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Alisertib demonstrates significant antitumor activity in bevacizumab resistant, patient derived orthotopic models of glioblastoma

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Abstract

Aurora A Kinase (AURKA), a member of the serine/threonine kinase family, plays a critical role in cell division, and it is widely overexpressed in a variety of tumors including glioblastoma (GBM). Alisertib (MLN8237) is an orally administered selective AURKA inhibitor with potent antiproliferative activity, currently undergoing clinical testing in different tumor types. *In vitro* evaluation of alisertib against the primary glioblastoma (GBM) lines GBM10, GBM6 and GBM39 showed significant antitumor activity with IC₅₀s ranging between 30–150 nM. Orthotopic xenografts of GBM39, GBM6 and GBM10 were established by implanting 3×10⁵ cells in the caudate nucleus of nude mice; animals were randomized to treatment with either alisertib 30 mg/kg/day or vehicle. In all three models, treatment with alisertib resulted in a statistically significant prolongation of survival (p<0.0001). Alisertib administered in these mice decreased phosphorylated, Aurora-A induced mitotic arrest, and significantly decreased histone H3 phosphorylation in tumors. The same primary glioma lines were previously characterized in orthotopic *in vivo* models for response to the anti-VEGF antibody bevacizumab; GBM6 and GBM39 were resistant to bevacizumab treatment. In conclusion, alisertib displays significant antitumor activity against primary GBM lines and xenografts, including patient derived GBM lines resistant to bevacizumab; these data support clinical translation in GBM.

Keywords

Aurora A; alisertib; GBM; bevacizumab; resistance

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Introduction

Glioblastoma (GBM) is a refractory malignancy, with a median survival of only 15–16 months [1,2], despite multimodality treatment including surgical resection, external beam radiation therapy and chemotherapy. Most patients will develop recurrent disease after completing initial therapy. Use of the VEGF targeting antibody bevacizumab still represents the mainstay of systemic treatment for recurrent disease in the US [3], but overall survival (OS) remains poor. Following progression on a bevacizumab containing regimen, no agent has demonstrated significant activity and prognosis remains uniformly dismal [4–6]. In the recently completed randomized phase III CABARET trial, median OS after progression on bevacizumab was only 3 to 3.4 mo, independent of bevacizumab continuation [7]. Therefore, there is an urgent need to develop novel therapeutic strategies for patients with recurrent GBM, including the clinically challenging setting of bevacizumab failure.

Alisertib (MLN8237) is an Aurora A kinase (AURKA) inhibitor with improved potency and selectivity as compared to earlier inhibitors such as MLN8054 [8]. In experimental models and in patients, alisertib inhibits proliferation of tumor cell lines and induces characteristic pharmacodynamic changes consistent with AURKA inhibition – increased mitotic index, reduced bipolar spindles, and increased chromosome alignment abnormalities [9,10,8]. Moreover, alisertib has demonstrated anti-tumor activity against multiple xenograft or disseminated tumor models with dramatic growth inhibition seen in lung (H460:91%) and colon (HCT-116:93%) models [8]. Alisertib's activity against primary brain tumors *in vitro* and *in vivo* has not been fully explored to date, however.

Emerging data supports a role for AURKA in glioma oncogenesis. The AURKA gene was amplified in 26% of gliomas and mRNA overexpression was identified in 60% of the specimens [11,12]. These initial studies suggested a possible correlation between the level of AURKA expression and glioma grade, with higher levels observed in glioblastoma (GBM). Multiple studies evaluating either AURKA mRNA or protein levels [13–15] have confirmed this observation, supporting possible associations of AURKA expression in glioma with outcome. Recently published data also implicates AURKA in the self-renewal and tumorigenicity of glioma-initiating cells that contribute to GBM recurrence and treatment resistance [16].

In vitro data also corroborate the importance of AURKA to GBM growth as the small molecule AURKA inhibitor C1368 was cytotoxic when tested against several GBM cell lines [15]. The Aurora kinase inhibitor VE-465 also decreased GBM cell viability via a caspase-independent pathway [17]. Cytotoxicity of alisertib was tested in a variety of low-passage primary GBM cell lines using a colony formation assay. Alisertib was cytotoxic with IC₅₀s ranging from 60 nM to 225 nM, with higher susceptibility being observed in the low-passage primary tumor cell lines. Longer drug exposure times were also associated with enhanced cytotoxicity [14]. Taken together, these data support that therapeutic development of AURKA inhibitors for GBM patients represents a direction worth pursuing.

