WP1066 Disrupts Janus Kinase-2 and Induces Caspase-Dependent Apoptosis in Acute Myelogenous Leukemia Cells

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Abstract
Several cytokines and growth factors that stimulate the proliferation of acute myelogenous leukemia (AML) cells transduce their signals by activating the transcription factor Janus-activated kinase 2 (JAK2). Accordingly, the inhibition of JAK2 or of its downstream signaling pathways suppresses the proliferation of AML cells. Because (E)-3-(6-bromopyridin-2-yl)-2-cyano-N-((50-1-phenethyl)acrylamide) (WP1066) is a novel analogue of the JAK2 inhibitor AG490, we tested its activity in AML cells and investigated its mechanism of action. Using clonogenic assays, we found that although WP1066 had a marginal effect on normal marrow progenitors, it inhibited the proliferation of AML colony-forming cells obtained from patients with newly diagnosed AML and that of the AML cell lines OCI-M2 and K562. WP1066 inhibited OCI-M2 cell multiplication by inducing accumulation of cells at the G0-G1 phase of the cell cycle. Similar to its parent compound AG490, WP1066 inhibited the phosphorylation of JAK2, but unlike AG490, WP1066 also degraded JAK2 protein, thereby blocking its downstream signal transducer and activator of transcription (STAT) and phosphoinositide-3-kinase pathways. These effects resulted in the activation of the caspase pathway. Incubation of both OCI-M2 and K562 cells with WP1066 activated caspase-3, induced cleavage of poly(ADP-ribose) polymerase, and caused caspase-dependent apoptotic cell death. Thus, WP1066 is a potent JAK2 inhibitor whose effects in AML and other hematologic malignancies merit further investigation. [Cancer Res 2007;67(23):11291–9]

Introduction
Acute myelogenous leukemia (AML) is the most common acute leukemia in adults. Despite the availability of numerous cytotoxic drug combinations for leukemia and the ongoing development of novel antileukemic agents, disease recurrence is common, and most patients will die of their disease, indicating a need for novel treatment strategies (1, 2). In recent years, researchers have begun studying a new generation of antineoplastic agents called signal transduction inhibitors in clinical trials. These drugs target growth factor- and cytokine-activated signal transduction pathways, thereby disrupting extracellular and intracellular signals that stimulate proliferation and provide these cells with a survival advantage.

Upon binding to their cellular receptors, hematopoietic growth factors and cytokines activate Janus-activated kinase (JAK) proteins. The JAK proteins are a family of four cytosolic tyrosine kinases (JAK1, JAK2, JAK3, and TYK2). Once activated, JAKs phosphorylate and activate cellular cytokine receptors and signaling molecules such as those regulated by the Src kinase cascade, the ras/mitogen-activated protein kinase pathway, phosphoinositide-3-kinase (PI3K), and signal transducer and activator of transcription (STAT; ref. 3). Specifically, JAK2 modulates leukemia-cell survival and proliferation and plays a major role in the pathogenesis of AML and other hematologic malignancies. For example, in AML cells bearing the translocation t(9;12), the oligomerization domain of the TEL gene combines with the catalytic domain of JAK2 and incites the leukemogenic process (4, 5). In myeloproliferative disorders and a subset of AML, a mutation enacting constitutive activation of JAK2 instigates leukemogenesis (6). In patients with AML, constitutive activation of either STAT or PI3K (the pathways that are activated by JAK2) carries a poor prognosis (7, 8). Therefore, inhibition of JAK2 should be a beneficial strategy for the treatment of AML (9).

In recent years, investigators studied the activity of several tyrosine phosphorylation inhibitors, called tyrophostins (10–12). Although one of those, the JAK2 inhibitor AG490, has been extensively studied, it has not been developed for clinical use. Therefore, researchers have synthesized several AG490 derivatives and tested their activity (9–13). In the present study, we investigated a novel AG490 analogue, (E)-3-(6-bromopyridin-2-yl)-2-cyano-N-((50-1-phenethyl)acrylamide) (WP1066), whose solubility and protein kinase-inhibitory profile suggested that this compound might be a good candidate for clinical development. We found that WP1066 inhibits JAK2 and its downstream STAT and PI3K pathways and induces caspase-dependent apoptosis of AML cells.

