Regression of glioma tumor growth in F98 and U87 rat glioma models by the Nitrone OKN-007

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Background. Glioblastoma multiforme, a World Health Organization grade IV glioma, has a poor prognosis in humans despite current treatment options. Here, we present magnetic resonance imaging (MRI) data regarding the regression of aggressive rat F98 gliomas and human U87 glioma xenografts after treatment with the nitrone compound OKN-007, a disulfonyl derivative of α-phenyl-tert-butyl nitrone.

Methods. MRI was used to assess tumor volumes in F98 and U87 gliomas, and bioluminescence imaging was used to measure tumor volumes in F98 gliomas encoded with the luciferase gene (F98luc). Immunohistochemistry was used to assess angiogenesis (vascular endothelial growth factor [VEGF] and microvessel density [MVD]), cell differentiation (carbonic anhydrase IX [CA-IX]), hypoxia (hypoxia-inducible factor-1α [HIF-1α]), cell proliferation (glucose transporter 1 [Glut-1] and MIB-1), proliferation index, and apoptosis (cleaved caspase 3) markers in F98 gliomas. VEGF, CA-IX, Glut-1, HIF-1α, and cleaved caspase 3 were assessed in U87 gliomas.

Results. Animal survival was found to be significantly increased (P < .001 for F98, P < .01 for U87) in the group that received OKN-007 treatment compared with the untreated groups. After MRI detection of F98 gliomas, OKN-007, administered orally, was found to decrease tumor growth (P < .05). U87 glioma volumes were found to significantly decrease (P < .05) after OKN-007 treatment, compared with untreated animals. OKN-007 administration resulted in significant decreases in tumor hypoxia (HIF-1α [P < .05] in both F98 and U87), angiogenesis (MVD [P < .05], but not VEGF, in F98 or U87), and cell proliferation (Glut-1 [P < .05 in F98, P < .01 in U87] and MIB-1 [P < .01] in F98) and caused a significant increase in apoptosis (cleaved caspase 3 [P < .001 in F98, P < .05 in U87]), compared with untreated animals.

Conclusions. OKN-007 may be considered as a promising therapeutic addition or alternative for the treatment of aggressive human gliomas.

Keywords: anti-glioma therapy, bioluminescence imaging, F98 rat glioma model, in vivo, magnetic resonance imaging, nitrone OKN-007, U87 xenografts.

Grade IV glioblastomas (GBMs) are the most malignant type of gliomas, with a mean survival time of ~15 months.1 High-grade gliomas (grades III and IV) are the most common primary brain tumors in adults, and their malignant nature ranks them fourth in incidence of cancer-related death.2 Standard treatment for GBM, which typically involves maximal safe resection followed by a combination of radiation and chemotherapy with temozolomide, has improved overall survival (but median survival has...
remained 15–18 months, and 5-year survival rates are <10%).3,4 Prognosis is even poorer for recurrent disease, with response rates for cytotoxic chemotherapy typically in the range of 5%-10%, and 6-month progression-free survival rates of <15%.4,5 In addition to cytotoxic chemotherapy, bevacizumab, an anti–vascular endothelial growth factor (VEGF) therapy, is used for treatment of recurrent glioblastoma,3,6 but clinical outcomes continue to remain suboptimal. Here, we report on the use of a nitronate compound, OKN-007 (disodium 4-[(tert-butyl-imino) methyl] benzene-1,3-disulfonate N-oxide or disufenton), which has shown promise in preclinical models for gliomas.

OKN-007 is a small molecule that can traverse the blood-brain barrier and has been previously shown to be anti-inflammatory, antioxidant, and proapoptotic.7,8 When administered orally, OKN-007 has recently been found by our group to be a novel anticancer therapy against C6 rodent gliomas,9 which are moderately aggressive.10 Assessment of OKN-007 in a more aggressive glioma model, such as the F98 model, and in human-derived U87 glioma cell xenografts, was the primary goal of this study. The F98 rat glioma model was considered because these gliomas have an infiltrative pattern of growth, have attributes associated with human GBM gliomas, and are classified as anaplastic malignant tumors.11,12 The F98 glioma cell line was obtained as a result of administering ethylnitrosourea (ENU) to pregnant rats whose progeny developed brain tumors12 and is commonly used as a preclinical rodent glioma model. Likewise, similar to GBM, U87 human glioma cell-derived tumors in immunocompromised rodents are highly cellular with atypia, such as mitotic figures and irregular nucleoli, and profuse neovascularization; however, unlike GBM, these tumors have a nondiffuse infiltrative growth pattern and a well-demarcated tumor mass surrounded by reactive astrocytes.10

