

Flavopiridol Increases Sensitization to Gemcitabine in Human Gastrointestinal Cancer Cell Lines and Correlates with Down-Regulation of Ribonucleotide Reductase M2 Subunit¹

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ABSTRACT

As a single agent, gemcitabine (2',2'-difluorodeoxycytidine) has shown minimal activity against gastrointestinal malignancies with only a modest improvement in survival in patients with pancreatic cancer. Recently, gemcitabine resistance has been associated with the up-regulation of mRNA and protein levels of the ribonucleotide reductase M2 subunit (RR-M2), a rate-limiting enzyme in DNA synthesis that is cell cycle regulated. In this study we show that flavopiridol, a cyclin-dependent kinase inhibitor, enhances the induction of apoptosis by gemcitabine in human pancreatic, gastric, and colon cancer cell lines. As determined by quantitative fluorescence microscopy, flavopiridol enhanced gemcitabine-induced apoptosis 10–15-fold in all of the cell lines tested in a sequence-dependent manner. This was confirmed by poly(ADP-ribose) polymerase cleavage and mitochondrial cytochrome *c* release. Colony formation assays confirmed the apoptotic rates, showing complete suppression of colony formation only after exposure to sequential treatment of G₂₄→F₂₄. This is associated with suppression of the RR-M2 protein. This appears to be related to down-regulation of E2F-1, a transcription factor that regulates RR-M2 transcription and hypophosphorylation of pRb. The proteasome inhibitor PS-341 could restore the protein levels of E2F-1 in G₂₄→F₂₄ treatment indicating that E2F-1 down-regulation is attributable to its increased degradation via ubiquitin-proteasome pathway. This also resulted in restoration of RR-M2 mRNA and protein. These results indicate that flavopiridol in gemcitabine-treated cells inhibits parts of the machinery necessary for the transcription induction of RR-M2. Thus, combining flavopiridol with gemcitabine may provide an important and novel new means of enhancing

the efficacy of gemcitabine in the treatment of gastrointestinal cancers.

INTRODUCTION

Gemcitabine (2',2'-dFdCyd³) is an analogue of deoxycytidine, which has demonstrated activity as a single agent or in combination with other chemotherapeutic agents against solid tumors, including non-small-cell lung cancer (1), head and neck cancer (2), hormone-refractory prostate cancer (3), breast cancer (4), and ovarian cancer (5). dFdCyd is phosphorylated by DCK to its 5'-monophosphate form (dFdCMP) and additionally metabolized by several other enzymes to 5'-diphosphate (dFdCDP) and 5'-triphosphate derivative (dFdCTP) (6, 7). dFdCTP is incorporated into the DNA causing masked chain termination (6, 7). Furthermore, dFdCDP inhibits RR enzyme activity. RR is a rate-limiting and central enzyme in DNA synthesis, because it converts ribonucleotide diphosphates into deoxyribonucleotide diphosphates (8, 9). Therefore, gemcitabine decreases the dNTP pool of the cell, causing a competitively higher incorporation of dFdCTP, as compared with dCTP, into the DNA during S phase. Furthermore, dFdCyd inhibits dCMP deaminase, which is an enzyme in the degradation pathway of gemcitabine. This results in an increased efficacy of gemcitabine.

RR consists of two subunits, M1 and M2. The M2 subunit is mainly involved in RR enzyme activity during S phase, and its protein and activity level had been correlated with DNA synthesis and invasive potential of a tumor cell (10, 11). Goan *et al.* (12) have recently shown that RR overexpression, but not DCK down-regulation, is a major mechanism of gemcitabine resistance. Increased RR activity, associated with gemcitabine treatment, has been shown to expand the dNTP pool, which competitively inhibits the incorporation of dFdCTP into the DNA. Furthermore, the expanded dNTP pool down-regulates the activity of DCK via a negative feedback mechanism, reducing the phosphorylation of gemcitabine (12). In addition, it has been hypothesized that an extended dNTP pool will increase gemcitabine degradation via a positive feedback mechanism of dCMP deaminase.

Despite its promising clinical activity and based on the frequent development of resistance, response rates to gemcitabine in gastrointestinal cancers of the pancreas, the stomach, and the colon still remain quite low with confirmed responses being <10% (13–19). Additional development of this drug will depend on new means to enhance its effectiveness.

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³ The abbreviations used are: dFdCyd, difluorodeoxycytidine; CDK, cyclin-dependent kinase; RR-M2, ribonucleotide reductase M2; RR, ribonucleotide reductase; PARP, poly(ADP-ribose) polymerase; DCK, deoxycytidine kinase; dNTP, deoxynucleotide triphosphate; [³H]dThd, [³H]thymidine; pRb, Retinoblastoma protein.

Flavopiridol is a synthetic flavone, identified in the National Cancer Institute drug screen, with antitumor efficacy observed in preclinical models (20) and in clinical studies in solid tumors (21), as well as in hematological neoplasms (22). Previous studies have shown that flavopiridol in nanomolar concentrations inhibits the activity of cdk1, 2, 4, and 6 (23, 24). *In vitro* flavopiridol has been shown to inhibit tumor cell growth through the blockade of cell cycle progression at G₁ or G₂ (20, 24). As a single agent in a 150–300-nM range, flavopiridol also inhibits the colony formation of a series of human cancer cell lines (25).

Flavopiridol has been shown to enhance the induction of apoptosis by paclitaxel and mitomycin C in MCF-7 (breast carcinoma) and MKN-74 (gastric adenocarcinoma) cells (26, 27). In the case of paclitaxel, this was highly sequence-dependent, such that paclitaxel needed to precede flavopiridol to achieve this effect. Ongoing clinical trials evaluating combinations of flavopiridol with chemotherapy, including paclitaxel and cisplatin, show promising clinical responses in a Phase I study. This includes a complete remission in a patient with carcinoma of the esophagus (28).

Flavopiridol has been shown to inhibit the transcription of cell cycle-specific genes (29). RR-M2 is regulated by E2F-1, a transcription factor. Because gemcitabine resistance is associated with the up-regulation of the S-phase enzyme RR-M2 we hypothesized that any means to down-regulate the expression RR-M2 should theoretically potentiate gemcitabine-induced apoptosis. In this study we show that flavopiridol potentiates gemcitabine-induced apoptosis in a sequence-dependent manner in pancreatic (Capan-2), as well as colorectal (HCT-116) and gastric cancer (MKN-74 and SK-GT-5) cells. This potentiation is correlated with the down-regulation of the RR-M2 subunit. This down-regulation is caused by the proteasome-mediated degradation of E2F-1 and the hypophosphorylation of pRb, events which are critical for the transcriptional regulation of RR-M2.

