The Janus Kinases Inhibitor AZD1480 Attenuates Growth of Small Cell Lung Cancers In Vitro and In Vivo

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Abstract

Purpose: The prognosis of small cell lung cancer (SCLC) is poor, and there has been very little progress in the medical treatment of SCLC in the past two decades. We investigated the potential of Janus-activated kinases (JAK) inhibitor, AZD1480, for treatment of SCLC in vitro and in vivo.

Experimental Design: JAK1 and JAK2 were inhibited by AZD1480 or siRNAs, and the effect of inhibition of JAK gene family on SCLC cell viability was evaluated. The effect of AZD1480 on cell-cycle distribution and apoptosis induction was studied. Antitumor effects of AZD1480 in tumor xenografts were assessed.

Results: AZD1480 significantly inhibited growth of six out of 13 SCLC cells with IC50s ranging from 0.73 to 3.08 μmol/L. Knocking down of JAK2 and JAK1 inhibited proliferation of Jak2-positive/Jak1-negative H82 cells and Jak1-positive/Jak2-negative GLC4 cells, respectively. Treatment of SCLC cells with AZD1480 for 24 hours resulted in an increase of 4N DNA content and histone 3 serine 10 phosphorylation, indicative of G2–M phase arrest. Moreover, SCLCs underwent apoptosis after AZD1480 treatment as exemplified by the downregulation of MCL1, the accumulation of cleaved caspase 3, cleaved PARP, and increase of annexin-V–positive cells. Finally, xenograft experiments showed that AZD1480 attenuated the growth of H82 and GLC4 tumors in mice, and we observed stronger apoptosis as well as decreased CD31-positive endothelial cells in H82 and GLC4 xenografts upon AZD1480 treatment.

Conclusions: JAK inhibitor AZD1480 attenuated growth of SCLC cells in vitro and in vivo. Clinical development of anti-JAKs therapies in SCLC warrants further investigation. Clin Cancer Res; 19(24); 6777–86. © 2013 AACR.

Introduction

Small cell lung cancer (SCLC), a smoking related cancer, accounts for 10% to 15% of lung cancers (1). SCLC has the propensity to develop early and widespread metastatic disease. Although approximately 20% of patients with SCLC limited to the thorax can be cured by concurrent chemoradiotherapy, patients with metastatic disease are incurable. There has been very little progress in the medical treatment for SCLC for the past two decades (2, 3), and no major drug discovery after the 1990s (4). The genomic alterations present in SCLC have been recently described in detail (13–15). We previously reported copy number alterations of the SCLC genome from 33 SCLC tumors using array-based comparative genomic hybridization (aCGH) analysis (16). A high copy gain of the JAK2 gene, defined as the copy number of the gene in the tumor was more than 8-folds the copy of the gene in the reference genome, was observed in one SCLC tumor, and the copy number gain of the JAK2 gene, defined as the copy number of gene in the tumor was more than the copy number in the reference genome, was observed in around 30% of SCLC tumors and cell lines. In an independent aCGH analysis across several tumor types (17), copy number gain of the JAK2 gene was observed in 42.5% of SCLC specimens, and high-level amplification of the JAK2 gene was observed in 5% of SCLC (17). In addition, copy number gain of the JAK2...
Translational Relevance
Small cell lung cancer (SCLC) is characterized by rapid growth and high lethality. There has been only very limited progress in the treatment of SCLC during the past two decades, and this has been mainly due to introduction of radiation to the chest and brain. Chemotherapy with cisplatin and etoposide remains the standard first-line treatment and topotecan is the standard second-line treatment. Here, we demonstrated that inhibition of Janus-activated kinases (JAK) by either the small-molecule inhibitor AZD1480 or siRNAs attenuated growth of SCLC cells. AZD1480 induced apoptosis of SCLC cells in vitro and attenuated growth of SCLC xenografts in vivo. Given the limited therapeutic options for advanced SCLC, our findings may influence clinical development of medical treatment of SCLC.

