Preclinical Cancer Therapy in a Mouse Model of Neurofibromatosis-1 Optic Glioma

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Abstract

Mouse models of human cancers afford unique opportunities to evaluate novel therapies in preclinical trials. For this purpose, we analyzed three genetically engineered mouse (GEM) models of low-grade glioma resulting from either inactivation of the neurofibromatosis-1 (Nf1) tumor suppressor gene or constitutive activation of KRas in glial cells. Based on tumor proliferation, location, and penetrance, we selected one of these Nf1 GEM models for preclinical drug evaluation. After detection of an optic glioma by manganese-enhanced magnetic resonance imaging, we randomized mice to either treatment or control groups. We first validated the Nf1 optic glioma model using conventional single-agent chemotherapy (temozolomide) currently used for children with low-grade glioma and showed that treatment resulted in decreased proliferation and increased apoptosis of tumor cells in vivo as well as reduced tumor volume. Because neurofibromin negatively regulates mammalian target of rapamycin (mTOR) signaling, we showed that pharmacologic mTOR inhibition in vivo led to decreased tumor cell proliferation in a dose-dependent fashion associated with a decrease in tumor volume. Interestingly, no additive effect of combined rapamycin and temozolomide treatment was observed. Lastly, to determine the effect of these therapies on the normal brain, we showed that treatments that affect tumor cell proliferation or apoptosis did not have a significant effect on the proliferation of progenitor cells within brain germinal zones. Collectively, these findings suggest that this Nf1 optic glioma model may be a potential preclinical benchmark for identifying novel therapies that have a high likelihood of success in human clinical trials. [Cancer Res 2008;68(5):1520–8]

Introduction

Low-grade gliomas account for 50% of all brain tumors in children from birth to 14 years of age (1). Compared with high-grade gliomas, comparatively little is known about the genetic changes that underlie the formation of pediatric low-grade gliomas. Some insights into the molecular pathogenesis of these tumors have derived from the study of children with the inherited tumor predisposition syndrome, neurofibromatosis type-1 (NF1). Young children with NF1 (mean age at diagnosis of 4.5 years) are prone to the development of low-grade astrocytomas that typically involve the optic nerves and optic chiasm (2–4). Although most optic gliomas in children with NF1 are grade I pilocytic astrocytomas, other low-grade gliomas may also be encountered (5). In the context of NF1, both WHO grade I and II gliomas exhibit low proliferative indices (typically less than <1–2%), are locally invasive, and exhibit infrequent nuclear atypia (6).

Despite the benign nature of these tumors, young children with NF1-associated optic glioma may develop vision loss as a result of optic nerve dysfunction or precocious puberty due to hypothalamic involvement (7, 8), necessitating treatment. Currently, symptomatic children are most commonly treated with either combined carboplatin and vincristine chemotherapy (9, 10) or temozolomide alone (11–13). Although chemotherapy results in durable responses in many patients, additional therapies are required for those children who exhibit clinically progressive disease. Unlike the case of high-grade gliomas, for which multiple genetic, xenograft, and cell culture models have supported extensive biological and preclinical investigations, low-grade gliomas have been considerably more challenging to model and study. One of the major technical obstacles has been the relative difficulty of establishing consistent low-grade glioma explant models. These explant models have been limited by their low engraftment rates and inconsistent growth patterns in vivo. To circumvent these problems with explant models, advances in mouse genetic engineering have facilitated the development of mice in which naturally occurring human cancer-causing genetic changes can be introduced into specific cell types (14–17).

To model human NF1-associated low-grade optic glioma in mice, we have taken advantage of LoxP-Cre strategies to conditionally inactivate the Nf1 gene in glial cells. The glial cell lineage was targeted in these mice using two independently generated human GFAP promoter constructs to express Cre recombinase. The first GFAP-Cre line (GFAP-Cre-m), developed by Messing and colleagues (18), results in Nf1 inactivation in GFAP+ cells beginning around embryonic day 11.5, whereas a second GFAP-Cre line (GFAP-Cre), developed in our laboratory, inactivates Nf1 in GFAP+ cells by embryonic day 14.5 (19). Despite the fact that both Cre lines use a similar human GFAP promoter, ~20% of Nf1+/− mice with conditional Nf1 gene inactivation using the GFAP-Cre-m line (Nf1+/−GFAP-wCKO mice) develop optic gliomas involving the retro-orbital optic nerve head (20), whereas ~100% of Nf1+/− mice with conditional Nf1 inactivation using our GFAP-Cre line (Nf1+/−GFAP+CKO mice) develop prechiasmatic and chiasmatic optic gliomas (21, 22). In addition, based on the observation that the Nf1 gene product, neurofibromin, functions to preferentially inhibit KRas activity in astrocytes (23), we generated Nf1+/− mice in which a constitutively active KRas allele (KRas*) is expressed in GFAP+ cells using our GFAP-Cre line. As reported for the original Nf1+/−GFAP+CKO mice, these Nf1+/−KRas+ mice also develop

