Prolonged Inhibition of Glioblastoma Xenograft Initiation and Clonogenic Growth following In Vivo Notch Blockade

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Abstract

**Purpose:** To examine the effects of clinically relevant pharmacologic Notch inhibition on glioblastoma xenografts.

**Experimental Design:** Murine orthotopic xenografts generated from temozolomide-sensitive and -resistant glioblastoma neurosphere lines were treated with the γ-secretase inhibitor MRK003. Tumor growth was tracked by weekly imaging, and the effects on animal survival and tumor proliferation were assessed, along with the expression of Notch targets, stem cell, and differentiation markers, and the biology of neurospheres isolated from previously treated xenografts and controls.

**Results:** Weekly MRK003 therapy resulted in significant reductions in growth as measured by imaging, as well as prolongation of survival. Microscopic examination confirmed a statistically significant reduction in cross-sectional tumor area and mitotic index in a MRK003-treated cohort as compared with controls. Expression of multiple Notch targets was reduced in the xenografts, along with neural stem/progenitor cell markers, whereas glial differentiation was induced. Neurospheres derived from MRK003-treated xenografts exhibited reduced clonogenicity and formed less aggressive secondary xenografts. Neurospheres isolated from treated xenografts remained sensitive to MRK003, suggesting that therapeutic resistance does not rapidly arise during in vivo Notch blockade.

**Conclusions:** Weekly oral delivery of MRK003 results in significant in vivo inhibition of Notch pathway activity, tumor growth, stem cell marker expression, and clonogenicity, providing preclinical support for the use of such compounds in patients with malignant brain tumors. Some of these effects can persist for some time after in vivo therapy is complete. Clin Cancer Res; 19(12); 3224–33. ©2013 AACR.

Introduction

Glioblastoma (GBM) is the most common primary malignancy of the central nervous system in adults, and more than 70% of patients die within 2 years of diagnosis (1). More effective therapies are urgently needed, including treatments which can target the subpopulation of primitive cancer stem cells (CSC) thought to be resistant to radiation and standard chemotherapies (2–4). It has been suggested that signaling pathways active in non-neoplastic neural stem cells are also required in stem-like glioma cells (5, 6), including Notch, a pathway also known to promote neural stem cell identity and survival in the fetal and adult brain (7–9).

Prior reports support the concept that Notch signaling is active in glioblastoma and other malignant gliomas and that Notch inhibition can slow the growth of tumor cells as well as sensitize them to radiation or chemotherapy (10–20). In many of these studies, in vitro Notch blockade resulted in reductions in clonogenic capacity or a decreased fraction of tumor cells expressing stem cell markers, suggesting that glioma CSCs have an ongoing requirement for pathway activity (13, 18, 19, 21, 22). The invasive capacity of gliomas has also been linked to Notch (23–25). Thus, targeting Notch signaling may affect several processes critical for the growth and spread of glioblastoma. Notch signaling can be initiated in gliomas by canonical and noncanonical pathway ligands, with hypoxia inducing their expression in neoplastic cells and perivascular stromal cells serving as another potential source (13, 19, 22, 26, 27, 28). Alternative mechanisms, such as activation of Notch signaling by perivascular nitric oxide, have also been shown (29).

Ligand binding to Notch induces cleavage of the transmembrane receptor by the γ-secretase complex, and several small-molecule γ-secretase inhibitors (GSI) which block this process have been used to treat glioblastoma cultures in vitro (13, 30–34). Much less is known, however, about the...
Translational Relevance

Glioblastoma are among the most aggressive solid tumors, and essentially no curative therapies currently exist. The Notch pathway represents an attractive new target, but testing of clinically relevant glioma models and therapeutic regimens has been limited. We report that orally delivered γ-secretase inhibitor MRK003 can effectively penetrate the brain and inhibit Notch activity, stem cell phenotype, and tumor growth in xenografts generated from glioblastoma neurospheres. Interestingly, neurospheres isolated from MRK003-treated xenografts show persistent suppression of clonogenicity and in vivo growth capacity, suggesting that Notch blockade can have long-term effects on glioma biology.

