Selective Sensitization of Transformed Cells to Flavopiridol-induced Apoptosis following Recruitment to S-Phase

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ABSTRACT

Flavopiridol is a potent inhibitor of cyclin-dependent kinases (cdks). In a large proportion of solid tumor cell lines, the initial response to flavopiridol is cell cycle arrest, NCI-H661 non-small cell lung cancer cells are representative of a subset of more sensitive cell lines in which apoptosis is observed during the first 24 h of drug exposure. Analysis of the apoptotic population indicates that cells in S-phase are preferentially dying. In addition, cells are sensitized to flavopiridol following recruitment to S-phase, whether accomplished by synchronization or by treatment with noncytotoxic concentrations of chemotherapeutic agents that impose an S-phase delay. Combinations of gemcitabine or cisplatin, followed by flavopiridol at concentrations that correlate with cdk inhibition, produce sequence-dependent cytotoxic synergy. A survey of paired cell lines, including WI38 diploid fibroblasts or normal human bronchial epithelial cells, along with their SV40-transformed counterparts, demonstrates that treatment with flavopiridol during S-phase is selectively cytotoxic to transformed cells. These data suggest that treatment during S-phase may maximize responses to flavopiridol and that the administration of flavopiridol after chemotherapy agents that cause S-phase accumulation may be an efficacious antitumor strategy.

INTRODUCTION

Cyclin-dependent kinases (cdks)³ comprise a family of enzymes that are the core components of the cell cycle machinery (1). ORDERLY cell cycle progression requires the scheduled activity of the cdks, governed in part by their association with cyclins, as well as by their state of phosphorylation. Cyclin D-dependent kinases 4 and 6, as well as cyclin E-cdk2 complexes, sequentially phosphorylate the product of the Rb susceptibility gene, Rb, to facilitate the G1-S transition (2). Cyclin A-cdk2 and cyclin B-cdk1 complexes are required for orderly S-phase progression and the G2-M transition, respectively (3). In addition, two groups of inhibitors, known as the Cip/Kip proteins and the INK4 proteins, also regulate cdk activity (4). Deregulated cdk activity is a hallmark of human cancer. Commonly, genetic and epigenetic events result in overexpression of cyclins or absent or diminished levels of cdk inhibitors, providing tumor cells with a selective growth advantage (5, 6). Ectopic expression of cdk inhibitors in tumor cell lines usually results in cell cycle arrest in G1 or G2 or both, and this has translated into therapeutic benefit in xenograft models with slowed tumor growth and improved host survival. In addition, in some cell systems, ectopic cdk inhibitor expression has produced cytotoxicity. For example, adenoviruses encoding p27Kip1 or p16INK4A have induced apoptotic cell death in vitro and tumor regression in vivo (reviewed in Ref. 7). These observations suggest that cdks are an attractive set of targets for novel antineoplastic drugs and have prompted great interest in the development of pharmacological cdk inhibitors that could produce similar antitumor effects.

Flavopiridol is the first potent small molecule inhibitor of cdks to reach clinical trial. The drug is structurally related to a natural alkaloid originally purified from the extract of the stem bark of Dysoxylum binecatiferum, a plant native to India. Flavopiridol inhibits multiple cdks, including cdc2 (cdk1), cdk2, cdk4, cdk6, and cdk7, as it has been shown to directly inhibit the kinase activity of cdk immune precipitates from exponentially growing cells with IC50s of 100–400 nM (8, 9). Structural studies have demonstrated that the aromatic portion of flavopiridol interferes with binding of ATP to the adenine-binding pocket of the cdks (10). Although other protein kinases are also inhibited, including protein kinase C and the epidermal growth factor receptor, the IC50s are 10 μM or greater, indicating that flavopiridol is specific for cdks at the nanomolar concentrations achieved in vivo. Furthermore, inhibition of cdk7 prevents the phosphorylation events necessary for activating the other cdks, so that the inhibition of cdks 1, 2, 4, and 6 is both direct and indirect. Transcriptional repression of cyclin D1 by flavopiridol also contributes indirectly to the inhibition of cdks 4 and 6 (11).