In this study, using orthotopic primary human GBM xenograft models, we demonstrated that orally administered alisertib significantly prolongs survival, including two animal models

which have demonstrated primary resistance to bevacizumab. Correlative studies confirmed target effects of alisertib on AURKA signaling, which supports effective CNS drug penetration in mice. Based on these data, clinical testing of alisertib in GBM patients is warranted.

Methods

Cell Culture

Primary GBM xenograft lines were passaged in the flanks of BALB/c nude mice (Charles River) as previously described [18]. For the *in vitro* experiments, tumors were excised from the mice, minced and subsequently plated onto flasks in DMEM containing 2.5% FBS and 1X Penn/Strep at 37°C, 5% CO₂ in a humidified environment. Following short term expansion, cells were removed with Tryple (Invitrogen) prior to plating *in vitro* for experiments (DMEM, 10% FBS, 1X Penn/Strep) or for orthotopic implantation into BALB/c nude mice.

MTT assays

GBM6, GBM10, GBM12 and GBM39 xenograft lines were plated in 96 well plates in DMEM containing 10%FBS and 1X, penicillin/streptomycin at a density of 10⁴ cells per well. Twenty-four hours after plating the cells were exposed to different concentrations of alisertib. Cell proliferation was measured by MTT assay (ATCC, Manassas, VA) at days 1, 2, 3, 7, and 10 post exposure to alisertib and the IC₅₀ was calculated for each xenograft line, as previously described [19].

GBM6 and GBM39 flank xenograft model

10⁶ GBM6 or GBM39 cells/mouse were mixed 1:2 with Matrigel (BD Biosciences, San Jose, CA) and injected subcutaneously into the flanks of BALB/c nude mice. Upon the establishment of palpable tumors mice were treated with either a placebo or alisertib (30mg/kg/day) by oral gavage. At pre-specified time points after treatment initiation day 1 (at 1 h and at 8 h post treatment), day 8 (at 1 and 8 h post treatment), day 14 (at 1 and 8 h post treatment), and day 21 (at 1 and 8 h post treatment) tumors were excised, formalin fixed and paraffin embedded. H&E sections were examined for cell morphology and subsequent sections were used for immunostaining.

Orthotopic tumor models

The effect of treatment with alisertib was examined in orthotopic GBM6, GBM39 and GBM10 xenografts by implanting 3×10^5 cells into the right caudate nucleus of BALB/c nude mice as previously described [18] using a small animal stereotactic frame. Starting at 2 wks post tumor implantation, mice were treated with alisertib (30mg/kg/day) (10–15 animals per group) or placebo (8–15 animals per group) by oral gavage for 22 days. Animals were euthanized as per IACUC guidelines when they developed neurologic symptoms or lost >10% of body weight. In a parallel experiment, orthotopic xenograft animals were euthanized at a similar schedule as for the GBM39 flank model, brains embedded in paraffin. H and E sections were examined for cell morphology and additional sections were used for immunohistochemistry. The effect of treatment with bevacizumab in the same

orthotopic xenograft models was examined by establishing orthotopic xenografts as described above, followed by treatment by intraperitoneal administration of bevacizumab, 5mg/kg, 2 times per week or placebo. Animals were euthanized as per IACUC guidelines when they developed neurologic symptoms or lost >10% of body weight.

Immunohistochemistry for phospho-Histone H3

Five-micron sections deriving from xenograft tumors were deparaffinized in CitriSolv (Fisher, Pittsburgh, PA). Slides were then rehydrated in graded ethanol baths, washed briefly with water and incubated with 0.6% H₂O₂ in methanol for 20 min. Sections were washed in 75% ethanol for 3 minutes, 50% ethanol for 3 minutes and distilled water for 3 minutes. Sections were placed in Antigen Retrieval Citra (Biogenex, San Ramon, CA) at room temperature followed by a 99°C water bath for 20 min, and subsequently placed on ice for 20 min prior to being transferred into PBS. Sections were blocked with 10% Normal Goat Serum (NGS) in PBS containing 0.01% Triton X 100. Rabbit anti-human p-Histone H3 (Ser 10)-R (Santa Cruz Biotechnology, Inc, Dallas, TX) (add KI67) was diluted 1:250 in PBS containing 10% NGS, applied to each section at 4°C overnight in a humidified chamber followed by three brief washes in PBS. Sections were exposed to a goat anti-rabbit IgG-B in PBS containing 10% NGS for 60 minutes, developed using the VectaStain Elite and DAB kits as per manufacturer directions (Vector, Burlingame, CA), and counterstained with hematoxylin for 30 seconds. Photographs were taken using a 40X objective in a Zeiss AxioPlan2 microscope.

