histological parameters and representative HE and collagen IV stained sections for each tumor model are shown in Table 3 and Figure 3 respectively. Quantitative analysis of the collagen IV immunostaining was not possible for the 9L and the CGL9 tumors, due to technical problems and high background signal respectively.

Common features of all tumor types, as shown on HE staining (Fig. 3), were a high cell density and the presence of edema. Edema at the periphery of the tumor appeared more pronounced for the 9L, CGL9 and U-87 MG tumors.

**GV1A1 model**

The GV1A1 tumors grew very rapidly (mean survival time = 13.3 ± 0.6 days) and reached a mean volume of 75 mm$^3$ within 9 days after tumor cell implantation. At this tumor growth stage, the GV1A1 tumor margins were sharply delineated on MR images and on HE staining, with some obvious tumor cell foci in the contiguous brain tissue on HE staining. The contrast of the GV1A1 tumors on T$_2$w images and ADC maps was very heterogeneous. On average, ADC was significantly higher in the tumor (1343 ± 40 µm$^2$.s$^{-1}$) than in the contralateral tissue (801 ± 43 µm$^2$.s$^{-1}$), and represented the highest value of the studied tumor types. The hyperintense ring observed on ADC maps was due to the development of pseudo-cysts,
frequently observed for this type of tumor at late growth stages and readily visible on the HE staining. The high mean ADC value in this tumor type might be partly caused by the presence of these pseudo-cystic regions. Cells with large nuclei were detected within and around the pseudo-cystic regions and might correspond to inflammatory cells. Enlarged vessels characterized the GV1A1 tumors as shown on both collagen IV staining and VSI MRI maps, leading to higher VSI_{histo} and VSI_{MRI} in the tumor than in the contralateral tissue (VSI_{histo}: 6.7 ± 0.1 vs 3.9 ± 0.3 µm, VSI_{MRI}: 14.0 ± 1.7 vs 6.1 ± 1.0 µm). The vessel density in the tumor was much lower than in the contralateral tissue (131 ± 69 vs 280 ± 27 mm^2) while the mean tumor BVf (2.5 ± 0.4 %) was slightly (but significantly) lower than the contralateral tissue (3.0 ± 0.4 %). Vascular surface exhibited the same tendency than BVf. Hœchst and collagen IV fluorescence indicated that all tumor vessels were perfused and most of them, especially the largest ones, were permeable (Hœchst staining area larger than that of collagen IV).

Little is known in the literature about this glioma model, especially regarding its morphological and vascular characteristics. This model has been mainly used to study the immunogenic response of the host with regards to glioma.\textsuperscript{23,33}

\textbf{9L model}

Unlike the GV1A1 model, the 9L model is a widely used experimental rat brain tumor model.\textsuperscript{34} The mean survival time of the 9L implanted rats was 15.0 ± 1.0 days. A 75 mm^3 volume was obtained within 11 days. Like GV1A1, the 9L tumor margins were sharply delineated on MR images and on HE staining, even though many tumor cell foci were clearly visible in the contiguous brain tissue on HE staining. 9L tumors were homogeneously hyperintense on both T\textsubscript{2}w images and ADC maps (tumor vs contralateral: 1210 ± 10 / 762 ± 13 µm\textsuperscript{2}.s\textsuperscript{-1}). These tumors were characterized by the presence of two types of cells, which differed in arrangement and in shape of nuclei. Some cells with round nuclei were
arranged in clusters, while the spindle-shaped cells with oblong nuclei and forming large bundles had a sarcomatoid appearance. These cells were embedded in collagen-rich stroma (as shown by the high noise level in the collagen IV sections). All these morphological characteristics were consistent with the literature.\textsuperscript{24,35-37} The ADC values in 9L gliosarcoma were always found around 1.4 times higher than in the contralateral tissue.\textsuperscript{36,37} Our ADC values were similar to those reported by Hall et al.\textsuperscript{38} Both BVf and VSI\textsubscript{MRI} were higher in the 9L tumors (4.0 ± 0.3 % and 8.6 ± 1.3 µm) than in the contralateral tissue (2.7 ± 0.2 % and 5.1 ± 0.5 µm). This was consistent with the qualitative analysis of immunohistological data. The vessel density in 9L tumors was reduced compared to the contralateral tissue (but not as dramatically as in the GV1A1 tumors) and the vessel diameters in the tumors were clearly larger than contralaterally. Most vessels were perfused and most of the perfused vessels were permeable. In the literature, the 9L gliosarcoma is described as a highly vascular tumor model with no sign of necrosis or ischemia.\textsuperscript{24,35} Using a stereological approach, Pathak et al.\textsuperscript{39} measured vascular volume in the 9L tumor and in the contralateral tissue (5.29 ± 1.67 % and 1.90 ± 0.39 %, respectively), which is of the same order than our BVf.

