Aurora-A inhibition offers a novel therapy effective against intracranial glioblastoma

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Abstract

Glioblastoma (GB) remains a devastating disease for which novel therapies are urgently needed. Here we report that the Aurora-A kinase inhibitor alisertib exhibits potent efficacy against GB neurosphere tumor stem-like cells in vitro and in vivo. Many GB neurosphere cells treated with alisertib for short periods undergo apoptosis, although, some regain proliferative activity upon drug removal. Extended treatment however results in complete and irreversible loss of tumor cell proliferation. Moreover, alisertib caused GB neurosphere cells to partially differentiate and enter senescence. These effects were also observed in glioma cells treated with the Aurora-A inhibitor TC-A2317 or anti-Aurora-A siRNA. Furthermore, alisertib extended median survival of mice bearing intracranial human GB neurosphere tumor xenografts. Alisertib exerted similar effects on GB neurosphere cells in vivo based on the presence of activated phosphoThr288Aurora-A, abnormal mitoses and increased cellular ploidy, consistent with on-target activity. Our results offer preclinical proof-of-concept for alisertib as a new therapeutic for glioma treatment.

Keywords

Aurora-A; alisertib; glioblastoma; neurospheres; orthotopic xenograft model

Introduction

Aurora-A (AURKA) is a serine/threonine kinase critical for centrosome duplication, spindle assembly and mitotic exit (1–3). Aurora-A also drives cell cycle progression by promoting cyclin B1, Wnt, myc and other pro-proliferative pathways (4–6). Aurora-A knock-down blocks cellular proliferation and may induce tetraploidy, apoptosis and senescence *in vitro* (7–9). Aurora-A kinase inhibitors have thus emerged as unique antiproliferative agents inhibiting mitosis and important signaling pathways.

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No conflicts of Interest

Alisertib (MLN8237) is a highly-selective Aurora-A kinase inhibitor in clinical trials for a variety of malignancies (10). Because novel approaches are urgently needed for the treatment of glioblastoma (GB), we tested alisertib against GB neurosphere tumor stem-like cells *in vitro* and *in vivo*.

Materials and Methods

Neurosphere Culture and Proliferation Assays

GB9, GB30 and GB169 neurosphere cells were derived from patient surgical samples at the Ohio State University Cancer Center under an IRB-approved protocol. Minced tissue was passed through 18- and 21-gauge needles to achieve single cell suspensions. Cells were cultured in DMEM/F12 containing N2 supplement (Invitrogen) and 20 ng/ml each of epidermal growth factor and basic fibroblast growth factor (R&D). Neurospheres were passaged by trituration with a 21-gauge needle. U87 cells obtained from the American Type Culture Collection were grown in DMEM/10% FCS. No additional authentication was performed on these cells.

The investigational Aurora-A inhibitor alisertib was provided by Takeda Pharmaceuticals. TC-A2317 (11) was from Tocris Bioscience. For growth curves, cells were plated at 10^4 cells/well in 24-well plates in the presence of drug or DMSO vehicle (0.0032 to 0.02%). Every 2 days 3 wells per condition were triturated and cells counted (Coulter model Z2). Metabolic activity was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide conversion to formazan (MTT assay). Cells were plated in 96-well plates at 10^4 cells/well in 100 μl of medium containing alisertib or DMSO. After 4 days at 37°C, 10 μl of MTT reagent (Roche) was added/well and plates processed per the manufacturer's instructions.

Apoptosis

Neurosphere cells were treated with 200 nM alisertib or DMSO and trypsinized at indicated intervals. One-half of the cells per 60 dish were pelleted and resuspended in 100 μl Annexin-V Binding Buffer (Invitrogen). Anti-Annexin-V-FITC (BD Pharmingen, 5 μl) was added, incubated at 25°C for 15 min, diluted to 500 μl with Binding Buffer and filtered through mesh. Binding was measured by flow cytometry (488 nm excitation).

β-galactosidase assay

Neurospheres were treated for 12 days with 200 nM alisertib or DMSO alone. Cytospins were prepared by centrifuging GB neurospheres onto charged glass slides (3 min at 800 rpm). Slides were fixed in 2% formaldehyde, 0.2% glutaraldehyde and stained for β-galactosidase activity using the Senescence β-Galactosidase Staining kit (Cell Signaling).

