Cancer Research

# γ-Secretase Inhibitors Enhance Temozolomide Treatment of Human Gliomas by Inhibiting Neurosphere Repopulation and Xenograft Recurrence

Candace A. Gilbert[1], Marie-Claire Daou[1], Richard P. Moser[2], and Alonzo H. Ross[1]

## Abstract

Malignant gliomas are treated with a combination of surgery, radiation, and temozolomide (TMZ), but these therapies ultimately fail due to tumor recurrence. In glioma cultures, TMZ treatment significantly decreases neurosphere formation; however, a small percentage of cells survive and repopulate the culture. A promising target for glioma therapy is the Notch signaling pathway. Notch activity is upregulated in many gliomas and can be suppressed using γ-secretase inhibitors (GSI). Using a neurosphere recovery assay and xenograft experiments, we analyzed if the addition of GSIs with TMZ treatment could inhibit repopulation and tumor recurrence. We show that TMZ + GSI treatment decreased neurosphere formation and inhibited neurosphere recovery. This enhancement of TMZ treatment occurred through inhibition of the Notch pathway and depended on the sequence of drug administration. In addition, *ex vivo* TMZ + GSI treatment of glioma xenografts in immunocompromised mice extended tumor latency and survival, and *in vivo* TMZ + GSI treatment blocked tumor progression in 50% of mice with preexisting tumors. These data show the importance of the Notch pathway in chemoprotection and repopulation of TMZ-treated gliomas. The addition of GSIs to current treatments is a promising approach to decrease brain tumor recurrence. *Cancer Res; 70(17); 6870–9. ©2010 AACR.*

## Introduction

Glioblastoma multiforme (GBM) is the most frequent and malignant form of brain tumor, making up 17% of all primary brain tumors in the United States, with an incidence of 3.17 cases per 100,000 persons per year (1). The current 5- and 10-year survival rates for GBM patients are 4.5% and 2.7%, respectively (1). GBM clinical treatment consists of a combination of surgical resection, radiotherapy, and chemotherapy. The chemotherapy drug temozolomide (TMZ) is an alkylating agent that readily penetrates the blood-brain barrier (2). TMZ is administered as both a concomitant and an adjuvant treatment to radiotherapy. This aggressive treatment increases the 2-year survival rate for GBM patients from 10.4% with radiotherapy alone to 26.5% (3). Cells that escape radiotherapy- and chemotherapy-induced cell death eventually reenter the cell cycle and contribute to local tumor recurrence. Despite advances in chemotherapy regimens, the median progression-free survival, which measures the time until tumor recurrence, is 6.9 months and the median overall survival is 14.6 months with TMZ and radiotherapy (3). Hence, there is a dire need to target the cells that evade current treatments.

Neurosphere cultures were originally developed for propagation of normal neural stem cells (4), and these methods are now applied to tumors (5–7). Neurosphere cultures maintain genetic profiles similar to the patients' tumors and form invasive intracranial xenografts in immunocompromised mice (8–10). Our laboratory developed a neurosphere recovery assay that measures neurosphere formation at three time points to assess the capacity of the culture to repopulate after chemotherapy (11). First, we assess the ability of the cells to form neurospheres shortly after treatment. Second, we count the number of neurospheres that form during a 1-week recovery period to determine if the surviving cells resume neurosphere formation. Third, we dissociate the neurospheres and count the number of secondary neurospheres that form to measure self-renewal (12). This neurosphere recovery assay provides a quantitative assay for culture repopulation following drug treatment. We previously showed that TMZ drastically diminished initial neurosphere formation in many glioma cultures; however, these cultures eventually recovered and formed a robust number of secondary neurospheres (11). The ability of TMZ-treated neurospheres to recover and repopulate the culture suggests that some cells undergo a transient cell cycle arrest, allowing them to evade cell death and eventually resume proliferation.

Notch signaling is a promising pathway to target glioma cells. The Notch receptors, their ligands, and downstream targets, including members of the Hairy enhancer of split

**Authors' Affiliations:** Departments of [1]Biochemistry and Molecular Pharmacology and [2]Neurosurgery, University of Massachusetts Medical School, Worcester, Massachusetts

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**Corresponding Author:** Alonzo H. Ross, Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation Street, Room 819, Worcester, MA 01605. Phone: 508-856-8016; Fax: 508-856-2003; E-mail: Alonzo.Ross@umassmed.edu.

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(Hes) and Hes-related protein (Hey) families, are commonly overexpressed in glioma tissue and cell lines (13–15). γ-Secretase inhibitors (GSI) are used to inhibit the Notch pathway in basic research and clinical trials (16). In glioma cultures, GSI treatment suppressed cell growth and decreased neurosphere formation and tumor growth of $CD133^+$ cells (17). Correspondingly, increased Notch signaling enhanced glioma cell survival (18). GSIs were also shown to sensitize glioma neurosphere cultures to radiation, thereby increasing the efficacy of radiotherapy (19).

In this study, we analyze if the combination of TMZ and GSIs enhances glioma therapy by inhibiting tumor repopulation and recurrence. In contrast to TMZ-only treatment, the TMZ + GSI treatment strongly inhibited neurosphere recovery. This was confirmed by the loss of secondary neurosphere formation in cultures treated with both TMZ and GSIs. In subcutaneous xenografts, *ex vivo* and *in vivo* TMZ + GSI treatment decreased tumor progression and increased survival. These data show the importance of the Notch pathway for chemoprotection in malignant gliomas. The addition of GSIs to the current care regimens for GBM patients is a promising new approach to decrease brain tumor recurrence.

## Materials and Methods

### Cell culture

Glioma cell lines converted to neurosphere cultures, U87NS and U373NS, and primary GBM lines, GS7-2 and GS8-26, were grown in serum-free defined medium consisting of DMEM/F-12 1:1 (Life Technologies, Inc.), B27 (Life Technologies), 15 mmol/L HEPES (Life Technologies), 20 ng/mL epidermal growth factor (Invitrogen), 20 ng/mL basic fibroblast growth factor (Invitrogen), and 1% penicillin-streptomycin (Life Technologies). Cultures were passaged using the pH dissociation method (20). Details of the converted and primary lines are described in Supplementary Materials and Methods.

### Drug treatment

TMZ and *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-5-phenylglycine *t*-butyl ester (DAPT) were purchased from Sigma-Aldrich. $N^2$-[(2*S*)-2-(3,5-Difluorophenyl)-2-hydroxyethanoyl]-$N^1$-[(7*S*)-5-methyl-6-oxo-6,7-dihydro-5*H*-dibenzo[*b*,*d*]azepin-7-yl]-L-alaninamide (LY411,575; ref. 21) was a gift from Lisa Minter and Barbara Osborne (University of Massachusetts-Amherst, Amherst, MA). Details of drug dosage are given in Supplementary Materials and Methods.

