

Inhibition of JAK Kinase Activity Enhances Fas-mediated Apoptosis but Reduces Cytotoxic Activity of Topoisomerase II Inhibitors in U266 Myeloma Cells¹

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ABSTRACT

Our previous work demonstrated that the Janus kinase (JAK)-Stat3 pathway regulates expression of Bcl-x_L in the U266 human multiple myeloma cell line and prevents Fas-mediated apoptosis. Inhibition of this pathway by the JAK selective kinase inhibitor AG490 or dominant-negative Stat3 protein results in down-regulation of Bcl-x_L expression and enhanced sensitivity to Fas-mediated apoptosis. Because Bcl-x_L has also been implicated in resistance to chemotherapeutic drugs, we investigated whether inhibition of the JAK-Stat3 pathway and subsequent reduction in Bcl-x_L expression would also enhance cytotoxic drug activity. Contrary to this prediction, pretreatment of U266 myeloma cells with AG490, followed by exposure to topoisomerase II-inhibiting agents, antagonized drug-induced apoptosis. This effect correlated with reduced cyclin D1 expression and cell cycle arrest. The cell cycle arrest following AG490 pretreatment further correlated with reduced mitoxantrone-induced DNA double-strand breaks and reduced cell death, findings consistent with the critical requirement of DNA damage for drug cytotoxicity. These studies demonstrate that inhibition of the JAK-Stat3 pathway can result in paradoxical effects relative to cytotoxic drug response. These paradoxical responses may be explained by the findings that JAK-Stat3 signaling regulates the expression of multiple genes involved in controlling cell proliferation and apoptosis. Thus, understanding the cellular context of inhibiting signal transduc-

tion pathways is essential for the design of novel combination therapies for cancer.

INTRODUCTION

Multiple myeloma is an incurable B-cell malignancy characterized by the accumulation of malignant plasma cells in the bone marrow (1, 2). Multiple myeloma patients frequently respond to initial chemotherapy treatment. However, most patients ultimately relapse because of the emergence of tumor cells that are unresponsive to a wide spectrum of anticancer agents. This phenomenon is known as MDR³ (3). Classical mechanisms of MDR include the expression of drug transporters, such as P-glycoprotein, MDR-related protein, or lung resistance protein; alteration of intracellular drug targets such as topoisomerase II; and enhanced drug detoxification. More recently, alterations in downstream mediators of programmed cell death have been shown to confer resistance to chemotherapeutic drugs, supporting the hypothesis that aberrant expression of antiapoptotic proteins as a consequence of genetic mutations may not only contribute to the initial pathogenesis of the disease but may also confer a *de novo* drug-resistant phenotype (4).

Originally identified as the product of a gene translocation in B-cell lymphomas, the Bcl-2 family of apoptotic mediators has been implicated in both the pathogenesis of malignant disease and tumor cell resistance to chemotherapeutic agents (5, 6). Antiapoptotic family members, including Bcl-x_L, Bcl-2, Mcl-1, and BAG-1, can form homodimers or heterodimers with proapoptotic factors such as Bax, Bad, Bid, and Bak to control mitochondrial permeability and cytochrome *c* release. Constitutive expression of high levels of the antiapoptotic factor Bcl-x_L prevents cytochrome *c* release and confers resistance to a wide range of chemotherapeutic drugs (7) and physiological signals for apoptosis (8). Thus, relative stoichiometric protein expression is one of the primary means of regulating Bcl-2 family activity.

Using the IL-6 dependent myeloma cell line, U266, we demonstrated previously that IL-6 signal transduction through JAKs and Stat3 results in constitutive expression of the antiapoptotic Bcl family member, Bcl-x_L (9). Inhibition of Stat3 activity with either a JAK kinase selective inhibitor, AG490 (10), or with a dominant-negative Stat3 construct (Stat3β) resulted in complete abrogation of Bcl-x_L expression and sensitized cells to Fas-mediated apoptosis. Because Bcl-x_L has been

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³ The abbreviations used are: MDR, multidrug resistance; IFN, interferon; STAT, signal transducers and activators of transcription; IL, interleukin; JAK, Janus kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CIs, confidence intervals; PI, propidium iodide; CDK, cyclin-dependent kinase.

implicated in resistance to chemotherapeutic drugs, we hypothesized that inhibition of Stat3 and subsequent down-regulation of *Bcl-x_L* gene expression may also enhance drug sensitivity in myeloma cells. If so, this would provide a rationale for the design of combination therapies to overcome drug resistance related to antiapoptotic signal transduction mechanisms.

The JAK/STAT pathway was originally discovered as an effector of normal IFN signaling; however, several recent studies (reviewed in Ref. 11) have demonstrated that constitutively activated STAT signaling directly contributes to oncogenesis. STAT proteins are involved in signaling by many cytokines and growth factors, including the IL-6 family of cytokines, IFN γ , EGF, and PDGF. Phosphorylation of a critical tyrosine residue activates STATs by stabilizing the association of two STAT monomers through reciprocal phosphotyrosine-SH2 interactions to form a dimer. Cytokine receptors, such as IL-6 or IFN receptors, which lack intrinsic tyrosine kinase activity, can recruit members of the JAK family cytoplasmic tyrosine kinases to act as intermediaries for activation of STATs (12, 13). Following activation and dimerization, STAT proteins translocate to the nucleus where they bind to specific DNA response elements and induce expression of STAT-regulated genes. STAT signaling has been implicated in the control of multiple cellular responses to diverse cytokines and growth factors, including cell proliferation and apoptosis (14, 15). Significantly, constitutive activation of STAT family members has been demonstrated in various human blood malignancies, including myeloma, lymphomas, and leukemias (9, 16–19), and in solid tumors, including breast carcinoma and head and neck cancers (20, 21), where they regulate the expression of genes implicated in tumor cell proliferation and survival. These observations support the development of molecular inhibitors of the JAK/STAT pathway as specific chemotherapeutic agents or adjunct therapies.

Combination therapy is a common strategy to circumvent drug resistance (22). For example, analogues of cyclosporin A, verapamil, and quinine have all been shown to inhibit the ATP-dependent drug efflux activity of P-glycoprotein (23). However, the dose-limiting toxicity of these agents limits their use in clinical practice. Recent advances in molecular genetics have provided opportunities to design highly specific inhibitors of signal transduction pathways that may enhance the efficacy of standard chemotherapy drugs by reducing or altering pathways associated with cell survival.

