Pyridone 6, A Pan-Janus–Activated Kinase Inhibitor, Induces Growth Inhibition of Multiple Myeloma Cells

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Abstract

Interleukin-6 (IL-6) and the subsequent Janus-activated kinase (JAK)-dependent signaling pathways play a critical role in the pathogenesis of multiple myeloma. Here, we compared the sensitivity and specificity of a novel pan-JAK inhibitor, tetracyclic pyridone 6 (P6), with that of AG490 in a panel of myeloma-derived cell lines. P6 induced growth arrest and subsequent apoptosis of the IL-6-dependent hybridoma and myeloma-derived cell lines (B9 and INA-6) grown either in IL-6-containing medium or in the presence of bone marrow–derived stromal cells (BMSC) using much lower concentrations of drug and with significantly faster kinetics than AG490. Myeloma-derived cell lines, which either express constitutively activated JAK/signal transducers and activators of transcription (STAT) 3 (U266) or are IL-6 growth stimulated (KMS11), are partially growth inhibited by P6. However, P6 does not inhibit the growth of myeloma-derived cell lines lacking activated JAKs/STATs nor does it inhibit mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase activity compared with AG490, which led to activation of ERK and induced robust apoptosis of all the examined cell lines. Finally, P6 inhibited the growth of primary myeloma patient samples grown in the presence of BMSCs. Thus, P6 is a more sensitive and specific inhibitor of JAK-STAT3 activity compared with AG490 and potently inhibited the growth of primary myeloma cells and myeloma-derived cell lines grown on BMSCs. (Cancer Res 2006; 66(19): 9714-21)

Introduction

Janus-activated kinases (JAK) are a group of tyrosine kinases that mediate cytokine signaling. Specifically, cytokine binding to their receptors leads to the activation of associated JAKs, which autophosphorylate and transphosphorylate cytokine receptors, creating binding sites for effectors of signaling, including the signal transducers and activators of transcription (STAT) proteins, which in turn become tyrosine phosphorylated by the JAKs (1). There is accumulating evidence that constitutive activation of different JAKs and STATs mediates neoplastic transformation and promotes abnormal cell proliferation in various malignancies (2, 3). Recently, a somatic activating mutation in the JAK2 tyrosine kinase (JAK2V617F) was identified in polycythemia vera, in other myeloproliferative disorders (MPD), and in a subset of leukemias (4–6). In addition, the TEL-JAK2 fusion protein (resulting in constitutive JAK2 activation) has been identified in patients with MPD, acute myelogenous leukemia, and acute lymphoblastic leukemia, and introduction of the TEL-JAK2 fusion protein in murine bone marrow led to fatal myeloproliferative and lymphoproliferative disease (7). Other mechanisms of constitutive JAK activation include the association of JAKs with other oncogenic tyrosine kinases, such as nucleophosmin-anaplastic lymphoma kinase, Src, and mutant epidermal growth factor receptor (2, 3, 8). Perhaps the most common mechanism causing abnormal JAK activation involved in malignant transformation is through dysregulated cytokine signaling, which is found in prostate and breast cancer as well as in hematologic malignancies, including multiple myeloma. Interleukin (IL)-6 is a cytokine required for terminal differentiation of B cells and plays a critical role in the pathogenesis and growth of multiple myeloma (9). IL-6 binds to the IL-6 receptor (IL-6R), leading to the dimerization of these chains activating the receptor-associated JAKs (JAK1, JAK2, and Tyk2). The JAKs trigger the phosphorylation of the signal-transducing gp130 chain of the IL-6R and activation of STAT3 protein and the Ras/mitogen-activated protein kinase (MAPK) pathway (10). STAT3 is found to be constitutively tyrosine phosphorylated in at least 50% of primary myeloma samples, whereas inhibition of STAT3 activity in myeloma-derived cell lines using IL-6R antagonists, dominant-negative STAT3 constructs, or the JAK2 inhibitor AG490 induces growth inhibition or apoptosis (11–17). These experimental observations highlight the potential therapeutic importance of JAK kinase blockade in the treatment of several malignancies, including myeloma. The tyrphostin AG490 was the first and best-characterized JAK tyrosine kinase inhibitor shown to inhibit the growth of cancer cells *in vitro* and *in vivo* (18, 19). However, high micromolar amounts of AG490 are required for growth inhibition and it remains unclear whether this inhibitor is indeed specific (20, 21). Recently, a tetracyclic pyridone 2-*tert*-butyl-9-fluoro-3,6-dihydro-7*H*-benz[h]-imidaz[4,5-*f*]isoquinoline-7-one, pyridone 6 (P6), was found to inhibit the JAKs in the low nanomolar range (IC_{50}, 1-15 nmol/L) and block IL-2-dependent proliferation of CTLL cells (22). P6 is a reversible ATP inhibitor, and when tested against many other kinases, IC_{50}s of >130 nmol/L were required. P6 was recently cocrystalized with the JAK2 kinase domain and binds via an induced fit mechanism, buried within a narrow ATP-binding pocket (23). Here, we examined, characterized, and compared the sensitivity and specificity of P6 with that of AG490 in a variety of myeloma-derived cell lines and determined that, whereas AG490 induced apoptosis and growth arrest of all the myeloma-derived cell lines (irrespective of JAK-STAT activation),

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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P6 only inhibited the growth of myeloma-derived cell lines expressing activated JAKs and STAT3 both in culture and grown on bone marrow–derived stromal cells (BMSC) as well as primary myeloma cells.

Materials and Methods

Cell culture, proliferation assays, and fluorescence-activated cell sorting analysis. B9 murine hybridoma cells were obtained from Keith Stewart (McLaughlin Centre for Molecular Medicine, Toronto, Ontario, Canada) and grown in Iscove's modified Dulbecco's medium supplemented with 2 mmol/L L-glutamine, penicillin/streptomycin (Invitrogen, Carlsbad, CA), 5% fetal bovine serum (FBS), and 1 ng/mL of recombinant human IL-6 (rhIL-6; R&D Systems, Minneapolis, MN). The B9-independent cell line was generated by serially diluting out the IL-6 over 14 days and enriching for the IL-6-independent cells by Ficoll-Hypaque (Becton Dickinson, Franklin Lakes, NJ) separation. The resulting B9(i) cells do not require IL-6 for growth. Murine bone marrow stromal cells (muBMSC; MS-5 cells) were obtained from Malcolm Moore (Memorial Sloan-Kettering Cancer Center, New York, NY) and grown in RPMI 1640 with 10% FCS and penicillin/streptomycin. RPMI 8226 cells were purchased from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 with L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, and 10% FBS. KMS11 and U266 were obtained from Peter L. Bergsagel (Mayo Clinic College of Medicine, Rochester, MN) and grown in RPMI 1640 with above-stated supplements. INA-6 myeloma cells were obtained from M. Gramatzki (University of Erlangen, Erlangen, Germany) and grown in RPMI 1640 with 10% FBS, L-glutamine, penicillin/streptomycin, and 200 units/mL rhIL-6. Human BMSCs (huBMSC) were isolated as previously described (24) and cultured in RPMI 1640 supplemented with 10% FBS, 5% horse serum, L-glutamine, penicillin/streptomycin, 1 mmol/L sodium pyruvate, 20 μg/mL L-asparagine, 50 nmol/L 2-mercaptoethanol, 10 mmol/L HEPES, and nonessential amino acids. For proliferation assays, 5×10^5 INA-6 cells were plated in 3 mL of medium in the presence of IL-6 or huBMSC, and 16 hours later, 0.5 μmol/L P6 or DMSO was added and cells were counted daily over 3 days using a hemocytometer after trypan blue staining. B9(d) and B9(i) cells (5×10^3) were plated in 200 μL/96-well dish in the presence of IL-6 or muBMSC and treated with either DMSO or increasing concentrations of P6 or AG490. KMS11, U266, H929, and RPMI-8226 cells (1×10^4) were plated in 200 μL/96-well dish and treated with P6, AG490, and/or cisplatin at 1 μg/mL. Cells were counted after 72 hours using a hemocytometer with trypan blue exclusion. Proliferation was also measured by Alamar Blue reduction (Biosource, Camarillo, CA) using a spectrofluorometric microtiter plate reader. Cell cycle progression and apoptosis was determined by flow cytometry using Annexin V-FITC (Clontech-Takara Bio, Palo Alto, CA) and propidium iodide. Patient multiple myeloma cells were purified from bone marrow aspirates from seven different multiple myeloma patients first by Ficoll-Hypaque density gradient centrifugation and then by negative selection (RosetteSep separation system, StemCell Technologies, Vancouver, British Columbia, Canada). The purity of multiple myeloma cells was confirmed by flow cytometric analysis using anti-CD138Ab (BD PharMingen, San Diego, CA). In all samples, >90% purity of $CD138^+$ cells was achieved. All patients gave informed written consent under an Institutional Review Board–approved protocol. Primary multiple myeloma cells were cultured in RPMI 1640 supplemented with 20% FCS, 100 units/mL penicillin, 100 g/mL streptomycin, 1 mmol/L sodium pyruvate, and 2 mmol/L glutamine. Primary multiple myeloma cells were plated on a confluent layer of BMSCs (HS-5 cells) in the presence of DMSO or P6 (0.5 μmol/L), and viable cell number was determined 48 hours later using a hemocytometer with trypan blue exclusion. All experiments were done in triplicate.