Here, we investigated the activity of the JAK inhibitor AZD1480 and JAK1/2 siRNA inhibition in SCLC models.

Materials and Methods

Cancer cell lines
The following SCLC cells have been studied: GLC4, NCI-H69, NCI-H82, NCI-H128, NCI-H146, NCI-H187, NCI-H526, NCI-N592, NCI-H620, NCI-H678, NCI-H792, NCI-H1173, DMS-114, and AC-3. DMS-114 was obtained from American Type Culture Collection, and the other cell lines were obtained as previously described (16). Cells were maintained in RPMI containing 10% FBS with the exception of DMS-114, which was maintained in Waymouth’s media containing 10% FBS. The cells were not tested and authenticated by the authors and were passed less than 10 times since obtaining the cells.

Real-time PCR
The mRNA expression of the JAK2 gene in SCLC cells was evaluated using Taqman gene expression assay (Applied Biosystems) following the manufacturer’s instruction. The glyceraldehyde-3-phosphate dehydrogenase gene was used as endogenous control. Relative expression of the JAK2 gene of each cell line was analyzed using the 2^(-ΔΔCT) value method and was calibrated to the expression level of the NCI-H69 cell line.

The copy number of the JAK2 gene was determined using Taqman copy number assay (Applied Biosystems), following manufacturer’s instruction. The ribonuclease P RNA component H1 (RPPH1) gene was used as endogenous control, which was labeled by VIC probe. The copy number was analyzed using CopyCaller version 1 software (Applied Biosystems). The copy number of the JAK2 gene in the reference genomic DNA was defined as 2.

Western blot analysis
Western blot analysis was performed as described previously (30). Antibodies were obtained from Cell Signaling Technology (β-tubulin, JAK1, JAK2, STAT3, phospho-STAT3, PARP, cleaved caspase-3, phospho-histone 3 serine 10, and MCL1), and Sigma-Aldrich Corporation (Actin).

Growth inhibition assays
AZD1480 was provided by AstraZeneca and INC16562 by Incyte Corporation. Cisplatin and etoposide were obtained from Sigma-Aldrich. SCLC cells were treated with various concentrations up to 10 μmol/L of AZD1480, cisplatin, or etoposide for 72 hours. Cell viability was determined by the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). The IC_{50} was the drug concentration at which 50% of the cells were viable comparing with the untreated cells.

To determine the synergistic effect of AZD1480 and cisplatin or AZD1480 and etoposide, GLC4 cells were treated with AZD1480 and cisplatin or AZD1480 and etoposide at 1:1 ratio. The combination index (CI) value was determined using CompuSyn software 1.0 (CompuSyn Inc.). Synergy was defined as a CI value less than 1.0 (31).
**Sequencing JAK2 V617F in SCLC cells**

JAK2 V617F were sequenced in the AC3, GLC4, NCI-H128, NCI-H187, NCI-N592, NCI-H620, NCI-H678, NCI-H792, and NCI-H1173 SCLC cells using real-time PCR assay as described previously (32). The forward primer was 5'-TGTAAAACGAGCTGTGTCGACTACGGTCAACTGCATGAAA-3' and the reverse primer was 5'-CAGGAAAACGGCTATGACCCATGCCAACTGTTAGCCAC-3'. The JAK2 mutational status of the NCI-H69, NCI-H82, NCI-H146, NCI-H526, and DMS-114 SCLC was available from the Broad-Novartis Cancer Cell Line Encyclopedia database (33).

**siRNA transfection**

siRNAs were obtained from Ambion (JAK1 and JAK2) and Dharmacon (JAK2 and control siRNA). siRNA transfection was performed using Peptide reagent (SignaGen Laboratories), following the manufacturer’s instructions with a final siRNA concentration of 5 nmol/L. Protein was extracted 48 hours after transfection, and cell-cycle analyses were performed 72 hours after transfection.

