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Flavopiridol Down-Regulates Antiapoptotic Proteins and Sensitizes Human Breast Cancer Cells to Epothilone B-induced Apoptosis

Sylvie Wittmann, Purva Bali, Sreenivasa Donapaty, Ramadevi Nimmanapalli, Fei Guo, Hirohito Yamaguchi, Mei Huang, Richard Jove, Hong Gang Wang, and Kapil Bhalla[1]

Department of Interdisciplinary Oncology, Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, Florida 33612

ABSTRACT

The molecular mechanisms underlying the cell cycle growth-inhibitory and apoptotic effects of flavopiridol (FP) were determined in human breast cancer cells. Treatment with FP caused accumulation in the G_1 phase of the cell cycle and induced apoptosis of SKBR-3 and MB-468 cells. This was associated with down-regulation of the levels of cyclins D1 and B1, as well as with inhibition of cyclin-dependent kinase (cdk) 1, cdk2, and cdk4. FP-induced apoptosis was accompanied by a conformational change and mitochondrial localization of Bax. This resulted in the accumulations of cytochrome *c*, Smac, and Omi/HtrA2 in the cytosol and induced the poly(ADP-ribose) polymerase cleavage activity of caspase-3. Treatment with FP also attenuated the mRNA and protein levels of XIAP, cIAP-2, Mcl-1, Bcl-x_L, and survivin. In MB-468 cells with overexpression of Bcl-2 (468/Bcl-2), FP-induced Bax conformational change and apoptosis were inhibited, whereas the FP-mediated decline in the levels of IAP proteins, Mcl-11 and Bcl-x_L remained unaltered. The effects of cotreatment with FP and the nontaxane tubulin-polymerizing agent epothilone (Epo) B were also determined in MB-468 cells. Sequential treatment with Epo B followed by FP induced significantly more apoptosis of MB-468 cells than treatment with the reverse sequence of FP followed by Epo B or treatment with either agent alone ($P < 0.05$). Treatment with Epo B followed by FP induced more Bax conformational change and was associated with a greater decline in the levels of XIAP, cIAP-2, Mcl-1, and Bcl-x_L. However, MB-468/Bcl-2 cells remained relatively resistant to Epo B followed by FP. Taken together, these findings suggest that the superior sequence-dependent anti-breast cancer activity of Epo B followed by FP may be due to FP-induced Bax conformational change and down-regulation of the antiapoptotic IAP, Bcl-x_L, and Mcl-1 proteins, but this treatment may not overcome the resistance to apoptosis of breast cancer cells conferred by overexpression of Bcl-2.

INTRODUCTION

FP[2] is a semisynthetic flavonoid, which is a potent small molecule inhibitor of multiple cdks, including cdk1 (cdc2), cdk2, cdk4, cdk6, and cdk7 (1). It inhibits cdks by binding to their ATP-binding pocket (2). FP also causes transcriptional repression of cyclin D1 (3). By inhibiting cdk7/cyclin H, it inhibits the phosphorylation of cdks at threonine 160/161 (1, 4). Collectively, these activities result in the arrest of the cell cycle in the G_1-S or G_2-M boundary and mediate the antiproliferative effect of FP in tumor cells (5). FP has also been shown to induce apoptosis of noncycling transformed cells in a p53-independent manner, regardless of the intracellular Bcl-2/Bax levels (6, 7). FP treatment reduced the levels of the antiapoptotic Bcl-2 and Mcl-1 proteins (8, 9). Recently, in human breast cancer cells, FP was shown to down-regulate c-erbB-2 and cyclin D1 levels as well as induce growth arrest and apoptosis (3, 10).

XIAP, cIAP-1 and -2, and survivin are prominent members of the IAP protein family that exert antiapoptotic effects by interfering with the processing and activities of the executioner caspase-3 and -7 (11–13). Whereas overexpression inhibits tumor cell apoptosis induced by anticancer agents, attenuation of the levels and activities of the IAP proteins sensitizes tumor cells to apoptosis induced by anticancer agents (14–16). For example, treatment with the antisense oligonucleotides targeting survivin, a single BIR domain-containing member of the IAP protein family, induced apoptosis and sensitized cancer cells to chemotherapy (17). Recently, FP was demonstrated to be a global inhibitor of mRNA transcription in diffuse large B-cell lymphoma OCI-LY3 cells (18). FP inhibited the mRNAs of c-myc, cyclin D2, Mcl-1, and the IAP family of proteins (11). FP was also shown to inhibit the activity of the transcription elongation factor p-TEFb, a complex of cyclins with cdk9, which phosphorylates the COOH terminus of the RNA polymerase II complex and promotes transcription elongation (19, 20). Although the precise mechanism underlying FP-mediated transcriptional repression remains to be elucidated, inhibition of transcriptional elongation and/or binding to duplex DNA by FP may explain this effect (21).

