Magnetic Resonance Spectroscopy for Detection of Choline Kinase Inhibition in the Treatment of Brain Tumors

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Abstract

Abnormal choline metabolism is a hallmark of cancer and is associated with oncogenesis and tumor progression. Increased choline is consistently observed in both preclinical tumor models and in human brain tumors by proton magnetic resonance spectroscopy (MRS). Thus, inhibition of choline metabolism using specific choline kinase inhibitors such as MN58b may be a promising new strategy for treatment of brain tumors. We demonstrate the efficacy of MN58b in suppressing phosphocholine production in three brain tumor cell lines. In vivo MRS studies of rats with intracranial F98-derived brain tumors showed a significant decrease in tumor total choline concentration after treatment with MN58b. High-resolution MRS of tissue extracts confirmed that this decrease was due to a significant reduction in phosphocholine. Concomitantly, a significant increase in poly-unsaturated lipid resonances was also observed in treated tumors, indicating apoptotic cell death. MRI-based volume measurements demonstrated a significant growth arrest in the MN58b-treated tumors in comparison with saline-treated controls. Histologically, MN58b-treated tumors showed decreased cell density, as well as increased apoptotic cells. These results suggest that inhibition of choline kinase can be used as an adjuvant to chemotherapy in the treatment of brain tumors and that decreases in total choline observed by MRS can be used as an effective pharmacodynamic biomarker of treatment response. Mol Cancer Ther; 14(4); 899–908. ©2015 AACR.

Introduction

Glioblastoma (GBM), the most common primary brain tumor in adults, is an aggressive and locally invasive tumor. Despite advances in surgery, radiotherapy, and chemotherapy, overall survival of patients affected by GBM has only marginally increased from 6 to 14 months in recent decades (1). This may partly be due to a lack of effective therapeutic options and limited availability of robust and sensitive methods to assess therapeutic response. As survival with GBM is short, it is critical to determine the efficacy of therapy early on in treatment. An increased understanding of the molecular mechanisms underlying oncogenesis has led contemporary drug discovery programs to be aimed predominantly at signal transduction pathways and molecules that drive cancer initiation and progression (2). Elevated choline kinase (ChoK) activity has been associated with enhanced synthesis of phosphocholine (PC) in many cancer cell types and has been proposed as a potential target for anticancer therapy (3). ChoK catalyzes the phosphorylation of choline, consuming ATP in the presence of Mg2+, yielding PC in a process mostly independent of the rate of net phosphatidylcholine biosynthesis (4–6). Several agents, such as growth factors, chemical carcinogens, and ras oncogenic transfection, induce ChoK activation in malignant cells, leading to an accumulation of PC (5).

A novel molecular therapeutic strategy focused on ChoK inhibition has recently been developed, resulting in the discovery of a group of compounds with inhibitory activity against ChoK (5, 7–9). The inhibition of ChoK using small-molecule inhibitors such as MN58b (5, 8) appears to be a promising new treatment strategy against solid tumors. MN58b is an anticancer drug that exhibits selective inhibition of ChoK activity, resulting in attenuated PC levels, reduced proliferation of cancer cells in vitro, and growth delay of human tumor xenografts (5, 8, 10–13).

There is a strong need to obtain precise surrogate biomarkers to effectively evaluate therapeutic response in GBM. 1H magnetic resonance spectroscopy (MRS) provides a novel way of noninvasively measuring changes in choline-containing tumor metabolites to serve as a pharmacodynamic biomarker of ChoK inhibition and therapeutic response. Al-Saffar and colleagues (5) have reported the efficacy of MN58b on subcutaneously implanted colon and breast cancer models; however, we are unaware of any in vivo MRS studies of brain tumor response to ChoK inhibition. Thus, the goal of the present study was to monitor changes in choline-containing metabolites in an intracranial model of rat glioma in response to treatment with the ChoK inhibitor, MN58b.

