Aurora-A Inhibition Offers a Novel Therapy Effective against Intracranial Glioblastoma

James R. Van Brocklyn\textsuperscript{1}, Jeffrey Wojton\textsuperscript{2}, Walter H. Meisen\textsuperscript{2}, David A. Kellough\textsuperscript{1}, Jeffery A. Ecsedy\textsuperscript{3}, Balveen Kaur\textsuperscript{2,4}, and Norman L. Lehman\textsuperscript{1,4}

Abstract

Glioblastoma 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 glioblastoma neurosphere tumor stem–like cells \textit{in vitro} and \textit{in vivo}. Many glioblastoma 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 glioblastoma 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 glioblastoma neurosphere tumor xenografts. Alisertib exerted similar effects on glioblastoma neurosphere cells \textit{in vivo} and resulted in markedly reduced activated phosphoThr288Aurora-A and increased abnormal mitoses and cellular ploidy, consistent with on-target activity. Our results offer preclinical proof-of-concept for alisertib as a new therapeutic for glioma treatment. Cancer Res; 74(19); 5364–70. ©2014 AACR.

Introduction

Aurora-A 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 knockdown blocks cellular proliferation and may induce tetraploidy, apoptosis, and senescence \textit{in vitro} (7–9). Aurora-A kinase inhibitors have, thus, emerged as unique antiproliferative agents inhibiting mitosis and important signaling pathways.

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, we tested alisertib against glioblastoma neurosphere tumor stem–like cells \textit{in vitro} and \textit{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 (Columbus, OH) under an Institutional Review Board–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 (Invitro-21) and 20 ng/mL each of epidermal growth factor and basic fibroblast growth factor (R&D Systems). 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 per well in 24-well plates in the presence of drug or DMSO vehicle (0.0032% DMSO and trypsinized at indicated intervals. One-half of the culture was added to the plates and processed per the manufacturer’s instructions.

Apoptosis

Neurosphere cells were treated with 200 nmol/L alisertib or DMSO and trypsinized at indicated intervals. One-half of the cells per 60-mm 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 minutes, 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 nmol/L alisertib or DMSO alone. Cytospins were prepared by centrifuging glioblastoma neurospheres onto charged glass slides (3 minutes 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 Technology).

Western blotting
Cells were lysed in RIPA containing 1 mmol/L phenylmethylsulfonylfluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 20 mmol/L β-glycerophosphate, and 1 mmol/L sodium orthovanadate. SDS-PAGE and Western blotting of PVDF membranes were performed using TBST and 4% non-fat dry milk. Primary antibodies against phosphoThr208-Aurora-A, Aurora-A (Abcam ab58949, 1:500; and ab13824, 1:10,000), CD44, p21, p27, Nestin (Millipore MAB5326; 1:1,000), GFAP or Nestin (Millipore MAB5326; 1:1,000), Olig2 (IBL 18953; 1:200), Aldh1L1 (NeuroMab 18-0226; 1:1,000), Mushashi-1 (R&D Systems MAB2628; 1:500), and β-catenin (Life Technologies 18-0226; 1:1,000), Olig2 (IBL 18953; 1:200), Aldh1LI (NeuroMab 75-140; 1:500), Mushashi-1 (R&D Systems MAB2628; 1:500), Nestin (Millipore MAB5326; 1:1,000), GFAP or β-actin (Santa Cruz Biotechnology sc-9065, 1:500; sc-47778, 1:10,000) were incubated overnight. Horseradish peroxidase (HRP)–conjugated secondary antibodies (Santa Cruz Biotechnology, 1:2000) were incubated for 1 hour at 25°C. Membranes were developed with Pierce Super Signal West Pico and exposed to X-ray film.

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

Mouse orthotopic xenograft model
All animal experiments were Institutional Animal Care and Use Committee 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) and evaluated via laparotomy. A midline incision was made, and a 1.0-mm burr hole was drilled at AP = 2.5 mm from the bregma. 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. 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-cyclodextrin, 1% sodium bicarbonate or vehicle alone twice daily 5 days per week. Mice were monitored daily and sacrificed according to Institutional Animal Care and Use Committee–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% H2O2 for 5 minutes at 25°C and treated with Target Retrieval Solution (Dako) for 25 minutes at 96°C. For anti-phospho-Thr208-Aurora-A, slides were further blocked with Protein Block Serum-Free (Dako) for 15 minutes at 25°C. Slides were incubated with anti-phospho-Thr208-Aurora-A (Abcam ab58949; 1:200) or anti–phosphohistone-H3 (Abcam ab5176; 1:400) for 1 hour at 25°C. Antibodies were detected using MACH 3 Rabbit HRP-Polymer (BioCare) and DAB (Dako). Slides were counter-stained 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 nmol/L 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 glioblastoma neurosphere cells with and without small-molecule Aurora-A inhibitors. Alisertib potently blocked proliferation of all three neurosphere lines tested (Fig. 1A). IC50 concentrations were 11, 24, and 32 nmol/L for GB169, GB9, and GB30 cells, respectively. Another Aurora-A inhibitor, TC-A2317 (11) was less potent, demonstrating IC50 concentrations of 170, 179 and 168 nmol/L for GB169, GB9, and GB30 cells, respectively. Alisertib IC50 concentrations are similar to slightly higher than those required to inhibit colony formation in two other glioblastoma neurosphere lines (14) and approximately 5- to 10-fold less than those effective against 10 conventional monolayer glioblastoma cell lines (15), suggesting greater efficacy against glioblastoma neurosphere cells.