Despite the recent progress in our understanding of its genetic and molecular basis, advanced and metastatic breast cancer remains incurable (22). Encouragingly, the microtubule-targeted tubulin-polymerizing agents, such as Taxol or Taxotere, have been demonstrated to exert a high level of clinical activity against metastatic breast cancer (23, 24). After intracellular uptake, tubulin-polymerizing agents bind to β-tubulin and promote tubulin polymerization and microtubule bundling (25). They also induce the activity of cdk1 and cause mitotic arrest followed by apoptosis of cancer cells (26, 27). Recently, the non-taxane Epo B was shown to be a promising anticancer agent, with demonstrable activity against cisplatin- or paclitaxel-resistant cancer cells (28). Treatment with Epo B was shown to trigger Bax conformational change, its relocation from the cytosol to mitochondria, cyt *c* release, caspase-3 activation, and apoptosis of human breast cancer cells (29). Overexpression of Bcl-2 or Bcl-x_L delayed these events and inhibited apoptosis induced by Epo B (26, 27). In several preclinical studies, sequence-dependent synergistic cytotoxic effects were observed when FP was administered after a variety of chemotherapeutic agents. These included paclitaxel, etoposide, cisplatin, gemcitabine, and cytarabine (30–32). However, thus far, neither the effects of FP on the antiapoptotic proteins (*i.e.*, IAP and Bcl-2 family members) nor its sensitizing effect on Epo B-induced apoptosis has been comprehensively studied in breast cancer cells. In the present studies, we demonstrate for the first time that clinically achievable levels of FP inhibit the activities of cdk1 and cdk2 and induce the Bax conformational change and cytosolic accumulation of cyt c, Omi/HtrA2 and Smac/DIABLO (33), resulting in growth arrest and apoptosis of breast cancer cells. Treatment with FP also caused transcriptional repression and a decline in the protein levels of XIAP, cIAP-2, Mcl-1, and Bcl-x_L. In addition, a sequence-dependent enhancement of Epo B-induced apoptosis of the breast cancer cells was observed when cells were treated with Epo B followed by FP.

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[1] To whom requests for reprints should be addressed, at Department of Interdisciplinary Oncology, Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, MRC 3 East, Room 3056, Tampa, FL 33612. Phone: (813) 903-6861; Fax: (813) 903-6817; E-mail: bhallakn@moffitt.usf.edu.

[2] The abbreviations used are: FP, flavopiridol; cdk, cyclin-dependent kinase; PARP, poly(ADP-ribose) polymerase; Epo, epothilone; IAP, inhibitors of apoptosis; cyt, cytochrome; ALLnL, *N*-acetyl leucyl-leucyl norlucinal; XIAP, x-linked IAP.

MATERIALS AND METHODS

Reagents. FP and Taxotere were a gift from Aventis Pharmaceuticals (Bridgewater, NJ). Epo B was a gift from Novartis Corp. (Basel, Switzerland). A stock solution of FP was made at concentration of 2 mM in DMSO, whereas Taxotere and Epo B were made at a concentration of 10 mM in DMSO and stored at −20°C. The proteosome inhibitor ALLnL was purchased from Sigma Chemical Co. (St. Louis, MO). Dr. Xiaodong Wang (University of Texas, Southwestern School of Medicine, Dallas, TX) kindly provided the anti-Smac/DIABLO (33). Polyclonal anti-cyclin D1 antibody was a gift from Dr. Warren Pledger (Moffitt Cancer Center, Tampa, FL; Ref. 34). Survivin polyclonal antibody was purchased from Alpha Diagnostic International (San Antonio, TX). Polyclonal anti-PARP was purchased from Cell Signaling (Beverly, MA). Anti-Bax, anti-cIAP-2, and anti-Mcl-1 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-XIAP and β-actin antibodies were purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada) and Sigma Chemical Co., respectively. Monoclonal anti-Bcl-x_L, anti-cyt *c*, and anti-CDK1 antibodies were purchased from BD PharMingen (San Diego, CA). Monoclonal anti-Bcl-2 and anti-cyclin B1 antibodies were purchased from Dako (Carpinteria, CA).

Cell Culture. The human breast cancer cell lines MB-468 and SKBR-3 were obtained from American Type Culture Collection (Manassas, VA) and maintained in the recommended culture medium. Briefly, MB-468 and SKBR-3 cells were maintained in Leibovitz's L-15 and McCoy's media, respectively (CellGro, Herndon, VA). MB-468 overexpressing Bcl-2 and the control cells were maintained in DMEM (29). Culture mediums were supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc., Grand Island, NY) at 37°C in a humidified 5% CO_2 incubator. The MB-468 cell line was maintained in a CO_2-free incubator.

Flow Cytometric Analysis of Cell Cycle Status and Apoptosis. The flow cytometric evaluation of cell cycle status and apoptosis was performed according to a previously described method (35). The percentage of cells in G_1, S, and G_2-M phases was calculated using Multicycle software (Phoenix Flow Systems, San Diego, CA).

Apoptosis Assessment by Annexin V Staining. After drug treatments, cells were resuspended in 100 μl of staining solution [containing annexin V fluorescein and propidium iodide in a HEPES buffer (annexin V-FITC; BD PharMingen). After incubation at room temperature for 15 min, cells were analyzed by flow cytometry (36). Annexin V binds to those cells that express phosphotidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of those cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells (stained only with annexin V) and necrotic cells [stained with both annexin V and propidium iodide (36)].

Preparation of S-100 and Western Analysis of Cytosolic Cyt *c*, Smac, and Omi. Untreated and drug-treated cells were harvested by centrifugation at 1,000 × *g* for 10 min at 4°C. The cell pellets were washed once with ice-cold PBS and resuspended with 5 volumes of buffer [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride] containing 250 mM sucrose. The cells were homogenized with a 22-gauge needle, and the homogenates were centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatants were further centrifuged at 100,000 × *g* for 30 min. The resulting supernatants (S-100) were collected, and the protein concentrations were determined by using the BCA protein assay reagent from Pierce Biotechnology Inc. (Rockford, IL). A total of 75 μg of the S-100 fraction was used for Western blot analysis of cyt *c*, Smac, and Omi/HtrA2 (35, 37, 38).

Western Analyses. Western analyses of XIAP, cIAP-2, Mcl-1, Bcl-x_L, Bcl-2, survivin, Bax, cdk1, cdk2, cdk4, cyclin B1, cyclin D1, and β-actin were performed using specific antisera or monoclonal antibodies (see above), as described previously (35). Horizontal scanning densitometry was performed on Western blots by using acquisition into Adobe Photoshop (Adobe Systems, Inc., San Jose, CA). The expression of β-actin was used as a control.

