Dynamic Imaging of Emerging Resistance during Cancer Therapy

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Abstract
One of the greatest challenges in developing therapeutic regimens is the inability to rapidly and objectively assess tumor response due to treatment. Moreover, tumor response to therapeutic intervention in many cases is transient, and progressive alterations within the tumor may mask the effectiveness of an initially successful therapy. The ability to detect these changes as they occur would allow timely initiation of alternative approaches, maximizing therapeutic outcome. We investigated the ability of diffusion magnetic resonance imaging (MRI) to provide a sensitive measure of tumor response throughout the course of treatment, possibly identifying changes in sensitivity to the therapy. Orthotopic 9L gliomas were subjected to two separate therapeutic regimens, with one group receiving a single 5-day cycle (1ω) of low-dose 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and a second group receiving two cycles at the same dose, bisected with 2 days of rest (2ω). Apparent diffusion coefficient maps were acquired before and throughout treatment to observe changes in water mobility, and these observations were correlated to standard measures of therapeutic response and outcome. Our results showed that diffusion MRI was indeed able to detect the emergence of a drug-resistant tumor subpopulation subsequent to an initially successful cycle of BCNU therapy, leading to minimal gains from a second cycle. These diffusion MRI findings were highly correlated with tumor growth delay, animal survival, and ex vivo growth inhibition assays showing emerging resistance in excised tumors. Overall, this study highlights the ability of diffusion MRI to provide sensitive dynamic assessment of therapy-induced response, allowing early opportunities for optimization of therapeutic protocols. (Cancer Res 2006; 66(9): 4687-92)

Introduction
Although advances in therapeutic intervention, such as surgical techniques, radiation therapy, and chemotherapy, have provided some success in managing brain neoplasms, the prognosis for highly aggressive tumors, such as glioblastoma multiforme, is still poor, with a median survival time of 9 months, and only 5% to 10% of patients surviving past 2 years (1). Both neurosurgery and radiotherapy have proven to be effective methods in treating gliomas; however, adjuvant chemotherapy has yielded mixed results and is still a subject of controversy (2). Studies have shown that tumors consist of subpopulations, which exhibit variable characteristics, growth rates, and drug sensitivities (3–5), which may account for variable and unpredictable response to chemotherapy. Even with optimal tumor response, there is a high rate of tumor recurrence of increased malignancy, which is frequently refractory to further treatment. A number of studies suggest that the treatment-refractory nature of these recurrences could result from previous chemotherapy (6–8). With tumors being such a dynamic entity, optimal therapeutic benefit presumably would require proficient management of the therapeutic regimen to account for these changes. This would require early, sensitive, and progressive assessment of therapeutic response.

Diffusion magnetic resonance imaging (MRI) has emerged as a powerful tool that has garnered attention as a quantitative approach for assessment of tumor response to therapeutic intervention (9). This technique exploits water mobility within tissues as a biomarker that highly correlates with cellularity (10–12). Destruction of tumor cells in response to therapy leads to a change in water mobility that is detectable by diffusion MRI. Moreover, these microscopic changes in the diffusibility of water occur well in advance of currently used macroscopic indicators of tumor status, such as changes in volume, performance status, or survival. Our studies, among others, have shown the capability of this approach to provide sensitive and rapid prospective assessment of therapeutic response, which has proven to be invaluable in the evaluation and development of experimental therapies (9, 12–18).

In this study, we employed the well-characterized orthotopic 9L glioma model as a system to evaluate therapeutic response to different 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) dosing regimens. Historically, this model has been shown to be an effective system for preclinical evaluation of therapeutic strategies, including BCNU. This study focused on the ability of diffusion MRI to provide a quantitative comparison of therapeutic response between subjects given either one 5-day cycle of BCNU given at 2 mg/kg/d (1ω), or two similar cycles of BCNU therapy separated by a 2-day rest period (2ω). Our results showed that not only did diffusion MRI provide an early prediction of therapeutic outcome, but was also sensitive enough to immediately detect the loss of therapeutic response to BCNU. These results were shown to correlate with survival and with ex vivo determination of the emergence of drug resistance in the treated tumors. These findings show that diffusion MRI is an effective tool for dynamic assessment of the dynamic changes in the sensitivity of a tumor to fractionated therapy.