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prechiasmatic and chiasmatic optic gliomas (23). We have previously characterized the Nf1+/−;GFAP−/−CKO optic glioma model in terms of tumor proliferation and natural history; however, there are no comparable data available for the other two strains.

Although the Nf1+/−;GFAP−/−CKO optic glioma model has been useful for defining the molecular and cellular pathogenesis of NF1 optic glioma formation and growth (24–27), its value in preclinical therapeutics has been largely unexplored (16). The objective of this study was to compare the three genetically engineered mouse (GEM) optic glioma strains and to select the most appropriate one for preclinical trials evaluating both conventional and biologically targeted therapies. Based on several criteria, the Nf1+/−;GFAP−/−CKO optic glioma model was chosen. Using magnetic resonance imaging (MRI) to identify mice with optic glioma, we show that conventional single-agent therapy with temozolomide (mTOR) pathway inhibition in astrocytes as well as in mouse and human optic gliomas leads to mammalian target of rapamycin (mTOR) pathway activation in Nf1 inactivation as well as conditional KRas activation. The first model (Nf1+/−;GFAP−/−CKO mice; ref. 21) was generated by successive breeding of Nf1+/−; mice with GFAP−/−;flox/flox mice (30) and GFAP-Cre mice (19). The second model (Nf1+/−;GFAP−/−CKO mice; ref. 20) was generated by successive breeding of Nf1+/−; mice with GFAP−/−;flox/flox mice (30) and GFAP-Cre;m mice (18). The third model (Nf1+/−;flox/flox mice; ref. 23) was generated by successive breeding of Nf1+/−; mice with LSL-KRasG12D mice (31) and GFAP-Cre;m mice (19). All mice were maintained on a C57BL/6 background.

Materials and Methods
Mice. Three independent GEM models of Nf1 optic glioma were used based on the timing and location of Nf1 inactivation as well as conditional KRas activation. The first model (Nf1+/−;GFAP−/−CKO mice; ref. 21) was generated by successive breeding of Nf1+/−; mice with GFAP−/−;flox/flox mice (30) and GFAP-Cre mice (19). The second model (Nf1+/−;GFAP−/−CKO mice; ref. 20) was generated by successive breeding of Nf1+/−; mice with GFAP−/−;flox/flox mice (30) and GFAP-Cre;m mice (18). The third model (Nf1+/−;flox/flox mice; ref. 23) was generated by successive breeding of Nf1+/−; mice with LSL-KRasG12D mice (31) and GFAP-Cre;m mice (19). All mice were maintained on a C57BL/6 background.

Chemicals. Temozolomide was purchased from the St. Louis Children’s Hospital pharmacy. Rapamycin was purchased from Santa Cruz Biotechnology. All other chemicals were obtained from Sigma.

Manganese-enhanced MRI. MR images were collected in an Oxford Instruments 4.7-T magnet (33 cm, clear bore) equipped with 15-cm inner diameter, actively shielded Oxford gradient coils (maximum gradient, 18 G/cm; rise time, 100 μs), and Tecron Model 3020 gradient power amplifiers. The gradient/ magnets are interfaced with a Varian INOVA console, and data were collected using a home-built 1.5-cm outer diameter surface coil (receive) and a Varian 9-cm inner diameter Helmholtz coil (transmit; ref. 32). Mouse body temperature was maintained at 37 ± 1°C using a heating pad formed by circulating warm water. Mice were anesthetized with isoflurane/O2 and maintained on isoflurane/O2 (1:1.5% (v/v)) during all imaging experiments.

