Suppression of glioblastoma by targeting the overactivated protein neddylation pathway

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Background. The neddylation pathway has been recently identified as an attractive anticancer target, and MLN4924, a specific NEDD8-activating enzyme inhibitor, has been developed as a first-in-class anticancer agent. However, neither the status of the neddylation pathway in glioblastoma (GBM) nor the effect of MLN4924 against GBM has been systematically investigated yet.

Methods. To measure the activation state of the neddylation pathway in GBM, expression of the NEDD8-activating enzyme (E1), NEDD8-conjugating enzyme (E2), and global protein neddylation in GBM tumor tissues versus adjacent tissues were examined by immunoblotting analysis and immunohistochemistry staining. To assess the therapeutic efficacy of neddylation inhibition in GBM, cell proliferation in vitro and tumor growth in vivo were determined upon neddylation inhibition by MLN4924, an investigational NEDD8-activating enzyme inhibitor.

Results. The neddylation pathway was overactivated in a majority of GBM tumor tissues when compared with adjacent normal tissues. The upregulation of this pathway in GBM tissues was positively correlated with high-grade disease and postoperative recurrence but was negatively associated with patient overall survival. MLN4924 treatment inhibited cullin neddylation, inactivated cullin-RING E3 ligase, and led to the accumulation of tumor-suppressive cullin-RING E3 ligase substrates to trigger cell-cycle arrest and senescence or apoptosis in a cell-line dependent manner. Moreover, inhibition of neddylation by MLN4924 significantly suppressed tumor growth in an orthotopic xenograft model of human GBM.

Conclusion. Our study indicates that an overactivated neddylation pathway may be involved in GBM progression and that inhibition of this oncogenic pathway is a potentially new therapeutic approach for GBM.

Keywords: cullin-RING E3 ligase, glioblastoma, MLN4924, neddylation, NEDD8.

Introduction

Glioblastoma (GBM) is the most malignant primary brain tumor. The prognosis for patients with GBM remains unsatisfactory as they are frequently treated with a combination of surgery, radiation, and traditional chemotherapy.1,2 In the last decades, great effort has been focused on understanding the molecular mechanisms of the initiation and progression of GBM.3 However, little progress has been achieved in the transition from understanding this disease to successfully treating it.4 Thus, it is urgent to identify novel anti-GBM targets and develop more effective therapeutic strategies for the treatment of GBM.
Protein homeostasis maintains normal cellular function. The ubiquitin-proteasome system (UPS) is responsible for regulating multiple biological processes through timely degradation of unwanted proteins. Because of its essential role in maintaining intracellular homeostasis, dysregulation of UPS is involved in the development of many diseases including cancer. Therefore, targeting UPS represents an attractive therapeutic strategy for human cancers. Bortezomib (also known as Velcade or PS-341) is a first-in-class general proteasome inhibitor, approved by the FDA for the treatment of patients with multiple myeloma or mantle cell lymphoma. However, the drug lacks specificity due to overall inhibition on the degradation of proteasome-mediated proteins, which may contribute to clinical toxicity. Thus, numerous efforts have been focused on improving specificity by targeting the components upstream of the proteasome, especially substrate-specific E3 ubiquitin ligase.