### Neurosphere recovery and secondary neurosphere assays

For the neurosphere assay, cells were plated as previously described (11). Immediately after plating, cells were treated with DMSO, DAPT-only, LY411,575 (LY)-only TMZ-only, TMZ + DAPT, or TMZ + LY. The initial neurospheres were counted on day 7 for the converted cell lines and on day 10 for the slower-growing primary lines. Neurosphere recovery was measured on day 14 or day 20. The neurospheres were dissociated and replated and secondary neurosphere formation was measured on day 21 or day 30. Details are described in Supplementary Materials and Methods.

For the samples labeled "pretreated," a single dose of DAPT was administered when the cells were plated, and then TMZ was added to the medium 24 hours later. For the "cotreated" samples, single doses of TMZ and DAPT were added simultaneously when the cells were plated. Finally, samples labeled "posttreated" were treated with TMZ, and then DAPT was added 24 hours later.

### Virus infections

NICD-pMIG (22) or pMIG vectors were cotransfected with retrovirus envelope and gag-pol vectors into HEK293T cells with FuGENE 6 (Roche Applied Science). Retrovirus was collected after 48 hours. Neurosphere cultures were infected in noncoated bacterial dishes to prevent the cells from becoming adherent in the presence of serum. Cells were incubated with virus and 8 μg/mL polybrene (Sigma-Aldrich) at 37°C for 6 hours. GFP-positive cells were sorted on a FACSAria (BD Biosciences).

### Subcutaneous xenografts: *ex vivo* drug treatment

U87NS and U373NS neurospheres were dissociated and $2.5 \times 10^4$ cells/mL were plated in defined medium and treated with DMSO, TMZ-only (200 μmol/L), DAPT-only (1 or 5 μmol/L), or TMZ + DAPT as described for recovery assays. After 7 days, $2.5 \times 10^5$ or $3 \times 10^6$ live cells were counted using trypan blue and resuspended in 100 μL of PBS. Cells were subcutaneously injected into the flanks of nude mice. Mice were monitored for tumor formation for up to 120 days postinjection and euthanized when tumors reached volumes of 1.5 to 2 $cm^3$.

### Subcutaneous xenografts: *in vivo* drug treatment

For the *in vivo* experiments, we used LY incorporated into 7012 Teklad LM-485 rodent chow (LY chow) at a concentration of 0.0275 g/kg (Harlan Laboratories, Inc.; ref. 23). U87NS cells ($10^6$) resuspended in 100 μL of PBS were subcutaneously injected into the flanks of male nude mice. When the tumor reached approximately 150 $mm^3$ [volume = (3/4) ($\pi$) (length/2) $(\text{width}/2)^2$], we began the following treatments: (*a*) DMSO control: 2 d of 100-μL DMSO/PBS (1:1) i.p. injections; (*b*) TMZ-only: i.p. injections of TMZ (20 mg/kg) in 100 μL of DMSO/PBS on days 1 and 2; (*c*) LY chow-only: 2 d of 100-μL DMSO/PBS i.p. injections; the mice were fed LY chow from days 3 to 12; (*d*) TMZ + LY chow: i.p. injections of TMZ (20 mg/kg) in 100 μL of DMSO/PBS on days 1 and 2. The mice were fed LY chow from days 3 to 12. Mice were observed for up to 150 days and euthanized when the tumor reached 1.5 to 2 $cm^3$.

## Results

### Glioma neurosphere cell lines express Notch receptors and downstream targets

Converted cell lines (U87NS and U373NS) and primary neurosphere cultures established from patients' GBMs (GS7-2 and GS8-26) express the mRNAs for *Notch1–Notch4* and the downstream targets *Hes1* and *Hey1* (Fig. 1A). Treatment with DAPT downregulated the mRNA levels of *Hes1* and *Hey1* (Fig. 1B). The DAPT concentration used was determined based on a 50% or greater knockdown of Notch targets. For

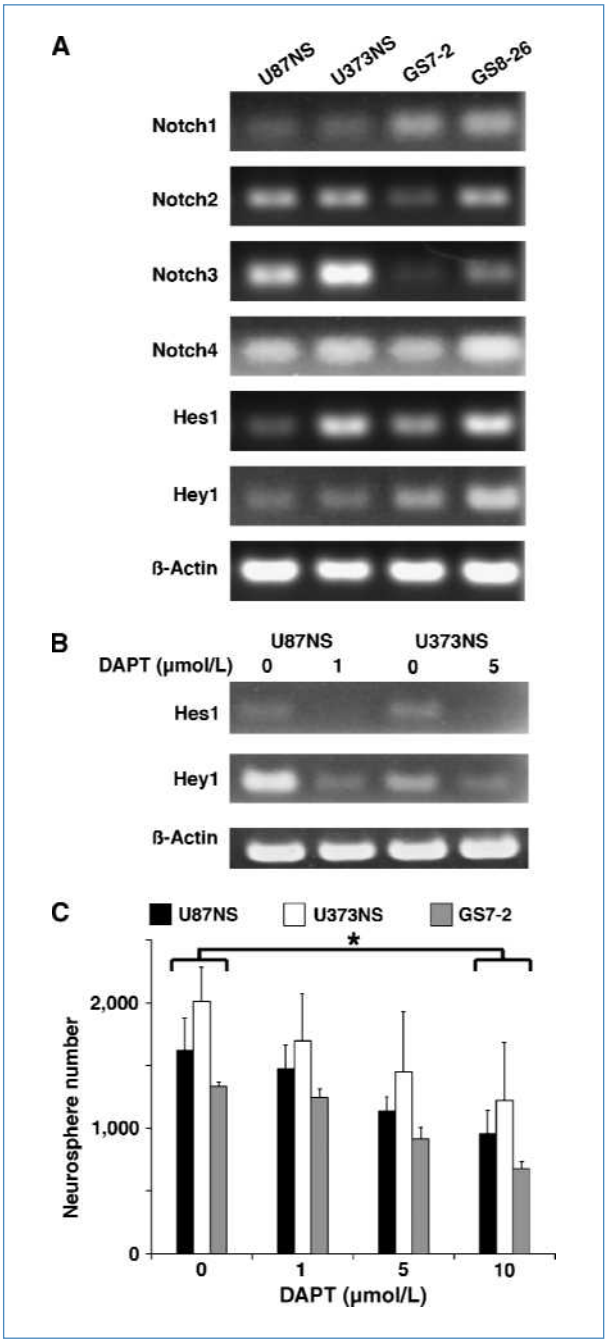


**Figure 1.** The Notch pathway is active in neurosphere cultures and is blocked with DAPT treatment. A, mRNA levels of the Notch receptors and downstream targets were measured by reverse transcription-PCR (RT-PCR). *Notch1–Notch4, Hes1*, and *Hey1* were detected in each neurosphere culture. β-Actin was used as a loading control. B, *Hes1* and *Hey1* expression were analyzed by RT-PCR 48 h after DAPT treatment. DAPT treatment decreased *Hes1* and *Hey1* mRNA levels by 72% and 76% in U87NS (1 μmol/L) and by 76% and 51% in U373NS (5 μmol/L), respectively. C, the DAPT titration curve showed that treatment with low concentrations (1–5 μmol/L) of DAPT-only resulted in minimal inhibition of neurosphere formation in U87NS, U373NS, or GS7-2 cultures. Columns, mean; bars, SD. DAPT administered at higher concentrations (10 μmol/L) significantly decreased neurosphere formation. Neurospheres were counted on day 7 for U87NS and U373NS and on day 10 for GS7-2. *, $P < 0.001$ (*t* test).