MATERIALS AND METHODS

Cell Culture and Treatment

The human gastric cancer cell line MKN-74 was graciously supplied by Dr. E. Tahara (Hiroshima University, Hiroshima, Japan). HCT-116 and Capan-2 cell lines were purchased from American Type Culture Collection (Manassas, Virginia). The gastric adenocarcinoma cell lines MKN-74 (p53 wild type) and SK-GT-5 (p53 mutated) were maintained in Eagle's minimal essential medium supplemented with 20% heat inactivated normal calf serum (Intergen; Ref. 30). The colorectal carcinoma cell line HCT-116 was maintained in RPMI 1640, and the pancreatic carcinoma Capan-2 cells were cultured in McCoy's 5a medium, both supplemented with 10% fetal bovine serum (Hyclone). All of the mediums were supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin and maintained at 37°C in a 5% carbon dioxide atmosphere. All of the cultures were tested as *Mycoplasma* free. If the cells were treated with the proteasome inhibitor PS341 (ProScript, Cambridge, MA) 20 nM of this drug were added during the last 24 h of treatment to

either drug-free or flavopiridol-containing medium unless otherwise specified.

QFM

The nuclear morphology of the cell (multinucleated and apoptotic) was determined by staining nuclear chromatin by 4',6'-Diamidino-2-phenylindole (Sigma Chemical Co., St. Louis, MO). Apoptotic cells have 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 described previously (26, 27). MKN-74, SK-GT-5, and Capan-2 were treated with 300 nM of flavopiridol and 100 nM of gemcitabine unless otherwise specified. HCT-116 cells were treated with 50 nM of gemcitabine and 150 nM of flavopiridol. The concentrations, where <10% of the cells were viable, were determined by Alamar blue assay. Briefly, cell lines were cultured for 48–72 h (~60% confluent) and treated according to the following conditions for 24 h: untreated (ND₂₄); flavopiridol (graciously supplied by Dr. Edward Sausville, National Cancer Institute, Bethesda, MD) alone (F₂₄); gemcitabine (Gemzar; Lilly, Indianapolis, IN) alone (G₂₄); flavopiridol and gemcitabine together [(G + F)₂₄]; flavopiridol for 24 h followed by removal of medium containing flavopiridol and addition of medium with gemcitabine for 24 h (F₂₄→G₂₄) or the same drugs given in reverse order (G₂₄→F₂₄); and either gemcitabine or flavopiridol for 24 h followed by removal of drug medium and addition of fresh medium without drug (G₂₄→ND₂₄, F₂₄→ND₂₄) for 24 h. 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 4',6-diamidino-2-phenylindole for 30 min at room temperature in the dark. 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 equipped with an epifluorescence condenser.

Colony Formation Assays

For colony formation studies 150 nM of flavopiridol and 10 nM of gemcitabine were used. The dosage of each single agent was selected that achieved submaximal growth inhibition in the colony formation assay. For each condition, 500-1000 cells were plated in at least duplicate samples on a 100-mm plate, and colony formation assays with both drugs were performed. After the cells attached to the bottom (~24 h after plating), the cells were treated for 24 h with either gemcitabine alone, flavopiridol alone, a combination of both drugs, or sequential application of the two drugs, each for 24 h. After treatment, drug-containing medium was removed, and cell were cultured ≤10 days in total. After 10 days without additional change of medium, the colonies were fixed and stained by adding 200 µl of 0.5% crystal violet (Sigma Chemical Co.) in 0.2 mM of citric acid for 60 min. Colonies with >50 cells were counted under a magnifier. The

number of colonies counted in the untreated condition were set 100%, and the number of colonies scored in the other conditions was related to untreated cells. The rate of colony formation was calculated as a mean value of duplicate samples with the SD of the mean.

[³H]dThd Incorporation

Triplicate 60-mm plates with 50–70% confluent MKN-74 cells were treated with either 300 nM of flavopiridol or drug-free medium. After 24 h, the medium was removed, and 100 nM of gemcitabine and 2 μ Ci/ml [³H]dThd (NEN, Boston, MA) were added for another 24 h. After the treatment, medium was removed and plates were washed once with ice-cold PBS and twice incubated with 1 ml of 5% trichloroacetic acid for 20 min on ice. After washing once with ice-cold PBS, cells were incubated with 1 ml of 0.25 N NaOH for 30 min in 37°C. The extract (0.8 ml) was added to 5 ml of scintiverse scintillation fluid (Fisher Scientific, Pittsburgh, PA) and analyzed for 10 min in a scintillation counter (Beckman). Duplicate parallel samples were treated similarly except without [³H]dThd and used for cell counts. The scintillation cpm were correlated to 1 million cells. The cpm/ 1×10^6 cells of ND₂₄→G₂₄ were set 100%, and the cpm/ 1×10^6 cells were calculated accordingly. The experiment was repeated once. The statistical significance of the experimental results was determined by the two-sided *t* test.

Immunoblot Analysis

For immunoblotting, cells were treated with gemcitabine and flavopiridol according to the conditions described above.

Preparation of Cell Extracts for Immunoblotting. For preparation of cell lysates, the floating and attached cells were lysed with buffer A containing 50 mM HEPES-KOH, (pH 7.5); 150 mM NaCl; 1 mM of 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 μ g/ml each of aprotinin and leupeptin. The cells were additionally disrupted by passing through a 27-gauge syringe 10 times, and lysates were clarified by centrifugation (20 min at 7500 \times *g* for 20 min at +4°C). The remaining supernatant was recovered, aliquoted, and frozen at –20°C.

Preparation of Cytosolic and Mitochondrial Fractions of Cytochrome *c*. The floating and the attached cells were harvested as described for QFM. After centrifugation, the cell pellet was incubated for 30 min on ice with 300 μ l of an iso-osmolar lysis buffer B, containing 10 mM 4-morpholinepropanesulfonic acid-buffer, 250 mM sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. Thereafter, cells were homogenized by 40 strokes of a glass dounce homogenizer with a B pestle, and the suspension was spun for 10 min with 500 \times *g* at +4°C. The supernatant was recovered and ultracentrifuged with 40,000 \times *g* for 16 min at +4°C. The supernatant was recovered again, aliquoted, and frozen at –20°C and was used as the cytosolic cell fraction. The remaining pellet was washed once with buffer B, equally ultracentrifuged, and the remaining pellet (mitochondrial fraction) was resuspended in 30 μ l of lysis buffer A. Samples were kept at –20°C.

