ANTICANCER RESEARCH *25*: 1841-1850 (2005)

WP-1034, A Novel JAK-STAT Inhibitor, with Proapoptotic and Antileukemic Activity in Acute Myeloid Leukemia (AML)

STEFAN FADERL, ALESSANDRA FERRAJOLI, DAVID HARRIS, QUIN VAN, WALDEMAR PRIEBE and ZEEV ESTROV

The Department of Leukemia of The University of Texas M.D. Anderson Cancer Center, Houston, Texas, U.S.A.

Abstract. *Cytokine stimulation induces proliferation and growth of acute myeloid leukemia (AML) blasts and high levels of cytokines have been associated with poor prognosis in AML. The Jak-Stat pathway constitutes a major mediator of cytokine activity. We investigated whether WP-1034, a novel Jak-Stat inhibitor, is active against AML blasts. OCIM2 and fresh AML cells were incubated with 1 to 6 μM WP-1034 to determine its effect on proliferation. WP-1034 effectively inhibited proliferation of OCIM2 cells and fresh AML samples. We then analyzed the expressions of Stat 1, 3, and 5, as well as Phospho-Stat 1, 3, and 5 by Western immunoblotting after incubation of OCIM2 cells without and with 1 to 10 μM WP-1034 for 2 hours, and at 5 μM from 20 minutes up to 4 hours and found that WP-1034 blocked Stat 3 and 5 activation. Analysis of cell cycle status by PI staining and flow cytometry showed that WP-1034 caused cell cycle arrest of OCIM2 cells in sub-G_0 phase. We then evaluated the induction of apoptosis of OCIM2 cells following incubation with WP-1034 at 3 to 6 μM by annexin V-CY5 assay and analyzed caspase 3 and PARP cleavage using Western immunoblotting. We found that WP-1034 induced apoptosis of OCIM2 cells and that induction of apoptosis involved cleavage of caspase 3 and the DNA repair enzyme poly (adenosine diphosphate [ADP]-ribose) polymerase (PARP). Taken together, our data suggest that WP-1034 is a potent inhibitor of AML cell proliferation by inhibition of Stat 3 and 5 and induction of caspase-dependent apoptosis.*

Corresponding author: Zeev Estrov, M.D., Department of Bioimmunotherapy, Box 422, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, U.S.A. Tel: (713) 794-1675, Fax: (713) 745-2374, e-mail: zestrov@mdanderson.org

Key Words: WP1034, acute myeloid leukemia, apoptosis, signal transduction, Jak-Stat.

Acute myeloid leukemia (AML) is a hematologic malignancy characterized by clonal proliferation of hematopoietic progenitor cells. The backbone of AML therapy is combination therapy including cytarabine (ara-C) in combination with an anthracycline. However, despite intensive treatment efforts and extensive clinical research with numerous other combinations of cytotoxic agents, the overall prognosis of patients with AML remains poor and the search for more effective therapies continues (1). More recently, targeted therapies such as inhibitors of tyrosine kinases (Flt3, c-kit, BCR-ABL) and other components of intracellular signaling pathways (*e.g.* Ras) have been developed and investigated in clinical trials (2, 3). Although clinical responses and evidence of biological activity have been demonstrated with some of these treatments, the intricate net of signaling pathways of AML blasts remains elusive and further dissection of the disease biology of AML is necessary to provide additional targets for therapy.

The Janus kinase-signal transduction and activator of transcription (Jak-Stat) signaling pathway is used by several cytokines and growth factors. Jak proteins are receptor-independent protein tyrosine kinases which, upon phosphorylation and activation, can generate several signaling pathways such as those regulated by ras, phosphatidylinositol 3-kinase (PI3K), and Stats (4). Abnormal activation of Stat 1, 3, 5 and 6 has been demonstrated in cells transformed by Src, Abl, and other oncoproteins (5-7). Spontaneous activation of Stat has been demonstrated in acute lymphoblastic leukemia (ALL) cells and constitutive DNA binding and tyrosine phosphorylation of Stat 1, 3 and 5 has been detected in patients with AML (8-10). The findings of constitutive activation of Stat proteins in AML cells suggest involvement of the Jak-Stat pathway in the process of transformation and proliferation of AML. Inhibition of the Jak-Stat pathway may, therefore, constitute a suitable target to abrogate AML cell proliferation.

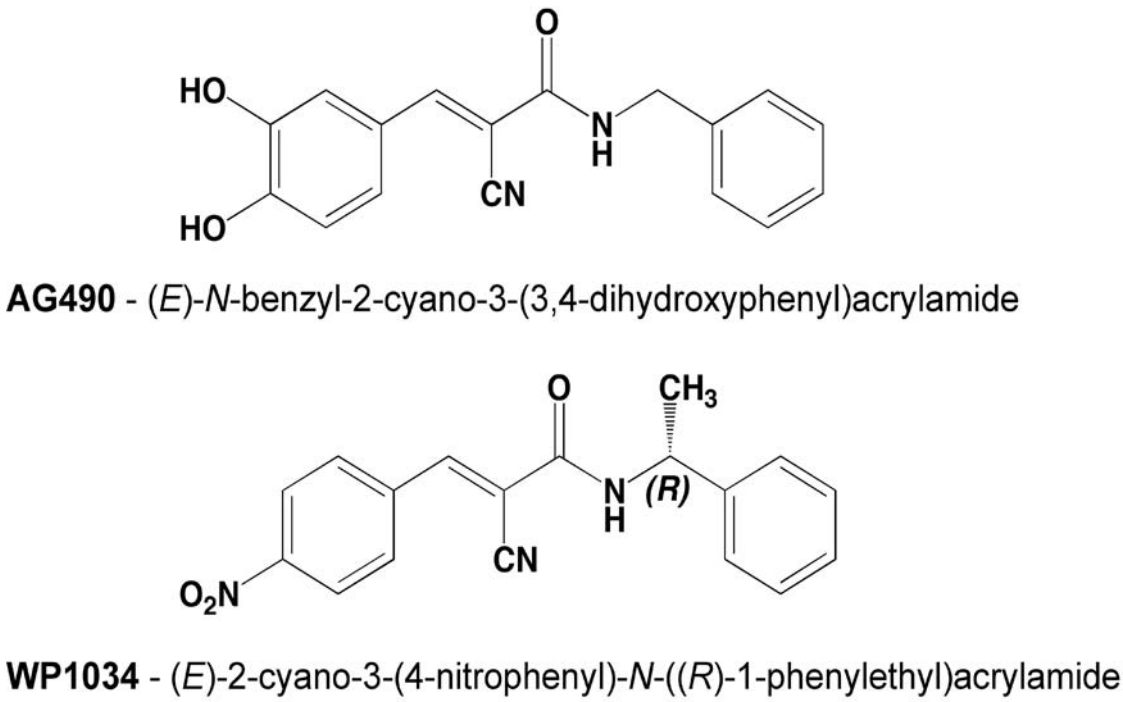

Figure 1. *Structure of AG490 and WP-1034.*

WP-1034 [(*E*)-2-cyano-3-(4-nitrophenyl)-*N*-((*R*)-1-phenylethyl) acrylamide] represents a further development of AG490 [(*E*)-*N*-benzyl-2-cyano-3-(3,4-dihydroxyphenyl) acrylamide] (11), a member of the tyrphostin family of tyrosine kinase inhibitors, which has been predominantly studied as an inhibitor of the Jak-Stat pathway (Figure 1). Here, we report the inhibitory effect of WP-1034 on the proliferation of OCIM2 and fresh AML cells and show that the activity of WP-1034 is mediated through inhibition of Stat activation, cell cycle arrest, and induction of apoptosis.