Immunoprecipitation of Conformationally Changed Bax. Cells are lysed in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid lysis buffer [150 mM NaCl, 10 mM HEPES (pH 7.4), and 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid] containing protease inhibitors. Immunoprecipitation is performed in lysis buffer by using 500 μg of total cell lysate and 2.5 μg of anti-Bax 6A7 monoclonal antibody (Sigma Chemical Co.). The resulting immune complexes and the supernatants were subjected to immunoblotting analysis with anti-Bax polyclonal rabbit antiserum, as described previously (29).

Cdk Kinase Assay. Cdk1, cdk2, and cdk4 kinase activities in the untreated and drug-treated cells were determined by using an immunoprecipitation kinase assay with reagents provided in a commercially available kit (Upstate Biotechnology, Lake Placid, NY) as described previously (26). For immunoprecipitation reaction, 3 μg of primary monoclonal anti-cdk1, anti-cdk2, or anti-cdk4 antibody were incubated with 200 μg of cell lysates on ice for 45 min. Precipitation of the antibody-antigen complex was accomplished by rotation overnight in the presence of 20 μl of protein G-agarose beads at 4°C (Roche, Indianapolis, IN). Immunoprecipitates were then incubated with substrate histone H1 protein in the presence of [γ-^{32}P]ATP and inhibitory mixture in kinase buffer, allowing immunoprecipitated active cdk1, cdk2, or cdk4 to phosphorylate histone H1. The radiolabeled substrate was read in a scintillation counter to estimate the relative cdk1 or cdk2 activity, as described by the manufacturer. An aliquot of the reaction was separated by SDS-PAGE and analyzed by autoradiography.

RNase Protection Assay. A RiboQuant Multi-Probe RNase Protection Assay System was used according to the manufacturer's instructions (BD PharMingen) and as described previously (39). Two probe sets, hAPO-2 (Bcl2 family: Bcl-x_L/$_S$, Bak, Bax, Bcl-2, and Mcl-1) and hAPO-5C (IAP family: survivin, XIAP, cIAP-1, and cIAP-2), were used for T7 RNA polymerase-directed synthesis of [α-^{32}P]UTP-labeled antisense RNA probes. Both probe sets contain the DNA templates L32 and glyceraldehyde-3-phosphate dehydrogenase used as internal controls. The probes (1×10^6 cpm/reaction) were hybridized with 20 μg of RNA isolated from breast cancer cells after treatment with FP at different time points using the RNeasy Mini kit (Qiagen, Valencia, CA). After overnight hybridization, samples were digested with RNase to remove single-stranded (nonhybridized) RNA. The remaining probes were resolved on a 5% denaturing polyacrylamide gel and analyzed by autoradiography.

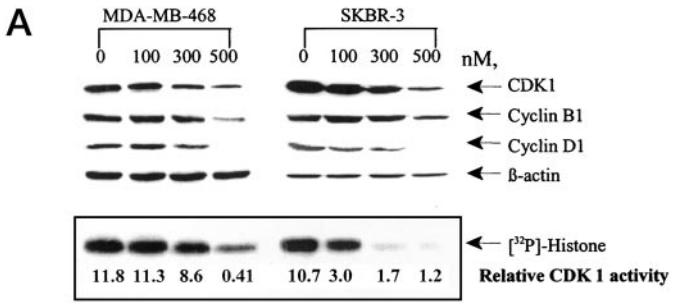
Preparation of Detergent-soluble and -insoluble Fractions. After the designated drug treatments, cells were lysed with TNSEV buffer [50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 100 mM NaCl, 1 mM sodium orthovanadate, and 1% NP40 containing 20 μg/ml aprotonin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 25 mM NAF, and 5 mM *N*-ethylmaleimide] (40). The insoluble fraction (pellet) was solubilized with SDS buffer [80 mM Tris (pH 6.8), 2% SDS, 100 mM DTT, and 10% glycerol]. Fifty μg of proteins from the NP40-soluble and -insoluble fractions were separated on a 7.5% SDS-polyacrylamide gel and analyzed by Western blotting.

Statistical Analysis. Significant differences between values obtained in a population of cells treated with different experimental conditions were determined using Student's *t* test.

RESULTS

FP Inhibits Cyclin D1 and B1 Levels and Activities of Cdks, Resulting in Cell Cycle G_1 Accumulation. To extend the reported findings from other cell types to human breast cancer cells (1–4), we first determined the cell cycle effects of clinically achievable concentrations of FP on breast cancer MB-468 and SKBR-3 cells. Fig. 1*A* shows that treatment with FP for 24 h induced a dose-dependent decline in the levels of cdk1 and cyclins D1 and B1 as well as inhibition of cdk1 activity. FP also inhibited the activity of cdk2 and cdk4 to a lesser extent in these cell types (data not shown). These inhibitory effects of FP on the cell cycle-regulatory proteins were associated with the accumulation of cells in the G_1 phase of the cell cycle, with a corresponding decline in the percentage of cells in the S and G_2-M phases (Fig. 1*B*).