Neddylation is a recently identified protein posttranslational modification pathway through which NEDD8 (neural precursor cell expressed, developmentally downregulated 8) is conjugated to target proteins for the regulation of protein functions. This process is catalyzed by a cascade of enzymes including NEDD8-activating enzyme (E1), NEDD8-conjugating enzyme (E2), and NEDD8 ligases (E3). The best known substrates of neddylation are a family of proteins called cullins, which are the essential components of multi-unit cullin-RING E3 ligase (CRL). CRL is the largest multi-unit ubiquitin ligase family and controls the turnover of numerous proteins with fundamental roles in physiological and pathological conditions including tumorigenesis and progression. Recently, several other cancer-related proteins (e.g., oncogenic mouse double minute 2 homolog (Mdm2), Hu antigen R (HUR), Smurf1, tumor suppressive p53, and TGF-β receptor II (TβRII)) have also been shown to be neddylated, highlighting the pivotal role of neddylation in carcinogenesis and progression. While the neddylation pathway was reported to be overactivated and contributed to the progression of several malignant human solid tumors including lung cancer, intrahepatic cholangiocarcinoma, and colon cancer, its role in the progression of brain tumors has not been well characterized. Recently, MLN4924, a small molecule inhibitor of Ned8-activating enzyme (NAE) was discovered via high-throughput screening. Structurally, MLN4924 inhibits NAE activity by binding to NAE at its active site to form a covalent Nedd8-MLN4924 adduct that is unable to move forward to subsequent intrasubstrate reactions and thus blocks protein neddylation. MLN4924 has proven significant anticancer efficacy as a single agent or in combination of chemotherapies. Because of its robust pharmacological efficacy and well-tolerated toxicity in preclinical studies, MLN4924 has been recently stepped up into several phase I clinical trials. Mechanistically, MLN4924 inhibits cullin neddylation, which is required for the activation of CRL. The inhibition of CRL, therefore, leads to the accumulation of tumor-suppressive CRL substrates to induce growth suppression and cell death. While the efficacy of MLN4924 has been evaluated in many types of human cancer, the therapeutic potential of this investigational NAE inhibitor has not been well addressed in GBM.

In this study, we reveal for the first time that the status of whole neddylation pathway is overactivated in GBM and further demonstrate the efficacy of MLN4924 in vitro and in vivo. Our findings highlight a critical role of the neddylation pathway in the development of GBM, which can serve as a potential therapeutic target for the treatment of GBM.

Materials and Methods

Cell Lines, Cultures, and Reagents

Human GBM cell lines U251and A172 cells were obtained from the American Type Culture Collection (ATCC), and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, HyClone) containing 10% fetal bovine serum (Biochrom AG) and 1% penicillin-streptomycin solution at 37°C with 5% CO2. Two 26S proteasome inhibitors, MG132 and bortezomib, were dissolved in dimethyl sulfoxide (DMSO) and kept at −20°C before use. MLN4924 was synthesized and used for in vitro and in vivo studies as previously described.

Cell Proliferation Assay and Cell Counting Assay

For the cell proliferation assay, cells were seeded in 96-well plates with 4000 cells per well in triplicate, cultured for 24 hours, and treated with DMSO or MLN4924 at various concentrations for 72 hours. Cell viability was determined by using the APTite kit (PerkinElmer) or Cell Counting Kit-8 (CCK-8, Beyotime) according to the manufacturer’s instructions. For cell counting assay, cells were seeded into 24-well plates with 2 × 104 cells per well, treated with DMSO or MLN4924 (0.1 μM), and counted with Cellometer Auto T4 (Nexcelom Bioscience) at indicated time points.

Cell Clonogenic Assay

Cells were seeded into 6-well plates in triplicate (300 cells per well). Twenty-four hours later, the old culture medium was replaced with fresh medium in the presence or absence of MLN4924, followed by incubation at 37°C for 12 days. The colonies on the plates were fixed with 4% paraformaldehyde and stained with crystal violet. Colonies with more than 50 cells were counted.

Transwell Migration Assay

A standard transwell migration assay using a transwell polycarbonate filter (8 μm pore size) (Corning Inc.) was performed to analyze the efficacy of MLN4924 on cell migration. Briefly, cells were pretreated with DMSO or MLN4924 at indicated concentrations, resuspended with serum-free DMEM, and adjusted to appropriate density. The suspension was added into the upper chambers with 100 μL per chamber. DMEM containing 20% fetal bovine serum was added into the lower chambers at 104 cells per chamber. The cells were cultured for 16 hours. Cells that had migrated to the underside of the upper chambers were stained with 0.1% crystal violet for 30 minutes. Cells that passed through the polycarbonate membrane of the transwells were counted under a Leica microscope.

