# Flavopiridol disrupts STAT3/DNA interactions, attenuates STAT3-directed transcription, and combines with the Jak kinase inhibitor AG490 to achieve cytotoxic synergy

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## Abstract

**Up-regulated signal transducers and activators of transcription (STAT)–mediated signaling is believed to contribute to the pathogenesis of a variety of solid and hematologic cancers. Consequently, inhibition of STAT-mediated signaling has recently been proposed as a potential new therapeutic approach to the treatment of cancers. Having shown previously that the pan–cyclin-dependent kinase inhibitor flavopiridol binds to DNA and seems to kill cancer cells via that process in some circumstances, we evaluated the hypothesis that flavopiridol might consequently disrupt STAT3/DNA interactions, attenuate STAT3-directed transcription, and down-regulate STAT3 downstream polypeptides, including the antiapoptotic polypeptide Mcl-1. SDS-PAGE/immunoblotting and reverse transcription-PCR were used to assess RNA and polypeptide levels, respectively. DNA cellulose affinity chromatography and a nuclear elution assay were used to evaluate the ability of flavopiridol to disrupt STAT3/DNA interactions. A STAT3 luciferase reporter assay was used to examine the ability of flavopiridol to attenuate STAT3-directed transcription. Colony-forming assays were used to assess cytotoxic synergy between flavopiridol and AG490. Flavopiridol was found to (*a*) disrupt STAT3/DNA interactions (DNA cellulose affinity chromatography and nuclear elution assay), (*b*) attenuate STAT3-directed transcription (STAT3 luciferase reporter assay), and (*c*) down-regulate the STAT3 downstream antiapoptotic polypeptide Mcl-1 at the transcriptional level (reverse transcription-PCR and SDS-PAGE/immunoblotting). Furthermore, flavopiridol, but not the microtubule inhibitor paclitaxel, could be combined with the STAT3 pathway inhibitor AG490 to achieve cytotoxic synergy in A549 human non–small cell lung cancer cells. Collectively, these data suggest that flavopiridol can attenuate STAT3-directed transcription in a targeted fashion and may therefore be exploitable clinically in the development of chemotherapy regimens combining flavopiridol and other inhibitors of STAT3 signaling pathways. [Mol Cancer Ther 2006;5(1):138–48]**

## Introduction

Signal transducers and activators of transcription (STAT) are transcription factors that shuttle from the cytoplasm to the nucleus in response to certain extracellular signals (1, 2). In particular, phosphorylation on specific tyrosine and serine residues induces monomeric cytoplasmic STAT proteins to dimerize and translocate to the nucleus, where they bind specific DNA sequences and activate transcription of several genes, including those encoding the antiapoptotic proteins Bcl-2, Bcl-$x_L$, and Mcl-1 (3, 4). Constitutive activation of STAT3 signaling has been observed in several neoplasms, including acute leukemias (5), breast cancer (6), and squamous cell carcinoma of the head and neck (7), mycosis fungoides (8), and large granular lymphocytic leukemias (9). Consequently, there is considerable interest in developing inhibitors of STAT3-mediated signaling (10–12).

Flavopiridol, a cytotoxic small-molecule antineoplastic agent being evaluated in phase I and II clinical trials (13–15), induces apoptosis *in vitro* in human cancer cell lines (16–25) and human chronic lymphocytic leukemia cells (23) and *in vivo* in human leukemia and lymphoma xenografts grown in nude mice (17). Consistent with these results, tumor regressions have been seen in human squamous cell and prostate carcinoma xenograft models (24, 26) and in human clinical trials (13–15). Although flavopiridol was initially identified as a cytostatic inhibitor of cyclin-dependent kinases (CDK; refs. 27–30), its ability to kill noncycling cells (14, 16, 18, 23) raises the possibility that molecular targets in addition to CDKs may be at least partially responsible for its cytotoxicity.

To date, four targets that might contribute to flavopiridol-induced cytotoxicity have been identified: CDKs (28–30), glycogen phosphorylase (31, 32), P-TEFb (a component of the complex regulating activation of RNA polymerase II via phosphorylation; refs. 33, 34), and DNA (35). Although CDK

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inhibition has been most extensively studied, it remains to be shown whether CDK inhibition alone is sufficient to account for flavopiridol-induced cytotoxicity. Glycogen phosphorylase is unlikely to be of clinical relevance because of the high flavopiridol concentrations necessary to inhibit the enzyme in cancer cells (31, 32). Potentially consistent with its ability to inhibit P-TEFb-regulated activation of RNA polymerase II (33, 34), flavopiridol has been found to down-regulate several cellular proteins (20, 36, 37). On the other hand, treatment of yeast with flavopiridol up-regulates 80% of transcripts that are affected (38), raising the possibility that effects on P-TEFb might not explain all of the transcriptional effects of flavopiridol.

Recent work from our laboratory suggests that DNA might also be an important target of flavopiridol (35). In particular, flavopiridol binds to DNA with an affinity approaching that of the intercalator doxorubicin (35). Analysis using the National Cancer Institute COMPARE program (an analytic tool for comparing profiles of the effects of various anticancer agents on 60 tumor cell lines *in vitro*) also indicates that the cytotoxic effects of flavopiridol strongly correlate with those of several additional DNA-interacting agents (35). Nonetheless, flavopiridol does not induce the types of DNA damage seen with many DNA-interacting antineoplastic agents (35). In particular, flavopiridol does not induce DNA strand breaks, inhibit topoisomerase I or II, or cause DNA-DNA or DNA-protein cross-links (35). These observations suggest that flavopiridol may have a unique mechanism of action but leave unresolved the issue of how drug-DNA interactions might contribute to the cytotoxicity of flavopiridol.

We now report that flavopiridol disrupts STAT3/DNA interactions, attenuates STAT3-directed transcription, and down-regulates the antiapoptotic STAT3 transcriptional target Mcl-1 at the mRNA and protein levels in a variety of neoplastic cell lines. In conjunction with recent results showing that Mcl-1 overexpression induces a high incidence of neoplastic transformation *in vivo* (39) and that Mcl-1 down-regulation is sufficient to induce apoptosis in Mcl-1-expressing cells (40, 41), the present observations identify a potential mechanistic explanation for flavopiridol-induced cytotoxicity. Equally important, these results suggest that flavopiridol may represent the first cytotoxic small molecule to reach human antineoplastic clinical trials that inflicts cytotoxicity via disruption of transcription factor/DNA interactions.

## Materials and Methods

### Reagents

Flavopiridol was kindly provided by the Pharmaceutical Resources Branch of the National Cancer Institute, whereas paclitaxel and AG490 were purchased from Sigma (St. Louis, MO).

### Cell Culture

Cells were cultured in the following media: A549 cells in RPMI 1640 containing 5% (v/v) fetal bovine serum; ML-1, K562, Jurkat, My-5, and HCT116 cells in RPMI 1640 containing 10% fetal bovine serum; OV17R cells in MEM containing 20% fetal bovine serum; PC-3 cells in F-12 medium containing 10% fetal bovine serum; and MDA-MB-468 cells in Iscove's modified essential medium containing 5% fetal bovine serum. All media contained 100 units/mL penicillin G, 100 μg/mL streptomycin, and 2 mmol/L glutamine. Cells were passaged once or twice weekly and maintained at 37°C in an atmosphere containing 95% air-5% $CO_2$ (v/v).

