Siomycin A targets brain tumor stem cells partially through a MELK-mediated pathway

Ichiro Nakano, Kaushal Joshi*, Koppany Visnyei*, Bin Hu, Momoko Watanabe, Diana Lam, Eric Wexler, Kuniyasu Saigusa, Yuko Nakamura, Dan R. Laks, Paul S. Mischel, Mariano Viapiano, and Harley I. Kornblum

Department of Neurological Surgery, James Comprehensive Cancer Center, The Ohio State University Medical Center, Columbus, Ohio (I.N., K.J., B.H., K.S., Y.N., M.V.), Departments of Molecular and Medical Pharmacology (K.V., M.W., D.R.L., H.I.K.), Pathology (P.S.M.), and Psychiatry (E.W., H.I.K.), David Geffen School of Medicine at UCLA, Los Angeles, California

Glioblastoma multiforme (GBM) is a devastating disease, and the current therapies have only palliative effect. Evidence is mounting to indicate that brain tumor stem cells (BTSCs) are a minority of tumor cells that are responsible for cancer initiation, propagation, and maintenance. Therapies that fail to eradicate BTSCs may ultimately lead to regrowth of residual BTSCs. However, BTSCs are relatively resistant to the current treatments. Development of novel therapeutic strategies that effectively eradicate BTSC are, therefore, essential. In a previous study, we used patient-derived GBM sphere cells (stemlike GBM cells) to enrich for BTSC and identified maternal embryonic leucine-zipper kinase (MELK) as a key regulator of survival of stemlike GBM cells in vitro. Here, we demonstrate that a thiazole antibiotic, siomycin A, potently reduced MELK expression and inhibited tumor growth in vivo. Treatment of stemlike GBM cells with siomycin A resulted in arrested self-renewal, decreased invasion, and induced apoptosis but had little effect on growth of the nonstem cells of matched tumors or normal neural stem/progenitor cells. MELK overexpression partially rescued the phenotype of siomycin A–treated stemlike GBM cells. In vivo, siomycin A pretreatment abraded the sizes of stemlike GBM cell–derived tumors in immunodeficient mice. Treatment with siomycin A of mice harboring intracranial tumors significantly prolonged their survival period compared with the control mice. Together, this study may be the first model to partially target stemlike GBM cells through a MELK-mediated pathway with siomycin A to pave the way for effective treatment of GBM.

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that FOXM1b plays a role in cell cycle progression of various cancers, including glioma.\textsuperscript{15,16} A study by Radhakrishnan et al. suggested that siomycin A treatment selectively induces apoptosis in transformed, but not in normal, lung fibroblasts in culture.\textsuperscript{15} Another study, by Bhat et al., suggested that siomycin A treatment induces apoptosis of melanoma cells.\textsuperscript{16} It is not clear, however, whether siomycin A triggers apoptosis such that it can inhibit tumor formation and/or growth of malignant gliomas or BTSCs.

Another potential key player in BTSC survival and proliferation is maternal embryonic leucine-zipper kinase (MELK), a member of the snf1/AMP-activated protein kinase family of serine-threonine kinases.\textsuperscript{17,18} The members of this family are largely associated with cell survival under nutrient starvation.\textsuperscript{19–23} However, unlike other family members, MELK appears to regulate the proliferation of somatic stem cells, as we have demonstrated for neural stem cells (NSCs).\textsuperscript{24} Several recent studies have also identified MELK as a potential target for certain somatic tumors, including colorectal, lung, and ovarian cancers.\textsuperscript{25,26} In GBM, MELK expression was inversely correlated with survival, and MELK was a key component of a transcriptional module in which a number of genes that modulate M-phase were coregulated.\textsuperscript{27–29} In vitro, MELK knockdown by small interfering RNA (siRNA) induced apoptosis of stemlike GBM cells.\textsuperscript{27} In contrast, MELK knockdown arrested proliferation of mouse neural precursors without causing a significant increase in apoptosis.\textsuperscript{24} Thus, MELK is likely a key regulator of BTSC survival but not of NSC survival, raising the possibility that MELK is an attractive therapeutic target.

