Preclinical Development

Interpreting Mammalian Target of Rapamycin and Cell Growth Inhibition in a Genetically Engineered Mouse Model of Nf1-Deficient Astrocytes

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Abstract

The identification of mammalian target of rapamycin (mTOR) as a major mediator of neurofibromatosis-1 (NF1) tumor growth has led to the initiation of clinical trials using rapamycin analogs. Previous studies from our laboratory have shown that durable responses to rapamycin treatment in a genetically engineered mouse model of Nf1 optic glioma require 20 mg/kg/day, whereas only transient tumor growth suppression was observed with 5 mg/kg/day rapamycin despite complete silencing of ribosomal S6 activity. To gain clinically relevant insights into the mechanism underlying this dose-dependent effect, we used Nf1-deficient glial cells in vitro and in vivo. First, there was an exponential relationship between blood and brain rapamycin levels. Second, we show that currently used biomarkers of mTOR pathway inhibition (phospho-S6, phospho-4EBP1, phospho-STAT3, and Jagged-1 levels) and tumor proliferation (Ki67) do not accurately reflect mTOR target inhibition or Nf1-deficient glial growth suppression. Third, the incomplete suppression of Nf1-deficient glial cell proliferation in vivo following 5 mg/kg/day rapamycin treatment reflects mTOR-mediated AKT activation, such that combined 5 mg/kg/day rapamycin and PI3-kinase (PI3K) inhibition or dual PI3K/mTOR inhibition recapitulates the growth suppressive effects of 20 mg/kg/day rapamycin. These new findings argue for the identification of more accurate biomarkers for rapamycin treatment response and provide reference preclinical data for comparing human rapamycin levels with target effects in the brain.

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Introduction

Individuals with the neurofibromatosis type 1 (NF1) inherited cancer syndrome are prone to the development of brain (glial cell) tumors that arise in young children along the optic pathway (optic pathway glioma; refs. 1, 2). NF1-associated optic pathway gliomas are World Health Organization grade I astrocytomas composed of glial fibrillary acidic protein (GFAP)-immunoreactive cells (3). These tumors have low proliferative indices and commonly exhibit indolent clinical behavior. Although many of these tumors do not require treatment, those that lead to reduced visual function are managed with carboplatin/vincristine chemotherapy. However, genotoxic therapies are concerning in the setting of a tumor predisposition syndrome. For this reason, numerous studies have focused on the identification of targeted therapies for these common tumors in children with NF1 (4, 5).

To develop preclinical small-animal models of NF1-associated optic glioma, we and others have used Nf1 mutant genetically engineered mice (GEM; refs. 6, 7). Nf1+/− mice with Nf1 gene inactivation in GFAP+ cells develop optic gliomas in the prechiasmatic optic nerve and chiasm by 3 months of age (8, 9). Similar to their human counterparts, these Nf1 mouse gliomas have low proliferative indices, and exhibit microglial infiltration and increased vascularity (9, 10). On the basis of their similarity to NF1-associated optic glioma, Nf1 GEM have been successfully used for proof-of-principle preclinical studies using chemotherapy (temozolomide) to show tumor shrinkage, reduced glioma proliferation, and increased tumor apoptosis (11).

Analysis of Nf1-deficient astrocytes and other Nf1-deficient cell types previously revealed that the NF1 protein, neurofibromin, functions to negatively regulate cell growth by inactivating the Ras proto-oncogene (12, 13). Neurofibromin contains a 300 amino acid residue domain with sequence similarity to members of the GTPase activating protein family of molecules that serve to accelerate the conversion of Ras from its active GTP-
bound to its inactive guanosine diphosphate-bound form (14–16). Subsequent studies further showed that neurofibromin Ras-mediated growth regulation operates through the mammalian target of rapamycin (mTOR) pathway (17, 18). In this regard, NFI-deficient human and mouse cells exhibit increased mTOR pathway activation as evidenced by high levels of activity of the mTOR effector, ribosomal S6. Using activation-specific phospho-S6 antibodies, several groups showed increased mTOR activation in human and mouse NFI-associated tumors, including human and mouse optic gliomas. This exciting finding prompted investigators to do preclinical studies using the rapamycin macrolide to inhibit mTOR activation and NFI-deficient tumor growth in vivo (11, 17, 19).