Materials and Methods

Drugs. WP-1034 was obtained as a lyophilized powder, dissolved in 1% DMSO, diluted with a 5% dextrose solution, and stored at –20˚C. AG490 (Sigma Chemicals, St. Louis, MO, USA) was dissolved in distilled water and further diluted in culture medium.

Cell lines. The AML cell line OCIM2 was provided by M.D. Minden (Ontario Cancer Institute, Toronto, ON, Canada) (12, 13). The OCIM2 cell line was established from a patient with erythroleukemia. It proliferates in the presence of culture medium and fetal calf serum (FCS) without exogenous growth factors. K562 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were maintained in RPMI 1640 culture medium (Gibco, Grand Island, NY, USA) supplemented with 10% FCS (Flow Laboratories, McLean, VA, USA). Cell cultures were grown in plastic tissue-culture dishes or flasks (Falcon Plastics; Becton Dickinson, Oxnard, CA, USA) and split twice weekly at different cell densities according to standard procedures using trypsin.

Table I. *Patients characteristics.*

UPN	Age/ Gender	Diagnosis	Karyotype	% Blasts		WBC*
				Marrow	Blood	
1	68/F	AML	-7	88	91	53.1
2	68/F	AML M5	Diploid	62	27	8.7
3	72/M	AML	Diploid	22	27	40.1
4	41/F	AML M1	Diploid#	87	83	86.6

*x 10^9/L, # later clonal evolution

Patient samples. Fresh bone marrow samples were obtained from 4 patients with AML (Table I). Samples were obtained and studies were performed with the patients' informed consent and the approval of the Institutional Review Board at the University of Texas M.D. Anderson Cancer Center, USA.

Western immunoblotting. Cell lysates were assayed for their protein concentration using the BCA Protein Assay Reagent (Pierce Chemical, Rockford, IL, USA). Each set of paired lysate samples was adjusted for the same protein concentration. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was then

conducted at constant wattage (10 W) in running buffer cooled to 4˚C (14). Stacking gels contained 4% (wt/vol) acrylamide, while separating gels contained 12% (wt/vol) acrylamide. Approximately 50 μg of sample protein was loaded into each of the appropriate lanes. Proteins separated with SDS-PAGE were transferred to nitrocellulose membranes; the transfers were performed overnight at 30 V in a cooled (4˚C) reservoir containing 25 mM Tris [tris (hydroxymethyl)aminomethane], 192 mM glycine, and 20% methanol (pH 8.3) transfer buffer (15). The nitrocellulose membranes were then removed from the blot apparatus and placed in a solution of Ponceau S stain (0.5% Ponceau S and 1% glacial acetic acid in water) to verify equal loading of protein in control and treated samples (16).

After membranes were stained for 5 minutes, they were rinsed for 2 minutes and examined. Equal loading of protein was verified, and the membranes were then rinsed for an additional 10 minutes and immunoscreened. The membranes were blocked with BLOTTO (5% dried milk dissolved in 50 mL of PBS) for at least 1 hour at room temperature. They were then washed 3 times in PBS plus 0.5% Tween 20. Next, the membranes were incubated for 1 to 12 hours with the appropriate antibodies (see below). After incubation, the membranes were rinsed 3 times in PBS containing 0.5% Tween 20 for 15 minutes each. The bound antibody was detected with the ECL Western Blotting Detection System (Amersham, Arlington Heights, IL, USA). The membranes were incubated with an anti-rabbit and anti-mouse horseradish peroxidase-labelled antibody at a concentration of 1:200 and 1:1750, respectively, in PBS plus 0.5% Tween 20 at room temperature for 1 hour. After this incubation, the membranes were washed in PBS containing 0.5% Tween 20, and bound antibody was detected according to the ECL protocol. Chemiluminescence of the membranes was detected with X-OMAT AR5 X-ray film (Kodak, Rochester, NY, USA) in stainless steel exposure cassettes (Sigma Chemical Co.).

The following antibodies were used for the respective proteins: monoclonal mouse anti-human CPP32 (Transduction Laboratories, Lexington, KY, USA) for detection of procaspase 3, rabbit anti-human cleaved caspase 3 (New England Bio Labs, Beverly, MA, USA) for detection of caspase 3, mouse anti-human poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP; Pharmingen, San Diego, CA, USA) for detection of PARP, mouse anti-human Stat 1, 3, 5 and pStat 1, 3, and 5 antibodies (Upstate Cell Signaling Solution, Charlottesville, VA, USA) for detection of Stat 1, 3, 5, and pStat 1, 3, and 5, respectively. Normal mouse immunoglobulin G (IgG) and rabbit IgG (Sigma) were used as controls. To confirm the detection of these proteins, we used lysates of Jurkat cells (ATCC; for the detection of procaspase 3, PARP, Stat and pStat proteins) and Hela cells (ATCC for the detection of cleaved caspase 3).

Cell cycle analysis. Cell cycle analysis was performed according to standard protocols. Briefly, 5 x 10^6 cells were pelleted following incubation with WP-1034. The cell pellets were washed and resuspended in 2 mL of 1% paraformaldehyde in PBS. Cells were incubated for 15 minutes at 4˚C and then washed again in PBS, resuspended in 2 mL of absolute ethanol, and stored at –20˚C until staining. The stored cells were then washed twice in PBS, resuspended in 0.5 mL of propidium iodide (PI) staining buffer (50 μg/mL PI, 10 μg/mL Rnase in PBS), and then incubated for 1 hour at room temperature in total darkness. Flow cytometric analysis was performed using a FACSCalibur and the CellQuest software program (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Data analysis was performed using CellQuest and the Modfit LT V2.0 software program (Verity Software House, Topsham, ME, USA).

