# Inhibition of Constitutive STAT3 Activity Sensitizes Resistant Non-Hodgkin's Lymphoma and Multiple Myeloma to Chemotherapeutic Drug-mediated Apoptosis[1]

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## ABSTRACT

**Hematopoietic malignancies have been shown to depend on cytokine growth factor autocrine/paracrine loops for growth and differentiation. This results in the constitutive activation of cytokine-mediated transcription factors like signal transducer and activators of transcription (STAT) 3 in non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM). Recent evidence demonstrates that cytokines also contribute to a drug-resistant phenotype in many tumor cell types. We hypothesized that inhibitors of the STAT3 pathway would sensitize drug-resistant and endogenous cytokine-dependent NHL and MM tumor cells to the cytotoxic effects of chemotherapeutic drugs. We examined an AIDS-related NHL cell line, 2F7, known to be dependent on interleukin (IL)-10 for survival and an MM cell line, U266, known to be dependent on IL-6 for survival. IL-10 and IL-6 signal the cells through the activation of Janus kinase (JAK)1 and JAK2, respectively. Thus, we investigated the effect of two chemical STAT3 pathway inhibitors, namely, piceatannol (JAK1/STAT3 inhibitor) and tyrphostin AG490 (JAK2/STAT3 inhibitor), on the tumor cells for sensitization to therapeutic drugs. We demonstrate by phosphoprotein immunoblotting analysis and electrophoretic mobility shift analysis that piceatannol and AG490 inhibit the constitutive activity of STAT3 in 2F7 and U266, respectively. Furthermore, piceatannol and AG490 sensitize 2F7 and U266 cells, respectively, to apoptosis by a range of therapeutic drugs including cisplatin, fludarabine, Adriamycin, and vinblastine. The specificity of the inhibitors was corroborated in experiments showing that piceatannol had no effect on U266 and, likewise, AG490 has no effect on 2F7. The sensitization observed by these inhibitors correlated with the inhibition of Bcl-2 expression in 2F7 and Bcl-xL expression in U266. Altogether, these results demonstrate that STAT3 pathway inhibitors are a novel class of chemotherapeutic sensitizing agents capable of reversing the drug-resistant phenotype of cytokine-dependent tumor cells.**

## INTRODUCTION

Drug resistance remains one of the most difficult barriers to overcome in patients with malignant disease. A great percentage of patients fail to respond to current treatment methods, such as chemotherapeutic drugs. Whereas chemotherapy initially results in remission, many suffer from tumor relapse arising from drug-resistant tumor cell clones. Resistance to conventional drugs necessitates the use of alternative strategies or adjuvant therapies. The study of drug resistance and cell survival factors in tumor cells has resulted in the identification of a number of potential targets for anticancer therapy (1, 2). Among those are cytokines involved in growth and survival, and the various proteins comprising their signaling pathways (3).

Our laboratory and others have demonstrated that the cytokines, IL-6[3] and IL-10, render tumor cells, in particular MM, prostate carcinoma, and NHL, resistant to the cytotoxic effects of chemotherapeutic drugs (4–8). Each cytokine is secreted endogenously by tumor cells to form autocrine/paracrine loops, which signals through surface receptors and induces a stronger drug-resistant phenotype. We have shown that neutralization of IL-10 by neutralizing antibody or the down-regulation of its transcription and secretion by the anti-CD20 antibody, Rituximab, sensitizes ARL cell lines to conventional chemotherapy (8). Similarly, IL-6 neutralization in MM renders tumor cells more susceptible to apoptotic stimuli, both *in vivo* and *in vitro* (4, 9). Recent work has implicated the regulation of antiapoptotic proteins as the underlying mechanism involved in conferring apoptotic resistance by survival-enhancing cytokines (10–17). These findings heightened the importance of delineating cytokine signaling in malignant tissue and the role it plays in regulating protective factors. Identification of novel targets in such pathways becomes important for disrupting the growth and survival signals critical for cancer outgrowth in the tumor microenvironment.

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[3] The abbreviations used are: IL, interleukin; NHL, non-Hodgkin's lymphoma; MM, multiple myeloma; JAK, Janus kinase; STAT, signal transducer and activators of transcription; ARL, AIDS-related lymphoma; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt; PI, propidium iodide; MDR, multidrug resistance; SIE, *sis*-inducible element; FIC, fractional index of cytotoxicity.

As with every member of the IL and IFN classification of signaling proteins, IL-6 and IL-10 signal transduction is mediated through the JAK/STAT pathway (18). JAKs (JAK1–3, TYK2) are cytosolic tyrosine kinases that bind the intracellular domains of cytokine receptors and become phosphorylated after the ligation of soluble cytokine (19). JAKs, in turn, phosphorylate the receptor chains, allowing for the docking of STAT proteins. Once bound to receptors, STATs are phosphorylated, dimerize, and translocate to the nucleus where they initiate transcription of target genes (20–23). Seven STAT proteins are currently known (STAT1–4, 5a, 5b, and 6), and each possesses the ability to bind multiple cytokine and growth factor receptors (18). IL-6 and IL-10 receptors are both bound by STAT3, although its recruitment to each receptor is facilitated by different JAKs. IL-10 receptor dimerization by bound IL-10 cytokine induces recruitment and activation of TYK2 and JAK1, whereas the IL-6 receptor binds and activates mainly TYK2 and JAK2 (24, 25). STAT3's involvement in both cytokine signaling pathways makes it an attractive target for intervention therapy in tumor cells exhibiting IL-6 and/or IL-10-dependent drug resistance. STAT3 is made additionally attractive by evidence that it is constitutively activated and thought to play a role in the oncogenicity in many primary tumors and tumor cell lines (26–32).

Activation of STAT1, 3, and 5 appears to have a role in cell growth and differentiation in hematopoietic cells, as well as in preventing apoptosis during cell activation (33, 34). STAT3, specifically, has been found constitutively activated in breast cancer, MM, head and neck cancer, leukemias, lymphomas, lung cancer, and prostate carcinoma (26, 28, 29, 35–38). Furthermore, we have shown that STAT3 is also activated in AIDS-related NHL (8). Recent findings in MM revealed that STAT3 activity confers resistance to Fas-mediated apoptosis (26), the mechanism of which involved the regulation of Bcl-xL by activated STAT3. We and others have also reported that Bcl-2 is regulated by STAT3 activity in lymphoid tumor cells (39–41). Because Bcl-xL and Bcl-2 have been shown to protect cells from chemotherapy-induced cell death (42), we presume that the aberrant activation of STAT3-dependent signaling pathways in MM and ARL may confer resistance to chemotherapy. On the basis of these previous findings, we hypothesize that endogenous cytokine secreted by tumor cells induces cytokine receptor signaling via the JAK/STAT pathway, and culminates in the STAT3-mediated activation of antiapoptotic factors and drug resistance. Therefore, we expect that inhibitors of STAT3 activation can render drug-resistant tumor cells sensitive to drug-mediated apoptosis.