Materials and Methods

Drugs. WP1066 (MW 356.22) was synthesized by Waldemar Priebe at the University of Texas M. D. Anderson Cancer Center and stored as a lyophilized powder. The lyophilized form of the drug was dissolved in 1% DMSO, diluted with a 5% dextrose solution, and stored at −20°C. The stored solution was diluted in tissue culture medium before use. The structure of WP1066 is shown in Fig. 1A.

Patient samples. Fresh bone marrow (BM) samples were obtained from four patients with AML at the University of Texas M. D. Anderson Cancer Center and three healthy volunteers (controls). The characteristics of the patients with AML are shown in Table 1. All studies were done after the M. D. Anderson Cancer Center Institutional Review Board approved the study and after the subjects gave their written informed consent to participate.

Cell lines. The AML cell lines OCI-M2 and OCI/AML3 were provided by M.D. Minden (Ontario Cancer Institute, Toronto, Ontario, Canada; refs. 14, 15),
and the AML cell lines HL60 and KG1 and the JAK2 V617F-positive erythroleukemia HEL cells were obtained from the American Type Culture Collection. Cells were maintained in RPMI 1640 (Life Technologies) supplemented with 10% FCS (Flow Laboratories), grown in plastic tissue culture flasks (Falcon Plastics; Becton Dickinson), and split twice weekly. Also, the human BCR-Abl–expressing Philadelphia chromosome (Ph)–positive AML cell line K562 was obtained from ATCC, and the human megakaryocytic leukemia cell line CMK, carrying a mutation of the JAK3 pseudokinase domain, was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ GmnH). The cells were grown in RPMI 1640 supplemented with 10% FCS in plastic tissue culture flasks (Falcon Plastics) and split twice weekly.

AML blast colony assay. The AML blast colony assay was done as described previously (16). Briefly, 1 × 10^5 nonadherent T-cell–depleted BM cells were plated in 0.8% methylcellulose (Fluka Chemical) in α medium (Life Technologies), supplemented with 10% FCS and 50 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; Immunex Corporation) and incubated in 35-mm Petri dishes (Nunc) in duplicate for 7 days at 37°C in a humidified atmosphere of 5% CO2 in air. WP1066 was diluted in isotonic 5% dextrose solution and added to the culture mixtures at the initiation of the cultures at concentrations of 0.5, 1.0, 2.0, and 3.0 μmol/L. The cultures were microscopically evaluated on day 7 of the culture. A blast colony was defined as a cluster of at least 40 cells. Individual colonies were microaspirated, smeared on glass slides, and stained with crystal violet.
stained to confirm their leukemic cell composition. The ability of this assay to identify AML blast colony cells rather than normal hematopoietic progenitor cells was previously confirmed by cytogenetic analysis of such colonies (17).

**Erythroid blast-forming unit and colony-forming unit-GM colony culture assay.** The erythroid blast-forming unit (BFU-E) colony assay and the granulocyte-macrophage colony-forming unit (CFU-GM) clonogenic assay were done as described previously (18). Briefly, 2 × 10^5 BM cells were cultured in 0.8% methylcellulose in Iscove’s modified Dulbecco’s medium (Life Technologies) supplemented with 10% FCS, 1.0 unit/mL human erythropoietin (Amgen), and 50 ng/mL recombinant human GM-CSF (Immunex Corporation) in the presence or absence of WP1066. One milliliter 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 number of BFU-Es, defined as an aggregate of more than 50 hemoglobinized cells or three or more erythroid subcolonies, and for the number of CFU-GM, defined as clusters of more than 50 granulocytes and/or monocyte/macrophage cells.

**Cell line clonogenic assay.** The cell line clonogenic assay was done as described previously (19). Briefly, OCI2M2 and K562 cells (2–4 clusters of more than 50 granulocytes and/or monocyte/macrophage cells. more erythroid subcolonies, and for the number of CFU-GM, defined as clusters of more than 50 granulocytes and/or monocyte/macrophage cells.