The responses to flavopiridol of many hematopoietic and epithelial cell lines have been characterized (12–15). Our previous studies focused on NSCLC cell lines (16). These are representative of a large proportion of solid tumor cell lines in which flavopiridol causes arrest at both the G1 and G2 phases of the cell cycle, consistent with its inhibition of cdks 2, 4, 6, and 1. This occurs within 24 h of drug exposure at concentrations as low as 200 nM. In addition, cytotoxic effects have been observed, resulting from p53-independent apoptosis. However, cell death usually follows cell cycle arrest and is delayed, maximally occurring at 72 h after the initiation of treatment (17). Cytotoxicity occurs to a small degree at concentrations approximating 300 nM and is more marked at concentrations of 500 nM and above. Flavopiridol has also been found to be cytotoxic to noncycling tumor cells (17). These observations raise the possibility that the primary response of many solid tumor cell lines to flavopiridol-mediated cdk inhibition is cytostatic growth arrest, and that delayed cytotoxicity is mediated by the effects of high concentrations of drug on other possible cellular targets.

In contrast, some solid tumor cell lines are more sensitive to flavopiridol. For example, treatment of head and neck squamous cell carcinoma cell lines (18) or NCI-H661 NSCLC cells (16) with 300 nM flavopiridol results in a decrease in S-phase that is accompanied by the onset of apoptosis within the first 24 h of drug exposure. Although cell cycle arrest and apoptosis may be occurring concomitantly, it is also possible that the predominant response of these cell lines is the preferential death of the S-phase population rather than G1 arrest. Here, we have analyzed the apoptotic population 24 h after flavopiridol treatment of NCI-H661 cells and have found this to be the case. In addition, these cells are further sensitized to flavopiridol if they are first recruited to S-phase before drug exposure. In these experiments, S-phase recruitment was achieved by release after hydroxyurea-induced synchronization at the G1-S boundary or by treatment with noncytotoxic doses of chemotherapy agents capable of imposing an
S-phase delay. S-phase recruitment prior to flavopiridol exposure similarly sensitizes A549 cells, which initially respond to flavopiridol alone with G1 arrest (16, 17). The data indicate sequence-dependent synergism between standard chemotherapy agents and concentrations of flavopiridol achievable in vivo that are associated with cdk inhibition. Moreover, we demonstrate that apoptosis mediated by flavopiridol during S-phase is selective for transformed cells. These results indicate that treatment during S-phase may maximize cytotoxic responses to flavopiridol, and that combinations with chemotherapy drugs that induce S-phase accumulation represent a promising avenue for the future clinical development of flavopiridol and other small molecule cdk inhibitors.

MATERIALS AND METHODS

Cell Culture. NCI-H661 and A549 NSCLC cell lines, WI38 human diploid fibroblasts, the SV40 virus-transformed subline W138/VA13, and the SV40 virus-immortalized human bronchial epithelial cell line BEAS-2B (19) were obtained from the American Type Culture Collection (Rockville, MD). NHBE cells at first passage were obtained from the Clonetics division of BioWhittaker (San Diego, CA and Walkersville, MD). NHBE and BEAS-2B were maintained in bronchial epithelial cell growth medium, commercially supplied by Clonetics. All other cell lines were maintained in DMEM supplemented with 10% bovine calf serum.

Drug Treatment. Cells were synchronized at the G1-S boundary by treatment with 1 mM hydroxyurea (Sigma Chemical Co.-Aldrich Co., St. Louis, MO) for 24 h. Mitotic arrest was achieved by treatment with 0.4 μM nociocodazole (Sigma Chemical Co.-Aldrich Co.) for 16 h. Flavopiridol was provided by Dr. Edward Sausville of the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. A 50 mM stock was prepared in DMSO and stored at −70°C. Drug was diluted in medium and used at final concentrations ranging from 150 to 500 nM. A 1 mg/ml aqueous solution of cisplatin was obtained from Eli Lilly and Co. (Indianapolis, IN) and was freshly reconstituted before each experiment in 0.9% NaCl to create a 100 mM stock solution. After filter sterilization, the stock solution was diluted in medium to final concentrations ranging from 10 nM to 30 μM. Concentrations of cisplatin and gemcitabine chosen for each cell line were those that were nontoxic and produced S-phase accumulation within 24 h. Cells were plated 24–30 h before drug treatment and were at subconfluent density at the time of drug exposure. In the case of sequential drug treatments, NHBE and BEAS-2B cells were rinsed in HBSS, the recommended buffer for these cells, and all other cells were rinsed in PBS before application of the second drug.

