Preclinical Development

Therapeutic Potential of AZD1480 for the Treatment of Human Glioblastoma

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Abstract

Aberrant activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway has been implicated in glioblastoma (GBM) progression. To develop a therapeutic strategy to inhibit STAT-3 signaling, we have evaluated the effects of AZD1480, a pharmacologic inhibitor of JAK1 and JAK2. In this study, the in vitro efficacy of AZD1480 was tested in human and murine glioma cell lines. AZD1480 treatment effectively blocks constitutive and stimulus-induced JAK1, JAK2, and STAT-3 phosphorylation in both human and murine glioma cells, and leads to a decrease in cell proliferation and induction of apoptosis. Furthermore, we used human xenograft GBM samples as models for the study of JAK/STAT-3 signaling in vivo, because human GBM samples propagated as xenografts in nude mice retain both the hallmark genetic alterations and the invasive phenotype seen in vivo. In these xenograft tumors, JAK2 and STAT-3 are constitutively active, but levels vary among tumors, which is consistent with the heterogeneity of GBMs. AZD1480 inhibits constitutive and stimulus-induced phosphorylation of JAK2 and STAT-3 in these GBM xenograft tumors in vitro, downstream gene expression, and inhibits cell proliferation. Furthermore, AZD1480 suppresses STAT-3 activation in the glioma-initiating cell population in GBM tumors. In vivo, AZD1480 inhibits the growth of subcutaneous tumors and increases survival of mice bearing intracranial GBM tumors by inhibiting STAT-3 activity, indicating that pharmacologic inhibition of the JAK/STAT-3 pathway by AZD1480 should be considered for study in the treatment of patients with GBM tumors.

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Introduction

Glioblastoma (GBM) is a challenging disease to treat (1, 2). Patients diagnosed with GBM have a median survival of 12 to 14 months, and most tumors have an aggressive rate of recurrence and resistance to existing treatments. Aberrant activation of signaling pathways has been implicated in GBM tumor progression including receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (1–4). Activation of the phosphoinositide 3-kinase pathway is also a common feature of GBM due to frequent loss of PTEN that causes dysregulated phosphoinositide 3-kinase pathway activity and an increase in downstream Akt signaling (5). Other pathways implicated in GBM initiation and/or progression include PKC (6), MAPK (7), Wnt (8), NF-κB (9, 10), and the Notch and Hedgehog pathways (11). Constitutive activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is linked to GBM tumor promotion and maintenance by promoting cell growth while inhibiting apoptosis (12).

The JAK/STAT pathway is involved in inflammation, proliferation, and invasion/migration (13). Activation of this pathway involves binding of a cytokine to its receptor, which leads to tyrosine phosphorylation of intracellular corresponding JAK kinases. This allows for recruitment and phosphorylation of STAT-3 by JAK kinases. Phosphorylated STAT-3 proteins dimerize, translocate to the nucleus, and initiate gene transcription. Cytokines of the interleukin (IL)-6 family, including IL-6, oncostatin M (OSM), leukemia inhibitory factor, ciliary neurotrophic factor, and IL-11, are potent activators of the JAK/STAT pathway. The JAK/STAT pathway, using JAK1 and JAK2, and activating predominantly STAT-3 (13). Common gene targets of STAT-3 include prosurvival molecules such as Bcl-2, Bcl-xL, survivin, c-ABL, and VEGF (13). STAT-3 is phosphorylated on tyrosine 705 and serine 727, and phosphorylation of both residues is required for maximal STAT-3 transcriptional activity (14).

The link between inflammation and cancer has been well established, and the JAK/STAT pathway, especially STAT-3, has been implicated in multiple cancers (15, 16).
STAT-3 is upregulated and aberrantly activated in many cancers including breast, colon, prostate, and GBM (17, 18), yet STAT-3 has a very low frequency of mutation (19). Aberrant activation of STAT-3 may be due to stimuli in the GBM microenvironment, such as IL-6, or by loss of negative regulators. IL-6 family members including IL-6 and OSM are upregulated in GBMs and corresponding tumor microenvironment (20). IL-6 gene amplification events occur in 40% to 50% of GBM patients, which is associated with decreased patient survival (21). Recently, it was shown that STAT-3 is a major transcription factor responsible for the mesenchymal subtype of GBMs (22). This subtype correlates with a more malignant phenotype and poor outcome compared with other GBM subtypes (23, 24).

