Effects of the JAK2 inhibitor, AZ960, on Pim/BAD/BCL-xL survival signaling in the human JAK2 V617F cell line SET-2

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Running Title: Effects of AZ960 on JAK2 survival signaling in SET-2 cells

Keywords: JAK2 V617F, STAT3/5, tyrosine kinase inhibitors, MPDs, BCL-xL, Pim1

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The JAK2 V617F mutation is believed to play a critical role in the pathogenesis of polycythemia vera, essential thrombocytopenia and idiopathic myelofibrosis. We have characterized a novel small molecule JAK2 inhibitor, AZ960, and used it as a tool to investigate the consequences of JAK2 V617F inhibition in the SET-2 cell line. AZ960 inhibits JAK2 kinase with a $K_i$ of 0.00045 $\mu$M in vitro and treatment of TEL-JAK2 driven Ba/F3 cells with AZ960 blocked STAT5 phosphorylation and potently inhibited cell proliferation (GI$_{50}$ = 0.025 $\mu$M). AZ960 demonstrated selectivity for TEL-JAK2 driven STAT5 phosphorylation and cell proliferation when compared to cell lines driven by similar fusions of the other JAK kinase family members. In the SET-2 human megakaryoblastic cell line, heterozygous for the JAK2 V617F allele, inhibition of JAK2 resulted in decreased STAT3/5 phosphorylation and inhibition of cell proliferation (GI$_{50}$ = 0.033 $\mu$M) predominately through the induction of mitochondrial-mediated apoptosis. We provide evidence that JAK2 inhibition induces apoptosis by direct and indirect regulation of the anti-apoptotic protein BCL-xL. Inhibition of JAK2 blocked BCL-xL mRNA expression resulting in a reduction of BCL-xL protein levels. Additionally, inhibition of JAK2 resulted in decreased Pim1/2 mRNA expression. Decreased Pim1 mRNA corresponded with a decrease in Pim1 protein levels and inhibition of BAD phosphorylation at Ser112. Finally, siRNA-mediated suppression of BCL-xL resulted in apoptotic cell death similar to the phenotype observed following JAK2 inhibition. These results suggest a model in which JAK2 promotes cell survival by signaling through the Pim/BAD/BCL-xL pathway.

INTRODUCTION

The Janus-associated kinase (JAK) family, comprised of four different protein tyrosine kinases JAK1, JAK2, JAK3, and TYK2, plays an important role in cellular survival, proliferation, and differentiation (1). Several groups have identified a unique acquired mutation in the JAK2 gene encoding a valine-to-phenylalanine substitution, V617F, that results in constitutive kinase activity and has been shown to promote deregulated hematopoiesis (2-4). JAK2 V617F is frequently detected in myeloproliferative disorders (MPDs), a group of clonal hematopoietic stem cell disorders that include polycythemia vera (PV), essential thrombocythemia (ET), and idiopathic myelofibrosis (IMF), all of which have the potential to transform to acute myeloid leukemia (AML; ref. (2)). JAK2 V617F is constitutively phosphorylated and able to activate downstream signaling in the absence of cytokine stimulation when transfected into factor-dependent cell lines (4). Furthermore, several groups have shown that hematopoietic stem cell expression of JAK2 V617F in the mouse adoptive transfer model results in a
polycythemic phenotype followed by myelofibrosis, demonstrating a critical role for aberrant JAK2 signaling in the pathogenesis of the disease (2).

JAK kinases are key mediators of signaling downstream of a variety of cytokine and/or growth factor receptors. In particular, JAKs phosphorylate the signal transducers and activators of transcription (STAT) family of proteins (1,5). Once phosphorylated, STATs dimerize and translocate to the nucleus where they bind DNA and regulate expression of target genes (6-8). Aberrant STAT signaling has been reported in MPDs (9) and a range of both hematologic and solid cancers (7,10). JAK/STAT signaling has been implicated in driving both cell cycle regulation and anti-apoptotic pathways by controlling the transcription of key genes involved in these processes (6,7).

The anti-apoptotic protein BCL-xL plays an important role in promoting cell survival and is a key transcriptional target of STAT3/5 (8,11,12). BCL-xL has been shown to play a role in normal megakaryocytopoiesis by regulating the survival of megakaryocytes and mature platelets (13). A role for BCL-xL in the growth of factor-independent erythroid colonies has also been described (14). Furthermore, a recent study found that megakaryocytes from PV and IMF patients express greater levels of BCL-xL compared to normal cells, and that high levels of BCL-xL correlate with decreased levels of apoptosis in IMF megakaryocytes (15). These studies suggest that BCL-xL plays an important role in the pathogenesis of MPDs.

Pim kinases (Pim1, Pim2 & Pim3) are key regulators of cell survival and are suspected to play important roles in hematologic diseases (16-19). Accumulating evidence indicates that Pim kinases are controlled at the transcriptional level downstream of STAT signaling (17-19). Pim1 and 2 have been shown to play important roles in cell survival downstream of constitutively active Fms-like tyrosine kinase 3 (Flt3)-internal tandem duplication (ITD) signaling in leukemia cells (20,21), and a recent report has shown that the pro-apoptotic Bel-2 family member BAD, is a principle component for transmitting cell survival signals downstream of Flt3-ITD/Pim1 signaling (22). Upregulation of Pim kinases 1, 2, and 3 can repress the activity of BAD by phosphorylation at Serine 112 (23-27). Phosphorylation at this site acts as a gatekeeper maintaining BAD in an inactive state, thus promoting cell survival (28). Unphosphorylated BAD forms a heterodimer with BCL-xL, promoting the displacement of Bax from BCL-xL, and subsequent induction of mitochondrial outer membrane permeabilization and apoptosis (11,29,30).