FP Induces Bax Conformational Change, Cytosolic Accumulation of Death Promoters, and Apoptosis. We next determined whether the perturbations in cell cycle status of the breast cancer cells induced by FP were accompanied by the molecular events associated with apoptosis. As shown in Fig. 2*A*, treatment of MB-468 cells with FP induced the conformational change in the cytosolic Bax, detected



B

Cell-Cycle effects of Flavopiridol (FP) in MDA-MB-468 and SKBR-3 cells

	% Cells		
	G0-G1	S	G2/M
MDA-MB-468			
control	42.09	30.95	26.96
100 nM FP	35.56	30.70	33.74
500 nM FP	58.61	17.23	24.16
SKBR-3			
control	44.13	26.91	28.97
100 nM FP	57.55	17.18	25.27
500 nM FP	57.42	18.02	24.57

Fig. 1. Treatment with FP inhibits the levels and activities of cell cycle-regulatory proteins and causes breast cancer cells to accumulate in the G_1 phase of the cell cycle. Cells were harvested after treatment with the indicated concentrations of FP for 24 h. After this, cell lysates were obtained to determine the levels of cyclin B1, cyclin D1, and cdk1 as well as the histone H1 kinase activity of cdk1 (*framed bands*; *numbers* represent the relative CDK1 activity for each condition; *A*), or the percentage of cells in the various phases of the cell cycle was determined by flow cytometry (data represent the mean of three experiments; *B*).

by immunoblot analysis with the anti-Bax 6A7 antibody, without affecting the total intracellular levels of Bax. The conformational change in Bax is known to trigger the mitochondrial relocation of Bax and the release of death promoters from the mitochondria (41). Fig. 2, *B* and *C*, demonstrates that, consistent with this, FP treatment induced the cytosolic accumulations of cyt *c* and IAP antagonists Smac and Omi/HtrA2, associated with the PARP cleavage activity of caspase-3 (37, 38). This resulted in a significant dose-dependent increase in the percentage of apoptotic cells that were positive for the expression of annexin V (Fig. 2*D*). Although not shown, these findings were also observed in SKBR-3 cells, where FP was also noted to down-regulate c-ErbB-2 protein levels.

FP Treatment Down-Regulates mRNA and Protein Levels of IAP and Bcl-2 Family in Breast Cancer Cells. In a previous study of genomic-scale measurement of mRNA turnover in human diffuse large B-cell lymphoma cells, FP was shown to inhibit gene expression broadly but particularly inhibited the mRNA expression of the genes encoding key cell cycle and apoptosis regulators (18). Therefore, we determined the effect of FP on the mRNA and protein levels of IAP and Bcl-2 families in breast cancer cells. Fig. 3, *A* and *B*, demonstrates that exposure of MB-468 cells to 500 nM FP for 8 h down-regulated the mRNA expression of XIAP, cIAP-2, Mcl-1, Bcl-x_L, Bcl-2 and survivin. A similar effect of FP was also observed in SKBR-3 cells (data not shown). Consistent with inhibition of transcription, treatment with 500 nM FP for 24 h also reduced the protein expressions of XIAP and cIAP-2, as well as that of Mcl-1 and Bcl-x_L, in the two cell types (Fig. 4). FP also lowered Bcl-2 levels in MB-468 cells, whereas Bcl-2 levels were undetectable in SKBR-3 cells. As noted above, treatment with FP reduced Her-2 expression in SKBR-3 cells (data not shown). Exposure to 500 nM FP for 24 h consistently lowered survivin expression in SKBR-3 cells (Fig. 4), but this effect was observed at higher levels of FP ($\geq$1.0 μM) in MB-468 cells (data not shown). We also determined the contribution of FP-induced proteasomal degradation to the decline in the intracellular levels of survivin *versus* Mcl-1 and cIAP-2 proteins. The effects of treatment with FP and/or the proteasomal inhibitor ALLnL on the detergent NP40-soluble- or -insoluble protein levels were determined (42). Fig. 5, *A* and *B*, demonstrates that, as compared with Mcl-1 and cIAP-2, FP treatment caused a more profound decline in the levels of survivin in the detergent-insoluble fraction, which contains misfolded proteins that are targeted for proteasomal degradation, than in the detergent-soluble fraction (40). Cotreatment with FP and ALLnL markedly reversed the decline in the survivin levels (more so in the detergent-insoluble *versus* -soluble fraction) as compared with the levels of cIAP-2 or Mcl-1. These findings suggest that treatment with FP promotes proteasomal degradation of proteins to a variable extent, *e.g.*, survivin was affected more than cIAP-2 or Mcl-1. Apart from transcriptional repression, this contributes to the FP-mediated overall decline in the levels of these proteins.