Propidium Iodide Staining and Fluorescence-activated Cell Sorting Analysis

Cells treated with DMSO or MLN4924 (0.3 μM) were harvested and fixed in 70% ethanol at −20°C overnight, stained with
propidium iodide (PI, 36 μg/mL, Sigma) containing RNase A (10 μg/mL, Sigma), at 37°C for 30 minutes, and then analyzed for cell-cycle profile by CyAn ADP (Beckman Coulter). Data were analyzed with ModFit LT software (Verity).24

AnnexinV-Fluorescein Isothiocyanate and PI Staining and Fluorescence-activated Cell Sorting Analysis
Cells treated with DMSO or MLN4924 (0.3 μM) were collected and stained with AnnexinV- fluorescein isothiocyanate (FITC) and PI using an Annexin V-FITC Apoptosis Detection Kit (Beckman Coulter) followed by flow cytometric analysis. Data were analyzed with Summit software (Verity).38

Immunoblot Analysis
Cells lysates were prepared for immunoblot (IB) analysis using antibodies against cullin1(Santa Cruz Biotechnology), total H2AX(t-H2AX), UBC12, UBA3, (Epitomics, Inc.), p-1Kbx(Ser32), p-H2AX(Ser139), p21, p27, CD11, ORC1, cleaved Caspase-3(c-Caspase3), cleaved PARP(c-PARP), WEE1, p-Histone H3(Ser10)(p-H3), NEDD8 (Cell Signaling Technology), NAE1, GAPDH (Sigma), and β-actin (CW810).24

SA-β-Galactosidase Staining
U251 cells were plated into a 60 mm dish at the appropriate density, cultured overnight, and treated with DMSO or MLN4924 (0.3 μM) for 96 hours. The expression of senescence-associated β-galactosidase (SA-β-Gal) was determined using SA-β-galactosidase staining kit (Beyotime) according to the manufacturer’s protocol.25

Immunohistochemical Staining of Human Glioma Tissues
Formalin-fixed and paraffin-embedded (FFPE) tissues from different grade gliomas were provided by the Department of Neurosurgery at Huashan Hospital, Shanghai, China (WHO grade I, n = 15; grade II, n = 14; grade III, n = 27; grade IV, n = 69; 10 pairs of primary and recurrent GBM). The procedure of immunohistochemical (IHC) staining was performed as previously described.39 In brief, after deparaffinization, antigen retrieval with pH 6.0 citrate buffers and microwave, the sections were then incubated with appropriate goat anti-human NEDD8 antibody (1:200, Cell Signaling Technology). Secondary antibodies, 3,3-diaminobenzidine, and hydrogen peroxide chromogen substrate (DAKO) were processed according to the manufacturer’s instructions. The sections were finally counterstained with hematoxylin and mounted. Immunoreactive score (IRS) was applied as described with IRS > 6 considered as high.40 Most patients received radiotherapy and chemotherapy with temozolomide. Clinicopathological parameters according to the expression of NEDD8 are shown in Supplementary materials (Supplementary Fig. S1). The study was approved by the Huashan Hospital Ethics Committee, and informed consent was obtained from each patient under institutional review board protocols.

Antitumor Effect of MLN4924 in Vivo
The orthotopic xenograft model of GBM was established by AntiCancer Biotech (Beijing, China) as described previously.51 Briefly, U251-RFP human GBM tissue, which had been previously grown subcutaneously in nude mice, was harvested and carefully inspected to remove necrotic tissue. The harvested tumor tissue was then equally divided into small pieces of 1 mm3 each. A single 1 mm piece of the above tumor tissue fragments was inserted into the incision on the cranium of each mouse. The tumor-bearing mice were randomized into 2 groups of 10, and each was treated with 10% Hydroxypropyl beta cyclodextrin (HPBDC) or MLN4924 (60 mg/kg, s.c.) twice a day, respectively, on a 7 days-on/2 days-off schedule for 4 cycles.16