Western blotting

Cells were lysed in RIPA containing 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 20 mM β-glycerosphosphate and 1 mM sodium orthovanadate. SDS-PAGE and western blotting of PVDF membranes was performed using TBST and 4% non-fat dry milk. Primary antibodies against phosphoThr[288]Aurora-A, Aurora-A (Abcam ab58494, 1:500; & ab13824,

1:500), CD44, p21, p27, cleaved PARP, BimEL, neurofilament-L (Cell Signaling 3570, 1:2000; 2947,1:1000; 3686, 1:1000; 9541, 1:1000; 2933, 1:1000; & 2837, 1:1000), β-catenin (Life Technologies 18-0226, 1:1000), Olig2 (IBL 18953, 1:200), Aldh1L1 (NeuroMab 75-140, 1:500), Mushashi-1 (R&D Systems MAB2628, 1:500), Nestin (Millipore MAB5326, 1:1000), GFAP or β-actin (Santa Cruz sc-9065, 1:500; sc-47778, 1:10,000) were incubated overnight. HRP-conjugated secondary antibodies (Santa Cruz, 1:2000) were incubated for 1 h at 25°C. Membranes were developed with Pierce Super Signal West Pico and exposed to X-ray film.

RNA interference

U87 glioblastoma cells were transfected with 300 pmol Dharmacon ON-TARGET plus SMART pool anti-human AURKA siRNA or Qiagen Allstars negative control siRNA, using Oligofectamine and Opti-MEM-I (Life Technologies).

Mouse orthotopic xenograft model

All animal experiments were IACUC approved (2013A0051). Female athymic mice (nu/nu genotype, 6–8 weeks, 20–25 g weight, NCI strain 01B74) were anesthetized with intraperitoneal ketamine (100 mg/kg)/xylazine (20 mg/kg) and fitted into stereotactic frames (David Kopf Instruments). A small incision was made just lateral to the midline and a 1.0 mm burr hole was drilled at AP= +1 mm, ML= −2.5 mm from the bregma. Two microliters of a single neurosphere cell suspension (50,000 cells/μl) was deposited at 1 μl/min into the right caudate nucleus at a depth of −3 mm from the dura. The burr hole was filled with bone wax and the incision closed (4–0 vicryl suture).

Beginning 5 days after implantation, mice were orally gavaged with 20 mg/kg alisertib dissolved in 10% (2–hydroxypropyl)-β-cylcodextrin, 1% sodium bicarbonate or vehicle alone twice daily 5 days/week. Mice were monitored daily and sacrificed according to IACUC-approved methods and criteria for neurologic symptoms.

Histology and Immunohistochemistry

FFPE sections (4 μm) of mouse brain tissue were stained with hematoxylin and eosin (H&E). For immunohistochemistry, sections were deparaffinized, rehydrated, blocked with 3% H_2O_2 for 5 min at 25°C and treated with Target Retrieval Solution (Dako) for 25 min at 96°C. For anti-phosphoThr[288]Aurora-A, slides were further blocked with Protein Block Serum-Free (Dako) for 15 min at 25°C. Slides were incubated with anti-phosphoThr[288]Aurora-A (Abcam ab58494, 1:200) or anti-phosphohistone-H3 (Abcam ab5176, 1:1,400) for 1 hour at 25°C. Antibodies were detected using MACH 3 Rabbit HRP-Polymer (Biocare) and DAB (Dako). Slides were counterstained with hematoxylin.

Control and alisertib-treated neurosphere cytospins were stained with H&E or immunostained with anti-β-catenin (BD Pharmingen # 610153, 1:2,500). For Feulgen staining, GB9 cells were treated for 5 days with 200 nM alisertib or DMSO and cytospins prepared. Slides were stained using the Blue Feulgen DNA Ploidy Analysis Staining Kit (ScyTek) and scanned with an Aperio XT ScanScope. Image analysis was performed with

Definiens Tissue Studio 3.6 (51,480 control neurosphere nuclei and 15,864 alisertib-treated neurosphere nuclei were examined).

Results and Discussion

Effect of Aurora-A inhibition on growth and survival of glioma neurosphere cells

Glioblastoma neurosphere cells demonstrate tumor stem cell-like properties and are considered to closely mimic the biologic behavior of naturally occurring gliomas (12, 13). We performed growth curve analysis of GB neurosphere cells with and without small molecule Aurora-A inhibitors. Alisertib potently blocked proliferation of all 3 neurosphere lines tested (Fig. 1A). IC_{50} concentrations were 11, 24 and 32 nM for GB169, GB9 and GB30 cells, respectively. Another Aurora-A inhibitor, TC-A2317 (11) was less potent, demonstrating IC_{50} concentrations of 170, 179 and 168 nM for these cells, respectively (Fig. S1A). Observed alisertib IC_{50} concentrations are similar to slightly higher than those required to inhibit colony formation in 2 other GB neurosphere lines (14) and approximately 5 to 10-fold less than those effective against 10 conventional monolayer GB cell lines (15) suggesting greater efficacy against GB neurosphere cells.