**Western immunoblotting.** Cell lysates were assayed for their protein concentrations using the BCA protein assay reagent (Pierce Chemical). Each set of paired lysate samples was adjusted for the same protein concentration. SDS-PAGE was then conducted at a constant wattage (10 W) in running buffer cooled to 4°C (19). The stacking gels contained 4% (wt/vol) acrylamide, and the separating gels contained 12% (wt/vol) acrylamide. Approximately 50 μg of sample protein was loaded onto each lane. Proteins separated by electrophoresis were transferred to nitrocellulose membranes; the transfers were done overnight at 30 V in a cooled (4°C) reservoir containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol (pH, 8.3) transfer buffer. The nitrocellulose membranes were then removed from the blot apparatus and placed in a Ponceau S staining solution (0.5% Ponceau S and 1% glacial acetic acid in water) to verify equal loading of protein in the control and treated samples (20–23). After the membranes were stained for 5 min, they were rinsed for 2 min and examined. Equal loading of protein was verified, and the membranes were rinsed for an additional 10 min and immunoscreened. The membranes were blocked with Blotto (5% dried milk dissolved in 50 mL of PBS) for at least 1 h at room temperature. They were then washed thrice in PBS plus 0.5% Tween 20. Next, the membranes were incubated for 1 to 12 h with the appropriate antibodies (described below). After incubation, the membranes were rinsed thrice in PBS containing 0.5% Tween 20 for 15 min each. The bound antibody was detected using the ECL Western Blotting Detection System (Amer sham). Chemiluminescence of the membranes was detected using X-OMAT AR5 X-ray film (Kodak) in stainless-steel exposure cassettes (Sigma Chemical Co.). Protein levels were scored using densitometry with the UltraScan XL laser densitometer (Pharmacia). Results were normalized by dividing the numerical value of each sample signal by the numerical value of the signal from the corresponding value of the β-actin that served as a control.

The following antibodies were used to detect the specified proteins: mouse antihuman JAK1, TYK2 and phosphorylated (p) CrkL (Becton Dickinson Translational Laboratories), rabbit antihuman CrkL, JAK2, JAK3, pJAK1 (p-Tyr1022/1023), and pJAK2 (p-Tyr1007/1008), Upstate Cell Signaling Solutions; rabbit antihuman pJAK3 (p-Tyr1046, Santa Cruz Biotechnology), rabbit antihuman pTYK2 (p-Tyr1014/1015), Cell Signaling Technologies, mouse antistatins, STAT3, STAT5, pSTAT3 (pTyg20), and pSTAT5 (pTyg136) antibodies (Upstate); mouse antiphosphorylated AKT (p-Ser473/477), nonphosphorylated AKT, and mouse antihuman poly(ADP-ribose) polymerase (PARP; Pharmingen); and monoclonal mouse antihuman CPP32 and mouse antihuman cleaved caspase-3 (New England BioLabs) against procaspase-3 and caspase-3. Normal mouse immunoglobulin G (IgG) and rabbit IgG (Sigma Chemical Co.) were used as controls. To confirm the detection of these proteins, lysates of Jurkat cells (ATCC; for the detection of JAK1, STAT3, STAT5, pSTAT3, pSTAT5, procaspase-3, and PARP proteins), Hela cells (ATCC; for the detection of cleaved caspase-3 and pTYK2), human endothelial cells (for the detection of TYK2), TF1 cells (ATCC; for the detection of JAK3) and NIH/3T3 cells treated with platelet-derived growth factor (Pharmingen) were used for the detection of phosphorylated AKT.