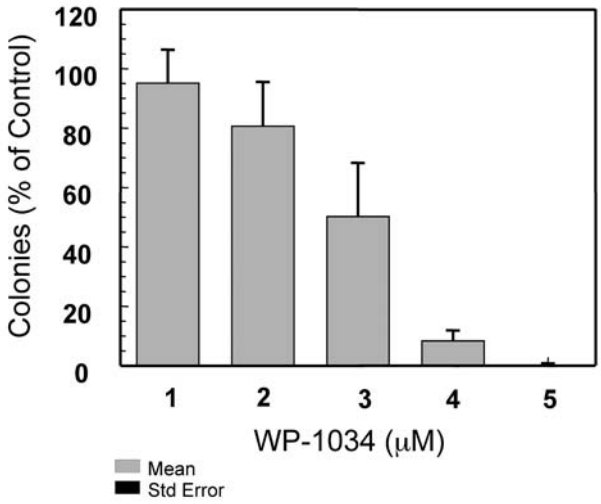

Figure 2. *Effect of WP-1034 on OCIM2 colony forming cell proliferation. Each data point represents the mean colony number in duplicate cultures. Representative data from 1 to 3 identical experiments are depicted.*

Annexin V assay for detection of apoptosis. To quantify the percentage of cells undergoing apoptosis, we used annexin V-CY5 (Pharmingen, San Diego, CA, USA), as previously described (17). Briefly, WP-1034-treated U266 cells were washed twice with cold PBS and then resuspended in binding buffer (10 nM HEPES [*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid], 140 nM NaCl, 5 nM $CaCl_2$, pH 7.4) at a density of 1 x 10^6 cells/μL. After incubation, 100 μL of the solution was transferred to a 5-mL culture tube to which 5 μL of annexin V-CY5 and 10 μL of PI were added. The tube was gently vortexed and incubated for 15 minutes at room temperature in total darkness. At the end of the incubation, 400 mL of binding buffer was added to the tube and the cells were analyzed immediately *via* flow cytometry. Flow cytometric analysis was performed with a FACSCalibur using CellQuest. Data analysis was performed with CellQuest and Modfit LT V2.0.

TUNEL assay for detection of apoptosis. The apoptosis detection system Fluorescein (Promega, Madison, WI, USA) was used to perform TdT-mediated dUTP nick-end labelling (TUNEL) (18). Briefly, OCIM2 cells were incubated with 4 μM of WP-1034 for 2 hours in the presence or absence of 50 μM of the caspase inhibitor Ac-DEVD-CHO (CalBiochem, La Jolla, CA, USA) (19). Cells fixed with 4% formaldehyde on slides were made permeable with 0.2% Triton-100 in PBS. After washing, the slides were treated with equilibration buffer (supplied with the TUNEL kit) and then incubated with with a TdT buffer (prepared according to the manufacturer's instructions) for 60 minutes and then with 2X SSC for 15 minutes. After washing, the slides were treated with anti-fade solution and then mounted on slides with glass cover slips and rubber cement. The slides were analyzed using a fluorescence microscope.

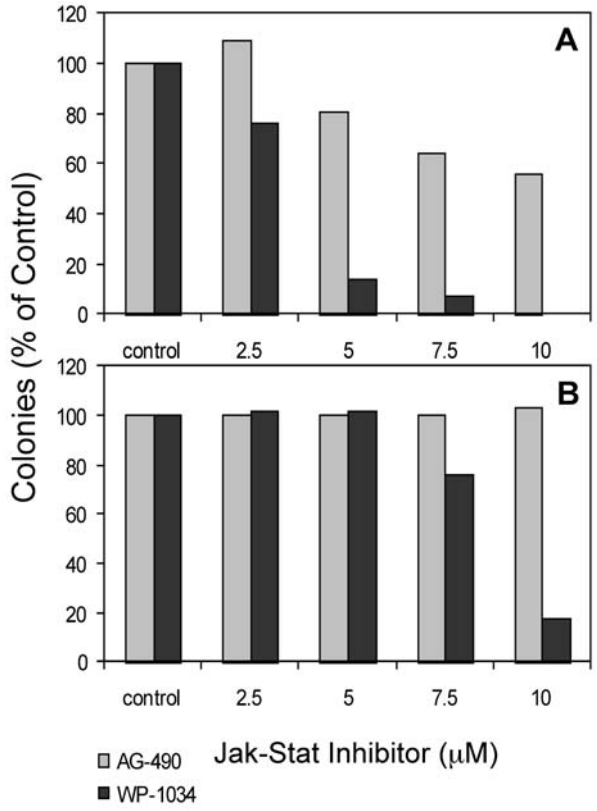

Figure 3. *Effect of WP-1034 and AG490 on K562 (A) and OCIM2 (B) colony-forming cell proliferation.*

Cell line clonogenic assays. The cell line clonogenic assay was performed as previously described (20). Briefly, OCIM2 cells (2-4 x 10^4 cells/mL) were cultured in 0.8% methylcellulose (Fluka Chemical, Ronkonkoma, NY, USA), 10% FCS, and RPMI 1640 medium in the presence of WP-1034, which was dissolved in ethanol at a final concentration of less than 0.1%. WP-1034 was then added at concentrations of 1, 2, 3, 4 and 5 μM. The cultures mixes were placed in 35-mm Petri dishes (Nunc, Naperville, IL, USA) in triplicate and maintained at 37˚C with 5% CO_2 in air in a humidified atmosphere. The colonies were counted after 7 days by using an inverted microscope. A colony was defined as a cluster of more than 40 cells.

AML blast colony assay. The AML blast colony assay was performed as previously described (21, 22). Briefly, 1 x 10^5 non-adherent T-cell depleted marrow cells were plated in 0.8% methylcellulose in a medium supplemented with 10% FCS and 50 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF; Immunex Corp, Seattle, WA, USA). WP-1034 was diluted in isotonic 5% dextrose solution and added at the initiation of the cultures at concentrations of 1, 2, 3, 4, 5 and 6 μM. The cultures were incubated in 35-mm Petri dishes in triplicate for 7 days at 37˚C in a humidified atmosphere of 5% CO_2 in air. AML blast colonies were microscopically evaluated on day 7 of culture. A blast colony was defined as a cluster of ≥20 cells. Individual colonies were plucked, smeared on glass slides, and stained to confirm their leukemic cell composition. That this assay identifies AML blast colony cells rather than normal hematopoietic progenitor cells has previously been demonstrated by cytogenetic analysis of these colonies (23).

Results

WP-1034 inhibits leukemia cell line colony proliferation. Myeloid leukemia cells respond to cytokine stimulation. Furthermore, high cytokine levels have been associated with poor prognosis in AML. As cytokines act through activation of the Jak-Stat pathway, we studied the effects of the Jak-Stat inhibitor WP-1034 on the proliferation of the acute myeloid leukemia cell line OCIM2. We found that WP-1034 suppressed the colony-forming growth of OCIM2 cells in a dose-dependent fashion at concentrations ranging from 1 to 5 μM (Figure 2). At concentrations of ≥4 μM, WP-1034 almost completely abolished cell proliferation. We then compared the effect of WP-1034 with that of AG490 on K562 and OCIM2 cells. In both cell lines, WP-1034 exerted a stronger inhibitory effect than did AG490. Whereas the proliferation of K562 cells was inhibited by >80% at WP-1034 concentrations of ≥5 μM, a concentration of at least 10 μM was necessary in OCIM2 cells to achieve the same degree of inhibition of proliferation. Of note, OCIM2 cells proved largely resistant to the cytotoxic effects of AG490 (Figure 3).