We also examined the impact of alisertib on GB neurospheres using MTT assays. Although alisertib reduced the metabolic activity of these cells, the magnitude of response was less than that seen by cell counts (Fig. 1B). Short exposure to highly antiproliferative concentrations of alisertib, *e.g.*, 200 nM, reduced total cellular metabolism by approximately 28 to 33% (Fig. 1B), similar to the fraction of cells in which an equivalent alisertib exposure induces apoptosis (Fig.s 1B & 1C). This suggests that despite attenuated proliferation, some tumor cells remained metabolically active following treatment. Therefore, the antiproliferative effect of Aurora-A kinase inhibition cannot be due solely to immediate tumor stem-like cell killing.

To test if alisertib-induced toxicity was due to increased apoptosis, we measured annexin-V staining in alisertib-treated normal human astrocytes and GB cells. Alisertib did not induce significant annexin-V staining in human astrocytes (Fig. S1B). In contrast, alisertib-treated GB neurosphere cells showed increased annexin-V staining indicative of apoptosis, reaching approximately 85% after 12 days of drug exposure (Fig. 1C & Fig. S1C).

Since alisertib caused only a limited amount of apoptosis after 6 days of exposure and did not completely prevent metabolic activity in GB neurospheres, we investigated if neurosphere cells could resume proliferation following treatment. GB neurospheres were exposed to alisertib continuously for 3 to 12 days, the drug washed out, and cells cultured with or without alisertib for an additional 3 days. Cell counts were then obtained to measure GB cell proliferative potential following drug treatment (Fig. 1D & Supp Fig. 1C). In a similar experiment, recovery of metabolic activity following extended alisertib exposure was examined by MTT assay (Fig. S1D).

If alisertib-treated cells retained proliferative capacity, total cell counts should increase when further cultured in drug-free media. As shown in Figures 1D and S1C, after 3 days of alisertib treatment GB neurosphere cells allowed to recover in drug-free media showed

increased numbers compared to continually treated cells. The difference in tumor cell numbers between cultures continually treated for 6 days and those treated for 6 days and allowed to recover in drug-free media was negligble however, indicating nearly complete loss of tumor cell proliferative potential. A similar reduction of recoverable MTT activity took longer, suggesting total senescence and/or death of a small population of persisting cells (15 to 20%) required extended drug exposure, but eventually occurred (Fig. S1D).

Effect of Aurora-A inhibition on morphology, differentiation and senescence of GB neurosphere cells

Aurora-A inhibitors had a dramatic effect on GB cell morphology after 2 weeks of exposure. Neurospheres contained fewer, but larger cells (Fig. 2A & S2). More cells were attached to the culture dish and many exhibited prominent astrocyte-like processes. Furthermore, alisertib caused a strong increase in β-galactosidase staining (Fig. 2B).

H&E-stained cytospins confirmed that individual alisertib-treated GB neurosphere cells were greatly enlarged (Fig. 2C). Quantitation of nuclear DNA by image analysis of Feulgen-stained (16) cytospins revealed that tumor nuclei mean area increased from 98.7 μm^2 in control cells to 416 μm^2 in alisertib-treated cells indicating failed cytokinesis and tetraploidy (Fig. 2D).

Aurora-A kinase activity depends on activation by autophosphorylation at threonine 288 (17). Western blots for phosphoThr^{288}Aurora-A show that phosphoThr^{288}Aurora-A is markedly decreased in alisertib- and TC-A2317-treated GB neurospheres verifying drug target-hit (Fig.s 3A, 3B & S3A).

To look for biochemical evidence of apoptosis, differentiation and senescence of GB neurosphere cells in response to Aurora-A inhibitors, we immunoblotted for proteins indicative of these processes. Consistent with Annexin-V staining (Fig. 1C), BimEL, a proapoptotic BH3-only protein negatively regulated by Aurora-A (18) and/or cleaved PARP were consistently elevated in alisertib- or TC-A2317-treated cells (Fig.s 3A, 3B & S3A).

Olig2, a transcription factor expressed in normal and neoplastic astrocytes and oligodendrocytes, was increased in all GB neurosphere lines treated with alisertib or TC-A2317 (Fig.s 3A, 3B & S3A). GFAP and Aldh1L1, markers of astrocytic differentiation (19), were also increased in response to alisertib (Fig.s 3A and 3B). Unlike neurosphere tumor stem-like cells, U87 glioma cells expressed high basal levels of GFAP however alisertib treatment prevented the decreased GFAP expression seen in U87 cells approaching confluence (Fig 3A). In agreement with observed elevated β-galactosidase activity (Fig. 2B), expression of the cellular senescence associated cyclin-dependent kinase inhibitor p21 (20) was strongly increased by alisertib (Fig. 3A & 3B) and TC-A2317 (Fig. S3A).

