WP1066, a Novel JAK2 Inhibitor, Suppresses Proliferation and Induces Apoptosis in Erythroid Human Cells Carrying the JAK2 V617F Mutation

Srdan Verstovsek,1 Taghi Manshouri,1 Alfonso Quintás-Cardama,1 David Harris,1 Jorge Cortes,1 Francis J. Giles,1 Hagop Kantarjian,1 Waldemar Priebe,2 and Zeev Estrov1

Abstract Purpose: The discovery of an activating somatic mutation in codon 617 of the gene encoding the Janus kinase (JAK)-2 (JAK2 V617F) in patients with myeloproliferative disorders has opened new avenues for the development of targeted therapies for these malignancies. However, no effective JAK2 inhibitors are currently available for clinical use.

Experimental Design: We investigated the activity of (E)-3-(6-bromopyridin-2-yl)-2-cyano-N’-(S0-1-phenylethyl)acrylamide (WP1066), a novel analogue of the JAK2 inhibitor AG490, in JAK2 V617F – positive erythroleukemia HEL cells and in blood cells from patients with polycythemia vera.

Results: We found that WP1066 significantly inhibited JAK2 and its downstream signal transducer and activator of transcription-3, signal transducer and activator of transcription-5, and extracellular signal-regulated kinase-1/2 pathways in a dose- and time-dependent manner. As a result, WP1066 concentrations in the low micromolar range induced time- and dose-dependent antiproliferative and proapoptotic effects in HEL cells. As expected, WP1066 inhibited the proliferation of peripheral blood hematopoietic progenitors of patients with polycythemia vera carrying the JAK2 V617F mutation in a dose-dependent manner.

Conclusions: Our data suggest that WP1066 is active both in vitro and ex vivo and should be further developed for the treatment of neoplasms expressing the JAK2 V617F mutation.

The myeloproliferative disorders polycythemia vera, essential thrombocythemia, and chronic idiopathic myelofibrosis are a group of heterogeneous diseases arising from the clonal transformation of a hematopoietic stem cell (1–3). A dominant gain-of-function mutation in the Janus kinase 2 (JAK2) gene in patients with myeloproliferative disorders, involving the substitution of valine for phenylalanine at position 617 within the JH2 domain of JAK2 (JAK2-V617F), was the first acquired somatic mutation to be described in these disorders (4–8). The V617F mutation induces constitutive activation of JAK2 (9) and its downstream signal transducer and activator of transcription (STAT)–5–mediated transcription, and as a result, growth factor independence and erythropoietin hypersensitivity (5, 7). The incidence of the JAK2 V617F mutation in patients with myeloproliferative disorders, as determined by allele-specific PCR, was 35% to 50% in chronic idiopathic myelofibrosis, 32% to 57% in essential thrombocythemia, and 74% to 97% in polycythemia vera (4, 7).

The four mammalian JAKs (JAK1, JAK2, JAK3, and TYK2) play an essential role in cytokine signaling in hematopoietic cells (9). JAK activation is mediated by ligand-induced aggregation of cytokine receptor–associated JAKs and their subsequent autophosphorylation at specific tyrosine residues. These phosphorylated tyrosine residues serve as docking sites for SH2 domain–containing proteins of the STAT family (9, 10). On binding of cytokines such as erythropoietin, interleukin-3, and granulocyte-macrophage colony stimulating factor, STAT monomers are phosphorylated by JAK2 and dimerize and translocate to the nucleus, where they modulate gene transcription. Deregulation of the JAK-STAT pathway has been observed to promote cell growth and prevent apoptosis in a variety of solid tumors and myeloid leukemias (11). Hence, JAK2 inhibitors are being actively sought for possible development as anticancer therapy.