**Flow cytometry**

Flow cytometry was used to study cell-cycle changes and apoptosis in SCLC cells upon AZD1480 treatment. Cell-cycle analysis was performed as previously described (30). In brief, cells were treated with AZD1480 at various concentrations for 24 hours, collected, and stained with propidium iodide before analysis on FACSCalibur (Becton, Dickinson and Company).

The occurrence of apoptosis was determined by the fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (Becton, Dickinson and Company) after cell treatment with AZD1480 at various concentrations for 48 hours.

**Immunofluorescence study**

Cells were incubated with various concentrations of AZD1480 for 24 hours. Two hundred thousand SCLC cells were collected, washed with PBS, then spun onto a slide at 800 rpm for 5 minutes using cytopsin. Spun cells were fixed with 2.5% paraformaldehyde for 10 minutes, permeated with cold 100% methanol for 10 minutes, and washed with 0.5% Triton X-100 for 2 minutes. Anti-phospho-histone 3 serine 10 rabbit antibody (1:1000, Cell Signaling Technology) was applied at room temperature for 3 hours, followed by anti-rabbit secondary antibody labeled by FITC for 90 minutes. Cellular DNA was then stained with 0.01% 4', 6-diamidino-2-phenylindole, and then cover slips were mounted.

**Xenograft studies**

Four million NCI-H82 or GLC-4 cells were injected subcutaneously in the flank of athymic nude mice. Once the xenograft reached 5 mm in diameter, mice were treated with either vehicle containing 0.5% hypermellose and 0.1% Tween-80 or AZD1480 60 mg/kg/d by oral gavage. Tumor size was measured every 3 days and calculated using the \( V = \frac{1}{2}L \times W^2 \) formula. Use of animals for the study was approved by NIH under protocol number MOB005.
Hematoxylin and eosin (H&E) stain and immunohistochemistry study of Ki-67 and CD31 in xenografts were performed as described previously (34). Anti-Ki-67 and anti-CD31 antibodies were from Santa Cruz and Novus Biologicals. Quantitations of necrosis [% necrotic cells in tumors; vehicle (n = 7) and AZD1480 (n = 6)] and Ki-67–positive cells [% Ki-67–positive cells in tumors; vehicle (n = 5) and AZD1480 (n = 6)] were performed by a pathologist (B. Kallakury.)

To evaluate the apoptosis, xenografts were lysed and the extent of apoptosis was determined using Caspase-Glo 3/7 Assay Systems (Promega).

Statistical analysis

We used the Spearman method to analyze correlations between variables. Comparisons of variables between two groups were performed using a Student t test and among three or more groups were performed using one-way ANOVA, followed by least significant difference test for post hoc analysis. P values less than 0.05 were regarded as significant.

Results

Expression of JAK2 is related to copy number of the gene in SCLC cells

We evaluated the expression of JAK1, JAK2, and their downstream molecule STAT3 as well as phospho-STAT3 in SCLC cells (Fig. 1A). Expression of JAK1 and JAK2 proteins was variable: little JAK1 expression was detected in H82 and H620 cells, and little JAK2 expression was detected in H187, GLC4, and AC3 cells. The expression of JAK2 mRNA and JAK2 protein were significantly associated with the copy number of the JAK2 gene (Fig. 1B and C and Supplementary Figure 2.)
Figure 3. G2–M cell-cycle arrest in SCLC cells upon AZD1480 treatment. A, cell-cycle analysis of N592, GLC4, and H82 cells upon AZD1480 treatment (percentages of each cell-cycle stage were averages of triplicate experiments). The images are representative cell-cycle analysis of N592 cells upon AZD1480 treatment. B, Western blot analysis of histone 3 serine 10 (H3S10) phosphorylation in N592 cells upon AZD1480 treatment. C, phospho-H3S10-positive N592 cells, determined by immunofluorescence upon AZD1480 treatment (100 cells were analyzed per each low-power field, and six low-power fields were analyzed per each concentration, vertical bar: SD). P = 0.004 among groups by one-way ANOVA. D, CI in GLC4 cells upon treatment with cisplatin plus AZD1480 and etoposide plus AZD1480.
Table S1). Although JAK2 V617F mutations have been frequently observed in myeloproliferative disorders (23), mutations in JAK genes (JAK1, JAK2, JAK3, and TYK2) were reported in less than 5% of lung cancer specimens (Fig. 1D), and we did not observe JAK2 V617F mutation in the 14 SCLC cells studied.