SDS-PAGE and Immunoblotting. Protein was quantified by spectrometric measurement of the protein content according to the manufacturer's instruction of the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Whole cell extracts (100–200 μ g), 50 μ g of the cytosolic, or 10 μ g of the mitochondrial fraction were resolved by either 8 or 15% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA). The equal loading of proteins per each lane was confirmed by amido black staining (Sigma Chemical Co.). The membranes were probed with mouse monoclonal antibodies specific to PARP (catalogue no. 66391A), cytochrome *c* (65981A; PharMingen, San Diego, CA), E2F-1 (sc-251), cyclin E (sc-247), cdk2 (sc163-G; Santa Cruz Biotechnology), pRb (MS-107-p), cyclin-D1 Ab-1 (MS-210-PO; Neomarkers, Fremont, CA), or with rat monoclonal antibodies specific to RR-M2 subunit (antihuman tubulin, MAS 077p; Accurate, Westbury, NY). The membranes were treated with a secondary horseradish peroxidase-conjugated antmouse (NA 931; Amersham Life Science) or antirat antibody (A-5795; Sigma Chemical Co.) for 1 h at room temperature. Detection was done by ECL chemiluminescence reagents (DuPont NEN Life Science Products, Boston, MA), according to the manufacturer's protocol. The levels of expressions were quantified using a densitometric scanning system. To calculate the ratio of mitochondrial/cytosolic cytochrome *c* (mit/cyt_{cyt.c}), quantification of autoradiographies was performed by computer densitometry in at least two different exposure times. The results were calculated as quotient of the mean density of the area measured by computer densitometry.

Kinase Activity Assay

Kinase assays were performed as described previously (31). Protein lysates were prepared as described above. In brief, 200 μ g of soluble protein were incubated with 1 μ g of anticyclin E (sc-248; Santa Cruz Biotechnology Inc.) at 4°C for 2 h. Immune complexes were then precipitated with 40 μ l of recombinant Protein A-agarose (Upstate Biotechnology, Waltham, MA) overnight at 4°C, washed three times with lysis buffer and twice with kinase assay 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 μ g/ml each aprotinin and leupeptin. The kinase assay was carried out by combining the washed protein beads with 20 μ l of kinase buffer plus 10 μ Ci of [γ -³²P]ATP, 15 μ M ATP, and 50 μ g/ml Histone H1 (catalogue no. 1140965 and catalogue no. 223 549, Boehringer Mannheim). The reaction was allowed to proceed for 20 min at 30°C and was terminated by adding 10 μ l of Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.2% 2-mercaptoethanol, 0.001% bromophenol blue) and boiling for 5 min. Products were resolved by 10% SDS-PAGE. The activity levels on autoradiographs were quantified using a densitometric scanning system.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from MKN-74 cells treated according to the above-mentioned treatment conditions with gemcitabine and flavopiridol by the cesium chloride method as

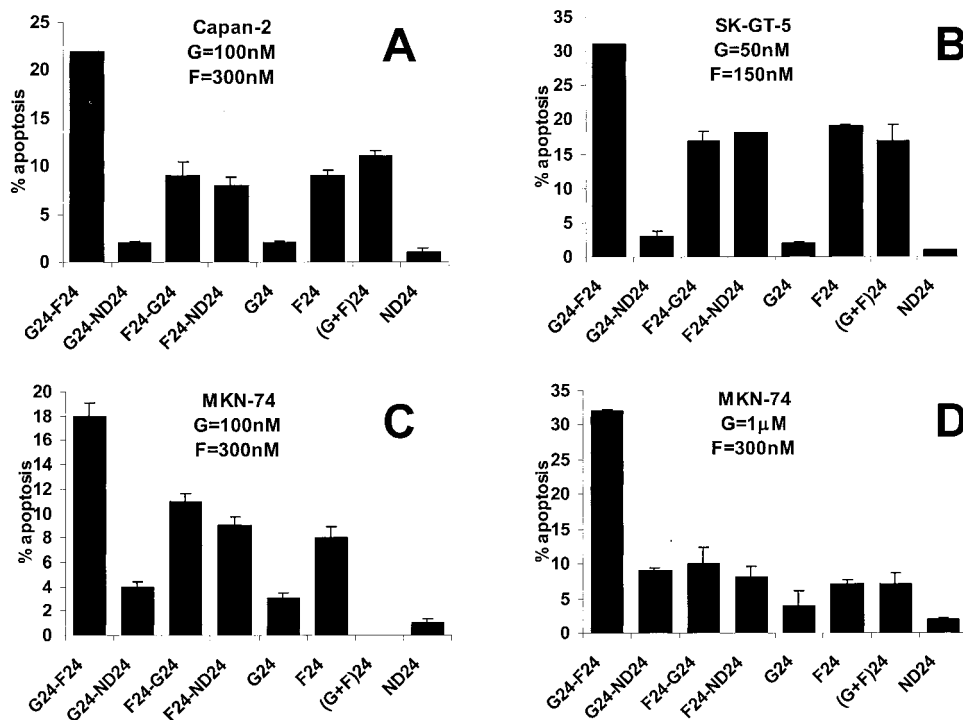


Fig. 1 Induction of apoptosis and dose dependency in gemcitabine- and flavopiridol-treated Capan-2 (A), SK-GT5 (B), and MKN-74 (C and D) cells. The cells were treated as described in "Materials and Methods." A, B, and C, all three cell lines show a sequential dependent 10–15-fold potentiation of apoptosis when gemcitabine (G_{24}) was followed by flavopiridol (F_{24}) compared with the other combinations or single drug treatment. C and D, an increase of the apoptotic rate can be achieved by an increase of the gemcitabine (G_{24}) concentration from 100 nM to 1 μ M in sequential treatment of MKN-74 cells with gemcitabine followed by flavopiridol (F_{24}).

described in current protocols in molecular biology. Twenty μ g of total RNA were then electrophoresed on a 1% agarose-phosphate buffer gel, blotted onto Hybond-N nylon membranes (Amersham), and RNA was cross-linked by an UV Stratilinker (120mJ; Stratagene). The membranes were hybridized with 32 P-labeled RR-M2 cDNA probe in Expresshyb hybridization solution (Clontech). The 1.17-kb human RR-M2 cDNA probe was obtained by *Bam*HI restriction from an *Escherichia coli*-based pCRII phagemid HRRM2 (American Type Culture Collection, Manassas, VA). The probe was labeled by random priming [32 P]-dCTP (50 μ Ci; NEN, Boston, MA) incorporation using a random-prime labeling kit (Amersham). The probe was purified by passing through Sephadex Quick Spin columns (Boehringer Mannheim).

mRNA Half-Life Assay

MKN-74 cells were treated with 100 nM of gemcitabine. After 24 h, the medium was replaced by either drug-free medium or 300 nM of flavopiridol-containing medium, both supplemented with 5 μ g/ml actinomycin D for another 3, 6, 12, or 24 h. Actinomycin D was used to prevent new initiation and continuous transcription of mRNA. After the experiment, RNA was extracted as described above.