Western blot, antibodies, immunoprecipitation, and electrophoretic mobility shift assay. Whole-cell, nuclear, and cytoplasmic extracts were prepared as previously described (25). Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA), and Western blot analysis was carried out by standard methods (26). Antibodies for Western blotting include the following: STAT3, phosphorylated STAT3, phosphorylated STAT1, phosphorylated extracellular signal-regulated kinase (ERK) 1/2, phosphorylated AKT, AKT (Cell Signaling, Beverly, MA), anti-phosphotyrosine 4G10, phosphorylated STAT5 (Upstate Biotechnology, Lake Placid, NY), STAT1 (BD Transduction Laboratories, San Diego, CA), STAT5, and ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA). JAK1 (BD Transduction Laboratories) and JAK2 (Upstate Biotechnology) were used for Western blot and immunoprecipitation as described previously (27). Anti-tubulin and actin were used for normalization (Sigma, St. Louis, MO). Electrophoretic mobility shift assay (EMSA) was carried out as described by using a high-affinity m67-binding probe using anti-STAT3 and anti-STAT1 for supershifting (28).

Reagents. Tetracyclic pyridone 2-*tert*-butyl-9-fluoro-3,6-dihydro-7*H*-benz[h]-imidaz[4,5-*f*]isoquinoline-7-one, P6 (synthesized by William Bornmann; ref. 22), AG490 (Calbiochem, San Diego, CA), DMSO, and cisplatin (Sigma, St. Louis, MO), U0126 10 mmol/L stock in DMSO (Cell Signaling).

Results

STAT3 activation in myeloma-derived cell lines. To compare the sensitivity and specificity of P6 with that of AG490, we examined several myeloma-derived cell lines with variable levels of activated JAKs-STAT3. JAK activation can lead to STAT3 phosphorylation, dimerization, translocation to the nucleus, and binding to DNA, which was examined by an EMSA in these cell lines (Fig. 1*A*). The IL-6-independent H929, RPMI 8226, and KMS11 myeloma-derived cell lines have almost undetectable levels of STAT3 DNA-binding activity (Fig. 1*A*). The murine-derived B9(d) hybridoma/plasmacytoma cell line is dependent on IL-6 for growth (29). Unlike normal cells that respond to IL-6 stimulation by transiently activating the JAK-STAT3 pathway, constitutive phosphorylation of STAT3 as determined by gel shift and Western blot was observed in this cell line when grown in IL-6-containing medium, suggesting an abnormality in the negative regulators of IL-6 signaling (Fig. 1*A* and *B*; data not shown). The STAT3-binding complex was supershifted by α-STAT3 antisera, whereas α-STAT1 antisera had no effect (Fig. 1*A*; data not shown). The B9(d) cell line was serially cultured in medium containing decreasing amounts of IL-6, which initially resulted in growth arrest and apoptosis of many cells. However, after several weeks, a heterogeneous population of cells was isolated, which grew independently of IL-6. These B9(i) cells express constitutively activated STAT3 and JAK2 (Fig. 1*A*; data not shown). B9-IL-6-independent cell lines were previously generated and found to contain constitutively activated JAK1, JAK2, and STAT3 (30). The U266 cell line contains robust levels of STAT3 protein bound to DNA (Fig. 1*A*) in agreement with the reported observation of autocrine/paracrine IL-6 production and constitutive STAT3 activation (16, 31, 32). The mobility of the STAT3 DNA-binding complex in the U266 cells is slightly faster compared with that of the B9 cells, which could be due to differential association of STAT3 with STAT1 or other proteins (Fig. 4*A*). INA-6 cells are a human IL-6-dependent myeloma-derived cell line expressing high levels of tyrosine-phosphorylated STAT3 (12–14).

P6 inhibits IL-6-mediated STAT3 tyrosine phosphorylation and cell proliferation in B9 cells. The sensitivity and specificity of P6 for JAK kinases was compared with that of AG490 by examining JAK-mediated STAT3 phosphorylation in the IL-6-dependent cell line B9(d). B9(d) cells grown in the presence of IL-6 contain high levels of tyrosine-phosphorylated STAT3 as determined by EMSA and Western blot analysis (Fig. 1*B*). P6 was added to B9(d) cells, and 20 hours later, a significant decrease in both DNA binding and phosphorylated STAT3 was observed using concentrations of 0.25 μmol/L, whereas 1 μmol/L resulted in