AZD1480, an ATP competitive inhibitor of JAK1 and JAK2, was recently shown to inhibit the growth of solid tumors including breast, ovarian, and prostate (25). AZD1480 inhibited constitutive and IL-6 induced STAT-3 activation and subsequent nuclear translocation. The ability of AZD1480 to effectively limit tumor volume was attributed to inhibition of STAT-3. In this study, we sought to determine the efficacy and potential antitumor effects of AZD1480 in GBMs, which have not been previously studied. We show that AZD1480 effectively inhibits JAK1,2/STAT-3 signaling in 2 human glioma cell lines, a murine glioma cell line, and human GBM xenografts. This inhibition of STAT-3 activation leads to a decrease in glioma cell proliferation and induction of apoptosis. In vivo, AZD1480 inhibited the growth of GBM xenografts propagated subcutaneously through decreased STAT-3 signaling. More importantly, AZD1480 treated mice bearing intracranial GBM xenografts had significantly longer survival times compared with vehicle-treated mice. Although future studies are necessary, this is the first report of the antitumor effects of AZD1480 in GBM, which show a therapeutic benefit for targeting JAK/STAT-3 signaling in GBMs.

### Materials and Methods

#### Reagents and cells

AZD1480 (Supplementary Fig. S1), a JAK1/2 inhibitor, was synthesized and provided by AstraZeneca (25, 26). Antibodies to phosphorylated STAT-3 (Y705 and S727), phosphorylated JAK1 (Y1022/1023), CD133 and Caspase-3 were from Cell Signaling Technologies; JAK1, JAK2, phosphorylated JAK2 (Y1007/1008), cyclin A and survivin from Santa Cruz; STAT-3 and PARP from BD Transduction Laboratories; and GAPDH from AbCam. Monoclonal antibodies to Bcl-2 and Bcl-xl were a generous gift of Dr. Tong Zhou (University of Alabama at Birmingham (UAB), Birmingham, AL). OSM, IL-6, and soluble IL-6R were purchased from R&D Systems. U87-MG, U251-MG, and 4C8 cells were maintained in Neurobasal media with B27 supplement, EGF (10 ng/mL), and FGF (25 ng/mL). 4C8 was established as described (27).

#### Xenograft GBM tumors

Human GBM xenograft tumors were maintained by the UAB Brain Tumor Core Facility with the approval (APN # 10090862) of the UAB Institutional Animal Care and Use Committee. Human GBM xenografts were analyzed by the Heflin Genomics Core Facility using the Applied Biosystems AmpF/STR system to screen 15 different STR markers, and determined to have identical STR patterns to that of the original patient’s tumor from which they were derived. Xenograft tumors were dissociated into single cells for brief cell culture analysis, snap frozen for protein isolation and immunoblotting, injected subcutaneously in the flank, or injected intracranially. Female athymic nude mice (6–8 weeks old) were used for all experiments. Flank tumors were removed, washed with PBS, minced, and disaggregated. Cells were passed through a 40 μm filter and plated in Neurobasal media with FBS (10%), amphotericin (1%), B27 Supplement, gentamycin (0.25%), L-glutamine (260 mmol/L), EGF (10 ng/mL), and FGF (10 ng/mL) and cultured as spheroids in suspension (29). Xenograft tumor cells were separated based on cell...
surface CD133 separation using the CD133 MicroBead kit (Miltenyi Biotec). Populations were verified by immunoblotting for CD133. Xenograft flank tumors were removed and snap frozen in liquid nitrogen and lysed in radioimmunoprecipitation lysis buffer with protease inhibitors using a tissue homogenizer, and 30 μg of protein was immunoblotted. For subcutaneously injected tumor experiments, xenograft tumors were roughly disaggregated and minced. Approximately 100 (xenograft X1066) or 200 μL (xenograft X1046) of tumor slurry was injected subcutaneously into the flanks of athymic nude mice. Tumor volume was measured using calipers and calculated using the following equation: \( v = \frac{0.5 \times \text{longest diameter} \times \text{shortest diameter}^2}{} \). On day 6, mice were randomized to vehicle control or AZD1480. Treatment was administered i.p. twice a day (6–8 hours apart) at 30 mg/kg per dose in sterile water. Dosing schedule included continual twice daily i.p. injections for the duration of the experiment. Mice were euthanized and tumors excised, divided, and snap frozen for analysis or formalin fixed and paraffin embedded. For intracranial injection, xenograft tumors were disaggregated into single cells, and approximately 5 × 10^5 cells in 5 μL of methylcellulose were injected 2 mm anterior and 1 mm lateral to the bregma at a depth of 2 mm over 2 minutes for adequate perfusion. Tumors were allowed to establish for 5 days before beginning daily i.p. injection of AZD1480 (50 mg/kg per mouse) in methylcellulose or vehicle on day 6. Treatment schedule consisted of 5 days of treatment followed by 2 days of rest for a total of 3 weeks. All mice were euthanized at moribund.

