Sequential Dependent Enhancement of Caspase Activation and Apoptosis by Flavopiridol on Paclitaxel-treated Human Gastric and Breast Cancer Cells

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ABSTRACT

Although in the past 10 years paclitaxel has emerged as a successful drug in cancer therapy, the overall response rate to this drug in patients with advanced metastatic disease remains low. Therefore, an understanding of the mechanism of the effect of paclitaxel on inducing apoptosis and the discovery of new ways to enhance the effect of paclitaxel will be critical to improving the therapeutic efficiency of this drug. In the present studies, we have determined that the cyclin-dependent kinase inhibitor flavopiridol significantly enhances paclitaxel-induced apoptosis in the human gastric and breast cancer cell lines MKN-74 and MCF-7. Flavopiridol enhances paclitaxel-induced apoptosis only when administered after paclitaxel treatment. The activation of caspases, specifically caspase 3, is enhanced by flavopiridol on paclitaxel-treated cells. In accordance with this, poly(ADP-ribose) polymerase cleavage is enhanced in combination therapy relative to single-agent paclitaxel. The induction of apoptosis, activation of caspase 3, and poly(ADP-ribose) polymerase cleavage in treatment regimens with paclitaxel and paclitaxel followed by flavopiridol were reversed by treatment with the caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, which supports the notion that caspases are the executioners of apoptosis in these processes. Paclitaxel alone causes transient mitotic arrest with activation of cdc-2 kinase. Cells exit mitosis in a specific time window without cytokinesis, with a decrease in cdc-2 kinase activity and MPM-2 labeling. Flavopiridol accelerates the mitotic exit when administered after paclitaxel treatment in association with a more rapid decrease in MPM-2 labeling. In contrast, pretreatment with flavopiridol prevents cells from entering mitosis by inhibiting cdc-2 kinase activity, thus antagonizing the paclitaxel effect. Therefore, in this study we show that potentiation of paclitaxel-induced apoptosis by flavopiridol is highly sequence dependent, such that mitotic entry and cdc-2 kinase activation by paclitaxel must precede flavopiridol therapy, and the synergistic effect of flavopiridol on paclitaxel-treated cells is due to enhancement in caspase activation.

INTRODUCTION

During the past 10 years, a series of new drugs has been introduced into the chemotherapy arena. Chief among these is the mitotic spindle inhibitor paclitaxel (Taxol). Paclitaxel has shown a broad range of activity in cancers of the breast, lung, and esophagus (1, 2). However, despite its success, the overall response rate for this drug still remains between 30 and 40% in patients with advanced metastatic disease. Therefore, identifying drugs that could potentiate the effect of paclitaxel will be critical in improving the therapeutic efficiency of this agent. One promising candidate in this line is flavopiridol, a synthetic flavone, which is currently undergoing Phase I and II clinical trials (3). Flavopiridol has been shown in vitro to inhibit tumor cell growth at nanomolar concentrations through blockade of cell cycle progression at G1 or G2 (4). It is a potent inhibitor of CDKs with respect to the ATP binding site. Inhibition of CDKs, including CDK-1 (cdc-2), -2, -4, and -7, and hypophosphorylation of pRb have also been reported (5, 6). We have previously reported that flavopiridol at nanomolar concentrations significantly enhances the induction of apoptosis by mitomycin C and paclitaxel in gastric and breast cancer cell lines (7, 8). Synergism between flavopiridol and paclitaxel has also been observed against A549 non-small cell lung cancer cells (9). These studies indicate that a combination of paclitaxel and flavopiridol is highly sequence dependent, such that paclitaxel should precede flavopiridol to achieve the maximal effect (8, 9). The critical events that decide the importance of sequential therapy are not known.