Materials and Methods
9L animal models. Rat 9L glioma cells were obtained from the Brain Tumor Research Center at the University of California at San Francisco. The cells were grown as monolayers in 10-cm2 sterile plastic flasks in DMEM with 10% fetal bovine serum, 100 IU/mL penicillin and 100 mg/mL streptomycin, and 2 mmol/L L-glutamine in an incubator held at 37°C and 7.5% CO2.
containing stained with sulforhodamine B (Sigma, St. Louis, MO). A microtiter plate untreated cells reached confluence, at which time the cells were fixed and the cells were treated with serial dilutions of BCNU ranging from 200 to reach eight times the initial volume compared with the control group. The initial pretreatment volume was also determined and subtracted by the

time for tumors from each treatment group, cessation of the experiment. The mean time for the control group tumors to grow to reach eight times the initial volume was determined. Subsequently, the mean time for tumors from each treatment group, 10 and 20a, to reach eight times the initial pretreatment volume was also determined and subtracted by the mean time of the control group to obtain the tumor growth delay value. Tumor growth delay represents the difference in days for treated groups to reach eight times the initial volume compared with the control group.

In vitro cytotoxicity assays. Growth inhibition was evaluated using the sulforhodamine B assay as previously described (22). Briefly, 9L cells were seeded at a density of 4,000 per well in 96-well plates. After 24 hours, the cells were treated with serial dilutions of BCNU ranging from 200 to 0.1 μmol/L or vehicle in complete medium. Cells were allowed to grow until untreated cells reached confluence, at which time the cells were fixed and stained with sulforhodamine B (Sigma, St. Louis, MO). A microtiter plate reader was used to measure the absorbance at 490 nm (V_max; Molecular Devices, Sunnyvale, CA). Data plotted represent the mean and SD of 20 BCNU (n = 8) and vehicle controls (n = 2) with each cell line done in quadruplicate.

Results

Diffusion-weighed MRI detects cellular response to fractionated BCNU therapy. Representative serial ADC maps (color overlaid on anatomic image) of controls (vehicle only), 10 and 20a-treated animals are shown in Fig. 1. Pretreatment ADC maps were similar, indicating that the treatment groups were well matched for ADC values. However, whereas tumor diffusion of the control animals remained essentially unchanged over time, an appreciable increase in ADC was observable in the BCNU-treated animals over the first 6 days. Moreover, a progressive decrease in diffusion (shift towards blue) was observed in both 10 and 20a groups that started on day 8, which subsequently reached and remained at baseline until the end of the experiment. Interestingly, despite receiving a second cycle of BCNU therapy, the 20a group similarly decreased in diffusion as with the 10a group through qualitatively comparing representative images from each group. Visual comparison of the serial MRI images also qualitatively revealed a modest retardation of tumor growth rate in BCNU-treated animals compared with the vehicle control group.

Figure 1. Anatomic images with color ADC maps from representative tumors over course of therapy. Normalized quantitative color ADC maps overlays of representative vehicle control tumor (left), 10a group (middle), and 20a group (right) from days 0 to 14 of therapy. Color intensities are directly related to ADC values (ADC units of 1 x 10^-3 mm²/s), with blue representing low diffusion and red representing high diffusion. The ADC value of the tumor in the control animal remained relatively constant throughout the study, whereas both 10a and 20a BCNU-treated tumors showed a much greater increase in tumor diffusion throughout treatment.
Figure 2. Mean ADC changes from serial acquisitions throughout therapy. Normalized ADC values from each group were plotted as a function of time. Arrows, 2 mg/kg BCNU injections, where the first cycle was given from days 0 to 4, and animals receiving a second cycle were injected from days 7 to 11. Changes in mean ADC of control groups remained fairly constant, whereas both BCNU-treated groups showed a significant rise in ADC after the initial cycle of BCNU therapy. Note that animals receiving a second cycle of BCNU therapy showed a progressive decrease in ADC throughout the second course of BCNU therapy.

Figure 2 presents quantitative analysis of the entire imaging data set. These data show a significant increase in the mean ADC beginning on day 2 after initiation of BCNU administration and peaking on day 6 in the 1o and 2o groups with values of 18% and 15% increase, respectively. Comparatively, control animals displayed a slight 3% increase in ADC on day 2, which subsequently subsided to baseline values and remained fairly constant throughout the rest of the experiment. Observed differences between control and treated groups were confirmed to be statistically significant as early as day 4 (1o, P < 0.04; 2o, P < 0.04), and statistical significance was maintained on day 6 (1o, P < 0.01; 2o, P < 0.01) and day 8 (1o, P < 0.03; 2o, P < 0.02). As expected, there was no significant difference in mean ADC change between the 1o- and 2o-treated groups over the first 8 days (P > 0.2, days 2-8). The mean ADC values of the 1o group started to decrease at day 7 probably due to completion of therapy and perhaps repopulation of tumor cells. Interestingly, although the second phase of BCNU therapy for the 2o group was initiated, a progressive drop in ADC was also observed starting on day 7 and progressed throughout the second cycle of dosing (days 7-11). This indicated that a decrease in extracellular space was occurring over time, which would be consistent with repopulation of the tumor mass, possibly due to the emergence of BCNU resistance.