Nevertheless, no effective inhibitors of JAK2 are currently available for clinical use. The tyrophostin AG490 (12) has been the most studied JAK2 inhibitor. AG490 inhibits JAK2/signal transducer and activator of transcription 3 (STAT3) signaling in vitro, but only at high concentrations (IC50 50-100 μmol/L), which has translated into limited activity in vivo (13–15). Several attempts at improving the properties of AG490 have led to the synthesis of related analogues (16–20). Our group has generated several JAK2 inhibitors. We found that one of these compounds, the AG490-related WP1066 analogue, inhibits
JAK2 in acute myeloid leukemia cells (19, 20). Therefore, we chose to test the activity of WP1066 in polycythemia vera. In the current study, we showed that WP1066 suppressed the proliferation of erythroid colony-forming cells obtained from patients with polycythemia vera carrying the JAK2 V617F mutation and inhibited JAK2 and its downstream STAT and the extracellular signal-regulated kinase (ERK)-1/2 pathways in erythroid leukemia HEL cells that express the JAK2 V617F isoform.

**Materials and Methods**

**Cells and study drugs.** HEL cells and the myeloid (HL60 and K562) and lymphoid (Raji and PEER) cell lines were purchased from the American Type Culture Collection. The cells were maintained in Iscove’s modified Dulbecco’s medium (Invitrogen), and the culture medium in which HEL cells were grown was also supplemented with 10% horse serum (Sigma). The human megakaryocytic leukemia cell line CMK, carrying a mutation of the JAK3 pseudo-kinase domain, was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH). Peripheral blood samples were obtained from five untreated patients with polycythemia vera and two healthy individuals who gave informed consent as part of an Institutional Review Board–approved protocol. Peripheral blood mononuclear cells were separated by Histopaque (density 1.077) gradient centrifugation and used immediately in experiments. AG490 was purchased from Sigma-Aldrich and ([6]-3-(6-bromopyridin-2-yl)-2-cyano-N-(50-phenylethyl)acrylamide (WP1066; Fig. 1A) was synthesized in Waldemar Prieß’s laboratory as lyophilized powder and dissolved in 1% DMSO, diluted with a 5% dextrose solution, and stored at -20°C until use.

**Growth inhibition assay.** The 3,4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium (MTT) assay was done using an MTT-based cell proliferation/cytotoxicity assay system (Promega). Briefly, fresh low-density peripheral blood cells and various cell lines at the logarithmic phase of their growth were washed twice in RPMI 1640 containing 10% FCS and counted in a hemocytometer. Cell viability was assessed by the trypan blue (0.1%) staining method. Equal numbers of viable cells (5 x 10^5 per well) were incubated in a total volume of 100 μL of RPMI 1640 supplemented with 10% FCS alone or with WP1066 at increasing concentrations; the incubations were continued for up to 72 h in 96-well flat-bottomed plates (Linbro; Flow Laboratories) at 37°C in a humidified 5% CO2 atmosphere. Experiments for each condition were done in triplicate. After incubation, 20 μL of CellTiter96 One Solution Reagent (Promega) were added to each well. The plates were then incubated for an additional 60 min at 37°C in a humidified 5% CO2 atmosphere. Immediately after incubation, absorbance was read using a 96-well plate reader at a wavelength of 490 nm.

**Blast-forming unit-erythroid and colony forming unit-granulocyte-macrophage clonogenic assay.** The blast-forming unit-erythroid (BFU-E) and colony forming unit-granulocyte-macrophage (CFU-GM) colony culture assay was done as previously described (21). Briefly, 2 x 10^5 mononuclear cells from the peripheral blood of patients with polycythemia vera were cultured in 0.8% methylcellulose in Iscove’s modified Dulbecco’s medium supplemented with 10% FCS, 1.0 units/mL human erythropoietin (Amgen), 50 ng/mL stem cell factor (Amgen), and 50 ng/mL granulocyte-macrophage colony stimulating factor. Next, 1 mL of the culture mixture was placed in 35-mm Petri dishes in duplicate and incubated at 37°C in a humidified atmosphere of 5% CO2 in air. All cultures were evaluated after 14 days for the presence of BFU-E and CFU-GM. A BFU-E was defined as an aggregate of >500 hemoglobinized cells or three or more erythroid subcolonies, whereas a CFU-GM was defined as a cluster of >50 granulocyte and/or monocyte/macrophage cells.