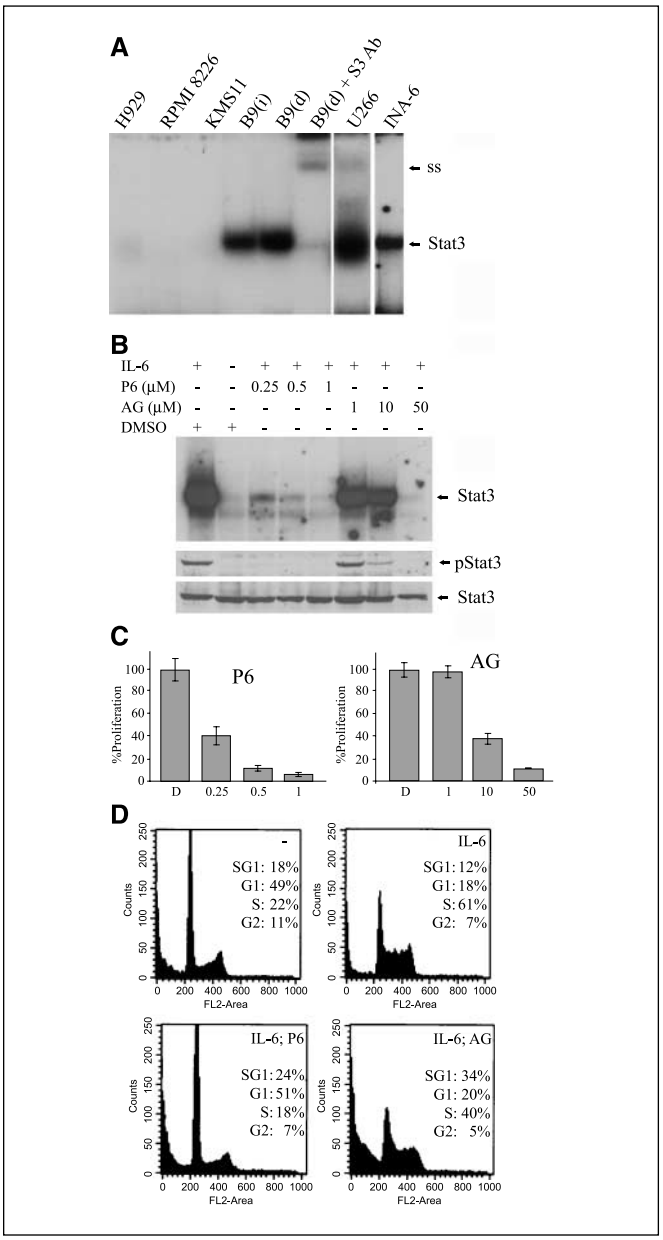

Figure 1. STAT3 activation by EMSA in myeloma-derived cell lines and P6 inhibits IL-6-mediated STAT3 tyrosine phosphorylation and cell proliferation in B9 cells at lower IC_{50} compared with AG490. *A,* nuclear extracts from H929, RPMI 8226, KMS11, B9(i), B9(d), U266, and INA-6 murine hybridoma and human myeloma-derived cell lines were analyzed by EMSA for STAT3 DNA-binding activity using a high-affinity binding site for STAT3 (m67). A supershift (*SS*) is observed following the addition of STAT3 antisera. *B,* nuclear extracts from B9(d) cells treated with IL-6 and increasing concentrations (μmol/L) of P6 or AG40 were examined by EMSA and Western blot for tyrosine-phosphorylated STAT3 (*pStat3*) and total STAT3 (*Stat3*). *C,* cell proliferation of B9 cells was measured after 3 days using Alamar Blue in the presence of increasing concentrations (μmol/L) of P6 or AG490. Within each experimental set, the proliferation of cells treated with DMSO was arbitrarily set as 100%. *Bars,* range of values derived from at least three independent experiments. *D,* flow cytometry analyses of B9 after 24 hours of incubation in the presence or absence of IL-6 and in the presence of P6 (0.5 μmol/L) or AG490 (50 μmol/L) using Annexin and propidium iodide. Percentage of cells in sub-G_1 (*SG1*), G_1, S, and G_2.

complete abrogation of phosphorylated STAT3. In contrast, 50 μmol/L AG490 was required to elicit a similar decrease in phosphorylated STAT3 (Fig. 1*B*). We compared the effect of P6 and AG490 on B9 cell proliferation (Fig. 1*C*). Treatment with P6 inhibited B9 cell proliferation in a dose-dependent manner (IC_{50}, 0.25 μmol/L), whereas 40-fold higher concentrations of AG490 (IC_{50}, 10 μmol/L) were required to achieve comparable levels of growth inhibition.

P6-induced growth inhibition of B9 cells mimics IL-6 deprivation. IL-6 deprivation for 24 hours of B9(d) cells resulted in a G_1 arrest and minimal induction of apoptosis (Fig. 1*D*; data not shown; ref. 33). If P6 inhibits JAKs, then its effects should mimic that of IL-6 removal in this cell line. B9 cells grown in the presence of IL-6 and P6 underwent a G_1 arrest and an induction of apoptosis (Fig. 1*D*; data not shown). In contrast, B9 cells grown in the presence of AG490 and IL-6 did not arrest in G_1 but rather underwent marked apoptosis, suggesting that the mechanism by which AG490 induces growth arrest is not simply by blocking IL-6-mediated signaling.

Kinetics and specificity of P6. To determine the kinetics and specificity of JAK-STAT3 inhibition by P6, IL-6 signaling was examined in the B9(d) cells. Removal of IL-6 from the culture medium of B9 cells led to a rapid decline in phosphorylated STAT3 over 3 to 6 hours (Fig. 2*B*). The addition of IL-6 to B9 cells, which have been starved of IL-6, resulted in robust and sustained phosphorylation of STAT3 (Fig. 2*A*). Pretreatment of B9 cells with P6 for 5 minutes effectively blocked IL-6-mediated phosphorylation of STAT3 in contrast to AG490, which was unable to block

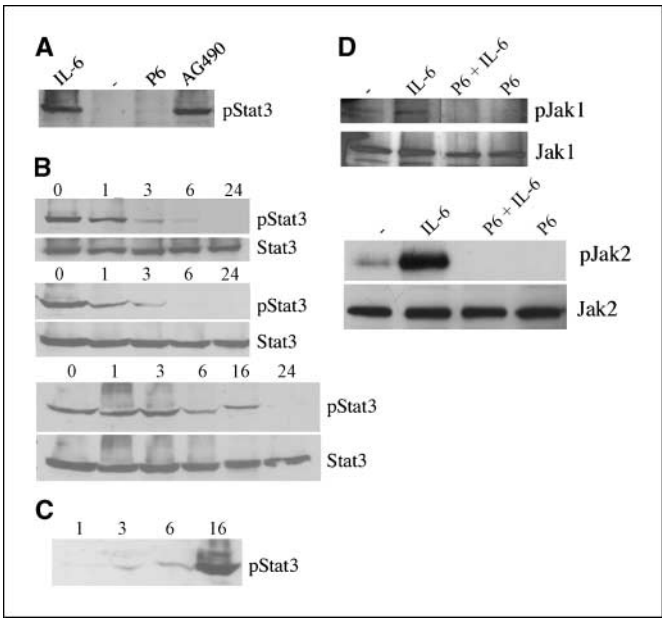

Figure 2. Kinetics and specificity of P6 and AG490 on JAK signaling in B9 cells. *A,* nuclear extracts from B9 cells starved of IL-6 (−) and treated with P6 (0.5 μmol/L) or AG490 (50 μmol/L) for 5 minutes before stimulation with IL-6 for 30 minutes (*IL-6*) were analyzed by Western blot for phosphorylated STAT3. *B,* nuclear extracts from B9 cells washed of IL-6 (at various times in hours) were examined by Western blot for phosphorylated STAT3 and reprobed with total STAT3. Nuclear extracts were isolated at the indicated times (hours) from B9 cells grown in the presence of IL-6 and P6 (*middle*) or AG490 (*bottom*) and analyzed for phosphorylated STAT3 and total STAT3 by Western blot analysis. *C,* extracts from B9 cells starved of IL-6 overnight, treated with P6 for 30 minutes, and then washed of P6 and, after 1, 3, 6, and 16 hours, stimulated with IL-6 for 20 minutes and analyzed by Western blot for phosphorylated STAT3 and reprobed with total STAT3. *D,* whole-cell extracts from B9 cells starved of IL-6 (−), treated with IL-6 for 30 minutes (*IL-6*), treated with both P6 (0.5 μmol/L) and IL-6 for 30 minutes (*P6+IL-6*) or P6 alone (P6), were analyzed for tyrosine-phosphorylated JAK1 and JAK2 by immunoprecipitation with α-JAK1 (*top*) or α-JAK2 (*bottom*) antisera followed by Western blot with anti-phosphotyrosine (*pJAK1*) or (*pJAK2*) and reprobed with total JAK1 (*Jak1*) or total JAK2 (*Jak2*).