**Phosphorylated JAK2 ELISA assay**

Approximately 65 μg of lysates from snap frozen xenograft samples were analyzed for phosphorylated JAK2 levels using the JAK2 [pYpY1007/1008] ELISA (Invitrogen).

**Annexin V/propidium iodide staining**

U251-MG cells were stained with Annexin V and propidium iodide (PI) using Clontecy ApoAlert Annexin V-FITC Apoptosis Kit, and examined by flow cytometry. The percentage of Annexin V-positive and PI-positive cells was determined by FlowJo 7.5.5 software.

**Quantitative reverse transcription-PCR**

Total RNA was isolated using TRIzol (Invitrogen), and approximately 1 μg of RNA per sample was used to generate cDNA by reverse transcription for PCR (RT-PCR). Predesigned Taqman primers (Applied Biosystems) were used to obtain quantitative PCR results using the Applied Biosystems StepOnePlus Real-Time PCR System Thermal Cycling Block and corresponding software analysis for data quantification (StepOneSoftware v2.1, Applied Biosystems). The following Taqman primers and the corresponding Gene Ref # were used: human c-Myc (Hs99999003_m1), human IL-6 (Hs00174131_m1), and human SOCS-3 (Hs02330328_s1). Eukaryotic 18s rRNA (Hs9999901_s1) was used as an endogenous control.

**Statistical analysis**

Student t test and Mann–Whitney rank sum tests were done for comparison of 2 values, ANOVA analysis was done on appropriate multivariable analyses using the Bonferroni test, and the log-rank test was used for Kaplan–Meier survival curves. \( P < 0.05 \) was considered statistically significant.

**Results**

**AZD1480 inhibits constitutive STAT-3 and JAK2 activation in glioma cells**

We sought to determine the inhibitory effect of AZD1480 on JAK/STAT-3 signaling in GBM tumor cells and potential antitumor effects. Two human glioma cell lines (U251-MG and U87-MG) and a murine glioma cell line (4C8) that all exhibit constitutive STAT-3 activation were used to determine the effects of AZD1480. Treatment of glioma cells with AZD1480 at 1 μmol/L blocked constitutive STAT-3 and JAK2 phosphorylation in all 3 glioma cell lines beginning as early as 30 minutes and lasting for at least 16 hours (Fig. 1A). Comparable results were observed using 0.5 μmol/L AZD1480 (data not shown). This shows that AZD1480 inhibits constitutive activation of STAT-3 in GBM cell lines.