subsequent experiments, U87NS and GS7-2 cultures were treated with 1 μmol/L DAPT, whereas U373NS and GS8-26 cultures were treated with 5 μmol/L DAPT.

### TMZ + DAPT treatment inhibits neurosphere recovery and secondary neurosphere formation

When administered alone, low concentrations of DAPT (1–5 μmol/L) decreased Notch pathway signaling (Fig. 1B), but had little to no affect on the number of neurospheres (Fig. 1C). In addition, low concentrations of DAPT did not affect the size of the neurospheres (Fig. 2B). In U87NS, U373NS, and GS7-2 cultures, treatment with 10 μmol/L DAPT decreased neurosphere formation by 41%, 39%, and 49%, respectively, compared with DMSO controls (Fig. 1C); however, the DAPT-treated cells resumed proliferation and formed secondary neurospheres (Supplementary Fig. S1).

To determine if DAPT enhances TMZ therapy, we examined the effect of combined treatment on neurosphere recovery (Fig. 2A). After treatment with TMZ-only and TMZ + DAPT, cultures had similar decreases in the number of initial neurospheres formed (Fig. 3A–D). TMZ-only and TMZ + DAPT treatments decreased initial neurosphere formation by 80% to 98% and 83% to 99%, respectively. Cultures were given an additional 7 or 10 days to recover in the absence of drugs. During this recovery period, the neurospheres that formed after TMZ-only treatment increased in size; however, the TMZ + DAPT–treated neurospheres remained the same in size (Fig. 2C). The number of neurospheres also increased after recovery in the TMZ-only–treated cultures, but this recovery was not observed in the TMZ + DAPT–treated cultures. After recovery from the TMZ-only treatment, U87NS showed a 2-fold increase and U373NS showed a 1.5-fold increase in the number of neurospheres (Fig. 3A and B). The primary neurosphere cultures also showed a recovery from the TMZ-only treatment: the number of GS7-2 neurospheres increased by 1.8-fold, and GS8-26 by 1.6-fold (Fig. 3C and D). In contrast, TMZ + DAPT effectively inhibited recovery for U87NS, U373NS, GS7-2, and GS8-26 (Fig. 3A–D). The number of neurospheres in these cultures was essentially the same after recovery on day 14 or day 20 relative to the number of initial neurospheres counted on day 7 or day 10.

To assess if the cultures retained cells capable of self-renewal, the initial neurospheres were dissociated to single cells and replated to measure secondary neurosphere formation. TMZ-only–treated cultures readily formed secondary neurospheres, but secondary neurosphere formation for TMZ + DAPT–treated cultures was significantly diminished. U87NS secondary neurosphere formation in the TMZ-only–treated culture was 36-fold greater ($P < 0.0001$) than the secondary neurosphere formation in the TMZ + DAPT–treated culture (Fig. 3A), and U373NS secondary neurosphere formation in the TMZ-only–treated culture was 23-fold greater ($P < 0.001$) than that in the TMZ + DAPT–treated culture (Fig. 3B). The primary cultures also had profuse secondary neurosphere formation after TMZ-only treatments, but minimal secondary neurosphere formation after TMZ + DAPT treatments. Secondary neurosphere formation was 45-fold greater ($P < 0.001$) in the GS7-2 TMZ-only–treated culture (Fig. 3C) and