Materials and Methods

Cell lines and culture

To assess the toxicity and efficacy of MN58b on growth inhibition of gliomas, we chose three rat brain tumor cell lines F98, 9L, and 908.
and 9L overexpressing EGFRviii (14). The F98, 9L, and 9L-EGFRviii glioma cell lines were maintained as adherent monolayers cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS (HyClone), 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer, Invitrogen), 200 U/mL penicillin, and 200 mg/mL streptomycin sulfate at 37°C in 5% CO2 in air. Cells were maintained in exponential growth phase by routine passage twice weekly at 3 × 10⁶ cells per T75 flask. 9L and F98 cell cultures were tested upon receipt from the laboratory of Dr. J. Biaglow (Department of Radiation Oncology at the University of Pennsylvania) in 1999 using the Rat Antibody Production Test performed by Charles River Laboratories and rescreened in 2005 using IMPACT III PCR profiling performed by RADIL. Cell lines were used within 6 months of reconstitution and tested bimonthly for mycoplasma. The 9L-EGFRviii cell line was cloned from the 9L cells in the laboratory of Dr. Donald M O’Rourke. Department of Neurosurgery, University of Pennsylvania. 9L-EGFRviii cell line was obtained from Dr. Donald M O’Rourke in 2010. No additional characterization has been performed on this cell line.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyloxazole bromide; thiazolyl blue (MTT) assay

The F98, 9L, and 9L-EGFRviii rat glioma cell lines were plated in quadruplicate in 96-well plates at 7.5 × 10⁴ cells per well and incubated overnight. Culture medium was replaced with media containing varying concentrations of MN58b. After 24 hours, 20 μL of 5 mg/mL MTT (Sigma-Aldrich) in sterile PBS was added, and the cells were incubated for 2 hours. The media/MTT mixture was removed and replaced with 150 μL DMSO (Fisher Scientific), shaken, and the absorbance read at 550 nm using a Spectra Max M5 plate reader (Molecular Devices). Background signal was read as absorbance at 690 nm and subtracted from each sample.

ChoK activity assay

For each cell line (F98, 9L, and 9L-EGFRviii), 5 × 10⁵ cells per well were seeded in a 6-well plate and incubated for 24 hours at 37°C. The exponentially growing cells were pulsed for 1 hour with 0.5 μCi/mL of [methyl-¹⁴C]-choline (Perkin Elmer) per well at 37°C followed by the addition of varying concentrations of MN58b, which was synthesized in house as previously described (13). After 2-hour treatment, the medium was removed and cells were washed twice with ice-cold PBS and fixed in 16% ice-cold trichloroacetic acid (Fisher Scientific). ChoK inhibition was probed at 2 hours because this time point has been previously found to be a time before significant loss in cell viability, thus providing a more accurate measurement of ChoK activity (13). Each sample was washed 3× in diethyl ether, lyophilized, and resuspended in water for thin layer chromatography (TLC) separation using a solvent system of NaCl/CH₃OH/NH₄OH (50:70:0.5). The TLC plate was analyzed by autoradiography using a FujiFilm FLA-7000 to detect radioactivity.

Perchloric acid extracts of F98 tumor cells

F98 cells were seeded (1 × 10⁶/mL, 150 cm² flasks) and incubated overnight, and media were aspirated and replaced with fresh media containing 0, 10, and 20 μmol/L MN58b. After 24-hour MN58b treatment, cells were trypsinized, washed, and an aliquot was removed for viability counts. The remaining cells were pelleted and homogenized in 3 volume of 6% perchloric acid (PCA), transferred into an Eppendorf tube and centrifuged (13,000 rpm, 30 minutes, 4°C), neutralized with 3 mol/L potassium hydroxide (KOH; Sigma-Aldrich), and the neutralized samples were lyophilized for 24 hours.

High-resolution NMR spectroscopy of tumor cell PCA extracts

Lyophilized samples were resuspended in 500 μL of deuterium oxide (D₂O; Sigma-Aldrich) including 0.5 mmol 3-(trimethylsilyl) 3,3,3-tetadeuteropropionic acid (TSP; Sigma-Aldrich), which was used as an internal concentration and chemical shift reference. The pD of the solution was adjusted to 7.0 using deuterium chloride (DCl, Sigma-Aldrich) or potassium deuterate oxide (KOD; Sigma-Aldrich) solutions using a standard pH meter and calibrated to actual pH by adding 0.41 to the measured pH (15). High-resolution nuclear magnetic resonance (NMR) spectroscopy was performed at 30°C using an 11.7 T, 55-mm (inner diameter) vertical bore spectrophotometer (Bruker). The sample was first transferred to a clean, dry 5-mm thin-walled NMR tube (Wilmad-LabGlass). Fully relaxed, one-dimensional proton spectra were acquired using a pulse-acquire sequence with the following parameters: 90° pulse, repetition time (TR) = 10.73 seconds, sweep width (SW) = 6,000 Hz, number of complex points (NP) = 32 K, and number of averages = 64, with scan time = 11 minutes 28 seconds per sample. Acquired spectroscopic data were transferred offline for further postprocessing and analysis.