**C6 model**

The C6 model is also widely used in experimental neuro-oncology.\textsuperscript{34} The C6 tumors grew slower than the GV1A1 and 9L tumors and exhibited a greater variability in tumor growth. The mean survival time was 28.3 ± 6.8 days. A 75 mm\textsuperscript{3} volume was obtained within 17 days. Like GV1A1 and 9L models, the C6 tumors appeared relatively well delineated on MR images as well as on HE staining, even though focal invasion into the contiguous brain tissue was obvious on HE staining. At this growth stage, the C6 tumors appeared relatively homogeneous in hypersignal on T\textsubscript{2}w images and ADC maps. The ADC values measured for the C6 tumors and contralateral tissues (1151 ± 80 µm\textsuperscript{2}.s\textsuperscript{-1} vs 791 ± 42 µm\textsuperscript{2}.s\textsuperscript{-1}) were close to those of the 9L model. The C6 tumors were characterized by the presence of few small...
necrotic foci, surrounded by small cells with dense nucleus and typical pseudopalisadic aspect of multiform glioblastoma. All these morphological characteristics were in agreement with the numerous studies describing the C6 glioma model.\textsuperscript{40} Compared to GV1A1 and 9L models, the C6 tumors exhibited the lowest vessel density ($32 \pm 13$ mm\(^2\)) and the highest VSI\textsubscript{h} (9.5 ± 1.5 µm) and VSI\textsubscript{MRI} (18.2 ± 1.7 µm). The C6 BVf (2.4 ± 0.4 %) tended to be lower than the contralateral one (2.7 ± 0.2 %) but, unlike the vascular surface, the difference was not significant. Most C6 vessels were perfused and the biggest ones were highly permeable. BVf, VSI\textsubscript{MRI} and VSI\textsubscript{h} values were similar to those previously reported\textsuperscript{29,41} and in good agreement with histological studies from Farrell et al.\textsuperscript{42} At the growth stage analyzed in the present study, the BVf values were somewhat heterogeneous in the tumor tissue but not as much as at later stages, where the periphery appeared hypervascularized unlike the tumor core.\textsuperscript{29,41}

\textbf{CGL3 model}

CGL3 tumors grew very slowly compared to all other tumor types. The mean survival time was 66.3 ± 5.0 days. A 75 mm\(^3\) volume was obtained within 49 days. CGL3 tumor margins were not as well delineated on HE staining than for the other tumor types. CGL3 tumors were homogeneous in hypersignal on T\textsubscript{2}w images and ADC maps. ADC values in CGL3 tumors (959 ± 80 µm\(^2\).s\(^{-1}\)) were significantly higher than contralateral ones (707 ± 65 µm\(^2\).s\(^{-1}\)). Cell densities lower than that of the other tumor types characterized CGL3 tumors, with cell nuclei of irregular shape. The size of CGL3 vessels (VSI\textsubscript{h} = 5.7 ± 1.6 µm, VSI\textsubscript{MRI} = 8.5 ± 0.9 µm) tended to be higher than in the contralateral tissue (VSI\textsubscript{h} = 4.4 ± 0.2 µm, VSI\textsubscript{MRI} = 6.7 ± 0.7 µm) but the difference was not significant. On collagen IV staining, it could be observed that the distribution of vessel diameters was different from all rat tumors: most vessels exhibited similar or slightly larger diameter than the contralateral vessels and only few of them were evidently large. BVf in CGL3 tumors
(4.0 ± 0.7 %) was slightly but significantly higher than contralaterally (3.2 ± 0.7 %), while the vascular surface was not significantly different. All vessels in CGL3 tumors were perfused but not permeable, similarly to normal brain vasculature.