WP-1034 blocks activation of Stat 3 and 5. The Jak-Stat pathway is activated by several hematopoietic growth factors and cytokines. Jak-Stat proteins play a crucial role in the regulation of cell cycle, apoptosis, differentiation and proliferation of cells. Constitutive activation of the Jak-Stat pathway has been implicated in abnormal growth and proliferation of cancer cells. It has been shown that especially Stat 3 and Stat 5 have been abnormally phosphorylated and activated in leukemogenesis. We, therefore, asked whether WP-1034 would inhibit phosphorylation of Stat proteins and whether Jak-Stat inhibition would be associated with cell cycle arrest, induction of apoptosis and inhibition of colony-forming cell proliferation of fresh AML cells.

To test this hypothesis, we incubated OCIM2 cells with increasing concentrations of WP-1034. We found that WP-1034 inhibited Stat 3 and Stat 5 phosphorylation in a time- and dose-dependent manner (Figure 4). In a time-response course, OCIM2 cells were incubated with 5 μM of WP-1034 for 20 minutes, 40 minutes, and 1, 2, 3 and 4 hours. Levels of phospho-Stat 3 and phospho-Stat 5 were reduced to undetectable levels after incubation times of ≥1 hour (Figure 4A). Incubation of OCIM2 cells with WP-1034 at concentrations of 1, 2.5, 5, 7.5 and 10 μM for 2 hours significantly down-regulated Stat 3 and Stat 5 phosphorylation (Figure 4B). These data suggest that WP-1034 effectively inhibits Jak-Stat signaling in AML cells. No effect was observed on levels of phospho-Stat 1.

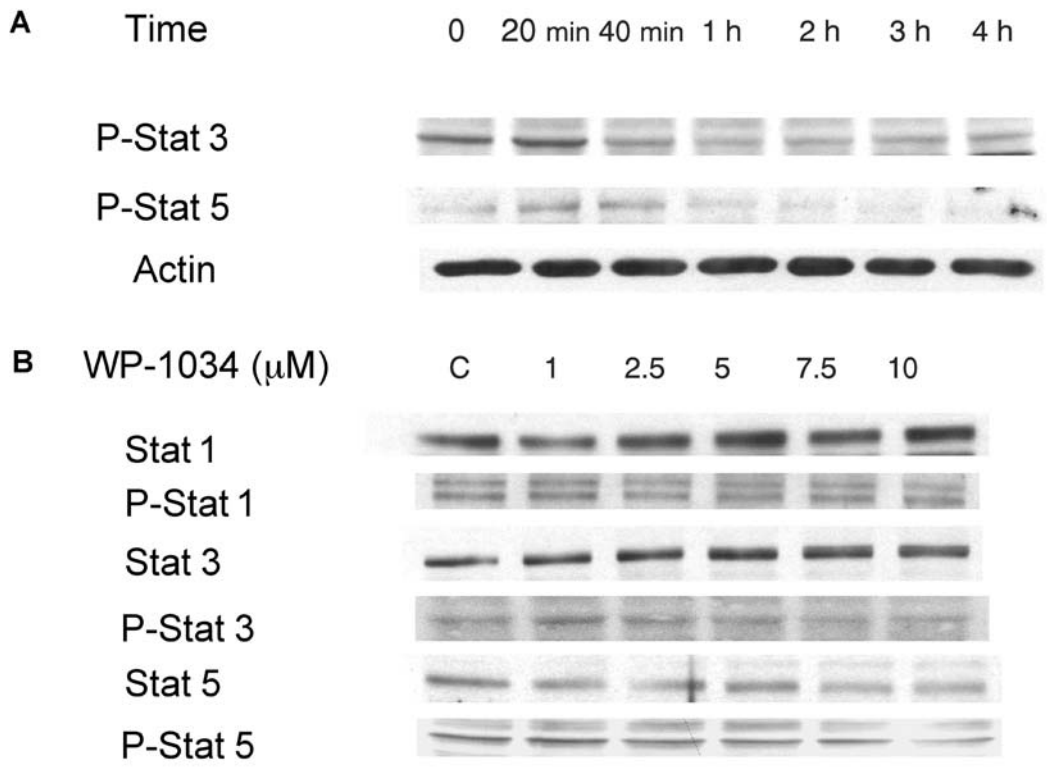

Figure 4. *Effect of WP-1034 on phosphorylation of Stat 1, 3, and 5. OCIM2 cells (1 x 10^7 cells/mL) were incubated at 5 μM for 20 min, 40 min, and 1, 2, 3 and 4 hours (A) and for 1 hour in RPMI medium supplemented with 10% FCS at increasing concentrations of WP-1034 (1, 2.5, 5, 7.5 and 10 μM) (B). Phosphorylated Stat 1, 3, and 5 were detected via Western immunoblotting, as described in the Materials and Methods section. Equal protein loading was confirmed by using anti-β-actin antibodies.*

WP-1034 induces cell cycle arrest in OCIM2 cells. Because WP-1034 blocked Stat 3 and Stat 5 phosphorylation and inhibited OCIM2 cell proliferation, we asked whether WP-1034 affected the progression of OCIM2 cells through the cell cycle. To answer this question, we incubated OCIM2 cells with 5 μM of WP-1034 for 2, 4, 6 and 16 hours and performed a cell cycle analysis using flow cytometry. We found that WP-1034 induced cell cycle arrest at Sub-G_0 phase at 6 hours of incubation, with around 23% of the cells accumulating at sub-G_0 phase at 16 hours of incubation (Figure 5).

WP-1034 induces apoptosis in OCIM2 cells. Because WP-1034 inhibited OCIM2 cell proliferation, blocked activation of Jak-Stat, and induced cell cycle arrest at the Sub-G_0 phase, we hypothesized that WP-1034 induces apoptotic cell death. To test this hypothesis, OCIM2 cells at the peak of their growth were incubated in the absence or presence of 3, 5 and 6 μM of WP-1034. Using the annexin V assay, we demonstrated that WP-1034 induced apoptosis in OCIM2 cells in a dose-dependent fashion (Figure 6). The percentage of cells undergoing apoptotic cell death increased from 9% (control) to 38% (6 μM WP-1034).

WP-1034-induced apoptosis involves activation of caspase 3 and cleavage of poly(ADP-ribose)polymerase (PARP). To validate these findings, we incubated OCIM2 cells with or without 4 μM of WP-1034 in the presence of absence of 50 μM of Ac-DEVD-CHO and used the TUNEL assay to detect apoptotic cells. We found that WP-1034 induced apoptotic cell death in OCIM2 cells and that the addition of the caspase inhibitor Ac-DEVD-CHO blocked OCIM2-induced apoptosis (Figure 7).

Apoptosis is triggered by the activation of a family of cysteine proteases, termed caspases, which are synthesized as latent intracellular proenzymes. Cleavage of the procaspase forms converts them into biologically active caspases. We, therefore, studied the effect of WP-1034 on activation of caspase 3, a downstream executioner caspase. We incubated OCIM2 cells without and with 1, 2.5, 5, 7.5 and 10 mM of WP-1034 for 1 hour and detected procaspase 3 and cleaved caspase 3 protein expression by Western immunoblotting (Figure 8). We found that WP-1034 induced dose-dependent caspase 3 cleavage. Activated caspase 3 abrogates the effect of substrates that protect cellular integrity, such as the DNA-repair enzyme PARP.