Animal model and tumor cell implantation

An allogeneic rat animal model was chosen to better understand the complexities of this disease in an immune-competent rat model (16). Studies have pointed to F98 orthotropic GBM models as one of the most realistic and cost-effective simulations of the human glioblastoma due to its low immunogeneity, accurate histologic representation of growth and infiltration, and refractory response to clinically relevant therapies (17). All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Six-week-old syngeneic female Fisher F344/NCr (120–150 g weight) rats (n = 13) were purchased from the NCI and housed in a temperature-controlled animal facility with a 12-hour light-dark cycle. General anesthesia was induced by intraperitoneal injection of ketamine/xylazine (80/8.0 mg/kg). The animal was placed on a stereotactic frame. A small burr hole was made 3-mm lateral and 3-mm posterior to the bregma and 2-mm deep into the right cerebral hemisphere using a drill bit. F98 tumor cells (5 × 10⁵) in a 10-μL suspension in PBS were inoculated over a 5-minute period with a Hamilton syringe and a 30-gauge needle using a stereotactic apparatus. After injection of the cell suspension, the needle was withdrawn slowly, and the wound was closed by suturing the skin. Animals were monitored periodically for 2 weeks after which baseline in vivo MRS experiments were performed.

In vivo MRI

Animal preparation for MRI scan. Animals were anesthetized with 3% isoflurane in oxygen and mounted on an animal holding cradle. The animal’s head was secured with a nose cone in an in-house-developed restraining device to minimize motion-induced artifacts. Subdural electrocardiogram (EKG) needle electrodes were placed in the forelimbs, and a respiration pillow was placed on the dorsal side of the body. A thermister was inserted into the rectum to monitor body temperature. EKG electrodes,
respiratory pillow, and thermister were connected to a small animal monitoring device (SA Instruments) to record vital signs, including the EKG, respiration, and core body temperature, during the scan. The cradle with the animal in position was then inserted inside a 35-mm inner diameter transmit-receive quadrature volume coil (M2M Imaging) and the coil was placed in the center of the magnet. During the scan, anesthesia was maintained using 1% to 1.5% isoflurane, and the animal body temperature was regulated at 37 ± 1°C by blowing warm air into the magnet bore via a hose connected to a thermostatically controlled warm air device (SA Instruments).

**In vivo single voxel spectroscopy.** Anatomical images and spectroscopy data were acquired from the brains of 6 normal and 13 intracranial tumor-bearing rats for baseline measurements. The *in vivo* MRS study was repeated on tumor-bearing animals after 5 days of MN58b (*n* = 10) or saline (*n* = 3) injection to evaluate treatment response. *In vivo* studies were performed on a 9.4 T horizontal bore magnet equipped with 40 G/cm gradients interfaced to an Agilent Direct-Drive console (Agilent) operating vnmrj 2.3.C software. Multislice spin echo and $T_2$-weighted anatomical images were acquired to localize the tumor and to plan the MRS voxel. A single voxel with dimensions of $3 \times 3 \times 3$ mm$^3$ was placed within the tumor, and a spectrum was acquired using a PRESS sequence with the following parameters: $TR = 3,000$ ms, echo time ($TE_1$) = 12.68 ms and $TE_2 = 10.01$ ms, number of averages = 128, $NP = 4,096$, and $SW = 4,000$ Hz, resulting in an acquisition time of 6 minutes 24 seconds. Water suppression was performed using the variable power and optimized relaxation delays (VAPOR) technique (18). An unsuppressed water spectrum was also acquired (with 8 averages) to serve as a reference for metabolite concentrations.