IL-6-mediated STAT3 phosphorylation (Fig. 2*A, lanes 3* and *4*). The addition of P6 to B9 cells grown in the continuous presence of IL-6 led to a decline in phosphorylated STAT3, which mirrored that of IL-6 withdrawal (Fig. 2*B*). In contrast, >16 hours of exposure to AG490 were required to completely inhibit phosphorylation of STAT3 (Fig. 2*B, bottom*). These data suggest that either AG490 is slowly taken-up by cells or it is converted over time into a different chemical, which eventually inhibits JAK activity. These hypotheses were addressed by incubating cells with AG490 for 1 hour, washing the cells and subsequently adding IL-6, and examining phosphorylated STAT3 levels. No inhibition of phosphorylated STAT3 was observed over a 24-hour period, suggesting a delay in uptake of AG490 (data not shown). The addition of P6 for 1 hour followed by washing the cells resulted in a decrease of phosphorylated STAT3 for 6 hours, but after 16 hours, no inhibition was observed (Fig. 2*C*). Thus, P6 is rapidly taken up by cells and has a half-life of at least 6 hours when cells are transiently pulsed with the drug. The addition of P6 to a cell line that is not growth inhibited by JAK inhibition allowed us to determine that P6 can inhibit JAK activity when added to cells in culture for at least 72 hours (data not shown). To test the ability of P6 to inhibit JAK activity, we examined the levels of tyrosine-phosphorylated JAK1 and JAK2 following IL-6 stimulation in the presence or absence of P6 (Fig. 2*D*). Both JAK1 and JAK2 phosphorylation were completely inhibited by P6 treatment, indicating that P6 can block JAK activity in cell culture. AG490 could also inhibit JAK1 and JAK2 phosphorylation in cell culture (Supplementary Fig. S1A). Furthermore, P6 directly inhibited JAK1 and JAK2 kinase activities *in vitro* by immunoprecipitation kinase assays (data not shown).

P6 inhibits constitutive STAT3 phosphorylation and the growth of B9(i) cells. We examined the ability of P6 to inhibit the growth of the IL-6-independent B9(i) cell line. Unlike the parental B9 cell line that loses its phosphorylated STAT3 signal within a few hours in the absence of IL-6, the B9(i) cells express constitutively activated STAT3 in the absence of IL-6 as assessed by examining STAT3 tyrosine phosphorylation (Fig. 3*A*). The addition of IL-6 to the B9(i) cells did not significantly enhance phosphorylated STAT3 levels (Fig. 3*A*). The mechanism of constitutive STAT3 phosphorylation in this cell line is not known, but it is unlikely that a cytokine, such as IL-6, is being secreted in the medium, as conditioned medium from the B9(i) cells was unable to stimulate STAT3 phosphorylation in the B9(d) cells (data not shown; ref. 30). P6 treatment of B9(i) cells inhibited constitutive STAT3 phosphorylation (Fig. 3*A*). These data suggest that STAT3 phosphorylation in this cell line is likely dependent on aberrant JAK activity, which is inhibited by P6. We tested the effect of P6 on the growth of B9(i) cells and observed a marked decrease in proliferation (Fig. 3*B*). AG490 treatment of the B9(i) cell line was also effective in inhibiting STAT3 activation and proliferation at concentrations of 50 μmol/L (data not shown). IL-6 signaling through JAKs can also lead to an increase in ERK activity in several cell lines (12, 13, 34). B9(i) and B9(d) cells, however, did not express phosphorylated ERK1/2 nor did the addition of IL-6 lead to activation of ERK1/2 (Fig. 3*C*; data not shown for B9(d); ref. 30). Surprisingly, treatment of the B9(i) cells with AG490 led to a marked increase in phosphorylated ERK1 levels, whereas P6 had no effect on ERK phosphorylation (Fig. 3*C*; data not shown). These results suggest that the mechanism of action of AG490 in cell culture is not specific for the JAK kinases.

Effect of P6 on the U266 and KMS11 cell lines. We also examined the effects of P6 on the growth of two other myeloma-derived cell lines U266 and KMS11, which vary in their dependency on IL-6 (32, 35–38). The U266 cell line produces its own IL-6, contains constitutively active STAT3, but is only partially dependent on IL-6 signaling for growth (16). Gel shift analysis of nuclear extracts obtained from the U266 cell line showed STAT3 DNA-binding activity, which was completely supershifted by an anti-STAT3 antibody and cold m67 oligonucleotides (Fig. 4*A*; data not shown). Antisera to STAT1 decreased a small fraction of the DNA-binding complex, suggesting that STAT1/STAT3 heterodimers were included in the complex (Fig. 4*A*). P6 treatment of the U266 cell line completely abrogated STAT3 DNA-binding activity in agreement with previous reports showing inhibition of STAT3 activity by AG490 in this cell line (16). Furthermore, P6 treatment of U266 cells led to a modest reduction in cell growth (Fig. 4*A*) as well as a partial G_1 arrest, which is consistent with other investigator's findings on the significance of IL-6 signaling on growth control in this cell line (16, 32, 39). It was previously reported that AG490 inhibited STAT3 activity in U266 cells without any effect on growth but sensitized these cells to cytotoxic drug-mediated apoptosis (39). Similarly, we observed that neither P6 nor cisplatin led to significant apoptosis but in combination was

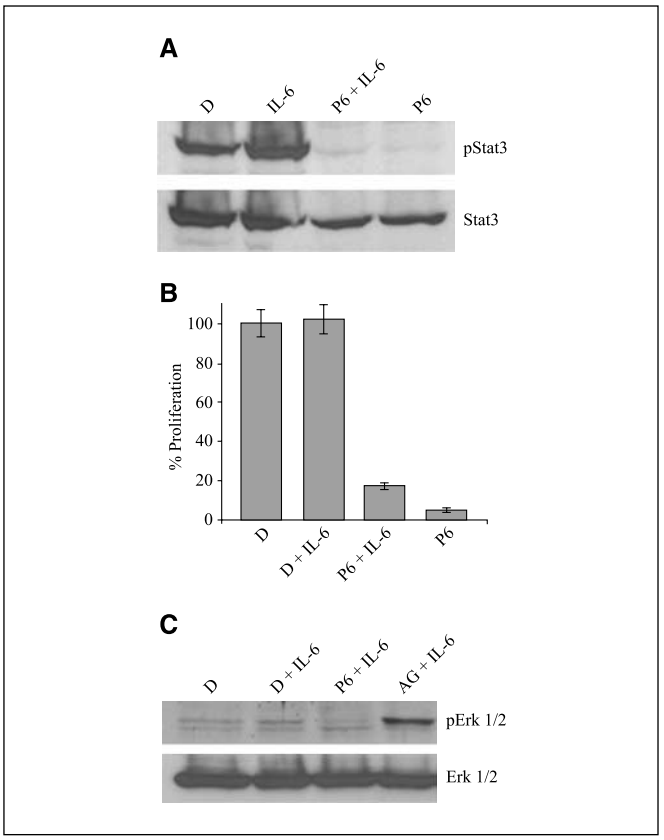

Figure 3. P6 inhibits constitutive STAT3 phosphorylation and growth of B9(i) cells. *A,* nuclear extracts from B9(i) cells were examined for tyrosine-phosphorylated STAT3 by Western blot analysis following treatment with P6 or DMSO (*D*) for 16 hours and then stimulation with IL-6 for 30 minutes. *B,* cell proliferation of B9(i) cells was measured after 3 days using Alamar Blue in the presence of DMSO, DMSO plus IL-6 (*D+IL-6*), P6 plus IL-6 (*P6+IL-6*) or P6 (0.5 μmol/L) alone. Within each experimental set, the proliferation of cells treated with DMSO was arbitrarily set as 100%. *Bars,* range of values derived from at least three independent experiments. *C,* whole-cell extracts from B9(i) cells treated with DMSO, DMSO plus IL-6, P6 (1 μmol/L) plus IL-6, or AG490 (50 μmol/L) plus IL-6 (*AG+IL-6*) for 2 hours were analyzed by Western blot for phosphorylated ERK1/2 (*pErk1/2*) and total ERK1/2 (*Erk1/2*).

synergistic for apoptosis (Fig. 4*A*). The IL-6-independent KMS11 cell line contains low levels of activated STAT3 as determined both by Western blot analysis and EMSA (Fig. 4*B*; data not shown). IL-6 stimulation led to a significant increase in activated STAT3, and the addition of P6 abrogated both the low-level constitutive and IL-6-inducible STAT3 activity (Fig. 4*B*). As was previously described, IL-6 stimulated the growth of KMS11 cells, which was completely inhibited by P6 (Fig. 4*B*; refs. 37, 40). These results confirm prior results showing that JAK-STAT3 activation is involved in the growth control of these two cell lines and that P6 inhibits this pathway.