In the diagnostic process for human gliomas, MRI plays an important role as an optimal imaging tool to provide information on brain tumor growth, vasculature, biochemical metabolism, and molecular changes in preclinical glioma models. Bioluminescence imaging relies on planar imaging principles to noninvasively image optical contrast at depths of several millimeters to centimeters with high sensitivity and submillimeter to millimeter resolution.13 This is attractive for the evaluation of transgene expression because reporter gene activity can be imaged in the same animal at various time points during tumor development. Tumor cells with a reporter gene for a luminescent enzyme, such as luciferase, can be imaged longitudinally during tumor growth or in response to therapeutic agents.13

In this study, MR and bioluminescence imaging methods were used to monitor tumor growth, and immunohistochemistry (IHC) was used to monitor levels of tumor markers for angiogenesis, cell differentiation or proliferation, and apoptosis in OKN-007-treated and untreated F98 glioma-bearing rats. Tumor growth (measured by MRI), IHC, and survival were also assessed in a U87 xenograft model in athymic rats.

**Methods**

**Rat Glioma Cell Implantation**

The study was conducted in compliance with the Oklahoma Medical Research Foundation and University of Utah Institutional Animal Care and Use Committees. The rat glioma cell implantation models were prepared as previously described.14,15 For the F98 model, F98 cells encoded with the luciferase gene were used. For this model, a total of 14 rats were used (6 untreated and 8 OKN-007-treated). For the U87 xenograft model, 10 rats were used (5 untreated and 5 OKN-007-treated). Both untreated and treated groups were stratified so that tumor sizes before initiation of treatment were similar.

**OKN-007 Treatment**

OKN-007 (2,4-disulfophenyl-N-tert-butyl nitrotrine) (Fig. 1A) was obtained from Ryss Laboratories (Union City, CA) and was administered to the rats in their drinking water at a concentration of 0.018% w/v. The treatment was given continuously until the end of the study, beginning 15 days after F98 glioma cell implantations (tumor volumes ≏30–50 mm3). Rats receiving normal drinking water were used as controls. The amount of OKN-007 consumed by either F98 or U87 glioma-bearing rats was determined by weighing water bottles each day (rats were singly housed). No significant deviations in the liquid volume uptake of compound were observed in either model, but there was greater uptake (~2-fold) in the U87 glioma-bearing athymic rnu/rnu rats, compared with the F98 glioma-bearing Fischer 344 rats (F98 glioma-bearing rats consumed ~10 mg/kg body weight/day, whereas rats with U87 xenograft gliomas consumed ~20 mg/kg body weight/day).

**Bioluminescence Imaging**

F98 glioma-bearing rats were anesthetized with an isoflurane vaporizer, injected intraperitoneally with 50 mg/kg firefly D-Luciferin (Xenogen Corp.; Alameda, CA) in 1× phosphate-buffered saline, and photographed 5 min after injection with an IVIS 100 imaging system (Xenogen) with an exposure time of 10 min, medium binning. Images were quantified using LivingImage software (Xenogen).

**MRI**

For both F98 and U87 glioma-bearing rats, MRI experiments were performed on a Bruker Biospec 7.0 Tesla/30-cm horizontal-bore magnet imaging system (Bruker Biospin; Ettlingen, Germany). Animals were immobilized using 1.5%-2.5% isoflurane and 0.8 L/min oxygen and placed in a 72-mm quadrature volume coil for signal transmission, and a surface coil was used for signal reception. Rat T2-weighted imaging was acquired using a rapid acquisition with relaxation enhancement (RARE) sequence with a repetition time (TR) of 3000 ms, an echo...
time (TE) of 63 ms, 20 transverse 1-mm–thick slices, a field of view of $3.5 \times 3.5$ cm$^2$, and an in-plane resolution of $137 \times 137$ μm$^2$.