### Immunoblotting

Cells grown to 50% confluence in triplicate 100-mm tissue culture dishes were washed four times *in situ* with 10 mL PBS and solubilized in alkylation buffer [6 mol/L guanidine HCl, 250 mmol/L Tris-HCl (pH 8.5 at 21°C), and 10 mmol/L EDTA supplemented immediately before use with 150 mmol/L β-mercaptoethanol and 1 mmol/L phenylmethylsulfonyl fluoride]. Samples were processed for SDS-PAGE and subsequent immunoblotting using techniques described previously (42). Antibodies were obtained as follows: anti-phospho-STAT1($Tyr^{701}$), anti-phospho-STAT3($Tyr^{705}$), anti-phospho-STAT3($Ser^{727}$), anti-phospho-STAT5($Tyr^{694}$), anti-phospho–extracellular signal-regulated kinase (ERK) 1/2($Thr^{202}/Tyr^{204}$), anti-phospho-ELK-1($Ser^{383}$), and anti-phospho-AKT($Ser^{473}$) from Cell Signaling (Beverly, MA); anti–cyclin D1 and anti-STAT5b from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Bcl-2 and anti-Bcl-$x_L$ from BD Transduction Laboratories (Lexington, KY); anti–cyclin A, anti-phospho–cyclic AMP–responsive element binding protein (CREB) ($Ser^{133}$), and anti-STAT1 from Upstate Biotechnology (Lake Placid, NY); anti-p21 and anti-p53 from Neomarkers (Union City, CA); anti-phospho–RNA polymerase II (antibody H5) and anti–RNA polymerase II (antibody 8WG16) from Convance (Richmond, CA); and anti-Mcl-1 from Dr. John Reed (Burnham Institute, La Jolla, CA).

### Reverse Transcription-PCR

Cellular RNA was isolated using Trizol reagent (Life Technologies, Rockville, MD) according to established procedures (43). Cellular RNA (2 μg), random primers (1 μL), and DEPC water (14 μL) were heated to 70°C for 5 minutes and cooled to 23°C. RNasin (1 μL), 5× reverse transcription buffer (5 μL; Promega, Madison, WI), 10 mmol/L deoxynucleotide triphosphates (1 μL), and 20 units avian myeloblastosis virus reverse transcriptase (1 μL; Promega) were then added. An aliquot of each resulting mixture was incubated for 10 minutes at 23°C followed by 60 minutes at 42°C. Aliquots (2 μL) were then supplemented with 10× PCR buffer (5 μL) containing 1.5 mmol/L $Mg^{2+}$, 10 mmol/L deoxynucleotide triphosphates (2 μL), respective forward and reverse primers (5 μL), distilled $H_2O$ (36 μL), and 5 units Taq polymerase (1 μL). PCR was done for 30 cycles with extension at 72°C (2 minutes), denaturation at 94°C (1 minute), and annealing 55°C (1 minute) using a Perkin-Elmer Cetus thermocycler (Wellesley, MA). After amplification, samples were subjected to electrophoresis on 1% agarose gels containing ethidium bromide, visualized under UV light, and photographed using a ChemiDoc Documentation System (Bio-Rad, Hercules, CA).

The following primers were used: β-actin, 5′-CTTAATGTCACGCACGATTTC-3′ (reverse) and 5′-ACGTTATGGTGATGATATCG-3′ (forward), 654-bp product; Mcl-1, 5′-CCGTCCAGCTCCTCTTCG-3′ (reverse), 5′-CGGACTCAACCTCTACTGTGG-3′ (forward), 354-bp product; topoisomerase I, 5′-TCTTGGGGTGGTTGCCGCG-3′ (reverse), 5′-CCGGATCCATGAGTGGGGACCACCTCCACAA-3′ (forward), 185-bp product; caspase-3, 5′-AAAGAATTCTTAGTGATAAAAATAGAGTTCTTTTGTGAG-3′ (reverse), 5′-AAAGGATCCTTAATAAAGGTATCCATGGAGAACACT-3′ (forward), 975-bp product; cyclin A, 5′-GAAGCAATGCACTGCAGC-3′ (forward) 5′-CAGATACAGGGTCTCTGC-3′ (reverse), 644-bp product; and cyclin D1, 5′-CATGCGCAAGATCGTCGC-3′ (forward) 5′-TCACTCTGGAGAGGAAGC-3′ (reverse), 544-bp product.

### DNA Cellulose Affinity Chromatography

Two hundred fifty to five hundred 100-mm dishes of subconfluent A549 cells were harvested by trypsinization. All subsequent steps were at 4°C. After three washes with ice-cold PBS, cells were suspended in 5 to 10 mL extraction buffer [1.7 mol/L NaCl, 1 mmol/L $Na_2EDTA$, 1 mmol/L β-mercaptoethanol, 20 mmol/L Tris-HCl, 100 mmol/L phenylmethylsulfonyl fluoride (pH 7.4)], disrupted by sonication, and sedimented. After polyethylene glycol (~6,000 molecular weight) was added to a final concentration of 10%, the sample was incubated for 15 minutes, sedimented to remove precipitated DNA, and dialyzed against buffer A [50 mmol/L NaCl, 1 mmol/L $Na_2EDTA$, 20 mmol/L Tris-HCl (pH 7.4)] for three changes of 5 hours each. After centrifugation, the supernatant was brought to a final concentration of 10% glycerol and 0.1% DMSO and applied to a duplex DNA-cellulose column (5 g; Sigma) that had been washed extensively with buffer B [50 mmol/L NaCl, 1 mmol/L $Na_2EDTA$, 1 mmol/L β-mercaptoethanol, 20 mmol/L Tris-HCl, 100 μmol/L phenylmethylsulfonyl fluoride, 0.1% DMSO (pH 7.4)] after initial treatment with buffer B containing 100 μg/mL bovine serum albumin to block nonspecific binding sites. After flow-through and wash fractions were collected (20-mL fractions), the column was eluted with buffer B containing increasing concentrations of flavopiridol followed by buffer B supplemented to 2 mol/L NaCl. The resulting fractions were dialyzed against buffer A, lyophilized, resuspended in SDS sample buffer containing 10% β-mercaptoethanol, subjected to SDS-PAGE (5–15% gradient) as described previously (42), transferred to nitrocellulose, and probed with antibodies as described in subsequent text.

### Nuclear Extraction Assay

Fifty 100-mm tissue culture plates of subconfluent A549 cells were harvested by trypsinization. All subsequent steps were at 4°C. Cells were sedimented, washed thrice with PBS, swelled for 20 minutes with nuclear isolation buffer [10 mmol/L Tris-HCl, 10 mmol/L NaCl, 3 mmol/L $MgSO_4$, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 100 units/mL Trasylol, 0.5 mmol/L EDTA (pH 7.4)], and homogenized in a Dounce homogenizer until cells were disrupted and nuclei were released (usually ~200 strokes). Nuclei were sedimented and extracted with 1-mL aliquots of nuclear extraction buffer [20 mmol/L HEPES, 0.2 mmol/L EDTA, 20% glycerol, 1 mmol/L DTT, 0.4 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL antipain, and leupeptin (pH 7.4)] containing increasing concentrations of flavopiridol followed by nuclear extraction buffer containing 400 mmol/L NaCl. The resulting extracts were dialyzed, lyophilized, resuspended SDS sample buffer, and subjected to SDS-PAGE followed by immunoblotting as described above.