**Treatment of tumor-bearing animals with MN58b**

After acquiring baseline *in vivo* MRS, 10 tumor-bearing animals were treated with MN58b at 2 mg/kg/day i.p. for 5 days. A separate cohort of tumor-bearing animals (*n* = 3) were treated with an i.p. injection of 1-ml saline daily for 5 days. *In vivo* MRS experiments were repeated as described above to evaluate the effect of MN58b treatment on tumor metabolism. Two animals died during the MN58b treatment, resulting in post-MN58b data from only 8 animals. The death of these animals was most likely due to the tumor burden, since no overt indications of toxicity were noted as a result of MN58b: loss of weight, change in grooming or social habits, fur ruffling, or change in eye color.

The tumor-bearing animals were anesthetized with an overdose of ketamine/xylazine after the completion of the final in vivo $^1$H MRS study. Following lack of deep tendon responses, the head was decapitated and the brain was removed from the skull. Tumor tissue and contralateral normal brain tissue were harvested from each animal under aseptic conditions in a laminar hood. A portion of the harvested tissue was immediately placed in a preweighed cryovial and stored at −70°C.

**PCA extracts and high-resolution NMR studies on tumor tissue**

PCA extracts and high-resolution NMR studies were performed on tissue samples from contralateral normal brain, saline-, and MN58b-treated tumor tissue on a 500 MHz vertical bore NMR scanner (Bruker) using the same experimental protocol as described for cell extracts.

**Histopathology**

A set of tissue samples was randomly selected from the saline- and MN58b-treated animals for histopathologic study. These samples were fixed in 10% neutral-buffered formalin for 24 hours and were fixed and dehydrated in ethanol, cleared in xylene, and embedded in paraffin blocks, which were cooled before sectioning. Consecutive 3-μm-thick serial sections were cut and stained with hematoxylin and eosin (H&E) or with an antibody against caspase-3 using methods previously described (19). Saline- and MN58b-treated tumor sections were examined for necrosis, hemorrhage, and cell density using H&E, and apoptosis using caspase-3 immunohistochemistry. H&E- and caspase-3-stained slides were scanned at ×20 and ×40 magnifications using the Aperio Scan Scope OS (Aperio Technology).

**Data quantification**

High-resolution NMR data from cells and tumor tissue extracts were analyzed using MestReNova (Mestrelab Research) to assess changes in PC and glycerophosphocholine (GPC) levels due to MN58b treatment. Metabolite peaks were identified on the basis of their chemical shifts (20). The following choline-containing metabolites were identified from their N-trimethyl (\(^{1}(N(CH_{3})_{3})\)) resonances: Cho 3.20 ppm (singlet); PC, 3.22 ppm (singlet); and GPC, 3.23 ppm (singlet) referenced to the internal standard TSP at 0.0 ppm after baseline correction. Metabolite concentrations were calculated from peak heights in $^1$H-MR spectra. The signal area in the proton spectrum is proportional to the concentration and the number of protons contributing to the signal. Because the spectra were fully relaxed and the line widths of all peaks were the same, absolute signal intensities can be determined from the peak heights by direct reference to TSP. The metabolite concentrations were calculated and corrected for the number of cells in the NMR experiment using the following formula: \([\text{metabolite peak height/number of protons}/\text{TSP peak height/number of TSP protons}] \times \text{TSP concentration/number of cells}\). The resonances at 3.2 ppm from the choline-containing metabolites are all composed of 9 protons, as is the 0.0 ppm resonance of TSP. The concentration of TSP was 1.93 μmol/l, and the number of cells for each experiment was in the range of 1.0 to 7.4 × 10$^7$ cells. For tissue extracts, we calculated the metabolite concentration per wet weight of the tissue, which ranged from 27.9 to 140 mg.

To assess the effect of MN58b on tumor growth, tumor volumes were measured using $T_2$-weighted images acquired at baseline and after 5 days of MN58b treatment. In-house custom software developed in the IDL programming environment (ITT Visual Information Solutions) was used to convert raw image data into an "analyze" file format. The analyze files were read into MRicro (1.39 version; McCasland Center for Brain Imaging) and the histopathology slides were scored for necrosis, hemorrhage, and cell density.
region of interest (ROI) tool used to draw whole tumor ROIs on multiple slices covering the tumor. The final volume was calculated as the sum of pixels from all the ROIs multiplied by the section thickness.