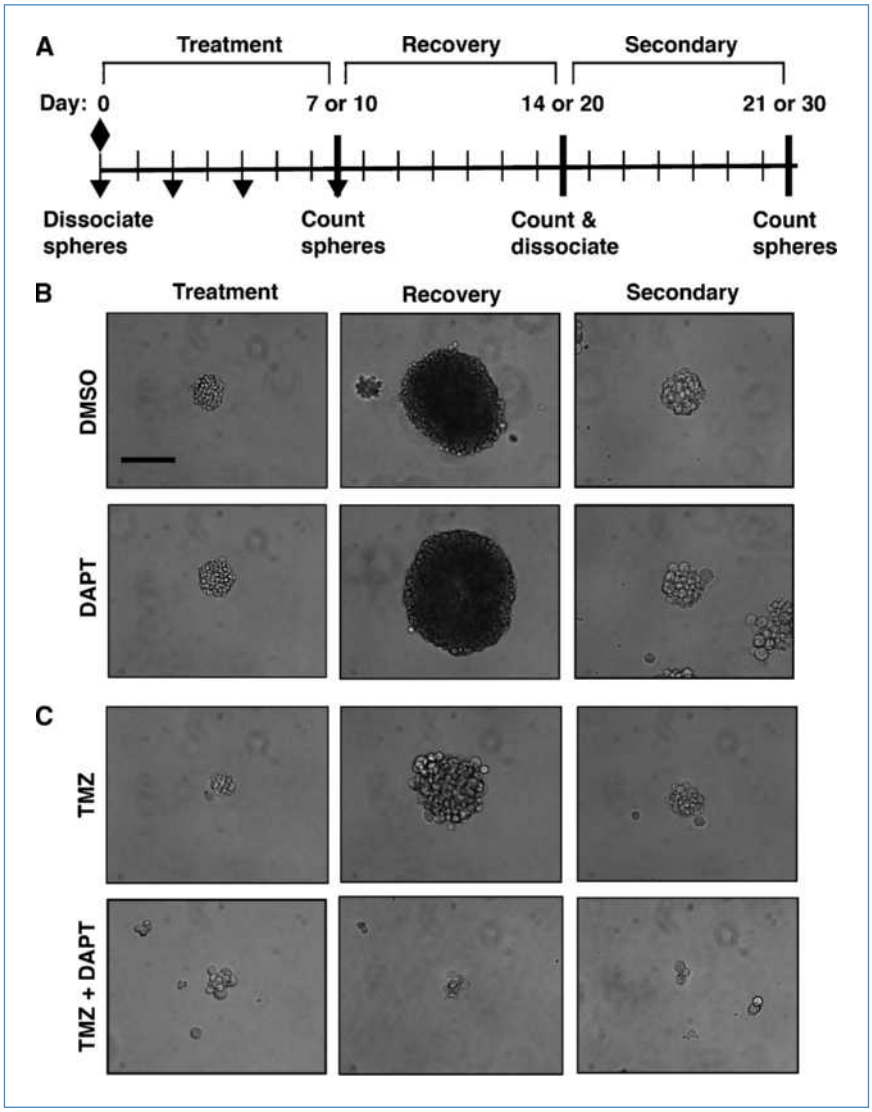


Figure 2. TMZ + DAPT treatment inhibits neurospheres from increasing in size. A, schematic for the neurosphere recovery assay. TMZ (⧫) is administered on day 0 after cells are dissociated and plated; DAPT (▼) is administered on days 0, 2, 4, and 7. B, representative micrographs of U87NS neurospheres with control DMSO and DAPT (1 μmol/L) treatments. Neurospheres treated with DAPT display a similar size compared with DMSO control cultures at treatment (day 7), recovery (day 14), and secondary (day 21) time points. C, representative micrographs of U87NS neurospheres with TMZ (200 μmol/L) treatment and TMZ (200 μmol/L) combined with DAPT (1 μmol/L) treatment. After the initial treatment (day 7), TMZ-only– and TMZ + DAPT–treated neurospheres were smaller than the control cultures. TMZ-only–treated neurospheres increased in size during the recovery period (day 14) and formed secondary neurospheres (day 21), whereas TMZ + DAPT–treated neurospheres did not increase in size or form secondary neurospheres. Bar, 50 μm.

25-fold greater ($P < 0.001$) in the GS8-26 TMZ-only–treated culture (Fig. 3D).

The number of cells in each neurosphere capable of self-renewal can be calculated by dividing the number of secondary neurospheres by the number of neurospheres formed during the recovery period. After recovery from TMZ-only treatment, there were an average of 8 and 3 cells per neurosphere that maintained self-renewal properties in the U87NS and U373NS cultures, respectively; however, in the TMZ + DAPT–treated cultures, there was only approximately 0.5 cell per neurosphere that was capable of self-renewal after the recovery period. In the primary lines treated with TMZ-only, each neurosphere from the GS7-2 and GS8-26 cultures contained a large number of cells capable of self-renewal, an average of 38 and 31 cells, respectively. In contrast, the average number of cells capable of self-renewal after TMZ + DAPT treatment decreased to only 2 cells per neurosphere in the GS7-2 and GS8-26 cultures.

To show that the lack of recovery and secondary neurosphere formation after TMZ + DAPT treatment was a specific response to the inhibition of γ-secretase activity, we repeated the neurosphere recovery assay with LY (21). When LY was administered to U87NS and U373NS cultures at various concentrations, there was a dose-dependent decrease in neurosphere formation (Supplementary Fig. S2A); however, the LY-only–treated cultures retained the ability to form secondary neurospheres (Supplementary Fig. S2B). In contrast, the combination of TMZ + LY significantly repressed recovery and secondary neurosphere formation (Supplementary Fig. S2C and D).

### Constitutive expression of Notch1 intracellular domain protects neurosphere cultures from TMZ + DAPT treatment

γ-Secretase cleaves other substrates in addition to the Notch receptors (24). To establish that DAPT enhances TMZ treatment by targeting the Notch pathway, we infected

U87NS and GS7-2 cells with a retrovirus to express the constitutively active Notch1 intracellular domain (NICD; ref. 22). Expression of functional NICD was confirmed by measuring increased mRNA levels of the downstream targets *Hes1* and *Hey1* (Fig. 4A). When NICD is constitutively expressed, the Notch pathway is not inhibited by GSI treatment (Supplementary Fig. S3). NICD-expressing U87NS and GS7-2 cells treated with TMZ-only were capable of recovery and robust secondary neurosphere formation, similar to the control cells expressing the empty vector (pMIG; Fig. 4B and C). Importantly, NICD expression attenuated the effects of TMZ + DAPT treatment, and the culture showed neurosphere recovery and robust secondary neurosphere formation. The control U87NS-pMIG TMZ-only–treated cells had a 1.9-fold recovery, but no increase was seen in the TMZ + DAPT–treated culture (Fig. 4B). U87NS-NICD cells showed a 2.3-fold recovery in TMZ-only–treated cultures and a 1.8-fold recovery in TMZ + DAPT–treated cultures. GS7-2-pMIG TMZ-only–treated cells showed a 2.1-fold increase in neurospheres during recovery, whereas TMZ + DAPT–treated cells showed no recovery (Fig. 4C). GS7-2-NICD cells showed a 2.6-fold recovery after TMZ-only treatment and a 2.8-fold recovery after TMZ + DAPT treatment.

Similar to the parental lines (Fig. 3A and C), U87NS-pMIG and GS7-2-pMIG cultures treated with TMZ-only had robust secondary neurosphere formation, but cultures treated with TMZ + DAPT had minimal secondary neurosphere formation (Fig. 4B and C). In contrast, U87NS-NICD and GS7-2-NICD cultures had robust secondary neurosphere formation for both TMZ-only and TMZ + DAPT treatments. When treated with TMZ + DAPT, U87NS-NICD secondary neurosphere formation was 61.2-fold greater ($P < 0.0001$) than U87NS-pMIG secondary neurosphere formation (Fig. 4B). In the GS7-2-NICD TMZ + DAPT–treated cultures, secondary neurosphere formation was 47.8-fold greater ($P < 0.0001$) than the secondary neurosphere formation in the GS7-2-pMIG TMZ + DAPT–treated cultures (Fig. 4C). Hence, constitutive NICD expression eliminates GSI enhancement of TMZ therapy, identifying the Notch pathway as the relevant GSI target.