Statistical Analysis

All of the experiments were performed 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 *t* test.

RESULTS

Flavopiridol Potentiates Apoptosis of Gemcitabine-treated Adenocarcinoma Cell Lines. To study the ability of flavopiridol on gemcitabine-treated cells to potentiate apoptosis, cells were treated with gemcitabine and flavopiridol with various schedules. As shown in Fig. 1, flavopiridol potentiates gemcitabine-induced apoptosis in Capan-2 (Fig. 1A), SK-GT-5 (Fig. 1B), and MKN-74 cells (Fig. 1, C and D) to a various degree as assessed by QFM. This potentiation appears to be sequence dependent such that gemcitabine followed by flavopiridol achieves the greatest potentiation of apoptosis. Capan-2 cells (Fig. 1A) treated with gemcitabine alone ($G_{24} \rightarrow ND_{24}$) or flavopiridol (F_{24}) underwent apoptosis in $2 \pm 0.2\%$ and $9 \pm 0.6\%$, respectively. However, when cells were treated sequentially with gemcitabine followed by flavopiridol ($G_{24} \rightarrow F_{24}$), $22 \pm 0.01\%$ of the cells underwent apoptosis ($P < 0.01$ compared with $G_{24} \rightarrow ND_{24}$ and $P = 0.03$ compared with F_{24}). The reverse sequence of flavopiridol followed by gemcitabine ($F_{24} \rightarrow G_{24}$) resulted in only $9 \pm 1.4\%$ apoptosis ($P < 0.01$ compared with $G_{24} \rightarrow F_{24}$). In Capan-2 cells treated with the concomitant combination of gemcitabine and flavopiridol [(G+F) $_{24}$], an apoptotic rate of $11 \pm 0.7\%$ was achieved ($P = 0.04$ compared with $G_{24} \rightarrow F_{24}$). Similar results were obtained for SK-GT-5 and MKN-74 cells, showing a significant potentiation if gemcitabine was followed by flavopiridol.

Because micromolar concentrations of gemcitabine are clinically achievable, we elected to treat MKN-74 cells with both micromolar (1 μ M; Fig. 1D) and nanomolar (100 nM; Fig. 1C) concentrations of gemcitabine and a fixed concentration of flavopiridol (300 nM). Sequential treatment of MKN74 cells

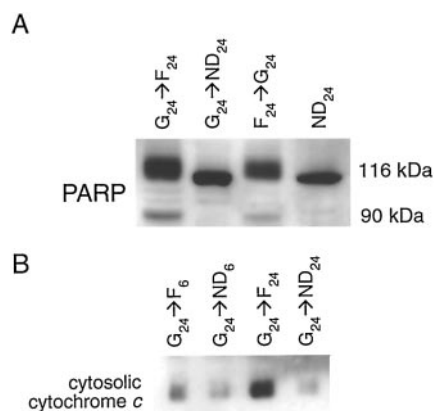


Fig. 2 PARP cleavage (A) and cytochrome *c* release (B) after treatment with gemcitabine and flavopiridol. Cells were treated with 100 nM gemcitabine (G) followed by either 300 nM flavopiridol (F) or no drug (ND) or with the reverse sequence (F→G) for the indicated time (6 or 24 h). After treatment cell extracts were prepared and analyzed by immunoblotting as described in “Materials and Methods.” A, cleavage of PARP to the apoptotic fragment (M_r 90,000) in Capan-2 cells, suggesting sequential therapy with $G_{24} \rightarrow F_{24}$ as the most effective combination. B, amount of cytosolic (cyt. c_{cyt}) in MKN-74 cells after treatment with gemcitabine followed by 6 or 24 h of flavopiridol. The ratios of mitochondrial to cytosolic cytochrome *c* show a 25-fold increase of cytochrome *c* release in $G_{24} \rightarrow F_{24}$ versus $G_{24} \rightarrow ND_{24}$, whereas there was only a 6-fold increase after treatment with $G_{24} \rightarrow F_6$ compared with $G_{24} \rightarrow ND_6$. The greatest cytochrome *c* release is observed in $G_{24} \rightarrow F_{24}$.

with an increased dose of 1 μM of gemcitabine followed by 300 nM of flavopiridol ($G_{24} \rightarrow F_{24}$) resulted in a higher rate of apoptotic cells ($32 \pm 0.2\%$) compared with treatment with 100 nM of gemcitabine followed by 300 nM of flavopiridol ($18 \pm 1.0\%$; $P = 0.038$). Interestingly, all of the other combinations with 1 μM of gemcitabine did not increase apoptotic rates compared with lower gemcitabine concentration.

Induction of Apoptosis Is Confirmed by PARP Cleavage and Mitochondrial Cytochrome *c* Release. To demonstrate that combination therapy results in higher induction of apoptosis, we analyzed the cleavage of the PARP, a substrate cleaved by caspases during apoptosis. As shown in Fig. 2A, in capan-2 cells, 24 h of 100 nM gemcitabine ($G_{24} \rightarrow ND_{24}$) treatment did not result in the PARP cleavage. Similar to our previous studies (26, 27), flavopiridol alone did not induce PARP cleavage (data not shown). However, sequential treatment of cells with gemcitabine followed by flavopiridol ($G_{24} \rightarrow F_{24}$) enhanced the PARP cleavage. In fact, as shown in Fig. 2A, a strong M_r 90,000 band developed in all of the gemcitabine/flavopiridol combinations ($G_{24} \rightarrow F_{24}$, $F_{24} \rightarrow G_{24}$). In keeping with this observation, sequential $G_{24} \rightarrow F_{24}$ induced the greater degree of apoptosis; the M_r 90,000 PARP cleavage product was increased 3-fold in the sequential therapy of $G_{24} \rightarrow F_{24}$ compared with $F_{24} \rightarrow G_{24}$, as determined by computer densitometry.