In vivo effects of GSIs on glioma stem cells and intracranial tumor growth.

We therefore used the GSI MRK003 to treat highly invasive, radiographically detectable orthotopic xenografts generated from GBM neurospheres (34). In a recent MK0572 GSI trial in patients with advanced solid tumors, a once-weekly dosing scheme was found to be optimal (35). MRK003 is structurally similar to MK0572 and is used in preclinical in vivo studies of both solid tumors and leukemia as it has superior pharmacokinetic properties in rodents (36–41). The optimal MRK003 dosing regimen in mice is 300 mg/kg once weekly by oral gavage, with maximal plasma drug concentrations seen 4 hours after administration and trough levels after 72 hours (42). Similarly, MK0572 has been shown in humans to reach peak concentrations in 3 to 8.4 hours, with target engagement by the drug estimated to last 48 to 96 hours in a once-a-week dosing regimen (35).

We focused on whether the GSI could suppress Notch activity and the expression of stem cell markers in glioma cells in vivo, the effects on xenograft growth and clonogenic potential, and whether therapeutic resistance emerged in neurospheres isolated from treated tumors. Interestingly, we found that when neurospheres are isolated from xenografts previously treated with MRK003, they show reduced clonogenic capacity in vitro and generate less aggressive tumors in vivo in the absence of additional therapy. This suggests that in vivo Notch blockade can cause prolonged suppression of glioblastoma xenograft initiating capacity even after treatment has stopped.

Materials and Methods

Cell culture and in vitro lentiviral labeling

The HSR-GBM1 neurosphere line was isolated by Galli and colleagues from a primary glioblastoma and was originally designated 020913 (43). The same group later isolated GBM line 040821. Both lines lack mutations in IDH1 and are lentivirally labeled with GFP (44). Five days after transduction, single cells were plated in 96-well plates at a density of 0.7 cell per well and individual colonies were obtained. The luciferase activity of every clone was measured using a luciferase reporter assay system (Promega) and expressed as relative luminescence units (RLU). Clones which had high RLU were designated HSR-GBM1-Luc or 040821-Luc and expanded for xenograft studies.

Intracranial xenografts

About 10^5 HSR-GBM1-Luc or 040821-Luc cells in 2 μL PBS dissociated to single cells were injected over 10 minutes into the right striatum of athymic (nu/nu) mice (Harlan) as previously described (45). Mice were monitored daily for neurologic changes, and tumor growth was tracked weekly using the Xenogen IVIS Spectrum optical Imaging Device and Living Image software (both from Calipers). Tumors were first identified by imaging from 4 to 6 weeks after injection and divided into vehicle and treatment groups containing lesions of comparable initial sizes. Animals in the treatment group received 300 mg/kg MRK003 provided by Merck Research Laboratories once weekly by oral gavage prepared as previously described (46), whereas the control group received vehicle. After sacrifice, brains were removed and bisected in a coronal plane at the level of tumor injection. One half of the brain was formalin-fixed, paraffin-embedded, and used for histopathologic analysis. Visually apparent tumor in the other portion was used for nucleic acid extraction and the generation of secondary tumor neurospheres. Microscopic examination, including mitotic counts and quantification of Ki67 (sc15402, 1:250, Santa Cruz Biologicals), PECAM (sc1506, 1:100, Santa Cruz Biologicals), and cleaved caspase-3 (#96618, 1:25, Cell Signaling Technology), was conducted by a board-certified neuropathologist masked with respect to treatment group (B.A. Orr or C.G. Eberhart). Measurement of cross-sectional tumor area at the injection site was conducted on the first hematoxylin and eosin (H&E)-stained slide cut from each xenograft block by B.A. Orr, who was masked with respect to treatment group, using Spot Basic Software (SPOT Imaging Solutions).