Western analysis for markers of stemness revealed that Musashi-1, an RNA-binding protein involved in stem cell self-renewal (21), the cancer stem cell marker CD44 (22) and β-catenin were all markedly decreased in alisertib-treated GB neurosphere cells (Fig.s 3A, 3B & S3B). β-catenin was also decreased by TC-A2317 (Fig. S3A). As β-catenin is required for CNS

progenitor self-renewal (23), these results may correlate with a decreased capacity for GB neurosphere cell self-renewal following Aurora-A inhibition.

The neural stem cell marker nestin was also decreased by alisertib in GB9 and GB30 cells (Fig. 3B). In contrast, nestin was increased after alisertib treatment in GB169 cells. Not all GB stem-like cells express specific stem and differentiation markers equally (12). Moreover, nestin expression is not exclusive to neural stem cells but is also found in regenerating neurons and glia (24), as well as many CNS neoplasms. Unlike in GB9 and GB30 cells, alisertib also increased neurofilament protein expression in GB169 cells (Fig. 3B) possibly correlating with increased nestin and induction of an early neuronal differentiation program in these cells. Thus apoptosis, differentiation, senescence and/or loss of neurosphere tumor cell stemness are likely factors contributing to the antiproliferative effect of Aurora-A inhibition in GB cells *in vitro*.

To investigate whether the above observed biochemical effects of alisertib on GB cells were Aurora-A specific, we transfected U87 glioma cells with anti-Aurora-A siRNAs. Aurora-A knockdown resulted in biochemical changes similar to those caused by alisertib including reduced CD44 and increased expression of cleaved PARP, GFAP and the senescence-associated cyclin-dependent kinase inhibitor p27 (Fig. 3C). Thus, the response of GB neurosphere cells to Aurora-A kinase inhibition or mRNA knockdown includes apoptosis, differentiation and senescence. Additionally, Aurora-A itself is involved in maintaining embryonic stem cells in the undifferentiated state (25). Thus, another mechanism in which Aurora-A inhibition may be effective against glioma cells could be by inducing a loss of stemness. This may be an important mechanism possibly leading to sensitization of gliomas to other therapies or inhibiting the emergence of therapy-resistant tumor cell clones.

Effect of alisertib on GB neurosphere orthotopic xenograft growth in vivo

To determine whether alisertib was effective against glioblastoma *in vivo* we treated nude mice containing GB30 and GB169 intracranial xenografts with the drug. As shown in Figure 4A, alisertib significantly extended the survival of mice with intracranial xenografts of both GB neurosphere lines. Mice with GB169 xenografts showed a 25.2% increase in median survival (87 days for alisertib-treated compared to 69.5 days for vehicle-treated, $p = 0.025$), while survival was increased by 33.3% in mice with GB30 xenografts (26 days for alisertib-treated compared to 19.5 days for vehicle-treated, $p < 0.0001$).

Examination of GB169 xenograft tumors from alisertib-treated mice revealed similar effects to those observed *in vitro*. As shown in Figure 4B, glioblastomas from alisertib-treated mice demonstrated enlarged nuclei and greatly decreased phosphoThr[288]Aurora-A compared to control mice (Fig. 4C). Furthermore, phosphohistone-H3 immunostaining revealed large numbers of abnormal arrested mitoses as previously described for Aurora-A inhibition (26), whereas mitoses in tumors from control mice more often resembled normal metaphase or telophase (Fig. 4D). These observations confirm that alisertib exhibited target-specific effects on GB cells *in vivo*.

In summary, the Aurora-A inhibitor alisertib potently inhibits proliferation of GB tumor neurosphere stem-like cells *in vitro* and demonstrates Aurora-A-specific target hit effects *in*