### STAT3 Luciferase Reporter Assay

Human K562 cells were cotransfected by electroporation with 10 μg pLuc-TK/STAT3 (kindly provided by Dr. Jacqueline Bromberg, Memorial Sloan-Kettering Cancer Center) and 2.2 μg pRL-TK (Promega) as a transfection efficiency control. Five hours after electroporation, cells were transferred to serum-free medium, and 250 nmol/L of the indicated drugs or DMSO diluent were added for a total exposure time of 22 hours. Human interleukin-6 (10 ng/mL) was added to the medium during the last 6 hours of drug exposure to induce STAT3 expression to increase the sensitivity of the assay. Cell lysates were collected according to the dual luciferase assay protocol (Promega). Sample light output was analyzed using a luminometer and resulting data were corrected for pRL-TK values and then normalized relative to diluent (DMSO) control. A value of 1 in this assay indicates no change from control, whereas values < 1 indicate attenuation of STAT3-directed transcription as assessed by this assay. The used drug concentrations in the displayed results were selected/optimized based on the ability of this concentration of flavopiridol to attenuate Mcl-1 levels without inducing appreciable apoptosis/cell death at the 22-hour time point. Both higher and lower concentrations of all tested agents were evaluated/explored in this assay, with the results seen at differing drug concentrations consistent with the displayed results.

### Evaluation of Cytotoxic Synergy

Evaluation of cytotoxic synergy was done as described previously (16). Briefly, 750 suspended A549 human non–small cell lung cancer cells obtained from trypsinization from stock flasks of subconfluent cell cultures were deposited into each of triplicate sets of 35-mm tissue culture plates and allowed to adhere overnight. Cells were then treated for 24 hours with diluent, flavopiridol, paclitaxel, or AG490 at the concentrations indicated in the text alone and in combination. After drug removal and washing, cells were allowed to proliferate in drug-free medium for 7 to 10 days and thereafter washed twice with serum-free PBS, stained with Coomassie blue, and then manually counted. Synergy was determined by median effect analysis using the CalcuSyn software program (Biosoft, Cambridge, United Kingdom).

## Results

### Flavopiridol Disrupts Transcription Factor/DNA Binding

Previous data have implicated DNA as a potential flavopiridol target but have also indicated that flavopiridol

does not induce DNA cross-links/strand breaks or inhibit topoisomerase I or II (see ref. 35; see Introduction). In view of these results, we assessed the possibility that flavopiridol might instead disrupt protein/DNA interactions. Initial experiments used DNA cellulose affinity chromatography (ref. 44; Fig. 1A). In these experiments, DNA/cellulose columns were preloaded with cellular lysate proteins and washed sequentially with flavopiridol-free mobile phase, increasing concentrations of flavopiridol, and high-salt buffer. As little as 500 nmol/L flavopiridol (a clinically achievable flavopiridol concentration; refs. 13, 14, 45) eluted STAT3 from the column. In contrast, several other polypeptides, including the transcription p53 as well as histone H1 and topoisomerase I, were not eluted by flavopiridol (Fig. 1A), thus indicating that STAT3 was eluted in this assay by flavopiridol with some degree of selectivity in comparison with several other DNA-binding proteins.

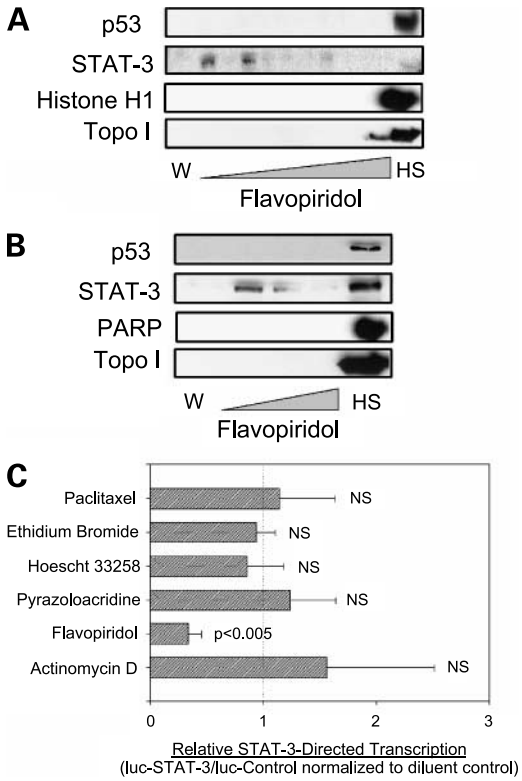


**Figure 1.** Flavopiridol disrupts STAT3/DNA interactions and attenuates STAT3-directed transcription. **A,** flavopiridol disrupts STAT3/DNA binding as assessed by DNA/cellulose affinity chromatography. A whole-cell lysate of A549 cells was loaded on a (duplex) DNA/cellulose column and eluted with progressively higher concentrations of flavopiridol (0.5 – 10 μmol/L). Eluted fractions were subjected to SDS-PAGE and probed as indicated by immunoblotting. *W,* flavopiridol-free column wash; *HS,* high-salt (2 mol/L NaCl) wash; *Topo I,* topoisomerase I. **B,** flavopiridol displaces STAT3 from isolated A549 cell nuclei. Nuclei were sequentially extracted with flavopiridol-free low-salt wash followed by increasing flavopiridol concentrations (0.5 – 10 μmol/L) and finally by high-salt (2 mol/L NaCl) buffer as described in the text. *PARP,* poly(ADP-ribose) polymerase. **A** and **B,** gel lanes were loaded based on equivalent volumes of eluted fractions. **C,** flavopiridol attenuates STAT3-directed transcription in intact A549 cells as assessed using a STAT3 luciferase reporter assay, whereas a variety of other DNA-interacting antineoplastic agents and the microtubule-interacting agent paclitaxel do not. Results were corrected using a cotransfected control *Renilla* reporter plasmid and normalized to diluent-treated controls. A value of 1 indicates no effects on reporter activity, whereas values <1 indicate specific attenuation of STAT3-mediated transcription. *Columns,* mean of four independent experiments; *bars,* SD. Statistical significance indicates the probability that the attained results are significantly different from 1 (the null hypothesis, two-sided confidence intervals). *NS,* not significant.

To examine whether flavopiridol might disrupt transcription factor/DNA binding under somewhat more physiologic conditions, a nuclear extraction assay (46) was employed (Fig. 1B). Nuclei isolated from A549 cells were extracted with low-salt extraction buffer containing progressively increasing concentrations of flavopiridol. Immunoblotting showed that flavopiridol displaced STAT3 from isolated cell nuclei, confirming that flavopiridol is capable of disrupting transcription factor/DNA interactions in a second *in vitro* system. Under identical conditions, other nuclear-resident proteins, including p53, topoisomerase I, and poly(ADP-ribose) polymerase, remained in the nuclei until eluted with high-salt buffer, again indicating some degree of selectivity in the elution of nuclear proteins by flavopiridol.