The phenotypic effect of JAK2 inhibition has been previously characterized in HEL cells, a JAK2 V617F positive erythroleukemic cell line (31-34); however, the mechanism underlying JAK2 V617F-driven cell survival has not been extensively investigated. In the present study, we have characterized the pharmacology of a novel JAK2 inhibitor, AZ960 and utilized it as a tool to evaluate the consequence of JAK2 V617F inhibition in the SET-2 cell line (35). The human megakaryoblastic cell line, SET-2, was established from the peripheral blood of a patient with leukemic transformation of ET (35,36). These cells are heterozygous for the JAK2 V617F allele and are able to proliferate independently of exogenous cytokines. Our results provide evidence that JAK2 inhibition induces a loss in mitochondrial transmembrane potential (Δψm) and apoptosis by direct and indirect regulation of the anti-apoptotic protein BCL-xL. Inhibition of JAK2 signaling blocked STAT5-mediated regulation of BCL-xL mRNA levels and resulted in reduced BCL-xL protein expression. Knockdown of BCL-xL induced a phenotype similar to that seen following JAK2 inhibition. Additionally, inhibition of JAK2 blocked production of the Pim1/2 kinases, and resulted in a corresponding decrease in BAD phosphorylation. These results suggest that JAK2 signals through the BCL-xL axis by directly regulating its expression and indirectly stabilizing its anti-apoptotic activity through the regulation of BAD.
MATERIAL & METHODS

Reagents. AZ960 (S)-5-fluoro-2-(1-(4-fluorophenyl)ethylamino)-6-(5-methyl-1H-pyrazol-3-ylamino) nicotinonitrile was synthesized by AstraZeneca R&D (Waltham, MA). Stock solutions were diluted in dimethylsulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO). The following primary antibodies were used: phospho (Y694)-STAT5 (BD Transduction Laboratories, San Jose, CA), STAT5 (Epitomics, Burlingame, CA and Cell Signaling Technology, Danvers, MA), Pim1 (12H8) and BAD (H-168, Santa Cruz Biotechnology, Santa Cruz, CA), phospho (Tyr705) STAT3, STAT3, phospho (Ser112)-BAD, BAD, cleaved PARP 19F4 and BCL-xL (Cell Signaling Technology).

Cell culture. SET-2 cells were purchased from DSMZ (Braunschweig, Germany) and cultured in RPMI containing 10% Fetal Bovine Serum (FBS, Sigma) and 1% L-glutamine (Invitrogen). The kinase domains of the JAK family kinases (JAK1, JAK2, JAK3 and TYK2) were fused with the dimerization domain of TEL and transfected into Ba/F3 cells (37). All engineered Ba/F3 cells were cultured in RPMI containing FBS (10%) and IL-3 (1 ng/ml, R&D Systems, Minneapolis, MN). For all experimental procedures engineered TEL-JAK family member Ba/F3 cell lines were washed three times with media and plated in the absence of IL-3.

Proliferation assay. Cellular proliferation was evaluated using the fluorometric/colorimetric Biosource AlamarBlue Assay (Invitrogen, Carlsbad, CA) and read in the Spectra Max Gemini EM microplate reader (Molecular Devices, Sunnyvale, CA). SET-2 cells were plated at 20,000 cells/well, TEL-JAK2 Ba/F3 cells at 2000 cells/well and all other TEL-JAKs at 5000 cells/well in 96-well plates. Cells were treated with compound 24h after plating and grown for 72h for SET-2 and 48h for TEL-JAK Ba/F3 cells. Following the indicated growth period Alamar Blue (10µl/well) was added, cells were incubated at 37°C/5% CO₂ for 2h, and fluorescence was measured at 545nm (excitation) and 600nm (emission). Data are normalized to percent of the control, and GI₅₀ values (the concentration that causes 50% growth inhibition) were calculated using XLfit4 version 4.2.2 for Microsoft Excel.

Caspase 3/7 activity assay. SET-2 cells were plated in white-walled 96 well plates at 10,000 cells/well. Twenty-four hour later, cells were treated and Caspase Glo 3/7 reagent (Promega, Madison, WI) was added at the indicated times according to the manufacturer’s protocol. Caspase activity was measured in the Tecan Ultra 384 microplate reader (Durham, NC).

Fluorescence-activated cell sorting (FACS) Analysis. Cells were seeded at 0.5x10⁶ cells/ml and treated with either vehicle control (DMSO) or AZ960 24h later. Following the indicated incubations times, cells were collected by centrifugation, resuspended in PBS, and stained with tetramethylrhodamine ethyl ester (TMRE, 0.15 µM, Sigma-Aldrich, St. Louis, MO) for 30min at 37°C for the detection of mitochondrial Δψₘ. Cells were then washed with PBS, resuspended in Annexin V binding buffer (BD Bioscience, San Jose, CA) and stained with Annexin V-FITC (BD Bioscience) for 15min on ice. Cells were stained with Topro3 (Molecular Probes, Carlsbad, CA) just prior to FACS analysis on the FacsCalibur. Twenty thousand cells were counted and data was analyzed with FlowJo 7.1.3 software (Tree Star, Inc., Ashland, OR).

Cell transfection and RNA interference. SET-2 cells were transfected with siRNAs using the Amaxa Nucleofector (Amaxa, Gaithersburg, MD) according to the manufacturer’s protocol. Briefly, 5 x 10⁶ cells per sample were transfected with 1µM siRNA using Amaxa Solution-V and program X-13. A GFP-expressing plasmid (Amaxa) was used to determine transfection efficiency. Silencer GAPDH siRNA, Negative Control #1 siRNA, Silencer Validated JAK2 siRNAs (607, 608, 609), Silencer Validated BCL-xL siRNA
(120717) and Silencer Pre-designed BCL-xL siRNAs (6876, 120716) were purchased from Ambion (Austin, TX). Proliferation assays using transfected cells were performed as described above. Twenty-four hours after transfection cell number and viability were determined using the Cellometer Auto T4 (Nexcelom Biosciences, Lawrence, MA), cells were plated at 20,000 cells/well in 96 well plates, and incubated for 72 before Alamar Blue detection.