The present study tested this hypothesis and examined the effects of inhibitors of STAT3 pathway activation, specifically piceatannol and tyrphostin AG490, on the sensitivity of ARL and MM to drug-mediated apoptosis. Piceatannol is a naturally occurring stilbene by-product from the seeds of Euphorbia lagascae (43) known to inhibit the tyrosine phosphorylation of STAT3 and STAT5, but not STAT1 or STAT2 (44). The inhibition of STAT3 by piceatannol is because of blocking of JAK1 kinase activity, which is induced by IL-10 in ARL. We also examined the effect of the STAT3 pathway inhibitor, tyrphostin AG490, which has been shown to inhibit the activation of STAT3 in hematopoietic tumor cells (31, 32, 45) and blocks STAT3 activation because of blocking of JAK2 kinase activity that is induced specifically by IL-6 in MM (46). AG490 is a member of the tyrphostin family of synthetic, low molecular weight compounds that selectively inhibit different protein tyrosine kinases (47). Thus, in this study we investigated (1) the effect of piceatannol and AG490 on the inhibition of STAT3 activity on 2F7 and U266 tumor cells (2), the effect of piceatannol and AG490 on the sensitization of 2F7 ARL and U266 MM tumor cells to drug (cisplatin, Adriamycin, fludarabine, and vinblastine) -mediated apoptosis, and (3) the effect of piceatannol and AG490 on the regulation of Bcl-2 family member proteins.

## MATERIALS AND METHODS

**Cell Culture.** The NHL B-cell line, 2F7, is of Burkitt's lymphoma histotope and was derived from a patient suffering from AIDS. It was graciously provided by Dr. Otoniel Martinez-Maza (Jonsson Comprehensive Cancer Center). The U266 cell line was derived from human MM and was kindly provided by Dr. James Berenson (Cedars Sinai Medical Center, Los Angeles, CA). Cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 10% heat inactivated fetal bovine serum (Gemini, Calabasas, CA). Supplemented medium is hereafter referred to as complete medium. Cells were maintained at 37°C in 5% atmospheric $CO_2$.

**Reagents.** Adriamycin, cisplatin, vinblastine, and verapamil were purchased from Sigma (St. Louis, MO). Fludarabine was kindly provided by Berlex (Richmond, CA). PBS (pH 7.4; Sigma) was used to solubilize and store stocks of Adriamycin (1 mg/ml), vinblastine (10 mg/ml), and fludarabine (1 mM) at 4°C. Cisplatin was dissolved in DMSO (Sigma) at 5 mg/ml before usage. The JAK2/STAT3 inhibitor, Tyrphostin AG490, was purchased from Alexis Corp (San Diego, CA). The JAK1/STAT3 inhibitor, piceatannol, was obtained from Calbiochem (San Diego, CA). The XTT cytotoxicity assay kit was purchased from Boehringer Mannheim (Indianapolis, IN).

Bcl-2 antibody was purchased from Dako (Carpinteria, CA), Bax and Bcl-xL antibodies were obtained from Santa Cruz (Santa Cruz, CA), and BAD antibody was purchased from Transduction Labs (Lexington, KY). Antibodies against STAT3 and phosphorylated STAT3 were obtained from New England Biolabs (Beverly, MA). Actin antibody was from Chemicon International (Temecula, CA). Secondary antimouse and antirabbit antibodies were purchased from New England BioLabs, Inc. Nitrocellulose blots were developed with LumiGlo (New England Biolabs, Inc.).

**Immunoblotting Analysis of Protein Expression.** After 24 h treatments to both cell lines with complete medium, piceatannol, or AG490, cells were collected and transferred to 1.5-ml microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) and pelleted for 10 s at 3,000 rpm in an Eppendorf microcentrifuge 5415 (Eppendorf Scientific Inc., Westbury, NY). Pellets were placed on ice and lysed in cold radioimmunoprecipitation assay buffer (1% NP40, 0.1% SDS, 0.5% deoxycholic acid, and 1× PBS) supplemented with protease inhibitor mixture tablets (Boehringer Mannheim) for 10 min. Lysates were sheered repeatedly using 1-cc insulin syringes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 14,000 rpm for 10 min at 4°C. Lysate supernatants were

transferred to new microcentrifuge tubes and quantified for protein concentration using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). An equal volume of sample buffer [6.2 mM Tris (pH 6.8), 2.3% SDS, 5% mercaptoethanol, 10% glycerol, and 0.02% bromphenol blue] was added to each lysate sample, then boiled for 10 min and stored at −80°C until immunoblotting was performed. Dilutions of 1:1,000 for primary antibodies to STAT3, phospo-STAT3, Bcl-2, Bcl-xL, Bax, and Bad were used. Actin and secondary antibodies were used at a dilution of 1:10,000.

**Electrophoretic Mobility Shift Assays.** To analyze the effect of piceatannol and AG490 on STAT3 binding in both 2F7 and U266 cells, we performed electrophoretic mobility gel shift assays (48). After 24 h of treatment, cells were pelleted and washed twice in ice-cold PBS. Cell membranes were subsequently lysed in 1 ml of NP40 lysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP40] on ice for 10 min. Samples were centrifuged at 2000 rpm for 5 min at 4°C in a microcentrifuge to pellet the nuclei. Supernatants were removed subsequently and discarded. Nuclei were resuspended in NP40 buffer for washing and rewashed twice in cold PBS. Nuclei were then pelleted and lysed in nuclear extraction buffer [20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT] and sonicated at 4°C for 30 min.

Nuclear lysates were incubated with $^{32}$P-labeled double stranded SIE probe (Ref. 49; forward: 5′-GAT CCA TTT CCC GTA AAT CA-3′; reverse: 5′-TGA TTT ACG GGA AAT GGA TC-3′) at room temperature for 15 min. Samples were run on 5% Tris-borate EDTA gels (Bio-Rad Laboratories) and exposed to radiography overnight at −80°C.