Image Scope viewing software and nuclear staining algorithm V.9 (version 9; Aperio Technologies, Inc.) were applied to quantify nuclear density from hematoxylin and caspase-3–positive nuclei from caspase-3 immunohistochemistry. Algorithm parameters were set to achieve concordance with manual scoring on a number of high-power fields, including intensity thresholds for positivity and parameters that control cell segmentation using the nuclear algorithm (22). Regions of tissue necrosis and staining artifacts were manually excluded. The algorithms calculate the percentage of weak (1+), medium (2+), and strong (3+) positive cells. The strong (3+) positive staining numbers were used to assess differences between saline- and MN58b-treated tumors.

Statistical analysis

Statistical analysis of the in vitro and in vivo 1H MRS data was conducted using a Student unpaired t test to evaluate the differences in metabolite concentration in response to MN58b treatment. A P value of ≤0.05 was considered to be statistically significant. All error bars shown in the figures represent mean ± SEM values. All statistical analyses were conducted using SPSS 16.0 (SPSS, Inc.).

Results

MN58b was screened for its ability to inhibit the viability of a panel of brain tumor cell lines using the MTT assay. MN58b treatment significantly reduced the viability of the F98, 9L, and 9L-EGFRviii cells in a dose dependent manner, with GI50s of 19.80 ± 2.80 μmol/L, 8.60 ± 3.00 μmol/L, and 46.85 ± 2.30 μmol/L, respectively (Fig. 1). The phosphorylation of 14C-labeled Cho (Rf = 0.07) was measured using TLC and autoradiography. 14C-PC (Rf = 0.14) production in the F98, 9L, and 9L-EGFRviii cells was quantified, plotted, and fitted to determine IC50s of 2.63 ± 0.65 μmol/L, 1.84 ± 0.53 μmol/L, and 1.85 ± 0.33 μmol/L, respectively (Fig. 2A and B). No statistical difference in ChoK inhibition was found between these groups. Because 14C-radio-tracing demonstrated similar treatment response from all three cell lines, we chose F98 cells for in vivo studies because they most closely resemble human GBM (14, 15). The 9L is a gliosarcoma and does not exhibit the necrotic areas seen in GBM (16). 9L-EGFRviii is similar to a GBM but is not as well characterized compared with the F98 tumor model.

Cellular extracts prepared from F98 cells treated with 0, 10, or 20 μmol/L MN58b were analyzed by NMR to determine changes in PC and GPC levels (Fig. 3A). Analysis of the choline-containing peaks showed lower PC levels in response to MN58b compared with saline-treated cells (Fig. 3B). We observed significantly reduced PC concentration in cells treated with both 10 μmol/L MN58b (5.85 ± 0.70 nmol/10⁷ cells) and 20 μmol/L MN58b (6.84 ± 3.51 nmol/10⁷ cells, P = 0.049) compared with saline-treated cells (18.85 ± 2.39 nmol/10⁷ cells, P = 0.004). We also observed higher GPC concentration in cells after 10 μmol/L MN58b treatment (6.64 ± 2.42 nmol/10⁷ cells, P = 0.911) and a further increase after 20 μmol/L (11.95 ± 7.50 nmol/10⁷ cells, P = 0.019).

Figure 1. MTT assay demonstrating inhibition of cellular viability of F98, 9L, and 9L-EGFRviii cell lines with MN58b. 24 hour MN58b exposure significantly reduced the viability of the F98, 9L, and 9L-EGFRviii cells in a dose-dependent manner. These data were normalized to untreated control cells and represent the average ± SEM of 3 separate experiments performed in quadruplicate.