Western immunoblotting. Cells were plated at 0.5x10^6 cells/ml in 6-well plates and treated 24 h later. Cells were lysed with SDS buffer (0.06M Tris-HCL, 1% SDS, and 10% glycerol) and protein concentration was determined using a BCA Protein Assay (Pierce, Rockford, IL). Protein samples (50 µg) were loaded onto NuPage Novex Gels (Invitrogen) and separated by electrophoresis according to the manufacturer’s protocol. Separated proteins were transferred to NuPage Nitrocellulose Membranes (Invitrogen), blocked in 5% non-fat dry milk, and then incubated with primary antibody overnight according to the manufacturers’ guidelines. Membranes were incubated with either anti-rabbit or-mouse-HRP conjugated secondary antibody for 1h (1:5000, Santa Cruz Biotechnology), and then exposed to SuperSignal West Dura Extended Duration Substrate (Pierce). In the cases when the LiCor Odyssey Infrared detection system was used, Licor blocking buffer and Licor anti-mouse and – rabbit-fluorescent conjugated secondary antibodies (1:15000, Licor Biosciences, Lincoln, NE) were used. Protein expression was quantified using the Licor Odyssey. IC50 values (the concentration that causes 50% inhibition) were calculated by plotting percent inhibition of the phospho-signal normalized to total protein signal using Xlfit4 version 4.2.2 for Microsoft Excel.

Gene expression. Total RNA was isolated from cells using the Qiagen Rneasy kit (Qiagen) and quantified using the Agilent Bioanalyzer (Agilent). Total RNA (50 ng) was reverse transcribed and amplified using AgPath One step RT-PCR reagents (Ambion) in the 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. TaqMan gene expression assays (Applied Biosystems) containing gene specific primer/probe sets for the following genes: Pim1 (NM_002648), Pim2 (NM_006875), Pim3 (NM_001001852), BCL-xL (NM_138578), and HPRT (NM_000194) were used for the detection of mRNA levels. Expression levels of tested genes were normalized to HPRT expression using the comparative Ct method (Applied Biosystems).

Enzyme biochemical assay and kinase profiling. Inhibition studies of AZ960 were performed using a recombinant JAK2 kinase (aa. 808-1132, Millipore, catalog # 14-640) at a peptide (Tyk2 peptide, Cell Signaling Technologies, MA, USA) concentration of 100nM and an ATP concentration of 15 µM. Concentrations of AZ960 ranging from 0.003 to 30 µM were used. The mode of inhibition (MOI) and inhibition constant (K_i) of AZ960 against JAK2 kinase were further evaluated by inhibition kinetics. Specifically, a series of JAK2 catalyzed reactions were set up in HEPES buffer (75mM, pH7.3) with a fixed concentration of peptide (FL-Ahx-IPTSPTTYFFKKK-COOH, Primm Biotech, MA, USA), and varied concentrations of ATP and AZ960. Each reaction’s progress was subsequently monitored by Caliper LC3000 system (Caliper Life Sciences, MA, USA), and the initial velocity of each reaction was extracted from the corresponding reaction time course. To define the MOI, initial velocities were plotted against corresponding ATP concentrations using Lineweaver-Burke plot and the characteristic convergence of the lines on y-axis demonstrated the competitiveness of AZ960 to ATP. Initial inspection of K_i by using Michaelis-Menten equation revealed that AZ960 is a tight-binding inhibitor of JAK2. Therefore, to precisely determine Ki of AZ960, the same set of data was fitted to Morrison’s equation (Eq. 1), and the resulting apparent K_i (K_i app) values were subsequently
fitted to equation 2 to determine the $K_I$ of AZ680 against JAK2 kinase.

$$vi/v0=1-(E+I+K_{app})/(E+I+K_{app}+A^*E*I)/2*E........Eq1$$

$$K_{app}=(S+Km)/(K_{in}+S/(a*K_I))...................Eq2$$

Where $vi$ is the initial velocity of a reaction with the inhibitor and $v0$ is that without the inhibitor; $E$ is the effective enzyme concentration; $I$ is the concentration of the inhibitor; $S$ is the concentration of ATP; $Km$ is the Michealis-Menten constant of ATP; and $a$ is the Dixon factor.

AZ960 was profiled against 83 kinases at three inhibitor concentrations (0.01, 0.10 and 1.0 µM) by Upstate Biotechnology (Billerica, MA) according to the manufacturers’ protocol.

Statistical analyses. Data were analyzed and graphed with either GraphPad prism version 2.01 (GraphPad Software, Inc., San Diego, CA) or Xlfit4 version 4.2.2 for Microsoft Excel. One-way analysis of variance and post hoc Bonferroni comparison statistical tests were used. Significance was set at $P < 0.05$.

RESULTS

AZ960 is a potent and selective inhibitor of JAK2. The pyrazolo nicotinonitrile, AZ960 (Fig. 1A), is a tight-binding ATP competitive inhibitor of JAK2 enzyme activity with a $K_I$ of 0.00045 µM. In enzymatic assays carried out at $K_m$ levels of ATP, AZ960 inhibited JAK2 enzyme activity with an $IC_{50}$ of <0.003 µM. JAK3 enzyme activity showed an $IC_{50}$ of 0.009 µM, demonstrating greater than 3-fold selectivity of AZ960 for JAK2 over JAK3 at $K_m$ for ATP. The kinase selectivity profile of AZ960 was further evaluated against a panel of 83 protein kinases at three inhibitor concentrations (0.01, 0.10 and 1.0 µM). The kinases were selected to represent the diversity of the kinase-based on kinase binding site similarity and the gatekeeper residue, a major determinant of small molecule kinase selectivity. AZ960 inhibited 11 kinases by greater than 50% at a concentration of 0.1 µM (Table 1). AZ960 inhibited 31 kinases less than 50% and showed no activity against the remaining 41 protein kinases tested at a concentration of 0.1 µM (supplemental data, Table 1).