In this study, we examined the use of the JAK kinase selective inhibitor, AG490, as a chemosensitizing agent in the U266 myeloma cell line. Although inhibition of *Bcl-x_L* by AG490 resulted in sensitization to Fas-induced apoptosis, this treatment protocol failed to sensitize the cells to either topoisomerase II inhibitors or alkylating agents. Rather, inhibition of the JAK signaling pathway by AG490 antagonized drug-induced apoptosis, and only the microtubule inhibitor vinblastine showed enhanced cytotoxicity following AG490 treatment. This effect correlated with a concurrent inhibition of cyclin D1 expression and induction of G₁ arrest, suggesting that the JAK/STAT pathway regulates the expression of multiple genes that influence response to chemotherapeutic drugs. Our study demonstrates the importance of understanding the complexity and cellular context of inhibiting signal transduction pathways believed to regulate antiapoptotic mechanisms that contribute to

drug resistance. This understanding has important implications for the design and development of agents that inhibit molecular targets in cancer cells.

MATERIALS AND METHODS

Cell Culture and Transfection. The U266 human myeloma cell line was obtained from ATCC (Rockville, MD) and maintained in RPMI 1640 (CellGro, Herndon, VA) with 10% FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine (Gemini Bio-Products, Inc., Calabasas, CA). U266 cells were transfected with a *Bcl-x_L*-Flag construct (kindly provided by Dr. Gabriel Nuñez, University of Michigan, Ann Arbor, MI) by using the TransIT-LT1 reagent (PanVera, Madison, WI) as described previously (9). Limiting dilution was used to select stable transfectants with high *Bcl-x_L* protein expression.

Western Blot Analysis. Cells were lysed in buffer composed of 50 mM Tris (pH 7.4), 5 mM EDTA, 150 mM NaCl, and 0.5% Triton X-100 containing 1 μ g/ml leupeptin and aprotinin and 1 mM phenylmethyl sulfonyl fluoride. The protein content of cell lysates was quantified by using the Bradford assay (Bio-Rad, Foster City, CA) and equal amounts of total protein dissolved in Laemmli SDS-PAGE sample buffer prior to separation by 15% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad), and Western blot analysis was performed by using standard techniques with enhanced chemiluminescence detection (Roche/Boehringer Mannheim, Indianapolis, IN). The antibodies used for immunoblotting were from the following sources: *Bcl-2* (Dako), *Bcl-x_L* (Santa Cruz Biotechnology, Santa Cruz, CA), Flag and NPT-II (5 Prime 3 Prime, Inc., Boulder, CO), and caspase 3 (generously provided by Dr. H. G. Wang of the H. Lee Moffitt Cancer Center). Blots were stripped and reprobed with anti- β -actin (Sigma Chemical Co., St. Louis, MO) to ensure equal protein loading (not shown).

Immunoprecipitation. Cells were lysed in immunoprecipitation buffer [50 mM HEPES (pH 7.2), 150 mM NaCl, 0.1% Tween 20, 1 mM EDTA, 1 mM EGTA, 0.1 mM orthovanadate, 0.5 mM sodium fluoride, 0.1 mM phenylmethyl sulfonyl fluoride, and 1 mM DTT] by using sonication for 5 s on ice. Following centrifugation to remove cellular debris, protein concentrations were determined by using the Bradford assay. Equal amounts of protein (200 μ g) were incubated with 5 μ l of anti-cyclin D1 antibody (Santa Cruz Biotechnology). After 2 h of rotation at 4°C, 30 μ l of Protein A/G Plus (Santa Cruz Biotechnology) were added for an additional 1 h. Tubes were centrifuged at 4000 rpm at 4°C, and precipitates were washed with ice-cold lysis buffer. Laemmli sample buffer was added to sedimented beads, heated to 100°C for 3 min, and the supernatants were separated on a 15% acrylamide gel. Proteins were then transferred to polyvinylidene difluoride membranes and immunoblotted with cyclin D1 antibody (Santa Cruz Biotechnology).

RNA Isolation and RNase Protection Assay. Total RNA was isolated by using the TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD). RNase protection was performed by using the PharMingen RiboQuant hAPO2c and hCyc-1 multitemplate probes according to the manufacturer's protocol (PharMingen, San Diego, CA). The probe was prepared

with ^{32}P incorporation by using *in vitro* transcription, and free nucleotide was removed on a G50 column (5 Prime 3 Prime). Purified probe was combined with 10 μg of total RNA and hybridized through a temperature range of 90°C–56°C over 16 h before RNase digestion. RNA hybrids were separated on a 5% denaturing gel, and protected fragments were quantitated with phosphor imaging by using Image Quant software (Molecular Dynamics, Sunnyvale, CA).

Cytotoxicity Assays. Cells were plated in 96-well microtiter plates at 15,000 cells/well in 0.2 ml of medium. Drugs were added with the cells at the time of plating. Melphalan (LPAM), doxorubicin, mitoxantrone, etoposide (VP-16), vinblastine, and Taxol were all obtained from Sigma-Aldrich (St. Louis, MO). Melphalan was dissolved in acidified ethanol, doxorubicin and mitoxantrone were dissolved in sterile ddH₂O, and VP-16 was dissolved in DMSO. AG490 was obtained from BioMol (Plymouth Meeting, PA) and dissolved in DMSO. After 96 h of incubation at 37°C, 50 μl of MTT dye (2 mg/ml in PBS; Sigma-Aldrich) were added to each well and incubated for 4 h. Plates were then centrifuged, the medium was aspirated, and 100 μl of DMSO were added to solubilize the formazan complex. Plates were mechanically agitated, and absorbance was measured at 540 nm by using a Dynex II Elisa plate reader (Dynatech). IC₅₀s were calculated by using linear regression analysis of percent survival *versus* log drug concentration.

Apoptosis Assays. Cells were plated in 24-well plates at 2×10^5 cells/well in 1 ml of medium. For analysis of Fas-induced apoptosis, cells were pretreated with 50 μM AG490 or DMSO vehicle control for 24 h, followed by 500 ng/ml agonistic anti-Fas antibody CH-11 (MBL International Corp., Watertown, MA) for 18 h or cytotoxic drug for 20–30 h. Apoptosis was measured by using staining with Annexin V-FITC (Bioss, Palo Alto, CA) and flow cytometry analysis on a FACScan cytometer and CellQuest software (Becton Dickinson, Mountain View, CA).