**Cytotoxicity Assay.** XTT assays were performed to determine cytotoxicity (50, 51). Tumor cells were seeded in 200 μl at $10^4$/well in 96-well tissue culture plates (Costar, Cambridge, MA). Cells were treated with complete medium, piceatannol (50 μM), or AG490 (10 μM) as single agents and in combination with chemotherapeutic drugs: cisplatin (1 μg/ml) and Adriamycin (1 μg/ml); fludarabine (20 μM) and vinblastine (0.1 μg/ml) for 2F7, and fludarabine (2 μM) and vinblastine (10 ng/ml) for U266. Drug concentrations were administered at subtoxic concentrations, based on drug titration. Cells grown in complete medium alone served as the assay baseline and negative control for cytotoxicity. Wells with 200 μl of complete medium only served as the plate blank. All of the samples were done in triplicate. After 24-h treatment at 37°C, the XTT dye was added to quantitate cell inhibition in each sample. The cytotoxicity induced by each treatment was calculated as the percentage of viability of each sample compared with the untreated cells.

$$\%\ Viability = \frac{(OD\ of\ treated\ samples)}{(OD\ of\ untreated\ cells)} \times 100\% \qquad (A)$$

**Statistical Analysis.** All of the values are presented as the mean ± SD of triplicate samples. ANOVA was used to test for significance. Pairwise analysis was performed by Bonferroni/Dunn post hoc test. $P < 0.05$ was considered to be significant.

**Isobolograms for Synergistic Cytotoxicity.** To establish whether the potentiation of chemotherapeutic drugs by STAT3 pathway inhibitors was more than additive, isobolograms (52) were performed using combinations of drugs and piceatannol or AG490 at various concentrations. Treatments on 2F7 cells included piceatannol (1, 5, 10, 20, 50, and 100 μM), cisplatin (0.01, 0.05, 0.1, 0.5, 1, and 5 μg/ml), vinblastine (5, 10, 50, 100, 500, and 1000 ng/ml), Adriamycin (0.01, 0.05, 0.1, 0.5, 1, and 5 μg/ml), and fludarabine (1, 5, 20, 50, and 100 μM). Treatments on U266 cells included AG490 (0.1, 0.5, 1, 5, 10, and 50 μM), vinblastine (0.5, 1, 5, 10, 50, and 100 ng/ml), and fludarabine (0.1, 0.5, 1, 5, and 20 μM) with cisplatin and Adriamycin at identical concentrations as listed for 2F7. Combinations yielding a cytotoxicity of 40% ± 5% were graphed as a percentage of the concentration of single agent alone that produced this amount of cytotoxicity.

$$FIC = \frac{Asample}{A50\%}, \frac{Bsample}{B50\%} \qquad (B)$$

**PI Analysis for Apoptosis.** To analyze apoptosis, as a measurement of DNA fragmentation, tumor cells were stained with PI (53) and analyzed on an Epics-XL MCL flow cytometer (Coulter, Miami, FL). Briefly, chemotherapeutic drugs were added to $10^6$ 2F7 cells seeded in complete medium or piceatannol (50 μM), or added to $10^6$ U266 cells seeded in complete medium or AG490 (10 μM). Drug concentrations were as follows: cisplatin (1 μg/ml), Adriamycin (1 μg/ml), fludarabine (20 μM), and vinblastine (0.1 μg/ml) for 2F7; and cisplatin (1 μg/ml), Adriamycin (1 μg/ml), fludarabine (2 μM), and vinblastine (10 ng/ml) for U266. After incubation, cells were washed twice in PBS/0.1% BSA. After washing, cells were permeabilized by resuspension in 500 μl of cold 75% ethanol and allowed to incubate at −20°C for 1 h. Cells were rewashed three times and resuspended in 100 μl of PI solution (100 μg/ml PI, 50 μg/ml RNase). PI staining was performed for 30 min at room temperature while light-protected. After incubation with PI solution, 500 μl of PBS/0.1% BSA was added per sample and analyzed by flow cytometry.

**Analysis of MDR-mediated Drug Efflux.** To determine whether STAT3 pathway inhibitors influence the cytotoxicity of drugs by inhibiting MDR-mediated drug efflux, $10^6$ 2F7 and U266 cells were treated with piceatannol (50 μM) and AG490 (10 μM), respectively, verapamil (100 μM), or complete medium alone for 1 h at 37°C. After incubation, Adriamycin (1 μg/ml) was added to each cell suspension. Cells were allowed to incubate in the presence of Adriamycin for 10 min. Cells were subsequently washed twice in PBS/0.1% BSA and analyzed by flow cytometry on a MoFlo Sorter (Cytomation, Fort Collins, CO), or washed in 37°C complete medium and incubated in medium for an additional 50 min. After 50 min, cells were washed twice in PBS/0.1% BSA and analyzed by flow cytometry. Intracellular accumulation of autofluorescing Adriamycin results in a higher mean fluorescence intensity per cell. Therefore, based on the fluorescence intensity of a cell population, one can determine the amount of Adriamycin that has been pumped out of the cells, which is representative of a functional MDR pump.

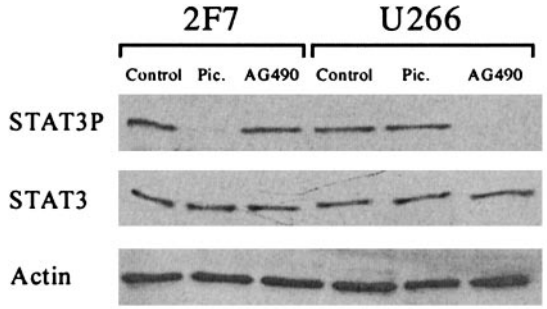


*Fig. 1* Constitutively activated STAT3 is inhibited by piceatannol in 2F7 cells and by AG490 in U266 cells. Tumor cells displayed constitutive phosphorylation of STAT3 (*STAT3P*) after 24 h of culture in complete medium (*control*), as analyzed by Western blotting. Piceatannol (50 μM) treatment inhibited STAT3 phosphorylation in 2F7 ARL cells after 24 h, but had no effect on STAT3 phosphorylation in U266 MM cells. Conversely, AG490 (10 μM) treatment abrogated STAT3 phosphorylation in U266 cells, but not in 2F7 cells. STAT3 protein expression remained unchanged after 24 h of treatment, indicating changed in phospo-STAT3 was not because of STAT3 down-regulation. Actin blotting was performed to ensure equal loading.

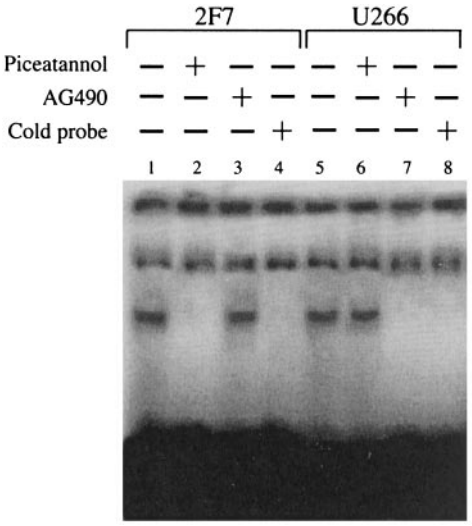


*Fig. 2* STAT3 DNA binding is inhibited by piceatannol and AG490 in 2F7 and U266, respectively. To analyze STAT3 binding inhibition, electrophoretic mobility shift assays were performed with $P^{32}$-labeled SIE probe and nuclear lysates from 2F7 and U266 cells treated with piceatannol (50 μM) or AG490 (10 μM) for 24 h as described in "Materials and Methods." Constitutive DNA binding of activated STAT3 was abrogated by piceatannol in 2F7 tumor cells (*Lane 2*), but not in U266 cells (*Lane 6*). In the presence of AG490, inhibition of STAT3 binding was seen in U266 tumor cells (*Lane 7*), not seen in 2F7 cells (*Lane 3*). The final lane of each tumor sample (*Lanes 4* and *8*) shows cold probe, at 10 times the concentration of labeled probe, was able to out-complete STAT3 binding in control lysates.