Selectivity within the JAK family of kinases was more thoroughly evaluated through the use of an isogenic cell line panel. The JH1 catalytic domains of JAK1, JAK2, JAK3 and TYK2 were fused with the oligomerization domain of the protein TEL, resulting in constitutive activation of the kinase activity and transformation of the Ba/F3 cell line (normally IL-3 dependent; ref. (37). These cell lines were used to measure the downstream phosphorylation of STAT5 and IL-3 independent proliferation in response to inhibitor treatment. AZ960 inhibited the phosphorylation of STAT5 in TEL-JAK2 cells with an average $(n=3)$ $IC_{50}$ of 0.015 ± 0.006 µM (Fig. 1B). AZ960 demonstrated 15 to 30-fold selectivity for TEL-JAK2-driven STAT5 phosphorylation compared to cell lines driven by other JAK kinase family members (TEL-JAK1, -JAK3 and -TYK2, Fig. 1B). Inhibition of TEL-JAK2-mediated STAT5 phosphorylation by AZ960 was closely correlated with potent inhibition of TEL-JAK2 driven cell proliferation, with an average $(n=4)$ $GI_{50}$ of 0.025 ± 0.001 µM (Fig. 1C). AZ960 was less potent in inhibiting the proliferation of the TEL-JAK1, -JAK3 and -Tyk2 cell lines with $GI_{50}$s of 0.230 ± 0.074, 0.279 ± 0.029 and 0.214 ± 0.151 µM, respectively (Fig.1C). Thus, AZ960 demonstrates nearly 10-fold selectivity compared to other JAK family members for cellular proliferation, consistent with inhibition of STAT5 phosphorylation.

Effects of JAK2 inhibition on STAT signaling and proliferation in SET-2 cells. The human megakaryoblastic cell line SET-2 is heterozygous for the JAK2 V617F mutation, and was used as a model to evaluate the effects of JAK2 inhibition on cell signaling and proliferation. SET-2 cells were treated with increasing concentrations of AZ960 and STAT3 and STAT5 phosphorylation were evaluated by western immunoblotting. As shown in Figure 2A, a dose-dependent decrease in both STAT3 and STAT5 phosphorylation levels was observed, with average $(n=3)$ $IC_{50}$s of 0.014 ± 0.007 and 0.022
± 0.009 µM, respectively. To determine the effects of JAK2 inhibition on SET-2 cell proliferation, the cells were treated with increasing concentrations of AZ960 for 72h. Similar to the effects on STAT3/5 phosphorylation, AZ960 potently inhibited SET-2 cell proliferation with an average (n=3) GI50 of 0.033 ± 0.020 µM (Fig. 2B), consistent with its activity in the TEL-JAK2 Ba/F3 cell line.

To confirm that the observed pharmacology of AZ960 in SET-2 cells is dependent on JAK2 inhibition, three siRNAs directed against JAK2 were evaluated for their effect on signaling and proliferation. Both a non-silencing siRNA (NS) and GAPDH siRNA were used as negative controls. JAK2 siRNA 1 and 2 showed a marked decrease in JAK2 protein expression as well as STAT5 phosphorylation, whereas JAK2 siRNA 3 had no effect on either JAK2 protein expression or STAT5 phosphorylation, thus JAK2 siRNA 3 provided an additional negative siRNA control (Fig. 2C). siRNA-mediated silencing of JAK2 by siRNAs 1 and 2 significantly reduced SET-2 cell proliferation as compared to the non-silencing control (p<0.05, Fig. 2D), similar to AZ960 (Fig. 2B). Taken together, these data indicate that inhibition of JAK2 in SET-2 cells results in inhibition of STAT phosphorylation and cellular proliferation. In addition, both AZ960 and JAK2-siRNA treatments result in a net cell loss that suggests induction of cell death.

Inhibition of JAK2 induces apoptotic signals in SET-2 cells. Several lines of evidence have indicated that JAK2 inhibition promotes cell cycle arrest and/or apoptosis (32-34). To better understand the phenotype of JAK2 inhibition, we evaluated the DNA content in SET-2 cells in response to AZ960. We found no increase in the G1 cell population, suggesting that AZ960 is not causing cell cycle arrest in SET-2 cells. However, AZ960 did induce a dose-dependent increase in the sub-G1 cell population at 48h (supplemental Fig. 1), suggesting that AZ960 is inducing cell death in SET-2 cells. To evaluate whether this involved the induction of apoptotic pathways, AZ960-treated SET-2 cells were assayed for caspase 3/7 activity and PARP cleavage. Treatment of SET-2 cells with AZ960 demonstrated both a time- and dose-dependent increase in caspase 3/7 activity and PARP cleavage (Fig. 3A), with significant induction of apoptosis detected within 16h of drug treatment as compared to the vehicle control (p<0.05, Fig. 3A, left panel), and at a concentration of 0.30 µM of AZ960 as compared to the vehicle control (p<0.05, Fig. 3A, right panel). To confirm that the observed induction of apoptosis by AZ960 in SET-2 cells was dependent on JAK2 inhibition, we examined PARP cleavage in SET-2 cells transfected with three siRNA’s directed against JAK2. Treatment of SET-2 cells with JAK2 siRNA 1 and 2 resulted in increased PARP cleavage, whereas JAK2 siRNA 3 did not have an affect on PARP cleavage (Fig. 3B). These results are consistent with the JAK2-siRNA proliferation data (Fig. 2), and suggest that JAK2 inhibition induces cell death through the induction of apoptosis in SET-2 cells.

BCL-xL is regulated by JAK2 and is important for cell survival. The anti-apoptotic protein BCL-xL is a well-known STAT3/5 transcriptional target (8,12), and may play a key role in the pathogenesis of MPDs by driving cell survival (14,15). Evaluation of BCL-xL gene expression showed a significant decrease in mRNA levels at both 4 and 24h after treatment with AZ960 as compared to the vehicle control (p<0.05, Fig. 4Ai). BCL-xL protein expression was only partially decreased 24 h after AZ960 treatment (Fig. 4Aii) and 48 h after treatment with JAK2 siRNA 1 and 2 (Fig. 4Aiii). The modest decrease in protein expression correlates with the 50% decrease in mRNA levels observed at 24h (Fig. 4Ai). Next, we sought to evaluate the impact of decreased BCL-xL expression on SET-2 cell survival by using siRNA-mediated suppression of BCL-xL. As shown in figure 4B, three siRNAs targeting BCL-xL markedly diminished BCL-xL protein expression and induced PARP cleavage in SET-2 cells. Decreased BCL-xL expression and induction of PARP cleavage is evident
within 24 h after transfection of the siRNAs (Fig. 4Bi), with near complete ablation of BCL-xL expression, and cleavage of PARP, by 48 h (Fig. 4Bii). The modest decrease seen in both JAK2 and actin levels may be a result of cell death. siRNA-mediated suppression of BCL-xL inhibited SET-2 cell proliferation (Fig. 4C), with notable cell killing, as observed for JAK2 inhibition (Fig. 2B and D).