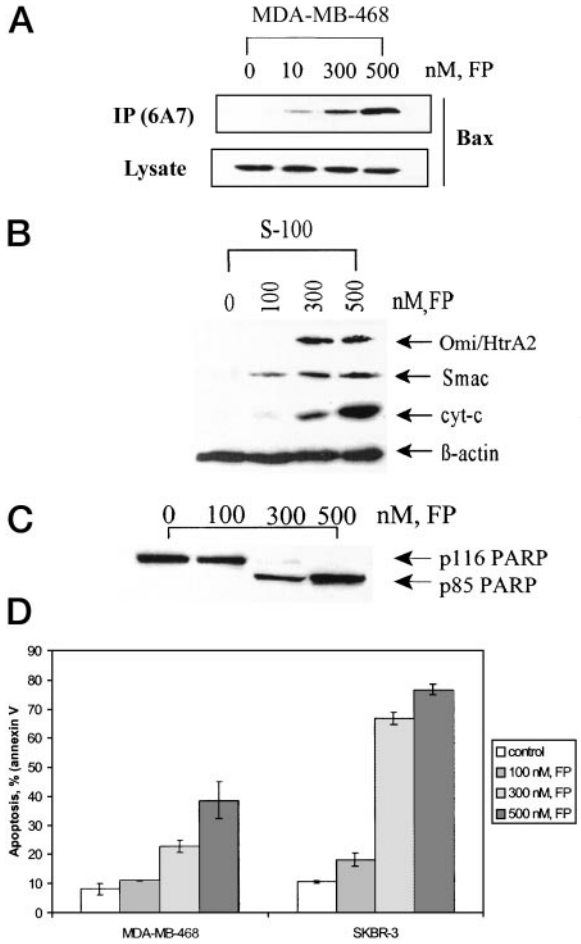


Fig. 2. FP induces Bax conformational change; cytosolic accumulation of cyt *c*, Smac, and Omi/HtrA2; PARP cleavage; and apoptosis of breast cancer cells. Cells were treated with the indicated concentrations of FP for 24 h. After this, to determine the levels of conformationally changed Bax protein, cell lysates were first immunoprecipitated with anti-Bax 6A7 monoclonal antibody and then immunoblotted with a polyclonal anti-Bax antibody (*A*). Cytosolic fractions (S-100) of untreated and FP-treated cells were used for immunoblot analyses of cytosolic cyt *c*, Smac, and Omi/HtrA2 (*B*). To determine the PARP cleavage activity of caspase-3, total cell lysates of untreated and FP-treated cells were also immunoblotted with the anti-PARP antibody (*C*). The percentage of apoptotic cells was determined by annexin V-FITC staining followed by flow cytometry, and *bar graphs* represent the means ± SE of three experiments (*D*).

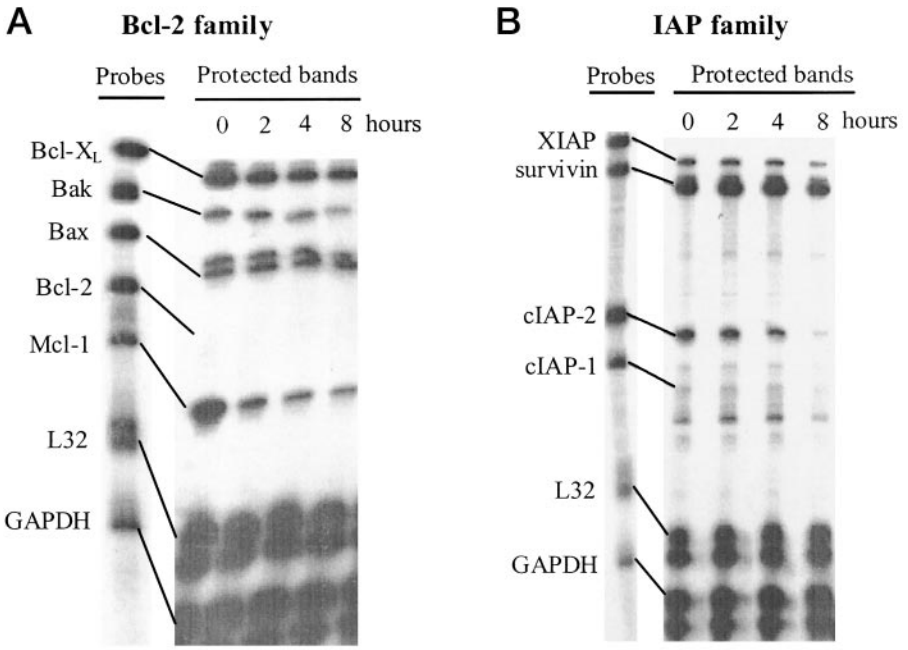


Fig. 3. FP reduces the mRNA levels of Bcl-2 and IAP protein family members. After treatment of MB-468 cells with 500 nM FP for the indicated time intervals, RNase protection assay was performed with 20 μg of total RNA using radiolabeled probes of Bcl-2 (*A*) or IAP (*B*) family obtained from RiboQuant multiprobe template sets.

Overexpression of Bcl-2 Inhibits FP-induced Apoptosis while Permitting FP-mediated Decline in the Levels of Other Antiapoptotic Proteins. Previous reports have suggested that in the B cell chronic lymphocytic leukemia (B-CLL) and myeloma cells, FP-induced apoptosis is associated with down-regulation of Bcl-2 levels. In other cell types, however, it has been shown to be independent of the intracellular levels of Bcl-2 (6–9, 43, 44). Fig. 6*A* demonstrates that treatment with 500 nM FP for 24 h did not affect the expression of Bcl-2 but considerably lowered the intracellular levels of XIAP, Mcl-1, and Bcl-x_L in 468/Bcl-2 cells. In contrast, as shown, in MB-468 cells, FP lowered the levels of all of these proteins, including those of Bcl-2. The ectopic overexpression of Bcl-2 also inhibited the FP-induced conformational change in Bax and apoptosis of 468/Bcl-2 *versus* MB-468 cells (Fig. 6, *B* and *C*).

Sequential Treatment with Epo B Followed by FP Enhances Apoptosis of MB-468 but not 468/Bcl-2 Cells. Next, we determined the effect of a combination of FP with Epo B against MB-468 cells. As shown in Fig. 7*A*, sequential treatment of MB-468 cells with Epo B for 18 h followed by FP for 24 h induced significantly more apoptosis than the reverse sequence or treatment with either agent alone. As compared with the reverse sequence, treatment with Epo B followed by FP maximized G_2-M accumulation and Bax conformational change. This was also associated with marked decline in the intracellular levels of XIAP and Mcl-1 (Fig. 7, *B–D*), as well as a decline in cIAP2 and Bcl-x_L (data not shown). These alterations collectively attenuated the restraint on apoptosis exerted by these proteins. By lowering G_2-M accumulation due to Epo B, pretreatment with FP inhibited the cytotoxic effects exerted by Epo B. A similar sequence-dependent superior apoptotic effect was also exerted by treatment with Taxotere followed by FP (data not shown). Sequential treatment with Epo B followed by FP was also tested against 468/Bcl-2 cells. G_2-M accumulation and apoptosis induced by Epo B followed by FP were significantly inhibited in 468/Bcl-2 *versus* MB-468 cells (Fig. 7, *A* and *B*; $P < 0.01$). Additionally, treatment with Epo B followed by FP did not induce Bax conformational change but down-regulated XIAP and Mcl-1 levels in 468/Bcl-2 cells (Fig. 7, *C* and *D*). Therefore, treatment with Epo B followed by FP also did not overcome the resistance to apoptosis of 468/Bcl-2 cells.