## RESULTS

**STAT3 Pathway Inhibitors, Piceatannol and AG490, Inhibit STAT3 Activation in a Cell Line-dependent Manner.** Although piceatannol and AG490 were both effective inhibitors of STAT3 activation, neither was capable of inhibiting the constitutive activation (phosphorylation) of STAT3 in both tumor cell lines (Fig. 1). This was expected because STAT3 activity in 2F7 is dependent on JAK1 signaling by IL-10 and inhibitable by piceatannol, whereas STAT3 activation in U266 is dependent on JAK2 signaling by IL-6 and is inhibitable by AG490. Both cell lines were treated with piceatannol (50 μM) or AG490 (10 μM) for 24 h and subjected to immunoblotting. Western blot analysis of STAT3 phosphorylation revealed that piceatannol was an effective inhibitor of STAT3 activation in the 2F7 cell line but not in the U266 cell line. Conversely, AG490 abrogated activation of STAT3 in U266 cells but not in 2F7 cells. Changes in phosphorylated STAT3 were not because of its down-regulation because blotting of STAT3 protein showed that levels remained constant. Blotting of actin was performed to ensure equal loading.

DNA binding activity and its inhibition by piceatannol and AG490 was examined by electrophoretic mobility shift assays using 2F7 and U266 nuclear extracts and the SIE as probe. Treatments with piceatannol (50 μM) and AG490 (10 μM) were carried out for 24 h. STAT3 binding proved to be inhibited in 2F7 cells on piceatannol treatment (Fig. 2, *Lane 2*), but not AG490 treatment (Fig. 2, *Lane 3*). Constitutive activation and DNA binding ability of STAT3 in U266 cells was hindered in the presence of AG490 (Fig. 2, *Lane 7*) but not with piceatannol (Fig. 2, *Lane 6*). The final lanes for each cell line (Fig. 2, *Lanes 4* and *8*) show that cold probe at 10 times the concentration of labeled probe was able to out-compete STAT3 binding in control lysates. These findings demonstrate that STAT3 binding inhibition by piceatannol and AG490 is dependent on whether JAK1 or JAK2 is inhibited and shows the specificity of the two inhibitors.

**Piceatannol and AG490 Potentiate the Efficacy of Chemotherapeutic Drugs.** To determine whether STAT3 pathway inhibitors could be used to enhance the sensitivity of tumor cells to chemotherapeutic drugs, we used two B-lymphocytic cell lines of which the resistance to apoptotic stimuli had been correlated with activated STAT3. The NHL cell line, 2F7, and the MM cell line, U266, demonstrated distinct responses to treatment with the STAT3 pathway inhibitors, piceatannol and AG490. The 2F7 cell line displayed no cytotoxicity after 24 h of treatment with piceatannol (50 μM) or AG490 (10 μM; Fig. 3). When treated with combinations of AG490 and therapeutic drugs, the 2F7 tumor cells did not undergo higher cytotoxicity than with treatment of drugs alone. However, when treated with drugs and piceatannol, the 2F7 tumor cells showed a significant decrease in cell viability. When U266 cells were treated with either STAT3 pathway inhibitor, their cell viability also did not change. In contrast to 2F7 cells, the U266 tumor cell line was only sensitized to therapeutic drugs by AG490 but not by piceatannol.

Isobolograms were conducted to evaluate the efficacy of drug-inhibitor combinations (Fig. 4). Experiments were performed on 2F7 and U266 with the panel of four chemotherapeutic drugs and their respective drug sensitizing inhibitors, piceatannol, and AG490. Isobolograms demonstrate that STAT3 pathway inhibitors sensitize the tumor cells in a synergistic manner after 24 h.

XTT metabolic assays used to quantitate viability cannot distinguish between apoptosis and inhibition of proliferation. PI

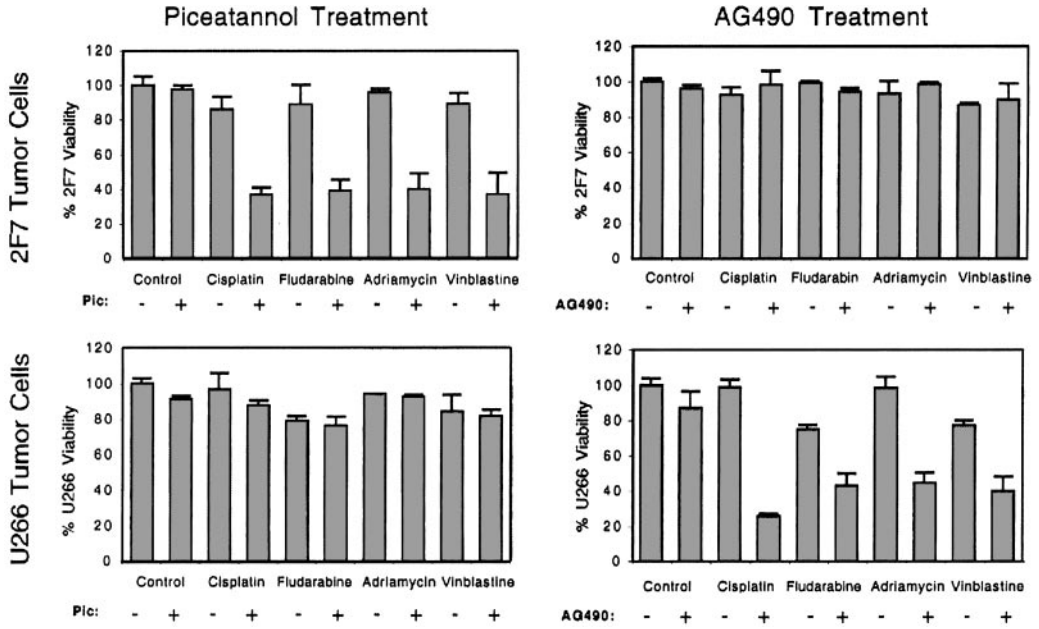


*Fig. 3* STAT3 chemical inhibitors potentiate the cytotoxic effect of chemotherapeutic drugs. Tumor cells ($10^4$) were treated with 50 μM of piceatannol, 10 μM AG490, or a combination of each inhibitor and each therapeutic drug at 37°C for 24 h. 2F7 treatments were as follows: cisplatin, 1 μg/ml; fludarabine, 20 μM; Adriamycin, 1 μg/ml; and vinblastine, 0.1 μg/ml. U266 treatments were: cisplatin, 1 μg/ml; fludarabine, 2 μM; Adriamycin, 1 μg/ml; and vinblastine, 10 ng/ml. Control samples were allowed to grow in complete medium alone. The XTT assay was used to determine cytotoxicity. The enhancement of cytotoxicity in piceatannol and drug combinations in 2F7 cells was determined to be statistically significant when compared with treatment with piceatannol or drug alone, $*P < 0.01$, but not in U266. The augmentation of cytotoxicity in AG490 and drug combinations in U266 cells was statistically significant when compared with treatment with AG490 or drug alone, $*P < 0.01$, but not in 2F7; *bars,* ± SD.