Here, we demonstrate that siomycin A reduces MELK expression and eradicates stemlike GBM cells by inducing apoptosis. In contrast, siomycin A does not affect survival or growth of the nonstem GBM counterpart or normal neural stem/progenitor cells derived from human fetal brains. When pretreated with siomycin A, in vivo tumor growth derived from stemlike GBM cells is significantly inhibited. Siomycin A treatment reduces MELK expression of stemlike GBM cells, while overexpression of MELK partially restores the phenotype of siomycin A treatment. These data demonstrate that siomycin A effectively decreases the number of stemlike GBM cells in vitro and growth of stemlike GBM cell–derived mouse intracranial tumors in vivo by acting partially through a MELK-mediated pathway.

Materials and Methods

Human Specimens and Tissue Culture

Twenty GBM samples were obtained from patients undergoing resection in accordance with a protocol approved by the UCLA Medical Center Institutional Review Board. Four surgical specimens of GBM and 1 sample of human fetal neural stem/progenitor cells were cultured as described.\textsuperscript{17,27,30} For the assays using spheres, all the experiments were performed with “younger” cells with less than 20 passages. Spheres were cultured in Dulbecco’s modified Eagle’s medium (DMEM/F12 supplemented with basic fibroblast growth factor (Peprotech), epidermal growth factor, B27, heparin (Sigma-Aldrich), penicillin/streptomycin (Invitrogen), and L-glutamine (Invitrogen). For the assays with serum-propagated cells, GBM1600 cells were maintained in DMEM/F12 with 10% fetal bovine serum (FBS), and cells within 10 passages were kept as frozen stocks. GBM1600 spheres were grown for 3 weeks from primary culture of GBM1600 cells that had been initially cultured in DMEM/F12 with 10% FBS. To verify the BTSC properties, the proportion of CD133-expressing cells, sphere-forming potential, and tumorigenic potential were determined on a monthly basis with all the tumor sphere samples throughout the entire period of this study. For the neurosphere-forming assay, cells were dissociated into single cells and plated into 96-well microwell plates in a 0.1-mL volume of growth media using serial dilutions, and the resultant sphere numbers were counted at 7–14 days. A cell line of normal human astrocytes immortalized with overexpression of human telomerase reverse transcriptase was obtained from American Type Culture Collection in 2008, cultured in DMEM/F12 with 10% FBS, and passaged when the culture was confluent.

Xenograft Studies

Three GBM samples (GBM1600, GBM146, and GBM157) were used for the xenotransplantation studies. GBM157 cells were stably expressing enhanced green fluorescence protein (EGFP) after infection with an EGFP-expressing lentiviral construct. Cells were then cultured for several weeks to ensure proper washout of viral particles. Prior to compound exposure, GBM spheres were dissociated and exposed to siomycin A or an equal concentration (0.1%) of dimethyl sulfoxide (DMSO) for 24 h. Cells were then washed, counted, and resuspended into DMEM/F12 only, at a density of 25,000 live cells/μL. An equal number of live cells (as determined by Trypan Blue stain) were then transplanted intracranially into animals. Animal experimentation was done with an institutional approval following guidelines of the National Institutes of Health. Severely immunocompromised non-obese diabetic–severe combined immunodeficiency (NOD-SCID) gamma (null) mice (Jackson Immunoresearch Laboratories) were used. The cell suspension was implanted using a Hamilton syringe, unilaterally, into the striatum of the mice, using the coordinates of 0.5 mm anterior from bregma, 2.2 mm lateral from the midline, and 3.0 mm below the pial surface. Animals were observed for neurological signs on a daily basis and sacrificed after 12 weeks. Following perfusion and postfixation with 4% paraformaldehyde (PFA), brains were cryosectioned at 20 μm.
**Tumor Size Measurement**

To calculate tumor size, all brains were cut in a uniform fashion, keeping every 10th section for size analysis. Sections containing EGFP-expressing GBM157 were scanned with a Typhoon Variable Mode Imager 9410 (GE Healthcare) by determining the tumor area on each section, based on the EGFP expression above background. To calculate tumor volume, the measured tumor area on each section was multiplied by the thickness of the section (20 μm) plus the thickness of the skipped sections between measured sections. Tumor volume was then expressed in cubic millimeters. For GBM1600, sections were stained with hematoxylin and eosin, using standard protocols, and visualized under a microscope. Photographic images were then evaluated using ImageQuant 5.2 software by drawing regions of interest around the tumor mass on each section. Tumor volume was calculated similarly to the EGFP-expressing sections described above. Statistical significance was determined by using an F-test followed by a T-test.