vitro and *in vivo*. It also significantly extends the survival of mice with intracranial GB neurosphere xenografts. Alisertib is therefore a promising new potential agent for the treatment of glioblastoma alone or in combination with other therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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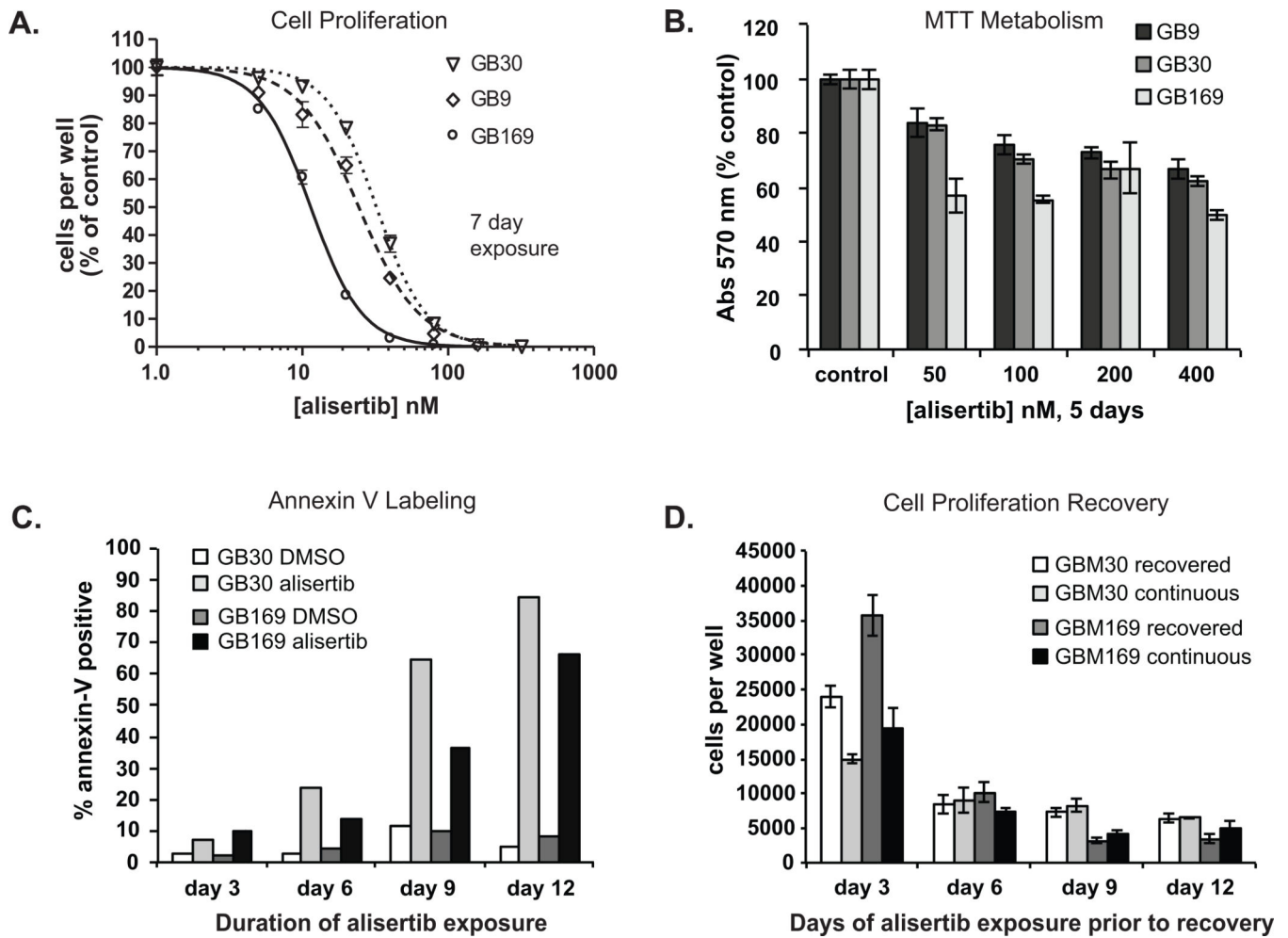
Figure 1. Effect of alisertib on proliferation and viability of glioblastoma neurosphere cells
A. GB neurospheres were cultured 7 days with various concentrations of alisertib or DMSO and counted. Means ± SD of triplicate wells are depicted. **B.** Neurospheres were treated for 5 days with alisertib or DMSO and MTT assays performed. Data are means ± SD of triplicate wells. Three experiments provided similar results. All alisertib treatments were significantly different from controls, $p > 0.05$. **C.