For contrast enhancement, mice were injected i.p. with 140 mg/kg manganese chloride followed by a s.c. injection of 0.5 mL saline to maintain hydration. Transaxial, T1-weighted, spin-echo images and diffusion-weighted MRI data were collected 16 to 48 h later (32). Apparent diffusion coefficients (ADC) and mean ADC values (<ADC>) describing average diffusion in three orthogonal directions, were computed according to standard equations (33). Optic nerve cross-sectional areas were measured following manual segmentation of the images using Imagej software.6

Preclinical paradigm. Nf1+/−;GFAP−/−CKO mice underwent manganese-enhanced MRI (MEMRI) at 10 to 12 weeks of age to confirm the presence of an optic glioma (Fig. 1) followed by randomization to either vehicle or temozolomide treatment (trial 1) or either vehicle or rapamycin treatment (trial 2) or rapamycin and temozolomide treatment (trial 3). In each trial, at least six mice were analyzed in each experimental arm. Temozolomide (42 mg/kg/d for 5 days) was given in the drinking water (n = 7 mice; ref. 34). Water consumption was monitored daily and the dose of temozolomide was adjusted accordingly. Controls were Nf1+/−;GFAP−/−CKO mice that consumed untreated drinking water (n = 6 mice). Three weeks later, mice were euthanized and their optic nerves/brains were removed for analysis. Rapamycin (5 or 20 mg/kg/d) was given as daily i.p. injections of a solution containing 5.2%. TWEEN 80 and 5.2% polyethylene glycol 400 (5 days per week for 2 weeks). Vehicle-treated mice were injected daily with an identical solution lacking rapamycin (n = 6). Four groups of treated mice were analyzed. The first two groups (n = 7 mice) were euthanized at the end of the second week of rapamycin treatment (rapamycin and high rapamycin), whereas the other two groups (n = 6 mice) were euthanized 2 weeks after the completion of rapamycin treatment (rapamycin + 2 weeks and high rapamycin + 2 weeks). Finally, 7 mice were treated simultaneously with temozolomide and rapamycin (5 mg/kg/d) for 5 days followed by rapamycin (5 mg/kg/d) alone for another 5 days. These animals were analyzed 2 weeks after the completion of the treatment.

Immunohistochemistry. Three hours before euthanasia, mice were injected with 50 mg/kg bromodeoxyuridine (BrdUrd). Perfusion, fixation, tissue sectioning, and immunostaining were performed as previously described (22). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) labeling was accomplished according to the manufacturer’s instructions (Roche Diagnostics). The number of BrdUrd-immunoreactive, Ki-67 (MB-1)-immunoreactive, and phospho-S6-immunoreactive cells as well as TUNEL-positive cells in the tumors was quantified by direct counting on three consecutive sections. The Ki-67 labeling indices were determined after counting the total number of nuclei visualized by hematoxylin counterstaining. Similar methods were used to quantitate cells in the mouse subventricular zone (SVZ) and dentate gyrus (DG). BrdUrd (Abcam, Inc.), Ki-67 (BD PharMingen), and phospho-S6 (Ser240/244, Cell Signaling Technology) antibodies were purchased from commercial sources.

Western blotting. Spleens were homogenized in a Dounce homogenizer and six of total protein, as determined using bicinchoninic acid reagents (Fisher), were separated by SDS-PAGE before immunoblotting. Phospho-S6 (Ser240/244), phospho-Akt (Ser473), phospho–p70S6 kinase (p70S6K; Thr389), Akt, S6, and p70S6K antibodies were purchased from Cell Signaling Technology. Blots were developed using chemiluminescence (Fisher) and exposure to BioMax film (Fisher).

Optic glioma measurements. The entire optic nerve with the eye and intact chiasm was microdissected and photographed with a scale bar. Prechiasmatic optic nerve diameters were measured at the chiasm and at −200, −400, and −600 μm anterior to the chiasm. To estimate the volume of the prechiasmatic optic nerve, and chiasm, the volumes (V1, V2, and V3) of the three 200-μm high truncated cones were combined using the diameter (D200), (D400), and (D600) values from each optic nerve measurement. The following equation (35) was used to calculate the total optic nerve volume:

\[
V = \frac{1}{12} \pi h(D_0^3 + D_2^2D_0 + D_2^2)
\]

Statistical analyses. The mean and SE were calculated and presented for each condition (treatment or mouse strain). A one-way ANOVA test was
performed to determine whether the means were significantly different among the various conditions. To compare individual treatment conditions to wild-type (WT) or untreated groups, unpaired t test (with Welch’s correction) analyses were performed. Statistical significance was set at \( P < 0.05 \).