**CGL9 model**

The mean survival time of CGL9 bearing *Nude* rats was 33.4 ± 1.9 days. A 75 mm³ volume was obtained within 23 days. The aspect of CGL9 tumors was very different from the other tumor types on MR images as well as on HE and immunohistological staining. On T₂w images and ADC maps tumors appeared respectively in hypersignal and in isosignal surrounded by a thin ring in hyposignal, surrounded itself by a thin ring in hypersignal. CGL9 tumors exhibited a unique aspect on HE sections compared to other tumor types and were highly infiltrative: the tumor core contained areas of normal tissue, which varied in size. Islets of tumor cells with round nuclei of different sizes were scattered among bundles of elongated tumor cells with oblong nuclei, seemingly streaming in a radial pattern with parallel bundles of normal tissue, which could be recognized by the presence of white matter bundles and a more intense staining with erythrosine, similar to normal brain tissue. A first ring of tissue with a cell density closer to that of normal tissue and the presence of numerous white matter bundles characterized the tumor margin. These features corresponded to the hyposignal observed around the tumor core on T₂w images and on ADC maps. A second ring of highly edematous tissue surrounded this margin and correlated with the ring in hypersignal on MR images. Another characteristic of CGL9 tumors was the high expression of collagen IV, which prevented quantitative analysis of blood vessels with our method. Unlike the preceding models, CGL9 ADC values (809 ± 30 μm².s⁻¹) were not significantly different from that of the contralateral tissue (738 ± 56 μm².s⁻¹). Both BVf and VSI_MRI were higher in the tumor (4.4 ± 0.3 % and 12.0 ± 1.0 μm) than in the contralateral tissue (2.8 ± 0.4 % and 5.7 ± 0.3 μm). Despite the high background due to overexpression of collagen IV, the density
of vessels appeared slightly reduced compared to the contralateral tissue but their diameter was larger. In addition, the orientation of CGL9 vessels was not random as in the other tumors: preferential longitudinal orientation from the center to the periphery of the tumor, mimicking a stellar organization. Hoechst histological data suggested that only a part of the vessels were perfused and most of them were permeable.

**U-87 MG model**

The U-87 MG high grade glioma is widely used for brain cancer research in orthotopic murine models, to a lesser extent in orthotopic rat model. The mean survival time of U-87 MG implanted rats was $26.5 \pm 1.9$ days. A $75 \text{ mm}^3$ volume was obtained within 17 days. U-87 MG tumors were well delimited on T$_2$w images and on HE staining. U-87 MG tumors were homogeneous in hypersignal on T$_2$w images. Unlike the other tumor models, the U-87 MG tumors were hardly detectable on ADC maps and, consistently, their ADC values $(734 \pm 44 \mu\text{m}^2\cdot\text{s}^{-1})$ were not significantly different from the contralateral ones $(706 \pm 43 \mu\text{m}^2\cdot\text{s}^{-1})$. A previous study by Sun et al. measured a higher ADC in U-87 MG glioma implanted in Nude mice brains $(1030 \pm 20 \mu\text{m}^2\cdot\text{s}^{-1})$ than in the contralateral tissue $(730 \pm 30 \mu\text{m}^2\cdot\text{s}^{-1})$. This discrepancy might be due to differences in cell batch, host animal origins, or tumor development stage. In a recent longitudinal study, we observed that the ADC of the U-87 MG glioma at early tumor growth stage was slightly higher than in the contralateral tissue and decreased towards the contralateral tissue ADC as tumor size became comparable to that observed in the present study (unpublished data). On HE staining, the U-87 MG tumors were very homogeneous with a high cell density and some tumor cells were aligned along vessels, forming thin bundles. The tumors were edematous but did not present any necrosis, or pseudo-cysts. Vessel density in U-87 MG tumors $(241 \pm 44 \text{ mm}^2)$ was similar to that of the contralateral tissue $(256 \pm 27 \text{ mm}^2)$. Vessel size was slightly higher in the tumor than in the contralateral vessels, the difference being significant on collagen IV
staining but not in MRI (p=0.54). BVf in U-87 MG tumors (5.3 ± 0.1 %) was significantly higher than in the contralateral tissue (2.3 ± 0.2 %). All vessels were perfused and permeable. In the literature, the U-87 MG orthotopic murine model is described as non-necrotic tumor with the most profuse vascularization,\textsuperscript{44} which is consistent with the present data obtained in *Nude* rats.