### Flavopiridol Disrupts STAT3-Directed Transcription

In view of the above data suggesting that flavopiridol might disrupt STAT/DNA interactions under cell-free conditions and in isolated cell nuclei, we next evaluated the possibility that flavopiridol might disrupt STAT3-directed transcription in intact cells using a STAT3 luciferase reporter (Fig. 1C). In these assays, a dual luciferase assay system was used to correct results for differences in transfection efficiency and nonspecific/global transcriptional effects. Whereas the microtubule-interacting agent paclitaxel and the DNA-interacting agents ethidium bromide, Hoescht 33258, pyrazoloacridine, and actinomycin D produced no selective effects on STAT3-directed transcription in this assay, flavopiridol significantly attenuated STAT3-directed transcription (Fig. 1C). It should be noted, however, that although actinomycin D was not seen to selectively attenuate STAT3-directed transcription in this assay, it decreased STAT3 and control reporter activities proportionally, presumably indicating nonspecific attenuation of transcription.

### Flavopiridol Down-Regulates Mcl-1 Message Levels

We next examined whether flavopiridol decreases messages that are transcriptionally regulated by STAT3. Because flavopiridol has been reported previously to inhibit transcription based on P-TEFb inhibition (33, 34), the effects of flavopiridol (250 nmol/L) were directly compared with the classic P-TEFb inhibitor 5,6-dichloro-1-β-D-ribobenzimidazole (DRB; 94 μmol/L). Messages that are regulated by STAT3 (Mcl-1, cyclin D1, and cyclin A) and messages that are not (topoisomerase I, caspase-3, and β-actin) were examined using reverse transcription-PCR.

As indicated in Fig. 2, results of these analyses showed that flavopiridol decreased levels of messages encoding Mcl-1, cyclin A, and cyclin D1. Whereas cyclin A and

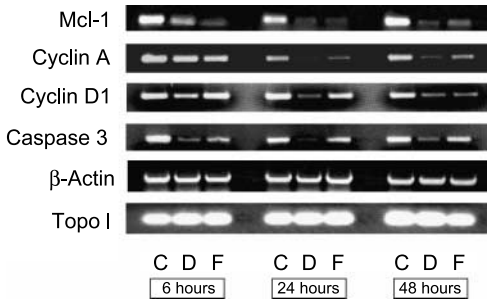


**Figure 2.** Flavopiridol rapidly attenuates levels of Mcl-1 message in A549 cells and has effects on message levels that differ from the classic P-TEFb inhibitor DRB. Although both flavopiridol and DRB down-regulate Mcl-1 message levels, DRB more dramatically attenuates cyclin A and caspase-3 message levels. *C,* control/diluent-treated cells; *D,* cells treated with 94 μmol/L DRB; *F,* cells treated with 250 nmol/L flavopiridol. Message levels were all assessed using reverse transcription-PCR. Representative of four independent experiments.

D1 mRNA levels declined only at later time points (most notably at 24 and 48 hours; Fig. 2), Mcl-1 mRNA levels declined dramatically after treatment with as little as 125 nmol/L flavopiridol for 6 hours, possibly reflecting the short half-life of Mcl-1 message (47).

Examination of additional messages indicated that the effects of flavopiridol and DRB were distinct at assessed concentrations. In particular, although both flavopiridol and DRB decreased Mcl-1 mRNA as early as 6 hours, only DRB consistently attenuated levels of caspase-3 message at that and at later time points (Fig. 2, representative of four independent experiments), clearly indicating differential effects of the two agents on transcripts. Likewise, DRB more dramatically down-regulated cyclin A and D1 messages in comparison with flavopiridol (Fig. 2). These observations, although not definitive, suggest that the effects of flavopiridol on transcription might be distinct from and more focal than DRB, consistent with the hypothesis that flavopiridol attenuates STAT3-directed transcription with some selectivity under these assay conditions.

### Flavopiridol Down-Regulates Proteins Downstream of STAT3

In view of data showing that flavopiridol can disrupt STAT3/DNA interactions (Fig. 1A and B), attenuate STAT3-directed transcription (Fig. 1C), and decrease STAT3-regulated messages (Fig. 2), we next examined whether flavopiridol might also attenuate levels of the corresponding cellular proteins. Of seven polypeptides known to be regulated by STAT3 (Mcl-1, cyclin A, cyclin D1, Bcl-$x_L$, Bcl-2, c-Myc, and p21; refs. 3, 4, 12), three (Mcl-1, cyclin D1, and Bcl-2) have been reported previously to be down-regulated by flavopiridol in selected cell types (20, 36, 37). Experiments in A549 cells showed that Mcl-1 declined more rapidly and dramatically than the other polypeptides in response to flavopiridol (Fig. 3), consistent with the down-regulation of Mcl-1 message observed in Fig. 2. Examination of eight additional cell lines (Fig. 4A), each of which is sensitive to flavopiridol-induced cytotoxicity (ref. 16; data not shown), revealed that Mcl-1 protein levels decreased in a dose-dependent manner in all of these lines, regardless of tumor type, after only 6 hours of treatment with as little as 125 nmol/L flavopiridol, a clinically achievable concentration (13, 14, 45).

In addition to having the ability to attenuate levels of Mcl-1 in cancer cells cultured under ambient growth

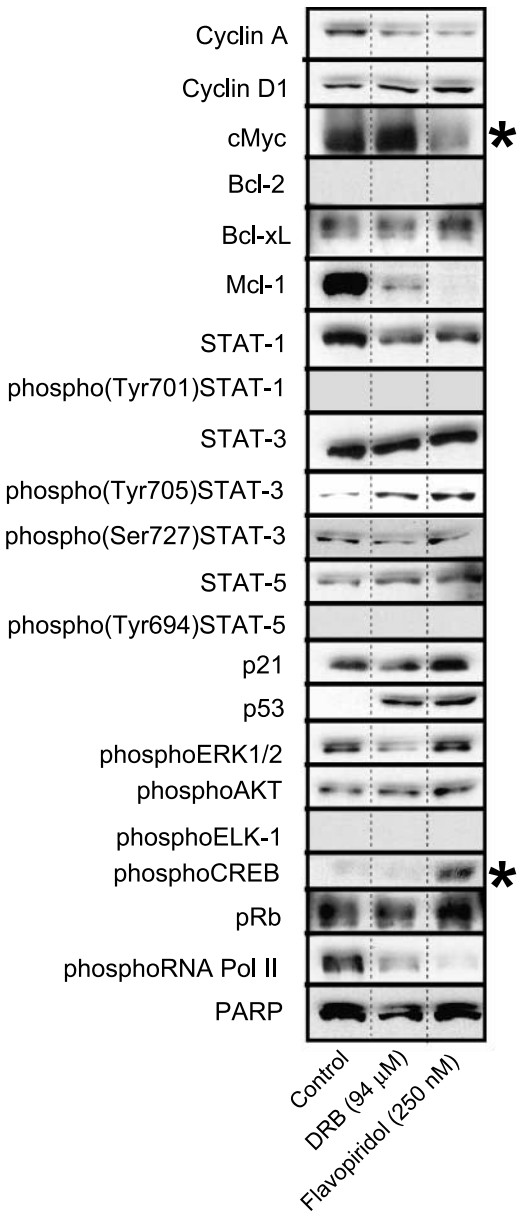


**Figure 3.** Effects of flavopiridol on levels of cellular proteins differ from those of the classic P-TEFb inhibitor DRB. Flavopiridol and DRB both down-regulate levels of Mcl-1 and phospho – RNA polymerase II, but only flavopiridol down-regulates c-Myc levels and up-regulates phospho-CREB (*asterisks*). Whole-cell extracts were subjected to SDS-PAGE (50 μg protein/lane) followed by immunoblotting as described in the text. The top pRb band corresponds to hyperphosphorylated Rb. Positive control lanes for those polypeptides found to be undetectable in assayed A549 cells showed used immunoblotting antibodies to be functional.