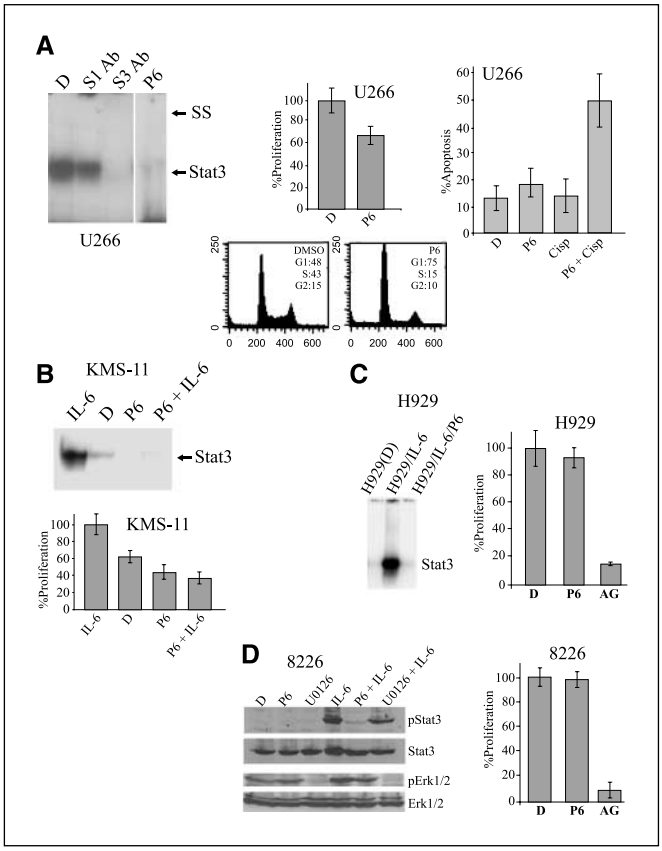

Figure 4. Effects of P6 on U266, KMS11, H929, and RPMI 8226 cells. *A,* nuclear extracts from U266 cells treated with DMSO or P6 (0.5 μmol/L) for 1 hour (P6) were analyzed for STAT3-binding activity by EMSA. Antisera to STAT1 (*S1Ab*) or STAT3 (*S3Ab*) were used to supershift components of the binding complex. Cell proliferation of U266 cells was measured after 3 days using Alamar Blue in the presence of DMSO or P6. Flow cytometry analyses of U266 cells after 48 hours of incubation in the presence or absence of P6 (0.5 μmol/L) using propidium iodide staining. Percentage of cells in sub-G_1, G_1, S, and G_2. U266 cells were assayed for apoptosis using Annexin V-FITC-propidium iodide staining after 72 hours of treatment with P6 (0.5 μmol/L) and cisplatin (1 μg/mL) or in combination. *B,* nuclear extracts from KMS11 cells treated with IL-6 for 30 minutes, DMSO, P6 (0.5 μmol/L), and P6 plus IL-6 analyzed by EMSA for STAT3 DNA-binding activity. Cell proliferation of KMS11 cells was measured after 3 days using Alamar Blue in the presence of IL-6, which was arbitrarily set as 100% proliferation, DMSO, P6 (0.5 μmol/L), and P6 plus IL-6. *C,* nuclear extracts from H929 cells (*H929/D*), IL-6 treated for 30 minutes (*H929/IL-6*), and IL-6 and P6 treated (*H929/IL-6/P6*) were analyzed by EMSA to examine STAT3 DNA-binding activity. Cell proliferation of H929 cells was measured after 3 days using Alamar Blue in the presence of DMSO, P6 (1 μmol/L), and AG490 (*AG*; 50 μmol/L). *D,* nuclear and whole-cell extracts from RPMI 8226 cells treated with DMSO, P6 (1 μmol/L), and U0126 (10 μmol/L) for 16 hours and then stimulated with IL-6 for 30 minutes were analyzed by Western blot for tyrosine-phosphorylated STAT3, total STAT3, phosphorylated ERK, and total ERK1/2. Cell proliferation of RPMI 8226 was measured after 3 days using Alamar Blue in the presence of DMSO, P6 (0.5 μmol/L), and AG490 (50 μmol/L). Within each experimental set (of the four cell lines examined), the proliferation of cells treated with DMSO was arbitrarily set as 100%. *Bars,* range of values derived from at least three independent experiments.

P6 does not affect the growth of cell lines lacking activated JAK-STAT3. The IL-6-independent H929 and RPMI 8226 myeloma-derived cell lines that harbor activating N-ras and K-ras mutations (respectively) are negative for STAT activation and are not growth stimulated by IL-6 (35, 41–43). We confirmed that H929 cells do not express phosphorylated STAT3, STAT1, STAT5, JAK1, and JAK2 (Figs. 1 and 4*C*; data not shown). Stimulation of H929 cells with IL-6 resulted in robust activation of STAT3, whereas the simultaneous addition of IL-6 and P6 abrogated STAT3-binding activity (Fig. 4*C*). P6 did not affect the growth of H929 cells in contrast to AG490, which led to both growth arrest and apoptosis of this cell line (Fig. 4*C*; data not shown). Similarly, the RPMI 8226 cell line lacks constitutively activated STAT3 (STAT1, STAT5, JAK1, and JAK2), whereas IL-6 stimulated the tyrosine phosphorylation of STAT3 (Fig. 4*D*; data not shown). P6 treatment did not affect proliferation of the RPMI 8226 cell line, whereas AG490 inhibited growth of these cells (Fig. 4*D*). Thus, P6 does not inhibit the growth of cells lacking activated JAK1/JAK2 and STAT1/STAT3/STAT5, whereas AG490 treatment resulted in profound growth inhibition of RPMI 8226 and H929 cell lines. The characterization of P6 showed that it behaves as a reversible and competitive inhibitor of ATP binding (20, 22, 23). Although P6 binds to the same highly conserved site found in tyrosine kinases, it showed specificity for the JAK family of tyrosine kinases. *In vitro*, the JAKs were inhibited at IC_{50}s of 1 to 15 nmol/L, whereas the dual specificity kinase MAPK/ERK kinase (MEK) was inhibited at an IC_{50} of 160 nmol/L, and 20 other kinases were inhibited more weakly than MEK (22). We subsequently examined the ability of P6 to inhibit MEK activity in cell culture. RPMI 8226 cells express a mutant form of K-ras, resulting in activation of MEK and phosphorylation of ERK. P6 did not affect levels of phosphorylated ERK1/2, whereas the MEK1/2 inhibitor U0126 led to complete abrogation of phosphorylated ERK1/2 (Fig. 4*D*). IL-6 stimulation through JAK activation resulted in increased levels of phosphorylated ERK1/2, which was restored to prestimulation levels with P6 (Fig. 4*D*). In contrast, 12-*O*-tetradecanoylphorbol-13-acetate stimulation of RPMI 8226 cells increased phosphorylated ERK1/2 levels, which were not affected by P6 (data not shown). Similar observations were made with H929 cells (data not shown). These data show that P6 specifically inhibits IL-6-stimulated JAK activation without affecting MEK activity.