**Results and Discussion**

**Analysis of mouse optic glioma models.** As previously reported by our laboratory (21–23) and by Parada’s group (20), \( N^{1+/-}/C0 \) mice with \( N^{1} \) loss or KRas activation in GFAP+ glial cells develop optic gliomas. Similar to human NF1-associated optic gliomas, the optic gliomas arising in \( N^{1+/-}/C0 \) mice produce focal expansion of the prechiasmatic optic nerves and chiasm, which is evident as focal enlargement on small-animal MRI (Fig. 1; ref. 32). Further, as is typical for the human disease, \( N^{1+/-}/C0 \) mouse optic gliomas have low proliferative indices (Fig. 1; ref. 22), nuclear atypia (Fig. 1), microglial infiltration (24), endothelial cell proliferation (22), as well as diffuse infiltration of the optic nerve (Fig. 1). Moreover, similar to their human counterparts, these tumors show a predilection for the optic nerve and exhibit maximal growth in young mice. However, none of these three models harbors the eosinophilic granular bodies, Rosenthal

![Figure 1.](image1.png)

**Figure 1.** \( N^{1+/-}/C0 \) mice develop optic gliomas similar to those observed in children with NF1. Top row, MRI (MEMRI) shows asymmetrically enlarged optic nerves in one representative \( N^{1+/-}/C0 \) mouse compared with control animals. Mouse optic gliomas display limited hypercellularity with occasional nuclear atypia and low mitotic activity. Bottom row, MRI shows asymmetrically enlarged optic nerves in a child with NF1 compared with MRI of the optic nerves from a healthy individual. Low cellularity and parenchymal infiltration as well as low proliferation rate are characteristics of the human tumors. White arrowheads point to the optic nerves. Black arrows point to atypical nuclei. Black arrowheads indicate MIB-1–labeled cells. Bar, 30 \( \mu \)m.

![Figure 2.](image2.png)

**Figure 2.** Comparison of the time course of optic glioma proliferation in the three GEM mouse strains. A, the number of Ki-67 (MIB-1)-positive cells in the prechiasmatic optic nerves and chiasm was higher in all three mouse strains compared with WT mice at 4 mo of age. At 2 mo of age, only the \( N^{1+/-}/C0 \) mice had a significant increase in optic glioma proliferation compared with WT mice. By 6 mo of age, there were no significant differences in proliferation compared with WT optic nerves. B, Ki-67 labeling indices display an identical trend for all mouse strains analyzed. At 4 mo of age, all three mouse strains exhibited a significant increase in the Ki-67 labeling index compared with WT mice. Columns, mean; bars, SE. *, significant differences \( (P < 0.05) \).
fibers, or microcystic structures commonly seen in the human counterpart, thus precluding their classification as juvenile pilocytic astrocytomas in the mouse. In this regard, these GEM optic gliomas most likely represent low-grade gliomas that are otherwise difficult to definitively classify further as either pilocytic (WHO grade I) or diffuse fibrillary (WHO grade II) astrocytomas; this is in fact similar to a subset of the astrocytomas encountered in children with NF1 (5). These lesions are not gliotic areas because gliosis contracts tissue and lacks the abnormal nuclei (atypia and pleomorphism) seen in tumor specimens.

To select an optimal model for preclinical evaluation, we analyzed the histology and natural history of the three previously published GEM models of optic pathway glioma. All three mouse models exhibit thickened prechiasmatic nerves and enlarged optic chiasms compared with age-matched WT mice, similar to children with NF1-associated optic glioma (Fig. 1). Whereas ~100% of the \( Nf1^{+/−} \) GFAP-CKO and \( Nf1^{+/−} \) KRas* mice harbored optic gliomas, a significantly lower percentage of the \( Nf1^{+/−} \) GFAP-mCKO mice developed optic gliomas in the prechiasmatic optic nerves and chiasm (Supplementary Fig. S1). These results are similar to those previously reported for the \( Nf1^{+/−} \) GFAP-CKO mouse model, in which only 20% of these mice had neoplastic lesions (20). In addition, \( Nf1^{+/−} \) GFAP-CKO and \( Nf1^{+/−} \) KRas* mice developed gliomas involving the prechiasmatic optic nerves and chiasm, whereas \( Nf1^{+/−} \) GFAP-mCKO mice more frequently develop retro-orbital optic gliomas near the optic nerve head. The differences in location and penetrance of the optic glioma phenotype in these three \( Nf1 \) mutant mouse strains underscore the importance of developing and comparing multiple mouse tumor models. Previous studies of human NF1-associated optic gliomas have shown that two thirds of optic gliomas involve the prechiasmatic optic nerves and chiasm (8, 36) similar to the \( Nf1^{+/−} \) GFAP-CKO and \( Nf1^{+/−} \) GFAP-mCKO mouse models, whereas 16% of tumors arise in an intraorbital/retro-orbital location (8).