Paclitaxel promotes microtubular aggregation and blocks cells in metaphase (10). The importance of mitotic block in induction of apoptosis in response to paclitaxel has been shown by various groups using antisense of cyclin B1 to abrogate cdc-2 kinase activity (11). The prevention of mitotic block also prevents cell death. However, the mechanism defining mitotic block to induce apoptosis by paclitaxel is not clearly understood.

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3The abbreviations used are: CDK, cyclin-dependent kinase; Rb, retinoblastoma; PARP, poly(ADP-ribose); DAPI, 4',6-diamidino-2-phenylindole; PI, propidium iodide; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.
Apoptosis is a programmed event that involves activation of a cascade of proteinases of the cysteine aspartyl protease gene family, the caspases (12, 13). Caspases play a key role in apoptosis by degrading several proteins essential for DNA repair and structural integrity (14–18). Paclitaxel-induced apoptosis has been suggested to proceed via activation of the caspase cascade (19, 20). Caspases 3 (CPP32/YAMA/apopain) and 7 (Mch3/ICE-LAP3) are activated on paclitaxel treatment in HeLa cells, resulting in cleavage of PARP, a Mr 116,000 proenzyme, into Mr 90,000 and 26,000 fragments (19). The failure to induce caspase has been correlated with resistance to paclitaxel, further supporting the importance of these enzymes in paclitaxel-induced apoptosis (19).

In this study, we have characterized the apoptotic and cell cycle events associated with combination therapy of paclitaxel and flavopiridol. Our results indicate that flavopiridol potentiates the paclitaxel-induced apoptosis when administered after paclitaxel by enhancing activation of caspases. The pretreatment of cells with flavopiridol inactivates the cdc-2 kinase, which prevents the mitotic arrest of paclitaxel from occurring in the context of a properly activated cdc-2 kinase, thus rendering the sequence of flavopiridol followed by paclitaxel combination inactive.

MATERIALS AND METHODS

Cell Culture. The human gastric cancer cell line MKN-74 was graciously supplied by Dr. E. Tahara (Hiroshima University, Hiroshima, Japan), and the MCF-7 cell line was purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in Eagle’s minimal essential medium and RPMI (respectively) supplemented with 20% heat-inactivated normal calf serum (Intergen, Purchase, NY), penicillin, and streptomycin at 37°C in 5% carbon dioxide. Both cultures were tested as Mycoplasma free.

Quantitative Fluorescence Microscopy. The nuclear morphology of the cell (multinucleated and apoptotic) was determined by staining nuclear chromatin with DAPI (Sigma Chemical Co., St. Louis, MO). Apoptotic cells contain condensed and fragmented chromatin. The percentage of apoptosis was determined by counting the cells and scoring for the incidence of apoptosis using an Olympus BH2-DM2U2UV Dichromatic Mirror cube filter (Olympus, Lake Success, NY). The protocol has been previously described (7). Briefly, MKN-74 and MCF-7 cells were cultured for 48–72 h (approximately 60% confluent) and treated according to the following conditions: untreated, 300 nM flavopiridol (graciously supplied by Dr. Edward Saussville, National Cancer Institute, Bethesda, MD) alone for 24 h, 100 nM paclitaxel (Taxol, Bristol-Myers Squibb, Princeton, NJ) alone for 18 h, 300 nM paclitaxel and 100 nM paclitaxel together for 18 h, 300 nM flavopiridol and 100 nM paclitaxel for 24 h followed by removal of medium containing flavopiridol and addition of medium with 100 nM paclitaxel for 18 h or the same drugs given in reverse order, and 100 nM paclitaxel for 18 h followed by removal of drug medium and addition of fresh medium without drug. In sequential therapy, the initially treated cells were washed with drug-free medium, and floating cells were collected and added back for subsequent treatment. At the end of treatment, adherent cells were trypsinized, pooled with floating cells, washed with PBS, and fixed in 3% paraformaldehyde for 10 min at room temperature. Cells were stained with DAPI for 30 min at room temperature in the dark. The aliquots of cells were taken to prepare slides, and duplicate samples of 400 cells each were counted and scored for the incidence of apoptotic chromatin condensation. The photomicrographs were obtained using a Zeiss Photomicroscope III (Oberkochen, Germany) equipped with an epifluorescence condenser.