**Antitumor activity of BCNU**

*In vitro cytotoxicity of BCNU*

The mean IC\textsubscript{50} determined for each cell line are shown in Table 4. *In vitro*, cell lines showed slightly different sensitivity to BCNU (from the less sensitive to the more sensitive: CGL9, U-87 MG, C6, CGL3 ≈ GV1A1, 9L).

*In vivo antitumor activity of BCNU*

The mean and median survival times for both control and BCNU-treated rats, and the increased life spans (ILS) for each tumor type are presented in Table 1. The lowest efficacy of BCNU treatment was found for U-87 MG and 9L tumors (no increase of the median and mean survival times). An ILS of around 30% was obtained for C6 and CGL3 models and 123% for GV1A1 tumors. BCNU treatment was the most efficient for CGL9 with an ILS higher than 230%.
Discussion

The aims of this study were to characterize tumor microstructure and microvasculature in 6 glioma models and to evaluate if MRI parameters reflecting the status of tumor vasculature, measured at the same tumor size and before treatment onset could describe the tumor phenotype and microvasculature and could be predictive of response to a chemotherapeutic agent.

For this purpose, the study was conducted using 3 rat and 3 human glioma models orthotopically implanted in the brain of rats. The 3 rat glioma models as well as the U-87 MG human glioma model were chosen as they have been extensively used in preclinical studies in oncology. We also included two human glioma models that were developed at Oncodesign because of their unique response and structural properties. The animals were treated with BCNU, a standard of care chemotherapeutic agent in the clinic for the treatment of gliomas.

Heterogeneity of glioma models

Only few studies in the literature have compared different glioma models. The genetic similarities and differences between the 4 most used rat glioma cell lines and human gliomas have been assessed. Tumor vascularization was also characterized in several glioma models using quantitative immunohistology. More recently, histopathological features of 3 xenografts and 1 syngeneic model were compared to those of spontaneous gliomas in dog. One recent paper reviews the most common syngeneic rat glioma models. In the present study, different morphological and vascular characteristics were found between the 6 glioma models. It shows that attention must be paid to relate the results of studies conducted using only one model to the particular characteristics of this model.
Heterogeneity of morphological characteristics assessed by histology

At the targeted tumor growth stage (i.e. mean tumor volume of about 75 mm$^3$), each glioma model presents typical morphological characteristics, almost allowing their identification in blind observation using immunohistology. Necrosis and pseudo-cysts are characteristic of glioblastomas, but only one glioma model, C6, showed some necrotic foci at this growth stage, a phenomenon that occurs later in all other models. Only GV1A1 exhibited pseudo-cysts at this tumor growth stage, which can also be found in high grade gliomas and might be correlated to inflammation. Clusters of gliomatous cells embedded between bundles of sarcomatoid cells characterized the 9L model. In the U-87 MG model, some tumor cells appeared aligned along vessels. CGL3 tumors were characterized by faint HE staining compared to other models, irregular shaped cell nuclei and absence of BBB disruption, which is generally observed in low-grade gliomas. Unlike the other models, the CGL9 was highly infiltrative, similarly to most human malignant gliomas.

Heterogeneity of microstructure and microvascular characteristics assessed by MRI

No significant difference in ADC, BVf and VSI$_{MRI}$ was found between contralateral tissues of the different models despite strain differences (Wistar, Fischer and Nude rats). Besides, the tumor phenotype could not be identified with certainty for all tumor models with only one of these measured parameters. Using ADC, BVf or VSI$_{MRI}$ alone, there were always two tumor models for which parameter values were similar to the contralateral ones (BVf for C6 and GV1A1, ADC for CGL9 and U-87 MG, VSI$_{MRI}$ for U-87 MG and CGL3). However figures 4C and 4D show that scatterplots of BVf vs ADC or VSI$_{MRI}$ vs BVf can be used to discriminate tumor from healthy tissue. To date, most clinical studies have assessed the value of BVf or ADC separately for diagnosis and great overlap has been found between normal tissue and tumors, between different tumor types and between tumor grades.$^{47,48}$ We show that the use of a combination of BVf, ADC or VSI$_{MRI}$, together with multivariate analysis
tools, is worthy of being further investigated as a biomarker for diagnosis and also for tumor recurrence identification.