We next studied the proliferation of the optic gliomas arising in each of the three GEM strains as a function of time. For these experiments, three to five mice per strain and time point were studied at 2, 4, and 6 months of age. We quantified the number of proliferating cells using either BrdUrd (data not shown) or Ki-67 immunostaining (Fig. 2) with equivalent results. These data are presented both as the total number of Ki-67+ cells (Fig. 2A) or the percentage of Ki-67+ cells (Ki-67 labeling index; Fig. 2B) with identical results. We chose to perform both measurements in these experiments because the total number of cells in the optic nerve/chiasm increases as a function of development in young mice. Similar to human NF1-associated gliomas, the proliferative index was low (~1%). The optic gliomas from all three models exhibited increased proliferation at 4 months followed by a decline to WT levels by 6 months. However, only the \( Nf1^{+/−} \) GFAP-CKO mouse strain exhibits increased proliferation at 2 months of age. Based on the time course of optic glioma proliferation, the high level of tumor penetrance, and the preferential localization to the prechiasmatic optic nerves and chiasm, the \( Nf1^{+/−} \) GFAP-CKO mouse model was chosen for the preclinical treatment studies.

We enrolled 10- to 12-week-old \( Nf1^{+/−} \) GFAP-CKO mice to approximate human clinical trials in which children are treated following the detection of an optic glioma. Similar to human clinical studies, we used MRI to identify tumors in living mice before randomization. Based on previous studies showing that maximal optic glioma proliferation in these mice occurs between 1 and 4 months of age, we elected to treat tumors detected in...
10- to 12-week-old Nf1+/−GFAP−/−CKO mice and examine the effect of treatment 4 weeks later.

Temozolomide treatment of Nf1+/−GFAP−/−CKO murine optic glioma results in increased apoptosis and decreased proliferation in vivo. One of the important criteria for an accurate small-animal model of human disease is showing that the disease in the GEM model responds similarly to therapies used to treat the human condition. Therefore, to validate the Nf1+/−GFAP−/−CKO optic glioma mouse model as a preclinical model of human NF1-associated optic glioma, we chose to determine the effect of temozolomide, as a single chemotherapeutic agent, on tumor growth. Before treatment, we routinely observed a 2.5- to 3-fold greater number of BrdUrd-immunoreactive (BrdUrd+) GFAP-immunoreactive cells (glia or astrocytes) using double-labeling immunohistochemistry in the optic nerve gliomas from Nf1+/−GFAP−/−CKO mice compared with WT optic nerves (Fig. 3A). Three weeks after treatment with temozolomide for 5 days, there was a significant decrease in the number of BrdUrd-immunoreactive cells (Fig. 3A) to levels similar to those seen in normal optic nerves. Because BrdUrd is not used to measure proliferation in human tumors, we quantified the number of proliferating tumor cells using Ki-67 (MIB-1). For these experiments, we present the data for the total number of Ki-67+ cells in each mouse tumor. Identical results were also obtained when the Ki-67 labeling index was calculated (data not shown). Following temozolomide treatment, we observed a significant decrease in the number of Ki-67−immunoreactive (proliferating) cells (Fig. 3B). Finally, because temozolomide treatment results in tumor cell death, we also examined programmed cell death (apoptosis) in treated and control optic glioma specimens. We observed an increase in the number of TUNEL-positive (apoptotic) cells following temozolomide treatment (Fig. 3C). WT optic nerves lacking optic gliomas were included in Fig. 3 as reference points for the levels of proliferation and apoptosis seen in tumors before and after treatment.