MMP-2/PI Bivariate Flow Cytometry. MMP-2 antibody recognizes epitopes shared by phosphoproteins appearing during mitosis. The positive labeling of MMP-2 correlates with entry into mitosis, whereas the dephosphorylation of these proteins correlates with anaphase onset (21). The cells containing 4 N DNA content and labeling positive for MMP-2 should be in M phase. MKN-74 and MCF-7 cells (1.4 × 10⁶/100 mm dish) were cultured for 48 h and treated with paclitaxel (100 nM) and flavopiridol (300 nM) as a single agent or sequentially by treating paclitaxel for 18 h followed by removal of drug and addition of either drug-free medium or medium containing 300 nM flavopiridol. The cells were harvested at specific time points by trypsinization, pooled with floating cells, and fixed with 70% ethanol. After washing with PBS containing 0.05% Tween 20 and 1% fetal bovine serum, cells were labeled with MMP-2 antibody (final concentration of 6 μg of MMP-2 antibody per ml) (Upstate Biotechnology, Lake Placid, NY) for 1 h at 4°C. Cells were washed twice with PBS and incubated with goat anti-mouse FITC (Boehringer Mannheim, Mannheim, Germany) for 1 h at room temperature in the dark. After washing twice with PBS, cells were resuspended in 5 μg/ml PI containing 50 μg/ml RNase A. Samples were analyzed on a FACSscan (Becton Dickinson, Mountain View, CA), and data were analyzed using CellQuest software. The MMP-2-positive (mitotic cells) show increased green fluorescence, thus shifting above the baseline of the dot plot (see Fig. 4A, boxes). Mitotic index was defined as percentage of MMP-2-positive cells.

cdc-2 Kinase Activity Assay. The MKN-74 and MCF-7 cells were treated with paclitaxel and flavopiridol as discussed for the MMP-2/PI protocol and lysed with buffer containing 50 mM HEPES-KOH (pH 7.5); 150 mM NaCl; 1 mM each EDTA, NaF, and DTT; 2.5 mM EGTA; 0.1% Tween 20; 10% glycerol; 10 mM β-glycerophosphate; 0.1 mM Na3VO4; 0.2 mM phenylmethylsulfonyl fluoride; and 10 μg/ml each aprotinin and leupeptin. The cells were further disrupted by passing through a 21-gauge syringe 10 times, and lysates were clarified by centrifugation (10 min at 10,000 × g). Soluble protein (200 μg) was incubated with 1 μg of anti-cyclin B1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C for 2 h. Immune complexes were then precipitated with 40 μl of immobilized protein A (Repligen, Needham, MA) overnight at 4°C and washed three times with lysis buffer and twice with kinase assay buffer [50 mM HEPES-KOH (pH 7.5), 10 mM MgCl2, and β-glycerophosphate, 1 mM DTT, 2.5 mM EGTA, 0.1 mM Na3VO4, and 1 mM NaF]. The kinase assay was carried out by combining the washed protein beads with 20 μl of kinase buffer plus 10 μCi of [γ-32P]ATP, 15 μM ATP, and 50 μg/ml histone H1 (Boehringer Mannheim, Mannheim, Germany). The reaction was allowed to proceed for 30 min at 30°C and was then terminated by adding 10 μl of Laemmli sample buffer and boiling for 5 min. Products were resolved by 10% SDS-PAGE.
The incorporated radioactivity was determined by Betascope 603 blot analyzer (Betagen Corp., Waltham, MA).