The size of tumors was measured by a whole-body fluorescence-imaging system twice a week as described.42 Briefly, whole-body imaging of tumor-bearing animals was conducted with an Olympus OV100 imaging system with 470 nm excitation light originating from an MT-20 light source. Emitted fluorescence was collected through appropriate filters, configured on a filter wheel with a DP70 CCD camera, and processed for contrast and brightness with Point Shop Pro 8 (Corel). At the end of the study, tumor tissues of mice were collected, photographed, and weighed. Animal studies were performed in accordance with animal protocol procedures and were approved by the AntiCancer institutional review committee.

Statistical Analysis
All data are represented as the mean ± SD. The statistical significance of differences between groups was assessed using GraphPad Prism5 software with the Student t test or 1-way ANOVA. The unpaired 2-tailed t test was used for comparing parameters between groups. Survival analysis was done using the Kaplan-Meier method and compared using the log-rank test. Three levels of significance (*P < .05, **P < .01, and ***P < .001) were used for all tests.

Results
Neddylation Pathway Is Overactivated and Correlated with the Progression of Gliomas
To evaluate the state of the neddylation pathway in GBM, we firstly determined the protein expression levels of NAE1, UBA3 (subunits of the E1 heterodimer) and UBC12 (E2) in GBM tumor tissues versus adjacent tissues (the peritumoral edema tissue) by IB analysis (Fig. 1A and Supplementary Fig. S3). The absence of tumor cells in the peritumoral edema tissue was confirmed by hematoxylin-and-eosin staining (Supplementary Fig. S2). We found that these neddylation enzymes were overexpressed in the majority of tumor tissues compared with adjacent tissues. Quantitative results showed that 72.2% of the tumor samples had a higher expression of NAE1 than adjacent tissues samples, whereas 78% of the GBM had a higher expression of UBC12 and UBA3. The overexpression of these neddylation enzymes suggests the neddylation pathway may play an important role in the development and progression of GBM.

Next, we determined the level of global protein neddylation in gliomas by IHC staining on FFPE samples of different grade gliomas using a specific NEDD8 antibody that recognizes NEDD8-conjugated proteins. We found that NEDD8 was strongly expressed in high-grade gliomas (WHO grades III and IV) and was gradually, weakly expressed in low-grade gliomas (WHO grades I and II) (Fig. 1B and C).
NEDD8 expression was further examined in 10 pairs of primary and recurrent GBM samples, and we found that NEDD8 was significantly upregulated in recurrent GBM (Fig. 1D, \(P = .0043\)). These data demonstrated that NEDD8 was gradually upregulated during glioma progression, indicating the activation of neddylation. Finally, we analyzed the relationship between global protein neddylation and patient survival and found that the overall survival of GBM patients with high NEDD8 expression (IRS > 6) was significantly shorter than those with low NEDD8 expression (log-rank \(P = .0273\), Fig. 1E).

The mean survival time of all GBM patients was 15 months, 16 months in the NEDD8 low-expression group (IRS \(\leq\) 6, \(n = 32\)), and 13 months in the NEDD8 high-expression group (IRS > 6, \(n = 37\)). Factors such as age, sex, and MGMT expression between these 2 groups were balanced, and the survival benefit of low NEDD8 may come from the differences in the KPS of the 2 groups (Supplementary Fig. S1).