**Reverse transcription-PCR.** Total RNA was extracted using TRI reagent (Sigma Chemical Co.). The RNA was resuspended in RNase-free water and frozen at −80°C in 5-μg aliquots until use. Reverse transcription-PCR (RT-PCR) was done using the Reverse Transcription System (Promega). Briefly, the aliquots of RNA were heated to 70°C for 10 min and then placed on ice. While the RNA was incubating, a 20-μL reaction mixture was prepared containing 5 mmol/L MgCl₂, 1 × transcription buffer [10 mmol/L tris(hydroxymethyl)amino methane-HCl (pH, 9.0 at 25°C), 50 mmol/L KCl, 0.1% Triton X-100], 1 mmol/L each deoxynucleotide triphosphate (dNTP), 1 unit/μL recombinant RNasin RNase inhibitor, 15 units/μg avian myeloblastosis virus (AMV) transcriptase, and 0.5 μg of random primers. The mixture was added to the RNA aliquot along with sufficient amount of random primers for reverse-transcribed free water for a final volume of 20 μL. The reaction mixture was incubated at 42°C for 1 h and then heated to 95°C for 5 min and held at 4°C for 5 min to inactivate the AMV transcriptase. The ssDNA was then amplified. Briefly, a 10-μl aliquot of the ssDNA was added to a reaction mixture containing 200 μmol/L dNTPs, 2 mmol/L MgCl₂, 1 × transcription buffer (described above), and 2.5 units of Taq DNA polymerase. Specific probes for JAK2 (forward, GGGTTTCCCTCAAGACGTGTGA; reverse, TCATTGCCTTCCTTTTCACAA), were added with nucleic acid-free water for a final reaction volume of 100 μL. The reaction mixture was amplified using a Techne Thermal Cycler (Techne Incorporated) for 40 cycles and then amplified at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. PCR products were detected on 2% agarose GEL gels (Invitrogen) containing ethidium bromide. The gels were visualized using a FluorChem 8900 imager (Alpha Innotech Corporation).

**Cell cycle analysis.** Cell cycle analysis was done according to standard protocols. Briefly, 5 × 10⁶ cells were pelleted after being incubated with WP1066. The cell pellets were washed and resuspended in 2 mL of 1% paraformaldehyde in PBS. Cells were incubated with or without WP1066 for 15 min at 4°C and then washed in PBS, resuspended in 2 mL of absolute ethanol, and stored at −20°C until they were needed for staining. The stored cells were then washed twice in PBS, resuspended in 0.5 mL of propidium iodide staining buffer (50 μg/mL propidium iodide and 10 μg/mL RNase in PBS), and incubated for 1 h at room temperature in total darkness. Flow-cytometric analysis was done using a FACScanB flow cytometer and the CellQuest software program (Becton Dickinson Immunocytometry Systems). Data analysis was done using the CellQuest and ModFit LT (version 2.0; Verity Software House) software programs.

**Annexin V assay for detection of apoptosis.** To quantify the cells undergoing apoptosis, the annexin V–FITC assay (PharMingen) was used as described previously (24). Briefly, WP1066-treated OCI2M2 and K562 cells were washed twice with cold PBS and then resuspended in binding buffer [10 mmol/L N-(2-hydroxyethyl)piperazine]-N₂'-2-ethanesulfonic acid, 

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140 nmol/L NaCl, and 5 nmol/L CaCl₂ (pH, 7.4) at a concentration of 1 × 10⁶ cells/mL. After incubation, 100 μL of each solution was transferred to a 5-mL culture tube, to which 5 μL of annexin V–FITC was added. The tubes were gently vortexed and incubated for 15 min at room temperature in total darkness. At the end of the incubation, 400 μL of binding buffer was added to each tube, and the cells were analyzed immediately using flow cytometry with a FACScalibur flow cytometer and the CellQuest software program. Data analysis was done using the CellQuest and ModFit LT (2.0).