Comparison of heterogeneity in tumor response using IDM analysis. In addition to monitoring mean ADC changes, evaluation of tumor treatment-induced heterogeneity in ADC values was accomplished for the 1o and 2o groups. The IDM approach provided a means to quantitatively analyze the heterogeneity of tumor response by tracking regional variations in diffusion (23). By comparing day 4 and 8 ADC maps to pretreatment images, representative scatter plots from vehicle controls (Fig. 3 A-D), 1o group (Fig. 3B and E), and 2o group (Fig. 3C and F) were generated to represent changes in ADC within specific voxels. The red, green, and blue data points represent the three different tumor regions based on IDM analysis of ADC change. Qualitatively, Fig. 1A and D shows that there was minimal increase in ADC for vehicle controls, whereas a larger fractional increase in ADC, as depicted by a more pronounced red region, was observed in both 1o and 2o groups. The percentage of the tumor falling into this region was then calculated to provide a quantitative measure for comparison between groups. In the control group, 11.7 ± 6.4% and 13.1 ± 1.5% of the tumor voxels were found to have an increase of at least 20 ADC units at days 4 and 8, respectively (Table 1). However, the 1o and 2o groups exhibited large increases with a mean of 37.6 ± 3.4% and 35.0 ± 4.1%, respectively, at day 4, which were both statistically significant versus controls (1o, P < 0.05; 2o, P < 0.05) but were not statistically significant to each other (P > 0.3). At day 8, significant regions of tumor response were also apparent in the 1o (33.5 ± 14.5%, P < 0.05 versus control) and 2o (26.1 ± 2.4%, P < 0.05 versus control) groups. More importantly, although the 2o group received a second cycle of BCNU therapy, this also failed to produce significant additional response over the 1o group based on IDM analysis (P > 0.2). This is consistent with the earlier finding, which showed minimal differences in mean ADC values at the same time point. Additionally, comparison of days 8 to 4 indicates a slight...
survival advantage (survival were determined as the time of survival from onset of therapy until Figure 4. Kaplan-Meier survival curves. Kaplan-Meier survival curves for animal shrinkage times of control versus 1\textsuperscript{o} group and 2\textsuperscript{o} group were analyzed using the fDM approach to determine the average percentage of tumor voxels exhibiting a significant increase (red pixels) in ADC (>20 \times 10^{-3} mm\textsuperscript{2}/s).

decrease in mean values for both 1\textsuperscript{o} and 2\textsuperscript{o} groups, correlating with the dynamic shift in ADC means. This decrease in ADC values suggests a decline in tumor responsiveness of the 2\textsuperscript{o} group to BCNU, although daily treatments were still being given.

**Minimal therapeutic gain with 2\textsuperscript{o} as compared with 1\textsuperscript{o}**. Therapeutic benefit of 1\textsuperscript{o} and 2\textsuperscript{o} dosing regimens were assessed using tumor volume measurements and animal survival. By using the low b factor images to measure tumor volumes (as described in Materials and Methods), changes in growth kinetics due to therapeutic intervention were calculated and represented as tumor growth delays. Comparing the treatment groups to control, 1\textsuperscript{o} and 2\textsuperscript{o} groups exhibited significant tumor growth delays of 4.05 \pm 0.25 days ($P < 0.001$ versus control) and 3.55 \pm 0.52 days ($P < 0.001$ versus control), respectively. However, significant differences in tumor growth delay between the 1\textsuperscript{o} and 2\textsuperscript{o} groups was not achieved ($P > 0.3$). Additionally, a Kaplan-Meier plot of animal survival probabilities, shown in Fig. 4, was generated by measuring the time differential between initiation of therapy to animal death due to tumor burden. The median survival time of control, 1\textsuperscript{o} group, and 2\textsuperscript{o} group was 9, 12.5, and 13 days, respectively. A Mantel-Cox log rank test was done, indicating statistical significance between survival times of control versus 1\textsuperscript{o} group ($P < 0.04$) and control versus 2\textsuperscript{o} group ($P < 0.001$). However, the 2\textsuperscript{o} group did not show improved survival compared with animals treated with a single cycle of BCNU ($1\textsuperscript{o}, P > 0.4$). This finding indicates that the inclusion of a second cycle of BCNU therapy had no discernible benefit although presumably still adding to systemic toxicity. Although therapeutic outcome is dependent on many factors, the results presented here show that diffusion MRI is predictive of therapeutic outcome by providing early detection of a loss in tumor response during the second treatment cycle as evidenced by changes in mean ADC values and fDM analysis.