Fluorescence-activated Cell Sorting Analysis. For analysis of DNA content, adherent cells were collected by trypsinization, combined with nonadherent cells, washed, and resuspended in 1 ml of PBS. An additional 1 ml of 80% ethanol was added, and cells were fixed overnight at 4°C. Fixed cells were centrifuged and resuspended in 0.5 ml of 500 μg/ml RNase A and incubated for 45 min at 37°C. Cells were centrifuged and resuspended in 1 ml of 69 μM propidium iodide in 38 mM sodium citrate and incubated at room temperature for a minimum of 30 min. Cells were analyzed for DNA content by flow cytometry using the ModFit program (Verity Software House, Topsham, ME).

Detection of Apoptosis by Flow Cytometry. A fluorescein apoptosis detection system was used (Promega Corp., Madison, WI). Briefly, after drug treatment, adherent cells were released by trypsinization, combined with nonadherent cells, collected by centrifugation, rinsed, and resuspended in 0.5 ml of PBS. Cells were fixed by adding 5 ml of 1% methanol-free formaldehyde for 20 min on ice. Cells were collected by centrifugation, rinsed twice, and resuspended in 0.5 ml of PBS and added to 70% ice-cold ethanol. After ethanol fixation, cells were rinsed and resuspended in PBS, and 5 × 10^5 cells were transferred to a conical tube. After centrifugation, the pellet was equilibrated in the manufacturer’s buffer and incubated with fluorescein-12-dUTP in the presence or absence of TdT for 90 min at 37°C, with protection from direct light exposure. The reaction was terminated by adding 1 ml of 20 mM EDTA.

Cells were washed twice in PBS containing 5 mg/ml BSA and 0.1% Triton X-100, and the cell pellet was finally resuspended in 0.5 ml of PBS containing 5 μg/ml propidium iodide and 50 μg/ml RNase A. After a 30-min room temperature incubation in the dark, cells were analyzed by two-color flow cytometry. Apoptosis was quantitated by the percentage of the population shifting to fluorescein positivity in the presence of TdT. For TUNEL assays, fluorescein intensity is shown on the X axis, and propidium iodide intensity is shown on the Y axis. When multiple experiments were used to generate graphs, error bars represent the SD.

BrdU Analysis. Flavopiridol-treated cells were pulse-labeled with BrdU for 2 h before collection. After BrdU labeling, adherent and nonadherent cells were combined, resuspended in 0.5 ml of PBS, and fixed in 5 ml of 70% ethanol, 50 mM glycine (pH 2.0). Samples were subjected to denaturation in 0.5 ml of 4 M HCl for 20 min and then incubated at 37°C for 1 h in 0.1 ml of PBS, containing 0.5% BSA, 0.1% Tween, and 20 μl of FITC-conjugated anti-BrdU antibody (clone BMG 6H8), obtained from Roche Molecular Biochemicals (Indianapolis, IN). After incubation, samples were centrifuged and resuspended in a 0.5-ml solution containing 500 μg/ml RNase A and 10 μg/ml propidium iodide in PBS. Cells were analyzed by two-color flow cytometry for BrdU incorporation and DNA content.

Assessment of Cell Viability. At the appropriate time after drug exposure, attached cells were released by trypsinization and combined with nonadherent cells. After centrifugation, cells were resuspended in PBS and treated with 0.2% trypsin blue, and trypan blue-excluding cells were counted using a hemocytometer. Viable cell counts were compared with the number of cells/dish at time 0 treatment. Viability was also confirmed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay.

RESULTS

Preferential Death of NCI-H661 Cells with S-Phase DNA Content in Response to Flavopiridol. Fig. 1A demonstrates the response of NCI-H661 cells to 300 nM flavopiridol for 24 h. After treatment,
there is an increase in cell number with \(G_1\) DNA content and a reduction in cell number with S-phase content, accompanied by a small sub-\(G_1\) peak (~15% of cells). The decrease in S-phase may be in part attributable to \(G_1\) arrest occurring in response to flavopiridol. However, the diminution in S-phase may also be attributable to the preferential death of S-phase cells. This is supported by a flow cytometry-based TUNEL assay (Fig. 1B). Approximately 25% of the cells shift to fluorescein positivity, and the apoptotic population is derived primarily from cells with an S-phase DNA content.