RNA extraction and quantitative PCR

Freshly dissected xenograft tissue was homogenized and nucleic acids extracted using TRIzol Reagent (Invitrogen). RNA was further purified using an RNeasy kit (Qiagen). Reverse transcription and quantitative PCR were carried out using ABI System (Applied Biosystems) and SYBR Green PCR Master Mix (Applied Biosystems) on an I-Cycler...
IQ Real-Time detection system (Bio-Rad) according to the manufacturer’s instructions. Expression levels were determined using the standard curve method with all expression levels being normalized to β-actin; all measurements were conducted in triplicate. The following primers were used: human Hes1: forward (F) 5'-AGTGAAGCA-CCTCCGGAAC-3', reverse (R) 5'-TCTACCTGTCTATG-CACTC-3'; human Hes5: forward (F) 5'-CCGGTGTTGGA- GAAGATC-3', reverse (R) 5'-TAGTCTGCTGACGCT- CTT-3'; human Hey1: forward (F) 5'-TCTACCTGTCTAGG- AGGCTTG-3', reverse (R) 5'-CGAAAATCCCAAACCTCC- GATA-3'; human Hey2: forward (F) 5'-GGATGCTCAGG- CAACAGGG-3', reverse (R) 5'-CAAGACGCTGTGCGCTCA- AAG-3'; human Nestin: forward (F) 5'-CAAGAGCCACTG- GAAGACCT-3', reverse (R) 5'-CCCTTCACCAGCCTTCTT- CC-3'; human CD133: forward (F) 5'-TCCACAGAATT- TACCTGACCTGG-3', reverse (R) 5'-CAGGAGAGGCA- GATGACCA-3'; human BMI1: forward (F) 5'-TGCTATG- TATGAGGAGAACCTT-3', reverse (R) 5'-TATTTCTATG- GAAGCTGACCATT-3'; human Nanog: forward (F) 5'-CC- TGTTATTGTGCGCTTG-3', reverse (R) 5'-GACAGCTTC- CCCGTCGACCCAT-3'; human SOX2: forward (F) 5'-CGATGATAATCCCTCTATG-3', reverse (R) 5'-CTGG- GCCAATACTTTCTCCT-3'; human MAP2: forward (F) 5'- AATGAGGCAGCAGGAAG-3', reverse (R) 5'-CATGGCC- CCTGCTGTCCTTGAGGTA-3'; human β-actin: forward (F) 5'-CCTCCGGAAC-3', reverse (R) 5'-GATCCACAGGAGTACCTTG-3' (28, 31).

Primary culture derived from intracranial xenografts and drug treatment assays
Tumor neurospheres were isolated from murine xenografts in as described for human specimens (43). After 7 to 10 days, new spheres that could undergo further passing were formed. For drug treatment assays, viable cells were plated at 2,500 cells/well in a 96-well plate containing serum-free neurosphere media and treated with indicated levels of MRK003. Viable cell biomass was measured 96 hours later using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega).

Clonogenic assays
Clonogenic assays were conducted as previously described (28), with passage 2 neurospheres derived from xenografts triturated into a single-cell suspension and 10^4 cells/5 mL seeded per well. At least 3 separate wells were analyzed per culture. Colonies were measured and counted after 3 weeks, using Motic Images Plus (Motic).

Statistical methods
Unless otherwise noted, in vitro and quantitative PCR data are shown as mean values with error bars representing SD for at a minimum 3 replicates, and experiments were repeated at least once. Comparison of mean values between groups was evaluated by unpaired, 2-tailed t test. Log-rank analysis of Kaplan–Meier curves was used to evaluate the survival. For all statistical methods, a P value less than 0.05 was considered significant. All tests were conducted by using GraphPad Prism 4 (GraphPad Software).

Results
Oral GSI slows intracranial glioblastoma xenografts growth and prolongs survival
Intracranial tumor xenografts were initiated using glioblastoma-derived neurospheres previously shown to form infiltrative malignant gliomas in nude mice (13, 28, 43, 45). We chose representative temozolomide-sensitive and -resistant neurosphere cultures for these studies. Growth of HSR-GBM1 cells was not inhibited by up to 150 μmol/L concentrations of temozolomide, whereas the 0.040821 line showed a profound dose-dependent reduction in growth (Fig. 1).