Cell Cycle Analysis. After treatment with AG490, cells were pulsed for 1 h with BrdUrd (Sigma) and fixed in 60% ethanol overnight at 4°C. Cells were consecutively incubated at 37°C with the following solutions: 0.04% pepsin for 1 h, 2 N hydrochloric acid for 1 h, and 0.1 M sodium borate for 10 s. Cells were then washed with PBTP solution (0.5% Tween 20 and 0.5% BSA in PBS) and resuspended in PBTP with 10 μl of anti-BrdUrd FITC (Becton Dickinson, Bedford, MA) for 1 h at room temperature. After being washed with PBTP, cells were resuspended in 500 μl of PBTP containing 10 $\mu\text{g/ml}$ PI (Sigma), 500 ng/ml Rnase A (Roche, Indianapolis, IN), and incubated at 37°C for 30 min prior to analysis by using flow cytometry. Cell cycle analysis was performed by using ModFit LP software (Becton Dickinson, Mountain View, CA).

Comet Assay. Cells were pretreated with 0.1% DMSO or 50 μM AG490 for 24 h followed by exposure to varying concentrations of mitoxantrone for 1 h. After a 1-h drug treatment, 5000 cells were washed with cold PBS and analyzed for DNA strand breaks by using the neutral Comet assay (24, 25). Briefly, cells were centrifuged, resuspended in 500 μl of cold PBS, and 1.5 ml of 1% agarose were added to each sample. The agarose-cell suspension was gently layered onto a frosted glass microscope slide and then placed in ice-cold lysis buffer containing 30 mM EDTA (pH 8.0), 0.5% SDS, and 0.25 mg/ml

proteinase K (Fisher Scientific, Norcross, GA). The samples were lysed for 1 h at 4°C and incubated 12–16 h for digestion of protein-DNA complexes. The agar slides were then reequilibrated in TBE [90 mM Tris-HCl, 90 mM boric acid, and 2 mM EDTA (pH 8.0)] for 2 h, followed by electrophoresis for 20 min at 25 V. DNA was stained with a 1:10,000 dilution of Syber Green (Molecular Probes, Eugene, OR) for 20 min and washed twice for 5 min in TBE. Fifty images per slide were captured on a Vysis fluorescent microscope and quantified by using Imagequant software (Molecular Dynamics, Sunnyvale, CA). The comet moment was calculated by using the following equation described by Kent *et al.* (24):

$$\text{Comet Moment} = \sum_{0-n} ((\text{Intensity of DNA at distance } X) * (\text{distance})) / \text{Intensity of total DNA.}$$

The mean comet moment value obtained from vehicle control samples was subtracted from the mean comet moment of each drug dosage, and CIs were determined by using ANOVA. The data shown are the means and 95% CIs of three independent experiments ($n = 50$ images for each dose of each independent experiment).

RESULTS

Ectopic Expression of Bcl-x_L Prevents Fas-mediated Apoptosis in Transfected U266 Cells Treated with AG490. We demonstrated previously that inhibition of the JAK/STAT signal pathway by either a JAK kinase inhibitor, AG490, or by transfection of a dominant-negative STAT (Stat3 β) construct reduces Bcl-x_L expression and sensitizes U266 myeloma cells to Fas-mediated apoptosis (9). To verify the role of Bcl-x_L in resistance to apoptosis, we transfected cells with an expression vector under the control of the mouse mammary tumor virus LTR and selected clones exhibiting stable overexpression of Bcl-x_L (Fig. 1a). Cells expressing high levels of ectopic Bcl-x_L protein were treated with AG490 to abolish expression of the endogenous Bcl-x_L protein and examined for sensitivity to Fas-mediated apoptosis. The tyrosine kinase inhibitor AG490 has been shown to prevent the phosphorylation and signaling activity of Jak2 but does not inhibit src family members including Src, Btk, Syk, Lck, or Lyn (10). This compound inhibits the growth and survival of cells with constitutive Jak kinase activity but has no significant effect on mitogen-stimulated normal B or T cells, B-cell lymphoma, T-cell leukemia, or IL-6-independent myeloma cells (9). The time and dose of AG490 treatment were as established previously (10) in the U266 cell line, based on the reported half-life of 12 h. As shown in Fig. 1b, 50 μM AG490 reduced the endogenous Bcl-x_L expression in both untransfected U266 cells and U266 cells transfected with the empty vector (U266/neo) but did not reduce the protein levels of ectopically-expressed Bcl-x_L in U266/Bclxl-1 cells.

We and others (9) have demonstrated that U266 myeloma cells display nearly complete resistance to Fas-mediated apoptosis. Consistent with previous reports, inhibition of endogenous Bcl-x_L with the JAK kinase inhibitor AG490 increased apoptosis to 76% (31% Fas-specific) in U266/neo cells. In contrast, U266/Bclxl-1 cells, which maintain high levels of Bcl-x_L expression because of the presence of the transgene, undergo only 9% Fas-specific apoptosis following exposure to AG490 and the agonistic anti-Fas antibody CH-11 (Fig. 2, a and

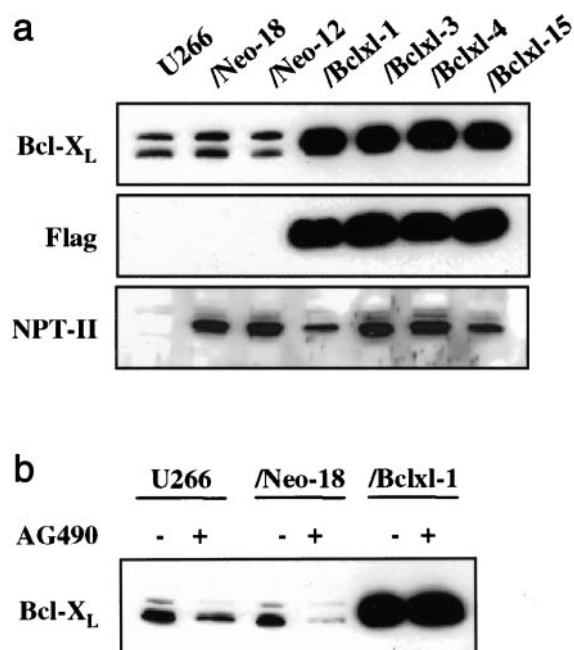


Fig. 1 The ectopic overexpression of Bcl-xL in U266 cells is unaffected by treatment with AG490. U266 cells were transfected with a Bcl-x_L-Flag fusion protein construct, selected with G418, and cloned by limiting dilution in the presence of G418. **a**, total protein was analyzed by using Western blot with antibodies against Bcl-x_L, Flag, and Neophosphotransferase (NPT II). The four high-expressing clones identified as U266/Bclxl-1, U266/Bclxl-3, U266/Bclxl-4, and U266/Bclxl-15 were selected for further analysis. U266/Neo-12 and U266/Neo-18 express Bcl-x_L levels equal to U266, however, NPT II expression demonstrates the presence of the Bcl-x_L construct in all selected clones. **b**, Western blot analysis of Bcl-x_L expression following treatment with DMSO (-) or AG490 (+) for 36 h. Blots shown are representative of a minimum of three separate experiments.