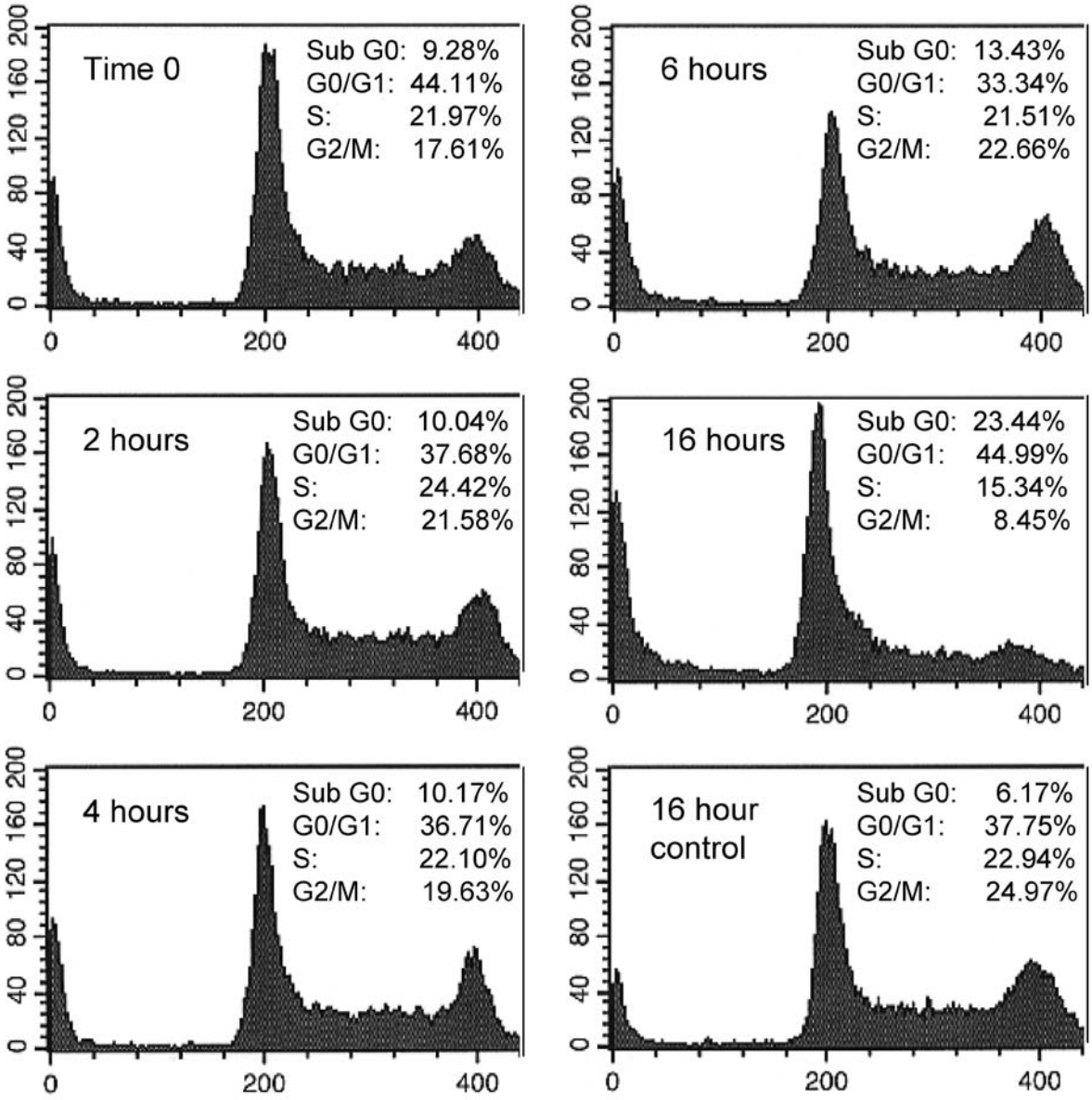

Figure 5. *Effect of WP-1034 on the cell cycle status of OCIM2 cells. Depicted are the percentages of cells in the Sub G_0, G_0/G_1, S, and G_2M-phases of the cell cycle after 2 to 16 hours of incubation with 4 μM WP-1034.*

Therefore, we incubated OCIM2 cells with WP-1034 at increasing concentrations. We found a dose-dependent increase in cleaved PARP protein levels (Figure 7).

WP-1034 inhibits leukemia colony-forming cell proliferation of fresh AML cells. We further investigated whether WP-1034 would also inhibit the proliferation of fresh AML samples from patient marrows. We, therefore, obtained marrow cells from 4 patients with AML (Table I) and studied the effects of WP-1034 in these cells using the AML blast colony assay. As shown in Figure 9A, we found that WP-1034 inhibited the proliferation of AML colony-forming blasts in a dose-dependent manner at concentrations ranging from 1 to 6 μM. We found that 5 μM of WP-1034 suppressed CML colony-forming blast proliferation at more than 50% and that proliferation was almost completely inhibited at 6 μM.

To elucidate whether WP-1034 acts specifically on AML blast proliferation and not on normal marrow cells, we incubated marrow cells from healthy volunteers with WP-1034 at concentrations of 4 to 6 μM. As demonstrated