In these studies, we previously showed that Nfi1 mouse optic glioma proliferation was reduced following rapamycin treatment. Treatment with 5 mg/kg/day rapamycin for 14 days resulted in reduced tumor proliferation using Ki67 (MIB-1) immunohistochemistry and attenuated mTOR pathway activation by phospho-S6 immunostaining; however, this effect was dependent on the continued presence of rapamycin, such that proliferation and mTOR activity returned to pretreatment levels in the presence of rapamycin, such that proliferation and mTOR activity returned to pretreatment levels upon cessation of rapamycin treatment. In this study, we measured rapamycin levels in the blood and brain in Nfi1 mutant mice following treatment with 0, 2, 5, and 20 mg/kg/day rapamycin, and correlated drug dose with mTOR pathway signaling and proliferation in vivo. We found that 5 mg/kg/day rapamycin dosing resulted in increased AKT activation, which was suppressed following 20 mg/kg/day rapamycin treatment. However, neither Ki67 nor phospho-S6 activity were robust biomarkers of the in vivo response to rapamycin. Instead, phospho-histone-H3 most strongly correlated with combined inhibition of both S6 and AKT phosphorylation. We recapitulated these in vivo results using Nfi1-deficient mouse low-grade glioma cells in vitro to show that combined treatment with rapamycin and the LY294002 PI3-kinase (PI3-K) inhibitor suppressed cell growth to levels seen with higher doses of rapamycin alone. Collectively, these data suggest that additional biomarkers will be required to adequately assess mTOR target inhibition and tumor proliferative responses to rapamycin treatment in vivo.

Materials and Methods

Mice

In this study, 5- to 6-week-old Nfi1GFAP-CKO mice (Nfi1flox/flox, GFAP-Cre) were used. These mice lack Nfi1 gene expression in GFAP+ (glial) cells, and were generated by successive intercrossing of Nfi1flox/flox and Nfi1flox/wt, GFAP-Cre mice as previously described (6). All mice were used in accordance with established and approved animal studies protocols at the Washington University School of Medicine. Mice were maintained on an inbred C57Bl/6 background.

mTOR inhibitor (rapamycin) treatment in vivo

Rapamycin (LC Laboratories) was administered at the indicated doses (2, 5, or 20 mg/kg) by daily intraperitoneal injections of rapamycin dissolved in ethanol (5 days per week for 2 weeks total). Vehicle-treated mice received daily injections of an identical solution lacking rapamycin. At least 5 mice were included in each treatment group. After the final injection, mice were euthanized. Blood samples were collected for rapamycin concentration determination, and then the mice were perfused with ice-cold normal saline. Brains were dissected and half of the brain was kept at −80°C to measure brain rapamycin concentrations. The remaining brain was divided into two parts for Western blotting and vibratome sectioning for Ki67 determinations.

Measurement of blood and brain rapamycin levels

Calibrators and quality controls were prepared by spiking known amounts of sirolimus into blank EDTA mouse blood or homogenized mouse brain tissue (Bioreclamation). sirolimus and the internal standard sirolimus-d3 were from Toronto Research Chemicals. Samples were extracted and analyzed using a modification of an online extraction liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/LC–MS/MS) assay previously described for the analysis of zotarolimus in blood and tissues (20). Mouse brains were weighed, high-performance liquid chromatography (HPLC)-grade water was added (1:3, v/v) and tissues were homogenized. For protein precipitation of 100 μL brain homogenates or 100 μL EDTA blood, 400 μL of ZnSO4, 7H2O (17.28 g/L) in 30:70 (v/v) HPLC-grade water/HPLC-grade methanol containing the internal standard sirolimus-d3 (5 ng/mL) was added. Samples were vortexed for 2.5 minutes at room temperature and centrifuged at 13,000 × g for 8 minutes at 4°C using a bench-top microfuge. The supernatant was transferred into 2 mL HPLC autosampler glass vials and 100 μL was injected into the LC/LC–MS/MS system (HPLC Agilent 1100 Series, Applied Biosystems/Sciex API 4000). HPLC I was used for online sample extraction, HPLC II for sample analysis and both were connected via a 6-port switching valve (20).