**AZD1480 treatment elicits functional antitumor effects in glioma cells**

Inhibition of STAT-3 signaling can decrease proliferation and induce apoptosis of glioma cells (30). U251-MG and 4C8 glioma cells were treated with AZD1480, which led to an inhibition of proliferation at a concentration of 10 μmol/L (Fig. 1B). This was also shown using the U87-MG cell line (data not shown). More importantly, we evaluated the ability of AZD1480 to inhibit proliferation of murine primary astrocytes and found no inhibitory effect at either a 1 or 10 μmol/L dose (Fig. 1B). This suggests that the functional effect of AZD1480 is specific to tumor cells without affecting normal glial cells. U251-MG cells were treated with AZD1480 (1 and 10 μmol/L) for 48 hours, stained with Annexin V and PI and analyzed by flow cytometry. AZD1480 induced apoptosis in a dose-dependent manner as seen by the increase in the percentage of Annexin V/PI positivity (Fig. 1C). The ability of AZD1480 to induce cell death was also evaluated by immunoblotting for the presence of cleaved PARP. Treatment with AZD1480 induced the cleavage of PARP (PARPc) at 24 hours, indicating induction of cell death (Fig. 1D). A common characteristic of transformed or malignant cells is the ability to grow in soft agar (31). We therefore determined the ability of AZD1480 to affect U251-MG growth as colonies in soft agar. Cells were plated in 0.4% agarose with media in the absence or presence of AZD1480 and colonies were stained and counted after...
AZD1480 inhibits JAK/STAT-3 signaling in glioma cells. A, cells were treated with AZD1480 for the indicated times, lysed and immunoblotted with the indicated antibodies. B, cells were treated with AZD1480 for the indicated times, and the WST-1 cell proliferation assay done. The 48 and 72 hours time points were statistically significant. Data represent mean ± SD, replicates of 3 (*, P < 0.001; ANOVA). C, U251-MG cells were treated with AZD1480 for 48 hours followed by Annexin V/PI staining and examined by flow cytometry. Data represent mean ± SD, replicates of 3 (*, P < 0.05; **, P < 0.001; ANOVA). Representative of 2 experiments. D, U251-MG cells were treated with AZD1480 for the indicated times, lysed and immunoblotted with the indicated antibodies. E, U251-MG cells were plated in 0.4% soft agar without or with AZD1480. Data represent mean ± SD (**, P < 0.001; ANOVA). Representative of 3 experiments.

AZD1480 Inhibition of Glioblastoma

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4 weeks. In a dose-dependent manner, AZD1480 prevented glioma cells from forming colonies (Fig. 1E).

AZD1480 prevents stimulus-induced phosphorylation of STAT-3 and downstream gene transcription

Cytokines present in the tumor microenvironment contribute to the malignancy and continual circuitry maintaining tumor growth and proliferation (16). Two members of the IL-6 family, OSM and IL-6, were used to activate JAK1,2/STAT-3 in glioma cell lines. AZD1480 prevented OSM-induced activation of JAK1,2/STAT-3 in a dose-dependent manner in all 3 glioma cell lines (Fig. 2A). Due to the greatly enhanced phosphorylation of STAT-3 following OSM stimulation, we have provided an appropriately exposed blot revealing the constitutive STAT-3 phosphorylation (Supplementary Fig. S2A). This inhibition was also observed following IL-6 stimulation.
To determine whether inhibition of STAT-3 phosphorylation correlated with inhibition of downstream gene expression, we tested the effect of AZD1480 on 3 targets of STAT-3: SOCS-3, c-Myc, and IL-6. Upon OSM stimulation, AZD1480 significantly prevented OSM-induced expression of SOCS-3, c-Myc, and IL-6 mRNA as shown by quantitative RT-PCR (Fig. 2B). AZD1480 inhibition of STAT-3 target genes was also verified using IL-6 as a stimulus (data not shown). We also tested the ability of AZD1480 to inhibit the NF-κB pathway, as a selectivity control. U87-MG glioma cells were incubated with AZD1480 (1 μmol/L) for 2 hours followed by treatment with TNF-α. Pretreatment with AZD1480 does not inhibit TNF-α-induced NF-κB p65 phosphorylation or expression of IL-8, a NF-κB driven gene (Supplementary Fig. S2C and D), supporting the absence of pleiotropic effects of AZD1480 on signaling pathways in glioma cells.