MLN4924 Specifically Inhibits Neddylation and Suppresses the Growth of GBM Cells

The overactivated neddylation pathway in GBM indicated it to be a potential therapeutic target. To evaluate the efficacy of MLN4924 in GBM cells, we determined the specificity of MLN4924 for inhibition of the neddylation pathway when compared with pan-proteasome inhibitors MG132 and bortezomib in U251 and A172 cells. We found that MLN4924, but neither MG132 nor bortezomib, specifically suppressed global protein neddylation and cullin neddylation (Fig. 2A), indicating that MLN4924 specifically blocked protein neddylation in GBM cells.
Fig. 2. MLN4924 specifically inhibits neddylation and suppresses the growth of GBM cells. (A) Specificity of the NAE inhibitor MLN4924 in inhibiting the neddylation pathway and suppressing CRL activity when compared with pan-proteasome inhibitors MG132 and bortezomib. U251 and A172 cells were treated with DMSO, MLN4924 (1 μM), MG132 (20 μM), and bortezomib (1 μM) for 1 hour, followed by immunoblot analysis to determine the change of global neddylation (top panel) and cullin1 neddylation with β-actin as a loading control (bottom panel). (B and C) U251 and A172 cells seeded into 96-well plates were cultured overnight and treated with MLN4924 at various concentrations for 72 hours to determine its therapeutic efficacy on cell proliferation followed by ATPlite and CCK8 cell viability assays, respectively (n = 3) (B). U251 and A172 cells seeded into 24-well plates were cultured overnight and treated with DMSO or MLN4924 (0.3 μM) for the indicated time points, followed by cell counting (n = 3) (C). (D and E) U251 and A172 cells were seeded into 6-well plates in duplicate and then grown in the presence or absence of MLN4924 for 12 days. The colonies with more than 50 cells were counted, following crystal violet staining (D). Transwell migration assay was performed to analyze the efficacy of MLN4924 on cell migration as described in the Methods section (E). *, P < .05; **, P < .01; ***, P < .001.
To determine the effects of MLN4924 on the proliferation of GBM cells, U251 and A172 cells were then treated with MLN4924 at indicated doses for 72 hours and subjected to ATPlite and CCK-8 cell viability assays. As shown in Fig. 2B, MLN4924 significantly inhibited cell proliferation in a dose-dependent manner in both cell lines. A time-course study (0–120 h) performed by cell-counting analysis confirmed that MLN4924 dramatically inhibited the proliferation of GBM cells (Fig. 2C). Moreover, cell clonogenic survival (Fig. 2D) and transwell cell migration (Fig. 2E and Supplementary Fig. S4) were both notably inhibited by MLN4924 in a dose-dependent manner. These findings demonstrated that the inhibition of the neddylation pathway with MLN4924 significantly suppresses the growth of GBM cells and therefore serves as an attractive anticancer target.

**MLN4924 Induces the Accumulation of CRL Substrates and Induces G2 Cell-cycle Arrest in GBM Cells**

To address the mechanism of the growth suppression of GBM cells by MLN4924, we detected the expression of a panel of CRL substrates in treated cells. As shown in Fig. 3A, cullin-1 neddylation was completely blocked by MLN4924, indicating the inactivation of CRL. As a result, p21 and p27 (2 well-known CRL substrates) were accumulated upon treatment. Meanwhile, NF-κB signaling inhibitor plxβα was also accumulated during MLN4924 treatment, indicating the inhibition of NF-κB signaling. Moreover, MLN4924 also induced the accumulation of DNA replication-licensing proteins CDT1 and ORC1 that initiated the upregulation of phosphorylated H2AX, a classical DNA damage marker. These findings indicated that accumulation of cell-cycle inhibitors, inactivation of NF-κB signaling, and induction of DNA damage resulting from neddylation inhibition may contribute to the suppression of cell proliferation after MLN4924 treatment.

Fluorescence-activated cell sorting analysis revealed that MLN4924 notably triggered G2/M cell-cycle arrest in GBM cells (Fig. 3B). To determine at which phase (G2 vs M) of the cell cycle the MLN4924-treated cells were arrested, we detected the expression of WEE1 (a well-defined CRL substrate and an inhibitor of G2-M phase transition) and p-H3 (a mitotic marker of M phase cells). As shown in Fig. 3C, MLN4924 induced significant accumulation of WEE1, whereas it decreased the expression of p-H3 sharply, indicating that G2 cell-cycle arrest was induced by MLN4924.