### Figure 2.

WP1066 down-regulates JAK2 and pJAK2 protein levels, but not mRNA. A, K562 (top) and OCIM2 (bottom) cells were incubated for 2 h in RPMI 1640 supplemented with 10% FCS and WP1066 at increasing concentrations (0.5–4.0 μmol/L; left) and with 3 μmol/L WP1066 for 1, 2, 3, 4, and 6 h (right). JAK2 and pJAK2 were detected using Western immunoblotting as described in Materials and Methods. Equal protein loading was confirmed using anti-α-actin antibodies. B, OCIM2 and K562 cells (1 × 10⁷ cells/mL) were incubated for 2 h in RPMI 1640 supplemented with 10% FCS and WP1066 (2 μmol/L). RT-PCR was done to measure JAK2 mRNA expression as described in Materials and Methods. As shown here, exposure to WP1066 did not change the level of JAK2 mRNA expression. C, K562 (top) and OCIM2 (bottom) cells were incubated for 2 h in RPMI 1640 supplemented with 10% FCS and WP1066 at increasing concentrations (0.5–4.0 μmol/L; left). JAK1, pJAK1, JAK3, pJAK3, TYK2, and pTYK2 were detected using Western immunoblotting as described in Materials and Methods. Equal protein loading was confirmed using anti-α-actin antibodies (data not shown). D, CMK cells were incubated for 2 h in RPMI 1640 supplemented with 10% FCS and WP1066 at increasing concentrations (0.5–4.0 μmol/L). JAK3 and pJAK3 were detected by Western immunoblotting as described above.
software programs. To determine whether WP1066-induced apoptosis was caspase dependent, OCIM2 cells were preincubated with 20 μl of the pan-caspase inhibitor Z-VAD-FMK (refs. 25, 26; PharMingen), and apoptotic cells were detected using the annexin V assay as described above.

Results

WP1066 inhibits AML blast colony-forming cell proliferation. To test our hypothesis, we first asked whether WP1066 would inhibit the proliferation of fresh AML progenitors obtained from patients. To answer this question, we obtained BM cells from the four patients with AML and studied the effects of WP1066 on AML blast colony-forming cells using the AML blast colony assay. We found that in spite of the significant patient heterogeneity, WP1066 inhibited the proliferation of AML blast colony-forming cells in a dose-dependent manner at concentrations ranging from 0.5 to 3.0 μmol/L (Fig. 1B). AML blast proliferation was completely abolished at concentrations >3 μmol/L.

WP1066 suppresses normal BM progenitor proliferation at increased concentrations. We next asked whether WP1066 selectively inhibits AML blast colony-forming cell proliferation or it also suppresses normal hematopoietic progenitor growth. We incubated BM cells obtained from the three healthy volunteers with WP1066 at concentrations of 0.5 to 3.0 μmol/L. We found that WP1066 inhibited the growth of normal BM BFU-E and CFU-GM progenitors at concentrations significantly higher than those required to inhibit the proliferation of AML progenitors. For example, at 2 μmol/L, WP1066 completely abolished AML blast colony growth, whereas it suppressed normal BM BFU-E growth by only an average of 22% and CFU-GM growth by only an average of 30% (Fig. 1C).

WP1066 inhibits AML colony-forming cell proliferation. We then sought to investigate the mechanism of action of WP1066. To do so, we first studied the effects of WP1066 on the proliferation of several AML cell lines. We found that the most sensitive lines were HEL cells that carry the JAK2 V617F mutation and OCIM2 and K562 cells in whom JAK2 is constitutively phosphorylated. WP1066 suppressed the colony-forming capacity of these cell types in a dose-dependent manner at concentrations ranging from 0.5 to 3.0 μmol/L with complete inhibition of colony formation at a concentration of 3.0 μmol/L. Notably, WP1066 did not inhibit the proliferation of the JAK3-dependent megakaryocytic leukemia CMK cells (Fig. 1D).

WP1066 degrades JAK2 protein. Next, we tested the effect of WP1066 on the levels of JAK2 protein in these AML cells. Specifically, we incubated OCIM2 and K562 cells with 0.5, 1.0, 2.0, 3.0, or 4.0 μmol/L WP1066 for 2 h and measured JAK2 and pJAK2 protein levels using Western immunoblotting. We found that WP1066 down-regulated the protein levels of both JAK2 and its phosphorylated form in a dose-dependent fashion (Fig. 2A, left). We then incubated the cells with 2 μmol/L WP1066 for 1, 2, 3, 4, or 6 h and found that WP1066 down-regulated JAK2 protein levels in a time-dependent manner in K562 cells, whereas in OCIM2 cells, WP1066 down-regulated only pJAK2 (Fig. 2A, right), suggesting that WP1066 induces both degradation of JAK2 protein and inhibition of its phosphorylation. We then studied the effect of WP1066 on JAK2 expression. We measured JAK2 mRNA expression using RT-PCR with both forward and reverse JAK2 primer sequences. We