**PCR specific for JAK2 V617F mutation.** Genomic DNA was extracted from peripheral blood mononuclear cells obtained from five patients with polycythemia vera. Exon 12 of the JAK2 gene was amplified from 200 ng of extracted DNA in a 50-μL PCR using the FAM-conjugated forward primer FAM-5'-GGGTTCCTCAGAAGCTGTA-3' and reverse primer 5'-TCAATTGGTTCCCTTTCACA-3' (Sigma-Genesys). After 5 min of hot start at 94°C, 10 PCR cycles were done at 94°C for

**Fig. 1.** Chemical structure of WP1066 and its effect on cellular viability. A, comparison of the chemical structures of the natural product caffeic acid benzyl ester (benzyl caffeate), its low-potency synthetic analogue AG490, and WP1066, a novel potent inhibitor of the JAK2/STAT signaling pathway. B, cells were exposed in vitro for 72 h to increasing concentrations of either WP1066 (B) or AG490 (C) and their proliferation and biological activity were measured by the MTT assay. HEL cells were significantly (paired t test) more sensitive to WP1066 than HL60 (P = 0.017), Raji (P = 0.007), PEER (P = 0.01), K562 (P = 0.006), CMK (P = 0.005), and normal (TM and RHN) low-density cells (P = 0.01 and P = 0.0045, consecutively). Representative experimental data from five independent experiments.
30 s, annealing at 57°C for 30 s, and extension at 72°C for 50 s. This was followed by 35 PCR cycles at 92°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and one final extension at 72°C for 10 min. The 460-bp amplified fragment was precipitated with isopropanol, washed with 70% ethanol, and redissolved in 20 μL of distilled water. The 460-bp fragment was digested with BamHI (New England Biolabs) overnight at 37°C and then analyzed on an ABI 3100 GenScan. The allele encoding the mutant JAK V617F remained undigested, whereas the allele encoding the wild-type JAK2 was digested into 241-bp, 189-bp, and 30-bp fragments.

Annexin V and propidium iodide staining. After treatment with WP1066 for 24, 48, and 72 h, cells were washed with PBS and resuspended in 100 μL of binding buffer [10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (pH 7.4), 0.15 mol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl2, and 1.8 mmol/L CaCl2], to which Annexin V-fluoroisothiocyanate had been added. Cells were then incubated for 15 min in the dark at room temperature. After incubation, cells were washed and then resuspended in 0.2 mL of binding buffer. Propidium iodide binding of Annexin V to apoptotic cells was analyzed in a flow cytometer (FACScan, Becton Dickinson) using CellQuest software (Becton Dickinson).

Measurement of mitochondrial transmembrane potential. After treatment with WP1066 for up to 72 h, cells were incubated with submicromolar concentrations of MitoTracker probes to evaluate changes in the potential of the mitochondrial membrane. MitoTracker probes passively diffuse across the plasma membrane and accumulate in mitochondria. Cells were stained with MitoTracker Red CMXRos and MTGreen FM (Molecular Probes). Briefly, cells were washed in Ca2+-free PBS, stained with MitoTracker dyes, and incubated for 1 h at 37°C in the dark. CMXRos and MTGreen are incorporated into mitochondria by force of the mitochondrial membrane potential and react with thiol residues to form covalent thiol ester bonds. Samples were run on a flow cytometer and were analyzed using CellQuest software (Becton Dickinson).

Detection of caspase-3 activity. To monitor caspase-3 and caspase-8 activities in living cells by flow cytometry, the fluorogenic software (Becton Dickinson). were run on a flow cytometer and were analyzed using CellQuest software (Becton Dickinson).