** GB neurospheres were treated with 200 nM alisertib for 3 to 12 days followed by Annexin V immunostaining. **D.** GB neurosphere cells were plated in 60 mm dishes (5×10^5/dish) and treated with 200 nM alisertib or DMSO (neurospheres in DMSO treatments were dispersed every 3 days to prevent overgrowth). Every 3 days one-half of the cells per time point dish were pelleted, washed with PBS and recultured in 24 well plates with 200 nM alisertib (continuous) or DMSO (recovered) for an additional 3 days and counted.
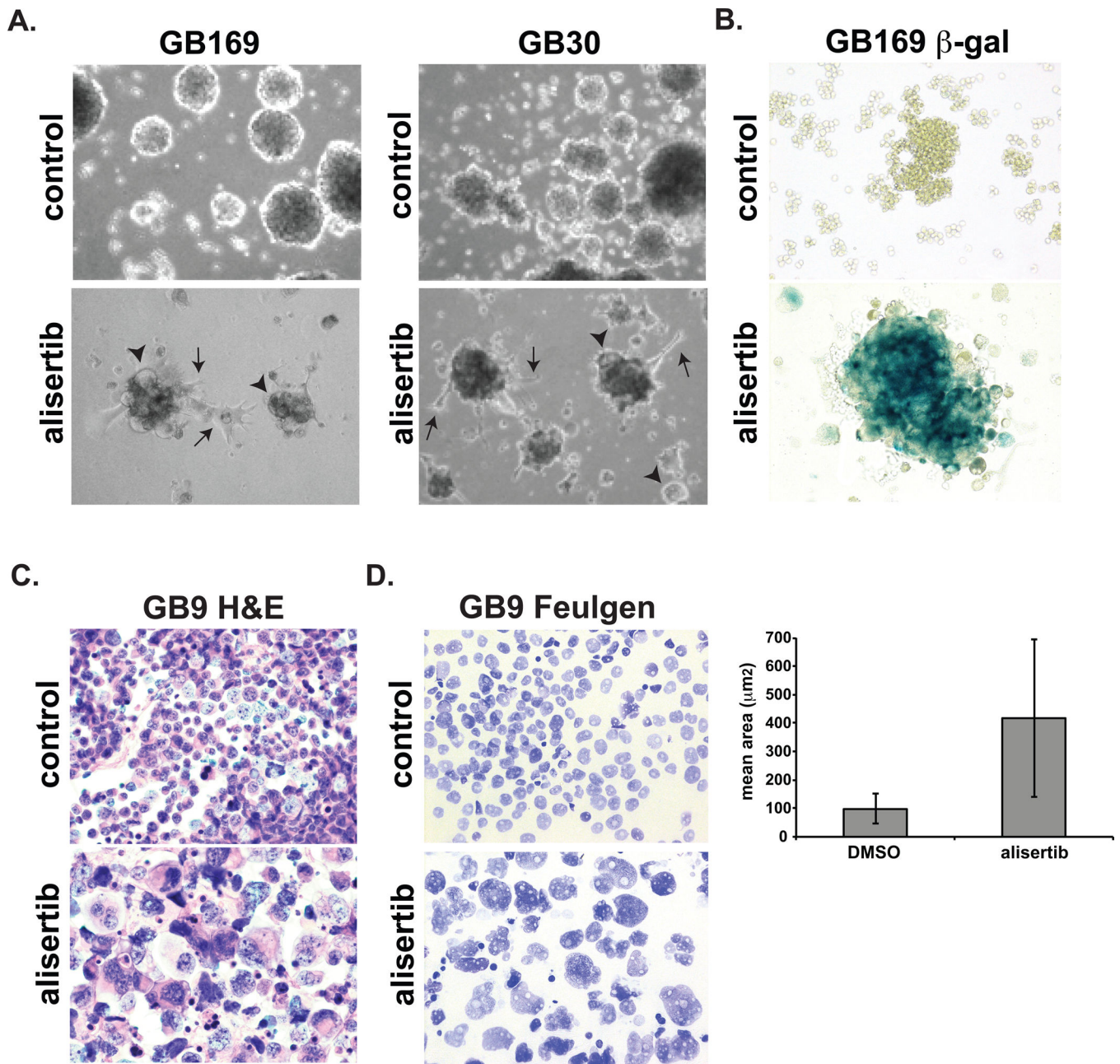
**Figure 2. Morphology of GB neurosphere cells treated with alisertib**
A. Glioblastoma neurospheres were treated with DMSO or 200 nM alisertib for 2 weeks and photographed. Many cells in alisertib-treated cultures exhibited cytomegaly (arrowheads) or astrocyte-like process (arrows). **B.