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

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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-G0 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.

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 oncogenes (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.

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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.

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

<table>
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<th>Age/Gender</th>
<th>Diagnosis</th>
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<th>Marrow</th>
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<th>WBC*</th>
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* x 10⁹/L, # later clonal evolution
performed using a FACSCalibur and the CellQuest software at room temperature in total darkness. Flow cytometric analysis was performed with SDS-PAGE gels containing 12% acrylamide and 4% Triton X-100 in a solution of 1× SDS-PAGE sample buffer. Approximately 25 μg of sample protein was loaded onto each gel. The gels were run at constant wattage (10 W) in running buffer cooled to 4°C. The separated proteins were transferred to nitrocellulose membranes using a semi-dry blot apparatus. The membranes were blocked with 5% dried milk dissolved in PBS for at least 1 hour at room temperature. They were then washed 3 times in PBS plus 0.5% Tween 20. 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 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, rabbit anti-human Stat 1, 3, and 5 antibodies (Upstate Cell Signaling Solution, Charlottesville, VA, USA) for detection of Stat 1, 3, and 5, and rabbit IgG (Sigma) was used as controls. To confirm the detection of these proteins, we used lysates of Jurkat cells (ATCC; for the detection of caspase 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 10⁶ 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 stained cells were 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 with CellQuest and the Modfit LT V2.0 software program (Verity Software House, Topsham, ME, USA).

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 mM NaCl, 5 mM CaCl₂, pH 7.4) at a density of 1 x 10⁶ cells/mL. 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 μL 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, OCI2M2 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 were fixed with 4% formaldehyde on slides and 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.
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% CO2 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 and 5 μM. The cultures were incubated in 35-mm Petri dishes (Nunc, Naperville, IL, USA) in triplicate and maintained at 37°C with 5% CO2 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 ≥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.
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-G0 phase at 6 hours of incubation, with around 23% of the cells accumulating at sub-G0 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-G0 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 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 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).

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).

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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

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![Figure 5. Effect of WP-1034 on the cell cycle status of OCIM2 cells. Depicted are the percentages of cells in the Sub G0, G0/G1, S, and G2/M-phases of the cell cycle after 2 to 16 hours of incubation with 4 μM WP-1034.](image)
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.
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 underline 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 pathophysiological 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


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