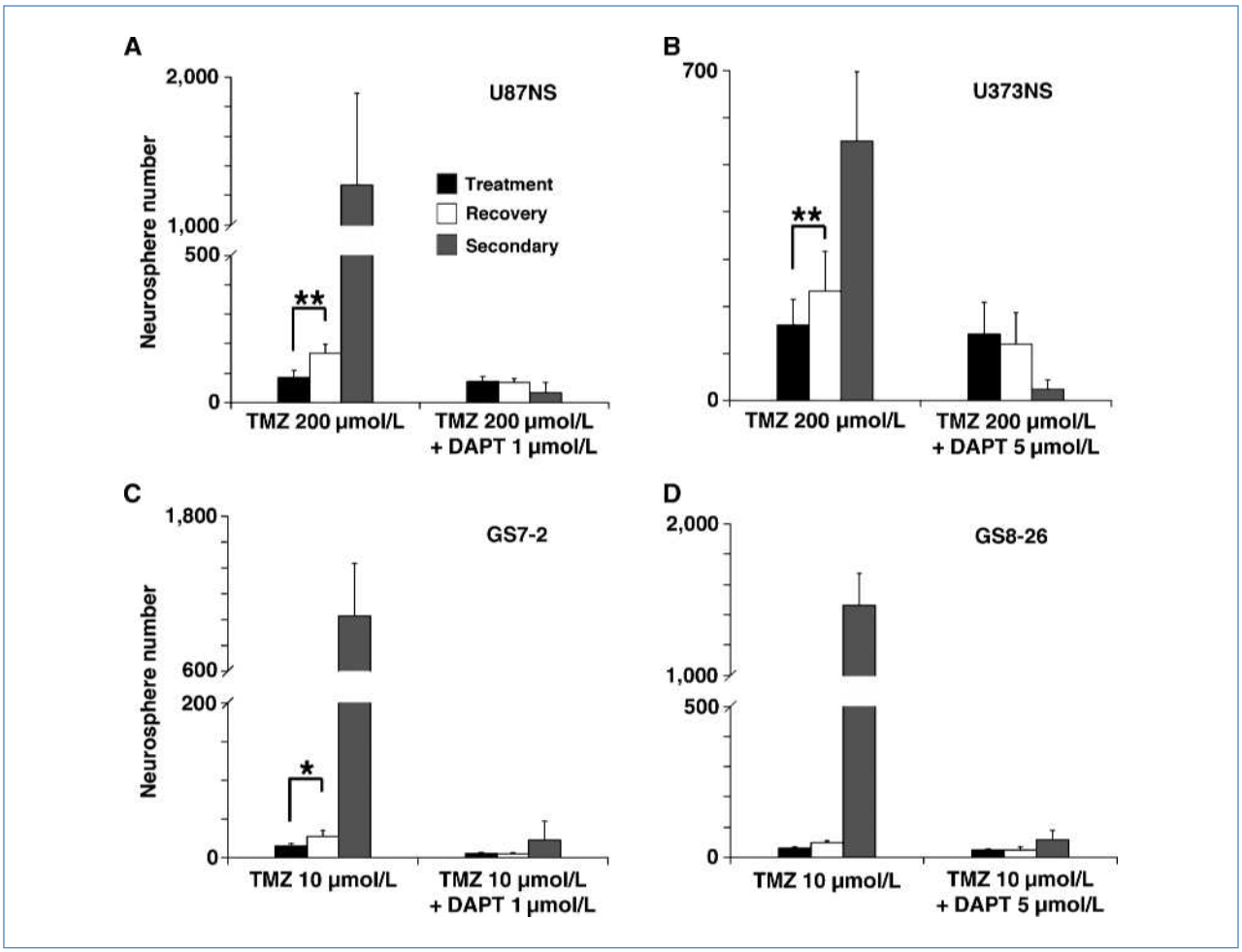


**Figure 3.** The neurosphere recovery assay shows that TMZ + DAPT treatment inhibits recovery and secondary neurosphere formation. Initial neurospheres were counted on day 7 for U87NS (A) and U373NS (B) cultures, or on day 10 for GS7-2 (C) and GS8-26 (D) cultures. Columns, mean; bars, SD. Recovery neurospheres were counted on day 14 or day 20 and secondary neurospheres were counted on day 21 or day 30. *, $P < 0.001$; **, $P < 0.0001$.

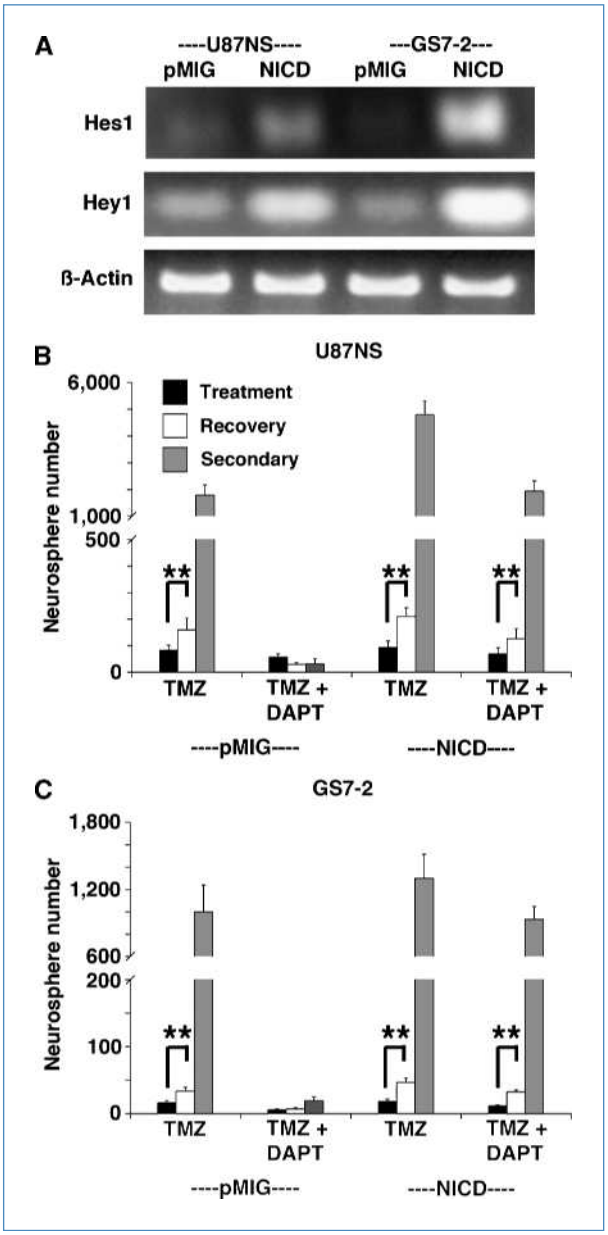


Figure 4. NICD-expressing cultures resume neurosphere formation following TMZ + DAPT treatment. A, RT-PCR analysis of *Hes1* and *Hey1* expression was used to confirm NICD activity. *Hes1* and *Hey1* expression increased in U87NS-NICD and GS7-2-NICD cultures compared with cultures expressing the empty vector (pMIG). B, treating U87NS-pMIG with TMZ + DAPT inhibited recovery, but U87NS-NICD cultures recovered after both TMZ-only and TMZ + DAPT treatment. C, in GS7-2-pMIG and GS7-2-NICD cultures treated with TMZ-only or TMZ + DAPT, cultures constitutively expressing NICD, but not those expressing the empty vector, were able to recover after TMZ + DAPT treatment. Refer to Fig. 3 for drug concentration. **, $P < 0.0001$.