**Data Processing**

Bioluminescence imaging data for monitoring F98 luciferase-expressing tumors was analyzed using Living Image, version 2.50, software (Caliper LifeSciences; Hopkinton, MA), to calculate mean radiance (p/sec/cm$^2$) in tumors.

From MR images, tumor volumes were obtained by compiling tumor areas from all slices that contained tumors, using ImageJ software (National Institutes of Health; version 1.40g). Tumor boundaries were easily distinguished in T2-weighted RARE images.

**IHC**

IHC was done for both the F98 and the U87 glioma-bearing rats.

**Hypoxia-inducible Factor-1α, VEGF, Carbonic Anhydrase IX, and Glucose Transporter 1.**—For hypoxia-inducible factor-1α (HIF-1α) IHC, the Catalyzed Signal Amplification System (DAKO; Carpinteria, CA),
based on streptavidin-biotin-horseradish peroxidase complex formation, was used according to the manufacturer’s recommended protocol. The primary antibody used was H11667 (Novus Biologicals; Littleton, CO), at a dilution of 1:1000. Nuclei were lightly counterstained with toluidine blue.

For VEGF, carbonic anhydrase IX (CA-IX), and glucose transporter 1 (Glut-1) specimens were incubated with either anti-VEGF Ab-1 polyclonal antibody (1:150 dilution; Calbiochem, Cambridge, MA), anti–CA-IX goat polyclonal antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti–Glut-1 (1:100 dilution, Santa Cruz Biotechnology). Secondary antibodies and avidin-biotin complex incubations were performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The final reaction was treatment of the sections in peroxidase substrate solution, 3,3′-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories). Counterstaining was done with toluidine blue. All slides were examined under 200× magnification with use of an Olympus BX41 microscope and were scored by an investigator blinded to the patient information and tumor grade. The immunohistochemical analyses of HIF-1α, VEGF, CA-IX, and Glut-1 were scored from 0 to 4 (0: 0% to <25%; 1: 25% to <50%; 2: 50% to <75%; 3: 75% to <100%; and 4: 100%) on the basis of the number of cells stained in a given field.16,17

Proliferation Index.—To determine the proliferation index (PI), the primary antibody, Ki-67 (clone MIB-1; dilution of 1:160), was used with a secondary antibody (dilution of 1:300; mouse Fab, Dako). Detection was done using the IView DAB detection kit, and the counterstain was done with hematoxylin (Ventana ES, Ventana Medical Systems). The PI was calculated by taking 6 random pictures representative of each slide at 400× (10 ocular × 40 objective) magnification with use of an Olympus Microfire camera. Images were transferred to Image-Pro Plus 5.0, and a LoPass large spectral filter was applied. With use of the count/size measurement feature, the MIB-1–stained cells (brown) were counted with the manual intensity range selection tool by histogram-based segmentation. The background-stained cells (blue) were counted in the same manner. The PI was calculated as the number of MIB-1–stained cells divided by the total number of cells in the field [(number of brown-stained cells)/(number of brown + number of blue-stained cells)].

MVD Index.—The slides for the MVD index analysis were prepared using the same steps as described above for the MIB-1 analysis, except that they were pretreated with Factor VIII (rabbit polyclonal) protease 2 (Ventana Medical Systems) and the primary antibody (dilution of 1:100). Slides were then soaked in a secondary antibody (dilution of 1:300; mouse Fab). The MVD index was calculated based on a previously published method16 using an Olympus Microfire camera.

Apoptotic Index.—To determine the apoptotic index (AI), sections were incubated with the primary antibody (cleaved caspase-3 rabbit polyclonal antibody) in 1:250 dilution. After incubating with ImmPRESS reagent, the sections were processed using the ImmPACT DAB peroxidase substrate solution. The AI was determined using a method similar to the that for PI measurements and was calculated as the number of cleaved caspase-3–stained cells divided by the total number of cells in the field.

Statistical Analyses

Statistical differences in MRI-detected tumor volumes, spectral peak intensities, and IHC scoring were analyzed between the treatment and control groups and between tumor and nontumor regions with use of an unpaired, 2-tailed Student’s t test using commercially available software (InStat; GraphPad Software, San Diego, CA). A P value <.05 was considered to indicate a statistically significant difference.