Immunoblot Analysis. Protein lysates prepared for kinase assays were used for immunoblotting. Fifty micrograms of soluble protein were resolved by either 8% or 10% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA). The equal loading of proteins was confirmed by amido black staining. The membranes were probed with mouse monoclonal antibodies specific to pRb, PARP, rabbit polyclonal antibody to caspase 3 (PharMingen, San Diego, CA), and mouse monoclonal cyclin B1 (kindly provided by Dr. Tim Hunt, Imperial Cancer Research Fund Clare Hall Laboratories, South Mimms, Herts, UK). The membranes were treated with a secondary sheep antimouse-horseradish peroxidase or donkey antirabbit-horseradish peroxidase antibody for 1 hour at room temperature. Detection was performed with enhanced chemiluminescence reagents (Dupont NEN Life Science Products, Boston, MA) according to the manufacturer’s protocol. The levels of expression were quantitated using a densitometric scanning system.

Statistical Analysis. All experiments were done in duplicate and were repeated at least three times unless otherwise indicated. The statistical significance of the experimental results was determined by the two-sided Student’s t test.

RESULTS

Flavopiridol Enhances Paclitaxel-induced Apoptosis in a Sequence-dependent Manner. Human gastric and breast cancer cells, MKN-74 and MCF-7, respectively, were treated with 100 nM paclitaxel (a clinically achievable concentration) and 300 nM flavopiridol (a concentration established to inhibit CDKs; Refs. 5 and 6) individually, concurrently and sequentially as paclitaxel for 18 h followed by flavopiridol for 24 h or as the same drugs given in reverse sequence. As determined by quantitative fluorescence microscopy, the combination of flavopiridol and paclitaxel together for 18 h increased the percentage of cells undergoing apoptosis from 3 ± 1% with flavopiridol alone (T18−ND24) to 8 ± 1% with the two drugs together for 24 h (P < 0.25; data not shown). In contrast, sequential treatment of MKN-74 cells with paclitaxel for 18 h followed by flavopiridol for 24 h induced apoptosis in 40 ± 2% (P < 0.005) of the cells (T18−3F24). Shorter exposure periods of flavopiridol (6–10 h) did not enhance paclitaxel-induced apoptosis. The percentage apoptosis by paclitaxel for 18 h followed by flavopiridol for 24 h was significantly greater than that observed with paclitaxel for 18 h followed by no drug for 24 h (15 ± 2%, P < 0.005; Fig. 1, T18−ND24). Under these treatment conditions (T18−ND24 and T18−F24), the viable cells acquired multinucleated phenotype (Fig. 1, Mu), a characteristic of paclitaxel treatment that is reported to result from aberrant mitosis (22). When the cells were treated in the reverse order such that flavopiridol treatment preceded paclitaxel treatment (F24→T18), only 8 ± 1% (P < 0.005) of the cells underwent apoptosis, which was not significantly different from the result obtained with flavopiridol for 24 h followed by no drug for 18 h. To address the possibility that with sequential therapy of paclitaxel followed by flavopiridol some cells may have lysed and hence inflated the observed proportion of live cells, the total (adherent

![Fig. 1](attachment://image.png)
The presence of 50\% with paclitaxel or paclitaxel followed by flavopiridol in the sequential therapy involved higher activation of caspases relative to single-agent paclitaxel, we analyzed both the cleavage of the PARP, a substrate cleaved by caspases during apoptosis, and the activation cleavage of caspase 3, a caspase shown to be involved in a variety of stimuli, including paclitaxel-induced apoptosis (17, 18). As shown in Fig. 2A, in MKN-74 cells, 18 h of 100 nM paclitaxel treatment did not result in the PARP cleavage. Twenty-four h of additional incubation in drug-free medium resulted in the appearance of the M₉ 90,000 apoptotic fragment of PARP (T₁₈→ND₂₄). Sequential treatment of cells with paclitaxel followed by flavopiridol (T₁₈→F₂₄) enhanced the PARP cleavage. As shown in Fig. 2A, a strong M₉ 90,000 band developed that was at least 10-fold greater in intensity than that for T₁₈→ND₂₄. In keeping with this observation, the M₉ 116,000 PARP proenzyme band was decreased 2–3-fold in the sequential therapy compared with paclitaxel alone, thus confirming the enhancement of apoptosis by this sequential combination. Treatment of MKN-74 cells with 100 nM paclitaxel and 300 nM flavopiridol together for 18 h or the reverse combination of flavopiridol followed by paclitaxel resulted in PARP cleavage that was less than that for paclitaxel alone (T₁₈→ND₂₄; Fig. 3).