DISCUSSION

Previous reports have highlighted the inhibitory effects of FP on the cdks and cell cycle progression through the G_1-S phase (1, 5). Using human lymphoma cells, a more recent study has demonstrated that FP is a global transcriptional repressor with more pronounced effects on the levels of mRNAs that have a relatively short half-life (1, 5, 18). In this report, we have focused on the effects of FP on human breast cancer cells, and we demonstrate that treatment with clinically achievable levels of FP lowers the levels of cyclin D1 and B1 and activities of cdks, resulting in the accumulation of cells in the G_1 phase. These studies also show for the first time that FP induces the conformational change and mitochondrial localization of Bax, which triggers the release of cyt *c*, Smac, and Omi/HtrA2 into the cytosol. In conjunction with the lowering of the apoptotic threshold due to down-regulation of the mRNA and protein expressions of Bcl-2 and IAP families of proteins, this promotes the activation of caspase-3 and apoptosis of breast cancer cells.

In the present studies, we found that FP treatment reduced the mRNA levels of Bcl-2 and the IAP family of proteins. FP is known to reduce the activity of the transcription elongation factor p-TEFb,

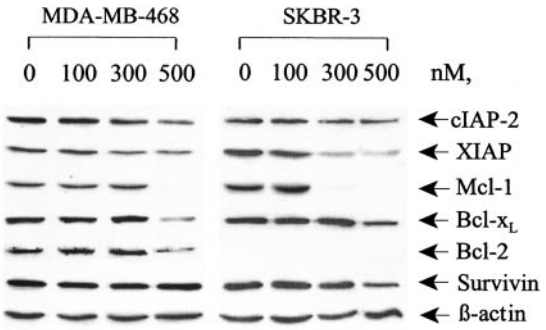


Fig. 4. FP treatment down-regulates the levels of Bcl-2 and IAP family of proteins. MB-468 and SKBR-3 cells were treated with the indicated concentrations of FP for 24 h. After this, the cells were harvested, and immunoblot analyses of cIAP-2, XIAP, Mcl-1, Bcl-x_L, Bcl-2, and survivin were performed using specific antibodies.

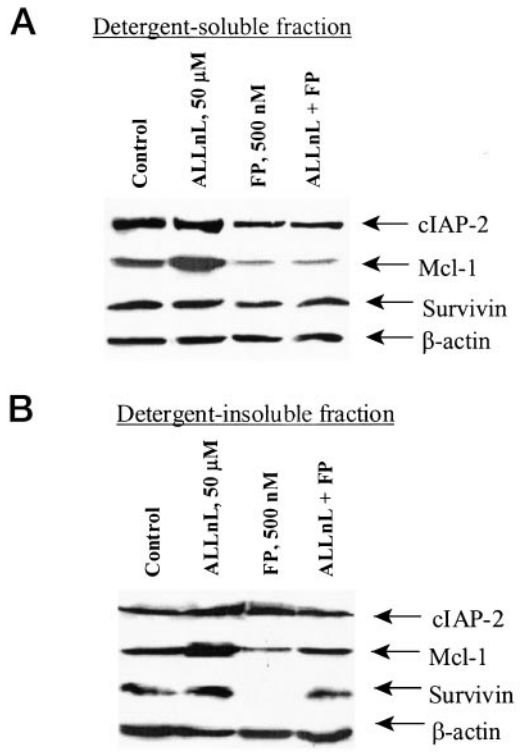


Fig. 5. A differential effect of proteasome inhibition by ALLnL on FP-mediated down-regulation of cIAP-2, Mcl-1, and survivin levels in the detergent-insoluble *versus* -soluble cell fractions. SKBR-3 cells were treated with 500 nM FP alone for 24 h, 50 μM ALLnL alone for 25 h, or pretreated with 50 μM ALLnL for 1 h followed by cotreatment with FP and ALLnL for an additional 24 h. After these treatments, cells were collected and lysed in NP40-containing buffer and centrifuged to obtain the supernatant containing the detergent-soluble fraction. The pellet containing the NP40-insoluble fraction was solubilized in SDS-containing buffer. Using specific antibodies, Western blot analyses of cIAP-2, Mcl-1, and survivin were performed on both the detergent-soluble (*A*) and the detergent-insoluble cellular fractions (*B*). The expression of β-actin served as the loading control.

which phosphorylates the COOH terminus of the RNA polymerase II complex that promotes transcription elongation, and thereby inhibits transcription elongation (19, 20). In addition, FP has also been shown to bind duplex DNA, which may interfere in the binding and transactivation due to the transcription factors nuclear factor-κB and signal transducers and activators of transcription 3 (21). FP has a well-documented global inhibitory effect on transcription. However, based on the turnover and half-life of the various mRNA species, the decline in the levels of specific mRNA is variable (18). This may explain the disparate effect of FP observed on the mRNA levels of XIAP, cIAP-2, Mcl-1, Bcl-x_L, and survivin. Treatment with FP was also noted to have a variable inhibitory effect on the protein levels of Bcl-2 and IAP families. This may be due in part to disparate translational control and proteasomal degradation of these proteins. For example, the FP-mediated decline in survivin levels may be due to increased proteasomal degradation of the unphosphorylated and inactive survivin. FP may inhibit survivin phosphorylation by inhibiting the activity of cdk1. The latter is known to phosphorylate survivin and preserve its activity necessary for maintaining the integrity of mitosis (13, 45). It is possible that the unphosphorylated and inactive survivin, which is misfolded and present in the detergent-insoluble cellular fraction (Fig. 5), may be particularly susceptible to proteasomal degradation. This may contribute to the FP-mediated decline in survivin levels. This is supported by the observation that cotreatment with the proteasomal inhibitor ALLnL and FP significantly restored the levels of survivin in the detergent-insoluble fraction of MB-468 cells.