staining for the detection of DNA fragmentation was used to discern between the two. PI analysis of 2F7 cells treated with piceatannol-drug combinations and U266 cells treated with AG490-drug combinations for 24 h show that the decrease in viability is because of induction of drug-mediated apoptosis, as illustrated in 2F7 flow panels (Fig. 5*A*). Apoptotic profiles for treatments in both cell lines are summarized in Table 1. The percentage of cells undergoing apoptosis is best represented in Fig. 5*B,* where killing is seen synergistically in combination treatments. Together, these findings substantiate the specificity of STAT3 inhibition by piceatannol and AG490, and corroborate drug sensitization to STAT3 activation levels shown by Western blotting and electrophoretic mobility shift assays (Figs. 1 and 2).

**Piceatannol and AG490 Sensitize Tumor Cells Independent of MDR-mediated Drug Efflux.** The presence of multiple drug resistance genes confers survival to tumor cells in the presence of various chemotherapeutic drugs (54, 55). We sought to determine whether the increased efficacy of therapeutic drugs by the STAT3 pathway inhibitors was, at least in part, because of blocking of drug efflux from the tumor cells by MDR proteins. Adriamycin was used as a marker drug to detect the efflux of therapeutic agents by MDR genes because of its ability to autofluoresce (56). Cells were pretreated with piceatannol, AG490, or verapamil. Verapamil is an MDR blocking agent (57) and was used as a control to show MDR protein pump inhibition. After pretreatment, cells were incubated in ADR to allow drug intake and to monitor the ability of the cell to discard the drug through pump-mediated efflux (Table 2). After 10 min, both cell lines showed a marked influx of Adriamycin. After 60 min, the 2F7 cells displayed a strong ability to pump out Adriamycin to near background levels. As expected, the presence of verapamil inhibited the MDR phenotype after 60 min. The U266 cell line showed a slower ability to decrease intracellular Adriamycin levels after 60 min. Moreover, verapamil did not significantly influence drug efflux in U266. Importantly, neither the presence of piceatannol in 2F7 cells nor the addition of AG490 on U266 cells influenced the intracellular levels of Adriamycin. Therefore, we conclude that the sensitization to chemotherapeutic agents by the STAT3 pathway inhibitors is intracellular-concentration independent, as well as MDR independent.

**STAT3 Pathway Inhibitors Down-Regulate Antiapoptotic Bcl-2 Family Members, Specifically Bcl-2 in 2F7 and Bcl-xL in U266.** Recent reports have identified STAT3 as a regulator of bcl-2 family members, Bcl-2 and Bcl-xL (5, 8, 26, 39). To confirm whether these antiapoptotic factors are effected by STAT3 inhibition and possibly involved in the reversal of drug resistance by STAT3 pathway inhibitors, Western blotting for their expression was performed. Both cell lines were treated with piceatannol or AG490 for 24 h. Western blot results show Bcl-2 inhibition in 2F7 cells by piceatannol treatment (Fig. 6), but not by AG490. No regulation of Bcl-2 was seen in U266 tumor cells. Blotting for Bcl-xL revealed that no regulation occurred in 2F7 with either inhibitor. Conversely, Bcl-xL was down-regulated in U266 cells by AG490, although piceatannol