**Survival Analysis of Intracranial Tumor Growth Experiments with Drug Injection**

At 4 days following transplantation of GBM30 cells into striatum of immunodeficient mice, either siomycin A or DMSO was injected into the tumor cavity. Treated mice were monitored closely, and the duration of their survival was recorded. Mice that showed signs of distress and morbidity were euthanized and considered as if they had died on that day. Survival rates were calculated as follows: survival rate (%) = (number of mice still alive / total number of experimental mice) x 100%. Log-rank analyses were performed for the statistics.

**Quantitative and Conventional Reverse Transcription–PCR**

RNA isolation, complementary DNA synthesis, and reverse transcription (RT)–PCR were performed as described.17,27

**Flow Cytometry**

GBM1600 spheres were used for flow cytometry and sorting of CD133 positive and negative enriched cells. Dissociated cells were incubated with the CD133 antibody (Clone AC141, Miltenyi Biotec) for 30 min and separated with phycoerythrin using fluorescence-activated cell sorting (FACS Aria, Becton Dickinson) with a purification-mode algorithm. Following separation, 100 cells per well were cultured in 96-well plates with either siomycin A or DMSO. For analytical flow cytometry, GBM samples were cultured in serum-free medium, and those younger than 20 passages were used for this study.17,27 Gating parameters were set by side and forward scatter to eliminate dead and aggregated cell populations.

**Apoptosis Assay**

GBM cells treated with siomycin A or DMSO for 48 h were dissociated into single cells. Analysis of the apoptotic cell population was performed using the Apoptosis Detection Kit (R&D Systems) according to the manufacturer’s instruction.

**Immunohistochemistry**

Brains of NOD-SCid gamma (null) mice were removed, fixed with ice cold 4% PFA in phosphate buffered saline (PBS), pH 7.4 overnight, sunk in 20% sucrose PBS, frozen in 4-methylbutane, and stored at −80°C until use. Sections were cryoprotected and sectioned at 20 μm. The following primary antibodies were used: Nestin (anti-Nestin, clone 10C2, 1:200, Mouse Monoclonal Antibody, MAB5326, Chemicon) and human nuclear antibody (anti-Nuclei, clone 235-1, 1:200, Mouse Monoclonal Antibody, MAB1281, Chemicon). The following secondary antibody was used: Alexa 568conjugated antibodies (1:1000, Molecular Probes, Invitrogen). In all cases, negative controls (no primary antibody) were used to ensure the specificity of the immunoreactivity. Photomicrographs were obtained using a microscope (model IX50; Olympus) fitted with a bright- and dark-field condenser using a digital camera (model C2020; Olympus). Digital images were processed, compiled, and adjusted with Adobe Photoshop 7.0.2 in order to accurately reflect direct observation.

**Western Blot**

Cells were lysed in ice-cold lysis buffer (0.1% Triton X-100, 20 mM Tris-HCl, pH 7.4, and 100 mM NaCl) containing protease-inhibitor cocktail (Sigma-Aldrich), and protein concentrations were determined by bicinchoninic acid protein assay according to the manufacturer’s protocol (Invitrogen). Twenty microgram of protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane (Invitrogen). The membrane was incubated with anti-MELK antibody (1:1000, Sigma-Aldrich), followed by incubation with horseradish peroxidase–conjugated anti-rabbit immunoglobulin antibody (1:1000, GE Healthcare). Detection was carried out with chemiluminescence using Super Signal West Pico Chemiluminescent Substrate (Invitrogen).
Transient Transfection

Transfection for dissociated GBM spheres was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol with modification as described. The effect of siomycin A (0.1 μM) on self-renewal of stemlike GBM cells was assessed by three rounds of low density (near clonal) sphere formation (1 cell/μL). Neurosphere-forming assay was performed as described above in serum-free medium with siomycin A or DMSO. Following dissociation of primary neurospheres into single cells, 100 GBM cells were again plated in the same manner to form secondary neurospheres. Likewise, tertiary neurospheres were established. The ratio of secondary or tertiary neurosphere number versus primary neurosphere number was assessed.

Neurosphere-Forming Assay

GBM cells or normal cells were plated in each well of 96-well plates with serum-free medium, and the number of neurospheres was counted at day 7. For siomycin A-pretreatment experiments, cells were incubated with siomycin A or DMSO for 3 h at 37°C, and an equal number of cells were seeded in 96-well plates after washout of drugs.