Rapamycin treatment of Nf1+/−GFAP−/−CKO murine optic glioma results in decreased proliferation in vivo. Neurofibromin, the Nf1 gene product, regulates cell growth in part by inhibiting the activity of the Ras proto-oncogene. Based on this regulatory property, anti-Ras pharmaceuticals were initially used to treat another nervous system tumor in patients with NF1 (37). Unfortunately, these drugs failed to show efficacy (38), suggesting that other biologically based targets need to be identified. Recent studies from our laboratory and others have shown that neurofibromin also negatively regulates the activity of the mTOR (26, 29, 39) and that mTOR inhibitors (e.g., rapamycin) can block the growth of Nf1−deficient murine astrocytes in vitro (26, 29). To determine whether this promising biologically targeted therapy would block Nf1+/−GFAP−/−CKO mouse optic glioma growth, we treated mice with either rapamycin (at 5 and 20 mg/kg/d) or vehicle daily for 2 consecutive weeks. Mice were euthanized either immediately after treatment or 2 weeks later. Rapamycin treatment had the predicted dose-dependent effect on mTOR pathway activation in vivo, shown by decreased activation (phosphorylation) of the downstream mTOR effector, ribosomal S6 (S6-P). Immediately following treatment, there was robust inhibition of S6-P in the tumors; however, this blockade became less significant 2 weeks later at the lower rapamycin dose (5 mg/kg/d; Fig. 4A). In contrast, treatment with 20 mg/kg/d rapamycin resulted in a sustained and durable inhibitory effect on S6 activation 2 weeks after the cessation of treatment (Fig. 4A).

Figure 4. Rapamycin treatment results in inhibition of mTOR activation and decreased tumor proliferation. A, immunostaining with activation-specific S6 antibodies (S6-P) shows that vehicle-treated tumor-bearing mice have significantly higher numbers of S6-P+ cells (arrowheads) in the prechiasmatic region compared with age-matched WT mice. Rapamycin treatment at both doses significantly reduces S6 activation; however, 2 wk after treatment with 5 mg/kg/d rapamycin, the percent of cells exhibiting S6 activation is similar to pretreatment values (rap+2w) but is significantly higher than observed in WT mice. In contrast, 20 mg/kg/d rapamycin resulted in a durable effect lasting for at least 2 wk following the treatment (high+2w). Inset, representative tumor cell with positive cytoplasmic S6-P labeling. B, immunostaining with Ki-67 (MIB-1) shows significantly decreased proliferation (Ki-67+ cells; arrowheads) in the optic nerve gliomas from rapamycin-treated mice compared with vehicle-treated mice after 2 wk with a return to pretreatment values after 2 additional wk (5 mg/kg/d rapamycin). As observed with the S6-P+ staining, 20 mg/kg/d rapamycin results in decreased tumor proliferation after drug cessation that is significantly lower than observed in vehicle-treated mice. Columns, mean; bars, SE. Bar, 40 μm.
Similarly, tumor proliferation was strongly inhibited by 5 mg/kg/d rapamycin treatment with a return to baseline following removal of drug (Fig. 4B). However, 20 mg/kg/d rapamycin treatment led to a sustained reduction in proliferation (Fig. 4B). No change in apoptosis was observed following rapamycin treatment (data not shown). These results show that rapamycin is a potent antineoplastic during treatment, but growth suppression is dependent on both the timing and dose of drug delivery. The lack of a sustained effect of the lower dose of rapamycin on tumor growth suggests tumorstatic, rather than tumorcidal, properties of this biological therapy and is consistent with early clinical studies on human low-grade tumors, including pilocytic astrocytoma, in which continued rapamycin administration resulted in tumor shrinkage (40).

Lastly, we sought to determine whether the combination of temozolomide and 5 mg/kg/d rapamycin would lead to further decreases in tumor proliferation or increases in tumor apoptosis. Analysis of optic nerve proliferation (Ki-67 staining) and apoptosis (TUNEL labeling) did not reveal any additional effect of combined therapy compared with temozolomide alone (data not shown). These results do not support a synergistic or additive effect of combination therapy with rapamycin and suggest that this combination treatment may have limited utility in the short term. It is possible that more durable responses would be observed with combination therapy; however, the tumor proliferative profile of the Nf1+/−;GFAP−/−CKO mouse model precludes an analysis of the effects of combinatorial therapies beyond 4 to 5 months of age.