**JAK2 regulates Pim1 and Pim2 expression and BAD phosphorylation in SET-2 cells.** Our studies show that JAK2 regulates BCL-xL expression and that BCL-xL plays a pivotal role in SET-2 cell survival. To better understand the mechanisms of AZ960-induced apoptosis and cell death we sought to determine if JAK2 signaling regulates BCL-xL function in addition to its expression. Pim survival kinases (Pim1, 2 and 3) have been implicated in the pathogenesis of hematologic and solid tumors and have been identified to be STAT target genes (16-19). All three Pim kinases are expressed in SET-2 cells, with the transcriptional level of Pim1 being greater than that of Pim2 and 3 (Fig. 5). As shown in Figure 5, the mRNA levels of Pim1 and Pim2 are significantly decreased in a dose-dependent manner in SET-2 cells following 4 and 24h of treatment with AZ960 as compared to the vehicle control (p<0.05). In contrast, no significant changes in Pim3 mRNA levels were observed in response to AZ960 treatment at either time-point (Fig. 5), suggesting that Pim1/2 are the primary Pim isoforms downstream of JAK2 signaling in SET-2 cells.

AZ960 disrupts mitochondrial Δψm and induces apoptosis in SET-2 cells. Since BCL-xL is known to mediate its anti-apoptotic effects through inhibition of mitochondrial cell death pathways (11), we evaluated whether inhibition of JAK2 signaling by AZ960 induced permeabilization of the mitochondrial membrane. FACS analysis was used to simultaneously measure the levels of phosphatidylserine (PS), an early marker of apoptosis detected with Annexin V, cell viability detected by the vital dye Topro3, and mitochondrial Δψm detected by the fluorescent dye TMRE. Since loss of mitochondrial Δψm is an early event in the apoptosis cascade, preceding increases in PS exposure (38), we assessed TMRE uptake in the viable (Annexin V-FITC negative/Topro3 negative) population. Treatment of SET-2 cells with AZ960 (0.3 μM) resulted in a 21, 31 and 56% decrease in the geometric mean of TMRE fluorescence at 16, 24 and 48h, respectively (Fig. 7A). AZ960 also increased the percentage of early apoptotic cells (Annexin V-FITC positive/Topro3 negative) from control levels of 5% to 12, 17 and 14% at 16, 24 and 48h, respectively (Fig. 7B). AZ960 also increased the percentage of early apoptotic cells (Annexin V-FITC positive/Topro3 negative) from control levels of 5% to 12, 17 and 14% at 16, 24 and 48h, respectively (Fig. 7B), and late apoptotic/necrotic cells (Annexin V-FITC positive/Topro3 positive) from control values of 30% to 37, 39 and 55% at 16, 24 and 48h,
respectively (Fig. 7B). Together, these data suggest that AZ960 induces cell death through the induction of mitochondrial-mediated apoptosis.

DISCUSSION

The discovery of the JAK2 V617F mutation in MPDs marked a major milestone in understanding the pathogenesis of these diseases. The JAK2 V617F mutation is detected in more than 95% of PV patients and in 50 to 60% of patients with ET or IMF as well as a minority of other MPDs and leukemias (2). The JAK/STAT signaling pathway has been implicated in modulating cell survival and apoptosis by regulating the gene expression of BCL-2 family members (1). Several studies have reported that dysregulation of these pathways are key drivers in the pathogenesis of hematologic diseases and malignancies (12,14,15,39,40). For instance, the aberrant expression of BCL-xL has been reported to contribute to the pathogenesis of both PV (14,40) and IMF (15). Data from our studies provide evidence that JAK2 promotes cell survival signals through the Pim/BAD/BCL-xL pathway and that inhibition of JAK2-mediated signaling through this pathway induces apoptosis in SET-2 cells.

In this report, we have characterized the pharmacology of AZ960, a potent inhibitor of JAK2 kinase activity. AZ960 selectively inhibited STAT5 phosphorylation and proliferation of TEL-JAK2 transformed Ba/F3 cells compared to cell lines containing other JAK-family-TEL fusion proteins. AZ960 and JAK2 siRNAs were utilized to examine JAK2 signaling in SET-2 megakaryoblastic cells heterozygous for the JAK2 V617F mutation. Both AZ960 and JAK2 siRNAs inhibited STAT5 phosphorylation and proliferation of SET-2 cells. Further phenotypic characterization of SET-2 cells demonstrated that pharmacologic inhibition of JAK2 kinase activity or loss of JAK2 protein results in the induction of apoptosis. AZ960 caused an increase in the sub-G1 cell population, and a near maximal induction of caspase 3/7 activity and PARP cleavage at 16h. Others have assessed the phenotype of JAK2 inhibition in JAK2 V617F-positive HEL cells, and reported either a G1 cell cycle arrest and/or induction of annexin-V positive, apoptotic cells (32-34,41). We did not observe a G1 cell cycle arrest in SET-2 cells treated with AZ960, suggesting the phenotypic outcome in response to JAK2 inhibition may vary in different cell types. The correspondence of small molecule and siRNA phenotypes further support the conclusion that our results are a direct outcome of blocking JAK2 signaling.

A key role for BCL-xL in megakaryocyte cell survival has recently been described in ex vivo analyses of megakaryocytes from IMF patients (15). BCL-xL is a well-known STAT3/5 target gene (8,12), whose mRNA and protein were shown here to be modulated by JAK2 inhibition. However, inhibition of JAK2 only resulted in a partial decrease in BCL-xL protein levels. To evaluate the possibility that BCL-xL may play a critical role in the survival of SET-2 cells downstream of JAK2 signaling we used siRNAs to knock down BCL-xL expression. siRNA-mediated suppression of BCL-xL induced cell death through the induction of apoptosis, similar to the effects of JAK2 inhibition with AZ960 or JAK2 siRNAs. Interestingly, only a partial decrease in BCL-xL expression is necessary to cause PARP cleavage, as can be seen 24 h after transfection of BCL-xL siRNAs, with greater responses at 48 h post-transfection (Fig. 4). These data coincide with the phenotypic responses observed in SET-2 cells following partial decreases in BCL-xL expression resulting from either AZ960 or JAK2 siRNA treatment. Together, these data suggest that SET-2 cells are highly dependent on BCL-xL for cell survival.