Caspase 3, a M₃ 32,000 proenzyme and an executioner caspase in paclitaxel-treated leukemic cells, yields upon activation M₉ 17,000 and 11,000 subunits. As predicted from PARP cleavage analysis, immunoblot analysis with caspase 3 antibody detected the activated M₉ 17,000 cleaved form in cells treated with T₁₈→ND₂₄ and T₁₈→F₂₄ (Fig. 2A). The intensity of the M₉ 17,000 band in combination therapy was at least 2-fold higher compared with single-agent paclitaxel.

Inhibition of Caspases Reverses the Induction of Apoptosis and PARP Cleavage by Paclitaxel and Combination Therapy. To further confirm the role of caspases responsible for the cleavage of PARP during paclitaxel and sequential combination therapy-induced apoptosis, the cells were treated with paclitaxel or paclitaxel followed by flavopiridol in the presence of 50 \( \mu \)M z-VAD-fmk, a caspase inhibitor. As shown in Fig. 2B, PARP cleavage was dramatically decreased with paclitaxel followed by flavopiridol in the presence of z-VAD-fmk. Consistent with this observation, the activation cleavage of caspase 3 was also reversed (Fig. 2B). Similar results were obtained with paclitaxel followed by no drug in the presence of 50 \( \mu \)M z-VAD-fmk (data not shown).

To investigate whether reversal of PARP and caspase 3 cleavage also resulted in reversal of apoptosis under these conditions, cells were treated with paclitaxel followed by either no drug or flavopiridol in the presence or absence of 50 \( \mu \)M z-VAD-fmk and stained with DAPI. As shown in Fig. 1, T₁₈→ND₂₄+z-VAD and T₁₈→F₂₄+z-VAD, addition of z-VAD-fmk completely reversed the induction of apoptosis (from 15% to <2% in paclitaxel followed by no drug therapy and from 40% to less than 5% in paclitaxel followed by flavopiridol therapy), with a majority of cells displaying a minimultinucleated phenotype. These results are consistent with the findings of Panvichian et al. (19), who indicate that inhibition of caspase activation results in minimultinucleation in HeLa cells and implicate this phenomenon in relative resistance to paclitaxel.