Fig. 2B shows the amount of cytosolic cytochrome *c* after treatment with G_{24} followed by flavopiridol or drug-free medium for 6 or 24 h. Compared with $G_{24} \rightarrow ND_6$, there was a 6-fold increase of cytosolic cytochrome *c* after addition of flavopiridol for 6 h. However, if flavopiridol was added for 24 h

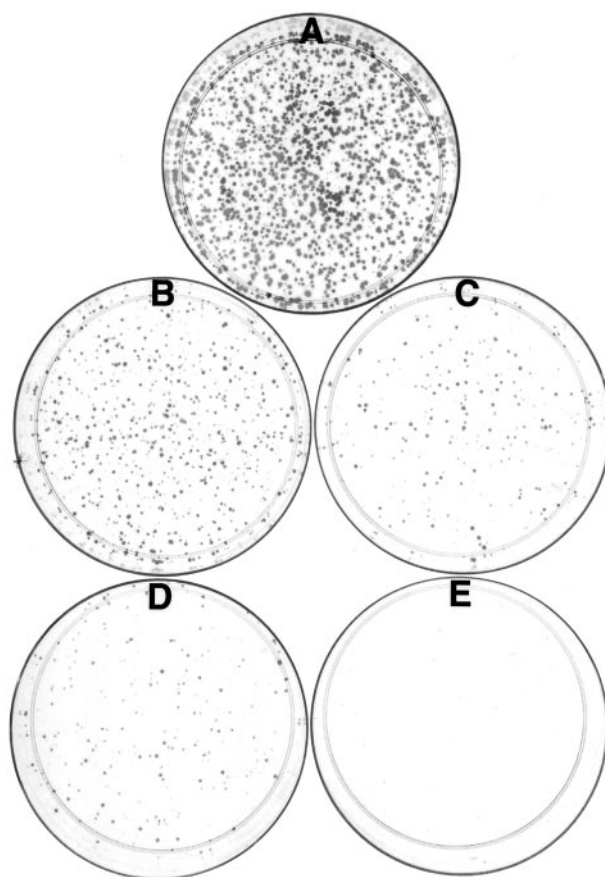


Fig. 3 Effect of treatment with gemcitabine and flavopiridol on colony formation in HCT-116 cells. Cells were treated, fixed, and analyzed as indicated in “Materials and Methods.” Compared with untreated cells (A), treatment with gemcitabine alone (B) or flavopiridol alone (C) reduced formation of colonies to $51 \pm 2\%$ and $19 \pm 1\%$, respectively. Colonies after single drug treatment (B and C) appeared to be smaller than in untreated plate (A). Combination treatment with gemcitabine and flavopiridol (D) resulted in an additional decrease of colony formation to $13 \pm 1\%$. Only in treatment with sequential gemcitabine followed by flavopiridol (E) was colony formation completely suppressed.

after pretreatment with 24 h of gemcitabine, there was a 25-fold increase of cytochrome *c* as compared with the $G_{24} \rightarrow ND_{24}$ control. This would confirm the greater enhancement of apoptosis by the sequential gemcitabine followed by flavopiridol combination.

Potential of Apoptosis by Flavopiridol Correlates with Suppression in Colony Formation. To confirm this data and to differentiate between cytotoxic and cytostatic effects of the drug combination, a colony formation assay was instituted. If only cytostatic effects of the drugs or the drug combinations predominate, delayed but additional growth of the colonies should be expected. To study the effect of the combination and sequential therapy, single drug concentrations were chosen that did not suppress colony formation completely. Thus, the gemcitabine concentration was lower than those used in the apoptotic assay. Fig. 3 shows the effect on colony formation in HCT116 cells after treatment with 10 nM of gemcitabine and 150 nM of flavopiridol. Compared with $100 \pm 1\%$ of colony

formation in untreated cells (no drug; Fig. 3A), treatment with gemcitabine alone (G_{24} ; Fig. 3B) or flavopiridol alone (F_{24} ; Fig. 3C) reduced formation of colonies to $51 \pm 2\%$ and $19 \pm 3\%$, respectively. On average, colonies after single drug treatment (G_{24} or F_{24}), especially after flavopiridol, appeared to be smaller than in untreated cells. Combination treatment with $(G + F)_{24}$ resulted in an additional decrease of colony formation to $13 \pm 1\%$ (Fig. 3D). However, the most dramatic effect was observed with sequential $G_{24} \rightarrow F_{24}$, where colony formation was completely suppressed (Fig. 3E).

The Lower Efficacy of the Reverse Sequence Is Supported by [3H]dThd Incorporation. Flavopiridol is known to arrest cells in G_1 or G_2 via inhibition of cyclin/cdks. It has been shown that the ability of a cell to incorporate gemcitabine is linked to its ability to enter the S phase of the cell cycle. This would suggest that a treatment preventing the S entry, such as pretreatment with flavopiridol (F_{24}), may result in a less efficient gemcitabine incorporation. This would at least in part explain the lower apoptotic rates that were achieved with the reverse sequence ($F_{24} \rightarrow G_{24}$). To test this hypothesis we instituted a [3H]dThd incorporation assay. Similar to gemcitabine, thymidine is incorporated into DNA during S phase. Before gemcitabine and [3H]dThd, cells were exposed to either flavopiridol or no drug-containing medium. There was a significantly ($P = 0.003$) reduced [3H]dThd incorporation after earlier treatment with flavopiridol, reducing its incorporation to 54% in ($F_{24} \rightarrow G_{24}$) as compared with ($ND_{24} \rightarrow G_{24}$).

Potentiation of Apoptosis Is Associated with a Down-Regulation of RR-M2 Subunit. It has been shown that gemcitabine-induced resistance is associated with an up-regulation of RR-M2 mRNA and protein levels (12). This will allow the cell to overcome the gemcitabine-induced down-regulation of RR function and, therefore, decrease the efficacy of gemcitabine in tumor treatment. To study the mechanism of potentiation of apoptosis by flavopiridol on gemcitabine-treated cells, we examined the protein levels of RR-M2 in MKN-74 and SK-GT-5 cells. As shown in Fig. 4A, treatment with flavopiridol as a single agent, as well as in combinations, resulted in a down-regulation of RR-M2 protein levels in both cell lines. In SK-GT-5 this down-regulation is complete in all of the flavopiridol-containing combinations or single drug treatments. In MKN-74, the extent of RR-M2 protein suppression with combination therapy is greatest for $G_{24} \rightarrow F_{24}$. Compared with $G_{24} \rightarrow ND_{24}$ only 30% protein expression is observed in $G_{24} \rightarrow F_{24}$. However, there is less down-regulation in the reverse or the concomitant treatment compared with SK-GT-5 cells. Furthermore, we observed a 1.5-fold up-regulation of RR-M2 protein in MKN-74 after single drug treatment with gemcitabine compared with untreated cells.