P6 inhibits B9(d), B9(i), INA-6, and patient CD138$^+$ cell proliferation in the presence of BMSCs. BMSCs play an essential role in supporting the survival and expansion of multiple myeloma cells (13, 14, 44). We therefore tested the effects of P6 treatment on B9(d) cells cocultured with muBMSCs. STAT3 was tyrosine phosphorylated in B9(d) cells grown in the presence of muBMSC, consistent with prior data indicating that BMSCs produce IL-6 and other growth factors (Fig. 5*A*; ref. 12). The addition of P6 inhibited phosphorylation of STAT3 (Fig. 5*A*). In addition to STAT3 activation, coculturing of myeloma-derived cell lines with BMSC has been shown to stimulate phosphorylation of MAPK (ERK1 and ERK2). B9(d) cells, however, grown in the presence of BMSC, did not express phosphorylated ERK1/2 (Fig. 5*A*) nor was IL-6 capable of inducing phosphorylation of ERK1/2 in B9(d) cells (data not shown). The growth analysis of B9 cells cocultured with BMSC revealed a dramatic decrease in cell proliferation with P6 treatment (Fig. 5*A*). P6 did not affect the growth or viability of the muBMSC

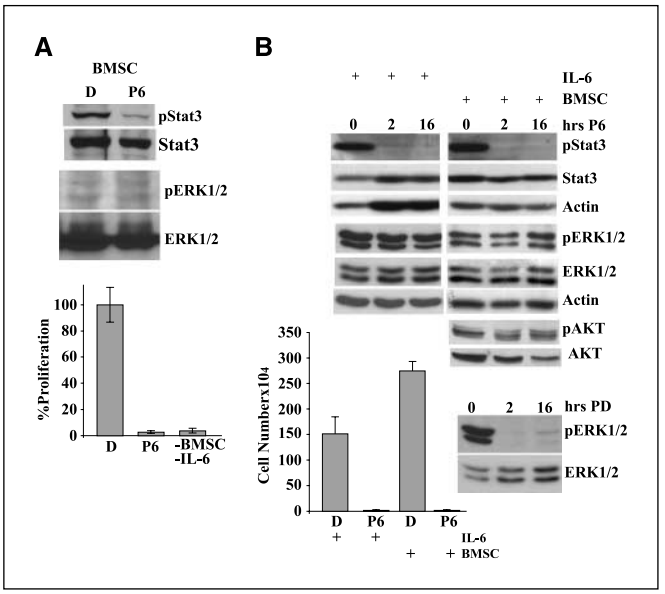

Figure 5. P6 inhibits B9(d), B9(i), and INA-6 cell proliferation in the presence of BMSCs. *A,* nuclear and whole-cell extracts from B9(d) cells grown on BMSCs for 48 hours then treated with DMSO or P6 (0.5 μmol/L) for 16 hours were examined by Western blot analysis for tyrosine-phosphorylated STAT3, total STAT3, phosphorylated ERK, and total ERK1/2. Cell proliferation of B9(d) cells grown on BMSC for 24 hours followed by treatment with either DMSO or P6 (0.5 μmol/L) for 3 days was determined by Alamar Blue. Within each experimental set, the proliferation of cells treated with DMSO was arbitrarily set as 100%. *Bars,* range of values derived from at least three independent experiments. *B,* nuclear and whole-cell extracts were isolated from INA-6 cells grown in the presence of either IL-6 or BMSCs treated for 2 or 16 hours with P6 (0.5 μmol/L) were examined by Western blot analysis for tyrosine-phosphorylated STAT3, total STAT3, actin, phosphorylated ERK, total ERK1/2, actin, phosphorylated AKT (*pAKT*), and total AKT. Cell proliferation of INA-6 cells grown in the presence of IL-6 or BMSC for 24 hours followed by treatment with either DMSO or P6 (0.5 μmol/L) for 3 days. Cells were counted using a hemocytometer after trypan blue staining. *Bars,* range of values derived from at least three independent experiments.

(data not shown). Similarly, B9(i) cells were cocultured on muBMSC and inhibition of growth and STAT3 phosphorylation was observed (data not shown).

We also examined the effect of P6 on the human IL-6-dependent myeloma cell line INA-6. This cell line was characterized as being dependent on either IL-6 or huBMSC for growth (12–14). Growth of INA-6 cells in the presence of IL-6 or huBMSC led to the tyrosine phosphorylation of STAT3 and JAK2, which was abrogated by P6 (Fig. 5*B*; Supplementary Fig. S2A). Equivalent levels of phosphorylated ERK1/2 were found in INA-6 cells when cultured with either IL-6 or huBMSC, which were not affected by P6 but were inhibited by treatment with the MEK inhibitor U0126 (Fig. 5*B*; data not shown). Similarly, phosphorylated AKT levels were not affected by P6 (Fig. 5*B*). P6 treatment of INA-6 cells cultured with IL-6 or huBMSC resulted in profound growth inhibition and G_1 arrest, whereas P6 did not affect the viability of the BMSC (Fig. 5*B*, Supplementary Fig. S2; data not shown). Thus, P6 effectively inhibited the growth of two myeloma-derived cell lines when grown in the presence of BMSC.

We evaluated the sensitivity of multiple myeloma cells to P6 from seven patients. Multiple myeloma cells (>90% $CD138^+$) were incubated with huBMSCs for 48 hours in the presence of either DMSO control or P6 (Fig. 6). Cell viability was decreased by >40% in five of seven samples but no significant effect on two of seven samples. Thus, in primary myeloma cells, P6 had a significant effect on the growth of cells.

Discussion

In this study, we examined the role of JAK kinase blockade in multiple myeloma–derived cell lines and compared the sensitivity and specificity of a novel pan-JAK inhibitor (P6) with that of the "gold standard" AG490. IL-6 signaling is known to play a role in the pathogenesis of myeloma (45). IL-6 stimulation can lead to the activation of JAK1, JAK2, and Tyk2, gp130, Src homology and collagen, and STAT3 to varying degrees as a function of cell type (42). We first examined cell lines that have a known dependence on IL-6 for survival (B9 or INA-6) and determined that (*a*) P6 inhibited growth in the nanomolar range compared with AG490 that required high micromolar concentrations, (*b*) P6 inhibited JAK activity within minutes following the addition of the compound in contrast to AG490 that required prolonged periods of incubation to impart its effects, (*c*) P6 retained activity in culture for >72 hours, and (*d*) the effects of P6 mimicked those of IL-6 withdrawal in the B9 cells, leading to a G_1 arrest in contrast to marked apoptosis induced by AG490. Furthermore, direct inhibition of JAK1 and JAK2 activity was observed in B9 and INA-6 cell lines by immunoprecipitation kinase assays and analysis of phosphorylated JAK levels in B9 cells treated with P6 (Fig. 2).