**Ex vivo BCNU sensitivity assays reveal acquired drug resistance in BCNU-treated tumors**. As previously described, ADC values in 2\textsuperscript{o}-treated animals failed to increase during the second cycle of therapy and were shown to correlate with a lack of additional therapeutic benefit. To investigate whether the lack of response in mean ADC during the second cycle of BCNU in the 2\textsuperscript{o} group was due to the emergence of a BCNU-resistant population, tumor cells were isolated from vehicle control tumors as well as tumors that had undergone two cycles of BCNU treatment. The sensitivity of these cells to BCNU was then evaluated using the sulforhodamine B growth inhibition assay to determine possible alterations in BCNU sensitivity of treated tumors compared with controls. As shown in Fig. 5, cell lines derived from 2\textsuperscript{o}-treated animals exhibited resistance to BCNU compared with control tumors. Calculated IC\textsubscript{50} showed a 2-fold decrease in sensitivity of the BCNU-treated tumors compared with vehicle controls (150.4 \pm 1.5 and 73.3 \pm 4.9 \mu mol/L, respectively). This observed increase in IC\textsubscript{50} of BCNU-treated tumors is comparable with previous reports, where 9L tumors have been shown to acquire resistance to chemotherapy following in vivo BCNU therapy (24).

**Discussion**

Current methods for gauging tumor response to therapy rely either on volumetric change over the course of therapeutic intervention or alterations in survival as indicators of efficacy. Our previous studies have validated the use of diffusion MRI as a non-invasive biomarker for evaluating anticancer efficacy (12, 13, 25). This approach has been shown to be universally applicable to a wide variety of models (11, 14, 16, 17, 26, 27) and is highly sensitive because microscopic alterations in tissue structure and physiology result in significant changes to water movement within the microenvironment (28, 29). Moreover, these have shown that the changes detected by diffusion MRI precede detectable alterations in tumor size or growth kinetics. Although these studies have validated the use of diffusion MRI for preclinical studies, only recently have studies provided direct clinical evidence that diffusion MRI is predictive of therapeutic outcome in patients with primary brain tumors (12, 30, 31). In our present study, we

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NOTE: Animals from control, 1\textsuperscript{o} group, and 2\textsuperscript{o} group were analyzed using the fDM approach to determine the average percentage of tumor voxels exhibiting a significant increase (red pixels) in ADC ($>20 \times 10^{-3} \text{mm}^2/s$).
A 2-fold shift in IC50 was observed in tumors from the 2t o20 for BCNU sensitivity. Using a previously described assay (Materials and Methods), cell lines were grown in varying BCNU concentrations ranging from 1 to 200 μmol/L and were then fixed to determine differences in total protein. A 2-fold shift in IC50 was observed in tumors from the 2o group (150.4 ± 1.9 and 73.8 ± 4.9 μmol/L, respectively).

show the use of diffusion MRI for allowing dynamic access into understanding therapeutic response by serially observing changes in ADC. The implications of this work provide a new direction for dynamic management of therapy where the ability to rapidly assess therapeutic efficacy during treatment could provide an opportunity to optimize initial regimens.

To test the use of diffusion MRI as a noninvasive, sensitive readout for optimization of dose and schedule of cancer therapy, we evaluated two separate schedules of BCNU therapy in an orthotopic 9L tumor model. In this study, diffusion MRI predicted initial therapeutic response, but more importantly, also detected a loss of tumor response during ongoing therapy. As shown in Fig. 2, there was a statistical difference in mean ADC change between BCNU-treated groups and control. However, comparison of the two treatment groups showed only slight differences in ADC profiles. The 1o group did have a more pronounced decline in ADC at the end of therapy compared with the 2o group, suggesting perhaps that a second cycle of therapy produced some level of tumor response. However, statistical significance of mean ADC change between the treated groups was never achieved throughout the course of the experiment. Nonetheless, this mirroring of ADC profiles between the 1o and 2o groups correctly predicted comparable tumor response. This provided the first indication that although the 2o group received a much higher total dose of BCNU, the more aggressive therapy did not lead to greater tumor cell death compared with the 1o group. Moreover, the decrease of ADC values in the 2o group before and throughout the second cycle of therapy indicated that the initial cycle of BCNU therapy altered tumor responsiveness to BCNU cytotoxicity during the second cycle.