Effect of Flavopiridol on Mitotic Exit after Paclitaxel Therapy. To further understand the sequence dependency of the paclitaxel and flavopiridol combination, we studied the effect of paclitaxel (as a single agent and in sequential therapy) on cell cycle events. To distinguish the G₂ and M cells in the 4 N peak, we labeled the cells with MPM-2 antibody, which recognizes epitopes shared by phosphoproteins appearing during mitosis. The two-dimensional flow cytometry was used to...
quantitate the population of cells in M phase. As shown in Fig. 4A, after 18 h of 100 nM paclitaxel treatment of MKN-74 cells (T18), the majority of cells (90%) accumulated with 4 N DNA content (the G2M population), with 71% of the cells staining positive for MPM-2 (mitotic index of 71). In untreated cells (ND24), only 3% of the population was positive for MPM-2 because of rapid turnover of cells from metaphase to anaphase. Twenty-four h after removal of paclitaxel (T18→ND24), the 4 N peak did not decrease. However, the mitotic index dropped dramatically, with only 13% of cells showing reactivity to MPM-2. These data indicate that, after paclitaxel treatment, cells enter, arrest, and then exit mitosis without undergoing cytokinesis (as shown by no change in the 4 N peak but a decrease in mitotic index). Similar results were obtained with MCF-7 cells (data not shown). Addition of flavopiridol to paclitaxel-treated cells (T18→F24) stimulated the mitotic exit, as determined by a rapid decrease in the number of MPM-2-positive cells. The quantitation of mitotic index with paclitaxel followed by flavopiridol (T18→F) and paclitaxel followed by no drug (T18→ND) is shown in Fig. 4B. At each time point examined, the mitotic index for T18→F was less than that for T18→ND. For example, the mitotic index decreased from 71% with T18 alone to 46% with T18→F. At a similar time point without flavopiridol (T18→ND), the mitotic index was not different (67%) from that for T18 alone (71%). In general, there was a 30–60% decrease in mitotic index after addition of flavopiridol (T18→F) compared with single drug (T18→ND) for each time point examined. The mitotic index was also decreased in reverse combination compared with paclitaxel alone (F24→T18 20% as compared with T18 alone (71%)). Previous studies have indicated that the mitotic block is critical for paclitaxel-induced apoptosis (11). Pretreatment with flavopiridol before paclitaxel prevents mitotic entry (as evidenced by the substantially lower mitotic index) and thus decreases the induction of apoptosis by paclitaxel in this combination therapy.

Effects of Flavopiridol on cdc-2 Kinase, Cyclin B1, and pRB after Paclitaxel Therapy. The exit from mitosis and progression into interphase requires the inactivation of cdc-2 kinase by degradation of cyclin B1 (23), dephosphorylation of cdc-2 at Thr161 (24), and/or phosphorylation at Thr14 and Tyr15 (25). We thus assayed cyclin B1-associated cdc-2 kinase activity by histone H1 phosphorylation, under these treatment conditions, in MCF-7 cells. As shown in Fig. 5B, 18-h paclitaxel treatment (T18) induced an increase in kinase activity by 5-fold as compared with untreated control cells (ND24). Twenty-four h after removal of paclitaxel (T18→ND24), however, kinase activity decreased dramatically by 50-fold, as compared with paclitaxel alone for 18 h. Flavopiridol is a competitive inhibitor of CDKs with respect to ATP. Flavopiridol treatment at 300 nM for 24 h inhibits the cdc-2 kinase activity by 10–20-fold in MKN-74 and MCF-7 cells (data not shown; Ref. 6). Therefore, as anticipated, addition of flavopiridol to paclitaxel-treated cells rapidly inactivated cdc-2 kinase. In fact, 24 h after removal of paclitaxel and addition of flavopiridol (T18→F24), there was virtually no activity left, whereas at a similar time point without flavopiridol (T18→ND24), a residual kinase activity could be detected. Other treatment conditions, including cotreatment or pretreatment of cells with flavopiridol before paclitaxel (F24→T18), blocked the activation of cdc-2 kinase by paclitaxel. Additionally, cdc-2 kinase activity was not reversed by z-VAD-fmk in MKN-74 cells treated with either paclitaxel or sequential combination therapy (data not shown), indicating that cdc-2 kinase either is an upstream regulator of the caspase activation pathway or is completely independent of this process.

A similar profile was observed for cyclin B1 protein expression in MCF-7 cells. It increased 5–6-fold after 18-h paclitaxel treatment compared with untreated controls (ND24) and decreased 25–30-fold with T18→ND24 and T18→F24 compared with 18-h paclitaxel treatment. Although the cdc-2 kinase activity was decreased more rapidly and to a greater extent for T18→F24 compared with T18→ND24, the decrease in cyclin B1 protein expression was similar in these two conditions. The difference in cdc-2 kinase activity may therefore be due to direct binding by flavopiridol to the ATP site of cdc-2, thus increasing the inactivation of the cdc-2 kinase activity by flavopiridol on paclitaxel-treated cells. With pretreatment of flavopiridol before paclitaxel (F24→T18), we did not observe any increase in the induction of cyclin B1 protein expression by paclitaxel (data not shown). Similar results for cdc-2 kinase activity and cyclin B1 protein expression were also obtained with MKN-74 cells.