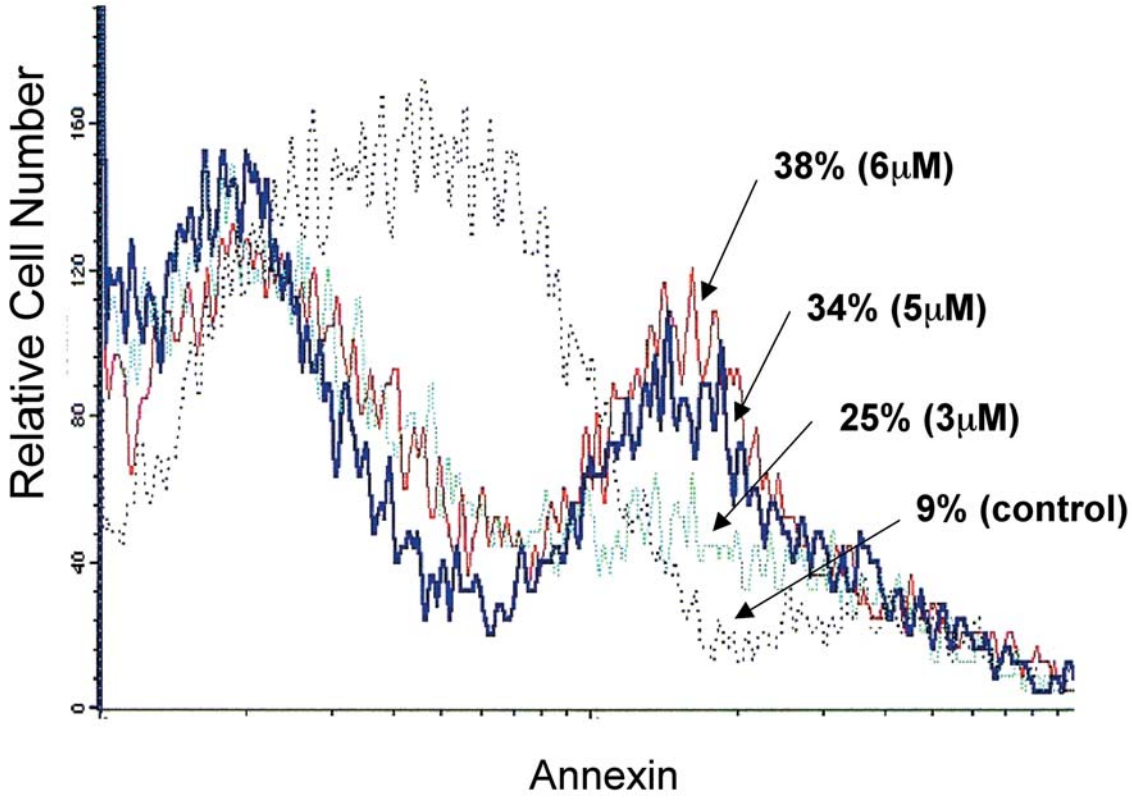

Figure 6. *WP-1014 induces apoptosis in OCIM2 cells. OCIM2 cells were incubated in the absence and presence of 3, 5 and 6 μM WP-1034. The fraction of cells undergoing apoptosis was detected by annexin V-CY5. The percentages depict the dose-dependent increase in the apoptotic cell fraction.*

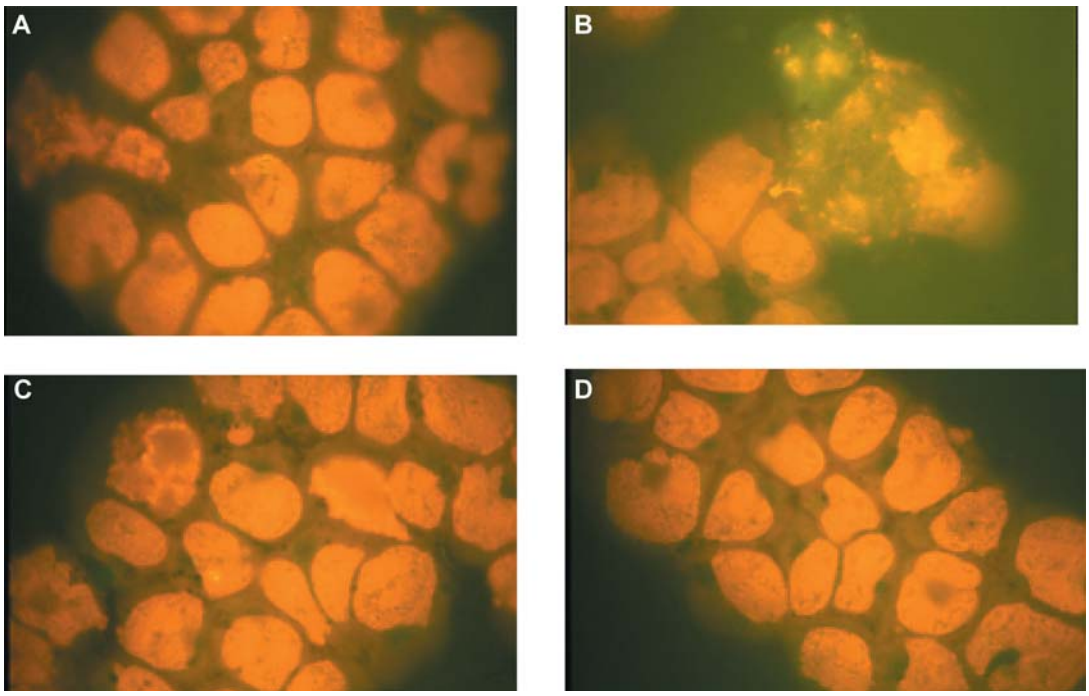

Figure 7. *Induction of apoptosis by WP-1034 in OCIM2 cells. OCIM2 cells were incubated for 2 hours with 4 μM of WP-1034 in the absence (B) and presence (D) of the caspase inhibitor Ac-DEVD-CHO at a concentration of 50 μM. Panel (A) shows a control with neither WP-1034 nor Ac-DEVD-CHO. Panel (D) shows cells incubated with Ac-DEVD-CHO, but without WP-1034. Apoptotic cells appear yellow.*

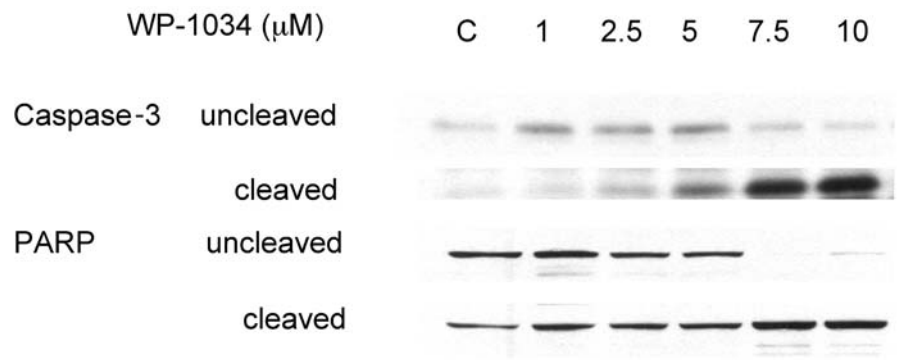

Figure 8. *Effect of WP-1034 on procaspase 3 and PARP cleavage. OCIM2 cells were incubated with 1, 2.5, 5, 7.5 and 10 μM of WP-1034 for 1 hour. The levels of procaspase 3, caspase 3, and uncleaved and cleaved PARP were detected via Western immunoblotting. The results demonstrate a dose-dependent increase in the cleaved caspase 3 (upper panel) and cleaved PARP levels (lower panel).*