b. Analysis of Fas surface expression revealed that all clones had equal levels of Fas surface receptor expression in the presence or absence of AG490, thereby excluding alterations in Fas expression as a mechanism of resistance to Fas-mediated apoptosis (data not shown). These results establish that Bcl-x_L expression, induced by Stat3 signaling, plays a primary role in resistance to Fas-mediated apoptosis in U266 myeloma cells.

Fas-mediated apoptosis has been shown to induce apoptosis through the activation of a proteolytic cascade of caspases (26). Bcl-x_L inhibits apoptosis by preventing the release of cytochrome *c* from the mitochondrial membrane and preventing the activation of downstream effector caspases (27, 28). To verify that AG490 treatment was promoting a caspase-dependent pathway of apoptosis and not inducing a general necrotic effect, we examined the activation of caspase 3 in U266 cells and Bcl-x_L-transfected clones in the presence or absence of AG490 and CH-11. As shown in Fig. 2c, treatment of U266 or U266/neo cells with CH-11 and AG490 individually results in minimal accumulation of the active caspase 3 cleavage products (p20/p17). However, in agreement with the increased apoptosis measured by using Annexin V staining, cleavage of caspase 3 is increased when AG490 and Fas are combined. In contrast, ectopic overexpression of Bcl-x_L inhibited both AG490 and

AG490 plus CH-11 and induced caspase 3 cleavage. These data demonstrate that ectopic Bcl-x_L prevents caspase activation induced by Fas activation and rescues cells from the AG490-associated reduction of Bcl-x_L and enhanced Fas-induced apoptosis.

Ectopic Overexpression of Bcl-x_L Confers Resistance to Chemotherapeutic Drug-induced Apoptosis. Previous studies have demonstrated that the antiapoptotic activity of Bcl-x_L can be drug type-specific (7, 29). Furthermore, the role of mitochondrial perturbation and cytochrome *c* release in the apoptotic pathway varies by cell type (30, 31). To examine the contribution of Bcl-x_L to drug resistance in U266 myeloma cells, we analyzed clones of U266/Bclxl cells with varying levels of Bcl-x_L protein expression (see Fig. 1). For all drugs tested, both U266/Bclxl-1 and U266/Bclxl-15 were found to be 20–50% more resistant to drug-induced apoptosis as compared with either the untransfected U266 cells or the empty vector control cells, U266/neo-12 (Fig. 3a). Additionally, Western blot analysis of caspase 3 activation demonstrated a dramatic inhibition of drug-mediated cleavage products in U266/Bclxl-1 cells expressing high levels of the Bcl-x_L protein (Fig. 3b). These data establish the important role of Bcl-x_L expression as a mechanism of resistance to the topoisomerase II inhibitors doxorubicin, mitoxantrone, and VP-16, and the alkylating agent melphalan, in U266 myeloma cells.

Treatment with AG490 Does Not Sensitize U266 Cells to Chemotherapeutic Drugs. Because Bcl-x_L expression confers resistance to chemotherapeutic drugs in U266 cells, we hypothesized that inhibition of Bcl-x_L expression by AG490 treatment would sensitize the cells to drug-induced apoptosis, similar to the effects we observed for Fas-mediated apoptosis. Using the protocol established previously to effectively eliminate Bcl-x_L expression and sensitize cells to Fas-mediated apoptosis, cells were pretreated with AG490 for 12 h, followed by exposure to the chemotherapeutic drugs doxorubicin, VP-16, mitoxantrone, melphalan, Taxol, or vinblastine. Doxorubicin, VP-16, and mitoxantrone induce DNA double-stranded breaks by stabilizing the cleavable complex of topoisomerase II, whereas melphalan creates DNA adducts. Taxol and vinblastine are both microtubule inhibitors. In contrast to the enhancement seen in response to Fas-mediated apoptosis, AG490 pretreatment did not potentiate drug-induced cell death, but actually antagonized the cytotoxicity of the DNA damaging agents (Fig. 4). Annexin V staining of apoptotic cells following 20–30 h of drug treatment demonstrated 10–40% reduction in cell death, with the greatest effect seen in the topoisomerase inhibitors doxorubicin, VP-16, and mitoxantrone. These data were confirmed by using MTT cytotoxicity assays. Melphalan cytotoxicity was reduced only at the highest concentration, and no significant difference was identified in cells treated with Taxol in the presence or absence of AG490. In contrast, pretreatment with 50 μM AG490 for 24 h followed by exposure to vinblastine resulted in 13–17% increased cell death compared with cells treated with vehicle control (<0.1% DMSO). Increased duration of AG490 pretreatment up to 48 h failed to alter the antagonistic effects of AG490 on topoisomerase II inhibitor-induced apoptosis, and no enhancement of drug activity was found with exposure to cytotoxic drug prior to AG490 treatment. A similar effect was seen in the U266/Bclxl cell lines, suggesting that the