Pharmacokinetics

Plasma samples (n = 5; 3-month-old male Fisher 344 rats) were collected and homogenized before high-performance liquid chromatography (HPLC) analysis. OKN-007 was extracted in methanol from plasma samples.18 Methanol (200 μL) was added to a 200-μL portion of plasma. This solution was transferred to a Millipore Ultraframe-MC centrifugal filtration unit and centrifuged for 30 min. The filtrate was injected onto the Shimadzu HPLC system consisting of an ACE-5 C-8 column. The mobile phase consisted of 94% potassium phosphate buffer (40 mM) and 6% acetonitrile (v/v). Absorbance at 297 nm was measured using a UV-Vis detector. A series of spiked standards were analyzed to develop a calibration curve based on peak area for the sample quantification and to validate the method. Concentrations of OKN-007 in plasma samples were calculated from the standard curve.

The mean plasma data collected up to 4 h after oral gavage were analyzed using nonlinear regression with the computer program Boomer (version 3.3.7, Mac OS X, http://www.boomer.org/, David Bourne, publisher, Oklahoma City, OK19). A number of oral administration models were considered, but the model presented in Fig. 1, an oral one-compartment model with a lag-time with equal weighting of data, was found to give the best-fit after evaluation of the weighted residual plots and the minimum value of Akaike’s information criterion.20 The model includes the first-order rate constants, k_a (absorption) and k_e1 (elimination), V/F, an apparent volume of distribution divided by bioavailability, and a lag time (Fig. 1B). Secondary parameters determined during the data analysis included the area under the plasma concentration versus time curve (AUC) and area under the concentration times time versus time curve (AUMC; using the trapezoidal rule), mean
residence time (MRT = AUMC/AUC), and oral clearance (CL/F = dose/AUC) (Fig. 1B).

Results

Pharmacokinetic data indicates that OKN-007 is essentially cleared 4–5 h after oral administration (9.18 µM bolus gavage administration of 10 mg/kg body weight) (Fig. 1). The mean C\text{max} in plasma was determined to be 1.316 µM/L after 1 h and eliminated with a half-time (t\text{1/2}) of 0.770 h (Fig. 1B and C).

For the entire study, animal survival was assessed by comparing OKN-007–treated animals with untreated animals. OKN-treated rats (F98: n = 8; U87: n = 5) were found to survive significantly longer (F98: P < .001; U87: P < .01), compared with untreated animals (F98: n = 4; U87: n = 5) (Fig. 2A and B).

Tumor growth was monitored using bioluminescence imaging with luciferase gene-containing F98 cells. Figure 3 depicts representative examples of bioluminescence imaging (mean radiance [p/s/cm²/sr]) data for F98 glioma-bearing rats that were either untreated (n = 6; representative animal at 19 days after intracerebral implantation of luciferase-transfected F98 cells) (Fig. 3A) or treated with OKN-007 (n = 8) (Fig. 3B).

Examples of totally regressed tumors are shown in Fig. 3B (19 days after cell implantation/4 days after the initiation of OKN-007 treatment, day 29 after implantation/14 days after initiation of treatment, and day 30 after implantation/15 days after initiation of treatment). Figure 3B also shows an animal with a slow response to OKN-007 (26 days after cell implantation/11 days after initiation of treatment). Mean radiance values for OKN-007–treated and untreated rats were evaluated including the total cohort of animals (n = 8) and again with only those animals that were responsive (n = 6). A significant difference (*P < .05) in the mean radiance was obtained when comparing untreated rats with the responsive OKN-treated rats.

Fig. 2. Survival of F98 (A) and U87 (B) glioma-bearing rats that were either untreated (UT) (F98: n = 4; U87: n = 5) or treated with OKN-007 (OKN; oral administration via the drinking water; 18 mg/kg/day; F98: n = 8; U87: n = 5). A significant difference (F98: ***P < .001; U87: **P < .01) was established when comparing the 2 treatment groups over the course of the study, where OKN-treated rats were found to survive longer. Treatment was started when tumors reached 20–50 mm³ in volume, which was at 11–15 days after intracerebral cell implantation of F98 or U87 cells.