We also examined the impact of alisertib on glioblastoma neurosphere cells 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, for example, 200 nmol/L, 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. 1B and C). 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 whether alisertib-induced toxicity was due to increased apoptosis, we measured Annexin-V staining in alisertib-treated normal human astrocytes and glioblastoma cells. Alisertib did not induce significant Annexin-V staining in human astrocytes (Supplementary Fig. S1B). In contrast,
alisertib-treated glioblastoma neurosphere cells showed increased Annexin-V staining indicative of apoptosis, reaching approximately 85% after 12 days of drug exposure (Fig. 1C and Supplementary Fig. S1C).

Because alisertib caused only a limited amount of apoptosis after 6 days of exposure and did not completely prevent metabolic activity in glioblastoma neurospheres, we investigated whether neurosphere cells could resume proliferation following treatment. Glioblastoma 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 glioblastoma cell proliferative potential following drug treatment (Fig. 1D and Supplementary Fig. S1C). In a similar experiment, recovery of metabolic activity following extended alisertib exposure was examined by MTT assay (Supplementary Fig. S1D).

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

Aurora-A inhibitors had a dramatic effect on glioblastoma cell morphology after 2 weeks of exposure. Neurospheres...
contained fewer, but larger cells (Fig. 2A and Supplementary Fig. 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 glioblastoma 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 μm² in control cells to 416 μm² 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 phosphoThr288Aurora-A show that phosphoThr288Aurora-A is markedly decreased in alisertib- and TC-A2317–treated glioblastoma neurosphere verifying drug target-hit (Fig. 3A and B; Supplementary Fig. S3A).

To look for biochemical evidence of apoptosis, differentiation, and senescence of glioblastoma 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. 3A and B; Supplementary Fig. S3A).

Olig2, a transcription factor expressed in normal and neoplastic astrocytes and oligodendrocytes, was increased in all glioblastoma neurosphere lines treated with alisertib or TC-A2317 (Fig. 3A and B; Supplementary Fig. S3A). GFAP and Aldh1L1, markers of astrocytic differentiation (19), were also increased in response to alisertib (Fig. 3A and B). 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 (CDK) inhibitor p21 (20) was strongly increased by alisertib (Fig. 3A and B) and TC-A2317 (Supplementary Fig. S3A).

Western blot 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 glioblastoma neurosphere cells (Fig. 3A and B; Supplementary Fig. S3B). β-Catenin was also decreased by TC-A2317 (Supplementary Fig. S3A). As β-catenin is required for CNS progenitor self-renewal (23), these results may correlate with a decreased capacity for glioblastoma 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 glioblastoma 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 glioblastoma cells in vitro.

To investigate whether the above observed biochemical effects of alisertib on glioblastoma 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 CDK inhibitor p27 (Fig. 3C). Thus, the response of glioblastoma neurosphere cells to Aurora-A kinase inhibition or mRNA knockdown includes apoptosis, differentiation, and senescence. In addition, 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 glioblastoma 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 Fig. 4A, alisertib significantly extended the survival of mice with intracranial xenografts of both glioblastoma neurosphere lines. Mice with GB169 xenografts showed a 25.2% increase in median survival (87 days for alisertib-treated mice compared to 69 days for control mice). These results suggest that alisertib may be a promising therapeutic agent for the treatment of glioblastoma.
compared with 69.5 days for vehicle-treated, \( P = 0.025 \), whereas survival was increased by 33.3% in mice with GB30 xenografts (26 days for alisertib-treated compared with 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 Fig. 4B, glioblastomas from alisertib-treated mice demonstrated enlarged nuclei and greatly decreased phosphoThr\(^{288}\)Aurora-A compared with 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 glioblastoma cells in vivo.

In summary, the Aurora-A inhibitor alisertib potently inhibits proliferation of glioblastoma 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 glioblastoma neurosphere xenografts. Alisertib is, therefore, a promising new potential agent for the treatment of glioblastoma alone or in combination with other therapies.

Disclosure of Potential Conflicts of Interest

N.L. Lehman received a commercial research grant from Takeda Pharmaceuticals International Corporation. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: J. Wojton, J.A. Ecsedy, B. Kaur, N.L. Lehman

Development of methodology: J. Wojton, B. Kaur, N.L. Lehman

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.R.V. Brocklyn, W.H. Meisen, D.A. Kellough, B. Kaur, N.L. Lehman

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.A. Kellough, J.A. Ecsedy, B. Kaur, N.L. Lehman

Writing, review, and/or revision of the manuscript: J.R.V. Brocklyn, W.H. Meisen, J.A. Ecsedy, B. Kaur, N.L. Lehman

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.L. Lehman

Study supervision: B. Kaur, N.L. Lehman

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Figure 4. A, alisertib extends survival of mice with glioblastoma 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 glioblastoma neurosphere cells in vivo. Mouse 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); B and C, magnifications, \( \times 400 \); D, \( \times 600 \).


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