Inhibition of Janus kinases decreases proliferation of a subset of SCLC cells

To explore the biologic importance of JAKs in SCLC, we treated SCLC cells with the pan-JAKs inhibitor AZD1480 (29). AZD1480 inhibited SCLC cell proliferation with an IC50 between 0.7 to 3.1 μmol/L in six out of 13 (46%) SCLC cell lines tested (Fig. 2A); no cytotoxic effect was reported in cancer cell lines of many solid tumors at these drug concentration (29). To test whether the inhibition of proliferation is AZD1480 specific, we treated N592 and H82 cells with INCB16562, a JAK1/2 inhibitor (35). The IC50 to INCB16562 was 2.8 μmol/L for NCI-N592 cells and 3.0 μmol/L for NCI-H82 cells (Fig. 2A), suggesting that proliferation of a portion of SCLC cells were abrogated by JAK inhibitors. The sensitivity to AZD1480 was unrelated to the protein expression of JAK1 (r = 0.18, P = 0.55 by Spearman method), JAK2 (r = −0.20, P = 0.50), or STAT3 (r = 0.10, P = 0.73; Supplementary Fig. S1). Upon AZD1480 treatment, STAT3 phosphorylation was inhibited in the AZD1480-sensitive GLC4 and NCI-N592 cells at a dose-dependent manner (Fig. 2C). For AZD1480-resistant cells, STAT3 phosphorylation was inhibited in the NCI-H128 cells but not in the NCI-H187 cells (Fig. 2B).

We further explored whether inhibition of JAKs attenuates proliferation of SCLC cells directly. Proliferation of NCI-H82 cells, which express JAK2 but little JAK1 proteins (Fig. 1B), was reduced upon treatment of the cells with two JAK2 siRNAs, compared with control siRNA (Fig. 2C). JAK1 siRNA did not influence expression of JAK2 protein in the NCI-H82 cells (Supplementary Fig. S2A), and JAK1 siRNA resulted in less inhibition of the proliferation of cells in comparison (Supplementary Fig. S2B).

In GLC4 cells, which express JAK1 but little JAK2 proteins (Fig. 1B), STAT3 was dephosphorylated upon JAK1 knockdown; proliferation of GLC4 cells was reduced upon treatment of cells with two JAK1 siRNAs compared with control siRNA (Fig. 2D). JAK2 siRNA did not influence expression of JAK1 protein as well as phospho-STAT3 protein in the GLC4 cells (Supplementary Fig. S2C), and it resulted in less inhibition of the proliferation of the cells (Supplementary Fig. S2D).
AZD1480 induces G2–M cell-cycle arrest and apoptosis in SCLC cells

AZD1480 caused significant dose-dependent accumulation of 4N DNA contents in NCI-N592, GLC4, and NCI-H82 cells (Fig. 3A), indicating G2–M arrest. Noticeably, a small portion of G2–M cells was in mitosis as exemplified by the dose-dependent increase of phospho-histone 3 serine 10 in N592 cells, as demonstrated by Western blot analysis (Fig. 3B) and immunofluorescence (Fig. 3C). Interestingly, although we did not observe changes in the cell cycle when we knockeddown JAK1 by siRNA in the GLC4 cells and JAK2 in the NCI-H82 cells, we observed increase of G2–M portion of the cells upon knocking down both JAK1 and JAK2 simultaneously in the cells (Supplementary Fig. S2E and S2F).