**Human xenograft GBM tumors exhibit constitutive JAK2/STAT-3 activation**

Human GBM xenograft tumors propagated in the flank of athymic nude mice retain the hallmark mutations seen in GBM (29). We tested several xenografts for activation of JAK2/STAT-3 signaling, and found that STAT-3 is phosphorylated on both tyrosine and serine residues in all xenograft samples tested (Fig. 3A). We also analyzed the levels of phosphorylated JAK2 by ELISA and found it to be activated as well (Fig. 3B). As expected, the levels of activation vary among tumors, which is also similar to human GBM heterogeneity (29). This is the first report of activated JAK2/STAT-3 in human GBM xenografts. The xenografts have been further analyzed for the following parameters: EGFR amplification/mutation, NF-κB status, molecular subtype, and% CD133+ cells (Supplementary Table S1). EGFR amplification varied among the xenograft tumors, while all had activated NF-κB, as assessed by immunoblotting for serine 276 phosphorylated p65 (data not shown). Important information has emerged regarding the identification and characterization of 4 subtypes of GBMs: Classical, Mesenchymal, Proneural, and Neural (24). Several of the xenografts studied have been analyzed for their genetic signatures, and have been classified as Proneural (GBM15, GBM12), Classical (GBM6), and Mesenchymal (GBM10; Supplementary Table S1). Lastly, the proportion of glioma-initiating cells, as assessed by staining for CD133 positive cells, is shown (Supplementary Table S1). These results reveal a striking heterogeneity in the percentage of CD133 positive cells in the xenografts. On the basis of our initial profiling results of JAK2/STAT-3 status among the GBM xenografts, we selected X1066, X1016, and X1046 that display high levels of activated STAT-3 to more extensively evaluate the antitumor role of AZD1480.

We next determined the ability of AZD1480 to affect JAK2/STAT-3 signaling in the GBM xenografts. AZD1480 effectively blocks constitutive STAT-3 and OSM-induced JAK1,2/STAT-3 signaling in X1066 xenograft tumor cells (Fig. 4A). Constitutive STAT-3 phosphorylation was inhibited with 1 μmol/L AZD1480 as early as 0.5 hour.
and as little as 0.5 μmol/L inhibited OSM-induced STAT-3 phosphorylation. Inhibition of constitutive and OSM-induced STAT-3 activation was confirmed in Xenografts X1046 and X1016, and also by using IL-6 as a stimulus (data not shown). AZD1480 prevented OSM-induced transcription of the STAT-3 target genes SOCS-3, c-Myc, and IL-6 (Fig. 4B). Xenograft X1016 tumor cell proliferation in cell culture was also inhibited by 10 μmol/L AZD1480 (Fig. 4C). These experiments validate AZD1480 as an effective inhibitor of JAK/STAT-3 signaling in human glioblastoma xenograft tumors in vivo.

Figure 3. Human xenograft GBM tumors exhibit constitutive JAK2/STAT-3 activation. A and B, fresh snap frozen xenograft tumor samples were lysed and immunoblotted with the indicated antibodies (A) or analyzed for phosphorylated JAK2 levels using ELISA (B).

Figure 4. AZD1480 inhibits JAK/STAT-3 signaling in human glioblastoma xenograft tumors in vitro. A, xenograft X1066 was disaggregated into single cells and treated with AZD1480 for the indicated times or treated with the indicated doses for 2 hours before stimulation with OSM (1 ng/mL) for 15 minutes. Cells were lysed and immunoblotted with the indicated antibodies. B, xenograft X1016 cells were disaggregated into single cells, treated with 1 μmol/L AZD1480 for 2 hours before stimulation with OSM (1 ng/mL) for 1 hour, and quantitative RT-PCR done. Data represent mean ± SD, replicates of 3 (**, P < 0.05; ***, P < 0.01; Student t test). C, xenograft X1016 was disaggregated into single cells, treated with AZD1480 for the indicated times, and the WST-1 cell proliferation assay was done. The 48 and 72 hours time points were statistically significant. Data represent mean ± SD, replicates of 3 (***, P < 0.01; ****, P < 0.001; ANOVA). D, xenograft X1066 was disaggregated into single cells and separated based on cell surface expression of CD133. Separated cells were then treated with AZD1480 (1 μmol/L) for 2 hours followed by treatment with OSM (10 ng/mL) for 15 minutes, lysed, and immunoblotted with the indicated antibodies.
human GBM xenografts. There have been reports of STAT-3 activation in glioma-initiating cells (32, 33). Xenograft X1066 was separated based on cell surface CD133 expression. We found that AZD1480 inhibited constitutive and OSM-induced STAT-3 phosphorylation in both CD133 negative and CD133 positive cell populations (Fig. 4D). This shows the potential for AZD1480 to inhibit STAT-3 activation not only in resident tumor cells, but also in the glioma-initiating cell population in GBMs.