For online sample cleanup, an extraction column (4.6 × 12.5 mm, 5 μm, Eclipse XDB-C8, Agilent) was used and samples were washed using 20% HPLC-grade methanol/80% HPLC-grade water + 0.1% formic acid delivered at a flow rate of 5 mL/min for 1 minute. The analytes were then backflushed onto a C8 analytical column (4.6 × 150 mm, 5 μm, Zorbax XDB-C8, Agilent) that was kept at 65°C. The following gradient was run: 87% methanol/13% 0.1% formic acid to 100% methanol within 2.0 minutes and then 100% methanol for an additional 1.5 minutes. The flow rate was 1 mL/min. The mass spectrometer was run in the...
positive MRM (multiple reaction monitoring) mode. The desolvation gas was heated to 600 °C, and the declustering potential was set to 160 V and the collision energy to 77 eV. The following ion transitions were monitored: m/z = 936.5—409.3 for sirolimus [M + Na]⁺ and m/z = 939.5—409.3 for the internal standard sirolimus-d3 [M + Na]⁺. The lower limit of quantitation in mouse brain tissue was 2 μg/g and in EDTA blood 0.5 ng/mL. The range of reliable response was 2 to 1,000 μg/g and 1 to 5,000 ng/mL, respectively (r > 0.99). The interday accuracy was between 85% and 115% and total imprecision was less than 15%. No relevant carryover, matrix interferences, and ion suppression/ ion enhancement were detected.

**Cell lines**

The mouse K4622 grade II glioma cell line was derived from a C57Bl/6 Nf1+/−; p53+/− (NPCis) mouse and was shown to be both Nf1− and p53-deficient (21). These cells were maintained in Dulbecco’s Modified Eagles’ Medium supplemented with 10% fetal bovine serum and 1% Pen-Strep.

**Pharmacologic inhibitors**

Rapamycin (LC Laboratories, catalog number R-5000; Supplementary Fig. S1A), NVP-BEZ235 (LC Laboratories, catalog number 915019-65-7; Supplementary Fig. S1B), and LY294002 (Calbiochem) were purchased from commercial sources. In vitro treatments were for 16 to 18 hours unless otherwise indicated. Experiments were done at least 3 times with identical results.

**Cell proliferation**

K4622 mouse glioma cells were plated (10,000 cells per well) in 24-well dishes and allowed to adhere for 24 hours followed by treatment with rapamycin, NVP-BEZ235, or LY294002 at the indicated concentrations. Cells were exposed to [3H]-thymidine (1 μCi/mL) for 4 hours. All assays were done 3 times with identical results (18, 21).

**Immunohistochemistry**

Brain tissues from rapamycin-treated or vehicle-treated mice were postfixed in 4% paraformaldehyde in PBS. Following sectioning on a vibratome, immunostaining with Ki67 (BD PharMingen) antibodies was done as previously described (6). The number of Ki67−immunoreactive cells in the dentate gyrus was quantified by direct counting on 3 consecutive sections from each mouse.

**Western blotting**

Brain tissues were harvested in NP-40 lysis buffer with protease and phosphatase inhibitors and homogenized by mechanical disruption. K4622 cells were lysed in standard NP-40 lysis buffer with protease and phosphatase inhibitors. Western blotting was done as previously described (22). All antibodies were purchased from Cell Signaling Technology and used at a 1:1,000 dilution unless otherwise stated. Primary phospho-histone-H3 (Ser10) antibody was purchased from Abcam, Inc. Following horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) incubation, detection was accomplished by enhanced chemiluminescence (Amersham Biosciences). Densitometry analysis was done with Gel-Pro Analyzer 4.0 software (Media Cybernetics) using α-tubulin (Sigma), β-actin or non–phospho-STAT3, AKT, S6, and 4EBP1 antibodies for normalization.

**Statistical analysis**

The measures of all biomarkers were summarized using the mean and SEM. The raw data were checked graphically and data transformation was done as necessary. The relationship between blood and brain rapamycin concentrations was described by linear regression analysis. For each biomarker, the differences of means among rapamycin dose levels were compared using 1-way ANOVA, followed by post hoc tests to compare individual treatment conditions to the wild-type or untreated group. To control the family-wise false-positive rate at the designed 0.05 level; however, such post hoc comparisons were conducted only if the overall difference in ANOVA was significant. All the analyses were implemented by the comprehensive statistical package R (http://cran.r-project.org/) and P < 0.05 were considered to be statistically significant.