The BCL-2 family member BAD interacts with other anti-apoptotic family members such as BCL-xL to neutralize their protective effects and activate the apoptotic machinery. Therefore, we sought to examine mechanisms downstream of JAK2 that may act to regulate BAD activity. Pim kinases are important...
downstream mediators of cytokine signal transduction pathways (17-19), and Pim1 and 2 have been shown to be responsible for the survival of transformed hematopoietic cells (20,42). A recent study reported that Pim1 was overexpressed in JAK2 V617F-positive ET patients compared to JAK2 V617F-negative patients, further supporting a role for Pim kinases in the pathogenesis of JAK2 V617F-driven MPDs (43). We have shown that inhibition of JAK2 signaling decreased mRNA levels of Pim1 and Pim2, but not Pim3, in SET-2 cells. These data are in agreement with Adams and colleagues (2006) who reported that Pim1 and Pim2, but not Pim3, mRNA levels were increased in TEL-JAK2 transformed Ba/F3 cells, compared to parental cells (20). Pim kinases control the activity of several downstream effectors such as the pro-apoptotic BCL-2 family member BAD at Ser112 (16,18). Phosphorylation of BAD at Ser112 inhibits its pro-apoptotic activity, thus promoting cellular survival (29). We have shown that inhibition of JAK2 signaling in SET-2 cells, by either AZ960 or JAK2 siRNAs, inhibits BAD phosphorylation. Taken together, these observations suggest that inhibition of JAK2 signaling effects both BCL-xL expression and regulation via the STAT/Pim/Bad pathway, implying a dual mechanism at work.

BCL-2 family members are important regulators of the intrinsic mitochondrial pathway of apoptosis (30); therefore, we evaluated whether AZ960 affects mitochondrial outer membrane permeability. AZ960 treatment resulted in a decrease in mitochondrial Δψm in the viable cell population prior to the exposure of PS, an early marker of apoptosis detected by Annexin V staining. This was associated with an increase in apoptosis and cell death, demonstrated by an increase in the Annexin V only and Annexin V/Topro3 positive cell populations, respectively. These data indicate that inhibition of JAK2 signaling triggers apoptosis through the mitochondrial pathway.

In this study, we have characterized a small molecule JAK2 kinase inhibitor that can be used as a tool to investigate JAK2 signaling. Our study provides a mechanistic rationale for the induction of mitochondrial-mediated apoptosis resulting from JAK2 inhibition in JAK2 V617F megakaryoblastic SET-2 cells (Fig. 8). Inhibition of JAK2, by either AZ960 or JAK2 siRNAs, results in suppression of STAT3/5 phosphorylation and downregulation of the anti-apoptotic STAT target gene BCL-xL, leading to apoptosis and cell death. BCL-xL appears to be key to promoting JAK2-mediated survival in SET-2 cells, since direct knockdown of the protein by siRNAs recapitulates the JAK2 inhibition phenotype. Furthermore, JAK2 inhibition also results in downregulation of Pim1 and 2 expression and a corresponding decrease in phosphorylation of the Pim kinase substrate BAD, known to inactivate BCL-xL in its unphosphorylated state. Thus, JAK2 survival signaling in SET-2 cells may involve complementary STAT-regulated pathways converging on BCL-xL, which both induce expression of the anti-apoptotic protein and stabilize its function by Pim1/2-mediated inhibitory phosphorylation of BAD.

ACKNOWLEDGMENTS

We thank Kevin Webster for his critical reading of the manuscript and helpful suggestions.
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Figure 1. Cellular selectivity of AZ960 for JAK family kinases. Ba/F3 cells were engineered to express constitutively active JAK kinases by fusing the kinase domain of JAK1, JAK2, JAK3 and TYK2 with the dimerization domain of TEL. (A) Chemical structure of AZ960 \( (C_{18}H_{16}F_2N_6) \). (B) TEL-JAK cells were treated with the indicated concentrations of AZ960 for 1 h and the levels of phospho-STAT5 were determined by western immunoblotting. Signal intensity was quantified using Licor Odyssey software. IC\(_{50}\) values were calculated from three independent experiments. (C) TEL-JAK cells were plated in 96-well plates, treated 24 h later with AZ960, and incubated for 48 h. Cell proliferation was determined using the Alamar Blue assay. Data is shown as mean of triplicates (±SD) across three separate plates from one representative experiment.

Figure 2. Inhibition of STAT3 and 5 phosphorylation and cell proliferation in SET-2 cells. (A) SET-2 cells were treated with AZ960 for 1 h and cell lysates were probed for STAT3 and 5 phosphorylation by western immunoblotting. Signal intensity was quantified using the Licor Odyssey and IC\(_{50}\) values were generated from three independent experiments. (B) SET-2 cells were plated in 96-well plates, treated 24 h later with AZ960, and incubated for 72 h. Cell proliferation was determined using the Alamar Blue assay. The mean of triplicates (±SD) across three separate plates from one representative experiment is shown. GI\(_{50}\) values were calculated from four independent experiments. (C) Three siRNAs targeting JAK2 were transfected into SET-2 cells using the Amaya Nucleofector system and protein expression was determined 48 h later. Both a non-silencing siRNA (NS) and GAPDH siRNA were used as negative controls. JAK2 siRNA 3 had no effect on JAK2 expression providing an additional negative siRNA control. (D) SET-2 cells were transfected with JAK2 siRNAs and triplicate samples were plated at 20,000 cells/well, in 96-well plates 24 h later. The cells were incubated for 72 h and proliferation was determined using the Alamar Blue assay. The mean of triplicates (±SD) is shown from a representative experiment. Experiments were repeated at least three times with similar results.