Antibodies used for Western immunoblotting. Rabbit anti-human antibodies specific for JAK2, JAK3, total STAT3, and total STAT5 and mouse monoclonal antibodies against phosphorylated (p) STAT3, pSTAT5, and anti-phosphophorysine (clone-4G10) were purchased from Upstate Biotechnology. Mouse anti-β-actin antibody (556433), mouse anti-human AKT, mouse anti-human pAKT, mouse anti-human pan-ERK, and mouse anti-human pERK1/2 (pT202/ pY204) were purchased from BD PharMingen. Monoclonal mouse anti–polypeptide (APD-ribose) polymerase (PARP) antibodies were obtained from Trevigen, Inc. Monoclonal mouse anti-caspase-3 (active and proactive) antibodies were purchased from ebioscience, and rabbit anti-human pIL1, rabbit anti-human JAK1, rabbit anti-human IL12, and rabbit anti-human pFLT3 were obtained from Cell Signaling. Agarose-conjugated protein A/G was obtained from Santa Cruz Biotechnology, Inc., and mouse anti–β-tubulin antibodies were obtained from Sigma-Aldrich.

Immunoprecipitation of JAK2, JAK3, and Western blotting. Cells (20 × 10^6 per sample) treated with WP1066 for different times and/or at different concentrations were collected, washed thrice with cold PBS, resuspended in 300 μL of lysis buffer [10 mmol/L sodium phosphate (pH 7.2), containing 100 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride, and 1× Roche complete mini-protease-inhibitor cocktail], and incubated on ice for 1 h. After centrifugation of the cell lysate at 14,000 rpm for 40 min at 4°C, the supernatant was removed and mixed with 15 μL of anti-JAK2 or anti-JAK3 rabbit antibody for 1 h on ice, followed by the addition of 50 μL of protein A/G agarose slurry and incubation overnight at 4°C under constant rotation. The antibody-protein complex was washed thrice, once with immunoprecipitation assay buffer [100 mmol/L NaCl in 10 mmol/L phosphate buffer (pH 7.2) containing 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, 5 mmol/L EDTA, and a cocktail of protease inhibitors], once with washing buffer [100 mmol/L NaCl in 10 mmol/L sodium phosphate (pH 7.2) and 0.1% Triton X-100], and once with 50 mmol/L Tris-HCl buffer (pH 7.5). The immunoprecipitated complex was eluted from the agarose with 2× loading buffer and run on a 9.5% SDS-PAGE gel. Western blotting was done overnight at 4°C. After the nitrocellulose membrane (Schleicher & Schuell) was blocked with 5% nonfat milk in PBS-0.01% Tween 20 for 5 h, membranes were incubated with mouse anti-phosphotyrosine antibody diluted in 5% nonfat milk (1:10,000) overnight at 4°C. The active bands for phosphorylated JAK2 and JAK3 were detected with horseradish peroxidase (HRP)–conjugated sheep anti-mouse antibody. Detection was done by enhanced chemiluminescence, as specified by the manufacturer (ECL, Amersham). The same membranes were stripped and reprobed with rabbit anti-JAK2 or anti-JAK3 antibodies diluted 1:5,000 and 1:1,000, respectively, in 5% milk in 1× PBS-0.01% Tween overnight at 4°C. The active bands for JAK2 and JAK3 were detected with HRP-conjugated donkey anti-rabbit antibody (Amersham).

Western immunoblotting. HEL cells were exposed to different concentrations of WP1066 for different periods of time. The cells were then collected, washed thrice with cold PBS, resuspended in 200 μL of lysis buffer [10 mmol/L sodium phosphate (pH 7.2) containing 100 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride, and 1× Roche complete mini-protease-inhibitor cocktail], and incubated on ice for 1 h. After centrifugation at 14,000 rpm for 30 min at 4°C, cell lysates were collected and the protein concentration was determined with Bio-Rad protein assay dye reagent (Bio-Rad). Western immunoblotting for pSTAT3, pSTAT5, and PIK3 was done by loading 50 μg of cell lysate onto a 10% Bio-Rad mini gel. After transferring and blocking the membrane with 5% nonfat milk for 5 h, it was probed with 0.1 μg/mL of mouse anti-pSTAT3, 1 μg/mL pSTAT5, and 1 μg/mL (v/v) of pJAK1 in 5% nonfat milk in 0.01% Tween overnight at 4°C. After the membrane was washed with PBS containing 0.01% Tween 20, active bands were detected using HRP-conjugated sheep anti-mouse or HRP-conjugated donkey anti-rabbit antibody. Detection was done using enhanced chemiluminescence, as specified by the manufacturer (ECL, Amersham). On detection of pSTAT3, pSTAT5, and pSTAT1, membranes were stripped, blocked, and reprobed with 1 μg/mL (v/v) of total STAT3, STAT5, and JAK1 antibody in 5% nonfat milk overnight at 4°C. After washing, active bands were detected using HRP-conjugated donkey anti-rabbit antibody. Finally, membranes were stripped and reprobed with mouse anti–β-tubulin antibody to check the equal loading of the protein.