**Results**

**Brain rapamycin levels are exponentially correlated with blood rapamycin levels**

One of the major obstacles in translating preclinical drug studies to human clinical trials is a relative paucity of reported blood and target tissue drug levels. In these studies, we sought to determine whether rapamycin crosses the blood–brain barrier and how brain tissue levels correlated with rapamycin dose and circulating levels. First, we measured rapamycin concentrations in the blood and brain tissues of 6- to 8-week-old Nf1+/−GFAPCKO mice either treated with rapamycin or vehicle daily for 2 consecutive weeks. Nf1+/−GFAPCKO mice were chosen as they lack neurofibromin expression in glial cells (astrocytes), but do not develop tumors. The optic glioma tumors in Nf1+/−GFAPCKO mice are too small for accurate determinations of rapamycin levels or drug target inhibition. Using LC/LC–MS/MS assay methods, we found dose-dependent increases in rapamycin concentrations in blood (Fig. 1A) and brain tissue (Fig. 1B). These observations showed that intraperitoneal rapamycin administration increases both blood and brain levels. However, brain rapamycin levels were exponentially correlated with blood rapamycin levels (Fig. 1C; R² = 0.8, P < 0.0001).

**Phospho-S6 is not a reliable biomarker of rapamycin inhibition in the brain**

Assessment of drug effects often involves the use of surrogate markers of drug target inhibition. Rapamycin is a robust inhibitor of mTOR signaling, and is most effective at blocking TORC1 activity (23–27). TORC1 signaling
involves raptor-mediated activation of ribosomal S6 kinase and S6. To this end, activation-specific phospho-S6 antibodies have been used as a surrogate biomarker of mTOR inhibition by rapamycin and rapamycin analogs.

First, we measured phospho-S6 levels in rapamycin-treated (2, 5, 20 mg/kg/day) or vehicle-treated Nf1GFAPCKO mouse brains. Rapamycin treatment at 5 and 20 mg/kg/day doses resulted in suppression of S6 activation.

Figure 1. Brain rapamycin levels are exponentially correlated with blood rapamycin levels. A, top, blood rapamycin concentration was increased with increasing dose as measured by LC/LC-MS/MS and represented as a column graph. Error bars represent the SEM. Bottom, representative scatter plot shows the linear regression analysis ($R^2 = 0.91, P < 0.0001$). B, rapamycin concentrations in brain tissue were increased as a function of dose (top). Error bars represent the SEM. Bottom, scatter plot shows the linear regression analysis ($R^2 = 0.91, P < 0.0001$). C, the relationship between blood and brain rapamycin was exponential ($R^2 = 0.8, P < 0.0001$). Data from 5 mice per treatment group were included and all the $R^2$ and $P$ values were obtained after logarithm data transformation. *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$.
using both phospho-S6-Ser\textsuperscript{235/236} (Fig. 2A) and phospho-S6-Ser\textsuperscript{240/244} (data not shown) antibodies after normalization to total S6 levels. No significant phospho-S6 reduction was observed at the 2 mg/kg/day rapamycin dose. Identical results were also obtained if the total number of phospho-S6–immunoreactive cells were quantitated by immunohistochemistry in the brains of \textit{Nf1}\textsuperscript{GFP}\textsuperscript{-/-} GFAPCKO mice following rapamycin treatment (data not shown).

Second, to additionally evaluate mTOR activity in the brain after rapamycin treatment, we examined Thr\textsuperscript{37/46} phosphorylation of the mTOR effector 4EBP1 (Fig. 2B). Similar to S6 activation, 4EBP1 phosphorylation was unchanged at the 2 mg/kg/day dose, whereas significant
reductions were observed both at 5 and 20 mg/kg/day rapamycin doses. Third, recent studies showed that mTOR regulates Jagged-1 levels in tuberous sclerosis complex-deficient cells (28). To determine whether Jagged-1 levels are elevated in Nf1-deficient primary astrocytes, we did Western blotting. We observed a 2-fold increase in Jagged-1 protein expression in Nf1−/− relative to wild-type astrocytes (Supplementary Fig. S2A). This increase in Jagged-1 protein expression was inhibited by rapamycin treatment; however, Jagged-1 levels were reduced in Nf1GFP/CKO mice treated with 2 mg/kg/day rapamycin—a dose at which no change in phospho-S6 expression was seen (Supplementary Fig. S2B).