Figure 2. Effect of MN58b on ChoK activity in tumor cells. Autoradiography of TLC-separated 14C-choline-containing metabolites (A) allows for the quantification of 14C-PC and demonstrates a dose-dependent reduction of choline phosphorylation in response to MN58b treatment. B, graph shows a dose-dependent reduction of ChoK activity in the F98, 9L, and 9L-EGFRviii cell lines. Error bars, ±SEM of 3 separate experiments.
P = 0.532) MN58b compared with saline-treated cells (6.27 ± 1.94 nmol/10^7 cells), but this difference was not statistically significant (Fig. 3B). Inhibition of the anabolic pathway of choline metabolism has been reported to increase GPC at the expense of PC (23). The PC/GPC ratio was thus calculated for each treatment group, and both 10 μmol/L (0.88 ± 0.39, P = 0.012) and 20 μmol/L MN58b (0.57 ± 0.08, P = 0.007) cells demonstrated significantly lower PC/GPC ratio as compared with saline-treated cells (3.01 ± 0.61; Fig. 3C).

In vitro MR spectra (Fig. 4A) were obtained for each tumor before and after saline or MN58b treatment. MN58b led to a significant growth arrest of F98 tumors as treated tumors only grew by 77% (P < 0.010) during the 5-day treatment period (Fig. 4B). The tCho concentration (1.08 ± 0.38 mmol/L) after MN58b treatment was significantly lower than tCho from the brains of rats used as normal controls (1.68 ± 0.23 mmol/L, P = 0.032), baseline values for the untreated tumor (1.95 ± 0.75 mmol/L, P = 0.005) as well as saline-treated tumors (1.92 ± 0.24 mmol/L, P = 0.010; Fig. 4A and C). When the data from individual animals were compared before and after MN58b treatment, a 44% reduction in tCho level was observed in MN58b-treated animals compared with baseline values.

We observed significantly increased Lip+Lac concentration in MN58b-treated tumors [8.79 ± 4.04 arbitrary units (AU)] compared with normal control brain (0.87 ± 0.67 AU, P = 0.002) as well as baseline tumor values (7.58 ± 3.29 AU, P = 0.048; Fig. 4D). There was no significant difference in the Lip+Lac concentration between MN58b-treated tumors and saline-treated tumor (11.87 ± 3.59 AU, P = 0.341; Fig. 4D). However, we observed an increase in the polyunsaturated fatty acyl chain resonance at 2.8 ppm after MN58b treatment (1.37 ± 0.46 AU, P = 0.018), baseline (0.51 ± 0.42 AU, P = 0.007) and saline-treated (0.52 ± 0.02 AU, P = 0.010), compared with control brain (0.03 ± 0.06 AU), respectively (Fig. 4A and E).

Similar to the results observed from F98 cells treated with MN58b, the ex vivo high-resolution NMR data from tissue extracts also demonstrated changes in PC and GPC values in MN58b-treated tumors (Fig. 5A). Significantly lower PC levels were observed in MN58b-treated tumors (0.33 ± 0.06 nmol/mg, P = 0.019) compared with saline-treated tumors (0.59 ± 0.06 nmol/mg). The saline-treated tumor demonstrated significantly higher PC (P = 0.030) than normal brain (0.30 ± 0.09 nmol/mg). Significantly higher GPC was also observed in MN58b-treated tumors (0.44 ± 0.05 nmol/mg, P = 0.009) compared with normal brain (0.23 ± 0.05 nmol/mg). The GPC in saline-treated tumors (0.40 ± 0.03 nmol/mg, P = 0.015) was significantly higher than contralateral normal brain. However, the GPC concentration between saline-treated and MN58b-treated tumor was not significantly different (P = 0.593). When the PC/GPC ratios were computed, a significantly lower PC/GPC ratio was observed in MN58b-treated (0.76 ± 0.09, P = 0.05) than saline-treated tumors (1.49 ± 0.25; Fig. 5B). However, there was no significant difference in the PC/GPC ratio between MN58b-treated (P = 0.124) and normal brain (1.41 ± 0.35; Fig. 5C).

Immunohistologic staining and quantitation were performed on a sample of saline- and MN58b-treated tumors. Quantitative analysis of hematoxylin staining demonstrated a 16% reduction in the total number of tumor cells in MN58b-treated (1.20 × 10^4 cells/mm^2) compared with saline-treated tumor (1.42 × 10^4 cells/mm^2, Fig. 6A–D). MN58b treatment was also associated with increased staining of CASPASE-3 indicative of apoptosis.
A 3.0-fold increase in caspase-3-positive cells was observed in MN58b-treated (4.3% positive cells) compared with saline-treated tumor tissue (1.4% positive cells).