In separate experiments, following treatment of HEL cells for different times and/or with different concentrations of WP1066, cells were washed twice with ice-cold PBS. The cell pellets were mildly resuspended with digitonin extraction buffer [10 mmol/L PIPES (pH 6.8), 0.015% digitonin, 300 mmol/L sucrose, 100 mmol/L NaCl, 3 mmol/L MgCl2, 5 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride supplemented with freshly added phosphatase inhibitors and protease inhibitors]. After incubation on ice for 20 min, samples were centrifuged at 14,000 rpm for 10 min. Supernatants containing cytosolic protein were transferred to a clean tube. Protein concentration was determined in the final supernatant using the Bio-Rad protein assay dye reagent following the manufacturer’s instructions. Samples were then stored in aliquots at -80°C. Western blotting for PARP was done.
by loading 50 μg of cell lysate on a 10% Bio-Rad mini gel, transferring and blocking with 5% nonfat milk for 5 h, and probing with 1 μg/mL of mouse anti-PARP antibody or 1 μg/mL of mouse anti-cytochrome c in 5% nonfat milk overnight at 4°C. After washing the membrane with PBS containing 0.01% Tween 20, active bands for PARP and cytochrome c were detected using HRP-conjugated sheep anti-mouse or HRP-conjugated donkey anti-rabbit antibody. Detection was done using enhanced chemiluminescence, as specified by the manufacturer (ECL, Amersham). After detection of PARP, the membranes were stripped, blocked, and reprobed with 1 μL/mL (v/v) of mouse anti–caspase-3, anti–caspase-9, or anti–cytochrome c antibody in 5% nonfat milk overnight at 4°C. After washing, active bands were detected using HRP-conjugated sheep anti-mouse antibody. Detection was done using enhanced chemiluminescence, as specified by the manufacturer (ECL, Amersham). After detection of PARP, the membranes were stripped, blocked, and reprobed with 1 μL/mL (v/v) of mouse anti–caspase-3, anti–caspase-9, or anti–cytochrome c antibody in 5% nonfat milk overnight at 4°C. After washing, active bands were detected using HRP-conjugated sheep anti-mouse antibody. Finally, membranes were stripped and reprobed with mouse anti–β-actin antibody to check the equal loading of the protein.

### Results

**WP1066 inhibits the growth of human HEL cells carrying the JAK2 V617F mutant isoform.** The cell line HEL carries the V617F mutation, which maps to the JH2 domain of JAK2 and induces constitutive JAK2 phosphorylation (4–8). The AG490 analogue WP1066 was designed to inhibit JAK2 tyrosine kinase activity (Fig. 1A). Accordingly, we investigated the effect of WP1066 on the proliferation of HEL cells expressing the mutant JAK2 V617F isoform using the MTT assay. We found that WP1066 markedly inhibited the growth of HEL cells in a dose-dependent manner (Fig. 1B). The IC_{50} value for inhibition of the proliferation of HEL cells was 2.3 μmol/L, whereas the IC_{80} and IC_{20} values were 3.8 and 0.8 μmol/L, respectively. Cellular viability of myeloid leukemia (HL60 and K562), JAK3-dependent megakaryocytic leukemia (CMK), T-cell lymphoma (Raji and PEER), and normal donor low-density cells (TM and RHN) was not affected by WP1066 (Fig. 1B), suggesting that the sensitivity to WP1066 is significantly higher in cells with a constitutive activating mutation of JAK2. Remarkably, unlike WP1066, AG490, added to culture at the same concentrations, slightly affected the spontaneous proliferation of those cell lines (Fig. 1C).