Both tumor growth and overall survival were used as endpoints in our initial analyses. To generate equivalent treatment groups and facilitate tracking of xenograft growth, cultures were transduced with a luciferase reporter (44). Mice were imaged weekly starting 4 weeks after the injection, and when HSR-GBM1 tumors were detected, mice were randomized into treatment and control groups of 10 and 9 animals with equal mean initial tumor sizes. Treatment began the next day, with 300 mg/kg MRK003 once weekly by oral gavage. Mouse body weight was monitored weekly, and no significant weight loss was noted in the GSI-treated animals as compared with controls until they became symptomatic due to increased tumor burden, suggesting that weekly administration of the GSI was well-tolerated.

Weekly imaging revealed growth in both the control and MRK003 treatment groups (Fig. 2A). However, xenografts in the GSI-treated group had a 49% slower growth rate than matched controls (P < 0.005; Fig. 2B). Overall survival from the point treatment was initiated was also significantly longer in animals receiving weekly MRK003 (P < 0.05; Fig. 2B). Xenografts initiated using 0.040821 glioblastoma neurospheres also showed a significant decrease in growth over 5 weeks measured using luciferase-based imaging (P < 0.05; Fig. 2C and D) as well as prolonged overall survival (P < 0.05; Fig. 2C and D) with MRK003 treatment. Thus, weekly GSI therapy can slow the intracranial growth of both temozolomide-sensitive and -resistant glioblastoma neurosphere lines and prolong animal survival.

Microscopic analysis of glioblastoma xenografts following Notch blockade
We conducted another in vivo study in which tumors were examined after the same treatment interval to facilitate direct comparison between treated and control lesions. Mice bearing HSR-GBM1 xenografts were randomized after tumor detection and given 5 weekly treatments with MRK003 or vehicle and then sacrificed 16 hours after the last oral dose. As before, weekly imaging of animals in the control and MRK003 treatment groups revealed that xenografts in the mice receiving the GSI grew significantly more slowly than matched controls (P < 0.05; Supplementary Fig. S1).
Brains were removed for microscopic and molecular analysis, and glioblastoma neurospheres were isolated from the xenografts so their functional properties could be assessed (Fig. 3A). A coronal cut was made through the injection site, and the microscopic cross-sectional area of each tumor was measured on an H&E-stained slide at this level (Fig. 3B). Consistent with the luciferase-based growth measurements, microscopic analysis revealed that xenografts in MRK003-treated mice had a mean 76% smaller tumor area at the level of injection than those in the vehicle-treated group ($P < 0.05$; Fig. 3B).

Microscopic analysis failed to highlight any pronounced difference in glioma cell morphology following treatment, but a mean 56% reduction in mitotic index was identified in the MRK003-treated mice ($P < 0.05$; Fig. 3C). Immunohistochemical analysis showed an 18% decrease in the Ki67 proliferation index in MRK003-treated tumors, but the difference was not statistically significant (data not shown). No regions of tumor necrosis were identified, and cleaved caspase-3 immunohistochemistry revealed a high apoptotic rate in all tumors with no significant difference between the treatment and control groups in 2 separate analyses (data not shown).

Finally, because Notch has been implicated in the control of angiogenesis and vascular density, we examined the number of PECAM1(CD31) immunopositive vessels in the xenografts. An increase of 42% was observed in the number of vessels in MRK003-treated cohort, but this trend was not statistically significant ($P = 0.08$; Supplementary Fig. S2A). We also measured expression of the pro-angiogenic factor VEGF in RNA extracted from the xenografts using quantitative PCR and human-specific primers so that only tumor-derived mRNA was analyzed. Again, however, the VEGF increase seen in tumors treated with MRK003 was not significant (Supplementary Fig. S2B). Nevertheless, our data are congruent with recent publications by several groups suggesting that GSI treatment leads to VEGF-driven aberrant growth of tumor blood vessels (47, 48).

