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

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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 stem–like cells in vitro and 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 in vitro 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 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 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 (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 (Invitrogen) 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%–0.02%). Every 2 days, three wells per condition were triturated and 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 10^3 cells per well in 100 μl of medium containing alisertib or DMSO. After 4 days at 37°C, 10 μl of MTT reagent (Roche) was added per well and plates 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 mmol/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 phenylmethylsulfonylfuoride, 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 phosphoThr288Aurora-A, Aurora-A (Abcam ab58494, 1:500; and ab13824, 1:500), CD44, p21, p27, cleaved PARP, BimEL, neurofilament-L (Cell Signaling Technology; 3570, 1:2,000; 2947, 1:1,000; 3686, 1:1,000; 9541, 1:1,000; 2933, 1:1,000; and 2837, 1:500), Mushashi-1 (R&D Systems MAB2628; 1:500), Olig2 (IBL 18953; 1:200), Aldh1L1 (NeuroMab 18-0226; 1:1,000), 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 h 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 xylazine (20 mg/kg) and were anesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (20 mg/kg) and then implanted with intraperitoneal injections of 103 cells with and without small-molecule Aurora-A inhibitors. Alisertib potentially blocked proliferation of all three neurosphere lines tested (Fig. 1A). IC50 concentrations were 11, 24, and 32 mmol/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 mmol/L for these cells, respectively (Supplementary Fig. S1A). Observed 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 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, for example, 200 mmol/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 neurospheres 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). 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
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.

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