To investigate whether the cells exiting mitosis express interphase markers, we examined the phosphorylation status of pRB. As shown for MKN-74 cells in Fig. 5A, under untreated conditions (ND24), pRB is mostly phosphorylated, which suggests that cells are cycling. After 18 h of paclitaxel treatment (T18) most of the pRB was in the phosphorylated form. Twenty-four h after paclitaxel removal (T18→ND24), most of the phosphorylated pRB had changed to a hypophosphorylated form. Addition of flavopiridol (T18→F24) to paclitaxel-treated cells induced complete hypophosphorylation of pRB. Similar results were obtained with MCF-7 cells.

Taken together, these results indicate that paclitaxel treatment induces transient mitotic block (i.e., increase in mitotic index), after which cells exit mitosis (i.e., decrease in mitotic

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**Fig. 3** Concurrent treatment [(T+F)18] or flavopiridol followed by paclitaxel treatment (F24→T18) does not enhance cleavage of PARP as compared with single-agent paclitaxel (T18). MKN-74 cells were treated with paclitaxel, paclitaxel followed by no drug (T18→ND24), flavopiridol followed by paclitaxel, and paclitaxel plus flavopiridol for the indicated times, and cell extracts were analyzed by immunoblotting for cleavage of PARP to the apoptotic fragment (Mr 90,000).
index) without undergoing cytokinesis (i.e., remain 4 N) and express G1 markers, a stage we refer to as pseudo-G1. Addition of flavopiridol after paclitaxel treatment accelerates the exit from mitotic block with a more rapid loss of mitotic cell cycle markers (i.e., decrease in MPM-2 reactivity) and induction of pseudo-G1 events (i.e., complete versus partial pRb hypophosphorylation). Pretreatment with flavopiridol before paclitaxel blocks the induction of cdc-2 kinase by paclitaxel and thus blocks entry of cells into M phase.

**DISCUSSION**

After our initial observation that flavopiridol significantly enhanced mitomycin C-induced apoptosis, we became interested in the combination of paclitaxel and flavopiridol. This combination is sequence dependent such that paclitaxel should precede flavopiridol treatment to achieve a synergistic effect. Our studies indicate that flavopiridol enhances paclitaxel-induced apoptosis by 2–3-fold. This would support the observations by Bible et al. (9) that indicate that paclitaxel followed by flavopiridol is more inhibitory to colony formation in A549 cells compared with concomitant or flavopiridol followed by paclitaxel therapy. In the present study, we have attempted to understand the mechanisms for the sequence dependency for induction of apoptosis. First, our results indicate that flavopiridol enhances activation of caspases on paclitaxel-treated cells, thus intensifying paclitaxel-induced apoptosis. Second, because paclitaxel-induced apoptosis is dependent on activation of cdc-2 kinase and mitotic block, pretreatment with flavopiridol blocks this effect, thus antagonizing the effect of paclitaxel on these cells.

It has been shown previously that after paclitaxel removal, HeLa cells exit mitotic block abnormally and undergo apoptosis (26). However, these observations were based on nuclear morphology and were insufficient to suggest that cells exit mitotic block without undergoing cytokinesis. In the present study, we show conclusively with biochemical analysis and cell cycle molecular markers that paclitaxel induces transient mitotic block in MKN-74 and MCF-7 cells, with elevation of cyclin B1 protein expression, cdc-2 kinase activity, and MPM-2 reactivity. The exit is observed with a decrease in these markers and induction of G1 markers. Because the 4 N peak on the histogram does not change, it indicates that cells exit mitosis without undergoing cytokinesis. The pRb is hypophosphorylated, which suggests that cells enter interphase with 4 N DNA content.