Because the cells arrested in G2–M by AZD1480 may survive from the acute drug treatment, we tested whether the combination with cisplatin or etoposide may show any synergistic inhibitory effect on SCLC cells. We observed that most CI values were less than 1.0 when AZD1480 was combined with either cisplatin or etoposide in GLC4 cells (Fig. 3D), suggesting a synergistic effect.

After AZD1480 treatment for 48 hours, we observed decreased expression of Mcl-1, a downstream molecule of STAT3, in N592 and GLC4 cells (Fig. 4A). We also demonstrated a dose-dependent decrease of uncleaved PARP expression as well as an increase of cleaved caspase-3 and PARP in NCI-N592 and GLC4 cells upon AZD1480 treatment, and an increase of cleaved caspase-3 in the NCI-H82 cells (Fig. 4B). In line with this result, an increase of Annexin-V–positive cells was also evident in NCI-N592 cells upon AZD1480 treatment (Fig. 4C and D). Taken together, these results suggest that AZD1480 induced apoptosis in SCLC cells.

AZD1480 attenuated the growth of SCLC xenografts

We evaluated the effect of AZD1480 in SCLC cells in vivo. We observed a decrease of xenograft growth upon AZD1480 treatment, in NCI-H82 cells (Fig. 5A) and GLC4 cells (Fig. 5B), compared with vehicle treatment. AZD1480 inhibited STAT3 phosphorylation in GLC4 xenografts (Fig. 5C).

On H&E staining, we observed trends of more necrotic cells in GLC4 as well as NCI-H82 xenografts treated with AZD1480 than vehicle. (Fig. 6A). We did not observe difference of Ki-67 staining between vehicle- and AZD1480-treated SCLC xenografts (43.6% and 39.2% in average in vehicle treated and AZD1480 treated, respectively, GLC4 xenografts, P = 0.34; 55% and 52% in NCI-H82 xenografts, P = 0.39; Fig. 6B). We also detected stronger signals of cleaved caspase-3/7 in AZD1480-treated SCLC xenografts, suggesting more apoptic cells in AZD1480-treated xenografts (P = 0.004 for GLC4 xenografts and P = 0.004 for NCI-H82 xenografts; Fig. 6C). We further detected decrease of CD31-positive endothelial cells in AZD1480-treated SCLC xenografts (P = 0.06 for GLC4 xenografts and P < 0.001 for NCI-H82 xenografts; Fig. 6D); our findings are consistent with a previous report of antiangiogenic effect of AZD1480 in vivo (36).
Here, we provide preclinical evidence of a potential value of JAKs as therapeutic targets in SCLC. AZD1480 was cytotoxic to SCLC cells at submicromolar concentrations. Incubation of SCLC cells with AZD1480 resulted in G2–M cell-cycle arrest and apoptosis. Finally, AZD1480 inhibited growth in vivo in two SCLC xenograft models. We also observed more necrosis and apoptosis in AZD1480-treated SCLC xenografts.

SCLC is characterized by a high number of genomic alterations and somatic gene mutations (14, 15). The mutation rate in SCLCs was 5.5 to 7.4 protein-changing mutations per million base pairs, which is higher than other solid tumors such as breast cancer, ovarian cancer, prostate cancer, and renal cancer (14, 15). Even though some candidate oncogenic drivers have recently been identified (14, 15), most of the mutated genes are not druggable. We searched the Catalogue of Somatic Mutations in Cancer (COSMIC) database and found that the frequencies of mutations in JAK1, JAK2, and JAK3 genes were very low: 4.8%, 0%, and 1.6%, respectively (Fig. 1D; ref. 37), and we did not detect JAK2 V617F mutation, a druggable mutation, in the 14 SCLC cells. Using next-generation sequencing techniques, Peifer and colleagues (14) and Rudin and colleagues (15) reported nonsynonymous mutations of the JAK1 gene in two out of 29 and one out of 42 SCLC specimens, respectively; no nonsynonymous mutation of the JAK2, JAK3, or TYK2 gene was detected in the two reports. Little is known on how these mutations influence the function of the proteins. High-level amplifications of JAK1 and JAK2 were reported in 2.5% and 5% of SCLC in the Tumorscape website, respectively (17), and we previously showed high copy amplification of the JAK2 gene in one out of 33 (3.3%) SCLC tumors (16). As we failed to observe correlations between sensitivity to AZD1480 and the expression of JAK1 or JAK2 proteins in SCLC cells, the factor(s) or target(s) predicting sensitivity to AZD1480 in SCLC remain unknown and warrant further studies.