**Temozolomide and rapamycin effects on optic glioma size.**

To determine whether the decrease in tumor growth was reflected in a change in the overall size of the tumor, we used two methods to calculate optic glioma size. Using a direct measurement method, we estimated the volume of the prechiasmatic portion of the dissected intact optic nerve (Fig. 5A, bottom). We observed a 20% reduction in total optic nerve volume when compared with untreated nerves, with the largest reductions observed following the temozolomide and 20 mg/kg/d rapamycin treatments. However, total optic nerve volume was decreased by only 10% following 5 mg/kg/d rapamycin treatment, which did not reach statistical significance (Fig. 5B). No further reductions in total optic nerve volume were observed following temozolomide plus rapamycin treatment. However, compared with WT optic nerves, there was a 40% increase in total optic nerve volume in untreated Nf1+/−;GFAP−/−CKO mice. Following temozolomide and 20 mg/kg/d rapamycin treatment, this additional volume contributed by the tumor was decreased by 45% and 60%, respectively. This degree of tumor volume reduction is consistent with clinical responses seen in children following successful treatment of solid tumors.

Second, we used MEMRI to estimate tumor volume; however, there was no comparable trend when comparing pretreatment and posttreatment prechiasmic optic nerve cross-sectional areas using this method (data not shown). The inability to use MEMRI to quantify small changes in tumor area likely reflects the thickness of the image slices (500 μm) relative to the tumor (~1 mm). Lastly, we saw no statistically significant change in tumor <ADC> following either temozolomide, rapamycin, or temozolomide and rapamycin treatment (data not shown). Ongoing studies aimed at reducing the slice thickness and collecting images at higher...
magnetic field strength (e.g., 11.7 T) may lead to improved quantification of tumor volume in vivo.

**Temozolomide and rapamycin have minimal effects on normal brain germinal zone cells.** We next assessed the effect of systemic chemotherapy on total body weight and brain germinal zone cell growth. First, in both temozolomide- and rapamycin-treated mice, we observed a reduction in total body weight (Fig. 6A). However, at 5 mg/kg/d rapamycin dose, the mice regained their weight within 2 weeks. In contrast, temozolomide-treated or 20 mg/kg/d rapamycin–treated animals exhibited minimal weight recovery 2 weeks after the completion of therapy. The combination of rapamycin and temozolomide did not result in further weight loss beyond what was observed with either drug alone. These findings indicate that these anticancer drugs are associated with significant systemic effects.

Second, we found that rapamycin treatment results in reduced mTOR pathway activation throughout the body, as evidenced by decreased p70S6K and S6 phosphorylation in the spleen (Fig. 6B). However, there was no significant effect of rapamycin treatment on the levels of Akt activation. In this regard, we found no evidence for feedback between mTOR blockade and Akt activity as previously reported in tumor cell lines (41, 42) or in cells or tumors lacking expression of the tuberous sclerosis complex genes (43, 44). The lack of feedback activation of Akt following rapamycin treatment suggests that mTOR inhibition is unlikely to result in compensatory changes in signaling within NF1-deficient cells that would counteract the inhibitory effects of rapamycin on NF1-associated optic glioma growth. Furthermore, these findings highlight differences between the consequences of NF1 and TSC gene inactivation, which both control mTOR signaling, on cell growth control.

**Figure 6.** Systemic effects of temozolomide and rapamycin. A, the body weight of drug-treated animals decreased during therapy. Rapamycin (20 mg/kg/d) treatment resulted in a statistically significant decrease in body weight immediately after treatment. *, *P* = 0.01. However, 2 wk after drug cessation, there is full and minimal recovery in the 5 and 20 mg/kg/d rapamycin–treated animals, respectively. B, Western immunoblotting of spleen lysates from rapamycin- and vehicle-treated mice shows inhibition of mTOR activation using phospho-p70S6K and phospho-S6 as surrogate markers. Rapamycin treatment did not change the phosphorylation (activation) status of Akt. Total protein levels are included as internal controls for equal protein loading. C, TUNEL staining shows no change in SVZ cell death in temozolomide-, rapamycin-, or combination-treated mice compared with controls. Representative photomicrographs of TUNEL labeling of cells in the SVZ. D, Ki-67 immunostaining shows no change in SVZ cell proliferation following temozolomide, rapamycin, or combination treatment compared with controls. Representative photomicrographs of Ki-67 immunostaining of cells in the SVZ. Columns, mean; bars, SE. Bar, 40 μm.
pathways, and suggest that loss of neurofibromin and tuberin/hamartin may activate mTOR differently. In this regard, previous studies from our laboratory have shown that Nf1 loss in astrocytes has no effect on cell size, whereas Tsc loss in astrocytes leads to increased soma size, an effect that is inhibited by rapamycin (29, 45).