To study whether the RR-M2 protein down-regulation is caused by a transcriptional down-regulation of RR-M2 mRNA, a Northern blot analysis was instituted. As described previously, human RR-M2 mRNA consists of two different species that differ in length. The 1.6-kb and the 3.4-kb mRNA are attributable to the alternate use of different polyadenylation signals, leading to various length of 3' noncoding sequences (9, 32). The results for MKN-74 cells are shown in Fig. 4B. As shown for the protein levels, flavopiridol, as a single agent as well as in combination treatment, results in a down-regulation of RR-M2

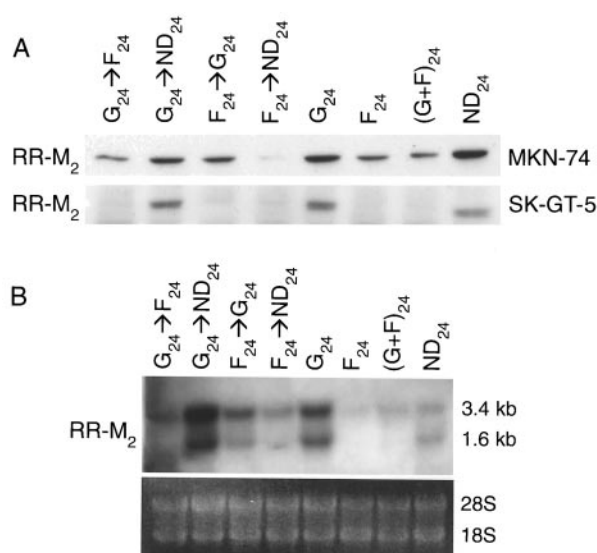


Fig. 4 Protein and mRNA levels of RR-M2 subunit after treatment with gemcitabine and flavopiridol. **A**, Western blot analysis in MKN-74 and SK-GT5 cells. Treatment with flavopiridol as a single agent as well as in combinations results in a down-regulation of RR-M2 protein in both cell lines. In SK-GT5, this down-regulation is complete in all flavopiridol-containing combinations or single-drug treatments. In MKN-74, the extent of RR-M2 protein after combination therapy with gemcitabine and flavopiridol is greatest for $G_{24} \rightarrow F_{24}$. Furthermore, there is an up-regulation of RR-M2 protein in MKN-74 after single-drug treatment with gemcitabine compared with untreated cells. **B**, Northern blot analysis of RR-M2 mRNA in MKN-74 cells. As shown for the protein levels, flavopiridol as a single agent as well as in combination treatment results in a down-regulation of RR-M2 mRNA. The greatest down-regulation after combination therapy with gemcitabine and flavopiridol is achieved with $G_{24} \rightarrow F_{24}$ compared with $G_{24} \rightarrow ND_{24}$. mRNA levels are increased after treatment with gemcitabine alone compared with untreated cells.

mRNA. The greatest down-regulation after combination therapy with gemcitabine and flavopiridol is achieved with $G_{24} \rightarrow F_{24}$ compared with $G_{24} \rightarrow ND_{24}$ (5-fold). The mRNA levels are increased after treatment with gemcitabine alone compared with untreated cells (3–5-fold). In the reverse sequence ($F_{24} \rightarrow G_{24}$), RR-M2 mRNA is 2-fold increased compared with $F_{24} \rightarrow ND_{24}$ suggesting an up-regulation by gemcitabine despite prior flavopiridol. These results support the sequence dependency of the combination of flavopiridol and gemcitabine.

Because the down-regulation of RR-M2 mRNA could result from a decreased mRNA stability, the mRNA half-life of RR-M2 was studied for ($G_{24} \rightarrow F_{24}$), because this was the most efficient treatment in terms of its ability to down-regulate RR-M2. Cells were treated with gemcitabine for 24 h followed by either flavopiridol or no drug-containing medium for another 3, 6, 12, or 24 h. To prevent new transcription of RR-M2 mRNA, actinomycin D was added during the second part of the treatment. There was no difference in mRNA stability after flavopiridol treatment compared with no treatment. However, mRNA levels decreased ~70% after 24 h of both treatments. Glycerinaldehyde-3-phosphate dehydrogenase mRNA half-life was used as a control.

These results suggest that the potentiation of gemcitabine-

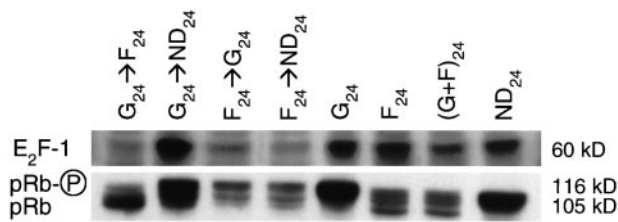


Fig. 5 Immunoblot analysis of E₂F-1 transcription factor and pRb in MKN-74 cells. Flavopiridol treatment results in a hypophosphorylation of pRb if cells are treated with F₂₄, (G + F)₂₄, or G₂₄→F₂₄ compared with untreated or gemcitabine-treated cells. This hypophosphorylation is greatest in the sequential treatment with G₂₄→F₂₄, whereas gemcitabine treatment shows the greatest amount of hyperphosphorylated pRb. Furthermore, a down-regulation of E₂F-1 and pRb protein levels can be observed in all flavopiridol combinations.

induced apoptosis by flavopiridol is associated with a down-regulation of RR-M2 protein as well as mRNA levels. Furthermore, the down-regulation is not caused by a decreased mRNA stability but appears to be truly transcriptional in nature. In MKN-74, this down-regulation appears to be sequence-dependent and, thus, correlates with the induction of apoptosis.