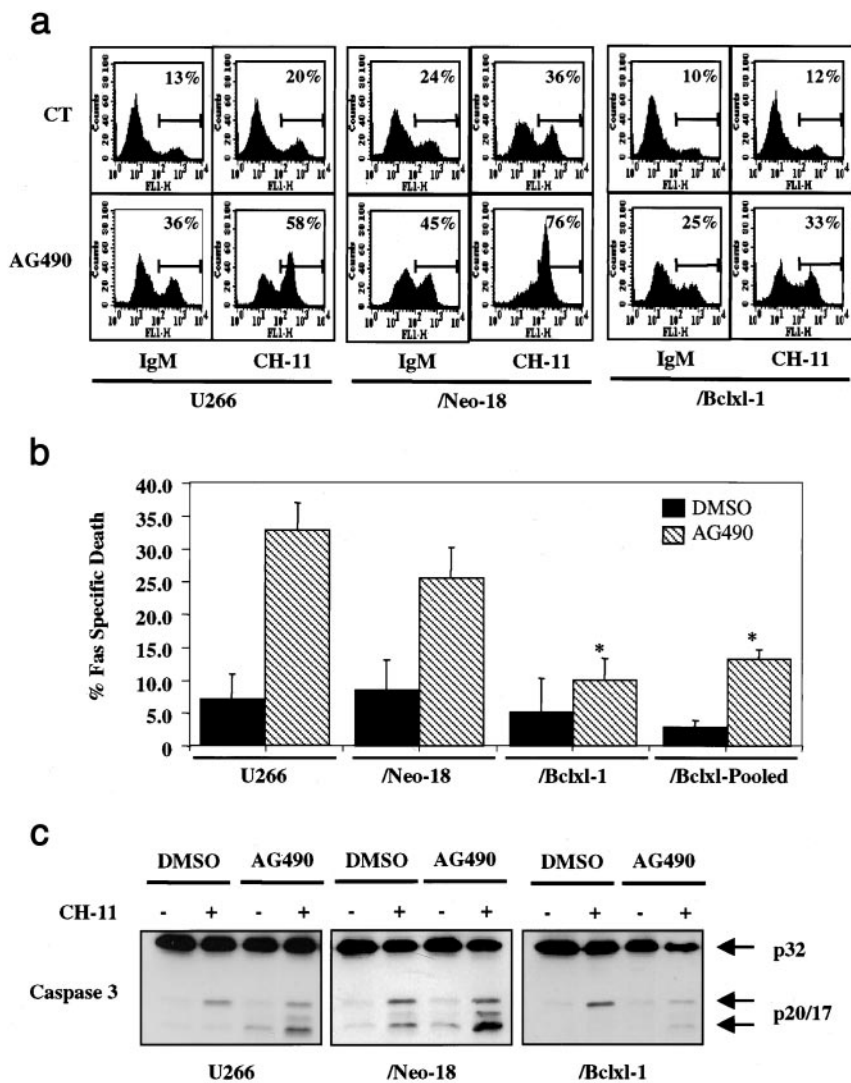


Fig. 2 Fas-mediated apoptosis following abrogation of endogenous Bcl-xL expression with AG490. Cells were pretreated with 50 μ M AG490 or DMSO for 24 h prior to the addition of the agonistic anti-Fas antibody CH-11. Following an additional 12 h of incubation, cells were stained with Annexin V-FITC and analyzed for apoptosis by flow cytometry. The percentage of Fas-specific apoptosis was calculated by subtracting vehicle control Annexin V-positive cells from AG490/Fas-induced Annexin V-positive cells. *a*, representative histogram of Annexin V-stained cells. Marker positioning is based on background fluorescence of DMSO control cells. *b*, mean Fas-specific apoptosis in the presence and absence of Bcl-xL expression. Isolated clones were combined in equal proportions and examined for Fas-mediated apoptosis (/Bcl-xL-pooled). *, statistically significant from U266 and/Neo-18, $P < 0.05$. *c*, Western blot analysis of caspase 3 cleavage in cells treated with AG490 and Fas. Cells were pretreated with 50 μ M AG490 or DMSO for 24 h prior to the addition of CH-11. Total protein was isolated and analyzed for caspase 3 cleavage by using Western blot analysis following an additional 12 h of incubation. p32 represents the uncleaved procaspase, and p21/p17 are the activated cleavage products of caspase 3.

cytoprotective activity of JAK kinase inhibition is independent of Bcl-xL expression (data not shown).

Inhibition of JAK/STAT Signal Transduction Reduces Cyclin D1 Expression and Leads to G₁ Arrest. IL-6 signal transduction through the JAK/STAT pathway has been shown to regulate the expression of several genes that contribute to the control of both cell survival and proliferation (reviewed in Refs. 32 and 33). Because the greatest protective effect of AG490 was seen in conjunction with doxorubicin, etoposide, and mitoxantrone, chemotherapeutic drugs that are classically considered to be more effective in cycling cells, we analyzed the effects of AG490 treatment on cell cycle progression. U266 cells were treated with AG490, and samples were collected for analysis over 72 h. BrdUrd incorporation and PI staining demonstrated an accumulation of cells in G₀/G₁-phase with a concurrent reduction in S-phase cells as early as 12 h following initial treatment, with a maximum effect at 48 h (Fig. 5). These data suggest that inhibition of the JAK/STAT signal transduction pathway in the U266 myeloma cell line altered the expression of genes required for cell cycle progression.

Cell cycle progression is controlled by cell cycle regulatory molecules including cyclins, CDKs, and CDK inhibitors (reviewed in Ref. 34). Mitogenic growth factors act during the G₁ phase of the cell cycle primarily through the induced expression of D-type cyclins and activation of cdk4 and cdk6. Recent studies have implicated Stat3 as a transcription factor in the expression of cyclin D1 (35). Therefore, we examined the effects of AG490-mediated inhibition of Stat3 on the expression of cyclin D1. RNase protection assay and Western blot analysis demonstrated that untreated and vehicle control-treated U266 cells express high levels of cyclin D1, whereas AG490-treated U266 cells showed a significant reduction in cyclin D1 mRNA and protein levels (Fig. 6). The reduction of cyclin D1 correlates with the accumulation of cells in G₁, suggesting that IL-6 signal transduction through the JAK/STAT pathway may contribute to cell cycle progression in U266 cells. Although we cannot rule out a direct inhibition of CDK activation by the tyrphostin, AG490, other studies have demonstrated that Stat3 directly regulates cyclin D1 expression (35, 36). These data suggest that inhibition of the JAK kinase pathway reduced the expression of