**Microstructure characteristics**

An inverse correlation between ADC and cell density in tumor tissues has often been reported in several studies.\(^8\) Low ADC in highly cellular tumor has been related to a restricted diffusion due to decreased free extracellular water volume, through an increase of cell density. However, in several clinical and experimental studies including ours,\(^41,48-51\) this correlation was not observed. ADC values are much higher in 9L than in U-87 MG tumors, although both models are highly cellular. On the contrary, CGL3 and U-87 MG models exhibited similar ADC values with very different cell density. Besides cell density, ADC values in glioma models might depend on other factors such as edema, necrosis, water exchange between intra- and extra-cellular compartments, and extracellular matrix composition.\(^52\)

**Microvascular characteristics**

All tumors exhibited similar or higher BVf and VSI\(_{MRI}\) than the contralateral tissue. These results are consistent with those of Schlageter et al. obtained on 5 different brain tumor models and using quantitative histology.\(^7\) For 4 models (GV1A1, C6, CGL3 and U-87 MG), it has been possible to perform quantitative analysis of fractional vascular surface and VSI\(_{histo}\) on brain sections after immunohistological labeling of the vessel basal lamina. A good correlation was found between the VSI values obtained by MRI and histology (Fig. 4A) and between BVf and fractional vascular surface (Fig. 4B). MRI provides VSI estimates larger by a factor about 2 however. This is consistent with previous studies,\(^29,41\) which found estimates of VSI larger when measured by MRI than when obtained by histology. This discrepancy may be caused by numerous biases in histology as well as in MRI. Histological estimates take into account all vessels while MRI estimates rely on perfused vessels only. Sample
preparation for histology might induce vessel dilation or compression. A 10 µm-thick histological slice may not accurately represent a 1 mm-thick MRI slice across a heterogeneous tumor. MRI estimates may be biased by the difference between the spatial configuration of real vessels (especially in tumors) and the idealized configuration (perfect cylinders) used in modeling or by macroscopic field inhomogeneities. It is hard to conclude whether the “true” values of the microvascular parameters are nearer to one or to the other experimental values (see Valable et al. for a more detailed discussion). However the good correlation between parameters determined by MRI and by histology suggests that they reflect similar microvascular characteristics, showing a good potential for MRI to assess these characteristics noninvasively.

Correlations between BVf and microvessel density have been previously reported in gliomas. In the present study, BVf values measured in the 6 glioma models were similar or higher than the contralateral tissue BVf. This does not necessarily mean that these tumors are as well or better oxygenated than healthy brain tissue. In some of these tumors (C6, GV1A1, CGL9 for example), vessel density (qualitatively or quantitatively assessed) was clearly lower than in the contralateral tissue. Thus many tumor cells were far from blood vessels suggesting that these tumors were probably not well oxygenated. They exhibited a normal or high BVf because the lower vessel density was compensated by a larger vessel size. Some tumors might also have a poor oxygenation status despite a normal BVf (and despite a normal vessel density) if the blood within the vessels is static (no blood flow) or if the blood flowing in the vessels is not well oxygenated. Thus BVf alone cannot be considered as a good marker of the oxygenation status of a tumor. Additional MRI accessible estimates like VSI\textsubscript{MRI}, tumor blood flow, blood oxygenation and vessel permeability are required to fully characterize \textit{in vivo} the vascular and oxygenation status of a tumor.
In vitro and in vivo sensitivity to BCNU

In vitro sensitivity to BCNU of the 6 tumor cell lines were similar (i.e. in the 10 – 200 µM range). However, in vivo studies showed that mean and median survival times did vary between tumor cell lines implanted in the brain of rats, despite a similar tumor size at treatment start. As an example, BCNU induced the complete healing of CGL9 tumor bearing rats (despite in vitro IC₅₀ higher than 150 µM), while it remained ineffective on 9L tumors (despite an in vitro IC₅₀ of around 30 µM). These results indicate that in vitro sensitivity was not predictive of the in vivo tumor sensitivity to the chemotherapeutic agent.