Although neurofibromin regulates cell growth through the mTOR pathway in many distinct cell types, we previously showed that increased Nf1-deficient astrocyte growth reflects raptor-dependent signaling through the Rac1/STAT3 pathway (21). In light of this tissue specificity, we next measured STAT3 activation using phospho-STAT3 (Ser727) antibodies. Consistent with the results obtained using phospho-S6 and phospho-Rac1/STAT3 pathway (21). In light of this tissue specificity, we next measured STAT3 activation using phospho-STAT3 (Ser727) antibodies. Consistent with the results obtained using phospho-S6 and phospho-4EBP1, STAT3 inhibition was only observed in the brain at the 5 and 20 mg/kg/day doses (Fig. 2C). Collectively, these data show that inhibition of mTOR downstream signaling in the brain requires at least 5 mg/kg/day rapamycin treatment.

**Ki67 does not accurately reflect rapamycin inhibition of glial cell proliferation in vivo**

Previous preclinical studies from our laboratory and by others have used Ki67 as a marker of tumor cell proliferation (11, 29). In these studies, we found that either the number of Ki67 cells or the percent of Ki67-positive cells in Nf1−/−/GFAP/CKO mouse optic glioma tumors or Nf1-deficient glial cells correlated well with other markers of cell proliferation in vivo, including bromodeoxyuridine incorporation (11). To determine whether Ki67 accurately reflected rapamycin inhibition of Nf1-deficient brain glial cell proliferation in vivo, we quantified the number of proliferating cells in a defined region of the dentate gyrus using standard Ki67 (MIB-1) immunostaining. Surprisingly, we observed a significant decrease in the number of Ki67-immunoreactive (proliferating) cells (Fig. 3A) at 2 mg/kg/day rapamycin. As shown earlier, 2 mg/kg/day rapamycin did not inhibit mTOR signaling. This finding suggests that Ki67 may not be the ideal marker to measure glial cell proliferation in preclinical Nf1 GEM low-grade glioma rapamycin drug studies. Using a less conventional marker of cell proliferation (proliferating cell nuclear antigen; PCNA), statistically significant reductions in cell proliferation were only observed following 20 mg/kg/day rapamycin treatment (Supplementary Fig. S3B). However, the low numbers of PCNA-immunoreactive cells present in each section limits the routine use of this marker for preclinical studies, consistent with other reports comparing these two biomarkers (30–33).

To identify more accurate markers of Nf1-deficient glial cell proliferation in vivo, we next measured cyclin D1 levels. Cyclin D1 was chosen on the basis of our previous studies, which showed that STAT3 regulates cyclin D1 expression in Nf1-deficient cells (21). Interestingly, we found that inhibition of cyclin D1 was only observed at 20 mg/kg/day rapamycin dosing (Fig. 3B). To confirm the apparent inhibition of cell growth only at 20 mg/kg/day rapamycin dosing, we measured phospho-histone H3 (HH3) levels using phospho-HH3 (Ser10) antibodies. Similar to the results obtained using cyclin D1 antibodies, we observed reduced phospho-HH3 only with 20 mg/kg/day rapamycin (Fig. 3C). Collectively, these results show that phospho-HH3 and cyclin D1 may be more accurate indicators of mTOR-mediated glial growth inhibition in the brain.

The inability of 5 mg/kg/day rapamycin to strongly inhibit glial proliferation in vivo reflects AKT hyperactivation

On the basis of the results described earlier, we sought to determine why equivalent mTOR pathway inhibition using several surrogate markers of mTOR activity was observed following 5 and 20 mg/kg/day rapamycin treatment; however, only 20 mg/kg/day rapamycin led to reduced glial cell proliferation in vivo. Previous studies have shown that rapamycin inhibition can paradoxically cause an increase AKT activation as a result of inhibition of TORC1-mediated AKT inhibition and/or TORC2-mediated AKT activation (34–37). Although this anomalous AKT activation has been reported in human Nf1-associated tumors (19, 29), this has not been previously observed in Nf1 GEM tumors or primary tissues (11, 38). To determine whether AKT activation was seen in the brain following rapamycin treatment, we measured AKT Ser473 phosphorylation in vivo, and found a 4-fold increase in AKT activation at 5 mg/kg/day, but not at 2 or 20 mg/kg/day. These results suggest that AKT activation might counteract the growth suppressive effects of rapamycin-mediated mTOR inhibition.