Figure 3. Inhibition of JAK2 induces apoptotic signals in SET-2 cells. (A) SET-2 cells were plated in 96-well plates, treated with AZ960 24 h later, and then incubated for the indicated times. Caspase 3/7 activity was measured using a Caspase Glo 3/7 luminescent assay, and PARP cleavage was determined by western immunoblotting with an antibody that detects both total and cleaved PARP (Cl-PARP). Cells were treated with either AZ960 (0.300 µM) for the indicated times (left panel) or at the indicated concentrations for 24 h (right panel). Caspase 3/7 activity is expressed relative to control and is shown as the mean of three independent experiments (±SD). PARP cleavage was assayed in three independent experiments with similar results; a representative image is shown. (B) SET-2 cells were transfected with JAK2 siRNAs, incubated for 48 h, and lysates were immunoblotted for total and cleaved PARP.

Figure 4. BCL-xL expression is regulated by JAK2 and is pivotal for SET-2 cell survival. (A) SET-2 cells were treated with the indicated concentrations of AZ960 and RNA was collected at 4 and 24 h. Relative BCL-xL mRNA levels were determined using real-time RT-PCR. The mRNA levels of BCL-xL were normalized to the mRNA levels of HPRT using the comparative Ct method according to the manufacturer’s protocol. Data are expressed as the mean (±SD) of two independent experiments in relative mRNA levels (arbitrary units, i). BCL-xL protein expression was assessed by western immunoblotting in SET-2 cells following treatment with either AZ960 for 24 h (ii) or JAK2-siRNAs for 48 h (iii). (B) SET-2 cells were transfected with BCL-xL siRNAs and incubated for either 24 or 48 h. Lysates were immunoblotted with the indicated antibodies. A representative image is shown from two independent experiments. Both a non-silencing siRNA (NS) and GAPDH siRNA were used as negative controls. (C) SET-2 cells
were transfected with BCL-xL siRNAs and triplicate samples were plated at 20,000 cells/well, in 96 well plates 24h later. The cells were incubated for 72h and proliferation was determined using the Alamar Blue assay. The mean of triplicate is shown from a representative experiment. NS: non-silencing siRNA control.

Figure 5. Pim1 and Pim2 gene expression is inhibited by AZ960 -mediated JAK2 inhibition. SET-2 cells were treated with the indicated drug concentrations and total RNA was isolated from cells at either 4 or 24h after treatment. Relative mRNA levels were determined using real time RT-PCR with ABI TaqMan gene expression assays. The mRNA levels of Pim1, 2 and 3 were normalized to the mRNA levels of HPRT using the comparative Ct method according to the manufacturer’s protocol. Data are expressed as relative mRNA levels (arbitrary units) and shown as mean (±SD) of two independent experiments.

Figure 6. Inhibition of JAK2 signaling blocks BAD phosphorylation in SET-2 cells. SET-2 cells were treated with either (A) AZ960 (0.300 µM) for the indicated times or (B) with AZ960 for 24 h before cell lysates were collected and probed with the indicated antibodies by western immunoblotting. SET-2 cell samples analyzed following treatment with AZ960 for 24 h shown in part B were the same samples tested for BCL-xL expression in Figure 4Aii. (C) SET-2 cells were transfected with JAK2-siRNAs, incubated for 48h, and lysates were immunoblotted with the indicated antibodies. NS: non-silencing siRNA control. A representative image from three independent experiments is shown.

Figure 7. AZ960 decreases Δψm and induces apoptosis in SET-2 cells. Cells were treated with AZ960 (0.3µM) for the indicated times, triple stained with Annexin V, Topro3 and TMRE, and then analyzed by flow cytometry. (A) Mitochondrial Δψm was evaluated by measuring the geometric mean of TMRE fluorescence in the viable cell population (Annexin V-FITC negative/Topro3 negative). (B) Percent of early apoptotic cells (Annexin V-FITC positive/Topro3 negative) and late apoptotic/necrotic cells (Annexin V-FITC positive/Topro3 positive) are shown. Experiments were repeated five times with similar results; representative data from one experiment is shown.

Figure 8. Model of JAK2-mediated survival signaling in SET-2 cells. Receptor-associated JAK2 phosphorylates STAT3 and 5, resulting in dimerization and translocation to the nucleus, where they regulate the expression of target genes. The survival kinases Pim1/2 and anti-apoptotic protein BCL-xL are upregulated by JAK2/STAT signaling. Pim1/2 may play a role in phosphorylating the pro-apoptotic protein BAD at Ser112, promoting binding of BAD to 14-3-3 proteins and its maintenance in an inactive state. Dephosphorylation of BAD in the absence of Pim1/2 activity results in heterodimerization with BCL-xL, and the displacement of Bax and/or Bak from BCL-xL. Free Bax/Bak oligomers induce mitochondrial permeabilization, release of cytochrome c, and induction of mitochondrial apoptotic signals through caspase activation. Thus AZ960 promotes apoptotic cell death in SET-2 cells by blocking JAK2-dependent pro-survival signals.
Table 1. Kinase selectivity profile of AZ960

| Kinase     | AZ960 | AZ960 | AZ960 |
|------------|-------|-------|-------|
|            | 0.01 µM | 0.10 µM | 1.0 µM |
| JAK2       | 4      | 3      | 3      |
| TrkA       | 9      | 2      | 1      |
| Aurora-A   | 27     | 18     | 6      |
| ARK5       | 35     | 6      | 3      |
| ALK        | 62     | 14     | 4      |
| CaMKII     | 71     | 29     | 6      |
| Flt4       | 76     | 22     | 2      |
| FGFR1      | 77     | 18     | 4      |
| LIMK1      | 78     | 38     | 10     |
| BrSK2      | 85     | 35     | 7      |
| FAK        | 87     | 17     | 6      |

AZ960 inhibited 31 of 83 kinases less than 50% at a concentration of 0.1 µM. AZ960 showed no activity on the remaining 41 kinases tested at a concentration of 0.1 µM (supplemental data, Table 1).
Figure 1

A.
Figure 1

B.

| AZ960 | 0 | 0.003 | 0.01 | 0.03 | 0.1 | 0.3 | 1.0 | 3.0 (µM) |
|-------|---|-------|------|------|-----|-----|-----|---------|
| Tel-JAK1 | P-STAT5 | T-STAT5 |
| Tel-JAK2 | P-STAT5 | T-STAT5 |
| Tel-JAK3 | P-STAT5 | T-STAT5 |
| Tel-TYK2 | P-STAT5 | T-STAT5 |
c. 