In Vitro Self-Renewal Assay

The effect of siomycin A (0.1 μM) on self-renewal of stemlike GBM cells was assessed by three rounds of low density (near clonal) sphere formation (1 cell/μL). Neurosphere-forming assay was performed as described above in serum-free medium with siomycin A or DMSO. Following dissociation of primary neurospheres into single cells, 100 GBM cells were again plated in the same manner to form secondary neurospheres. Likewise, tertiary neurospheres were established. The ratio of secondary or tertiary neurosphere number versus primary neurosphere number was assessed.

Brain Slice Invasion and Short-Term Cell Viability Assays

GBM cells were dissociated and cultured until they formed neurospheres of 300 to 400 μm diameter, at which point they were treated with 1 μM siomycin A or the same concentration of DMSO for 24 h. Coronal brain slices obtained from neonatal mice were prepared for organotypic culture as described and maintained in drug-free, serum-free medium for the duration of the experiment. The same number of cells from dissociated neurospheres was manually placed on the brain slices and imaged by fluorescence microscopy every 24 h. Quantification of the area and maximum distance of cell dispersion were performed as described using the software ImageJ v.1.43. Experiments were performed in triplicate, using 8–10 aggregates per experimental condition, and analyzed by 2-way ANOVA for repeated measures. In parallel experiments, cells treated with siomycin A or DMSO were dissociated and seeded at 4000 cells/200 μL in 96-well plates. Cell viability was then analyzed by measuring the reduction of a soluble tetrazolium salt (Cell Titer kit, Promega, Fitchburg, WI) according to the manufacturer’s instructions.

Drug Treatment

Siomycin A was obtained through the Developmental Therapeutics Program NCI/NIH. The other small molecule inhibitors were purchased from Calbiochem, and the dose of each compound for GBM sphere formation was determined according to the manufacturer’s information (http://www.emdbiosciences.com/Products/BrowseProductsByCategory.asp?catid=2315). In each experiment, the same concentration of DMSO with the drugs of interest was used as the control samples.

Results

MELK mRNA Was Highly Expressed by GBM Tissues

First, we evaluated MELK messenger RNA (mRNA) in GBM tissues and normal brain tissues by quantitative RT-PCR with 16 autopsy samples. In order to avoid contamination of infiltrating tumor cells, normal brain tissues were removed from the white matter of the contralateral side of the tumors. The results demonstrated that the average expression of MELK mRNA was 3.4 times higher in GBM tissues than in normal tissues (Fig. 1A).

MELK mRNA Was Upregulated in Stemlike GBM Cells

We then postulated that MELK mRNA expression may serve to identify BTSC inhibitors for two reasons: (1) knockdown of MELK expression with siRNA promotes apoptosis of stemlike GBM cells but not mouse neural stem/progenitor cells, and (2) MELK expression is inversely correlated with GBM patient survival. To test this hypothesis, neurosphere cultures were established from 4 GBM samples (GBM146, 157, 177, and 1600) in serum-free medium. The first 3 sphere samples were directly derived from surgical specimens, and GBM1600 spheres were derived from their serum-propagated cultures (Fig. 1B). In all cases, GBM cells in serum-free medium (neurosphere [NS]), but not their serum-propagated counterparts, possessed a strong tumorigenic ability in vivo (Fig. 1C, panel a) and clonogenic and differentiation potential in vitro (data not shown). These data suggest that GBM NS are enriched with BTSCs. The histopathology of the stemlike GBM cell–derived intracranial tumors in immunodeficient mice recapitulated that of the parental tumors (Fig. 1C, panel b). Similar to those in the majority of human GBM specimens, formed tumors in mouse brains derived from GBM cells in serum-free medium were highly immunoreactive to an immature neural marker, Nestin, while a remnant of transplanted serum-propagated GBM cells in mouse brains were negative for Nestin (Fig. 1C, panel c). The proportion of putative BTSC marker CD133–expressing cells was significantly higher in the serum-free condition than in the serum-containing condition in both primary GBM samples and GBM1600, although the proportion of CD133-positive cells in GBM1600 was much smaller than in the primary GBM samples (Fig. 1C, panel d). It is likely due to the exposure of GBM1600 cells to serum-containing medium at the initial stage of establishing the culture. To verify whether BTSCs express
MELK, we used RT-PCR to compare MELK expression in GBM157 cells under serum-free and serum-containing conditions, as well as GBM1600 cells separated based on CD133 expression. In agreement with our previous data using other samples, MELK expression was elevated in the BTSC-enriched conditions: GBM157 NS (Fig. 1D, panel a) and CD133-positive GBM1600 cells (Fig. 1D, panel b).