To establish an in vitro model for these observations, we examined AKT activation, mTOR activity, and cell proliferation in Nf1-deficient K4622 glioma cells treated over a wide range of rapamycin concentrations from 0.001 to 500 nmol/L (data not shown). On the basis of this dose range, we selected 3 rapamycin doses (0.001, 0.75, and 10 nmol/L) to parallel the effects of 2, 5, and 20 mg/kg/day rapamycin treatment. Similar to the results obtained following 5 mg/kg/day rapamycin treatment in vivo, we found that 0.75 nmol/L rapamycin resulted in a 5-fold increase in AKT activation compared with vehicle control, whereas no change in AKT Ser473 phosphorylation was observed at 0.001 or 10 nmol/L rapamycin (Fig. 4A). No change in AKT phosphorylation at residue Thr-308 was observed following 0.75 nmol/L rapamycin treatment (Supplementary Fig. S4A), and no increase in AKT Thr-308 phosphorylation was found in Nf1-deficient astrocytes relative to their wild-type controls (data not
shown). In addition, S6-Ser\textsuperscript{235/236}, STAT3-Ser\textsuperscript{727}, and 4EBP1-Thr\textsuperscript{37/46} phosphorylation was decreased in a rapamycin dose-dependent fashion (Fig. 4B).

Next, to determine whether rapamycin inhibits Nf1\textsuperscript{-/-} deficient glioma cell proliferation \textit{in vitro}, we examined the effect of rapamycin treatment (0.001, 0.75, and 10 nmol/L) on \textsuperscript{3}H-thymidine incorporation and histone-H3-Ser\textsuperscript{10} phosphorylation. Similar to the \textit{in vivo} results described earlier, we observed a maximum inhibitory effect on cell growth and histone-H3 phosphorylation.
Figure 4. The inability of 5 mg/kg/day rapamycin to inhibit glial proliferation in vivo reflects AKT hyperactivation. A, top, increased AKT-Ser\(^{473}\) phosphorylation was observed after 5 mg/kg/day rapamycin treatment, but not 2 or 20 mg/kg/day rapamycin treatment. Equal protein loading was confirmed by total AKT immunoblotting. *, \(P = 0.01\). B, K4622 cells were treated either with ethanol (vehicle) or 3 different concentrations of rapamycin (0.001, 0.75, and 10 nmol/L). Western blot analysis of phospho-S6 (Ser\(^{235/236}\)), STAT3 (Ser\(^{727}\)), 4EBP1 (Thr\(^{37/46}\)), and AKT (Ser\(^{473}\)) levels in Nf1-deficient K4622 glioma cells following rapamycin treatment. Total S6, STAT3, 4EBP1, AKT, and \(\alpha\)-tubulin served as internal loading controls. C, 0.001, 0.75, and 10 nmol/L rapamycin treatment reduced K4622 glioma cell growth by 14%, 35.6% (\(P < 0.05\)), and 78% (\(P < 0.0001\)), respectively, as measured by \([\text{H}]\)-thymidine incorporation. *, a statistically significant difference. D, Western blotting of K4622 cell lysates shows inhibition of phospho-histone H3 activation following rapamycin treatment. \(\alpha\)-Tubulin was included as an internal loading control. Relative density (R.D.) values are included at the bottom of the corresponding blots.
following 10 nmol/L rapamycin treatment (Fig. 4C and D), with no further inhibition observed at 100 or 500 nmol/L doses (Supplementary Fig. S5). Collectively, these results suggest that effective brain glial cell growth inhibition by rapamycin requires doses that inhibit both TORC1 (S6, 4EBP1, and STAT3) and TORC2 (AKT) downstream pathways.