### Treatment schedules for single doses of TMZ and DAPT affect neurosphere recovery

We tested if single DAPT doses administered before, during, or after TMZ treatment would have distinct effects. TMZ and DAPT were administered to U87NS and GS7-2 neurosphere cultures with three treatment schedules (Fig. 5A). Interestingly, pretreatment with DAPT decreased the efficacy of TMZ. Initial neurosphere formation was 7.2- and 2.7-fold greater than neurosphere formation in TMZ-only–treated U87NS and GS7-2 cultures, respectively (Fig. 5B and C). When dissociated, the pretreated and cotreated samples formed a large number of secondary neurospheres; however, posttreated samples had minimal secondary neurosphere formation (Fig. 5B and C). Secondary neurosphere formation was significantly greater in TMZ-only, pretreated, and cotreated cultures compared with posttreated cultures. Secondary neurosphere formation in U87NS cultures was 5.7-fold

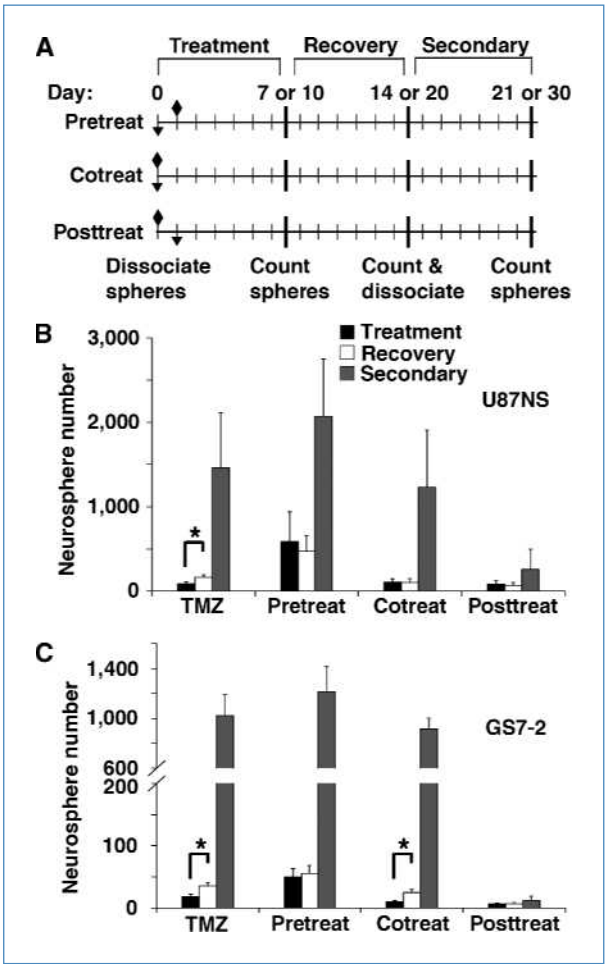


Figure 5. The treatment schedule of DAPT and TMZ affects neurosphere recovery. A, schematic of the recovery assay treatment schedules. TMZ (⧫) and DAPT (▼) treatments were administered with DAPT 24 h before TMZ (Pretreat), simultaneously with TMZ (Cotreat), or 24 h after TMZ (Posttreat). B and C, U87NS cultures (B) and GS7-2 cultures (C) were treated with different DAPT treatment schedules and recovery was analyzed. Initial neurospheres were counted on day 7 or day 10, recovery neurospheres were counted on day 14 or day 20, and secondary neurospheres were counted on day 21 or day 30. Columns, mean; bars, SD. Secondary neurosphere formation was inhibited only in cultures treated with the posttreatment schedule. Refer to Fig. 3 for drug concentration. *, $P < 0.001$ (*t* test).

greater ($P < 0.001$) with TMZ-only treatment, 8.1-fold greater ($P < 0.001$) with DAPT pretreatment, and 4.8-fold greater ($P < 0.01$) with cotreatment, relative to secondary neurosphere formation after DAPT posttreatment (Fig. 5B). The inhibition of GS7-2 secondary neurosphere formation was also greatest with posttreatment. Secondary neurosphere formation in the GS7-2 cultures was 85.7-fold greater ($P < 0.0001$) with TMZ-only treatment, 98.5-fold greater ($P < 0.0001$) with DAPT pretreatment, and 72.8-fold greater ($P < 0.0001$) with cotreatment, when compared with the DAPT posttreatment (Fig. 5C). These results led to two observations. First, TMZ + DAPT treatment acts through a specific, sequence-dependent mechanism. Second, these results provide insight for *in vivo* treatment schedules.

### TMZ + DAPT *ex vivo* treatment greatly reduces tumor initiation

We tested if neurosphere recovery correlated with the ability of cells to initiate tumors in a subcutaneous xenograft model. U87NS cells were treated *in vitro* with DMSO, DAPT-only, TMZ-only, or TMZ + DAPT (Fig. 6A; Supplementary Table S1). Live cells ($2.5 \times 10^5$) were s.c. injected into nude mice, and tumor initiation was observed when a palpable tumor formed. DMSO and DAPT-only *ex vivo* treated cells showed similar tumor incidence (6 of 7 and 7 of 7, respectively) and average latencies of 15 and 14 days, respectively. TMZ-only–treated cells had an increased tumor latency of 32 days, but the tumor incidence (6 of 7 mice) was similar to that of control xenografts. Impressively, none of the mice injected with