In the present studies, the ectopic overexpression of Bcl-2 did not inhibit the FP-mediated decline in the levels of XIAP and Mcl-1, but it inhibited FP-induced conformational change in Bax and apoptosis of MB-468 cells. This may be because treatment with FP had no effect on Bcl-2 levels in MB-468/Bcl-2 but markedly lowered Bcl-2 levels in MB-468 cells. This suggests that high Bcl-2 levels exert an overriding antiapoptotic effect in 468/Bcl-2, which remains in effect despite a considerable decline in the levels of the other antiapoptotic proteins. Unlike previously reported findings in other cell types, these observations also suggest that FP treatment may not be able to overcome the resistance to apoptosis of breast cancer cells exerted by high intracellular levels of Bcl-2.

The separate inhibitory effects of FP on G_1-S transition and on the levels of the antiapoptotic Bcl-2 and IAP proteins may also explain the sequence-dependent increase in the cytotoxic effects observed after treatment with Epo B followed by FP, but not by the reverse sequence of treatment with these agents. When treatment with FP precedes Epo B, FP interferes with Epo B-induced G_2-M transition and mitotic arrest that has to precede and is necessary for the engagement of the mitochondrial pathway of apoptosis (26, 29). Thus, sequential treatment with FP followed by Epo B results in the attenuation of the cytotoxic effects of drug combination. Conversely, when treatment with FP follows Epo B, the latter is able to trigger mitotic arrest followed by Bax conformational change and apoptosis. In this sequence, FP also lowers the protein levels of the Bcl-2 and IAP family, thereby attenuating their antiapoptotic control. A similar sequence-dependent effect of treatment with Taxol followed by FP was observed by others (30, 32), and a similar sequence-dependent

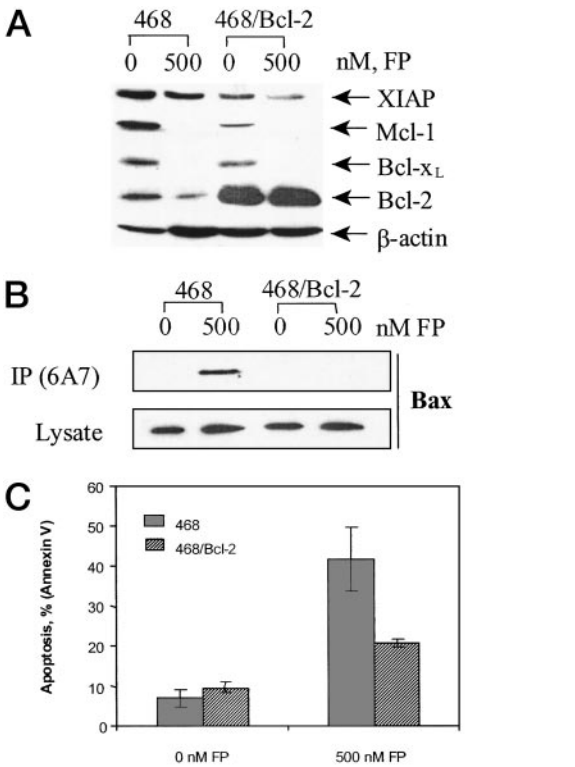
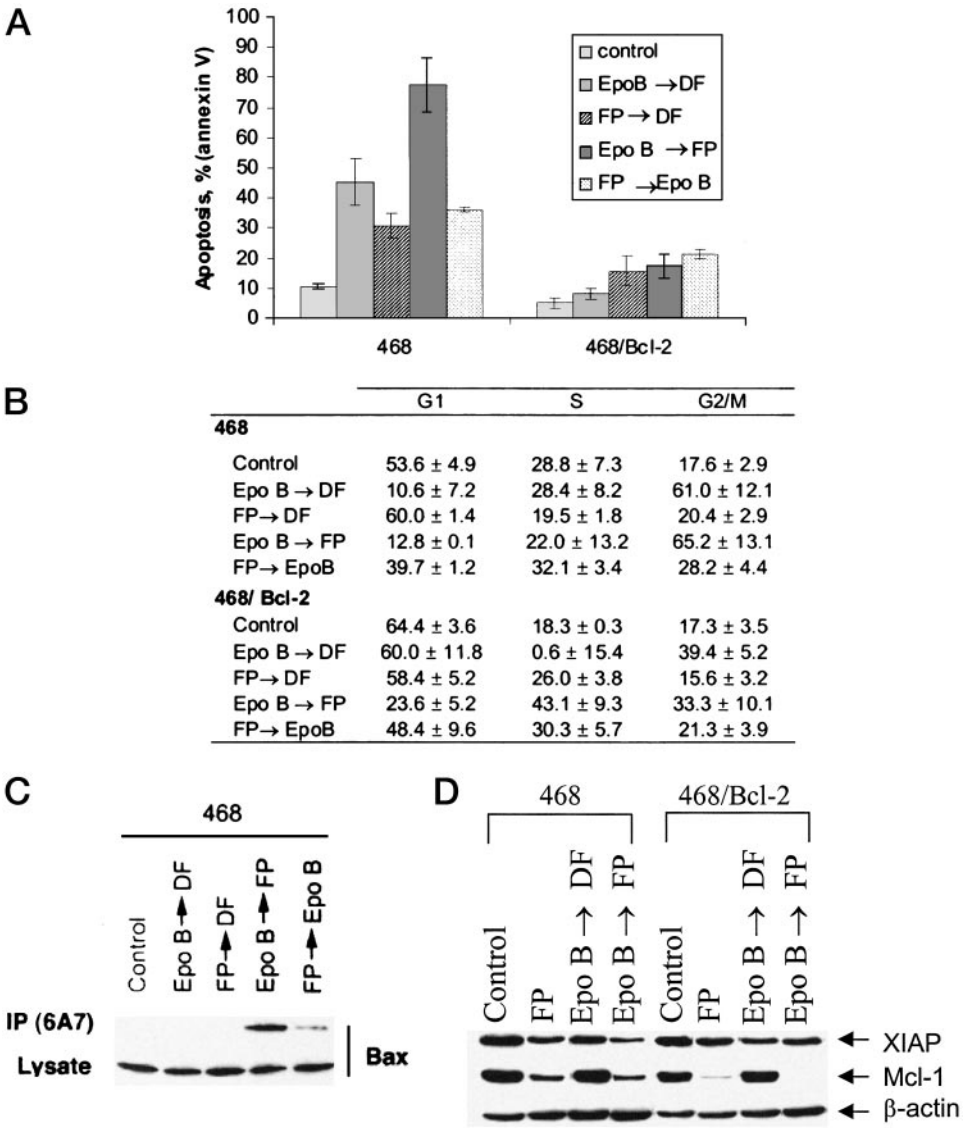