We demonstrated that AZD1480 inhibits SCLC viability in vitro and xenograft growth in vivo. It has been reported that AZD1480 inhibited xenograft growth of multiple solid tumor cell lines at least partly through its antiangiogenic effect (29, 36), whereas only a few reports suggested direct cytotoxicity of AZD1480 in cancer cells. AZD1480 induced apoptosis in multiple myeloma cells by inhibiting JAK2 and FGFR3 (38). In solid tumors, McFarland and colleagues demonstrated that 10 μmol/L AZD1480 resulted in apoptosis of U251-MG glioblastoma cells (39). Here, we showed that AZD1480 induced G2–M cell-cycle arrest and apoptosis in SCLC cells at submicromolar concentrations (Figs. 3 and 4). SCLC is a neuroendocrine tumor (40) and is rather distinct from other carcinomas for several reasons. Genetically SCLC tumors carry more mutations than other carcinomas and displays typical cigarette-related G:C ! T:A transversions (15). Our study provides the first preclinical evidence of a potential value of JAKs as therapeutic targets in SCLC.
evidence that inhibition of JAK may be a valid strategy to explore for the treatment of SCLCs.

We observed that AZD1480 slowed SCLC xenograft growth but did not induce regression of the xenografts (Fig. 5). Similar findings were reported in other molecular targeted therapies to treat SCLC tumors when used alone. Shoemaker and colleagues demonstrated that the Bcl-2 family inhibitor navitoclax (ABT-263) induced xenograft shrinkage of H146, H889, and H1963 SCLC cells and inhibited tumor growth rate in eight other SCLC cell lines (41). Although the Hedgehog inhibitor NVP-LDE225 only slowed xenograft growth of LX22 SCLC cells when given alone, it significantly enhanced antitumor activity of carboplatin and etoposide (42). We also observed synergistic effects between AZD1480 and chemotherapeutic drugs such as cisplatin or etoposide in GLC4 cells (Fig. 3D). As SCLC tumors tend to grow rapidly, decreased growth rate of xenografts by AZD1480 monotherapy may not be translated into marked clinical benefit of patients with SCLC. The antitumor effect of AZD1480 in combination with traditional chemotherapies on the other hand warrants further investigation.

AZD1480 is a multikinase inhibitor with potencies against TrkA, JAK2, Aurora-A, and Flt4 (29). To exclude the antitumor effect of AZD1480 specific, we showed that INC16562, another JAK inhibitor, inhibited proliferation of SCLC cells at micromolar level. We showed that AZD1480 resulted in increased activities of caspase-3/7 and decreased number of CD31-positive endothelial cells in SCLC xenografts (Fig. 6D), suggesting that both direct cytotoxicity and antiangiogenesis may contribute to the effect of AZD1480 on SCLC xenografts (36). We also demonstrated that knocking down JAK1 and/or JAK2 in SCLC cells inhibited cell proliferation (Fig. 2C and D) as well as G2-M arrest of the cells (Supplementary Fig. S2E and S2F). Considering the potential off-target effects of siRNAs, we do not exclude the possibility that targets in addition to JAKs contribute to the effect of AZD1480 in SCLC cells. In conclusion, JAKs inhibitor AZD1480 attenuated SCLC growth in vitro and in vivo. Clinical development of anti-JAK therapies, including AZD1480, in SCLC is warranted.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: J.-H. Lee, Y. Wang, G. Giaccone
Development of methodology: G. Giaccone
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