Fig. 3. Bioluminescence imaging (mean radiance [p/s/cm²/sr]) of F98 glioma-bearing rats that were either (A) untreated (UT; n = 4; representative animals 19 days after intracerebral implantation of F98 cells) or (B) treated with OKN-007 (OKN; oral administration via the drinking water; n = 8). F98 cells were transfected with the luciferase gene. Totally regressed tumors are shown at 19 days after cell implantation/4 days after the initiation of OKN-007 treatment (Bi); at day 29 after implantation/14 days after initiation of treatment) (Bii); and at day 30 after implantation/15 days after initiation of treatment (Biv). (Biii) Slow response to OKN-007 (26 days after cell implantation/11 days after initiation of treatment). (C) The mean radiance of OKN-treated rats was evaluated including the total cohort of animals (n = 8) and again with only those animals that were responsive (n = 6). A significant difference (*P < .05) in the mean radiance was obtained when comparing untreated rats with the responsive OKN-treated rats.
are shown in Fig. 3C. OKN-treated rats were evaluated, including the total cohort of animals (n = 8), with no significant decrease in treated animals (P = .095). If only those animals that were more responsive to treatment (the majority of animals; ie, n = 6) were included, there was a significant decrease (P < .05) in the mean radiance for these OKN-treated rats (n = 6) observed, compared with untreated rats (n = 6).

Comparative image assessment of tumor growth in treated and untreated F98 glioma-bearing rats was done using morphological MRI. Figure 4 shows representative T2-weighted MR images of untreated rats (Fig. 4A) at 23–24 days after intracerebral implantation of F98 cells (n = 4) and OKN-007 (OKN)–treated F98 glioma-bearing rats (Fig. 4B) at 29–30 days (14–15 days after initiation of treatment) and 23–24 days (8–9 days after initiation of treatment; n = 8). Examples of the most responsive and slow-responsive rats are shown. Figure 4C presents quantitative mean F98 tumor volumes, calculated from MR images, which indicate a significant decrease in tumor volumes of OKN-treated rats (P < .05 for the total treated cohort of animals) at 20–34 days after cell implantation, compared with untreated animals (at 15–24 days after cell implantation). If the most responsive rats (the majority; ie, n = 6) are compared with untreated rats, the significance (P < .001) for decreased tumor volumes is much higher. Figure 4D shows that quantitative mean U87 tumor volumes, obtained from MR images, were significantly decreased (P < .05) in OKN-treated rats (at 35–61 days after cell implantation), compared with untreated animals (at 18–34 days after cell implantation). Because of the lower number of animals (n = 5 for each group) in the U87 study, the response differences in animals were not evaluated (ie, only total data are presented).

Molecular assessment of tumor markers associated with tumor growth, such as cell differentiation, cell proliferation, angiogenesis, and apoptosis, were performed using IHC. Figure 5 shows representative IHC-stained histological brain tissue slices from untreated or OKN-007–treated F98 or U87 glioma-bearing rats. From the hematoxylin and eosin–stained tissue slides, it was evident that there was a decrease in brain cell population after OKN treatment in both F98 and U87 gliomas (Fig. 5A and B, respectively). Representative decreases in the staining of markers for cell proliferation (Glut-1) (Fig. 5A for F98 gliomas and 5B for U87 gliomas), hypoxia (HIF-1α) (Fig. 5A for F98 gliomas and 5B for U87 gliomas), angiogenesis (MVD [Fig. 5A for F98 gliomas] and VEGF [Fig. 5A for F98 gliomas]), and cell differentiation (CA-IX) (Fig. 5A for F98 gliomas) are shown after OKN treatment. It was also observed that OKN-007–treated rats had increased apoptosis, as measured by increased levels of IHC staining for cleaved caspase 3 (Fig. 5A for F98 gliomas and 5B for U87 gliomas). Mean IHC and IHC index scores are shown in Fig. 6 for VEGF, CA-IX, Glut-1, and HIF-1α (Fig. 6A) and for MVD (angiogenesis index) and MIB-1 (cell proliferation index) (Fig. 6B) in untreated (n = 5) and OKN-treated F98 glioma-bearing rats (n = 5). For U87 gliomas, mean IHC scores are shown for VEGF, CA-IX, Glut-1, and HIF-1α (Fig. 6C). Significant decreases in OKN-treated rats (n = 5) were observed for Glut-1 (P < .05 for F98 gliomas and P < .01 for U87 gliomas) and HIF-1α (P < .05 for both F98 and U87 gliomas), MVD (P < .05 for F98 gliomas), and MIB-1 (P < .01 for F98 gliomas), compared with untreated animals (n = 5). A significant increase in the apoptotic index (P < .001 for F98 gliomas and P < .05 for U87 gliomas) was also obtained for OKN-007–treated glioma-bearing rat brain tumor tissues, compared with untreated glioma tissues.