Siomycin A Reduced MELK Expression in Stemlike GBM Cells

We next sought to identify compounds that decrease MELK expression in stemlike GBM cells. We incubated GBM146 spheres with siomycin A (1 μM) along with 54 other compounds that we have previously identified as inhibitors of GBM cells (manuscript in preparation).
Following incubation with each compound for 24 h, MELK mRNA expression was investigated with quantitative RT-PCR (Fig. 2A). Siomycin A was identified as one of the compounds that strongly diminished MELK expression. We then extended this MELK expression analysis to several different cell cultures to confirm the inhibitory effect of siomycin A on MELK mRNA expression. Basal level of MELK expression was higher in spheres than in serum-propagated cells in GBM1600 in 2 separate wells. (B) RT-PCR representing MELK and FOXM1b expression in GBM1600 spheres (panel a) and normal neurospheres (panel b) following siomycin A treatment for indicated period. Data were confirmed by 3 independent results. Abbreviations: SC, serum-propagated cells; NS, neurospheres in serum-free medium; h, hours; SM, siomycin A.

Siomycin A Treatment Eliminated Stemlike GBM Cells, While It Did Not Significantly Affect Survival of Normal Neural Stem/Progenitor Cells

We utilized the aforementioned GBM specimens to determine the effects of siomycin A treatment on stemlike GBM cells. Incubation of GBM spheres with siomycin A inhibited survival with a half maximal inhibitory concentration (IC50) of approximately 1 μM (Supplemental Fig. 5). Unlike the results with siomycin A, none of the tested compounds displayed significant inhibitory effect on sphere-forming GBM cells. Siomycin A treatment also inhibited sphere formation from cultures derived from GBM1600 cells that had been previously propagated in serum-containing medium (GBM1600SC) (Fig. 3B, panel c). In contrast, siomycin A treatment for 3 h did not significantly alter the neurosphere-forming ability of normal human astrocytes or neural stem/progenitor cells derived from fetal cerebral cortices in serum-free medium (Fig. 3B, panel d).

Phenotype of Siomycin A Treatment Was Partially Rescued by MELK Overexpression

In order to validate whether the action of siomycin A in stemlike GBM cells is mediated through downregulation of MELK, we combined siomycin A treatment and overexpression of MELK in GBM spheres. RT-PCR confirmed the overexpression of MELK following transfection in GBM146 cells propagated in serum-free medium with or without siomycin A (Fig. 3C, panel a). At 6-h post-transfection, GBM cells were plated at the same density with either DMSO or siomycin A in serum-free medium. Treatment of these GBM cells with 1 μM siomycin A resulted in a significant reduction in sphere numbers to 23% of controls (Fig. 3C, panel b). Although MELK overexpression alone yielded only a marginal increase in the DMSO-treated sample, MELK overexpression significantly masked the effects of siomycin A treatment, such that the number of neurospheres recovered to 76% of DMSO-treated controls, and approximately 3-fold greater than siomycin A–treated green fluorescence protein–expressing samples (Fig. 3C, panel b, c). These data suggest that siomycin A acts, at least partially, through a MELK-mediated pathway. On the other hand, FOXM1b overexpression did not yield any significant positive effect on sphere forming capacity of siomycin A–treated GBM146 cells (Supplemental Fig. 4).

We then examined the ability of MELK to overcome inhibition of various pathway inhibitors (Supplemental Fig. 5). We first compared the impact of these compounds on the growth of stemlike GBM146 cells (Supplemental Fig. 5A). Unlike the results with siomycin A, none of the tested compounds displayed significant...
growth suppression of GBM146 spheres when the dose of each compound was equivalent to reach their IC50 (see Methods). When the treatment doses were increased up to 20 times, the numbers of formed spheres were reduced in a dose-dependent manner to varying degrees with the Akt inhibitor X, AG1296 (an inhibitor of platelet-derived growth factor receptor), and SU6656 (an Src inhibitor). We then evaluated the impact of MELK overexpression on the stemlike GBM146 cells treated with these compounds (Supplemental Fig. 5B). In contrast to the result with siomycin A, MELK overexpression did not exhibit any significant effect on phenotype with the tested compounds in sphere formation derived from GBM146 cells (Supplemental Fig. 5B). These data suggest that MELK does not mediate its effects by activating these signaling pathways and, by inference, that siomycin A does not exert its actions by inhibiting them.