**MLN4924 Induces Senescence or Apoptosis in a Cell Line-dependent Manner**

During the investigation of mechanistic basis for the inhibitory effect of MLN4924, we observed that MLN4924 induced an enlarged and flattened cellular shape in U251 cells (Fig. 4A, top panel), which suggested that the cells underwent senescence. To validate the phenomenon, senescence-associated β-galactosidase (SA-β-gal; a classical biochemical marker of senescence) was determined by SA-β-gal staining in these cells. As expected, a substantial fraction of MLN4924-treated U251 cells were positively stained (Fig. 4A, bottom panel), which demonstrated that MLN4924 triggered senescence in U251 cells. In contrast, A172 cells displayed apoptosis-like morphology with cell shrinkage (Fig. 4B) upon MLN4924 treatment, which was further validated by apoptosis analysis with AnnexinV/PI staining (Fig. 4C). Moreover, MLN4924 treatment induced the expression of cleaved-Caspase3 and cleaved-PARP (2 classical biochemical markers of apoptotic induction) in apoptotic A172 but not in senescent U251 cells (Fig. 4D). These findings demonstrated that neddylation inhibition by MLN4924 suppressed the growth of GBM cells by inducing senescence or apoptosis in a cell context-dependent manner.

**MLN4924 Suppresses the Growth of Orthotopic Xenograft in Human GBM**

To evaluate the antitumor effect of MLN4924 in vivo, the physiologically relevant orthotopic xenograft model of U251-RFP GBM was established and treated with MLN4924. The kinetic growth of tumors was consecutively monitored via a fluorescence-based imaging system. Representative kinetic images of tumors showed that MLN4924-treated tumors grew slowly, whereas control tumors grew rapidly (Fig. 5Aa and B). At the end of treatment, tumors were collected, photographed, and weighed. As shown in Fig. 5C, the size of MLN4924-treated tumors was remarkably smaller than that of control tumors, and the tumor weight of treated mice was significantly lower than that of control mice (P < .001). During the entire treatment, no obvious adverse effects were observed in the tumor-bearing mice. The dose of MLN4924 was well tolerated, with an average weight loss <15% (data not shown) for all dose groups at the end of treatment. These findings demonstrated the in vivo efficacy of MLN4924 on GBM.

To explore the in vivo mechanisms of MLN4924, we performed IHC analysis to determine the expression of Ki67, a classical cell proliferation marker, and p21, a well-known CRL substrate and tumor suppressor. As shown in Fig. 5D, Ki67 was significantly decreased, while p21 was increased after MLN4924 treatment. Meanwhile, we extracted proteins from treated and control tumors to determine the protein expression levels of NAE1, UBA3, UBC12, and the global protein neddylation. As shown in Fig. 5E, MLN4924 significantly inhibited the global protein neddylation, and a sharp decrease of NAE1 was observed after treatment. Taken together, these findings demonstrate a significant anticancer efficacy of MLN4924 in vivo.

**Discussion**

GBM is one of the most malignant tumors. Although much effort has been put into treating GBM, the prognosis is still poor. We and others have previously reported that the neddylation pathway was also overactivated in several other types of human cancer including lung cancer, intrahepatic cholangiocarcinoma, colon cancer, and liver cancer. In this study, we found that the neddylation pathway was overactivated and correlated with the malignancy of GBM tissues such as high-grade glioma, recurrence and poor patient survival. Specifically, analysis of the overall survival of GBM patients with NEDD8 expression (global protein neddylation) shows that NEDD8 expression is significantly correlated with a poor prognosis in GBM and highlights the vital role of NEDD8 in GBM. Further identification of NEDD8 expression in GBM subtypes by in-depth bioinformatic analysis might be rewarding.
Another important factor in neddylation cascade, UBC12/UBE2M, also shows correlation with prognosis (data not shown) according to the REMBRANDT database but needs further confirmation. These findings highlight the critical role of the neddylation pathway in regulating human GBM carcinogenesis.