![Graph showing the effect of AZ960 (μM) on Percent Net Growth for different TEL-JAK proteins: TEL-JAK1, TEL-JAK2, TEL-JAK3, and TEL-TYK2. The x-axis represents AZ960 concentration (μM) ranging from 0.001 to 100, and the y-axis represents Percent Net Growth ranging from -25 to 125. The graph shows a decrease in Percent Net Growth as AZ960 concentration increases for all TEL-JAK proteins.]
Figure 2

A.

| AZ960          | 0 | 0.003 | 0.01 | 0.03 | 0.1 | 0.3 | 1.0 | 3.0 (µM) |
|----------------|---|-------|------|------|-----|-----|-----|----------|
| P-STAT3        |   |       |      |      |     |     |     |          |
| T-STAT3        |   |       |      |      |     |     |     |          |
| P-STAT5        |   |       |      |      |     |     |     |          |
| T-STAT5        |   |       |      |      |     |     |     |          |

B.

Percent Growth of Control

AZ960 (µM)
Figure 3

A.

Time post-AZ960 treatment (0.300μM) AZ960 treatment (24h)

Caspase 3/7 Activity (fold change over control)

| Time (h) | 4 | 8 | 16 | 24 |
|----------|---|---|----|----|
| AZ960 (µM) |
| 0.03 | ![Image](image1.png) | ![Image](image2.png) |
| 0.10 | ![Image](image3.png) | ![Image](image4.png) |
| 0.30 | ![Image](image5.png) | ![Image](image6.png) |

B.

| siRNAs | NS | Gap | 1 | 2 | 3 |
|---------|----|-----|---|---|---|
| JAK2    | ![Image](image7.png) | ![Image](image8.png) |
| PARP    | ![Image](image9.png) | ![Image](image10.png) |
| Cleaved PARP | ![Image](image11.png) | ![Image](image12.png) |
Figure 4

A.

i

![Bar graph showing BCL-xL mRNA levels](image)

BCL-xL mRNA levels (arbitrary units)

0 0.03 0.1 0.3 0 0.03 0.1 0.3

4h 24h

AZ960 (µM)

ii

| AZ960 | BCL-xL | Actin |
|-------|--------|-------|
| 0     | 0.03   | 0.1   | 0.3   |

iii

| siRNAs | JAK2 |
|--------|------|
| NS, Gap, 1, 2, 3 | BCL-xL, Actin |

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Figure 4

B.

i. 24h post-transfection

| siRNAs     | NS | Gap | 1 | 2 | 3 |
|------------|----|-----|---|---|---|
| BCL-xL     |    |     |   |   |   |
| PARP       |    |     |   |   |   |
| Cleaved PARP |   |     |   |   |   |
| JAK2       |    |     |   |   |   |
| Actin      |    |     |   |   |   |

ii. 48h post-transfection

| siRNAs     | NS | Gap | 1 | 2 | 3 |
|------------|----|-----|---|---|---|
| BCL-xL     |    |     |   |   |   |
| PARP       |    |     |   |   |   |
| Cleaved PARP |   |     |   |   |   |
| JAK2       |    |     |   |   |   |
| Actin      |    |     |   |   |   |

C.

![Fluorescence graph](image-url)
Figure 5

- **Pim1 mRNA levels**
  - AZ960 (μM): 0, 0.03, 0.1, 0.3
  - 4h: 600, 400, 200, 0
  - 24h: 600, 400, 200, 0

- **Pim2 mRNA levels**
  - AZ960 (μM): 0, 0.03, 0.1, 0.3
  - 4h: 50, 25, 5, 0
  - 24h: 75, 50, 25, 0

- **Pim3 mRNA levels**
  - AZ960 (μM): 0, 0.03, 0.1, 0.3
  - 4h: 110, 100, 100, 100
  - 24h: 110, 100, 100, 100

* indicates statistically significant differences.
Figure 6

A.

| Time post-AZ960 (0.30 µM) | 0 | 1 | 4 | 8 | 24 (h) |
|---------------------------|---|---|---|---|--------|
| P-STAT3                  |   |   |   |   |        |
| T-STAT3                  |   |   |   |   |        |
| P-STAT5                  |   |   |   |   |        |
| T-STAT5                  |   |   |   |   |        |
| Pim1                     |   |   |   |   |        |
| P-BAD                    |   |   |   |   |        |
| T-BAD                    |   |   |   |   |        |
| Actin                    |   |   |   |   |        |

B.

| AZ960 | 0 | 0.030 | 0.10 | 0.30 (µM) |
|-------|---|-------|------|----------|
| P-STAT5 |   |       |      |          |
| T-STAT5 |   |       |      |          |
| Pim1    |   |       |      |          |
| P-BAD   |   |       |      |          |
| T-BAD   |   |       |      |          |
| Actin   |   |       |      |          |
Figure 6

C.

| siRNAs      | NS | Gap | 1   | 2   | 3   |
|-------------|----|-----|-----|-----|-----|
| JAK2        |    |     |     |     |     |
| P-STAT5     |    |     |     |     |     |
| T-STAT5     |    |     |     |     |     |
| Pim1        |    |     |     |     |     |
| P-BAD       |    |     |     |     |     |
| T-BAD       |    |     |     |     |     |
| Actin       |    |     |     |     |     |
Figure 7

A.

| Treatment                  | TMRE Fluorescence (geometric mean) |
|----------------------------|-----------------------------------|
| Vehicle control (DMSO)     | 59                                |
| AZ960 16h                  | 47                                |
| AZ960 24h                  | 41                                |
| AZ960 48h                  | 26                                |

B.

Annexin V-FITC
Effects of the JAK2 inhibitor, AZ960, on Pim/BAD/BCL-xL survival signaling in the human JAK2 V617F cell line SET-2
Joseph M. Gozgit, Geraldine Bebernitz, Pankaj Patil, Minwei Ye, Julie Parmentier, Jiaquan Wu, Nancy Su, Tao Wang, Stephanos Ioannidis, Audrey Davies, Dennis Huszar and Michael Zinda

J. Biol. Chem. published online September 4, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M803813200

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