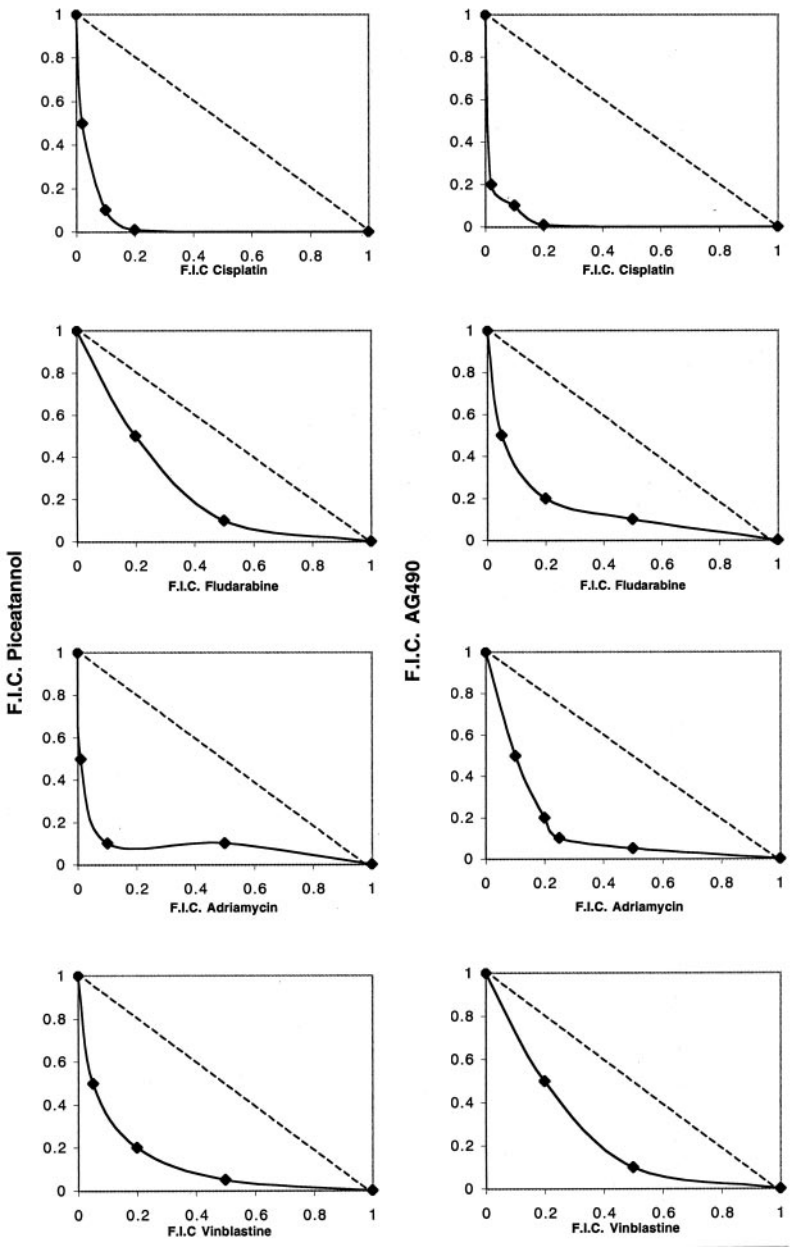


*Fig. 4* 2F7 cells were treated with a concentration curve of each drug in combination with a concentration curve of piceatannol, whereas U266 cells were treated with an AG490 concentration curve with various drug concentrations, as described in "Materials and Methods." The FIC represents the concentrations from combinations yielding a percent cytotoxicity of 40% ± 5% graphed as a percentage of single agent concentrations alone yielding 40% ± 5%; FIC = Asample/A30%, Bsample/B30%. Isobolograms show a clear synergistic effect using piceatannol with drugs in 2F7 cells and using AG490 with drugs in U266 cells.

had no effect. Blotting was also performed for two other Bcl-2 family members, Bax and Bad. Regulation was not observed in either protein by inhibitors in either cell line. Actin blotting was performed to ensure equal loading among cell line samples.

## DISCUSSION

Our findings demonstrate that chemical inhibitors of the STAT3 pathway possess the ability to potentiate the cytotoxic effects of chemotherapeutic drugs in NHL and MM. The STAT3 pathway inhibitors, piceatannol and tyrphostin AG490, sensitize the NHL cell line, 2F7, and the MM cell line, U266, respectively, to a battery of chemotherapeutic drugs, including cisplatin, fludarabine, Adriamycin, and vinblastine. Inhibition of STAT3 by piceatannol in 2F7 cells resulted in the specific down-regulation of Bcl-2, whereas AG490 inhibition of STAT3 in U266 cells resulted in the specific inhibition of Bcl-xL expression. Neither inhibitor was shown to influence intracellular levels of Adriamycin, thus, suggesting that sensitization is MDR-independent. Taken together, this evidence supports the potential clinical use of STAT3 pathway inhibitors as novel chemotherapeutic adjuvants for the sensitization of drug-resistant tumor cells.

Fig. 7 schematically shows the role of cytokines in protecting the tumor cells against drug-mediated apoptosis, and the role of STAT3 pathway inhibitors in reversal of resistance and sensitization to drug-mediated cytotoxicity. Activation of cyto-

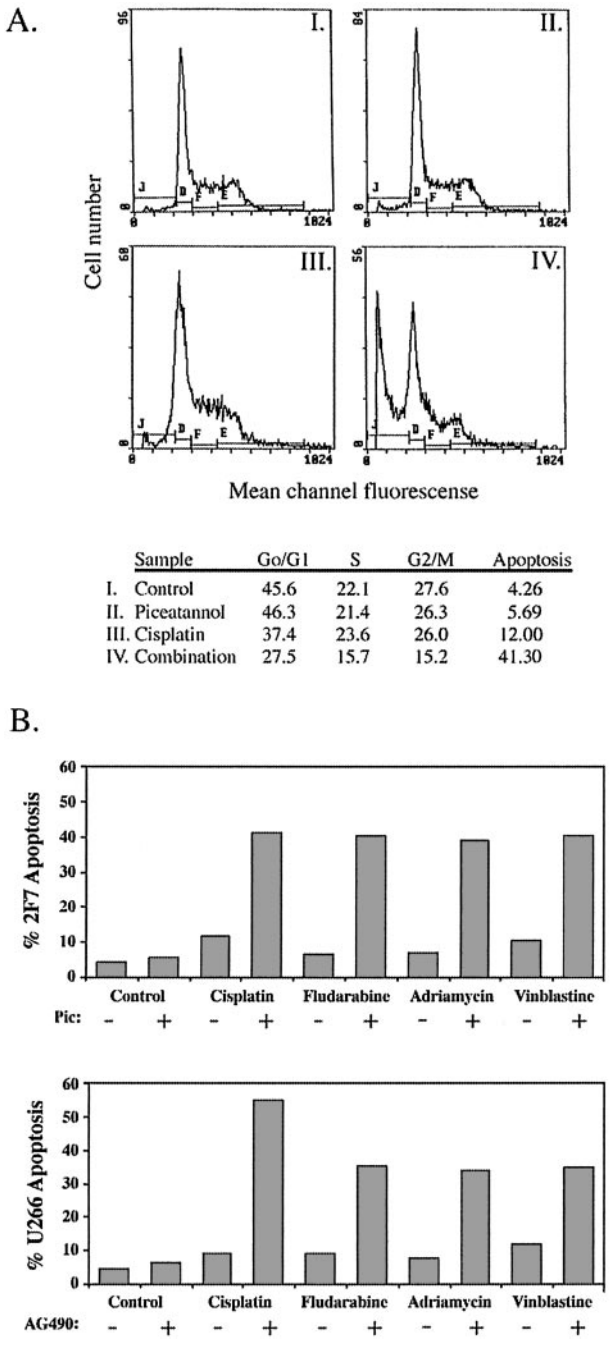


*Fig. 5* Tumors cells are sensitized to drug-induce apoptosis. 2F7 tumor cell lines were treated with piceatannol (50 μM), cisplatin (1 μg/ml), fludarabine (20 μM), Adriamycin (1 μg/ml), vinblastine (0.1 μg/ml) or a combination of piceatannol and drugs for 24 h. U266 tumor cell lines were treated with AG490 (10 μM), cisplatin (1 μg/ml), fludarabine (2 μM), Adriamycin (1 μg/ml), vinblastine (10 ng/ml) or a combination of AG490 and drugs for 24 h. Cells were stained with PI and analyzed by flow cytometry. *A*, 2F7 cells treated with piceatannol and cisplatin alone showed small changes in the number of apoptotic cells above that of control cells. However, the combination of piceatannol and cisplatin resulted in synergistic apoptosis. Similar results were observed with all drugs, as well as in AG490/drug combinations in U266 (summarized in Table 1). *B*, a graphical representation of apoptosis values from Table 1 demonstrates the synergistic augmentation of drug-induced killing. Values are representative of three independent experiments.