We also examined cell lines that are growth stimulated with IL-6 (KMS11) or produce their own IL-6, resulting in constitutive JAK-STAT3 activation (U266). We showed that P6 inhibited IL-6-stimulated proliferation of KMS11 cells consistent with a requirement for JAK kinase activity in cytokine-induced proliferation. It has been suggested that, for IL-6 to induce proliferation of myeloma cells (i.e., variants of the U266 cell line), the CD45 antigen needs to be expressed, leading to enhanced lyn kinase activity (46–48). We do not know whether IL-6-induced proliferation of KMS11 cells is also dependent on CD45 expression nor do we know whether blockade of IL-6 signaling by P6 could alter lyn activity through inhibition of JAKs. We have examined src activity in several epithelial-derived cell lines as well as BaF3 cells transformed with TEL-platelet-derived growth factor receptor (PDGFR)

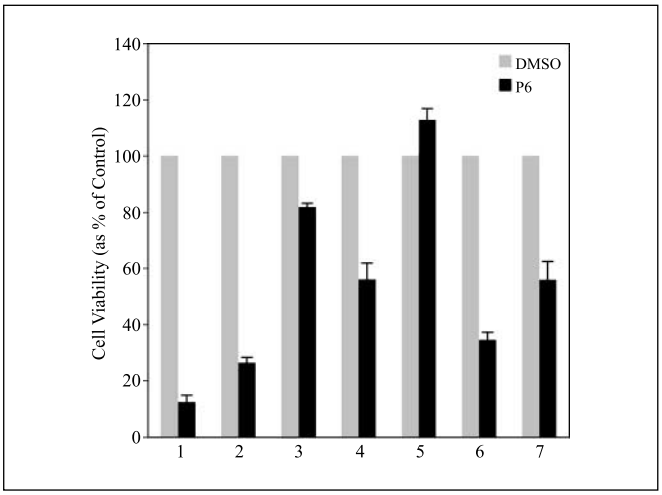

Figure 6. P6 inhibits patient multiple myeloma cell proliferation in the presence of BMSCs. Bone marrow–derived multiple myeloma cells (>90% $CD138^+$) were incubated with huBMSCs for 48 hours in the presence of either DMSO control or P6 (0.5 μmol/L). Cell viability was determined for each sample. *Columns,* mean (n = 3); *bars,* SD. A >40% decrease in cell viability was observed for patient samples 1, 2, 4, 6, and 7.

or BCR-Abl and determined that P6 does not inhibit c-src, TEL-PDGFR, or BCR-Abl kinase activity nor the growth of these cell lines, furthering the evidence that P6 is specific for JAK kinases.[4] Additionally, the U266 cells we examined contain constitutively phosphorylated STAT3, and the addition of IL-6 does not enhance their proliferative capacity nor is there a pronounced increase in phosphorylated STAT3 (data not shown). As was previously described, blockade of the IL6-JAK-STAT3 pathway in the U266 cell line does not affect the proliferative capacity of the cells but enhances the proapoptotic and antiproliferative effects of FAS or chemotherapeutic agents (16, 39). Similarly, P6 did not have a significant effect on the growth of U266 cells, but in combination with cisplatin, we observed pronounced apoptosis (Fig. 4*A*).

The specificity of P6 for JAKs was examined in several other cell lines, notably H929 and RPMI 8226. These cell lines do not express activated forms of JAK1, JAK2, STAT3, STAT1, or STAT5; thus, not surprisingly, P6 did not affect their growth in contrast to AG490, which led to both growth arrest and apoptosis of these cell lines, highlighting the lack of specificity of AG490 in these cell lines (Fig. 4*C* and *D*). H929 and RPMI 8226 cells express phosphorylated ERK1/2 as a consequence of aberrant Ras signaling, and P6 had no effect on phosphorylated ERK levels in these cell lines, showing that P6 does not inhibit the Ras-MEK-MAPK pathway in cell culture. In contrast, AG490 treatment of RPMI 8226 cells and the B9(i) cells resulted in increased phosphorylated ERK levels for reasons that are unclear (Fig. 3*C*; data not shown). IL-6 stimulation of RPMI 8226 cells, however, led to the activation of JAK2 and STAT3 as well as a slight increase in phosphorylated ERK levels, which were blocked by P6 (Fig. 4*D*). Thus, only IL-6/gp130/JAK-mediated increases in phosphorylated ERK are inhibited by P6.

Elegant work by Bargou's group has examined the growth and survival of primary myeloma cells and cell lines cocultured in the presence of BMSCs, which is an *in vitro* culture system that approximates the *in vivo* growth of myeloma cells (12–14). They observed that INA-6 cells cocultured with BMSC mediated strong activation of the MEK-ERK pathway in an IL-6-independent manner. Furthermore, blockade of both STAT3 and MEK-ERK pathways was required to induce apoptosis of this cell line as well as primary myeloma samples. We examined the growth of B9(d), B9(i), and INA-6 cells cocultured with BMSC and determined that P6 inhibited the growth of all three cell lines with a concomitant decrease in phosphorylated STAT3 levels but no effect on phosphorylated ERK levels. Our observations differ from those of Bargou's work in that, despite the lack of inhibition of the MEK-ERK pathway, we observe growth arrest of INA-6 cells cocultured with BMSC. One explanation is that phosphorylated ERK levels are similar in our INA-6 cells as a function of IL-6 or coculturing with BMSC, whereas phosphorylated STAT3 levels are strongly induced by both and thus the resultant dependency on the JAK-STAT3 pathway and perhaps less on the MEK-ERK pathway. Alternatively, P6 is inhibiting other signaling molecules, which may be critical for the growth and survival of these cells. Of note, P6 had no effect on phosphorylated AKT levels in the INA-6 cells (Fig. 5*B*). In addition to IL-6 the bone marrow microenvironment is known to produce a myriad of other growth factors and cytokines, which may obviate a requirement for IL-6 but may also signal in a JAK-dependent manner. Indeed several novel compounds, which have been used successfully in the treatment of myeloma (notably thalidomide), may in part function by inhibiting cytokine signaling leading to a reduction in JAK-STAT3 activity (49). Finally, we examined the effects of P6 on the growth of primary myeloma samples and determined that P6 had a significant effect on cell viability in the majority of the samples. In summary, our results highlight the differences between AG490 and P6 and clearly show in these cell lines that P6 is a more sensitive and specific inhibitor of JAK activity. Despite recent therapeutic advances, multiple myeloma remains an incurable hematologic malignancy. New therapies are needed, particularly for aggressive medullary variants and proliferative extramedullary manifestations of disease. P6 has significant *in vitro* activity as we have shown. These preclinical data provide the basis for clinical trials in myeloma and for further study of P6 activity in myeloma patients as it relates to JAK activation status.

[4] M. Berishaj, unpublished observation.

Acknowledgments

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References

1. Leonard WJ, O'Shea JJ. Jaks and STATs: biological implications. Annu Rev Immunol 1998;16:293–322.
2. Silva CM. Role of STATs as downstream signal transducers in Src family kinase-mediated tumorigenesis. Oncogene 2004;23:8017–23.
3. Boudny V, Kovarik J. JAK/STAT signaling pathways and cancer. Janus kinases/signal transducers and activators of transcription. Neoplasma 2002;49:349–55.
4. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 2005;365:1054–61.
5. Levine RL, Loriaux M, Huntly BJ, et al. The JAK2V617F activating mutation occurs in chronic myelomonocytic leukemia and acute myeloid leukemia, but not in acute lymphoblastic leukemia or chronic lymphocytic leukemia. Blood 2005;106:3375–9.
6. Levine RL, Wadleigh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 2005;7:387–97.
7. Lacronique V, Boureux A, Valle VD, et al. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. Science 1997;278:1309–12.
8. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305:1163–7.
9. Kishimoto T. Interleukin-6: from basic science to medicine—40 years in immunology. Annu Rev Immunol 2005;23:1–21.
10. Hirano T. Interleukin 6 and its receptor: ten years later. Int Rev Immunol 1998;16:249–84.
11. Bharti AC, Shishodia S, Reuben JM, et al. Nuclear factor-κB and STAT3 are constitutively active in CD138$^+$ cells derived from multiple myeloma patients, and suppression of these transcription factors leads to apoptosis. Blood 2004;103:3175–84.
12. Chatterjee M, Honemann D, Lentzsch S, et al. In the presence of bone marrow stromal cells human multiple myeloma cells become independent of the IL-6/gp130/STAT3 pathway. Blood 2002;100:3311–8.
13. Chatterjee M, Stuhmer T, Herrmann P, Bommert K, Dorken B, Bargou RC. Combined disruption of both the MEK/ERK and the IL-6R/STAT3 pathways is required to induce apoptosis of multiple myeloma cells in the presence of bone marrow stromal cells. Blood 2004;104:3712–21.
14. Honemann D, Chatterjee M, Savino R, et al. The IL-6 receptor antagonist SANT-7 overcomes bone marrow