Discussion

Both MR and bioluminescence imaging are ideal methods for monitoring tumor growth. The advantage of bioluminescence imaging is that tumor cells are tagged with the luciferase gene, and only tumor cells are visualized, without the inclusion of inflammatory regions (eg, edema). Only a 2-dimensional representation of the tumor mass is obtained, however; therefore calculating tumor volumes is difficult, and any necrotic lesions will be discounted from the observed tumor mass. Another limitation of bioluminescence imaging is that metabolism of luciferin and photon emission can occur in non-dividing cells that are still viable, and therefore, loss of luminescent signal may not always correlate with tumor cell killing.21 MR imaging provides actual 3-dimensional volume measurements and includes necrotic lesions in total tumor volume calculations. Methods used for small animal MR imaging are also relevant to the clinical procedures used to assist in the diagnosis of gliomas. In our study, when MR and bioluminescence imaging were compared, the bioluminescence imaging data for OKN-treated F98 glioma-bearing rats indicated that there was no significant difference in tumor volumes between the treatment groups; however, with MRI (T2-weighted RARE), the total treatment group was found to have significantly lower tumor volumes, compared with untreated animals. If the slow-responsive animals (n = 2) were removed and only the most responsive treated rats (n = 6) were included, there was a significant decrease (P < .05) in mean radiance in the bioluminescence images, compared with the untreated group. Likewise, there was a more significant decrease (P < .001) in F98 tumor volumes detected by MRI in the more responsive animals than in untreated rats. Future studies should be done to elucidate why there were a few slow-responsive OKN-007–treated animals and whether the response differences are reflected in IHC and/or molecular analyses.

Pharmacokinetics indicated that OKN-007 reached a plasma level of 1.3 μM/L in 1 h after a bolus dose of 10 mg/kg administered by gavage. In humans administered OKN-007 (0.5–0.8 mg/kg/h), when this compound was considered as a stroke therapeutic, intravenous infusion over 24 h resulted in a plasma concentration of 30 μM/L.22–24 No serious adverse events were reported in the study.
IHC assessment of commonly studied tumor markers for cell proliferation or differentiation, hypoxia, angiogenesis, and apoptosis indicated that OKN-007 was able to significantly decrease cell proliferation (Glut-1 and MIB-1) but not cell differentiation (CA-IX) (Fig. 6), decrease angiogenesis (MVD, but not VEGF), decrease hypoxia (HIF-1α), and increase apoptosis (cleaved caspase 3), compared with untreated controls. OKN-007–induced decreases in Glut-1 and HIF-1α levels seem to be similar in both F98 and U87 glioma models, whereas increased apoptosis seems to be more elevated in the F98 gliomas, compared with the U87 tumors. Microarray analysis is currently underway in our research groups to elucidate specific genetic alterations associated with OKN-007 treatment in preclinical models for gliomas.