Oral GSI inhibits Notch pathway activity and expression of stem cell markers

To confirm that oral GSI can penetrate the brain and block Notch signaling, we measured expression of established pathway targets in tumor-derived mRNA in all 3 xenograft cohorts. This included the HSR-GBM1 survival trial "XG1" with 9 vehicle and 10 MRK003-treated animals, the HSR-GBM1 "XG2" cohort in which animals were treated with vehicle ($n = 5$) or MRK003 ($n = 7$) for equal lengths of time, and the 040821 XG1 survival cohort comprising 7 vehicle and 7 MRK003-treated mice. Tumors in all of these MRK003-treated groups had significant 47% to 75% reductions in the mRNA levels of the Notch target Hes5 (Fig. 4A). Statistically significant reductions of 30% or more were seen in the target Hes1 in the HSR-GBM1 XG1 and 040821 XG1 groups. Finally, the MRK003-treated HSR-GBM1 XG1 and XG2 tumors showed significant reductions in Hey1 of 37% and 66%, respectively. In contrast, Hey2 did not respond significantly to GSI treatment in any trial. Thus, orally delivered MRK003 inhibits the Notch pathway in intracranial glioma xenografts, and the bulk of tumor cells do not become refractory to pathway blockade over 5 or more weeks of in vivo treatment.

We and others have previously shown that in vitro pharmacologic blockade of Notch signaling can deplete stem-like glioblastoma cells (13, 14, 18, 31, 49). To test the in vivo requirement for Notch in glioma stem cells, we measured mRNA levels for stem/progenitor, glial, and neuronal markers in the same groups described above. The mean mRNA levels of most stem cell markers were lowered in the treated xenografts, although because expression of stem cell markers in the vehicle-treated cohorts varied quite a bit, many of
these reductions did not reach the level of statistical significance. Nanog levels showed significant 42% to 75% reductions in all 3 MRK003-treated cohorts, nestin levels significant 36% to 43% reductions in 2 of 3, and CD133 a significant 46% reduction in one (Fig. 4B). Finally, mean mRNA levels of glial fibrillary acidic protein (GFAP) and neuronal (MAP2) differentiation markers showed significant GFAP increases of 165% to 201% in the 2 HSR-GBM1 studies, and a significant 28% increase of the neuronal marker MAP2 in 040821 XG1 xenografts following Notch blockade (Fig. 4C). These findings suggest that oral GSI inhibits the expression of stem cell markers and induces glial differentiation in glioblastoma xenografts.

**Prolonged suppression of clonogenicity and in vivo growth capacity following Notch blockade in xenografts**

We cultured neurospheres from the xenografts, propagating them with no in vitro GSI therapy. Consistent with the concept that in vivo Notch blockade had depleted stem-like cells required for long-term tumor growth, neurospheres could only be propagated from 3 of 7 MRK003 XG2–treated tumors as compared with 4 of 5 vehicle-treated ones. To further investigate growth potential of neurospheres derived from treated and untreated xenografts, we conducted clonogenic assays in all these re-isolated neurosphere lines at the second passage. Interestingly, even after 2 passages in media without any GSI added, neurospheres derived from MRK003-treated xenografts exhibited a significant reduction in clonogenic potential as compared with vehicle-treated ones (Fig. 5A, \( P < 0.0001 \), analysis of combined data from triplicate studies for each of the 7 xenograft-derived lines). Mean neurosphere size was reduced from 144 to 45 \( \mu m \) in cells previously treated with MRK003 (Fig. 5A). We have previously shown that only spheres over 100 \( \mu m \) can be serially passaged (28), suggesting that smaller ones are not derived from true stem cells.
To rule out the possibility that limited clonogenicity of secondary spheres was due to lingering effects on cell cycle, we examined the distribution of cells in G0–G1, S-phase, and G2–M using flow cytometry, with essentially identical results in spheres from MRK003- and vehicle-treated xenografts (Supplementary Fig. S3A). Differing degrees of contamination from murine cells could also potentially affect functional analyses, but immunostaining of neurospheres derived from MRK003- and vehicle-treated xenografts conducted alongside parental cells using a human-specific antibody did not show a significant number of murine cells in the cultures (Supplementary Fig. S3B).

We also examined clonogenic potential in neurospheres derived from 040821 xenografts, with all 7 vehicle and 6 of 7 MRK003-treated tumors giving rise to cultures. The mean neurosphere size was decreased from 124 to 93 μm by prior in vivo Notch blockade, with approximately 4 times as many large (>100 μm) spheres arising from vehicle-treated xenografts (Fig. 5B, P < 0.05). Our studies analyzing neurospheres derived from treated and untreated xenografts conducted alongside parental cells using a human-specific antibody did not show a significant number of murine cells in the cultures (Supplementary Fig. S3B).