** Alisertib-treated and control GB neurosphere cytospins were stained for β-galactosidase activity, **C.** H&E or **D**. Feulgen for DNA content. The histogram depicts mean area ± SD of Feulgen stained nuclei determined by image analysis. Magnifications: C–D, 400×.
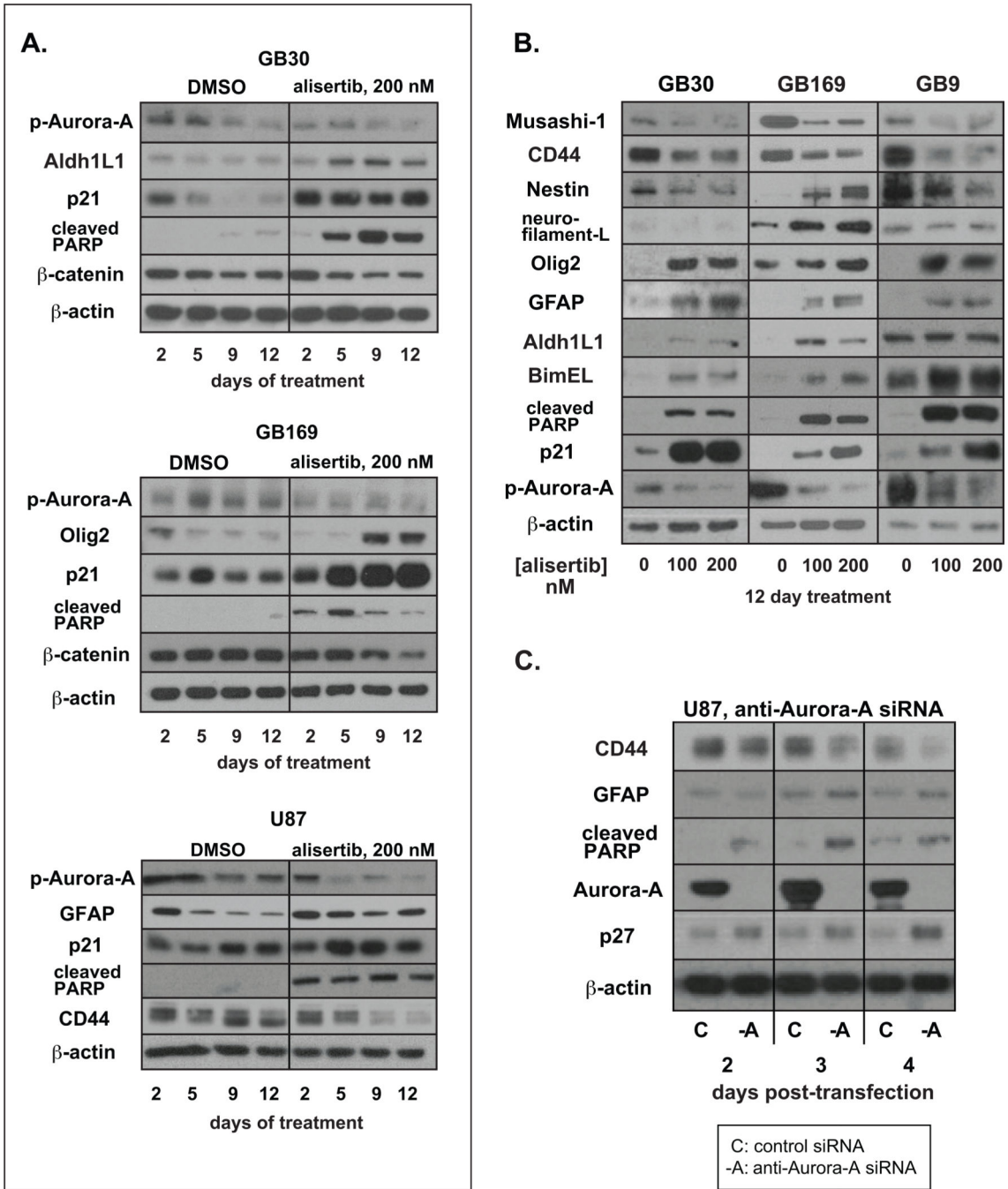
Figure 3. Effect of alisertib on senescence, apoptosis and stem markers in GB neurospheres
A. Glioblastoma cells were treated with 200 nM alisertib or DMSO for the indicated times and lysates immunoblotted. **B.** Neurospheres were treated with DMSO or 100 or 200 nM alisertib for 12 days and lysates immunoblotted. **C.** U87 cells were transfected with control or anti-Aurora-A siRNAs. Lysates were prepared 2, 3 and 4 days post-transfection.