Transcriptional Down-Regulation of RR-M2 mRNA Is Associated with a Flavopiridol-dependent Hypophosphorylation of pRb and with the Down-Regulation of Cell Cycle Regulating Proteins including pRb, E2F-1, Cyclin D1, and Cyclin E by Proteasome Degradation. It has been shown that RR-M2 is an E2F-1-regulated gene (33). During late G₁, pRb is hyperphosphorylated by the cyclin E/cdk2 kinase and releases E2F-1 from the E2F-1/pRb complex. Free E2F-1 binds to the DNA and initiates transcription of RR-M2. To explain the down-regulation of RR-M2 mRNA, we studied the effect of the sequential treatment with (G₂₄→F₂₄) on E2F-1, pRb, and other cell cycle proteins involved in the regulation of the G₁ and S phase of the cell cycle.

Fig. 5 shows an immunoblot analysis of E₂F-1 and pRb in MKN-74 cells. Flavopiridol treatment results in a hypophosphorylation of pRb if cells are treated with F₂₄, (G + F)₂₄, or G₂₄→F₂₄ compared with untreated or gemcitabine treated cells. The hypophosphorylation of pRb is greatest in the sequential treatment with G₂₄→F₂₄, as indicated by the complete loss of the top M_r 116,000 band and an increase of the lower bands (M_r ~105,000). Treatment with gemcitabine alone shows the greatest amount of hyperphosphorylated pRb. Furthermore, a down-regulation of E2F-1 and pRb protein levels was observed in all of the combination treatments containing flavopiridol. Because E2F-1 and pRb are regulated by the ubiquitin/proteasome pathway, we studied the effect of PS341, a proteasome inhibitor, on the expression of E2F-1, RR-M2 and pRb in MKN-74 cells treated with G₂₄→F₂₄. As shown in Fig. 6A, the expression levels of all three proteins increase 2–3-fold after addition of PS341 during treatment with flavopiridol [G₂₄→(F+PS341)₂₄]. This would indicate that flavopiridol enhances degradation of E2F-1 and pRb via the ubiquitin/proteasome system. Because RR-M2 synthesis depends on E2F-1, restoration of the E2F-1 protein could increase the levels of RR-M2 protein via up-regulation of RR-M2 mRNA. To test this hypothesis, we instituted a Northern blot analysis for RR-M2 mRNA after treatment

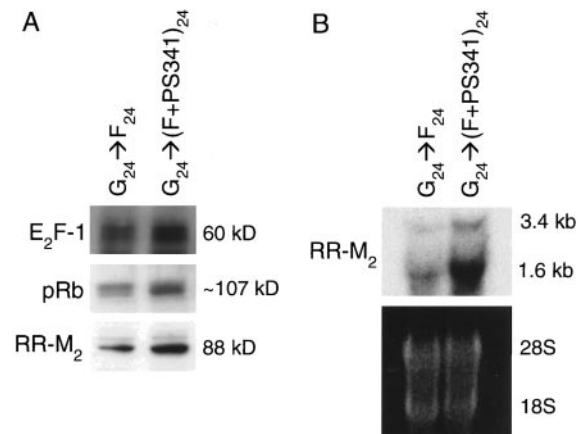


Fig. 6 The proteasome inhibitor PS341 restores E2F-1 and pRb protein as well as RR-M2 protein and mRNA levels that were down-regulated by flavopiridol treatment in MKN-74 cells. **A**, Western blot analysis of E2F-1, pRb, and RR-M2 protein after treatment with G₂₄→F₂₄ or G₂₄→(F + PS341)₂₄. All three proteins are up-regulated after addition of PS341 during treatment with flavopiridol. **B**, PS341 restored RR-M2 mRNA expression. Northern blot analysis of RR-M2 mRNA levels. MKN-74 cells were treated with G₂₄→F₂₄ or G₂₄→(F + PS341)₂₄. To achieve this effect, a lower flavopiridol concentration of 150 nM was administered together with a higher PS341 concentration of 30 nM.

of MKN-74 with G₂₄→F₂₄ and G₂₄→(F + PS341)₂₄. As shown in Fig. 6B, PS341 restored RR-M2 mRNA expression. These results suggest that RR-M2 mRNA and protein levels are controlled by the E2F-1/pRb transcription system. The hyperphosphorylation of pRb is mediated by cyclin D/cdk2, 4, and 6, as well as cyclin E/cdk2. Western blot analysis (Fig. 7A) shows a down-regulation of cyclin D₁ and cyclin E protein levels after sequential treatment with G₂₄→F₂₄ compared with G₂₄→ND₂₄, whereas cdk2 protein levels are not affected. This down-regulation can be antagonized by addition of PS341 during treatment with flavopiridol [G₂₄→(F+PS341)₂₄]. Furthermore, as shown in Fig. 7B, flavopiridol down-regulates cyclin E/cdk2 kinase activity after treatment with G₂₄→F₂₄ compared with G₂₄→ND₂₄ or untreated cells. The decreases in cyclin E/cdk2 as well as in cyclin D/cdk4 and 6 kinase activities would result in the hypophosphorylation of pRb. Because E2F-1 binds to hypophosphorylated pRb, this would contribute to the additional sequestration of E2F-1. Collectively, these results indicate that flavopiridol on gemcitabine-treated cells results in sequestering of E2F-1 by two pathways: (a) increased proteasome mediated degradation; and (b) pRb hypophosphorylation. The end result is down-regulation of RR-M2 transcription, and this may explain the enhancement of gemcitabine-induced apoptosis by flavopiridol.

DISCUSSION

In this study we show that flavopiridol potentiates gemcitabine-induced apoptosis in a sequence-dependent manner in pancreatic (Capan-2), as well as colorectal (HCT-116) and gastric cancer (MKN-74 and SK-GT-5) cells. Sequential treatment of gemcitabine followed by flavopiridol (G₂₄→F₂₄) results in a 10–15-fold increase of apoptotic rates of these adenocarcinoma