Several clinical studies have highlighted the heterogeneity in the response to treatment within the same pathological subgroup.⁴ The present study shows similar heterogeneity in responses to BCNU treatment between the 6 glioma models, in vitro and in vivo. In addition, BCNU responses in vitro and in vivo were not correlated (Tables 1 and 4). The CGL9 model, which was the less sensitive to BCNU in vitro, was the most sensitive in vivo and vice versa for the 9L tumor. The heterogeneity in the response to treatment observed in vitro and in vivo might be related to different genetic characteristics of the 6 tumor cell lines used⁴⁵,⁵⁷ and to microenvironmental factors. For example, in human glioblastomas, MGMT activity and EGFR expression have been found correlated to the sensitivity to alkylating agents.⁴,⁵ Other factors related to tumor microenvironment, such as low oxygenation, a consequence of poor perfusion and insufficient blood vessel supply, may exert a selective pressure leading to different genetic characteristics and thus different response to therapy from the original cultured cells. In addition, low perfusion and blood brain barrier permeability might contribute to poor local drug delivery after intravenous administration and thus to the different responses observed between the different tumor models and between in vitro and in vivo results.
A second result brought by our study is that no correlation was found between the ILS and the pre-treatment value of any MR parameter, alone or in combination, showing that these parameters were not predictive of treatment response. Canonical correlation analysis was performed to try and find the best linear combination of $VSI_{MRI}$, ADC and BVf that could correlate with ILS, yielding a highest correlation coefficient of $r^2 = 0.2$. In the literature, low pre-treatment ADC value was found predictive of therapeutic efficacy (chemotherapy alone or combined with radiotherapy) for human rectal carcinoma and for experimental colon and mammary carcinoma. Regarding brain tumors, two studies reported a predictive value of pretreatment ADC, but chemosensitivity was associated to a low ADC values in case of human oligodendroglioma and to high ADC value in case of a 9L glioma subcutaneously implanted in rats. An index of perfusion, combining information about permeability surface area product and blood volume/flow, measured before treatment onset was found of predictive value for therapy outcome (radiotherapy or chemoradiotherapy) in patients with rectal carcinoma and uterine cervical carcinoma. Conversely, none of the parameters measured before treatment using DCE-MRI was found of predictive value for head and neck cancers. For brain tumors and before treatment onset, low value of a parameter combining blood volume and extravascular extracellular volume was predictive of response to radiotherapy in human gliomas while high blood volume was found predictive of chemosensitivity in oligodendroglioma.

The low predictivity of pretreatment MR parameters we observed might be due to differences between experimental and human tumors (most studies from the literature were performed on human tumors), to different end-points (ILS in our study versus tumor size at different time points after treatment onset in most studies) or to the type of tumor (brain tumor versus other tumor types). If pretreatment ADC, BVf and $VSI_{MRI}$ do not appear to be predictive markers of chemotherapy response for brain tumors, several studies have shown their value when
measured after treatment onset as early markers of therapeutic efficacy. This will be the subject of further investigations in our group.

**Conclusion**

In conclusion, the present study shows that the six studied glioma models exhibited different characteristics in terms of morphology and vasculature, as well as different responses to BCNU treatment, despite a similar tumor size. This variability has to be taken into account in the design of preclinical studies to test the efficacy of anticancer drugs against glioma models.

When measured before treatment onset, neither ADC, nor BVf nor VSI\textsubscript{MRI} alone or in combination were predictive of the \textit{in vivo} response to BCNU treatment in these glioma models. Other parameters such as blood flow, vessel permeability, hypoxia status or interstitial fluid pressure might be pertinent to relate antitumoral effects to changes in tumor vasculature.

Our results also indicate that the vascular network of a tumor cannot be described by only one parameter such as BVf. VSI\textsubscript{MRI} was also found of interest, as BVf depends on both vessel density and size. In the present experiment, the tumor phenotype was better described by combining BVf with VSI\textsubscript{MRI} and BVf with ADC than by any of these parameters alone. This emphasizes the need for a multiparametric approach to characterize brain tumors, for which MRI is a promising imaging modality.

**Acknowledgements**

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References


of tissue water as determined by diffusion-weighted 1H-NMR spectroscopy in vivo. 


Figure captions

Figure 1: Heterogeneity between tumor models observed by MRI.
Examples of T2-weighted morphological images (T2w), ADC, BVf and VSI MRI maps from a representative rat for each glioma model. D9(6) means that imaging was done 9 days after tumor cell injection on 6 rats. The 6 glioma models exhibited different microstructure and vascular characteristics.

Figure 2: Comparison of mean values of the MRI estimated parameters between tumor models and with contralateral tissue.
The MR parameters ADC (A), VSI MRI (B) and BVf (C) were estimated for each glioma model and the contralateral tissue regions of interest. The data are expressed as mean ± SD for each group. *: p<0.05, between tumor and contralateral tissue. All MR parameters are similar in contralateral tissue between the 6 models, unlike the tumor tissues, which appeared very different.