Immunohistochemistry for phospho Aurora-A

Formalin fixed, paraffin embedded tumor xenografts were sectioned at 5 µm and stained for anti-Phospho-Aurora A (phospho T288) protein. Antigen was retrieved on-line with the Leica Bond RX stainer (Leica, Buffalo, IL) using Epitope Retrieval Solution 1 (Citrate based solution, pH 6). The tissues were incubated in Rodent Block M by Biocare RBM961L for 30min. Primary anti-Phospho-Aurora A antibody was diluted in Dako background reducing diluent and incubated for 30min. Staining was conducted using polymer refine detection kit from Leica that includes DAB per manufacturer's recommendation. Primary anti-Phospho-Aurora A (phospho T288) antibody (Abcam, ab58494) was used at 1:300 dilutions. A Zeiss AxioPlan 2 microscope and Axiovision software (version 4.7) were used to generate images under bright field configured with 20x PlanNEOFLUAR (NA 0.50) and 40x N-ACHROPLAN (NA 0.65).

Results

Assessment of therapeutic effect *in vitro*

In order to determine *in vitro* activity of alisertib against primary GBM lines, MTT assays were performed to determine the IC₅₀, by exposing the indicated cells to 0–1µM of alisertib. IC₅₀ ranged from 30–95nM (IC₅₀ GBM39 = 30nM, IC₅₀ GBM6 = 37nM, IC₅₀ GBM12 = 46nM, IC₅₀ GBM10 = 95 nM). Next, primary GBM cells were treated with alisertib at their respective IC₅₀, and cell proliferation was monitored over time. Significant *in vitro* antitumor activity was observed in all cell lines tested (Figure 1a, b, c). Of note, exposure to alisertib can cause cell senescence with maintenance of low level metabolic activity, thus the

observed significant *in vitro* activity using the MTT assay could actually represent an underestimation.

Assessment of therapeutic effect *in vivo*

In order to assess sensitivity to bevacizumab, an agent commonly used in recurrent GBM treatment, GBM6, GBM10 and GBM39 orthotopic xenografts were treated with bevacizumab versus placebo (10 animals per group). Treatment of GBM10 xenografts with bevacizumab led to significant prolongation of survival (50 vs 23 days, $p<0.0001$). In contrast, there was no difference in GBM39 (25 vs 24 days, $p=0.9$) or GBM6 (43 vs 45 days, $p=0.12$) xenografts, indicating primary resistance to bevacizumab (Figure 2). In order to evaluate the *in vivo* activity of alisertib, orthotopic xenograft tumor models in BALB/c nude mice were established by implanting 3×10^5 GBM6, GBM10 or GBM39 xenograft cells into the right caudate nucleus and treating for 22 days with alisertib by oral gavage (30mg/kg/day) or vehicle. Significant prolongation of survival was noted in all three models ($P<0.0001$) following treatment with alisertib versus vehicle controls (Figure 3).

Histologic findings

Histologic examination of formalin fixed, paraffin embedded tissue sections, obtained at prespecified euthanasia time points revealed the accumulation of polyploid cells, consistent with Aurora A inhibition (Figure 4a, b). Immunohistochemical staining of these sections, in both flank and orthotopic models, demonstrated significant decrease in phospho Aurora A staining consistent with target inhibition (Figure 4c, d). The similar findings of AURKA target inhibition both in flank and orthotopic models indicate that alisertib can effectively cross the blood-brain barrier in primary glioma tumors, further supporting the translational relevance of our findings. Similarly, there was significant decrease in Phospho Histone-H3 expression in treated tumors, as compared to untreated controls on days 1 (1 and 8 hours after alisertib administration), 8, 14 and 21, indicating decrease in tumor mitotic activity/proliferation (Figure 4e, f, g, h).

Discussion

Aurora serine/threonine kinases are key mitotic regulators required for the maintenance of chromosomal stability [20,21] by being involved in many steps as cells enter and progress through mitosis. In mammalian cells Aurora kinases consist of three members, Aurora-A, -B and -C that are expressed in a cell cycle dependent fashion. Aurora-A is localized primarily on centrosomes, spindle poles, and transiently along the spindle microtubules as cell progress through mitosis [22,21]. Given the critical role of Aurora-A in cell mitosis, several small molecule inhibitors have been developed and currently tested clinically [23].