The data thus far indicate that siomycin A treatment preferentially inhibits growth of stemlike GBM cells in...
vitro. Inhibition of sphere formation can result from either arrest of proliferation or induction of cell death of the sphere-forming GBM cells. To evaluate the impact of siomycin A treatment on cell death of stemlike GBM cells, we assessed apoptotic populations in the GBM spheres treated with either siomycin A or DMSO by flow cytometry with anti–Annexin V antibody (AV) and propidium iodide (PI). In all 3 primary GBM sphere samples (GBM146, 137, and 177) as well as in GBM1600 spheres, siomycin A treatment resulted in a significant increase of both early (AV+; PI−) and late (AV+; PI+) apoptotic populations (Fig. 4A, panels a and b). In contrast, any apparent increase in apoptotic populations was not observed with siomycin A treatment on serum-propagated nonstem GBM1600 cells (Fig. 4A, panel c) or normal neural stem/progenitor cells (Fig. 4A, panel d). Figure 3A, panel e depicts the results as bar graphs. These findings support the hypothesis that one mechanism by which siomycin A treatment selectively acts on stemlike GBM cells is through apoptotic cell death.

Use of the CD133 antigen (AC133) as a BTSC marker is controversial.7,34,35 However, we have found it useful to enrich for sphere-forming cells in the 4 samples being used in this study (Fig. 1B and C, and data not shown). Therefore, we further investigated the effects of siomycin A on the CD133-positive fraction of cells in these GBM samples. We harvested CD133-positive and CD13-negative GBM1600 cells following separation by fluorescence-activated cell sorting and subsequently, examined the effect of siomycin A treatment on clonal sphere formation from each fraction of cells. As expected, only CD133-positive cells were capable of forming secondary spheres in the control (DMSO) condition (Fig. 4B). Incubation of CD133-positive cells with siomycin A significantly diminished their potential to form spheres (Fig. 4B, panel c).

Self-Renewal of Stemlike GBM Cells Was Inhibited by Siomycin A Treatment

The data suggest that siomycin A treatment inhibits survival and growth of stemlike GBM cells (Figs. 3 and 4). Previously, we demonstrated that MELK regulates the self-renewal of stemlike GBM cells in vitro.27 Therefore, we next sought to determine the effect of siomycin A on this process. One means of estimating self-renewal is through the use of serial clonal assays to test multiple rounds of sphere-forming capacity.31,36 Therefore, we compared the effect of siomycin A treatment on secondary and tertiary sphere formation, using low-density cultures, with its effect on primary sphere formation derived from the GBM samples (GBM1600 and GBM146). In both samples, treatment of stemlike GBM cells with 0.1 μM of siomycin A, a concentration below that which induced significant apoptosis resulted in a greater impact on secondary sphere formation than on primary sphere formation (Fig. 4C). Impact on tertiary sphere formation was even more prominent (Fig. 4C). Taken together, siomycin A treatment targets survival and self-renewal of stemlike GBM cells, while the nonstem GBM cells and normal neural stem/progenitor cells are relatively resistant to the treatment.

Short-Term Treatment with Siomycin A Reduced Stemlike GBM Cell Invasion and Viability

To assess the effect of pretreatment with siomycin A on cell invasion, we tested stemlike GBM cells using an assay for cell invasion on organotypic cultures of brain tissue. These cultures mimic the brain cytoarchitecture and its natural barriers to cell movement (Fig. 5A).32 The stemlike cells in GBM146 and GBM157 were pretreated with 1 μM siomycin A or DMSO for 24 h, seeded on brain slices, and followed by fluorescence microscopy during 24–96 h as described.32 Both stemlike GBM samples dispersed aggressively through brain tissue in the absence of the drug (Fig. 5B and C). In contrast, GBM spheres pretreated with siomycin A failed to invade and exhibited significant size regression and reduction of fluorescence after 72 h (Fig. 5C). These data indicated that siomycin A treatment resulted in arrest of invasion and induction of subsequent cell death (Fig. 5C). Furthermore, pretreatment with siomycin A significantly reduced the mRNA expression of the proteases known to be involved in glioma invasion, such as MMP2 and MMP9 (Fig. 5D), supporting the anti-invasive effect of siomycin A.