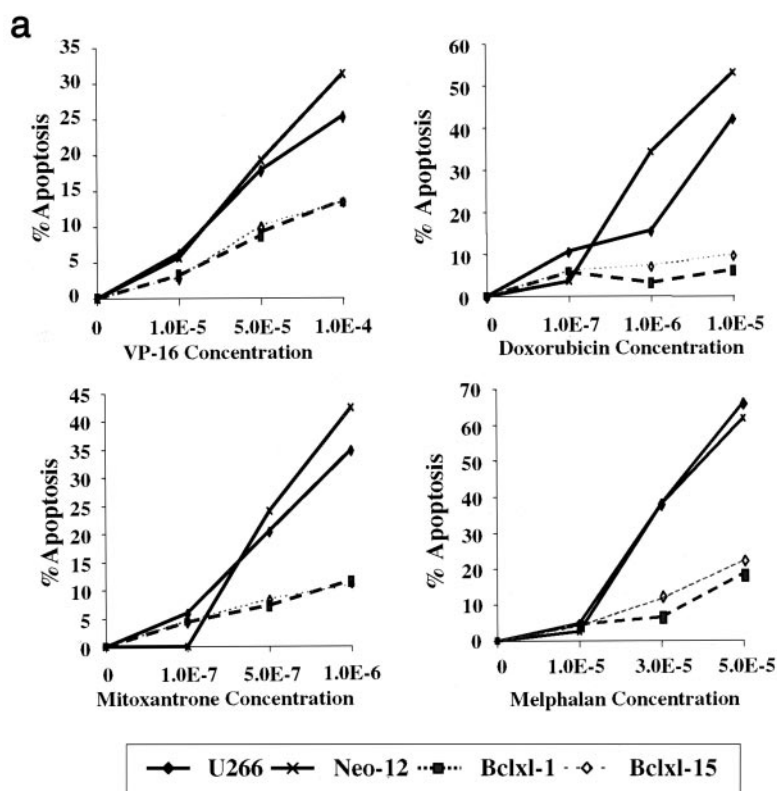
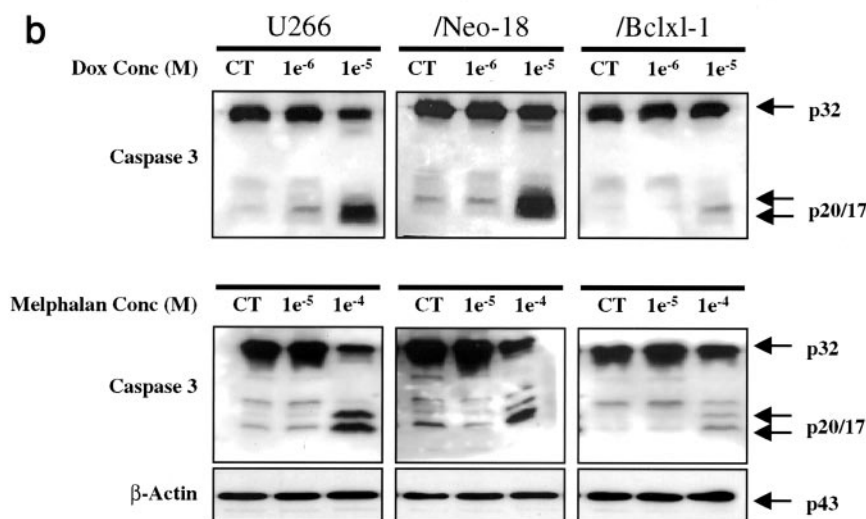


Fig. 3 Drug-induced apoptosis in U266 myeloma cells overexpressing Bcl-x_L. *a*, cells were treated with the indicated concentration of doxorubicin, melphalan, mitoxantrone, or VP-16 for 24 h followed by staining with Annexin V-FITC and analysis by using flow cytometry. The percentage of drug-specific apoptosis was calculated by subtracting vehicle control Annexin V-positive cells from drug-induced Annexin V-positive cells. *b*, Western blot analysis of caspase 3 cleavage in cells treated with doxorubicin or melphalan. Cells were incubated with drug for 24 h, lysed, and whole cell extracts were analyzed by using Western blot. Data are representative of three independent experiments.



cyclin D1, resulting in cell cycle arrest and resistance to chemotherapeutic drugs, especially topoisomerase II inhibitors.

AG490-mediated G₁ Arrest Reduces Mitoxantrone-induced DNA Double-Stranded Breaks. Cytotoxic drugs that inhibit topoisomerase II are generally believed to initiate cell death programs by inducing double-stranded breaks through stabilization of the DNA-topoisomerase II-cleavable complex during DNA synthesis (37). These agents are typically most effective during S-phase when topoisomerase II activity is great,

whereas cells arrested in G₁ are less susceptible to the activity of cytotoxic drugs that target a specific phase of the cell cycle (38). To examine the mechanism of drug resistance conferred by AG490 pretreatment, we used the neutral Comet assay to examine DNA double-stranded breaks following exposure to mitoxantrone. U266 myeloma cells were pretreated with 50 μ M AG490 for 24 h followed by 1 h of exposure to 0.1, 1, or 10 μ M mitoxantrone (Fig. 7). Comparison of the mean comet moment in AG490-treated cells *versus* DMSO vehicle control showed a