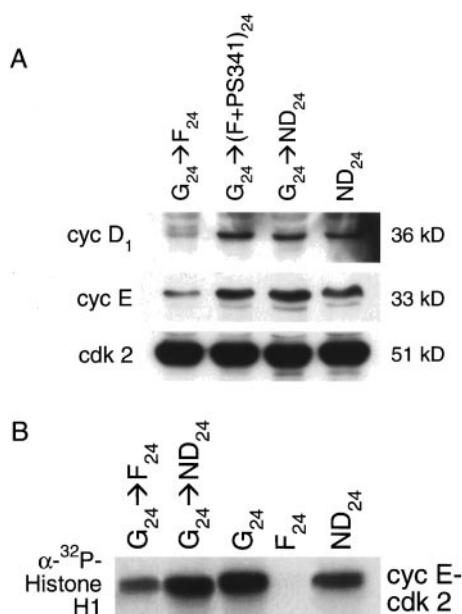


Fig. 7 Effects of flavopiridol treatment on cell cycle regulating proteins and kinases in MKN-74 cells. **A**, Western blot analysis shows a down-regulation of cyclin D₁ and cyclin E protein levels after sequential treatment with G₂₄→F₂₄ compared with G₂₄→ND₂₄, whereas cdk2 is not affected. This down-regulation can be antagonized by addition of PS341 during treatment with flavopiridol. **B**, cyclin E/cdk2 kinase activity in HCT-116. Cells were treated as indicated in “Materials and Methods” with gemcitabine, flavopiridol, or combinations of both drugs. Protein lysates were analyzed for their ability to phosphorylate the substrate histone H1. Addition of flavopiridol results in a down-regulation of Cyclin E/cdk2 activity, whereas treatment with gemcitabine results in an up-regulation compared with untreated cells.

cell lines, as compared with treatment with gemcitabine alone. This is greater than the potential of flavopiridol to increase induction of apoptosis in mitomycin- and paclitaxel-treated gastric (MKN-74) and breast (MDA-MB-468 and MCF-7) cancer cells (26, 27).

Clinically, micromolar concentrations of gemcitabine can be achieved (34). In our studies, this effect was dose-dependent such that a micromolar concentration of gemcitabine resulted in the greatest apoptotic rates, especially in sequential treatment of G₂₄→F₂₄. The fact that nanomolar concentrations of gemcitabine could also induce apoptosis and suppress colony formation would suggest that lower concentrations of gemcitabine could be used in combination with flavopiridol and still improve its clinical efficacy.

During apoptosis signaling, mitochondrial damage occurs at an early stage, resulting in release of cytochrome *c*, which is an upstream activator of caspases (35). Cytochrome *c* release was observed in all of the gemcitabine and flavopiridol combinations and was not observed in untreated or single drug-treated cells. Treatment with G₂₄→F₂₄ resulted in the highest release. Interestingly, there was only a minor degree of cytochrome *c* release for treatment schedules administering flavopiridol for 6 h. This indicates that the potentiation of apoptosis by flavopiridol *in vitro* is temporally related to the duration of flavopiridol exposure. This effect is greatest with >6 h (*i.e.*, 24 h in our

study) of flavopiridol treatment and shows the dynamics by which apoptosis is induced over time.

Recently it has been shown that RR overexpression is a major mechanism of gemcitabine resistance (12). Yet, it is not fully understood how gemcitabine influences this pathway. The increase in RR-M2 induced by gemcitabine in these studies is quite rapid. We observed a 3–5-fold up-regulation of RR-M2 mRNA, as well as a 1.5-fold up-regulation of M2 protein levels after only 24 h of gemcitabine treatment.

Flavopiridol on gemcitabine treated cells induces a transcriptional down-regulation of the RR-M2 subunit. As shown by actinomycin D studies, RR-M2 mRNA stability was not influenced by flavopiridol. However, under other treatment conditions, flavopiridol has been associated with a dramatic decrease in VEGF mRNA stability under hypoxic conditions (36) and with cyclin D1 down-regulation (29). In our system, the down-regulation of RR-M2 can be attributed to the inhibition of the E2F-1/pRb transcription system. In addition, there may be loss of RR-M2 protein by its direct degradation via the ubiquitin/proteasome system.

Interestingly the RR-M2 mRNA expression after treatment with gemcitabine alone (G₂₄→ND₂₄) was ~5-fold higher than after G₂₄→F₂₄, whereas the M2 protein expression after G₂₄→ND₂₄ was only 1.5-fold higher than after addition of flavopiridol (G₂₄→F₂₄). This correlates with observations in wild-type and gemcitabine-resistant human oropharyngeal KB cells (12) as well as in hydroxyurea-resistant clones of this cell line (10). A 9-fold higher degree of expression of RR-M2 mRNA and a 2-fold higher expression of protein levels have been observed in the resistant cell line compared with the wild-type cell line (10, 12). This mRNA up-regulation has been shown for RR-M2 but not for RR-M1, which remains nearly constant for all of the cell lines (12). The decrease of mRNA and protein we achieved with addition of flavopiridol on gemcitabine-treated cells was comparable with the level of RR mRNA and protein expression shown by Goan *et al.* (12) in gemcitabine-sensitive KB cells. This again supports the model that flavopiridol sensitizes cells to gemcitabine by down-regulating RR-M2 levels. The fact that RR is also increased in hydroxyurea-resistant cells indicates that suppression of RR by flavopiridol may result in enhancing the sensitivity to hydroxyurea as well.

To test that the down-regulation of RR-M2 mRNA with G₂₄→F₂₄ was at least contributing to the induction of apoptosis, we elected to restore RR-M2 mRNA by preventing E2F-1 protein degradation with the ubiquitin/proteasome inhibitor PS341. Unfortunately, under these treatment conditions, PS341 as a single agent also induces significant apoptosis in these cell lines (data not shown). Therefore, although RR-M2 mRNA and protein were restored by PS-341 in the sequential therapy of G₂₄→F₂₄, we were unable to appreciate a significant reversal in apoptosis in the PS-341-treated cells.

These results do not address other mechanisms by which flavopiridol may enhance sensitization to gemcitabine including deoxycytidine deaminase, DNA polymerase, or an decreased DCK activity based on the expanded dNTP pool after up-regulation of RR. The latter would decrease the efficacy of gemcitabine phosphorylation. Although these mechanisms may be involved in the effect of flavopiridol on gemcitabine-treated

cells, these studies are beyond the scope of this study. Furthermore, flavopiridol has been shown to potentiate apoptosis in different cell lines and with different chemotherapeutic agents (20–22, 25–28, 31). Therefore, this is not the only mechanism by which flavopiridol may enhance chemotherapy-induced apoptosis.

In this study we show that flavopiridol enhances the toxicity of gemcitabine by enhancing the induction of apoptosis. Gemcitabine alone remains a drug with only modest levels of clinical activity in gastrointestinal cancers. Therefore, the use of flavopiridol in combination with gemcitabine may provide a novel means to increase its activity, even at nanomolar concentrations of the drug. On the basis of these results, clinical trials with sequential therapy of gemcitabine followed by flavopiridol have now been initiated.

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