**Discussion**

In this study, the effect of the choline kinase inhibitor MN58b on choline metabolism was examined in a glioma model, and a significant decrease in tCho was observed resulting from decreased levels of PC. Elevated ChoK activity in tumor cells and increased tCho levels in cells and untreated tumors suggest that increased tCho in tumors is primarily driven by elevated ChoK. The significant decrease in cellular ChoK activity after treatment with MN58b indicates that inhibition of ChoK may be an effective adjuvant in the treatment of gliomas. MRI and MRS studies of MN58b-treated orthotopic glioma tumors demonstrated significant tumor growth inhibition and reduction in tCho levels. These findings were corroborated with the histologic finding of elevated apoptotic cells within MN58b-treated tumors, indicating the efficacy of MN58b as a potential therapeutic agent in the treatment of gliomas.

Overexpression and increased ChoK activity resulting in production of PC have been detected in several human tumor-derived cancer cell lines and tissue biopsies, including lung, colon, and...
and prostate (9–12, 24, 25). Decreased tumor tCho is usually associated with a positive response to conventional chemotherapy in cancer and suggests a role for choline compounds as potential biomarkers to assess treatment response (26). Recent studies have explored the possibility of altering the expression or activity of enzymes involved in choline metabolism as a novel therapeutic target for cancer treatment (11, 27). Inhibition of ChoK is regarded as an attractive cancer treatment strategy (28), and changes in tCho due to ChoK activity as detected by MRS can be used as a noninvasive pharmacodynamic marker of therapeutic response. MRS is an efficient technique for discrimination between brain lesions and for following treatment response (29, 30). It was not known, however, whether gliomas respond to ChoK inhibition therapy and whether MRS is capable of validating tCho as a surrogate marker of treatment response.

We studied three different glioma cell lines in vitro and observed their sensitivity to MN58b via MTT and ChoK assays. We observed the phosphorylation of 14C-labeled choline using a TLC-based ChoK assay and found inhibition of ChoK activity in a dose-dependent manner. Although MN58b inhibited 14C-PC production with similar potency in each of the three cell lines, the EGFRviii mutation imparted some resistance in terms of viability compared with wild-type 9L cells, which express very little EGFR. The observation of decreased ChoK activity and increased cellular toxicity in all three cell lines led us to explore ChoK inhibition in vivo in a rat model of brain cancer.

Higher choline levels may be suggestive of increased malignant potential (23), increased membrane turnover (31), or activation of oncogenic signaling (32). The tCho signal is commonly increased in malignant gliomas and is also associated with processes that either promote cellular proliferation or induce cell death that can be observed as changes in the tCho peak in MRS (29). In cell extracts using high-resolution 1H NMR, we observed a decrease in PC and nonsignificant increase in GPC in response to MN58b treatment, showing the effectiveness of ChoK inhibition on choline metabolite levels. In vivo, a significant MN58b-induced decrease in tCho was observed with 1H MRS indicating inhibition of ChoK (Fig. 4A and B). MN58b treatment caused significant reduction of the PC/GPC ratio in both cellular and tumor tissue extracts, which is consistent with previously reported results. The...
The in vivo data followed the same general trend as observed with ex vivo extracts. MN58b caused a decrease in tCho in vivo compared with saline treatment that was paralleled by a decrease in PC and in PC/GPC ratio in extracts. Although tCho was not significantly different in normal brain versus baseline tumor spectra in vivo, both PC and GPC were elevated in the extract spectra of tumors relative to contralateral tissue. Interestingly, the contralateral PC/GPC ratio was also not significantly different from saline-treated tumor extracts even though their absolute levels were much lower. This variability was possibly due to heterogeneity of the tumor sampled with single voxel MRS and the relatively broad spectral line width. In addition, gliomas are often histopathologically heterogeneous and have components of varying grades of malignancy and necrosis within the tumor. A decrease in tCho and lactate, as well as an increase in lipid, would be expected in necrotic tumor tissue.