Pretreatment with Siomycin A Reduced In Vivo Growth of Stemlike GBM Cells

The in vitro data indicate that siomycin A treatment inhibits the survival and proliferation of BTSC in GBM. However, one of the important aspects of a tumor stem cell is its ability to form and propagate tumors in vivo. We therefore investigated whether or not pretreatment with siomycin A on stemlike GBM cells results in a reduction of tumor formation and growth in vivo. When 10^5 GBM157 sphere cells were transplanted into the striatum of NOD-SCid gamma (null) mice, large intracranial tumors were formed within 12 weeks (Fig. 6A). We treated GBM157 sphere cells with either siomycin A or DMSO for 24 h prior to implantation. At 12 weeks post-implantation, 3 out of 4 mice showed lack of tumor formation with pretreatment of siomycin A, resulting in significant reduction of the average tumor size (Fig. 6A and B and Supplemental Fig. 6). The result was similar with another transplantation using GBM1600 cells (Fig. 6C and D and Supplemental Fig. 6) and GBM146 (data not shown), although statistical significance was not met due to the relatively small number of implanted mice in each group. Collectively, siomycin A eliminates growth of stemlike GBM cells both in vitro and in vivo.
Intratumoral Injection of Siomycin A Yielded Improved Prognosis of Mice Bearing Intracranial Tumors Derived from GBM Spheres

Our eventual goal is to determine therapeutic benefit with siomycin A on patients with malignant glioma. To meet this goal, we sought to address a preclinical test with mice bearing intracranial tumors derived from GBM spheres. In order to evaluate the effect on survival of intracranial tumor-bearing mice, we used one of the most aggressive GBM sphere samples, GBM30. Without treatment mice with GBM30-derived tumors died approximately within 14 days of transplantation. When we injected siomycin A into tumor lesions at day 4 post-transplantation, treated mice survived significantly longer than the DMSO-injected group (median

Fig. 4. Siomycin A treatment reduces self-renewal and induces apoptosis of stemlike GBM cells. (A) Representative flow cytometry detecting apoptotic populations in indicated samples (panels a–d) using anti-Annexin V antibody (AV) and propidium iodide (PI). Primary spheres (NS) are derived from GBM146 (panel a). The graph (panel e) indicates the overall results. (B) Representative flow cytometry confirming the separation of GBM1600 cells based on the CD133 signal intensities (panel a, b). The graph (panel c) describes the effect of siomycin A treatment on sphere formation derived from CD133+ and CD133− GBM1600 cells treated with either DMSO or siomycin A (1 μM). (C) The effect of siomycin A (0.1 μM) on self-renewal of GBM cells (GBM1600 and GBM146) is assessed by comparing the numbers of secondary neurospheres generated from primary neurosphere (2 NS/1 NS) as well as the number of tertiary neurospheres generated from primary neurospheres (3 NS/1 NS). *P < .05, ANOVA followed by post-hoc t-test. Results represented as mean ± SEM.
survival day 21 days) (Fig. 6E). The lower dose treatment did not exhibit any difference in survival period. Taken together, both ex vivo and in vivo treatment of mouse intracranial tumors with siomycin A resulted in decreased tumor growth and/or prolonged survival of tumor bearing mice.

Discussion

Current treatment for malignant glioma is only palliative. Recent studies have suggested that the resistance of BTSCs to current therapies is, at least in part, related to the failures of current treatment. Therefore, the development of novel therapies to effectively target BTSCs is imperative. One potential avenue of treatment is to take advantage of the similarities between BTSCs and their somatic counterparts, NSCs. However, therapies that target key pathways for the survival of both cell types might have unintended effects of killing normal NSCs. Therefore, a worthy treatment would be to target genes and signaling pathways that are required for BTSCs but not for normal NSCs.

MELK is expressed by several organ-specific stem cells, including NSCs. However, our previous study has suggested that NSCs do not require MELK for their survival, while their self-renewal and proliferation depends on MELK. In contrast, knockdown of MELK in stemlike GBM cells resulted in apoptosis. These data support our hypothesis that MELK is one attractive target for BTSC.