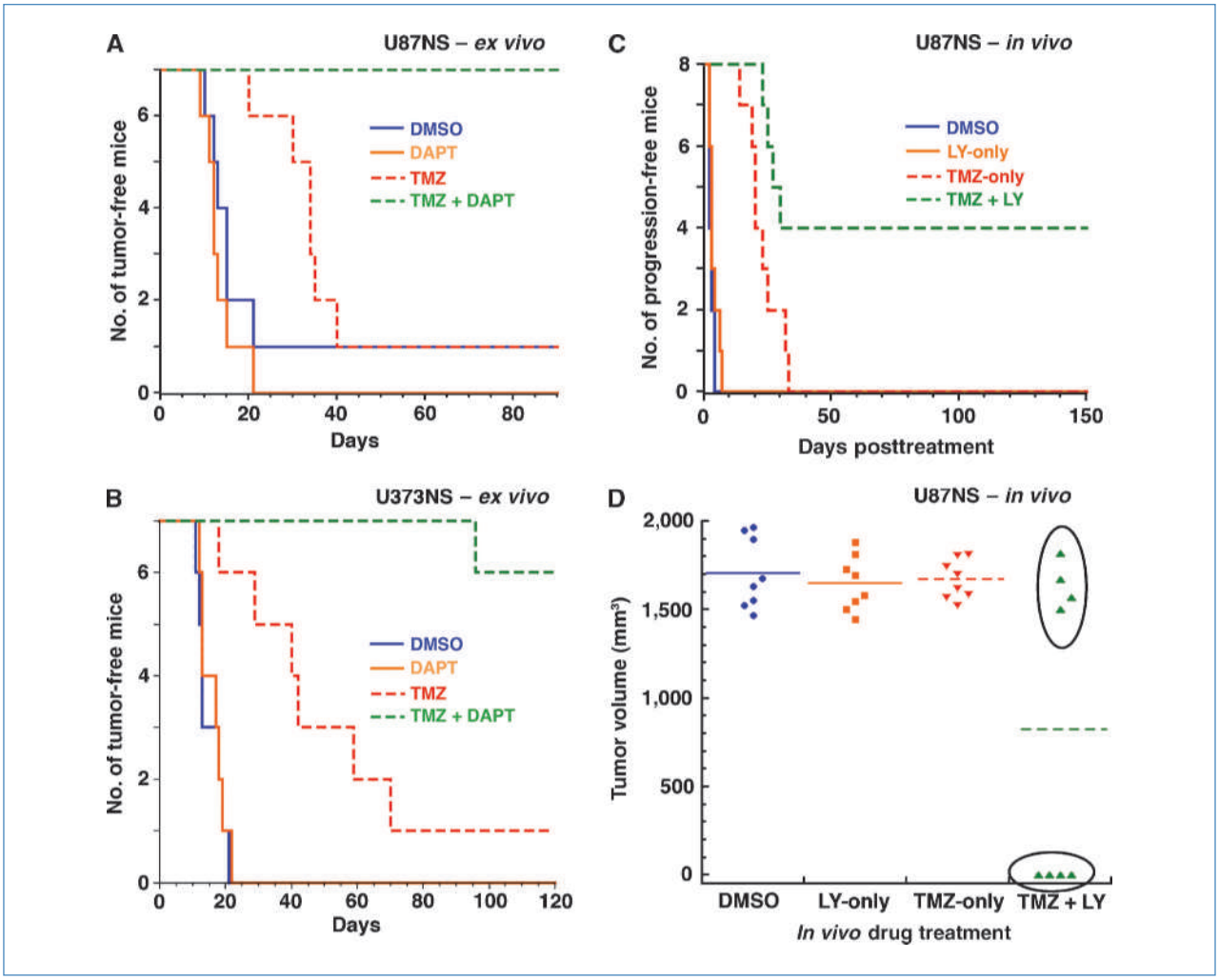


Figure 6. TMZ + DAPT treatment decreases tumorigenicity. A, U87NS cells were treated *ex vivo* with DMSO, 1 μmol/L DAPT, 200 μmol/L TMZ, or 200 μmol/L TMZ + 1 μmol/L DAPT, and 7 d after treatment, mice were s.c. injected with $2.5 \times 10^5$ live cells. TMZ treatment increased latency, but xenografts still formed. TMZ + DAPT inhibited xenograft formation. B, U373NS cells were treated *ex vivo*, and mice were s.c. injected with $3 \times 10^6$ live cells. Treatment with 200 μmol/L TMZ increased latency, but tumors still formed. Treatment with 200 μmol/L TMZ + 5 μmol/L DAPT decreased xenograft formation. C, *in vivo* TMZ treatment increased tumor latency in U87NS xenografts, but 100% of the mice succumbed to progression. TMZ + LY chow treatment completely blocked progression in 50% of the mice. D, tumor volumes at the time of sacrifice. The U87NS TMZ + LY chow–treated xenografts that lacked progression had no palpable tumor at 150 d posttreatment.

TMZ + DAPT–treated cells (0 of 7 mice) formed tumors, even after 90 days. When a higher number of live U87NS cells ($3 \times 10^6$) were injected, we saw a similar trend (Supplementary Table S1). Mice with $3 \times 10^6$ cells of U87NS DMSO (2 of 2 mice) and DAPT-only (2 of 2 mice) xenografts developed palpable tumors at 3 and 4 days, respectively, and 3 of 4 mice with TMZ-only–treated cells formed tumors with an average latency of 25 days. With this higher number of cells injected, U87NS TMZ + DAPT xenografts formed tumors in only 1 of 4 mice with a longer latency of 43 days.

U373NS cultures were treated with DMSO, DAPT-only, TMZ-only, or TMZ + DAPT, and $3 \times 10^6$ live cells were injected s.c. into nude mice (Fig. 6B; Supplementary Table S1). The control DMSO cells formed palpable tumors in an average of 15 days for 7 of 7 xenografts, and DAPT-only–treated cells formed tumors in an average of 16 days for 7 of 7 xenografts. *Ex vivo* treatment with TMZ-only increased the latency of tumor formation; however, the tumor incidence was similar to the DMSO control xenografts. Palpable tumors formed for 6 of 7 TMZ-treated U373NS xenografts in an average of 43 days. *Ex vivo* treatment with TMZ + DAPT greatly reduced tumor formation in mice. Only 1 of 7 mice formed a tumor in the TMZ + DAPT U373NS xenografts with an extended latency of 96 days. The tumor-free mice were observed for up to 120 days before sacrifice. These *ex vivo* experiments show the potency of TMZ + DAPT combined treatment in reducing tumor formation.

**TMZ + LY *in vivo* treatment inhibits tumor regrowth**

We tested the effect of *in vivo* TMZ + GSI treatments on preexisting subcutaneous glioma xenografts using LY chow (23). A 10-day diet of LY chow significantly decreased the mRNA levels of the Notch targets *Hes1* and *Hey1* (Supplementary Fig. S4). Mice were s.c. injected with $10^6$ U87NS cells and treated when the tumors reached a volume of approximately 150 $mm^3$. When the tumor volume was double the original volume from the start of the drug treatments, we judged the xenograft as progressing. The DMSO control and LY chow-only cohorts did not have any delay in tumor progression (Fig. 6C). TMZ treatment initially had decreased tumor volumes (Supplementary Fig. S5A). However, the TMZ-only–treated tumors progressed in 8 of 8 xenografts, and tumor volume doubled in an average of 23 ± 7 days after treatment (Fig. 6C). These tumors had a normal growth rate and were sacrificed between 23 and 39 days posttreatment. Impressively, 4 of the 8 mice treated with TMZ + LY chow displayed no tumor progression (Fig. 6C). In the other 4 of the 8 mice treated with TMZ + LY chow, tumor progression occurred in an average of 26 ± 3 days (Supplementary Fig. S5B), and mice were euthanized between 24 and 33 days posttreatment. The TMZ + LY chow mice that did not have tumor progression displayed a complete loss of a palpable tumor and remained tumor-free until euthanized at 150 days (Fig. 6D). In these mice, no tumor masses were evident by gross dissection and examination of H&E-stained sections (Supplementary Fig. S6A). Hence, the TMZ + LY chow treatment had a dramatic effect on preexisting tumors by curing 50% of the mice. During drug administration, toxicity was determined by weight loss. TMZ-only and TMZ + LY chow cohorts initially showed a slight weight loss after TMZ injections (Supplementary Fig. S6B). However, the TMZ-only and TMZ + LY chow mice returned to their starting body weight, and no significant weight difference was observed throughout the remainder of the treatment. This shows that the mice tolerated the LY chow alone and the combination of the TMZ + LY chow. The lack of overall weight loss also suggests that the mice on LY chow diets did not significantly reduce their food consumption compared with control mice and received the estimated average dose of 5 mg/kg/d of LY (23).