Maximal Nf1-deficient glial cell growth inhibition requires both mTOR and AKT silencing

Because maximal glial growth inhibition was only observed when both raptor-mediated (S6, 4EBP1, and STAT3) and rictor-mediated (AKT) mTOR downstream pathways were silenced by rapamycin treatment, we next sought to determine whether combined rapamycin and AKT inhibition could suppress Nf1−/− glioma cell growth similar to "high dose" (10 nmol/L) rapamycin treatment. In these experiments, we examined the effect of combined mTOR (rapamycin) and PI3-K inhibitor (LY294002) treatment on [3H]-thymidine incorporation and S6-Ser235/236, STAT3-Ser727, and AKT-Ser473 phosphorylation in K4622 Nf1-deficient mouse glioma cells. Following 0.75 nmol/L rapamycin treatment, S6-Ser235/236 AKT-Ser473 and STAT3-Ser727 phosphorylation (Fig. 5A) was significantly attenuated, and cell proliferation (Fig. 5B) was reduced by approximately 36%. Whereas pharmacologic inhibition of PI3K (30 μmol/L) reduced K4622 glioma cell growth by 50%, combined 0.750 nmol/L rapamycin and 30 μmol/L LY294002 treatment resulted in an 81% reduction in cell proliferation (Fig. 5B), similar to what was obtained following 10 nmol/L rapamycin treatment. This reduction in cell growth was not the result of increased apoptosis, as assessed by cleaved PARP immunoblotting (Supplementary Fig. S4).

Next, we used the NVP-BEZ235 dual kinase inhibitor to target both PI3K and mTOR (39, 40). Consistent with the results obtained following combined LY294002 and rapamycin treatment, AKT and mTOR (S6, STAT3, and 4EBP1) phosphorylation were blocked by NVP-BEZ235 at all doses examined (Fig. 6A). As described earlier, AKT phosphorylation on residue Ser-473, but not Thr-308, was inhibited by NVP-BEZ235 treatment. The observed suppression of AKT and mTOR activities resulted in inhibition of Nf1-deficient K4622 glioma cell growth (Fig. 6B). Collectively, these results show that combined PI3K and mTOR blockade effectively inhibits Nf1-deficient glioma proliferation.

Discussion

Preclinical cancer studies are designed to provide instructive information that informs human clinical trials. These small-animal drug trials aim to assess drug efficacy in the intact animal, correlate target inhibition with tumor growth suppression, and/or validate surrogate biomarkers of treatment success. The ability to achieve these objectives is highly dependent on the availability of accurate models that duplicate key
features of the human condition coupled with a rigorous evaluation of potential drug therapies. In the case of NF1-associated tumors, including optic glioma and malignant peripheral nerve sheath tumor (MPNST), rapamycin inhibits tumor growth in vivo (11, 19, 29, 38). However, in these various models, there were significant differences in the doses of rapamycin used (1 mg/kg/day to 20 mg/kg/day) and the effects of rapamycin on mTOR signaling. This variation could reflect differences in tumor species of origin [mouse (11, 38) versus human (19, 29)], host species immunocompetence [GEM (11, 38) versus tumor explants into immunocompromised mice (19, 29)], or tumor location [brain (11, 38) versus body (19, 29, 38)]. Similarly, these differences could result from disparities in rapamycin bioavailability. In this study, we did a series of experiments to address some of these critical issues.

First, we sought to define the relationship between blood and brain rapamycin levels and drug dose. Although we observed a dose-dependent increase in both blood and brain rapamycin concentrations with escalating rapamycin doses, the relationship between blood and brain rapamycin levels deserves further discussion. Most NF1 mouse preclinical studies have used rapamycin doses between 1 and 10 mg/kg/day (11, 38). In the brain, there was a linear relationship between blood and brain rapamycin concentrations at the lower doses (2 and 5 mg/kg/day); however, we observed an exponential relationship at the higher dose (20 mg/kg/day). This result suggests that rapamycin brain delivery may involve different order kinetics than that observed for peripheral tissues. It is well established that p-glycoprotein as an efflux transporter is a critical component of the blood–brain barrier (41). As such, it limits the entry of drugs that are substrates, such as rapamycin, into the brain (42). Our data may indicate that at the highest rapamycin dose (20 mg/kg) systemic rapamycin exposure exceeds the capacity of brain barrier efflux transporters, leading to the

Figure 6. Treatment with the dual PI3K/mTOR inhibitor NVP-BEZ235 inhibits NF1-deficient glioma cell growth. A, NF1-deficient K4622 glioma cells were treated with dimethyl sulfoxide (vehicle) or 3 different concentrations of NVP-BEZ235 (0.5, 1, and 2.5 μmol/L). Western blot analysis following NVP-BEZ235 treatment revealed reduced AKT (Ser473), S6 (Ser235/236), STAT3 (Ser727), and 4EBP1 (Thr37/46) phosphorylation. Total S6, AKT, 4EBP1, and STAT3 served as internal protein loading controls. Relative density (R.D.) values are included at the bottom of the corresponding blots. B, NVP-BEZ235 treatment reduced K4622 glioma cell growth by approximately 90% (P < 0.0001), similar to combined treatment with LY294002 and rapamycin. *, a statistically significant difference.