To confirm durable alterations affecting the initiation and/or propagation of tumors following in vivo Notch blockade, we expanded passage 2 neurospheres derived from treated and untreated HSR-GBM1 tumors and injected equal numbers of viable cells into the brains of nu/nu mice, then tracked their growth with no additional therapy. Three lines derived from MRK003-treated xenografts and three from vehicle-treated xenografts were injected into 5 animals each, and the 2 resulting cohorts of 15 animals were followed by weekly luminescence imaging and sacrificed when they became symptomatic. The growth of xenografts derived from tumors previously treated with MRK003 was slower than controls, although the difference was not statistically significant (P = 0.08, Fig. 5C). However, a significant prolongation in survival was seen among the cohort generated with cells previously treated with MRK003 (P < 0.05, Fig. 5C). We analyzed expression of Notch targets and stem and differentiation markers in these secondary HSR-GBM1 “XG3” xenografts and found that markers of Notch activity (Hes1, Hes5, Hey1) remained suppressed in the absence of ongoing therapy (Fig. 4A). The stem cell markers CD133 and SOX2 also were significantly lower (Fig. 4B), but interestingly the differentiation factors GFAP showed a significant change opposite that seen in the prior round of xenografts (Fig. 4C).

GBM neurospheres derived from xenografts previously treated with MRK003 remain sensitive to GSI

Hedgehog pathway blockade can rapidly result in the emergence of resistant tumor cells (50, 51). To determine...
whether in vivo Notch blockade also efficiently promotes resistance, we treated the cultured neurospheres derived from both treated and untreated tumors, as well as parental cells, with different concentration of MRK003 for 96 hours in vitro and measured viable cell mass. Neurospheres from MRK003-treated HSR-GBM1 and 040821 xenografts remained sensitive to MRK003 in vitro, suggesting that the period of in vivo therapy had not resulted in broad resistance to Notch inhibition (Fig. 5D).

Discussion

Notch signaling is important for the initiation and growth of a wide range of tumors (52–55), and a number of small-molecule inhibitors of the γ-secretase complex have been developed to target this pathway. One challenge has been ameliorating the gastrointestinal (GI) side effects of Notch inhibition, which results in goblet cell metaplasia (56, 57). Two recently published phase I trials of the GSIs RO4929097 and MK0752 in patients with advanced solid malignancies tested a range of dosing schedules, in the hopes that intermittent pathway blockade would show more manageable toxicities (35, 58).

In the MK0752 trial, a once-a-week dosing regimen was associated with notably less GI toxicity and fatigue (35). Interestingly, while patients with 18 different types of solid neoplasms were treated, most of which were from outside the brain, clinical activity was essentially restricted to intra-cranial gliomas of various types. This highlights the potential of Notch inhibitors as novel therapies for gliomas and the need to better understand their mechanisms of action using clinically relevant in vivo models and dosing schedules.

Our goals were to determine whether weekly oral administration of the GSI MRK003 could effectively inhibit Notch signaling in orthotopic glioblastoma xenografts and to examine its effects on tumor growth and clonogenic potential. Weekly oral administration of MRK003 was effective in inhibiting the growth of xenografts generated from both temozolomide-sensitive and -resistant neurosphere lines as measured by serial imaging. Survival was also significantly longer in treated animals, consistent with the recent report of biologic response in some patients with glioma (35).

In addition to these studies using survival as an endpoint, we examined another cohort treated for equivalent periods of time with MRK003 or vehicle. In these xenografts, MRK003 treatment was associated with a significantly lower mitotic rate and smaller cross-sectional area. Quantitative PCR analysis of all the xenograft trials showed decreased expression of a number of Notch targets, indicating that the small-molecule pathway inhibitor is able to effectively penetrate the brain and block pathway activity. The levels of several stem cell markers were also decreased, suggesting a potential effect on cancer stem cells.