**WP1066 inhibits BFU-E colony-forming cell proliferation.** It has recently been reported that 74% to 97% of patients with polycythemia vera carry the JAK2 V617F mutation (4–7). Therefore, we obtained peripheral blood from patients with polycythemia vera and evaluated the ex vivo effect of WP1066 on hematopoietic colony-forming cell proliferation. We first determined by PCR that the JAK2 V617F mutant was present in all polycythemia vera blood samples. The V617F mutation maps to the JH2 pseudo-kinase domain of JAK2, resulting in constitutive tyrosine phosphorylation and elimination of the cutting site for the restriction enzyme BsaXI. As shown in Fig. 2, wild-type alleles were identified by demonstration of 241-bp, 189-bp, and 30-bp BsaXI digestion products. We then tested the effect of WP1066 on hematopoietic colony-forming cell proliferation.
Peripheral blood mononuclear cells from the patients with polycythemia vera were plated in semisolid media in the presence of optimal concentrations of erythropoietin, granulocyte-macrophage colony stimulating factor, and stem cell factor for 14 days, with or without increasing concentrations (ranging from 0.5 to 3.0 $\mu$mol/L) of WP1066. We found that exposure to WP1066 resulted in a reduction in the number of both BFU-E and CFU-GM (Fig. 2B). However, the inhibitory effect of WP1066 was significantly more pronounced in BFU-E than in CFU-GM colonies, likely reflecting a higher dependency of erythroid colonies on JAK2 V617F signaling for proliferation.

WP1066 activates the caspase pathway and induces apoptosis in HEL cells. To delineate the mechanism of action of WP1066 on cells expressing JAK2 V617F mutation, we used HEL cells. We found that exposure of HEL cells to escalating concentrations of WP1066 for different periods of time induced apoptotic cell death in HEL cells, as evidenced by Annexin V staining (Fig. 3A and B). In contrast, WP1066 did not induce apoptosis either of the JAK3-dependent megakaryocytic leukemia cell line CMK or of low-density peripheral blood cells obtained from a healthy individual (Fig. 3B). Then, we incubated HEL cells with submicromolar concentrations of MitoTracker probes and analyzed changes in the mitochondrial membrane potential and the uptake of MitoTracker CMXRos and MTGreen dye using flow cytometry. As with Annexin V, we found that after 24, 48, and 72 h of exposure, WP1066 induced time- and dose-dependent apoptosis in HEL cells (Fig. 3C). Exposure of HEL cells to escalating concentrations of WP1066 for increasing periods of time also resulted in increased activation of caspase-3 (Fig. 3D), suggesting that WP1066 induced caspase-dependent apoptosis. Indeed, several features
of the WP1066-induced apoptosis supported this observation. Exposure of HEL cells to either escalating doses of WP1066 over a fixed period of time or a fixed WP1066 dose at different time points resulted in the dose-dependent (Fig. 4A) as well as time-dependent (Fig. 4B) cleavage of PARP. In addition, we showed that WP1066 induced cytochrome c release in HEL cells in a time-dependent manner (Fig. 4B), and this effect was accompanied by cleavage of procaspase-3 (Fig. 4B).