*Table 1*

Piceatannol and AG490 sensitize 2F7 and U266 tumor cells, respectively, to chemotherapeutic drugs within 24 h

Tumor cells were treated for 24 h with piceatannol, AG490, cisplatin, fludarabine, Adriamycin, or vinblastine, or combinations there of, as listed. After treatment, cells were stained with PI, as described in "Materials and Methods," and analyzed by flow cytometric analysis. Numbers are percentage of population in given cell cycle phase or undergoing apoptosis.

| | $G_o/G_1$ | S | $G_2/M$ | Apoptosis |
|---|---|---|---|---|
| 2F7 Treatment | | | | |
| Control | 45.6 | 22.1 | 27.6 | 4.26 |
| Piceatannol (50 μM) | 46.3 | 21.4 | 26.3 | 5.69 |
| Cisplatin (1 μg/ml) | 37.4 | 23.6 | 26.0 | 12.00 |
| + Piceatannol | 27.5 | 15.7 | 15.2 | 41.30 |
| Fludarabine (20 μM) | 43.5 | 22.3 | 27.2 | 6.60 |
| + Piceatannol | 28.7 | 15.5 | 15.0 | 40.30 |
| Adriamycin (1 μg/ml) | 41.7 | 22.1 | 26.8 | 7.13 |
| + Piceatannol | 26.8 | 16.7 | 17.0 | 38.80 |
| Vinblastine (0.1 μg/ml) | 61.0 | 10.6 | 16.9 | 10.70 |
| + Piceatannol | 28.0 | 15.5 | 15.9 | 40.10 |
| U266 Treatment | | | | |
| Control | 63.7 | 13.5 | 18.3 | 4.64 |
| AG490 (10 μM) | 71.1 | 6.7 | 15.7 | 6.21 |
| Cisplatin (1 μg/ml) | 63.1 | 10.8 | 16.5 | 9.29 |
| + AG490 | 34.6 | 4.6 | 5.6 | 54.90 |
| Fludarabine (2 μM) | 68.2 | 8.0 | 14.5 | 9.22 |
| + AG490 | 40.1 | 16.2 | 8.4 | 35.10 |
| Adriamycin (1 μg/ml) | 67.0 | 9.6 | 15.1 | 7.84 |
| + AG490 | 37.8 | 13.5 | 14.4 | 34.10 |
| Vinblastine (10 ng/ml) | 61.6 | 10.4 | 16.0 | 11.70 |
| + AG490 | 37.5 | 12.9 | 14.8 | 34.60 |

*Table 2*

STAT3 pathway inhibitors sensitize 2F7 and U266 tumor cells independent of drug efflux

Tumor cells were treated for 1 h with piceatannol, AG490, or verapamil, or combinations there of, as listed. After treatment, cells were incubated with Adriamycin (ADR), as described in "Materials and Methods," and analyzed by flow cytometric analysis. Numbers are percentage of population displaying auto fluorescence, an indication of intracellular ADR accumulation.

| | 10 min | 60 min |
|---|---|---|
| 2F7 Treatment | | |
| Complete medium only | 1.98 | 2.15 |
| Medium + ADR (1 μg/ml) | 96.6 | 6.8 |
| Piceatannol (50 μM) + ADR | 99.0 | 7.8 |
| Verapamil (100 μM) + ADR | 96.8 | 86.2 |
| U266 Treatment | | |
| Complete medium only | 1.11 | 1.35 |
| Medium + ADR (1 μg/ml) | 97.3 | 57.4 |
| AG490 (10 μM) + ADR | 98.5 | 45.2 |
| Verapamil (100 μM) + ADR | 98.4 | 61.2 |

kine receptors induces the activation of JAKs bound to the intracellular receptor chains. In the case of IL-10R, TYK2 activation results in the phosphorylation of JAK1. Thereafter, JAK1 phosphorylates tyrosines on the IL-10R terminus. The phosphorylated tyrosines bind STAT3 molecules through affinity to their SH2 domains, allowing JAK1 to tyrosine (Y) phosphorylate STAT3. Once phosphorylated, STAT3 monomers dimerize, translocate to the nucleus, and transcribe *bcl-2*. IL-6R

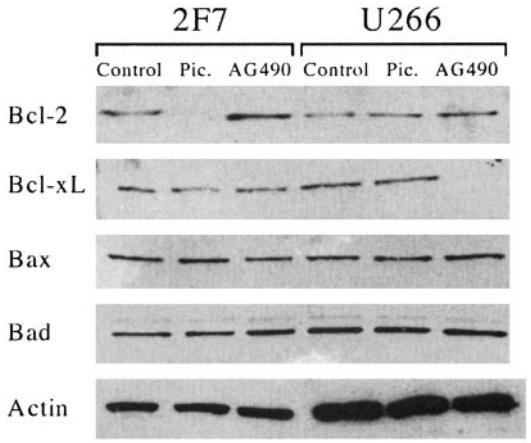


*Fig. 6* Bcl-2 and Bcl-xL are selectively down-regulated by piceatannol and AG490, respectively. Bcl-2, Bcl-xL, Bax, and Bad expressions were analyzed in both 2F7 and U266 cells after 24 h of treatment with piceatannol (50 μM) and AG490 (10 μM). Bcl-2 was shown to decrease in piceatannol-treated 2F7 cells but not in U266 cells. AG490 was shown to inhibit expression of Bcl-xL in U266 cells but had no effect in 2F7. Regulation of Bcl-2 in 2F7 and Bcl-xL in U266 by their respective inhibitors was specific, as neither Bax nor Bad were seen to have altered protein expression after 24 h in either cell line, regardless of inhibitor.

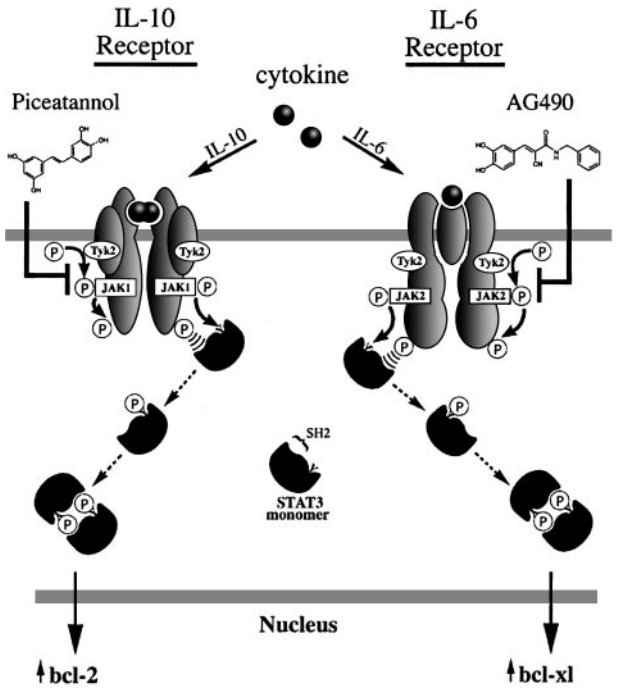


*Fig. 7* Proposed model for drug sensitization by chemical STAT3 pathway inhibitors. IL-6 and IL-10 cytokines bind their receptors and induce a cascade of phosphorylation events that lead to the activation of the STAT3 transcription factor. Activated STAT3 translocates to the nucleus where it targets antiapoptotic genes most likely involved in drug resistance. Diagram is discussed in detail within the discussion.

activation induces activation of TYK2 and JAK2. Similar to JAK1, JAK2 phosphorylates IL-6 receptor chains resulting in bound STAT3 and its phosphorylation by JAK2. STAT3 dimers then target *bcl-x* for transcription and Bcl-xL protein overexpression. Although the specific mechanisms of piceatannol and AG490 are still unclear, it is presumed that each inhibits TYK2 in its ability to activate any receptor associated JAK, *e.g.*, JAK1 in the case of IL-10R and JAK2 in IL-6R. This inhibition abrogates the overexpression of STAT3-dependent genes involved in chemotherapeutic drug resistance.