Aurora-A overexpression in high grade gliomas including GBM and its impact on multiple signaling pathways involved in growth differentiation [14,24–26] supported the rationale of testing the Aurora-A inhibitor alisertib in primary GBM lines and xenografts. Management of recurrent GBM represents an ongoing challenge [27]. Despite a plethora of novel therapeutic agents approved for other malignancies, the paucity of effective agents in GBM treatment is striking, with bevacizumab being the last agent approved in the US in 2010.

GBM patients at recurrence have invariably failed temozolomide and radiation; available therapeutic options such as nitrosoureas (van den bent, Lancet, 2015), or alternative schedules of temozolomide (RESCUE) [28] have demonstrated only modest efficacy. The recently completed EORTC 26101 trial demonstrated an improvement in progression free survival with a bevacizumab/lomustine combination as compared to lomustine, but no overall survival gain in recurrent GBM patients [29]. Of note, median overall survival in that trial was only 8 to 9 months indicating that no major improvements have occurred in the management of recurrent GBM, and again highlighting the need for novel more effective approaches.

The data we present here show significant antitumor activity of alisertib *in vitro* and *in vivo* against all three primary glioma lines and xenograft models, including two xenograft models that are resistant to bevacizumab treatment. These data are especially promising given the dismal prognosis of patients who have progressed to bevacizumab with a median overall survival in a range of 3 to 4 months in a recently completed randomized phase III trial [7], independent of bevacizumab treatment continuation. The characteristic morphologic effect of AURKA inhibition with presence of large polyploid cells was observed in treated tumors. In addition, immunohistochemical analysis confirmed inhibition of the target pathway with decrease in phospho A staining and decrease in phospho-histone H3, not only in flank, but also orthotopic xenografts: the latter indicates that systemic administration of alisertib can effectively inhibit the AURKA target in the CNS. It is of note, that the patient derived orthotopic xenografts employed in this manuscript have been shown to recapitulate not only the molecular characteristics but also the invasiveness of the patient tumor they derive from [18], which further supports the translational implications of these data.

Other investigators have demonstrated activity of alisertib against orthotopic models deriving from neurosphere cells with stem cell-like properties, the subpopulation of cells being viewed as the most difficult to eradicate and responsible for recurrence following chemotherapy and radiation [30]. Cell line data also indicates additive or synergistic effect with temozolomide and ionizing radiation [31]. Although *in vivo* demonstration of synergy with temozolomide and radiation is at this point missing, this represents a direction that also merits further preclinical investigation.

In summary, our preclinical data support the promise of alisertib as a novel therapeutic agent against bevacizumab resistant gliomas; clinical trials testing alisertib in this patient population are warranted.

Acknowledgments

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Figure 1.

(a, b, c) Significant cell killing of primary GBM lines GBM6, GBM10, and GBM39 was demonstrated *in vitro* by MTT assays.

Figure 2.

(a, b, c) In order to assess sensitivity to bevacizumab GBM6, GBM10 and GBM39 xenografts were orthotopically established in BALB/c nude mice and treated with bevacizumab vs placebo. Bevacizumab led to significant prolongation or survival of GBM10 xenografts, but not in the other two orthotopic models.

Figure 3.

(a, b, c) GBM6, GBM10 and GBM39 orthotopic tumor models were used to evaluate the therapeutic effect of MLN *in vivo*. Survival was significantly extended in mice treated with alisertib as compared to placebo treated controls in all three GBM tumor models ($P < 0.0001$ in all models).

Figure 4.

(a, b) Microscopic examination of hematoxylin and eosin stained sections from orthotopic tumor models demonstrate the accumulation of aneuploid cells following treatment with alisertib (c, d). Decrease in phosphorylated Aurora-A by IHC on day 1 at 8 hours post treatment with alisertib (d) as compared to baseline (c) consistent with successful targeting of Aurora-A signaling. (e, f, g, h). Tumor sections from both the GBM39 flank and orthotopic tumor models were subjected to immunostaining for phospho-Histone H3, a marker of mitotic activity. There was significant decrease in staining in alisertib treated animals (f, h) as compared to placebo treated samples (e, g), both in the flank (e, f) and orthotopic models (g, h) providing further evidence that alisertib is able to cross the blood brain barrier, and exert its antiproliferative effect in CNS tumors. Figures e, f, g, h were obtained on day 1 at 8 h following treatment with alisertib. Similar findings were observed on days 8, 14 and 21.