We have previously shown that OKN-007 has a dramatic effect on regressing tumor formation in a rat C6 glioma model.9,25 Morphological MRI was used to calculate tumor volumes; diffusion-weighted imaging was used to measure apparent diffusion coefficients, which are used to assess changes in water diffusion because of tissue structural alterations; perfusion-weighted MRI was used to characterize tissue perfusion rates, which can provide information on alterations in the vascular capillary bed. Rats treated with OKN-007 after tumors were visualized by MRI were found to have significantly decreased tumor volumes (~3-fold, *P < .05), compared with untreated controls.
decreased apparent diffusion coefficients (~20%, $P < .05$), and increased tissue perfusion rates (~60%, $P < .05$) in tumors, compared with nontreated rats. OKN-007 was administered in the drinking water at 10 mg/kg/day, starting when tumors had reached ~50 mm$^3$ in volume (about day 15 after intracerebral...
Fig. 6. (A) Mean IHC scores for VEGF, CA-IX, Glut-1, and HIF-1α (± SD) in untreated (dark gray bars; n = 5) and OKN-treated (light gray bars; n = 5) F98 glioma-bearing rats. Significant decreases (*P < .05) were observed for Glut-1 and HIF-1α in OKN-treated rats, compared with untreated animals. (B) Mean IHC index scores (± SD) for MVD (angiogenesis index), MIB-1 (cell proliferation index), and apoptosis (cleaved caspase 3) in untreated (dark gray bars; n = 5) and OKN-treated (light gray bars; n = 5) F98 glioma-bearing rats. (C) Mean IHC scores for VEGF, CA-IX, Glut-1, and HIF-1α (± SD) in untreated (dark gray bars; n = 5) and OKN-treated (light gray bars; n = 5) U87 glioma-bearing rats. (D) Mean IHC index scores (± SD) for apoptosis (cleaved caspase 3) in untreated (dark gray bars; n = 5) and OKN-treated (light gray bars; n = 5) U87 glioma-bearing rats. Significant decreases were observed for Glut-1 (*P < .05 in F98 gliomas, and **P < .01 in U87 gliomas) and HIF-1α (*P < .05 for both F98 and U87 gliomas) in OKN-treated rats, compared with untreated animals. Significant decreases were observed for MVD (*P < .05) and MIB-1 (**P < .01) in F98 OKN-treated rats when compared with untreated animals. A significant increase in apoptosis (***P < .001 for F98 gliomas and *P < .05 for U87 gliomas) was also observed in OKN-treated rats, compared with untreated animals.
implantation of rat C6 glioma cells) and continued for a total of 10 days.9 One group of rats was euthanized after the 10-day treatment period, and a second group was monitored for an additional 25 days after the treatment period.9 In the cohort of animals that were treated for 10 days and then euthanized, survival was 100% (P < .0001), whereas for the rats that were monitored for an additional 25 days, survival was >80% (P < .001).9 In the present study, both the rat F98 and the human U87 cell models were used to assess the effect of OKN-007 on the more aggressive F98 glioma model and a human GBM-derived cell xenograft model. The F98 glioma model is more infiltrative than the C6 model12,26 and is also synergetic in Fisher 344 rats.10,12 The U87 xenograft model has some characteristics that are associated with GBM, such as high cellularity with atypia (eg, mitotic figures and irregular nucleoli) and profuse neovascularization.10 We can conclude from this study that OKN-007 has the ability to cause glioma regression in aggressive rodent tumor models (F98 and U87) and in moderate gliomas (C6).

Currently, the known pharmacological effects of the nitrones are primarily anti-inflammatory in nature, in addition to their ability to scavenge free radicals. The parent nitrone compound α-phenyl N-tert-butylnitroamine (PBN) is known to inhibit the expression of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and nuclear factor kappaB (NF-κB).8 OKN-007 (formerly known as NXY-059) has been found to be neuroprotective in a rat transient middle cerebral artery occlusion model for neurological ischemia-reperfusion injury.22 The neuroprotective effect of OKN-007 has been suggested to be attributed in part to its ability to restore functionality of the brain endothelium.27 The anticancer activity of PBN-nitrones has been attributed potentially to the suppression of nitric oxide production, suppression of iNOS expression, suppression of S-nitrosylation of critical proteins (caspases, Bcl-2, OGG1 DNA repair enzyme, and PTEN tumor suppressor protein), which are involved in shutting down apoptosis, enhanced mutation events, and enhanced Akt-mediated signaling in oncogenesis, respectively), and inhibition of NF-κB activation.7

OKN-007 was shown in this study to decrease cell proliferation (MIB-1 and Glut-1), angiogenesis (MVD), and hypoxia (HIF-1α) and to increase apoptosis (cleaved caspase 3). OKN-007 may be an ideal antitumor therapeutic agent because of its multiple targets (cell proliferation, angiogenesis [although not necessarily through the VEGF pathway], and apoptosis). OKN-007 may be an ideal therapeutic alternative or may compliment current therapeutic protocols, which include surgical resection, radiation and chemotherapies, and bevacizumab (Avastin; anti-VEGF) treatment.

### Supplementary Material

Supplementary material is available at Neuro-Oncology Journal online (http://neuro-oncology.oxfordjournals.org/).

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### References