**Treatment with AZD1480 inhibits GBM tumor growth in vivo**

Because the overall goal is to develop a potential therapeutic agent for GBM patients, we evaluated the ability of AZD1480 to inhibit glioma growth in vivo. We first tested AZD1480 using a subcutaneously implanted xenograft model. Xenograft X1046 was injected subcutaneously into athymic nude mice, and starting at day 6, mice received twice daily i.p. injections of AZD1480 (30 mg/kg per dose) or vehicle control for a total of 3 weeks. At day 29 all mice were euthanized and tumors removed for analysis. AZD1480 significantly inhibited subcutaneous tumor growth compared with vehicle-treated mice (Fig. 5A). No significant weight loss or decrease in the total number of red blood cells was observed during AZD1480 treatment (data not shown). Tumors were analyzed by immunoblotting for effectiveness of AZD1480 on inhibition of STAT-3 phosphorylation. All tumors treated with AZD1480 had little or no STAT-3 tyrosine or serine phosphorylation compared with control-treated tumors (Fig. 5B). The levels of phosphorylated JAK2 also seem slightly decreased in AZD1480-treated tumors. We also observed a decrease in several growth promoting proteins including cyclin A, Bcl-2, and survivin in the flank tumors treated with AZD1480, while Bcl-XL expression was not affected (Fig. 5C). This suggests that AZD1480 inhibition of tumor growth can be attributed to an inhibition of STAT-3 activity. Following the same protocol, we verified the inhibition of tumor growth by AZD1480 using another xenograft tumor, X1066 (Fig. 5D). At day 21, all
mice were euthanized and flank tumors removed for analysis. Excised tumors were significantly smaller in weight than control-treated tumors (Fig. 5E), and expression of IL-6 was also significantly decreased in AZD1480-treated tumors (Fig. 5F), consistent with the interpretation that AZD1480 is inhibiting tumor growth *in vivo* due to inhibition of STAT-3 signaling and subsequent gene transcription.

The ability of AZD1480 to inhibit tumor growth and increase survival in an intracranial model of glioma was next examined. Xenograft X1046 was stereotactically injected into the brains of 20 athymic nude mice. The tumor was allowed to establish for 5 days before starting treatment. On day 6, AZD1480 (50 mg/kg per mouse) or vehicle control was administered orally once a day for 3 weeks with the endpoint measuring survival. The mice treated with AZD1480 had significantly increased survival when compared with vehicle-treated mice (Fig. 6A). The intracranial model of glioma was evaluated using another xenograft, X1016, as described above. As shown in Fig. 6B, mice receiving AZD1480 treatment survived significantly longer than those receiving vehicle control. It should be noted that xenograft X1046 is more sensitive to the effects of AZD1480 compared with xenograft X1016, which will be addressed in the Discussion.

Discussion

Here, we report our findings of AZD1480, a JAK1,2 inhibitor, and the antitumor effects in GBM tumors both *in vitro* and *in vivo*. AZD1480 inhibited constitutive and stimulus-enhanced JAK/STAT-3 signaling in 3 established GBM cell lines. AZD1480 also reduced the expression of several downstream gene targets of STAT-3; c-Myc, SOCS3, and IL-6, and elicited antitumor functional effects in glioma cells as seen by a decrease in proliferation, inhibition of soft agar colony formation and an induction of apoptosis.

We conducted studies using primary human GBM samples that are maintained as subcutaneously propagated xenograft tumors (29). A panel of 8 xenograft tumors was examined, and we found that JAK2 and STAT-3 activation was evident in all tumors, albeit the levels of activation vary among tumors. This heterogeneity is similar to what is seen in patient human samples (18). Both STAT-3 residues were phosphorylated in the xenografts, suggesting the presence of a transcriptionally active STAT-3 protein (14). Several of the xenografts were tested for responsiveness to AZD1480. AZD1480 effectively inhibited constitutive and stimulus-induced STAT-3 signaling, gene expression, and significantly inhibited proliferation of the xenograft cells.