Inhibition of protein neddylation by the NAE inhibitor MLN4924 has emerged as a promising anticancer agent.\(^{31,45,46}\) MLN4924 is currently in phase I clinical trials for several tumor types and hematological malignancies.\(^{31,32}\) In this study, we assessed and validated the efficacy of MLN4924 in GBM cells in vitro and in a physiologically relevant orthotopic xenograft of human GBM in vivo. It is well known that GBM treatment is blunted by the existence of the blood brain barrier, which may block the delivery of drugs into tumor tissues and therefore represent a main obstacle to traditional chemotherapeutic treatment of GBM.\(^{47}\) The advantages of MLN4924, such as its low molecular weight, high lipid solubility (http://integrity.thomson-pharma.com), and impressive efficacy on the clinically
relevant GBM model indicated the potential value of future clinical investigation of MLN4924 for the treatment of GBM.

We and others have previously demonstrated that MLN4924 inhibits NAE activity, abrogates cullin neddylation, and inactivates CRL, resulting in the accumulation of tumor-suppressive CRL substrates inducing DNA damage, cell-cycle arrest, apoptosis, or senescence in human cancer cells. In this study, we found that the antitumor mechanisms of MLN4924 in GBM generally concur with those observed in other types of cancer cells (Fig. 5F). Interestingly, GBM cells died of apoptosis or senescence in a cell context-dependent manner. We and others have demonstrated that senescence induced by MLN4924 was largely dependent on tumor suppressors such as p21 and p27 (2 classical CRL substrates that are accumulated in almost all tested cells upon neddylation inhibition), while the induction of apoptosis by MLN4924 might be attributed to the cell line-dependent induction of proapoptotic proteins such as Bik and Noxa. Further studies are required to address the mechanisms for cell-line dependent induction of these proapoptotic proteins that determine the cell fate (apoptosis vs senescence) upon neddylation inhibition by MLN4924.

Together, our studies provide a proof of evidence for future clinical investigation of MLN4924 in the treatment of human GBM. Moreover, the overactivation status of the neddylation pathway might serve as a potential index for the enrollment of appropriate patient populations for neddylation inhibitors in clinical trials. Finally, the rational combination of other antitumor agents, such as traditional chemoradiotherapy, may further improve the therapeutic effect of MLN4924 in GBM based on their independent antitumor activities.
Fig. 5. MLN4924 suppresses the growth of orthotopic xenograft of human glioma. (A and B) MLN4924 inhibited tumor growth measured by fluorescence imaging system. Nude mice bearing glioma xenografts with U251-RFP cells were administered MLN4924 at 60 mg/kg s.c. twice daily on a 7-days-on/2-days-off schedule for 4 cycles. Tumor size was monitored twice a week with fluorescence imaging system. Five out of 10 pictures are shown (A). Abbreviation: D, day). The data were converted to tumor growth curves by ModFit LT software, and the duration of treatment was visualized with arrows in Fig. 5B. *, P < .05; **, P < .01. (C) MLN4924 significantly reduced tumor volume. Mice were euthanized at day 35 after treatment (the end of study, n = 10). Tumor tissues of mice were collected, photographed, and weighed. P < .01. (D) ki67 and p21 staining of the glioma sections. (E) Immunoblotting analysis to determine the expression of NAE1, UBA3, UBC12 and the global protein neddylation in the treated and control tumors. (F) A working model. Inhibition of overactivated neddylation pathway with the NAE inhibitor MLN4924 blocks protein neddylation (especially cullin neddylation), inactivates CRL, results in the accumulation of tumor-suppressive CRL substrates, and induces cell-cycle arrest, senescence, or apoptosis to inhibit tumor cell growth.
on the findings that the combination of chemoradiotherapy could significantly enhance the efficacy of MLN4924 in several types of human cancers.29,30,49

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

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