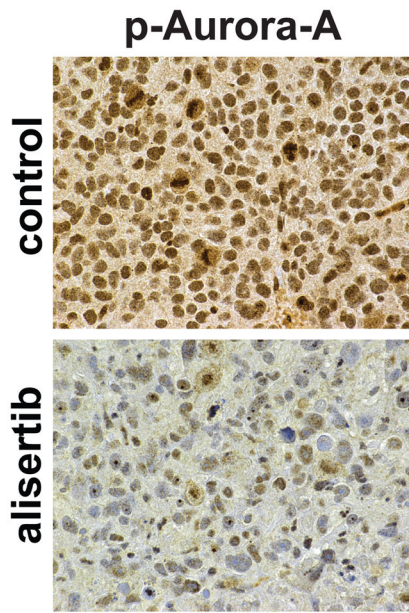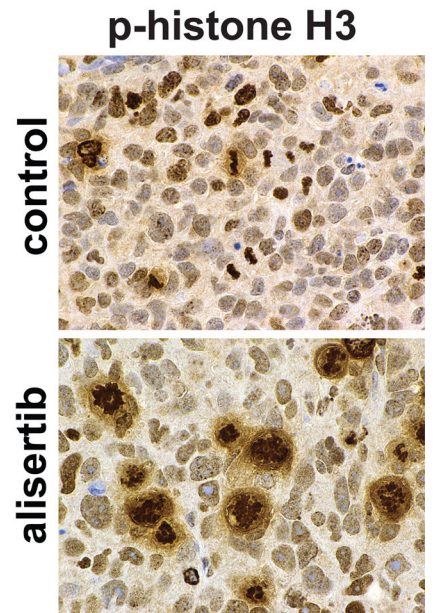
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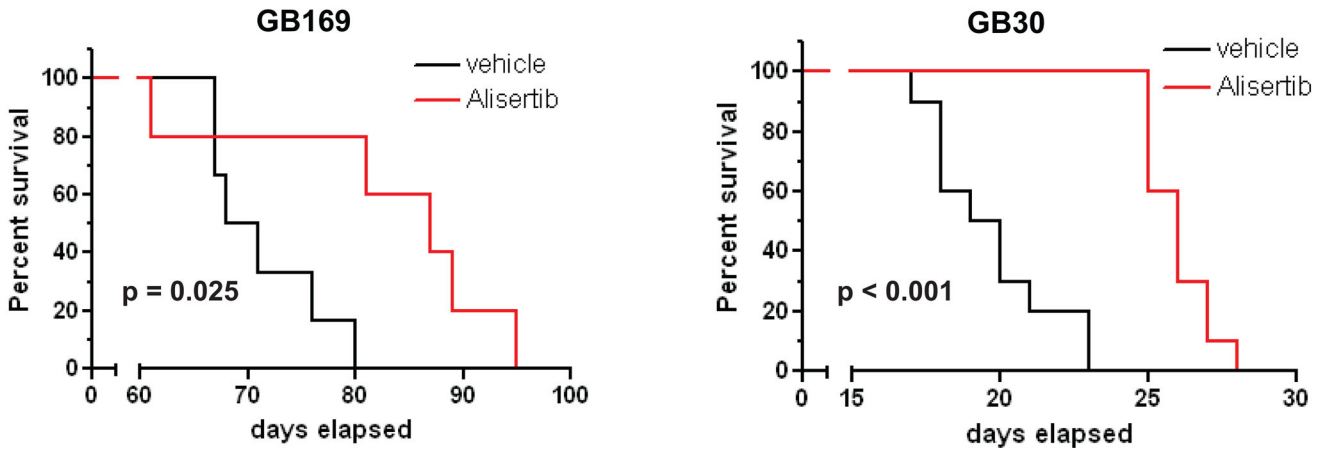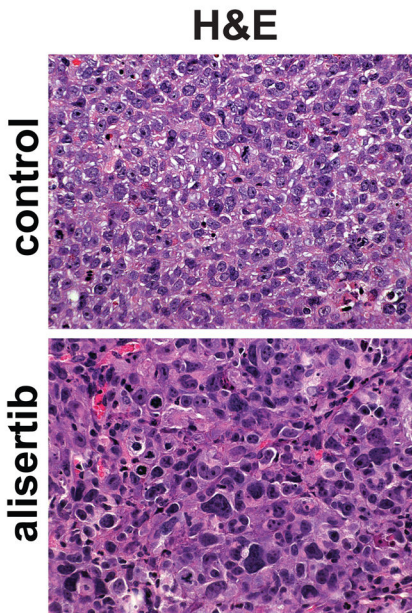
B.   **C.**   **D.**

Figure 4.
A. Alisertib extends survival of mice with GB neurosphere intracranial xenografts.
Kaplan-Meier curves for mice with GB169 or GB30 xenografts treated with 20 mg/kg
alisertib twice daily or vehicle alone are shown. **B.–D. Effects of alisertib on GB
neurosphere cells *in vivo*.** Mice with GB169 xenografts were given vehicle or alisertib (20
mg/kg twice daily) for 2 weeks and sacrificed. FFPE tumor sections were stained for H&E

(**B**) phosphoThr^{288}Aurora-A (**C**) or phosphohistone-H3 (**D**). Magnifications: B–C, 400×; D, 600×.