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**Figure 3.** WP1066 inhibits the phosphorylation of STAT3, STAT5, and AKT, but not that of CrkL. A, OCIM2 and K562 cells (1 × 10^7 cells/mL) were incubated for 2 h in RPMI 1640 supplemented with 10% FCS and WP1066 at increasing concentrations (0.5–4.0 μmol/L). STAT3, STAT5, and AKT and their phosphorylated forms were detected in cell lysates using Western immunoblotting as described in Materials and Methods. Equal protein loading was confirmed using anti–α-actin antibodies (data not shown). B, K562 cells were incubated for 2 h in RPMI 1640 supplemented with 10% FCS and WP1066 at increasing concentrations (0.5–4.0 μmol/L). CrkL and pCrkL were detected in cell lysates using Western immunoblotting as described above.
found no significant changes in mRNA expression levels in either K562 or OCIM2 cells after exposure to 3 µmol/L WP1066 for 4 h (Fig. 2B). These data suggested that in addition to inhibiting JAK2 phosphorylation, WP1066 degrades JAK2 protein. To test whether this effect is specific to JAK2, we incubated OCIM2 and K562 cells with 0.5, 1.0, 2.0, 3.0, or 4.0 µmol/L WP1066 for 2 h and measured the protein levels of JAK1, JAK3, TYK2, and their phosphorylated forms using Western immunoblotting. As shown in Fig. 2C, we found that incubation of K562 cells with 3.0 and 4.0 µmol/L WP1066 resulted in the down-regulation of JAK3 protein levels. A similar exposure of OCIM2 cells to WP1066 did not affect JAK3 and pJAK3 protein levels. Furthermore, incubation with WP1066 did not induce any significant change either in the levels of JAK1, TYK2, or their phosphorylated forms, suggesting that WP1066 specifically reduces the protein levels of JAK2 in OCIM2 cells and of both JAK2 and JAK3 in K562 cells. To further delineate the effect of WP1066 on JAK3 protein levels, we incubated the JAK3-dependent megakaryocytic leukemia CMK cells with 0.5, 1.0, 2.0, 3.0, or 4.0 µmol/L WP1066 for 2 h and measured the levels of JAK3 and pJAK3 by Western immunoblotting. We found that WP1066 did not affect the protein levels of JAK3 and pJAK3 of CMK cells (Fig. 2D).

WP1066 inhibits the phosphorylation of STAT3, STAT5, and AKT. JAK2 phosphorylates both STAT3 and STAT5, prompting the translocation of these transcription factors to the nucleus. To determine whether WP1066 inhibits this downstream effect, we incubated OCIM2 and K562 cells with WP1066 at increasing concentrations. We found that WP1066 inhibited the phosphorylation of STAT3 and STAT5 in a dose-dependent manner. Incubation of OCIM2 and K562 cells with WP1066 at a concentration of 0.5, 1.0, 2.0, 3.0, or 4.0 µmol/L for 2 h resulted in significant down-regulation of constitutive STAT3 and STAT5 phosphorylation in both cell lines (Fig. 3A). Because JAK2 also activates the PI3K/AKT pathway, we sought to determine whether WP1066 suppresses phosphorylation of AKT. We found that, similar to STAT3 and STAT5, AKT was constitutively activated in both OCIM2 and K562 cells, and that incubation of these cells with 0.5 to 4.0 µmol/L WP1066 for 2 h inhibited the phosphorylation of AKT (Fig. 3A). Because the proliferation of K562 cells was shown to be driven by the protein kinase BCR-Abl, we asked whether WP1066 inhibits the activity of BCR-Abl. We found that WP1066 did not affect the levels of pCrkL, suggesting that the effect of WP1066 is not mediated through the inhibition of BCR-Abl (Fig. 3B).

WP1066 induces cell cycle arrest in OCIM2 cells. Because WP1066 inhibited the proliferation of AML cells, we wondered how this JAK2 inhibitor would affect the progression of OCIM2 cells through the cell cycle. We incubated OCIM2 cells with or without 2 µmol/L WP1066 for 2, 6, or 14 h and did cell cycle analysis using flow cytometry. We found that exposure to WP1066 induced the accumulation of OCIM2 cells in the G0-G1 phase of the cell cycle. As shown in Fig. 4, after incubation for 14 h, only 9.0% of the WP1066-treated cells reached the G2-M phase.