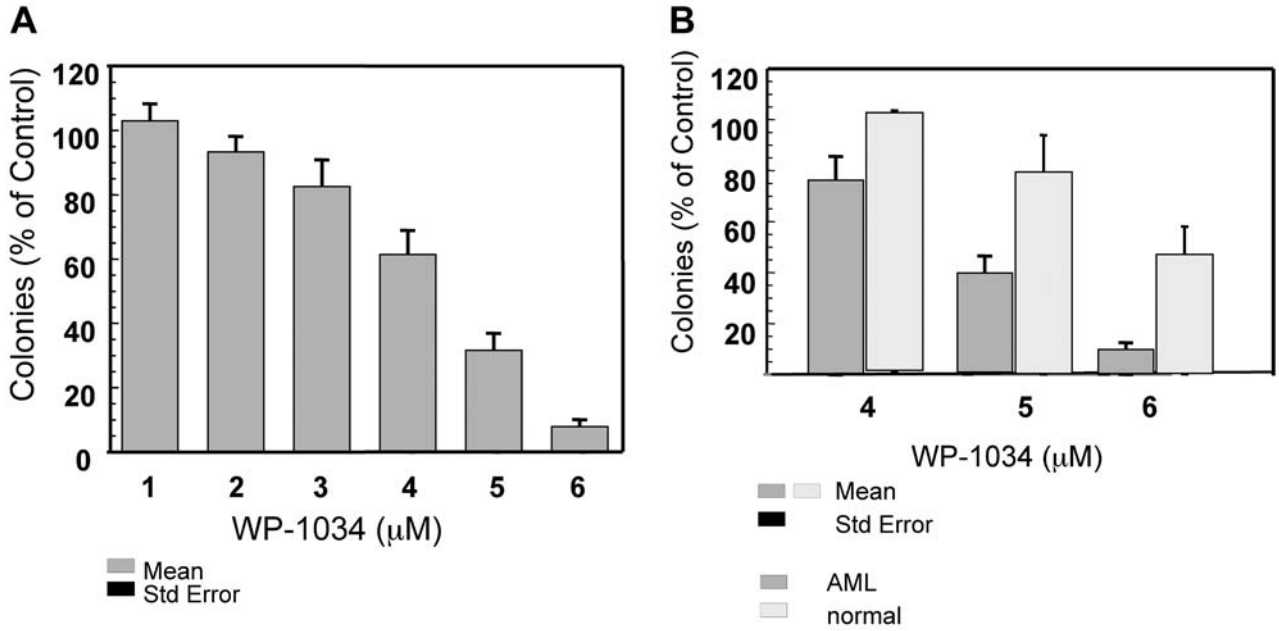

Figure 9. *Effect of WP-1034 on proliferation of AML colony-forming cells obtained from 4 AML patients. (A) After fractionation, AML cells were cultured in the presence of WP-1034 at concentrations ranging from 1 to 6 μM. AML colonies are presented as percentages of control. Each data point represents the mean value of duplicate and triplicate measurements +/- SD. (B) Effect of WP-1034 on AML cells and marrow cells from healthy volunteers at WP-1034 concentrations of 4 to 6 μM.*

in Figure 9B, we found a dose-dependent inhibition of the growth of healthy marrow CFU-GM between 4 to 6 μM of WP-1034. However, the inhibition of normal marrow cells was less than the inhibition of AML blast cells at the same concentrations of WP-1034.

Discussion

Treatment outcomes in AML are poor. Despite numerous clinical trials of cytotoxic drug combinations, novel and targeted agents such as tyrosine kinase inhibitors, farnesyltransferase inhibitors, or antiangiogenesis agents among others, disease recurrence is common and most patients will die from their disease.

In this study, we presented the antileukemic activity of a novel synthetic inhibitor of the Jak-Stat pathway, (*E*)-*N*-benzyl-2-cyano-3-(3,4-dihydrophenyl)acrylamide (WP-1034). We showed inhibition of proliferation of OCIM2 AML cell lines as well as fresh samples from AML patients in a dose-dependent fashion with almost complete abolition of cell

growth at WP-1034 concentrations ≥4 μM in cell lines and ≥6 μM in patient samples. Comparing the growth-inhibiting activity of WP-1034 in AML marrow samples and samples from healthy volunteers, the antiproliferative effect of the Jak-Stat inhibitor was more pronounced in the leukemia samples. We then demonstrated that incubation of cells with WP-1034 blocked activation of Stat 3 and Stat 5 in a dose- and time-dependent fashion, induced cell cycle arrest and triggered apoptosis, which appears to be mediated by a caspase-dependent pathway.

The significance of Jak-Stat as a signaling pathway for regulation of important cellular functions has long been understood (24-26). Jak-Stat signaling pathways were initially discovered from studies of interferon signaling. It has since become clear that a large number of cytokines, growth factors and hormones can activate Jaks and Stats. Upon ligand binding to the receptor, Jaks associate with the activated receptors, whereby they in turn activate members of the Stat family through phosphorylation on a single tyrosine residue. Activated Stats then form dimers, translocate to the nucleus, bind to specific response elements in the promoter regions of genes and, hence, trigger transcription of a gene profile that will be responsible for the respective phenotype of the cell. Although other pathways exist, Jak-Stat has been recognized as one of the most important signaling pathways downstream of cytokine receptors (27). Modulating the activity of Jak-Stat therefore provides the possibility to influence a cell's reaction to cytokine stimulation, establishing an attractive target for leukemia cells as well.

Several cytokines, such as GM-CSF and interleukin (IL)-6, induce growth and proliferation of AML cells and protect them from apoptotic cell death (28). In addition, high levels of hematopoietic growth factors have been detected in AML patients and are associated with a poor prognosis (29, 30). An association of cytokine activation with Jak-Stat in leukemias has been demonstrated in several papers. Schuringa *et al.* have shown that, in 25% of AML patients, Stat 3 was constitutively phosphorylated and that Stat 3 was constitutively bound to DNA response elements (31). Constitutive Stat 3 activation was caused by high secretion of IL-6 from the AML blasts and thus stimulated Jak-Stat pathways in an autocrine and/or paracrine manner. In addition to Stat 3, constitutive DNA binding and phosphorylation of Stat 1 and Stat 5 has also been detected in samples from AML patients (8-10, 32). These data support a crucial role of Jak-Stat in the process of proliferation and transformation of myeloid leukemia cells and thus provide a rationale for attempts to block cytokine activation *via* that path. In this sense, our data of successful growth inhibition in the presence of Jak-Stat inhibition fit well and further underlie the significance of Jak-Stat in these cells as a potential therapeutic target.

Although inhibition of Jak-Stat results in the blocking of Stat activation, induction of apoptosis and eventually inhibition of growth and proliferation of AML cells, regulation of cytokine stimulation through intracellular signaling pathways such as Jak-Stat is certainly far more complex. As reported by our group and others, the constitutive expression of growth-stimulatory signals is associated with activation of negative feedback loops such as increased expression of suppressors of cytokine signaling (SOCS) (33, 34). How inhibition of Jak-Stat influences these control mechanisms and, hence, the growth property of AML cells is unknown, as much as is the functionality of these feedback loops in myeloid leukemia cells. Furthermore, activation of Stat 3 has also been shown to be a critical step in a cascade of events leading to terminal differentiation of M1 myeloid leukemia cells by Minumi *et al.* (35). M1 cells were induced to growth arrest and terminal differentiation into macrophages by Stat activation through IL-6 and Leukemia Inhibitory factor (LIF). In M1 cells that constitutively expressed dominant negative forms of Stat 3, no induction of differentiation could be demonstrated. Although these data appear contradictory, they most likely reflect a far more significant biologic heterogeneity of myeloid leukemia cells than could be captured in blocking a single intracellular signaling pathway. It should also be remembered that cell lines represent an artificial system and are less suited than fresh patient samples to mirror pathophysiologic processes relevant to the disease.