Activated STAT-3 induces the expression of a wide array of genes that promote antiapoptotic behavior, drug resistance, cell migration and invasion, angiogenesis, and evasion of antitumor immunity (13). AZD1480 potently inhibited IL-6 and OSM induction of c-Myc and SOCS3 in glioma cells and GBM xenograft tumors. Of interest was the observation that expression of IL-6 was also inhibited by AZD1480. IL-6 has traditionally been considered to be an NF-κB responsive gene, particularly in response to TNF-α (34). NF-κB is constitutively activated in GBMs, and associated with apoptotic resistance and poor disease prognosis (12, 28, 35). The elevated levels of IL-6 detected in many cancers have been thought to result from activation of the NF-κB pathway (36). Our findings show that IL-6 and OSM activation of STAT-3 promotes IL-6 expression by GBM cells, indicating that IL-6 is also a STAT-3 target.
gene. Both NF-κB and STAT-3 activate IL-6, as well as other genes that promote cell survival, growth, angiogenesis, invasiveness, and motility (12, 36). The complex cross-talk between the NF-κB and JAK/STAT pathways is beginning to be elucidated, and data illustrate that the JAK/STAT/NF-κB axis is critical for tumor progression (36–38). Given the interdependency of the 2 pathways, inhibitors such as AZD1480 may attenuate NF-κB activation in vivo in the tumor microenvironment, and suppressing the JAK/STAT pathway. This remains to be evaluated in GBM.

The cancer stem cell hypothesis with regards to GBMs remains a complicated and challenging issue (39, 40), although it is clear that glioma-initiating cells are critical for tumor propagation, angiogenesis, invasion, and therapeutic resistance. CD133 was originally identified to be a "restrictive" initiating cell marker for GBM and necessary for tumorigenesis (41, 42). However, reports have illustrated that CD133 negative cells are also tumorigenic in vivo, showing that cell surface markers to identify cancer initiating cell populations are more complicated and dynamic than originally thought (43, 44). In our studies, we did not wish to restrict the cancer-initiating cell population to cells that express CD133, as we realize that other markers, such as SSEA-1 may be important (45). We revealed that AZD1480 is an effective inhibitor of STAT-3 signaling in both populations of glioma-initiating cells, regardless of CD133 expression status. The importance of STAT-3 in maintenance of glioma-initiating cells phenotype has been recently elucidated (32, 33, 44). Our results indicate that AZD1480 can target the glioma-initiating cell population in addition to resident tumor cells, thus having the potential to be a very effective therapeutic agent for patients with GBM.

In vivo, we found that AZD1480 inhibited xenograft tumor growth in a flank model using xenografts X1046 and X1066. This inhibition of growth correlated with decreased STAT-3 activation, indicating that AZD1480 treatment is preventing the transcriptional activity of STAT-3. This was accompanied by a decrease in expression of cyclin A, Bcl-2, survivin, and IL-6. In orthotropic tumor models in which GBM xenografts were intracranially injected, AZD1480-treated mice displayed significantly longer survival times than vehicle-treated mice. It should be noted that the mice were only treated for a total of 3 weeks, thus, longer duration of AZD1480 treatment may yield an even greater increase in survival of the mice. These findings are also suggestive that AZD1480, administered orally, has efficacy in the central nervous system. We also observed that in the intracranial model, xenograft X1046 was more sensitive to AZD1480 therapy compared with X1016. One noticeable difference between the 2 xenografts is that X1016 has amplified EGFR, while X1046 does not (Supplementary Table S1; data not shown). One hypothesis is that GBM tumors with amplified EGFR will require combination therapy with JAK and EGFR inhibitors for optimal response. Monotherapy of GBM patients with EGFR inhibitors does not provide improved radiographic responses or survival benefits (46), emphasizing a need for combination cancer therapies.

The current therapy for GBM tumors includes partial surgical resection, radiation, and chemotherapy, as it has been shown that treatment with radiation and the DNA alkylating agent temozolomide significantly increased survival in patients (47). However, these tumors eventually recur yielding these advances ultimately unsuccessful. Combination therapies, including receptor tyrosine kinase inhibitors and antiangiogenic agents, are currently being explored as therapeutic approaches against the invasive and resistant nature of these tumors (1). In fact, preclinical studies combining STAT-3 inhibitors with tyrosine kinase inhibitors, including EGFR and Src, report synergistic antitumor effects (48). Our results, along with other investigative reports, suggest AZD1480 may potentially be an effective antitumor agent when combined with current therapies available for GBM.

Disclosure of Potential Conflicts of Interest

E.N. Benveniste is a scientific advisor for The Sontag Foundation, and D. Huszar is a full-time employee of AstraZeneca and holds company stock.

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