Functional analyses using tissue harvested from treated and untreated xenografts support the notion that in vivo Notch inhibition depletes clonogenic capacity. Neurospheres from MRK003-treated HSR-GBM1 and 040821 xenografts remained significantly lower 2 passages after they had been isolated from xenografts, with no ongoing therapy in

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Figure 4. Oral MRK003 treatment affects proliferation and expression of Notch targets and neural stem cell and differentiation markers in glioblastoma xenografts.
Further proof of the durable suppression of tumor growth capacity came when we re-injected equal numbers of HSR-GBM1 cells isolated from MRK003- or vehicle-treated xenografts back into the brains of \( \text{nu/nu} \) mice. Despite the lack of any treatment of the in vitro cultures or secondary xenografts, prior anti-Notch therapy was associated with significant prolongation of survival. These secondary xenograft assays support our previously published in vitro clonogenic results and suggest that in vivo Notch inhibition can have effects on tumor initiation and growth that persist for a period of time after treatment. The suppression of stem cell markers suggests that this could be due, at least in part, to depletion of stem-like cells from tumors, although other mechanisms are also possible, such as variation of stem cell marker expression in conjunction with proliferation changes.

**Figure 5.** Persistent reduction in clonogenic capacity and in vivo tumor growth, but no induction of GSI resistance, following MRK003 treatment of xenografts. A, neurospheres derived from HSR-GBM1 XG2 MRK003-treated xenografts formed colonies in methylcellulose that were significantly smaller and less numerous than those derived from vehicle-treated tumors. Average neurosphere size shifted from 144 \( \mu \)m to less than 50 \( \mu \)m \((P < 0.0001)\), and the number of spheres over 100 \( \mu \)m in size was reduced 98% \((P < 0.0001)\). B, similarly, neurospheres derived from MRK003-treated 040821 XG1 showed significant decreases in the mean size and the number of large spheres. C, HSR-GBM1 XG2-derived neurospheres were also injected into \( \text{nu/nu} \) mice and allowed to form XG3 xenografts with no further therapy. Mean tumor growth as measured by luciferase imaging was 71% slower in animals with xenografts generated from spheres isolated from previously MRK003-treated mice, but this difference was not statistically significant \((P = 0.08)\). However, tumors initiated from neurospheres derived from previously MRK003-treated animals resulted in significantly prolonged survival (median survival: 78 vs. 60 days, log-rank test: \( P < 0.05 \)). D, growth over 96 hours in culture in the presence of increasing levels of MRK003 was measured in parental neurospheres or those derived from HSR-GBM1 XG2 and 040821 XG1 xenografts. GBM neurospheres derived from tumors treated in vivo did not show any increase in resistance to repeat Notch blockade in vitro.
Encouragingly, broad resistance to the GSI did not emerge following in vitro therapy, as neurospheres isolated from treated xenografts remained sensitive to MRK003. However, it seems likely that GSI therapy will need to be combined with other treatments if long-term survival of patients with glioblastoma is to be achieved. It has been suggested that Notch inhibition in conjunction with temozolomide chemotherapy (14, 15, 17, 18) or radiation (17, 59) can more effectively treat malignant gliomas than these standard therapies alone. We have also previously suggested on the basis of in vitro studies that targeting both Notch and Hedgehog simultaneously may increase therapeutic efficacy (31).

In summary, oral delivery of the GSI MRK003 results in significant inhibition of Notch pathway activity in intracranial xenografts generated from glioblastoma neurospheres and can therefore increase the growth of both temozolomide-sensitive and -resistant malignant gliomas. Treatment was associated with suppression of stem cell markers and induction of differentiation markers in tumor xenografts, as well as reduced mitotic activity. Interestingly, a persistent reduction in clonogenic and in vivo tumor growth capacity was noted after xenograft treatment was stopped, suggesting that Notch blockade can have prolonged effects on glioma biology.

Disclosure of Potential Conflicts of Interest

C.G. Eberhart discloses a patent licensed to Stemline Therapeutics and has a commercial research grant from Merck. No potential conflicts of interest were disclosed by the other authors.

References


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