In conclusion, we present evidence of the impact of Jak-Stat inhibition on the growth and proliferation of AML cells using a new and potent protein kinase inhibitor of Jak-Stat. Its significance will eventually have to be further substantiated in clinical trials.

References

1 Weick JK, Kopecky KJ, Appelbaum FR *et al*: A randomized investigation of high-dose *versus* standard-dose cytosine arabinoside with daunorubicin in patients with previously untreated acute myeloid leukemia: a Southwest Oncology Group Study. Blood *88*: 2841-2851, 1996.

2 Lancet JE and Karp JE: Farnesyltransferase inhibitors in hematologic malignancies: new horizons in therapy. Blood *102*: 3880-3889, 2003.

3 Sawyers CL: Finding the next Gleevec: FLT3 targeted kinase inhibitor therapy for acute myeloid leukemia. Cancer Cell *1*: 413-415, 2002.

4 Leonard WJ: Role of Jak kinases and STATs in cytokine signal transduction. Int J Hematol *73*: 271-277, 2001.

5 Tuyt LML, Bregman K, Lummen C *et al*: Differential binding activity of the transcription factor LIL-STAT in immature and differentiated normal and leukemia myeloid cells. Blood *92*: 1364-1373, 1998.

6 Garcia R and Jove R: Activation of STAT transcription factors in oncogenic tyrosine kinase signaling. J Biomed Sci *5*: 79-85, 1998.

7 Zong C, Yan R, August A *et al*: Unique signal transduction of Eyk: constitutive stimulation of the JAK-STAT pathway by an oncogenic receptor-type tyrosine kinase. EMBO J *15*: 4515-4525, 1996.

8 Gouilleux GV, Gouilleux F, Desaint C *et al*: STAT-related transcription factors are constitutively activated in peripheral blood cells from acute leukemia patients. Blood *87*: 1692-1697, 1996.
9 Gouilleux GV, Debierre GF, Gouilleux F *et al*: Activated Stat related transcription factors in acute leukemia. Leuk Lymphoma *28*: 83-88, 1997.
10 Xia Z, Baer MR, Block AW *et al*: Expression of signal transducers and activators of transcription proteins in acute myeloid leukemia blasts. Cancer Res *58*: 3173-3180, 1998.
11 Savell J, Ma Y, Morrow KS *et al*: AG490 inhibits G1-S traverse in BALB/c-3T3 cells following either mitogenic stimulation or exogenous expression of E2F-1. Mol Cancer Ther *3*: 205-213, 2004.
12 Papayannopoulou T, Nakamoto B, Kurachi S *et al*: Surface antigen profile and globin phenotype of two new human erythroleukemia lines: characterization and interpretation. Blood *72*: 1029-1038, 1988.
13 Wang C, Curtis JE, Minden MD and McCulloch EA: Expression of retinoic acid receptor gene in myeloid leukemia cells. Leukemia *3*: 236-249, 1989.
14 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bactriophage T4. Nature *227*: 680-685, 1970.
15 Towbin H, Staehelin T and Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA *76*: 4350-4354, 1979.
16 Gershoni JM and Palade GE: Protein blotting: principles and applications. Anal Biochem *131*: 1-15, 1983.
17 Vermes I, Haanen C, Steffens-Nakken H and Reutelinsperger C: A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein-labelled annexin V. Immunol Method *184*: 39-51, 1995.
18 Estrov Z, Manna SK, Harris D *et al*: Phenylarsine oxide blocks interleukin-1-induced activation of the nuclear transcription factor NF-kB, inhibits proliferation, and induces apoptosis of acute myelogenous leukemia cells. Blood *94*: 2844-2853, 1999.
19 Thornberry NA, Peterson EP, Ahao JJ *et al*: Inactivation of interleukin-1 beta converting enzyme by peptide (acyloxy) methyl ketones. Biochemistry *33*: 3934-3940, 1994.
20 Estrov Z, Cohen A, Gelfand EW and Freedman MH: Synergistic antiproliferative effects on HL-60 cells: deferoxamine enhances cytosine arabinoside, mehtotrexate, and daunorubicin cytotoxicity. Am J Pediatr Hematol Oncol *10*: 288-291,1988.
21 Buick RN, Till JE and McCulloch EA: Colony assay for proliferative blast cells circulating in myeloblastic leukemia. Lancet *1*: 862-863, 1977.
22 Minden MD, Buick RN and McCulloch EA: Separation of blast cells and T lymphocyte progenitors in the blood of patients with acute myeloblastic leukemia. Blood *54*: 186-195, 1979.
23 Freedman MH, Chan HS and Chang L: Childhodd erythro-leukemia: studies on pathogenesis using colony assays. Am J Pediatr Hematol Oncol *8*: 2-7, 1986.
24 Heim H: The Jak-STAT pathway: cytokine signaling from the receptor to the nucleus. J Recept Signal Transduct Res *19*: 75-120, 1999.
25 Aringer M, Cheng A, Nelson JW *et al*: Janus kinases and their role in growth and disease. Life Siences *64*: 2173-2186, 1999.
26 Imada K and Leonard WJ: The Jak-STAT pathway. Mol Immunol *37*: 2-11, 2000.
27 Leonard WJ: Role of Jak kinases and STATs in cytokine signal transduction. Int J Hematol *73*: 271-277, 2001.
28 Liu R, Liu CB, Mohi MG *et al*: Analysis of mechanisms involved in the prevention of gamma irradiation-induced apoptosis by hGM-CSF. Oncogene *19*: 571-579, 2000.
29 Lowenberg B, van Putten WL, Tou IP *et al*: Autonomous proliferation of leukemia cells *in vitro* as a determinant of prognosis in adult acute myeloid leukemia. N Engl J Med *328*: 614-619, 1993.
30 Estrov Z, Kurzrock R and Talpaz M: Cytokines and their antagonists in myeloid disorders. Semin Hematol *32*: 220-231, 1994.
31 Schuringa J-J, Wierenga ATJ, Kruijer W and Vellenga E: Constitutive Stat3, Try705, and Ser727 phosphorylation in acute myeloid leukemia cells caused by the autocrine secretion of interleukin-6. Blood *95*: 3765-3770, 2000.
32 Baskiewicz-Masiuk M and Machalinksi B: The role of STAT5 proteins in the proliferation and apoptosis of the CML and AML cells. Eur J Haematol *72*: 420-429, 2004.
33 Metcalf D: The SOCS-1 story. Exp Hematol *27*: 1715-1723, 1999.
34 Faderl S, Harris Q, Van Q *et al*: Granulocyte-macrophage colony-stimulating factor (GM-CSF) induces antiapoptotic and proapoptotic signals in acute myeloid leukemia. Blood *102*: 630-637, 2003.
35 Minami M, Inoue M, Wei S *et al*: STAT3 activation is a critical step in pg130-mediated terminal differentiation and growth arrest of a myeloid cell line. Proc Natl Acad Sci *93*: 3963-3966, 1996.

Received February 28, 2005
Accepted March 15, 2005