STAT3 is found constitutively activated in a variety of tumor cell types (22). This abnormal increase in activation implicated STAT3 as not only a factor in the onset of oncogenesis, but also an antiapoptotic player in the resistance to cell-mediated cytotoxicity and chemotherapeutic drug treatment. Subsequent studies have revealed a dual pattern of STAT3 involvement in tumorigenicity (22). It is well established that various oncogenes induce the phosphorylation and activation of STAT3, a process independent of external stimuli from cytokines or growth factors (58–62). Conversely, other model systems have revealed the ability of STAT3 to activate antiapoptotic and proliferation-associated proteins, such as Bcl-xL, Bcl-2, Mcl-1, c-myc, Cyclin D1, and $p21^{WAF-1}$ (26, 41, 63–66). Compounded by evidence that STAT3-using cytokines confer a drug-resistant phenotype in some tumor cells, STAT3 becomes an attractive target for intervention therapy.

Our findings with STAT3 pathway inhibitors did not demonstrate induction of spontaneous apoptosis, but rather sensitization of tumor cells to drug-mediated apoptosis. However, several reports demonstrate that inhibition of STAT3 by chemical inhibitors, dominant-negative transfection, antisense therapy, and transfection of STAT3 inhibitor proteins results in tumor cell apoptosis *in vitro* (41, 44, 45, 63, 67, 68). Vos *et al.* (46) reported that AG490 suppresses cell proliferation and induces apoptosis in MM cell lines through inhibition of JAK2 kinase, ERK2, and STAT3 phosphorylation. Epling-Burnetti *et al.* (63) reported that leukemic large granular lymphocytic leukemia from 19 patients displayed high levels of activated STAT3. Treatment with AG490 induced apoptosis with corresponding decrease in STAT3 DNA binding activity. Although apoptosis was independent of Bcl-xL and Bcl-2 expression, the Bcl-2 family member, Mcl-1, was significantly reduced by AG490 treatment. Activated STAT3 was shown to bind an SIE-related element in the murine mcl-1 promoter. Importantly, it has been shown that STAT3 inhibition is not detrimental to normal tissue *in vitro* or in animal models (45, 69). However, two points of concern are whether the high levels of chemical inhibitors necessary to induce apoptosis in tumor cell lines will prove nontoxic in humans and, unlike in mouse models where treatments were carried out to 9 weeks, whether long-term exposure to inhibitors will culminate into, as yet, unknown consequences and prove intolerable in patients.

Whereas the goal of previous reports has been to use STAT3 inhibition as a means to induce cytotoxicity, we propose the use of STAT3 pathway inhibitors at subtoxic levels in combination with chemotherapeutic drug regimens. As shown here, subtoxic levels of inhibitors can sensitize drug-resistant tumor cells and serve as adjuvant therapy to current treatment standards. Voorzanger *et al.* (70) demonstrated that IL-6 and IL-10 can act as cooperative growth

factors in NHL. This lends validity to the idea of inhibitor mixtures in patients with high serum levels of cytokines known to perpetuate cell growth. Furthermore, the effective use of neutralization antibodies against cytokines or their receptors is hindered because of their immunogenicity and expense to generate (3). Thus, nonpeptide inhibitors of STAT3 activation offer a better alternative to anticytokine therapy. Conceivably, inactivation of STAT3 in tumor cells may also sensitize them to killing by the immune system, as evidenced by AG490-mediated sensitization of U266 to Fas-mediated killing reported in Catlett-Falcone *et al.* (26).

Discrepancies between 2F7 and U266 with respect to their response to STAT3 pathway inhibitors, in particular the specificity of STAT3 to Bcl-2 regulation in NHL and specificity to Bcl-xL in MM, is a major point of interest. Both cell lines have been shown to depend on endogenous cytokine for survival when apoptotically stimulated. IL-6 and IL-10 play a role in the antiapoptotic phenotype of U266 and 2F7, respectively. Whereas both cytokines use STAT3 as an integral component of their signaling, alternative receptor signaling pathways and differing modes of STAT3 recruitment may also influence the ultimate effect on gene and protein expression. For example, IL-6 receptor signaling has been shown to involve the Ras-mitogen-activated protein kinase pathway, mediated through the gp130 signal transducer (33, 71). Mitogen-activated protein kinase signaling can interfere with downstream signaling of STAT3 (72). The absence of such a pathway in IL-10 receptor signaling may provide one reason for discrepancies in gene alteration. Perhaps more importantly is the JAK involvement in STAT3 recruitment to the intracellular cytokine receptor chains. Piceatannol is a strong inhibitor of JAK1 activation, whereas AG490 has no influence on JAK1, but can inhibit JAK2 and, to some extent, JAK3. The importance of JAK1 *versus* JAK2 activation of STAT3 in the regulation of STAT3-dependent protein expression remains unclear. Moreover, the identification of STAT3 and JAK2 adapter proteins (73, 74) raises interest in the level of their involvement in gene expression, STAT3 DNA binding specificity, and response to cytokine.

Conceivably, the observation that STAT3 pathway inhibitors sensitize drug-resistant hematopoietic tumor cells to chemotherapeutic drugs can be translated to solid tumors. We have shown previously that IL-6 confers resistance to prostate carcinoma cells against cisplatin and etoposide cytotoxicity (6). It has also been documented that prostate tumor cells can possess constitutively activated STAT3 and that its inhibition by dominant-negative transfection suppresses cell growth (31). It stands to reason that STAT3 is a principal mediator of IL-6-dependent enhancement of survival in the presence of apoptotic stimuli in these tumor cells. Therefore, IL-6 signal disruption by STAT3 inhibition may prove to be an effective alternative to chemotherapy alone.

In conclusion, our findings demonstrate that chemical inhibitors of the STAT3 pathway are a novel class of compounds capable of reversing drug resistance in lymphoid-derived tumors. Our study also suggests the potential use of STAT3 pathway inhibitors *in vivo* in the treatment of drug-resistant hematopoietic tumor cells.

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