1H MRS is highly sensitive to alterations in the unsaturated state of lipid acyl chains. Increased fatty acyl chain resonances in MR-visible lipids have been correlated with malignancy and necrosis in human brain tumors (33–35), and an increase in polyunsaturated fatty acids (PUFA; refs. 36, 37), along with a decrease in lactate (38, 39), is typically observed in tumors after treatment. We observed an increase in the 1.3 ppm resonance in both untreated and treated tumors compared with baseline, but increased PUFA resonance at 2.8 ppm was observed only in treated tumors. The increased PUFA resonance (Fig. 4A and C) suggests induction of apoptosis in the tumor after MN58b treatment, which was confirmed by caspase-3 staining of tumor tissue sections. The increased saturated fatty acyl chain peak at 1.3 ppm may reflect the onset of necrosis in both treated and untreated tumors. Increased MR-visible lipids have been reported in stressed cells before cell death and suggest that the presence of lipid may reflect cellular responses to environmental or drug-induced stress (37, 40–44).

Our in vivo MRS findings (decreased tCho and tumor growth arrest) are consistent with the histologic results in which MN58b-treated tumors had a reduction in the number of viable cells (Fig. 6). This indicates that MN58b not only inhibits ChoK activity but also leads to cell death and growth arrest, as is confirmed by the MTT assay in vitro and tumor size measurements by in vivo MRI. Despite significant growth arrest, we did not observe a significant reduction of tumor volume with MN58b treatment, which may in part be due to a suboptimal dose schedule of MN58b or route of administration (i.p. vs. i.v. or oral gavage) of the drug during treatment. Caspase-3 staining did confirm an increase in apoptosis after MN58b treatment which was consistently associated with increased PUFA resonances at 2.8 ppm (Figs. 4A and 6F and H). Similarly, a reduction in hematoxylin-stained cells was noted in MN58b-treated tumors, indicating the efficacy of the drug (Fig. 6B and D). These findings suggest the potential role of MN58b as a complimentary therapeutic in the treatment of patients with GBM.

Hyoxia is known to exist in brain tumors (45). Hypoxic tumor microenvironments pose a problem for radiotherapy and chemotherapy; cancer cells located in hypoxic environments undergo adaptive responses, which render them resistant to radiotherapy and chemotherapy, resulting in recurrence (46). Such resistance to treatment contributes to the incidence of cancer recurrence. Variations in the extent and degree of hypoxia/necrosis in combination with variable angiogenic patterns represent a considerable problem in radiotherapeutics and antiangiogenic management of GBM. Glunde and colleagues. (46) reported increased total choline and ChoK activity in a prostate cancer model and established a correlation between hypoxia, choline metabolites, and ChoK activity. These authors also suggested that hypoxia-inducible factor-1 activation of hypoxia response elements within the putative ChoK promoter region can increase ChoK expression within hypoxic environments, consequently increasing cellular PC and tCho levels within these environments (46). It is known that GBMs are more heterogeneous and hypoxic compared with other types of brain tumors. Thus, it is plausible to hypothesize that inhibition of ChoK activity by a specific ChoK inhibitor may help in reducing the survival pathways which allow GBM tumors to thrive in hypoxic microenvironments, making them more sensitive for chemotherapy and radiotherapy. However, Bansal and colleagues (47) have reported a decrease in choline phosphorylation in hypoxic prostate cancer cells which may result from other isoforms of hypoxia response elements affecting ChoK activity in human brain tumors. There is intensifying urgency to find new drugs for the treatment of brain tumors. Development of anticancer agents is the key focus of several research studies on developing specific molecular targets against the malignant phenotype (5, 48, 49) with the ultimate goal of improving activity and therapeutic selectivity for tumor versus normal cells.

**Conclusion**

In conclusion, this study demonstrated that 1H MRS can be used to detect a decrease in tCho that is associated with the inhibition of ChoK activity by MN58b in gliomas. These effects were seen in both cultured cells and in tumor xenografts. Significant reduction in tCho, elevated PUFA, and increased apoptotic marker caspase-3 after MN58b treatment demonstrate the potential of ChoK inhibition in GBM treatment. Monitoring metabolic changes with MRS may provide a noninvasive pharmacodynamic marker for ChoK inhibition and treatment response in brain tumors.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Kumar, S.P. Arlauckas, S. Saksena, G. Verma, E.J. Delikatny, H. Poptani
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Choline Kinase Inhibition for Treatment of Brain Tumors

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