WP1066 induces apoptosis in OCIM2 and K562 cells. Because WP1066 inhibited OCIM2 and K562 cell proliferation, inhibited JAK2, and induced cell cycle arrest at the G0-G1 phase, we asked whether WP1066 induces apoptotic cell death in AML cells. To address this, we incubated OCIM2 and K562 cells at the peak of their growth with 1, 2, or 3 µmol/L WP1066 for 4 h. Using the annexin V–FITC assay, we found that WP1066 induced apoptosis in both OCIM2 and K562 cells in a dose-dependent fashion (Fig. 5A). Specifically, we noted that the percentage of cells undergoing apoptosis increased with increasing concentrations of WP1066.
apoptotic cell death increased from 5.9% in the OCIM2 control cells to 51.7% in the treated OCIM2 cells and from 9.1% in the K562 control cells to 41.9% in the treated K562 cells. WP1066-induced apoptosis was caspase dependent because it was partially reversed by treatment with the pan-caspase inhibitor Z-VAD-FMK (Fig. 5B).

Incubation of OCIM2 cells with Z-VAD-FMK did not affect their apoptotic rate, whereas preincubation of these cells with Z-VAD-FMK before treatment with WP1066 lowered their apoptotic rate from 71.5% to 43.0% (the background apoptotic rate in this experiment was 22.8%).

**WP1066 activates procaspase-3 and cleaves PARP.** Because cleavage of procaspases converts them into biologically active caspases, which in turn triggers apoptosis (27), we investigated the effect of treatment with WP1066 on the activation of caspase-3, a downstream executioner caspase in the apoptotic pathway. We incubated OCIM2 cells with Z-VAD-FMK did not affect their apoptotic rate, whereas preincubation of these cells with Z-VAD-FMK before treatment with WP1066 lowered their apoptotic rate from 71.5% to 43.0% (the background apoptotic rate in this experiment was 22.8%).

WP1066-induced apoptosis was caspase dependent because it was partially reversed by treatment with the pan-caspase inhibitor Z-VAD-FMK (Fig. 5B). Incubation of OCIM2 cells with Z-VAD-FMK did not affect their apoptotic rate, whereas preincubation of these cells with Z-VAD-FMK before treatment with WP1066 lowered their apoptotic rate from 71.5% to 43.0% (the background apoptotic rate in this experiment was 22.8%).

**Discussion**

Cytoplasmic JAK proteins are crucial components of diverse signal transduction pathways that govern cellular survival, proliferation, differentiation, and apoptosis (3). In AML cells bearing the translocation t(9;12) (5), the catalytic domain of JAK2 is activated by the TEL gene and, in myeloproliferative disorders and rare cases of AML, by a gain of function mutation (6, 28–30). Notably, data accumulated in recent years show that constitutive activation of either JAK2 (4, 5) or its downstream signaling pathways is associated with poor outcome in patients with AML (6–8). Taken together, these data suggest that JAK2 is a therapeutic target in AML and prompted us to investigate the antileukemic activity of the novel AG490 analogue WP1066.
We first studied the effects of treatment with WP1066 on fresh BM cells obtained from patients with AML. We found that this treatment inhibited the proliferation of AML blast colony-forming cells, and that the antiproliferative effect of WP1066 was more pronounced in AML-CFU-GM and BFU-E colony-forming cells.

To elucidate the mechanism of action of WP1066, we investigated its effects on the AML cell lines OCI-M2 and K562. Using a clonogenic assay, we found that WP1066 completely abrogated colony growth of both cell lines at a concentration of 2 µmol/L. This effect was induced by arresting the progression of leukemia cells through the cell cycle at G0-G1 phase and by inducing caspase-dependent apoptotic cell death. Confirming our hypothesis, we found that WP1066 inhibited JAK2 phosphorylation. However, to our surprise, we found that unlike its protein kinase inhibitor parent compound AG490 (11, 12), WP1066 also degraded JAK2 protein without affecting its mRNA expression. Remarkably, WP1066 did not significantly affect protein levels of other JAK family members except those of JAK3 in K562 cells. Incubation of OCI-M2 cells with WP1066 at concentrations that induced JAK2 protein degradation did not reduce the levels either of JAK1, JAK3, TYK2, or their phosphorylated forms. Furthermore, incubation of K562 cells with WP1066 neither reduced the levels of JAK1 and TYK2, nor of their phosphorylated forms, indicating specificity. As expected, this effect resulted in the inhibition of two of the signal transduction pathways activated by JAK2. WP1066 inhibited the phosphorylation of STAT3 and STAT5 and that of AKT.