## Discussion

With current GBM treatment, tumor recurrence is highly probable. The neurosphere recovery assay of our laboratory shows that the glioma cells that survive chemotherapy can repopulate neurosphere cultures and form tumors (11). Neurosphere cultures are useful *in vitro* to study glioma response to drug treatments because the neurospheres resemble the phenotypes and genotypes of the patients' tumors (8, 10). In addition, we found that the adherent glioma cell lines grown as serum cultures are more sensitive to TMZ than the neurosphere cultures and do not recover (data not shown). In contrast, when neurospheres are treated with clinically relevant concentrations of TMZ, a small number of cells survive, recover from the chemotherapy, and repopulate the cultures.

The Notch pathway is active in gliomas and is inhibited with GSI treatment. Low concentrations of GSIs alone (1 and 5 μmol/L) did not have a significant effect on neurosphere formation (Fig. 1C). These results are consistent with those of Wang and colleagues (19), which showed that low concentrations of DAPT or L685,458 only moderately reduced cell growth. However, a recent publication showed that the potent GSI-18 inhibited neurosphere formation and xenograft growth (25). In the presence of higher concentrations of DAPT and LY, a dose-dependent response was observed. At 10 μmol/L, the GSIs had a moderate effect on initial neurosphere formation, but these cells retained their ability to form secondary neurospheres. It seems that GSI-only treatment initially impedes the proliferation of neurosphere cells, but these cells are capable of recovery. On the other hand, we showed that low concentrations of two GSIs, DAPT and LY, enhanced TMZ treatment (Figs. 2 and 3; Supplementary Fig. S2). Neurosphere recovery was inhibited and tumor formation was greatly reduced with TMZ + GSI treatment. Additionally, when the remaining neurospheres were dissociated and replated, we found that the cells from TMZ + GSI–treated cultures were no longer capable of self-renewal based on their inability to form secondary neurospheres. The mechanism for the permanent suppression of neurosphere formation with TMZ + GSI treatment is under study in our laboratory. The specific population of cells that are targeted by TMZ + GSI treatment is unknown. Research in the growing cancer stem cell field shows that GBM stem cells exhibit chemoresistance and radioresistance (26–28). Because Notch activity is associated with GBM stem cell function and survival (13, 29), and the cells that survive TMZ-only treatment are capable of self-renewal (Fig. 3) and tumor

initiation (Fig. 6), it is probable that the cells targeted by TMZ + GSI treatment possess a cancer stem cell phenotype. There is controversy about the current GBM stem cell markers, such as CD133 (30), and further progress in this field will be necessary to determine if the TMZ + GSI–responsive cells and the cancer stem cells are the same population.

Inhibiting the repopulation of the neurosphere cultures is dependent on the sequence of TMZ and GSI treatments (Fig. 5). With single doses of DAPT, secondary neurosphere formation was inhibited only when DAPT was administered after TMZ (posttreated). Simultaneous treatment of TMZ and DAPT did not significantly inhibit self-renewal. When DAPT was administered before TMZ (pretreated), secondary neurosphere formation was similar to that of the TMZ-only–treated cells. Pretreatment also resulted in a higher number of initial neurospheres formed. These results were important in determining *in vivo* treatment schedules for mice and will be valuable in translating this research into the clinic. Because current treatments also include radiotherapy, we ultimately need to add radiation to our TMZ + GSI treatment schedule. Recently, it was found that TMZ and radiation are additive when TMZ is administered before radiation (31). GSIs can also enhance radiation-induced cell death when administered within 24 hours before or after radiotherapy (19). However, our results show that GSI treatment before TMZ can diminish the efficacy of the chemotherapy, and to inhibit neurosphere and tumor formation, TMZ should be administered before GSIs. Further experiments are necessary to determine how irradiation will contribute to combination therapy with TMZ and GSIs and the sequence of treatments that will provide the most efficient therapy.

Our mouse experiments show that the addition of GSIs to TMZ treatment can significantly enhance the survival of mice with glioma xenografts. *Ex vivo* treatment of U87NS and U373NS cultures with TMZ + DAPT greatly decreased tumorigenicity (Fig. 6A and B). The *in vivo* treatments showed that TMZ-only treatment of preexisting tumors was not a sufficient therapy because it only temporarily blocked tumor progression. In 50% of the treated mice, TMZ + LY chow treatment completely halted tumor progression and culminated in the loss of a palpable tumor (Fig. 6C and D). In the other 50% of treated mice, there was substantial tumor volume at the time of sacrifice. This variability may result from several sources. In the mice that have a shorter latency, the TMZ concentration may not be high enough to induce a cell cycle arrest in all of the cells capable of recovery, which could hinder GSI enhancement (Supplementary Fig. S5A and B). Also, a slight variability in the food consumption between mice in the TMZ + LY chow cohort could explain the heterogeneous response. These observations emphasize the need for personalized treatment with regard to drug dosing. The response to the *in vivo* treatment schedule was analogous to the response to the DAPT posttreatment schedule in the neurosphere recovery assay, which showed that TMZ + GSI treatments permanently blocked culture repopulation and tumor regrowth. These studies suggest a role for TMZ + GSI therapy to reduce recurrences in patients with low tumor burden after surgical resection of the bulk tumor.

We believe that these studies have great potential for clinical translation because most or all GBMs have active Notch signaling (refs. 13, 14, 18; Fig. 1A), and all the lines in this study responded to the TMZ + GSI treatment (Figs. 3 and 6; Supplementary Fig. S2). In addition, TMZ is already the chemotherapy drug of choice for GBMs (3), and GSIs are in clinical trials (16, 32–34). An additional benefit of the combined treatment with TMZ + GSI is that lower concentrations of the GSI can be used, and in culture, a single dose of GSI is sufficient to enhance TMZ therapy. These may be important clinical factors because GSIs can cause cytotoxicity in the gastrointestinal tract (35); however, low GSI doses and intermittent treatment schedules diminish these side effects (16). It is also possible that more specific inhibitors, such as anti-Notch receptor antibodies (36), could be used in conjunction with TMZ. In contrast to the reversible effects of GSI-only or TMZ-only treatment, TMZ + GSI has an apparently permanent effect on neurosphere and tumor formation. This response has the potential to enhance clinical TMZ therapy by inhibiting glioma recurrence.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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