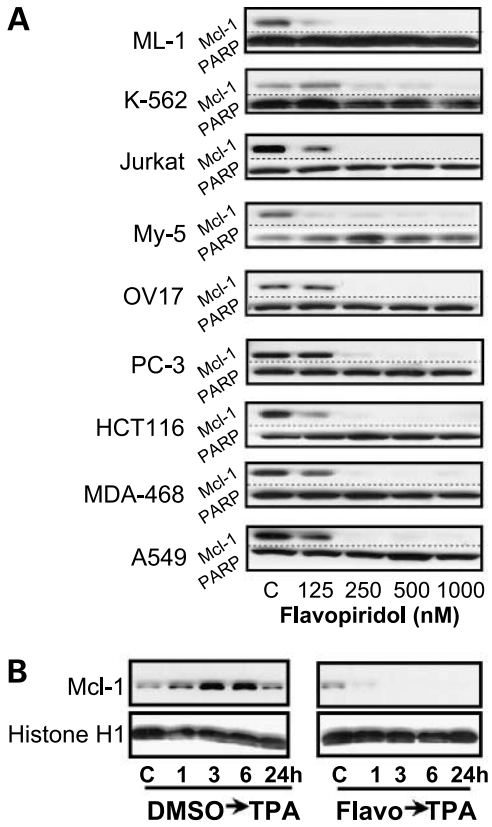


**Figure 4.** Flavopiridol down-regulates Mcl-1 in flavopiridol-sensitive human cancer cell lines and abrogates 12-*O*-tetradecanoylphorbol-13-acetate–induced increases in Mcl-1. **A**, treatment with as little as 125 nmol/L flavopiridol for 6 h attenuated Mcl-1 levels in nine different flavopiridol-sensitive human cancer cell lines. **B**, 12-*O*-tetradecanoylphorbol-13-acetate treatment of A549 cells results in increased Mcl-1 levels (*left*), whereas pretreatment with 250 or 500 nmol/L flavopiridol (data from 500 nmol/L treatment are shown) abrogates 12-*O*-tetradecanoylphorbol-13-acetate (*TPA*)–induced potentiation of Mcl-1. **A** and **B**, gel lanes were loaded based on equivalent amounts of protein (50 μg), and levels of polypeptides of interest were assessed by SDS-PAGE and immunoblotting. Representative of four separate experiments.

conditions, flavopiridol can also abrogate induced increases of Mcl-1. For example, the increases in levels of Mcl-1 induced by 12-*O*-tetradecanoylphorbol-13-acetate stimulation of A549 cells (Fig. 4B, *left*) were blocked by pretreatment of cells with flavopiridol (Fig. 4B, *right*). These data indicate that flavopiridol is capable of attenuating Mcl-1 levels not only under basal growth conditions but also under conditions of cellular stress.

Although cellular level of c-Myc and cyclin A also declined in some lines (e.g., Fig. 3), these decreases were variable and less dramatic. Levels of cyclin D1 and Bcl-$x_L$ also declined in response to flavopiridol treatment, but these decreases were not observed until 24 to 48 hours after the initiation of flavopiridol treatment (data not shown). Hence, we confirmed that the levels of five of seven polypeptides known to be transcriptionally regulated by STAT3 were attenuated in response to flavopiridol exposure.

Because flavopiridol is known under some circumstances to inhibit the activating phosphorylation of RNA polymerase II via inhibition of P-TEFb, potentially globally down-regulating transcription (33, 34), we compared the effects of flavopiridol with those produced by the classic P-TEFb inhibitor DRB in A549 cells to determine whether the effects of the two agents might be similar or, instead, distinct. Whereas flavopiridol treatment attenuated levels of c-Myc and increased levels of phospho-CREB, DRB did not (Fig. 3, *asterisks*). Hence, flavopiridol and the P-TEFb inhibitor DRB vary not only with respect to their effects on message levels but also with respect to their effects on various polypeptides. Collectively, these data suggest that the two agents may differ with regard to the mechanisms by which they exert their cellular effects, supporting results shown in Fig. 1, suggesting that flavopiridol may at least in some circumstances affect transcription with some selectivity.

### Flavopiridol-Induced Down-Regulation of Mcl-1 Does Not Result from Effects on Phospho-STAT3, Phospho-CREB, Phospho-ELK-1, Phospho-ERK1/2, Phospho-AKT, pRb, or E2F-1

Previous studies have implicated three transcription factors in the regulation of Mcl-1 levels: phospho-STAT3 (48), phospho-CREB (49), and phospho-ELK-1 (47). To determine whether flavopiridol-induced Mcl-1 down-regulation might be attributable to alterations in one of these activated transcription factors rather than disruption of STAT3/DNA interactions, we examined the effects of flavopiridol on the cellular levels of phospho-STAT3($Tyr^{705}$), phospho-STAT3($Ser^{727}$), phospho-CREB($Ser^{133}$), and phospho-ELK-1($Ser^{383}$) (Figs. 3 and 5A). Flavopiridol caused a (dose-dependent) increase, rather than a decrease, in phosphorylation of STAT3 on $Tyr^{705}$ and $Ser^{727}$ under the same conditions that down-regulated Mcl-1 (Figs. 3 and 5A). Although A549 cells had no detectable levels of phospho-ELK-1 either before or after treatment with flavopiridol, phospho-CREB was also up-regulated in response to flavopiridol treatment (Figs. 3 and 5A). Although the increased phosphorylation of STAT3 and CREB is unexplained at present (but might reflect feedback up-regulation as a consequence of altered downstream mRNA or polypeptide levels), these results clearly rule out decreased levels of the three known transcriptional regulators of Mcl-1 as a cause for flavopiridol-induced Mcl-1 down-regulation.

To determine whether flavopiridol-induced STAT3 phosphorylation occurs in other cell lines, we examined phospho-STAT3 levels in ML-1, K562, Jurkat, My-5, OV17R, PC-3, HCT116, and MDA-MB-468 cells treated with 250 nmol/L flavopiridol for 6 hours. Phospho-STAT3 levels were increased in five of nine assayed lines (A549, OV17R, PC-3, HCT116, and MDA-468; data not shown) and unaffected in the remainder, confirming that decreased levels of phosphorylated (active) STAT3 are not responsible for flavopiridol-induced Mcl-1 down-regulation in any of the cell lines. Similar experiments also failed to implicate diminished phosphorylation of CREB or ELK-1 in flavopiridol-induced Mcl-1 down-regulation in any of the cell lines (data not shown).