MELK has also been strongly associated with cancers. We and others have demonstrated that
MELK is highly expressed in malignant gliomas and is also related to patient outcome, such that patients with higher levels of MELK mRNA have shorter survival periods than those with lower levels of MELK expression. Some studies have shown that MELK plays a functional role in other cancer cells. For example, MELK knockdown decreased in vitro proliferation and anchorage-independent growth of cell lines derived from pancreatic and breast cancer, as well as the in vivo growth of transformed fibroblasts in a subcutaneous xenograft model. Lin et al. provided further evidence that the action of MELK on cancer cell growth is associated with resistance to apoptosis through the inhibition of a pro-apoptotic function of Bcl-G. In our prior study, we found that MELK is expressed in stemlike GBM cells and knockdown of MELK by siRNA results in apoptosis. These findings led us to hypothesize that upregulated MELK expression is a hallmark of survival for cancer stem cells, and targeting MELK may kill BTSCs while sparing normal NSCs. Thus, we sought inhibitors of a MELK-mediated pathway in BTSCs.

Toward the goal of developing novel GBM therapies directed at a MELK-mediated pathway, we analyzed the effect of small molecules on MELK expression in GBM spheres and identified siomycin A as a compound that suppresses MELK expression in GBM spheres. Previously, others demonstrated that siomycin A treatment leads to apoptosis of transformed, but not normal fibroblasts. In agreement with their study and
the phenotype of MELK knockdown of normal stem/progenitor cells and stemlike GBM cells, our data suggested that siomycin A treatment significantly reduces cell survival and self-renewal (Fig. 4) of stemlike GBM cells, with little toxicity to normal neural stem/progenitor cells (Figs. 3 and 4). This cytotoxicity of siomycin A on stemlike GBM cells was coupled to a significant anti-invasive effect in organotypic brain cultures (Fig. 5), resulting in a marked reduction of tumor progression in vivo (Fig. 6).

However, several open questions still remain. For example, it is unlikely that MELK is the only downstream target of siomycin A. Therefore, the action of siomycin A on other pathways may result in unanticipated toxicity. In addition, ex vivo siomycin A treatment inhibited but did not completely prevent tumor formation in one of the lines (GBM1600 in Fig. 6). It is entirely possible that there are siomycin A–resistant stemlike GBM cells. Our in vitro experiments may target only one type of BTSC in GBM, while there may be multiple different kinds of cells with tumor-initiating potential, both among tumors derived from different patients and within a single tumor. Lastly, the lack of demonstrable effect on neurosphere formation of normal stem/progenitor cells or immortalized astrocytes does not completely rule out a more subtle effect on them, a possibility that will need to be explored. Future studies are required to answer these questions.

Our studies demonstrate that siomycin A acts, at least in part, via a MELK-mediated pathway, but they do not clearly define the link between MELK and FOXM1b in BTSC. A previous study suggested that the action of siomycin A on FOXM1b has two distinct mechanisms: (1) regulation of the abundance of FOXM1b mRNA and protein and (2) direct inhibition of phosphorylation of the FOXM1b protein. We found that overexpression of MELK, but not FOXM1b, overrode the inhibitory effect of siomycin A on sphere formation derived from GBM cells (Fig. 2 and Supplemental Fig. 4). If direct inhibition of phosphorylation is the predominant mechanism of siomycin A on FOXM1b in GBM stem cells, then exogenously expressed FOXM1b may not rescue the phenotype of siomycin A–treated stemlike GBM cells. In addition, MELK and FOXM1b may play different roles in BTSCs and their derivatives. In fact, it is not clear yet whether FOXM1b is required for proliferation and/or survival of BTSCs in GBM.

Given that GBM is resistant to the current therapies and BTSCs in GBM may contribute to, at least in part, the therapy resistance, identifying novel therapies that efficiently eradicate BTSCs are crucial.

Our findings indicate siomycin A as a potent inhibitor for a MELK-mediated pathway in stemlike GBM cells. Treatment with siomycin A significantly inhibits survival, proliferation, self-renewal, and invasion of stemlike GBM cells in vitro and significantly reduces tumor growth in vivo. Furthermore, siomycin A treatment has little or no effect on survival or growth of nonstem cells in GBM or normal neural stem/progenitor cells in vitro. These results may provide a rationale to design novel therapeutic strategies that effectively and selectively target BTSCs in GBM with less toxicity to somatic cells. Future work will elucidate the mechanism of siomycin A action as well as the role of MELK and FOXM1b in BTSC.

Supplementary Material

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

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