Figure 3: Heterogeneity between tumor models observed by histology and immunohistology.
Representative photographs of the whole tumor for each tumor model stained with HE (A), collagen IV (B) and Hoechst (C). For each staining, higher magnifications of characteristic properties are showed in insert. All tumor models exhibited different cellular and vascular characteristics.
Black scale bar = 50 µm and white scale bar = 200 µm

Figure 4: Correlations between MR and histological parameters or between MR parameters
Scatter plots of histological parameters vs MR parameters: VSI\textsubscript{histo} vs VSI\textsubscript{MRI} (A) and vascular area vs BVf (B). One point represents either one tumor model (black symbols) or one contralateral tissue (white symbols). This correlation has been possible only for 4 glioma models. The black line illustrates the best linear fit for tumor measurements only.

Scatter plots of MR parameters in 2 of the 3 possible projection planes. Scatter plot of BVf as a function of ADC (C). Scatter plot of VSI\textsubscript{MRI} as a function of BVf (D). Human and rodent tumors are identified (black circles and black triangles). Tumor and contralateral mean values over all models are presented as white and black diamonds respectively. A discriminant line (dot line) between tumor and healthy brain (open circle) is displayed. It was computed using Linear Discriminant Analysis and shows the increased classification power brought by multiparametric MRI.
<table>
<thead>
<tr>
<th>Tumors</th>
<th>Treatment Groups</th>
<th>Number of rats</th>
<th>treatment days</th>
<th>Mean survival time (days)</th>
<th>Median survival Time (days)</th>
<th>ILS %</th>
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<td>D9, D23</td>
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<td>Control</td>
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<tr>
<td>CGL9</td>
<td>Control</td>
<td>5</td>
<td>D23, D37</td>
<td>33.4 ± 1.9</td>
<td>&gt;113*</td>
<td>&gt;230%</td>
</tr>
<tr>
<td></td>
<td>BCNU</td>
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<td></td>
<td>&gt;113*</td>
<td>&gt;113*</td>
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<td>D17</td>
<td>26.0 ± 2.9</td>
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</table>

**Table 1:** Number of rats in each group, and days of BCNU administration (D0 is the day of tumor cell injection), mean (± SD) and median survival times (in days) for Control and BCNU-treated groups, and increase life span (ILS).

* CGL9 tumor-bearing rats were terminated on D113 (no tumor development observed at necropsy).
<table>
<thead>
<tr>
<th>Tumors</th>
<th>n</th>
<th>ADC (µm².s⁻¹)</th>
<th>BVf (%)</th>
<th>VSI (µm)</th>
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<td></td>
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<td>Tumor</td>
<td>Contralateral brain</td>
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<td>GV1A1</td>
<td>6</td>
<td>801 ± 43</td>
<td>1343 ± 40</td>
<td>3.0 ± 0.4</td>
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<td>4</td>
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<td>1210 ± 10</td>
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<td>7</td>
<td>706 ± 43</td>
<td>734 ± 44</td>
<td>2.3 ± 0.2</td>
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Table 2: Quantitative MRI data. Apparent Diffusion Coefficient (ADC), Blood Volume fraction (BVf) and Vessel Size Index (VSI) in the contralateral striatum and in tumor, n indicates the number of animals in each group. Data are expressed as mean ± SD.
Table 3: Quantitative histological data: Vessel density, VSI<sub>histo</sub> and vascular surface determined for each tumor model on contralateral striatum (n<sub>1</sub> animals) and tumor (n<sub>2</sub> animals).
<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Tumor Origin</th>
<th>IC₅₀ (µM)</th>
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<tbody>
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<tr>
<td>U-87 MG</td>
<td>Human</td>
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</table>

**Table 4:** In vitro cytotoxicity (determined from 3 independent experiments) of a 96 h BCNU treatment measured by MTT assays (Mean ± SD IC₅₀) in a panel of 3 rats and 3 human glioma cell lines.
Figure 4

A) $y = 0.303x + 3.374$, $R^2 = 0.812$

B) $y = 3.238x - 6.336$, $R^2 = 0.900$

C) $VSI_{MRI}$ vs. $VSI_{hist}$

D) $ADC$ vs. $BVf$