Several reports have also implicated signaling through the ERK and AKT pathways in the regulation of Mcl-1 (50–54). Immunoblotting with reagents that recognize phospho-ERK1/2(Thr$^{202}$/Tyr$^{204}$) and phospho-AKT(Ser$^{473}$) suggested that flavopiridol had no effect on activation phosphorylation of AKT and that at higher concentrations flavopiridol actually increases levels of ERK1/2 phosphorylation (Figs. 3 and 5A). Hence, decreased signaling through AKT and/or ERK1/2 does not seem to be responsible for the observed flavopiridol-induced down-regulation of Mcl-1.

Finally, based on a recent report that the transcription factor E2F-1 directly represses the Mcl-1 promoter (55), we examined the possibility that flavopiridol might alter levels of hyperphosphorylated Rb (Figs. 3 and 5A) and/or nuclear levels of E2F-1 (Fig. 5B). Whereas Rb was unaffected, nuclear E2F-1 levels declined slightly (rather than increasing), hence also eliminating this pathway as a means of flavopiridol-induced down-regulation of Mcl-1. Furthermore, because Rb phosphorylation is regulated by CDK4 and CDK6, there is no indication that the ability of flavopiridol to inhibit these CDKs is playing a role in Mcl-1 down-regulation through the Rb/E2F-1 pathway.

In summary, extensive evaluation failed to provide evidence that flavopiridol altered known transcriptional regulators of Mcl-1 in a manner that could account for flavopiridol-induced down-regulation of Mcl-1 message and protein. Available evidence, therefore, suggests that flavopiridol-induced down-regulation of Mcl-1 occurs at, or downstream of, the level of phospho-STAT3-induced transcription.

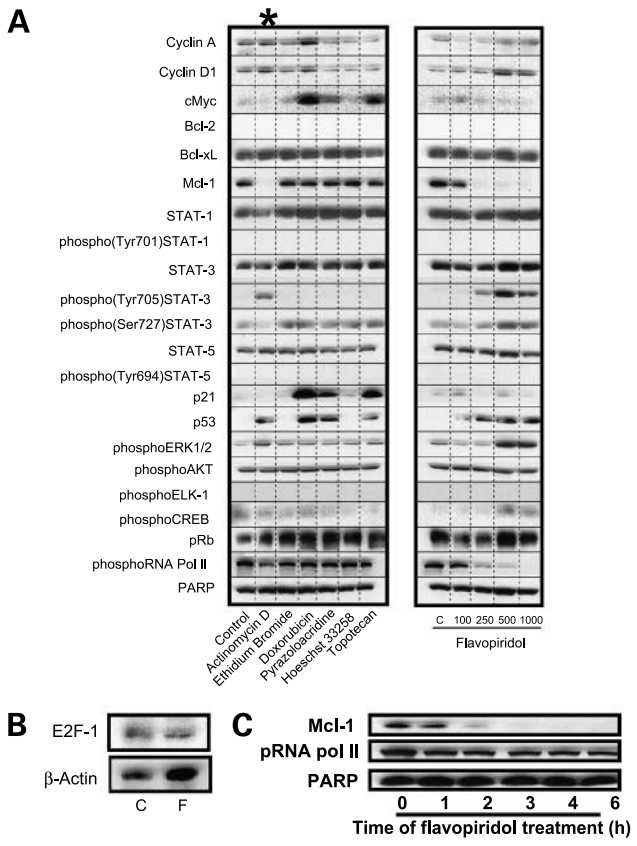


**Figure 5.** Effects of flavopiridol on levels of cellular polypeptides were similar to those of actinomycin D but distinct from those of other DNA-interacting agents. **A,** both flavopiridol and actinomycin D down-regulated Mcl-1 and up-regulated phospho-STAT3 and p53 levels, whereas other DNA-interacting agents did not. **B,** 250 nmol/L flavopiridol treatment of A549 cells for 6 h did not up-regulate levels of nuclear E2F-1. **C,** in at least some cancer cell lines (OV17R shown here), flavopiridol-induced attenuation of Mcl-1 occurs in the absence of effects on polysomal RNA polymerase II levels. Concentrations of DNA-interacting agents and flavopiridol in (**A**, *left,* and **C**) were all 500 nmol/L. Poly(ADP-ribose) polymerase and actin are shown as loading controls, and lanes were loaded by cellular protein level (50 μg). Polypeptide levels were assessed using SDS-PAGE and immunoblotting. Nuclei for **B** were isolated as described in the text.

### Cellular Effects of Flavopiridol Are Distinct from Other DNA-Interacting Antineoplastic Agents

To determine whether other DNA-interacting agents might similarly down-regulate Mcl-1, we first compared the effects of flavopiridol, ethidium bromide, Hoechst 33258, pyrazoloacridine, topotecan, actinomycin D, and doxorubicin on Mcl-1 and phospho-STAT3 levels in A549 cells (Fig. 5A). Of all tested DNA-interacting agents, only flavopiridol and actinomycin D (indicated with asterisk) attenuated Mcl-1 levels (Fig. 5A).

### Flavopiridol-Induced Mcl-1 Down-Regulation Is Independent of Altered RNA Polymerase II Phosphorylation in Some Cancer Cell Lines

Because flavopiridol has been reported previously to diminish the activating phosphorylation of RNA polymerase II by inhibiting P-TEFb (33, 34), we also examined the effects of flavopiridol on phospho–RNA polymerase II levels in a variety of cancer cell lines. In some cell lines (e.g., A549 cells), we observed that flavopiridol down-regulated RNA polymerase II phosphorylation in parallel with Mcl-1 down-regulation (Figs. 3 and 5A). In other cell lines (e.g., OV17R), however, we found that flavopiridol-induced Mcl-1 down-regulation occurred under conditions that did not produce RNA polymerase II dephosphorylation (Fig. 5C). Therefore, in at least in some cell lines, Mcl-1 down-regulation does not seem to be attributable to flavopiridol-induced decreases in RNA polymerase II phosphorylation, again consistent with the hypothesis that under at least some circumstances flavopiridol seems to affect transcription focally rather than globally.