Fig. 6. Ectopic overexpression of Bcl-2 inhibits FP-induced Bax conformational change and apoptosis but not the down-regulation of XIAP, Mcl-1, and Bcl-x_L proteins. Control (*468*) or MB-468 cells with ectopic overexpression of Bcl-2 (*468/Bcl-2*) were treated with 500 nM FP for 24 h. After this, cell lysates were immunoblotted with specific antibodies against XIAP, Mcl-1, Bcl-x_L, and Bcl-2 (*A*). To determine Bax conformational change, cell lysates were also immunoprecipitated (*IP*) with anti-Bax 6A7 antibody followed by immunoblotting with the polyclonal anti-Bax antibody (*B*). Alternatively, untreated and FP-treated cells were assessed for the percentage of apoptotic cells by staining with annexin V followed by flow cytometry (*C*). Values represent the mean $\pm$ SE of three experiments.



	G1	S	G2/M
468			
Control	53.6 ± 4.9	28.8 ± 7.3	17.6 ± 2.9
Epo B → DF	10.6 ± 7.2	28.4 ± 8.2	61.0 ± 12.1
FP→ DF	60.0 ± 1.4	19.5 ± 1.8	20.4 ± 2.9
Epo B → FP	12.8 ± 0.1	22.0 ± 13.2	65.2 ± 13.1
FP→ EpoB	39.7 ± 1.2	32.1 ± 3.4	28.2 ± 4.4
468/ Bcl-2			
Control	64.4 ± 3.6	18.3 ± 0.3	17.3 ± 3.5
Epo B → DF	60.0 ± 11.8	0.6 ± 15.4	39.4 ± 5.2
FP→ DF	58.4 ± 5.2	26.0 ± 3.8	15.6 ± 3.2
Epo B → FP	23.6 ± 5.2	43.1 ± 9.3	33.3 ± 10.1
FP→ EpoB	48.4 ± 9.6	30.3 ± 5.7	21.3 ± 3.9

Fig. 7. Superior apoptotic effects of sequential treatment with Epo B followed by FP observed in the control MB-468 (*468*) cells, but not in 468/Bcl-2 cells. 468 and 468/Bcl-2 cells were either treated with Epo B (20 nM) or FP (300 nM) for 18 h followed by incubation in the drug-free (*DF*) medium for 24 h or treated with Epo B (20 nM for 18 h) followed by FP (300 nM for 24 h) or the sequence of the drugs in reverse (*A*, *B*, and *D*). After these treatments, either the percentage of apoptotic cells or the percentage of cells in the various phases of the cell cycle was determined after staining with annexin V or propidium iodide and performing flow cytometry (*A* and *B*). 468 and 468/Bcl-2 cell lysates were obtained and subjected to immunoblot analyses using specific antibodies against XIAP and Mcl-1 (*D*), with the expression of β-actin serving as the loading control. For Bax conformational change analysis, the treatment duration for Epo B (50 nM) and FP (500 nM) was 8 and 16 h, respectively, before immunoprecipitation/immunoblotting analysis (*C*). Values of the percentage of apoptotic cells represented by the *bar graphs* (*A*) and the percentage of cells in the various phases of the cell cycle (*B*) are the mean ± SE of three experiments.

effect of treatment with Taxotere followed by FP has been observed by us in human breast cancer cells (data not shown). It is noteworthy that ectopic overexpression of Bcl-2 inhibited apoptosis of MB-468/Bcl-2 cells due to treatment with Epo B followed by FP. This treatment failed to induce Bax conformational change in 468/Bcl-2 cells, although the levels of XIAP and Mcl-1 declined in these cells. This suggests that the superior activity of the sequential treatment with Epo B followed by FP does not extend to breast cancer cells that possess high levels of Bcl-2.

In summary, the present findings underscore the sequence-dependent cytotoxic effects of a combination of EpoB followed by FP in human breast cancer cells. Although it is diminished in cells with high intracellular levels of Bcl-2, the efficacy of this combination against human breast cancer cells may be dependent on the FP-mediated decline in the mRNA and protein levels of antiapoptotic Bcl-2 and IAP families. To verify these observations *in vivo*, preclinical studies in mice and/or clinical studies against human breast cancers are planned.

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Sylvie Wittmann, Purva Bali, Sreenivasa Donapaty, et al.

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