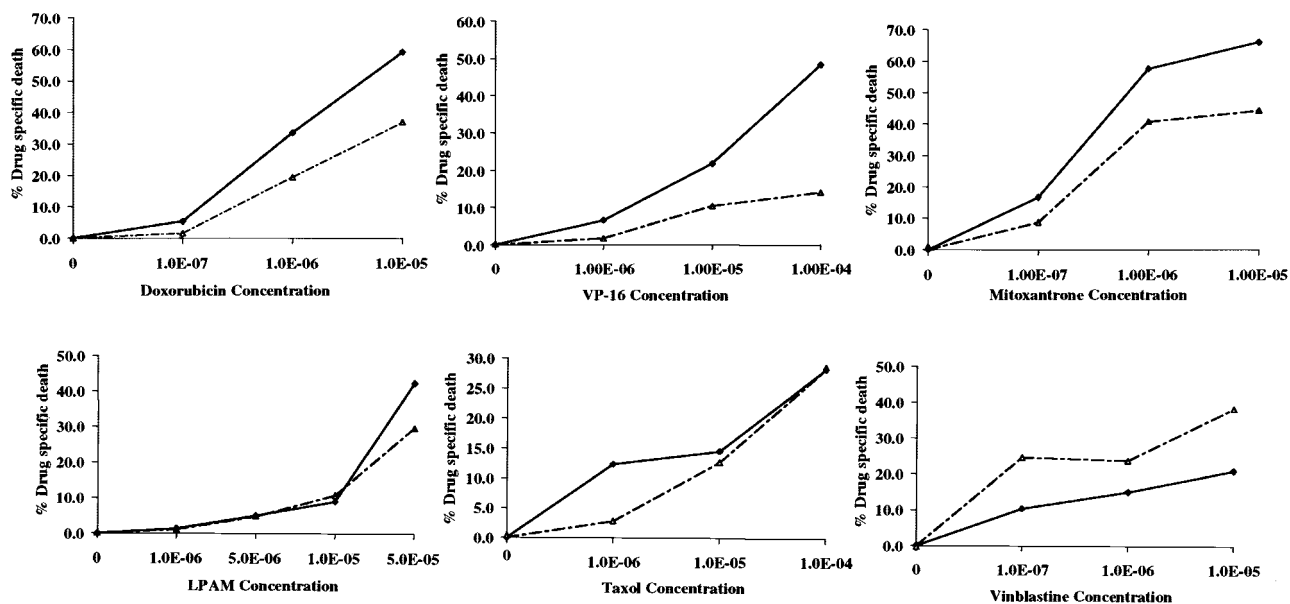


Fig. 4 Drug-induced apoptosis following inhibition of the Jak/Stat signal transduction pathway with AG490. U266 cells were pretreated with AG490 for 12 h and exposed to cytotoxic drugs for an additional 20–30 h, followed by Annexin V staining and analysis by using flow cytometry. The percentage of drug-specific apoptosis was calculated by using the equation (control apoptosis-specific apoptosis)/100-control. Data are representative of three independent experiments. Significance was determined by using the Student's two-tailed *t* test. -◆- DMSO -△- AG490.

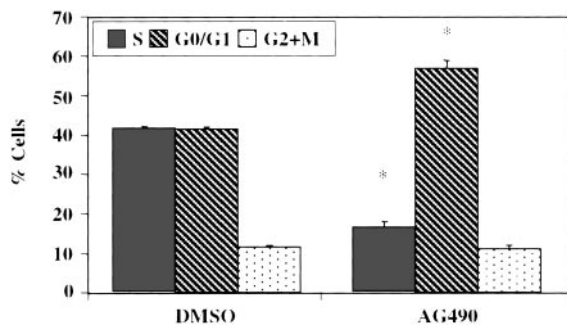


Fig. 5 Cell cycle analysis following AG490 treatment. Cells were treated with 50 μ M AG490 for 36 h and analyzed for cell cycle progression by using anti-BrdUrd antibody and PI. Bars, SD; *n* = 6 independent experiments; *, statistically significant from DMSO control, where *P* < 0.05 by using the two-tailed Student's *t* test.

significant reduction in DNA double-stranded breaks over all doses of mitoxantrone tested (within 95% confidence limits by using ANOVA over three independent experiments). These values correlate well with the reduced cytotoxicity of mitoxantrone measured by using both Annexin V staining and MTT analysis in AG490-treated cells.

DISCUSSION

In multiple myeloma, resistance to Fas-mediated apoptosis may allow myeloma cells to escape immune surveillance from cytotoxic lymphocytes, accumulate in the bone marrow, and acquire transforming mutations. Therefore, understanding the mechanisms that allow these tumor cells to survive will be critical to the effective treatment of this disease. We have

demonstrated previously that inhibition of IL-6 receptor signal transduction through the JAK/STAT pathway in the human myeloma cell line U266 results in down-regulation of Bcl-x_L and increased sensitivity to Fas-mediated apoptosis (9). In this study, we show that ectopic overexpression of Bcl-x_L will rescue cells from Fas-mediated apoptosis secondary to inhibition of endogenous Bcl-x_L expression, conclusively demonstrating that Bcl-x_L is causative in resistance to Fas-mediated apoptosis in these cells.

Fas and cytotoxic drugs have been shown to share common pathways associated with programmed cell death (8, 29, 39). Some of these programmed cell death pathways are inhibited by antiapoptotic proteins, including Bcl-x_L. Consistent with this, we demonstrate that ectopic overexpression of Bcl-x_L increases the resistance of the U266 myeloma cells to cytotoxic drugs. Therefore, we hypothesized that inhibition of the JAK/STAT pathway and subsequent inhibition of Bcl-x_L expression would sensitize cells not only to Fas-induced but also to cytotoxic drug-induced apoptosis. However, despite complete abrogation of Bcl-x_L expression by the JAK specific kinase inhibitor AG490, we found no enhancement of the activity of drugs that damage DNA. Rather, the activity of the topoisomerase II inhibitors was unexpectedly antagonized. This effect is likely related to the multiplicity of genes regulated by the Stat3 transcription factor, including the cell cycle regulatory protein, cyclin D1, in addition to genes like Bcl-x_L that control apoptosis.

IL-6 is the major growth factor for multiple myeloma cells, and IL-6 receptor-mediated activation of the Ras-mitogen-activated protein kinase pathway has been suggested as a mechanism of mitogenesis in myeloma cells (40). Our data indicate the involvement of the JAK-Stat3 pathway in regulating the proliferation of myeloma cells. The D-type cyclins are key regulators

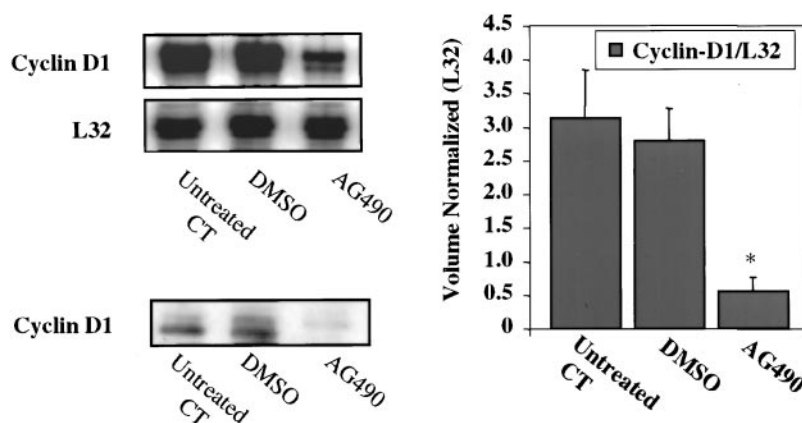


Fig. 6 AG490 effects on cyclin D1 expression. Cells were treated with 50 μM AG490 for 36 h, and RNA was isolated and analyzed by using RNase protection assay. *a*, autoradiograph of cyclin D1 mRNA and the housekeeping gene *L32* in the presence and absence of AG490. RNA levels were quantitated by using phosphor imaging, and cyclin D1 expression levels were normalized to the housekeeping gene *L32*. Bars, SD; $n = 3$ independent experiments. *, statistically significant from DMSO control, where $P < 0.05$ by using the two-tailed Student's *t* test. Western blot analysis of cyclin D1 protein expression. *b*, total protein was isolated from untreated, DMSO vehicle control, and AG490-treated cells, and equal amounts of protein were immunoprecipitated and immunostained with an anti-cyclin D1 antibody.