### Flavopiridol Can Be Combined with the Jak Inhibitor AG490 to Achieve Cytotoxic Synergy in A549 Cells

Having assembled much data in support of the hypothesis that flavopiridol binds to DNA (35), disrupts STAT3/DNA binding (Fig. 1A and B), attenuates STAT3-directed transcription (Fig. 1C), and reduces levels of downstream survival-critical polypeptides, including Mcl-1 (Figs. 2–5), and further that this phenomenon might under some circumstances be responsible for flavopiridol-induced cell death, we hypothesized that flavopiridol might be combined with other inhibitors of the Jak/STAT3 signaling cascade to achieve cytotoxic synergy. As depicted in Fig. 6,

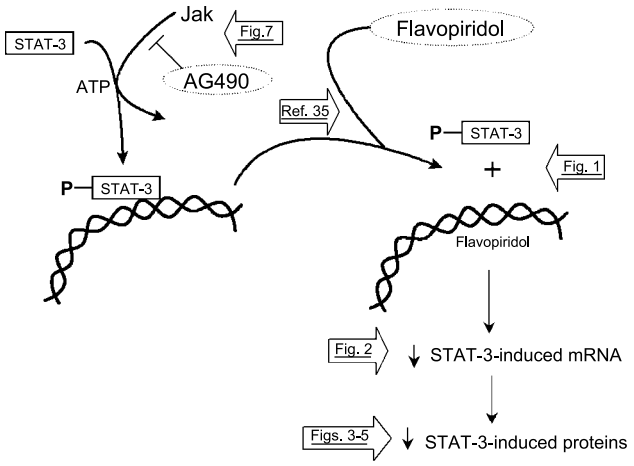


**Figure 6.** A model depicting the putative effects of flavopiridol and AG490 on STAT3-mediated transcription.

we reasoned that inhibition of a single survival-critical signaling pathway at two distinct points might yield synergistic, rather than just additive, effects. To test this hypothesis, we examined cytotoxic synergy resulting between flavopiridol and the Jak inhibitor AG490 as well as between paclitaxel and AG490 (as a ''negative'' control, given that paclitaxel does not effect the Jak/STAT3 signaling pathway) using median effect analyses (Fig. 7). In these analyses, combination indices < 1 are indicative of synergy. Whereas the combination of flavopiridol and AG490 produced cytotoxic synergy, consistently yielding combination indices in the 0.2 to 0.5 range (Fig. 7A), the combination of paclitaxel and AG490 did not produce similar evidence of synergy, consistently yielding combination indices clustered around 1, indicating additivity (Fig. 7B). Furthermore, immunoblotting experiments indicated that the attenuation of phospho-STAT3 levels by AG490 closely paralleled its cytotoxicity and was achieved in the AG490 concentration ranges used in these experiments (data not shown). These data are not only consistent with the possibility that AG490 and flavopiridol may synergistically affect the same survival-critical signaling pathway (putatively Jak/STAT3) but may additionally have further implications for the future development of clinical trials combining flavopiridol with other inhibitors of the Jak/STAT3 signaling pathway.

## Discussion

Results presented above support the following model of flavopiridol action (Fig. 6): (*a*) flavopiridol enters cancer cells and binds to dsDNA (35); (*b*) flavopiridol binding to DNA disrupts interactions of STAT3 with its consensus DNA-binding sequence (Fig. 1A and B); (*c*) consequent disruption of STAT3/DNA binding attenuates transcription of STAT3-regulated messages (Fig. 1C), including Mcl-1; and (*d*) resulting decreases in mRNA levels (Fig. 2) lead to down-regulation of several downstream polypeptides, including Mcl-1 (Figs. 3–5). This model can potentially explain a variety of previously seemingly disparate observations related to the action flavopiridol, including the new observation that flavopiridol can be combined with the Jak inhibitor AG490 to produce cytotoxic synergy (Fig. 7).

Previous studies have shown that flavopiridol can down-regulate cyclin D1 (36), Bcl-2 (20), and Mcl-1 (37) but leave levels of many other cellular polypeptides largely unaffected. Others have proposed that these alterations reflect the consequences of global attenuation of transcription as a consequence of flavopiridol-induced inhibition of P-TEFb, the activating kinase of RNA polymerase II (33, 34). We were intrigued, however, by the fact that cyclin D1, Bcl-2, and Mcl-1 are transcriptionally regulated by STAT3 (3, 12), leading to the present studies. In view of results presented in Figs. 1 to 5, the down-regulation of these polypeptides may reflect, at least in part, impaired STAT3-regulated transcription, providing an alternative potential mechanistic explanation for the observed transcription effects of flavopiridol. As indicated in Figs. 2 to 5, however, down-regulation of Mcl-1 message and protein levels seems to be earlier and more dramatic than down-regulation of cyclin D1. This might reflect both a shorter half-life of Mcl-1 message (47, 56) and possibly a greater reliance of Mcl-1 transcription on constitutive STAT3 activation rather than other pathways in the cell lines examined. In support of this latter possibility, we observed that constitutive ERK phosphorylation was much lower in the A549 cell line than in several other cell lines.[3] Constitutive phosphorylation of the ERK substrate ELK-1 was also undetectable in these cells (Figs. 3 and 5A).

In the analysis of presented experimental results, it should be noted that the flavopiridol concentration required to disrupt STAT3/DNA binding in the DNA cellulose affinity binding assay and the nuclear extraction assay shown in Fig. 1A and B were somewhat higher (500 nmol/L) than those required to produced effects in intact cells (125–250 nmol/L). Given that the assays presented in Fig. 1A and B did not use intact cells, however, these experiments make the assumption that intracellular, and further intranuclear, flavopiridol concentrations may be roughly equivalent to applied extracellular flavopiridol concentrations, and this may or may not be the case in a particular model system. Prior analyses quantitating intracellular flavopiridol concentrations suggest that flavopiridol is not concentrated in cells (42). Because flavopiridol levels have apparently not yet been quantitated in cell nuclei, it is presently impossible to know what intranuclear flavopiridol levels might be attained in response to applied extracellular flavopiridol concentrations. It is nonetheless generally encouraging, however, that the effects seen in Fig. 1A and B were observed in the same general concentration ranges that produce STAT3 and Mcl-1 effects as well as cytotoxicity in intact cells.

[3] T.J. Kottke and S.H. Kaufmann, unpublished observations.