**WP1066 inhibits the phosphorylation of JAK2, STAT3, STAT5, and ERK1/2 without affecting the phosphorylation of JAK1 and JAK3.** We then asked whether the antiproliferative and proapoptotic effects of WP1066 could result from the inhibition of JAK2. JAK2 is constitutively phosphorylated in HEL cells because these cells carry the V617F gain-of-function mutation. As expected, we found that incubation of HEL cells with increasing concentrations of WP1066 for 3 h resulted in concentration-dependent inhibition of JAK2 phosphorylation, without affecting the phosphorylation of JAK1 and JAK3 (Fig. 5A), whereas treatment with a fixed dose of WP1066 for up to 48 h (Fig. 5B) induced a time-dependent decrease in JAK2 phosphorylation.
phosphorylation. Notably, we observed a small reduction in the amount of total JAK2 on exposure to WP1066, suggesting that in addition to inhibiting JAK2 phosphorylation, WP1066 might cause JAK2 protein degradation, as we previously found in acute myelogenous leukemia cells (19). Because WP1066 inhibited JAK2, we reasoned that downstream signal transduction events that are critical in promoting JAK2-mediated cell survival might also be inhibited by WP1066. We incubated HEL cells with either escalating concentrations of WP1066 for 24 h (Fig. 6A) or a fixed dose of WP1066 for up to 48 h (Fig. 6B) and examined the phosphorylation status of STAT3, STAT5, and ERK1/2. We found that incubation with WP1066 down-regulated the phosphorylation of STAT3, STAT5, and ERK1/2. Exposure to WP1066 completely abrogated the phosphorylated forms of both STAT3 and STAT5. Likewise, exposure of HEL cells to increasing concentrations of WP1066 led to decreased phosphorylation of ERK1/2, without affecting the levels of total ERK1/2 protein (Fig. 6A). The ERK1/2 signaling pathway plays a major role in the control of diverse cellular processes such as proliferation, survival, differentiation, and motility. Significant ERK1/2 dephosphorylation was observed after 24 h of treatment with WP1066 at 1 μmol/L (Fig. 6B). Exposure of HEL cells to 3 μmol/L (~IC₅₀) of WP1066 led to complete inhibition of pERK1/2 (Fig. 6B). Remarkably, similar to JAK1 and JAK3, WP1066 did not affect the phosphorylation of FLT3, further confirming its specificity (Fig. 6B).

**Discussion**

Our experiments provide evidence of the marked activity of WP1066 against JAK2 V617F–bearing HEL cells, with an IC₅₀ value of 2.3 μmol/L. This antiproliferative activity was associated with the inhibition of the tyrosine kinase activity of the mutant JAK2 and its downstream effectors STAT3 and STAT5. In proliferation assays, WP1066 inhibited erythroleukemia HEL cell proliferation in a time- and dose-dependent manner. More importantly, in a clonogenic assay, exposure to WP1066 resulted in significant ex vivo inhibitory activity in primary peripheral blood mononuclear cells expressing JAK2 V617F from patients with polycythemia vera.

The JAK2 V617F mutation, which is found in ~90% of patients with polycythemia vera, has become a hallmark of this...
JAK2 is a member of the JAK family of membrane-bound tyrosine kinases whose activity is crucial for cytokine signaling. Functionally, JAK2 has been implicated in several critical biological activities including cellular proliferation, survival, and developmental processes. Although involved in the signaling of several cytokines and growth factors, JAK2 activity is essential in erythropoiesis, and JAK2 knockout results in embryonic death. JAK2+ mouse embryos die around day 12.5 postcoitum as a result of ineffective erythropoiesis (10). Two years ago, a clonal mutation in the JH2 pseudo-kinase domain of the JAK2 gene, causing a valine-to-phenylalanine substitution at amino acid position 617, was identified. This JAK2 V617F mutation occurs in the majority of patients with polycythemia vera and less frequently in other myeloproliferative disorders (4–7). This gain-of-function mutation induces constitutive JAK2 tyrosine kinase activity, resulting in erythropoietin-independent erythrocytosis in polycythemia vera and myeloid cell expansion in other myeloproliferative disorders (4–8). Therefore, inhibition of JAK2 has become an attractive target for the treatment of polycythemia vera and, possibly, other myeloproliferative disorders.