stromal cell-mediated drug resistance of multiple myeloma cells. Int J Cancer 2001;93:674–80.
15. De Vos J, Jourdan M, Tarte K, Jasmin C, Klein B. JAK2 tyrosine kinase inhibitor tyrphostin AG490 downregulates the mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT) pathways and induces apoptosis in myeloma cells. Br J Haematol 2000;109:823–8.
16. Catlett-Falcone R, Landowski TH, Oshiro MM, et al. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity 1999;10:105–15.
17. Oshiro MM, Landowski TH, Catlett-Falcone R, et al. Inhibition of JAK kinase activity enhances Fas-mediated apoptosis but reduces cytotoxic activity of topoisomerase II inhibitors in U266 myeloma cells. Clin Cancer Res 2001;7:4262–71.
18. Meydan N, Grunberger T, Dadi H, et al. Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. Nature 1996;379:645–8.
19. Levitzki A. Targeting signal transduction for disease therapy. Curr Opin Cell Biol 1996;8:239–44.
20. Thompson JE. JAK protein kinase inhibitors. Drug News Perspect 2005;18:305–10.
21. O'Shea JJ, Park H, Pesu M, Borie D, Changelian P. New strategies for immunosuppression: interfering with cytokines by targeting the Jak/Stat pathway. Curr Opin Rheumatol 2005;17:305–11.
22. Thompson J, Cubbon R, Cummings R, et al. Photochemical preparation of a pyridone containing tetracycle: a Jak protein kinase inhibitor. Bioorganics Med Chem Lett 2002;12:1219–23.
23. Lucet IS, Fantino E, Styles M, et al. The structural basis of Janus kinase 2 inhibition by a potent and specific pan-Janus kinase inhibitor. Blood 2005;107:176–83.
24. Aman MJ, Keller U, Derigs G, Mohamadzadeh M, Huber C, Peschel C. Regulation of cytokine expression by interferon-α in human bone marrow stromal cells: inhibition of hematopoietic growth factors and induction of interleukin-1 receptor antagonist. Blood 1994;84:4142–50.
25. Wen Z, Zhong Z, Darnell JE, Jr. Maximal activation of transcription of Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell 1995;82:241–50.
26. Ausubel FM, Brent R, Kingston RE, et al. Current protocols in molecular biology. Hoboken (NJ): John Wiley & Sons, Inc.; 1994.
27. Limnander A, Danial NN, Rothman PB. v-Abl signaling disrupts SOCS-1 function in transformed pre-B cells. Mol Cell 2004;15:329–41.
28. Bromberg J, Wrzeszczynska M, Devgan G, et al. Stat3 as an oncogene. Cell 1999;98:295–303.
29. Schwarze MM, Hawley RG. Prevention of myeloma cell apoptosis by ectopic bcl-2 expression or interleukin 6-mediated up-regulation of bcl-xL. Cancer Res 1995;55:2262–5.
30. Rawat R, Rainey GJ, Thompson CD, Frazier-Jessen MR, Brown RT, Nordan RP. Constitutive activation of STAT3 is associated with the acquisition of an interleukin 6-independent phenotype by murine plasmacytomas and hybridomas. Blood 2000;96:3514–21.
31. Klein B, Zhang XG, Jourdan M, et al. Paracrine rather than autocrine regulation of myeloma-cell growth and differentiation by interleukin-6. Blood 1989;73:517–26.
32. Schwab G, Siegall CB, Aarden LA, Neckers LM, Nordan RP. Characterization of an interleukin-6-mediated autocrine growth loop in the human multiple myeloma cell line, U266. Blood 1991;77:587–93.
33. Plowright EE, Li Z, Bergsagel PL, et al. Ectopic expression of fibroblast growth factor receptor 3 promotes myeloma cell proliferation and prevents apoptosis. Blood 2000;95:992–8.
34. Fukada T, Hibi M, Yamanaka Y, et al. Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. Immunity 1996;5:449–60.
35. Ogata A, Chauhan D, Urashima M, Teoh G, Treon SP, Anderson KC. Blockade of mitogen-activated protein kinase cascade signaling in interleukin 6-independent multiple myeloma cells. Clin Cancer Res 1997;3:1017–22.
36. Keller ET, Ershler WB. Effect of IL-6 receptor antisense oligodeoxynucleotide on *in vitro* proliferation of myeloma cells. J Immunol 1995;154:4091–8.
37. Ishikawa H, Tsuyama N, Liu S, et al. Accelerated proliferation of myeloma cells by interleukin-6 cooperating with fibroblast growth factor receptor 3-mediated signals. Oncogene 2005;24:6328–32.
38. Kawano MM, Ishikawa H, Tsuyama N, et al. Growth mechanism of human myeloma cells by interleukin-6. Int J Hematol 2002;76 Suppl 1:329–33.
39. Alas S, Bonavida B. Inhibition of constitutive STAT3 activity sensitizes resistant non-Hodgkin's lymphoma and multiple myeloma to chemotherapeutic drug-mediated apoptosis. Clin Cancer Res 2003;9:316–26.
40. Shirato L, Otsuki T, Yamada O, et al. Down regulation of protein kinase C during growth enhancement induced by interleukin-6 on a human myeloma cell line, KMS-11. Cancer Lett 1996;107:131–6.
41. Ernst TJ, Gazdar A, Ritz J, Shipp MA. Identification of a second transforming gene, rasn, in a human multiple myeloma line with a rearranged c-myc allele. Blood 1988;72:1163–7.
42. Ogata A, Chauhan D, Teoh G, et al. IL-6 triggers cell growth via the Ras-dependent mitogen-activated protein kinase cascade. J Immunol 1997;159:2212–21.
43. Zhang B, Fenton RG. Proliferation of IL-6-independent multiple myeloma does not require the activity of extracellular signal-regulated kinases (ERK1/2). J Cell Physiol 2002;193:42–54.
44. Hallek M, Bergsagel PL, Anderson KC. Multiple myeloma: increasing evidence for a multistep transformation process. Blood 1998;91:3–21.
45. Tassone P, Neri P, Burger R, et al. Combination therapy with interleukin-6 receptor superantagonist Sant7 and dexamethasone induces antitumor effects in a novel SCID-hu *in vivo* model of human multiple myeloma. Clin Cancer Res 2005;11:4251–8.
46. Zhou Q, Yao Y, Ericson SG. The protein tyrosine phosphatase CD45 is required for interleukin 6 signaling in U266 myeloma cells. Int J Hematol 2004;79:63–73.
47. Ishikawa H, Tsuyama N, Abroun S, et al. Requirements of src family kinase activity associated with CD45 for myeloma cell proliferation by interleukin-6. Blood 2002;99:2172–8.
48. Ishikawa H, Mahmoud MS, Fujii R, Abroun S, Kawano MM. Proliferation of immature myeloma cells by interleukin-6 is associated with CD45 expression in human multiple myeloma. Leuk Lymphoma 2000;39:51–5.
49. Kumar S, Raje N, Hideshima T, et al. Antimyeloma activity of two novel N-substituted and tetraflourinated thalidomide analogs. Leukemia 2005;19:1253–61.

Pyridone 6, A Pan-Janus–Activated Kinase Inhibitor, Induces Growth Inhibition of Multiple Myeloma Cells

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