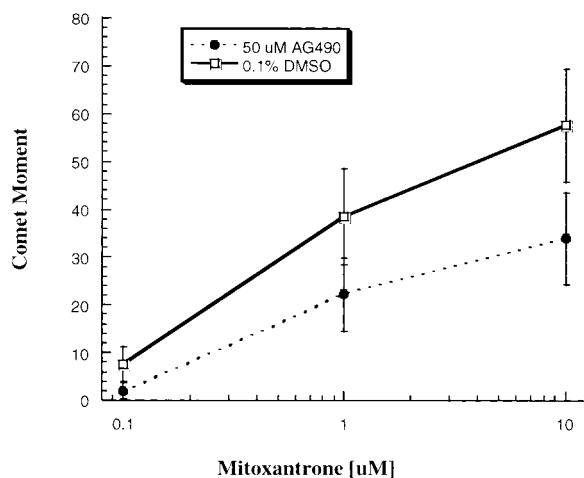


Fig. 7 Pretreatment with AG490 reduces DNA double-stranded breaks. U266 myeloma cells were pretreated with 50 μM AG490 or 0.1% DMSO vehicle control for 24 h, followed by 1-h exposure to the indicated dose of mitoxantrone. DNA double-stranded breaks were analyzed by using the neutral comet assay, and the comet moment was calculated as described in "Materials and Methods." The data shown are the mean values and 95% CIs of three independent experiments ($n = 50$ images for each dose of each independent experiment). Cells pretreated with AG490 demonstrated a significant (Student's *t* test, $P < 0.01$) reduction in mitoxantrone-induced DNA damage.

of cell cycle progression for G_1 to S-phase. In particular, cyclin D1 associates primarily with Cdk4 and Cdk6 promoting translocation to the nucleus and phosphorylation of Rb. Previous investigators have demonstrated that the JAK-Stat3 pathway regulates expression of cyclin D1 and thereby may regulate cell cycle progression (35, 36). Consistent with these observations and our earlier report that Stat3 is constitutively active in a majority of myeloma patients, our present findings support a role for the IL-6 induced JAK-Stat3 signal transduction pathway

in regulating cyclin D1 expression and cell cycle progression (9). Simultaneous treatment of the U266 myeloma cells with AG490 and IL-6 did not overcome the AG490-induced cell cycle arrest and only partially reversed the cytoprotective effects of AG490 in the cell cycle-dependent drugs (data not shown).

Signal transduction pathways represent novel molecular targets for therapeutic intervention either alone or in combination with standard chemotherapeutic regimens (41, 42). It could be predicted that inhibiting signal transduction pathways that regulate the expression of antiapoptotic genes would result in enhanced drug-induced apoptosis. In this study, we first demonstrated that cytotoxic drug activity was reduced in U266 cells selected for high levels of ectopic Bcl- x_L expression. This finding, in combination with our previous report that inhibition of the JAK kinase pathway by using the JAK specific kinase inhibitor AG490 results in reduced Bcl- x_L expression and increased Fas-induced apoptosis, led us to hypothesize that pretreating cells with AG490 would enhance drug-induced apoptosis. However, despite our reducing Bcl- x_L with AG490, this pretreatment failed to enhance the cytotoxic activity of the topoisomerase II inhibitors doxorubicin, VP-16, and mitoxantrone, the microtubule stabilizer Taxol, or the alkylating agent melphalan. Our data suggest that although inhibition of a specific signal transduction pathway may enhance some mediators of apoptosis, such as Fas-mediated apoptosis, it may actually reduce the efficacy of certain cytotoxic agents. This may be because signaling pathways like JAK/STAT control not only cell survival but also cell proliferation.

DNA topoisomerases are essential nuclear enzymes involved in DNA replication. Topoisomerase II produces double-stranded breaks by forming a covalent intermediate between topoisomerase II and DNA, the so-called cleavable complex. Topoisomerase inhibitors, including doxorubicin, mitoxantrone, and VP-16, stabilize the cleavable complex, resulting in collision of the replication fork, double-strand breaks, and cell death (reviewed in Ref. 37). Thus, cells arrested in G_1 are less sus-

ceptible to the activity of drugs that target a specific phase of the cell cycle, leading to a reduction in the cytotoxic response (38). Accordingly, U266 myeloma cells that were arrested in the G₁ phase of the cell cycle following exposure to AG490 incurred less DNA damage by the topoisomerase II inhibitor mitoxantrone, correlating with reduced cell death. In contrast, cytotoxicity of the alkylating agent melphalan is less dependent on cell cycle progression and was not significantly affected by AG490, whereas vinblastine, which binds to tubulin with high affinity and prevents microtubule assembly, was enhanced by pretreatment with AG490.

Our results demonstrate that a thorough understanding of how specific signal transduction pathways regulate downstream gene expression is necessary to design strategies for using targeted therapy to enhance cancer cell killing. In this particular case, inhibition of the JAK kinase pathway by treatment with AG490-enhanced Fas-induced apoptosis by decreasing Bcl-x_L expression but paradoxically reduced cytotoxic drug activity, particularly for topoisomerase II inhibitors, presumably by causing a cell cycle arrest associated with reduced cyclin D1 expression. However, these findings do not preclude the possibility that use of JAK-Stat3 inhibitors after cytoreduction with chemotherapy may prolong *in vivo* responses by preventing regrowth of surviving cancer cells. For example, we demonstrated previously that JAK-Stat3 inhibitors can accentuate the activity of immune therapy in animal models (43). Future studies will examine the possibility that administering inhibitors of JAK-Stat3 following chemotherapy may prolong treatment response.

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Inhibition of JAK Kinase Activity Enhances Fas-mediated Apoptosis but Reduces Cytotoxic Activity of Topoisomerase II Inhibitors in U266 Myeloma Cells

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