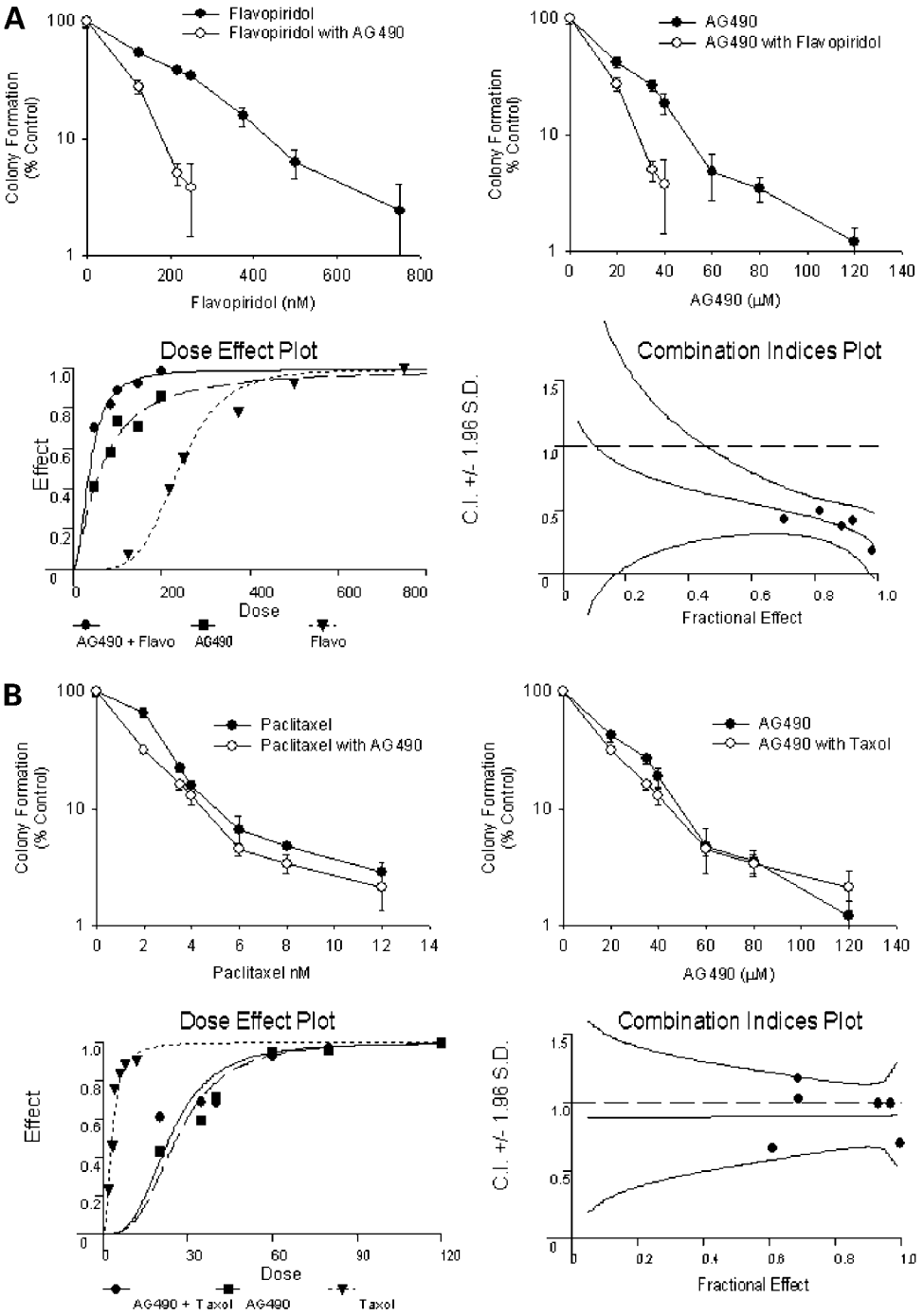


**Figure 7.** Flavopiridol and the Jak inhibitor AG490 can be combined to produce cytotoxic synergy, whereas the microtubule-interacting agent paclitaxel (Taxol) and AG490 combine to yield only additive effects. Representative median effect, dose effect, and combination indices plots for flavopiridol combined with AG490 (**A**) and paclitaxel combined with AG490 (**B**). Colony-forming assays using A549 cells supplemented using median effect analyses served as the basis for the displayed analyses. In combination index plots, combination indices $<1$ indicate synergy.

To our knowledge, this is the first report of a small molecule that can attenuate STAT3-mediated transcription by disrupting the binding of STAT3 to its consensus DNA-binding sequence. STAT3 is a downstream mediator of a variety of prosurvival cytokine signaling pathways (48, 50), and up-regulation of STAT3 signaling either by constitutive activation or by up-regulated cytokine signaling has been shown in a wide variety of cancers (3–7, 12, 57, 58). Additionally, up-regulated STAT3 signaling is believed to contribute to the neoplastic phenotype of many cancers by stimulating proliferation and attenuating apoptosis via its effects on downstream polypeptides, including Mcl-1, c-Myc, cyclins, and Bcl-$x_L$ (3–7, 12, 57, 58). Previous studies have shown that inhibition of constitutively activated STAT3 signaling using antisense oligonucleotides or the Jak inhibitor AG490 can inhibit tumor growth both *in vitro* and *in vivo*, providing direct support for the contention that inhibition of STAT3-mediated signaling represents a

potentially important approach for antineoplastic drug development (3–7, 12, 57, 58). The ability of a small molecule to prevent STAT3 binding to its consensus sequence provides a new means for examining the importance of this pathway in the clinical setting. This is illustrated by the observation that flavopiridol can be combined with an inhibitor of STAT3 signaling at another point in the pathway (AG490) to achieve cytotoxic synergy.

As is the case with STAT3 (see Introduction), mounting evidence indicates that Mcl-1 has an important role in cancer (59). First, Mcl-1 is often up-regulated in cancer cells (59). Second, Mcl-1 overexpression results in a high incidence of murine tumors (39). Third, down-regulation of Mcl-1 can trigger apoptosis in the absence of other proapoptotic stimuli (40). These observations suggest that the flavopiridol-induced cytotoxicity documented in previous studies (16–25) may be attributable, at least in part, to the down-regulation of antiapoptotic polypeptides, such as Mcl-1. Consistent with this possibility, we observed marked inhibition of Mcl-1 expression in a variety of flavopiridol-sensitive cancer cell lines *in vitro* (Fig. 4A). These results do not, of course, rule out the possibility that inhibition of STAT3/DNA binding (Fig. 1) might result in down-regulation of additional survival-critical antiapoptotic polypeptides as well.

Two reports implicate P-TEFb as a potentially important target of flavopiridol (33, 34) and further suggest that this mechanism may account for the transcriptional effects of the drug. The present studies confirm that phosphorylation of RNA polymerase II is decreased in response to flavopiridol treatment (Figs. 3 and 5A). Addition experiments, however, suggest that inhibition of P-TEFb seems not to be the sole mechanism by which flavopiridol down-regulates target proteins. First, the effects of flavopiridol and the classic P-TEFb inhibitor DRB are distinct, with flavopiridol having much more limited effects at the mRNA and protein levels (Figs. 2 and 3). Second, flavopiridol down-regulates Mcl-1 under conditions that do not affect RNA polymerase II phosphorylation in some cancer cell lines (Fig. 5C). Third, flavopiridol was found to attenuate STAT3-directed transcription in a luciferase reporter assay relative to a control reporter plasmid (Fig. 1C), indicating at least some degree of selectivity of transcriptional effects of the agent. Thus, at least a portion of the effects of flavopiridol are apparently independent of P-TEFb inhibition. Nonetheless, the relative contributions of P-TEFb inhibition and disruption of transcription factor/DNA binding to flavopiridol-induced down-regulation of transcription in various circumstances remain to be more fully elucidated. Additionally, the relevance of the ability of flavopiridol to disrupt STAT3-directed transcription *in vitro* to observed effects in human clinical trials remains to be clarified through targeted translational studies.

In summary, the present studies indicate that flavopiridol can disrupt STAT3/DNA interactions, attenuate STAT3-directed transcription, and down-regulate levels of the STAT3 transcriptional target Mcl-1 at the transcriptional level. These observations provide a potential explanation for at least some of the transcriptional effects of flavopiridol independent of its effects on P-TEFb. Additionally, the observation that flavopiridol can be combined with another STAT3 pathway inhibitor (AG490) to achieve cytotoxic synergy has potential implication for future combination clinical trials involving flavopiridol. Perhaps most importantly, these collective observations not only provide a potential explanation for many of the previously unexplained effects of flavopiridol but also raise the possibility that flavopiridol might represent the first of a new class of target-directed small-molecule antineoplastic agents capable of disrupting survival-critical transcription factor/DNA interactions with some degree of selectivity.

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# Flavopiridol disrupts STAT3/DNA interactions, attenuates STAT3-directed transcription, and combines with the Jak kinase inhibitor AG490 to achieve cytotoxic synergy

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