The STATs belong to a family of latent cytoplasmic transcription factors involved in the response of normal and leukemic hematopoietic cells to a variety of extracellular signals (31–33). Several growth factors and cytokines such as GM-CSF and interleukin-3, interleukin-4, and interleukin-6 are abundant in patients with AML. These proteins activate JAKs and, as a result, the STAT pathway inducing proliferative and antiapoptotic effects in AML cells (31–33). Indeed, several studies have shown constitutive tyrosine phosphorylation and DNA binding of STAT3 and STAT5 in patients with AML and detected abnormal activation of STAT1, STAT3, STAT5, and STAT6 in cells transformed by Src, Abl, and other oncoproteins (34–36). Although growth factors and cytokines might transduce their signal from their corresponding cytoplasmic receptors to the nucleus by engaging other pathways, JAK-STAT pathway stimulates OCIM2 cell proliferation (13). In the present study, we found that WP1066 suppressed the phosphorylation of STAT3 and STAT5. Remarkably, a similar effect was recently observed in malignant glioma (39).

The tyrphostin AG490 has been thought to be a specific JAK2 inhibitor. However, several studies suggested that AG490 directly inhibits STATs without affecting JAK activity (40, 41). Therefore, we tested the effect of WP1066 on another JAK2-activated pathway. Lipid second messengers derived from polyphosphoinositide metabolism, such as PI3K, play a pivotal role in multiple cell-signaling networks. Several biological effects of PI3K are mediated through the activation of the downstream serine/threonine protein kinase AKT (protein kinase B; ref. 42). The PI3K signaling pathway is frequently activated in blast cells of patients with AML and contributes to the survival and proliferation of these cells. Furthermore, constitutive activation of the PI3K pathway correlates with decreased survival in patients with AML (8). Notably, in a previous study, we found that inhibition of the PI3K pathway suppresses proliferation and induces apoptosis of AML cells (43). In the present study, we found that WP1066 suppressed the phosphorylation of AKT, suggesting that WP1066 inactivated JAK2 and, as a result, inhibited two of the JAK2-activated signaling pathways.

We previously established that STAT3 and STAT5 are constitutively phosphorylated in OCI-M2 cells, and that activation of the JAK-STAT pathway stimulates OCI-M2 cell proliferation (13). In contrast, there is no consensus as to which signaling pathways are engaged in stimulating the proliferation of K562 cells. Similar to our group, other investigators found that JAK2 and STAT5 are constitutively activated in K562 cells (44, 45). Similarly, other researchers found that both STAT3 and STAT5 were constitutively tyrosine phosphorylated and transcriptionally active in K562 (46–48). Although the current belief is that STAT5 is directly activated by BCR-Abl (45–47, 49), Skorski et al. (50) reported that PI3K is constitutively activated by BCR-Abl, and Samanta et al. (48) found that JAK2 plays a crucial role in the proliferation of K562 cells. Our data showing constitutive phosphorylation of JAK2 and AKT support these reports. However, because WP1066 inhibited the JAK-STAT and PI3K pathways without affecting the levels of pCrkL, it is unlikely that BCR-Abl induces JAK2 phosphorylation.

In conclusion, we showed that the novel AG490 analogue WP1066 inhibited JAK2 and its downstream STAT and PI3K pathways. By doing so, WP1066 blocked the progression of AML cells through the cell cycle, inhibited AML cell proliferation, and induced caspase-dependent apoptotic cell death in AML cells. These findings suggest that WP1066 is a potent agent whose antineoplastic properties and in vivo activity warrant further investigation.

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