Flavopiridol after paclitaxel therapy accelerates mitotic exit by inhibiting cdc-2 kinase.

It has been shown that paclitaxel concentration sufficient to induce mitotic block by stabilizing the microtubule dynamics, rather than by alteration of the microtubule mass, induces apoptosis (26). The importance of mitotic block in induction of apoptosis in response to paclitaxel has also been shown by various groups using antisense of cyclin B1 to abrogate the cdc-2 kinase activity (11). The prevention of mitotic block also prevents cell death. Flavopiridol, a competitive inhibitor of CDKs with respect to ATP, prevents entry into S and M phases (5, 6). When administered together or before paclitaxel, flavopiridol overrides the paclitaxel effect of cdc-2 kinase activation and inhibits entry into M phase, a prerequisite for paclitaxel-induced apoptosis. Previous studies have suggested that concurrent treatment with inhibitors of kinase, such as 2-amin-
Flavopiridol potentiates paclitaxel-induced apoptosis

opurine, and phosphatases, such as sodium orthovanadate, prevent the paclitaxel-induced cytotoxicity (27). Flavopiridol is a specific inhibitor of CDKs, whereas 2-aminopurine is a general protein kinase inhibitor. Thus, the inhibition of paclitaxel-induced cytotoxicity by flavopiridol (by cdc-2 kinase inactivation) confirms the previous notion regarding the importance of mitotic block and exit in paclitaxel-induced apoptosis and provides further explanation for the ineffectiveness of the reverse combination (F24 → T18).

Flavopiridol potentiates the paclitaxel-induced apoptosis when administered in sequence such that 18-h treatment with paclitaxel is followed by 24-h treatment with flavopiridol. Shorter durations of paclitaxel (i.e., 3 h) are also as effective, as long as the interval between initiation of paclitaxel and the start of flavopiridol is 18–24 h (data not shown). The downstream events that execute the paclitaxel-induced cell death involve activation of caspases. MKN-74 cells treated with paclitaxel show activation of caspase 3, and addition of caspase inhibitor z-VAD-fmk reverses the cleavage of caspase 3 and induction of apoptosis. In combination therapy with paclitaxel followed by flavopiridol, there was enhancement of caspase 3 activation and cleavage of PARP. This enhancement in activation of caspase can explain the increase in induction of apoptosis with this sequential combination. In fact, in the reverse combination of flavopiridol followed by paclitaxel, there was less cleavage of PARP and induction of apoptosis as compared with paclitaxel alone (T18 → ND24), which indicates that pretreatment of flavopiridol antagonizes the paclitaxel effect. The induction of apoptosis and cleavage of caspase 3 and PARP in sequential paclitaxel followed by flavopiridol therapy was significantly reversed by z-VAD-fmk, which suggests that activation of caspases, including caspase 3, is important in execution of cell death with this combination therapy. As z-VAD-fmk inhibits other caspases, including caspase 7, the role of these caspases in combination therapy is a subject of ongoing laboratory investigation. Whether this is due to a direct effect of flavopiridol on the activation of caspases or to suppression of caspase inhibitors (i.e., XIAP, survivin, and so forth) remains to be investigated.

A Phase I study of sequential paclitaxel followed by flavopiridol therapy is now underway at our center. On the basis of these preclinical data, we elected to treat patients with a fixed dose of paclitaxel infused over 3 or 24 h on day 1 followed by flavopiridol administered over 24 h starting on day 2. To date, we have observed clinical activity in patients with recurrent esophagus and prostate cancer at nanomolar concentrations of paclitaxel and flavopiridol that are similar to those used in these in vitro studies. These exciting clinical results represent a new direction in cancer therapy. Increased caspase activation by flavopiridol on paclitaxel-treated cells appears to represent a molecular event that is critical for the enhancement of apoptosis in this sequential combination therapy.

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