The most widely studied JAK2 kinase inhibitor is AG490 (12–16). AG490 was found to inhibit the in vitro proliferation of tumor-derived cell lines (22, 23) and the in vivo growth of JAK2-dependent human leukemia (15) and multiple myeloma in mouse models (24). However, high doses (50–100 μmol/L) were needed to induce significant tumor regression. A series of novel AG490 analogues were found to exert potent JAK2-STAT inhibitory activity (20, 25–29). Specifically, these small molecules inhibited wild-type and mutant JAK2 and STAT3 activation, abrogated the expression of Bcl-2, Bcl-XL, survivin, and Mcl-1, and as in our study, induced apoptotic cell death at low micromolar concentrations (19, 20, 25–29). In recent years, our group has synthesized AG490 analogues with a structure similar to that of benzyl caffeate, a natural product present in honeybee propolis (25–29). One of these compounds, WP1066, was found to inhibit the JAK-STAT pathway and induce apoptosis in a variety of tumor types both in vitro and in murine models (19, 21, 25, 26, 29, 30). STAT3 and STAT5 are cytoplasmic transcription factors that are activated by JAK2. On phosphorylation, both STATs translocate to the nucleus, bind to DNA, and activate transcription factors that activate proliferation and inhibit apoptosis (31, 32). WP1066 and similar analogues block the STAT pathway by inhibiting the phosphorylation of JAK2 and by inducing a rapid and specific JAK2 degradation (19) through a proteolytic mechanism that is not affected by serine/threonine protease inhibitors, proteasome inhibitors, calpain, or lysosomal proteolytic factors. Our data show that WP1066 induces this effect in HEL cells as well.

Erythropoietin-independent endogenous erythroid colony formation is one of the biologic hallmarks of polycythemia vera (13, 34–36). In contrast to the remarkable inhibition of HEL cell proliferation, WP1066 failed to completely abrogate BFU-E colony formation at the concentration range tested. Several factors may have contributed to this discrepancy. Polycythemia vera progenitors exhibit hypersensitivity to several cytokines and growth factors such as stem cell factor, erythropoietin, and granulocyte-macrophage colony stimulating factor (37–40). In this study, clonogenic assays were carried out in the presence of high concentrations of hematopoietic growth factors. Thus, either these factors could partially overcome the inhibitory effects of WP1066 or the surviving colony-forming cells originated from the normal hematopoietic clone. The latter possibility is supported by our recent data showing that hematologically normal bone marrow–derived BFU-E and CFU-GM are minimally affected by the same concentrations of WP1066 (21). Finally, given the significantly higher inhibition of BFU-E relative to CFU-GM, it is possible that CFU-GM proliferation in polycythemia vera is driven by activation of other pathways in addition to JAK2 kinase constitutively activated by the V617F mutation.

The JAK2 V617F mutation induces constitutive activation of JAK2 (5), and as a result, JAK2 activates several transcription factors. We found constitutive phosphorylation of STAT3, STAT5, and ERK1/2 in the erythroleukemia HEL cells expressing the JAK2 V617F mutation. This effect is not surprising because erythropoietin receptors have been shown to activate STAT, ERK, and the mitogen-activated protein kinase pathways (41). Exposure of HEL cells to WP1066 led to a rapid and durable dose- and time-dependent inhibition of the phosphorylation of STAT3 and STAT5 before caspase cleavage and the induction of apoptosis, suggesting that abrogation of STAT activation is responsible for the proapoptotic effect of WP1066. Importantly, similar to its effect on acute myelogenous leukemia cells (22), WP1066 selectively inhibited JAK2 without affecting the phosphorylation or the total protein levels of other JAK family kinases such as JAK1 and JAK3.

In conclusion, we have shown that WP1066 is an effective inhibitor of JAK2 and its downstream STAT and mitogen-activated protein kinase/ERK signaling pathways. The inhibition of polycythemia vera hematopoietic precursor proliferation, the activation of the caspase pathway, and the induction of apoptosis in erythroleukemia cells suggest that WP1066 might prove to be a useful agent for the treatment of patients with myeloproliferative disorders carrying the JAK2 V617F mutation.

Acknowledgments

We thank Dawn Chalae for editing the manuscript.

References

WP1066, a Novel JAK2 Inhibitor, Suppresses Proliferation and Induces Apoptosis in Erythroid Human Cells Carrying the JAK2 V617F Mutation

Srdan Verstovsek, Taghi Manshouri, Alfonso Quintás-Cardama, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/14/3/788

Cited articles
This article cites 37 articles, 9 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/14/3/788.full#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/14/3/788.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/14/3/788.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.