Although we observed a dose-dependent increase in both blood and brain rapamycin concentrations with escalating rapamycin doses, the relationship between blood and brain rapamycin levels deserves further discussion. Most NF1 mouse preclinical studies have used rapamycin doses between 1 and 10 mg/kg/day (11, 38). In the brain, there was a linear relationship between blood and brain rapamycin concentrations at the lower doses (2 and 5 mg/kg/day); however, we observed an exponential relationship at the higher dose (20 mg/kg/day). This result suggests that rapamycin brain delivery may involve different order kinetics than that observed for peripheral tissues. It is well established that p-glycoprotein as an efflux transporter is a critical component of the blood–brain barrier (41). As such, it limits the entry of drugs that are substrates, such as rapamycin, into the brain (42). Our data may indicate that at the highest rapamycin dose (20 mg/kg) systemic rapamycin exposure exceeds the capacity of brain barrier efflux transporters, leading to the
observed exponential increase in brain rapamycin concentrations.

Next, we evaluated mTOR target inhibition in the brain following rapamycin treatment, and were surprised to find that phospho-S6 inhibition did not correlate with reduced Nf1-deficient glial cell proliferation. This is consistent with in vitro studies using Nf1-deficient NPCis glioma cells or primary brain astrocytes (Supplementary Fig. S6A and B). In these experiments, more than 90% reduction in phospho-S6 levels (using either phospho-Ser235/236 or phospho-S6-Ser240/244 antibodies) was seen at rapamycin doses 10-fold lower than doses required to inhibit Nf1-deficient glial cell proliferation. Similarly, other mTOR downstream effectors, including STAT3 and 4EBP1, showed similar relationships between target inhibition and growth suppression. These findings suggest that maximal rapamycin-mediated growth suppression involves more than mTOR/raptor downstream target inhibition.

It is also possible that the inability of mTOR/raptor inhibition (phospho-S6/4EBP1/STAT3 levels) to predict mTOR-mediated growth suppression reflects rapamycin-induced mTOR-dependent AKT activation. Following 5 mg/kg/day rapamycin treatment, there is increased AKT Ser473 phosphorylation in the brains of Nf1<sup>GFP<sup>−/−</sup></sup>-deficient GEM optic glioma (11). For this reason, we sought to identify proliferation biomarkers that correlate with the sustained tumor growth suppression. In this study, we found that phospho-histone-H3 and cyclin D1 more accurately reflected reduced Nf1-deficient glial cell growth in response to rapamycin in vitro and in vivo. The finding that cyclin D1 levels paralleled growth inhibition has also been noted in Nf1-deficient MPNST preclinical studies (38). To this end, reexpression of the GTPase activating domain of neurofibromin was sufficient to restore cyclin D1 levels to normal.

As we attempt to leverage the emerging experience with GEM preclinical therapeutic studies to design more effective drug regimens for brain tumors, it becomes increasingly important to validate surrogate markers of disease activity and target inhibition in relevant small-animal models. The findings described in this study suggest that blood rapamycin levels do not accurately reflect brain concentrations and that currently used mTOR signaling biomarkers inadequately correlate with inhibition of Nf1<sup>−/−</sup> glial cell proliferation. Moreover, as suggested by other preclinical studies on glioma and MPNST (39, 43), dual kinase inhibitors that block both PI3K and mTOR function may have particular efficacy in the treatment of NF1-associated glioma. Future studies should focus on identifying additional surrogate biomarkers for Nf1-deficient tumors suitable for preclinical and eventually clinical studies targeting the AKT/mTOR signaling pathway (39, 43–48). The availability of more accurate biomarkers may increase the utility of preclinical mouse models in informing human clinical drug trials.

Disclosure of Potential Conflicts of Interest

D.H. Gutmann: University of Michigan; NFi gene patent.

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