In addition to assessing the effects of these treatments on tumor growth, we also examined the effect of chemotherapy on proliferating cells in brain germinal zones. In 3-month-old mice, neurogenesis largely results from proliferation within the SVZ and hippocampus (46). Four weeks after the beginning of treatment with temozolomide, rapamycin, or the combination of the two drugs, we found that apoptosis in SVZ cells (Fig. 6C) and in DG cells (data not shown) was minimally affected. Similarly, these treatments resulted in no change in SVZ (Fig. 6D) or DG (data not shown) cell proliferation. Collectively, these findings show that treatments sufficient to inhibit proliferation or induce apoptosis in tumor cells did not have a significant effect on the proliferation or apoptosis of the cells in postnatal neurogenic regions.

The use of Nf1+/−/GFAP−/−CKO mice for preclinical drug evaluation. As we enter into an era of biologically targeted therapeutics, it becomes increasingly important to use accurate small-animal cancer models as preclinical benchmarks that can accurately predict therapeutic responses in patients. There are several advantages of the Nf1 optic glioma model. First, tumors arise in a predictable location (prechiasmatic optic nerves and chiasm) with near 100% penetrance, as observed in children with NFI-associated optic gliomas (8, 36). Second, the optic gliomas are readily detectable using small-animal MRI in 15 min and limited image analysis time, allowing for reasonable experimental throughput (32). Third, the mouse optic gliomas share histologic features with the human disease and show similar growth patterns to those seen in children with NFI. Fourth, the initiating genetic event is known (Nf1 inactivation) without the introduction of an oncogenic mutation, such that the Nf1−/− glia in the optic gliomas are able to respond to growth-promoting signals from the brain microenvironment similar to human NFI-associated optic pathway gliomas (24, 27). In this regard, these tumors do not develop escape mechanisms and other genetic/epigenetic changes that may reflect constitutive intracellular signaling pathway activation associated with oncogene expression. Fifth, these tumors arise in immuno-competent mice, allowing investigators to define the role of brain immune system cells (e.g., microglia) in gliomagenesis and growth. Based on recent data, it is possible that future therapies might target microglia and/or microglia-produced factors that promote Nf1−/− astrocyte growth and Nf1 glioma growth. Lastly, the optic gliomas in the Nf1+/−/GFAP−/−CKO mouse exhibit increased cell death, decreased cell proliferation, and reduced tumor volume in response to conventional chemotherapy used for children with optic gliomas.

We have also used the Nf1+/−/GFAP−/−CKO optic glioma model to show target inhibition (inactivation of mTOR signaling following rapamycin treatment), mechanism of therapy effect (increased apoptosis versus reduced proliferation), and the effect of treatments on the normal brain. The ability to study these extratumoral effects is critical when considering chemotherapy in children whose brains are continuing to develop. It should be recognized that the challenges in evaluating low-grade gliomas include their slow growth rate, the minimal shrinkage following treatment, and optimizing tumor suppression during the periods of maximal tumor growth. Unlike high-grade gliomas, where significant tumor shrinkage and survival are end points for successful treatment outcome, children with NFI-associated optic gliomas do not typically exhibit significant tumor shrinkage following chemotherapy and rarely die as a direct result of their brain tumors. Moreover, these tumors tend to grow for a defined period during early childhood and uncommonly continue to grow in children older than 7 years of age. In this regard, therapies need to be designed that target the tumor during its period of rapid growth to avoid the incorrect conclusion that therapy resulted in tumor stabilization after the period of maximal tumor proliferation. For all of these reasons, it is important to recalibrate our expectations for low-grade glioma mouse models based on the unique behavior of human low-grade gliomas.

The use of Nf1+/−/GFAP−/−CKO mouse optic glioma model may afford more accurate predictive information relevant to the human condition, as it closely recapitulates most of the genetic and cellular abnormalities seen in NFI-associated optic pathway gliomas. In addition to this model, it will be important to develop additional GEM that phenocopy more clinically aggressive NFI-associated optic gliomas, such as those that develop in older children (47). The availability of robust small-animal models of low-grade glioma, such as the Nf1+/−/GFAP−/−CKO optic glioma mouse model, provides unique opportunities to examine additional therapies alone or in combination before the treatment of children with nervous system tumors.

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