fDM analysis of ADC change has been validated as an invaluable clinical tool for stratifying tumor response and predicting patient outcome in cases of primary brain neoplasms (29, 30). More importantly, the fDM approach provides an avenue to account for heterogeneity inherent of solid tumors and their response to therapy (23). Using this methodology, a quantitative assessment of therapeutic tumor response is obtained by measurements of the changes in regional fluctuations in ADC values within the whole tumor. In this study, we showed (Fig. 3; Table 1) that treated tumors displayed a significant tumor response versus controls, as evidenced by an increase of the tumor fraction with elevated ADC values. However, no statistical difference in tumor response, based on fDM analysis, was observed between the 1o and 2o groups at day 4 (P > 0.3). Moreover, at day 8, whereas therapy was still in progress for the 2o group, similar tumor response was observed between the 1o and 2o groups, suggesting that the second cycle of therapy provided minimal therapeutic benefit over tumors receiving only a single cycle.

Tumor growth delays and a Kaplan-Meier plot of animal survival were generated to ascertain the therapeutic value obtained from two cycles of BCNU therapy (2o) versus a single cycle (1o). As previously described, although treated groups showed a significant tumor growth delay compared with control tumors (1a, 4.05 ± 0.25 days; 2o, 3.55 ± 0.52 days), no significant difference was discernable between the 1o and 2o groups (P > 0.3). Moreover, as shown in Fig. 4, the 2o group exhibited negligible survival advantage compared with the 1o group, where median survival times were 13 and 12.5 days, respectively. This correlated with the diffusion data where a progressive decrease in mean ADC change during the second phase of therapy showed a decrease in cellular response to BCNU. Moreover, fDM analysis of treated tumors showed that the 1o and 2o groups had minimal differences in voxels of increased ADC at both days 4 and 8, thereby indicating that a second cycle of BCNU therapy did not provide an appreciable gain in tumor response. These findings reveal that diffusion MRI is effective as an early predictive biomarker for detection of diminished tumor response to a second cycle of BCNU therapy. This was confirmed by a lack in survival benefit of the 2o group over the 1o group.

With both diffusion MRI and animal survival studies indicating diminishing response of 2o tumors to BCNU therapy, we investigated whether BCNU-treated tumors did in fact acquire resistance to BCNU. Representative tumors from the 2o and vehicle control groups were excised, and cell lines were derived to establish BCNU sensitivity. Using a growth inhibition assay (22), we showed that BCNU-treated tumors were 2-fold more resistant to BCNU than control tumors with an IC50 of 150.4 ± 1.9 versus 73.8 ± 4.9 μmol/L, respectively. These findings parallel previous reports documenting the development of BCNU-resistant 9L tumors induced by in vivo BCNU therapy (23, 31) and confirms that diffusion MRI predicted loss of tumor response over time, which corresponded to diminished therapeutic benefit.

In summary, this report shows that diffusion MRI can provide a rapid assessment of therapy-induced tumor response to fractionated BCNU as well as the dynamic detection of the emergence of resistance. The findings of this study provide an example of the potential for diffusion MRI to play a major role in the preclinical development of therapeutic regimens with dynamic assessment of therapeutic effect driving efficient optimization of treatment regimens within a single experiment, dramatically increasing the efficiency of preclinical experimentation. Moreover, the data presented also clearly points to the potential application of diffusion MRI technologies for dynamic optimization of therapeutic outcomes of individual patients in the clinic.

Figure 5. Sulforhodamine B growth inhibition assay for BCNU sensitivity. Cell lines were developed from excised 2o and vehicle control tumors and assayed for BCNU sensitivity. Using a previously described assay (Materials and Methods), cell lines were grown in varying BCNU concentrations ranging from 1 to 200 μmol/L and were then fixed to determine differences in total protein. A 2-fold shift in IC50 was observed in tumors from the 2o group (150.4 ± 1.9 and 73.8 ± 4.9 μmol/L, respectively).

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