Flavopiridol-mediated Apoptosis Is Potentiated following Recruitment of Cells to S-Phase. The TUNEL analysis in Fig. 1 suggests that S-phase cells may be the most sensitive to flavopiridol, and that cells may be rendered more sensitive if they are recruited to S-phase before treatment. To test this hypothesis, NCI-H661 cells were treated with hydroxyurea to synchronize them at the \(G_1\)-S boundary and then released into DMSO or 300 nM flavopiridol. As shown in Fig. 2A, after release from a hydroxyurea-induced \(G_1\)-S block, progression through S-phase is markedly inhibited in the presence of flavopiridol. In addition, with time, an increasing number of cells undergo apoptosis, including almost the entire cell population by 24 h. This does not occur in cells released into DMSO, which continue to cycle without significant cell death. BrdU analysis at 6 and 12 h after release into flavopiridol indicates that the majority of the population is in S-phase when cell death is first detected. Quantitation of the results over multiple experiments (Fig. 2B) indicates that cells are more sensitive to flavopiridol when they are treated during S-phase.

Fig. 2. Recruitment to S-phase sensitizes NCI-H661 cells to flavopiridol. A, cells were treated with 1 mM hydroxyurea for 24 h (1H-24 hrs) to synchronize them at the \(G_1\)-S boundary. After a PBS wash, cells were then released into DMSO (1H/DMSO) or 300 nM flavopiridol (1H/300F). At the time points indicated, adherent and nonadherent cells were pooled and analyzed for cell cycle content (left-hand panels) and for apoptosis by TUNEL assay (middle panels). BrdU analysis of cells at 6 and 12 h (right-hand panels) after release into flavopiridol indicates that a large proportion of cells are in S-phase at the time cell death is first detected. In the BrdU analyses, fluorescein is on the Y axis, and propidium iodide is on the X axis. The BrdU-positive population is clearly identified when comparison is made with a sample to which no BrdU had been added. B, quantitation of apoptosis detected in cells treated as indicated, confirming that cells are sensitized to flavopiridol when they are treated after release from a hydroxyurea-induced cell cycle block. Data from five experiments were used to generate the graphs.
Cell Cycle Arrest in A549 Cells following Flavopiridol Exposure. NCI-H661 cells are representative of a subset of solid tumor cell lines that undergo apoptosis within 24 h when asynchronously growing cell populations are treated with flavopiridol alone. In contrast, many other solid tumor cell lines, including A549 cells, are less sensitive. In these cells, flavopiridol treatment initially results in cessation of cell growth, with G1 and G2 arrest over the first 24–48 h of drug exposure (Fig. 3A, top panels). Cell death is only observed at later time points (i.e., 72 h) and is most marked at concentrations ≥500 nM (16, 17). The shorter S-phase duration correlates with the absence of early apoptosis after flavopiridol exposure; A549 cells most likely undergo arrest at the G1 and G2 boundaries before the apoptosis of S-phase cells ensues.

A549 Cells Are Sensitized to Flavopiridol following S-Phase Recruitment. Although the initial responses of A549 and NCI-H661 cells to flavopiridol differ, A549 cells are similarly sensitized after treatment with flavopiridol alone. In contrast, many other solid tumor cell lines, including A549 cells, are less sensitive. In these cells, flavopiridol treatment initially results in cessation of cell growth, with G1 and G2 arrest over the first 24–48 h of drug exposure (Fig. 3A, top panels). Cell death is only observed at later time points (i.e., 72 h) and is most marked at concentrations ≥500 nM (16, 17). The initial G1 arrest in A549 cells is further demonstrated when they are exposed to flavopiridol after release from a nocodazole-induced mitotic block (Fig. 3B, upper panels). After the removal of nocodazole, both A549 and NCI-H661 cells released into DMSO enter S-phase at ~12 h. By 14 h, A549 cells released into flavopiridol demonstrate a block in G1. In contrast, G2 arrest is incomplete in the presence of flavopiridol in NCI-H661 cells (Fig. 3B, lower panels). Although NCI-H661 cells released into flavopiridol do not traverse the G1-S boundary as readily as control-treated cells, they are clearly capable of entering S-phase. At later time points after release from nocodazole into flavopiridol, the cell cycle patterns are not appreciably changed; A549 cells continue to demonstrate G1 arrest, whereas NCI-H661 cells continue to enter S-phase with additional accumulation of cells with a sub-G1 DNA content (data not shown). This difference may permit continued S-phase entry to occur in NCI-H661 cells in the presence of flavopiridol so that exposure to drug occurs during the phase when cells are most sensitive.

To further explain the lack of apoptosis observed in asynchronous A549 cells within the first 24 h after flavopiridol exposure, we examined the duration of S-phase traversal in these cells after release from a hydroxyurea-induced block at the G1-S boundary (Fig. 3C). Two h after release from hydroxyurea, nearly the entire cell population is in early S-phase. By 7–8 h, the majority of A549 cells have left S-phase by 7–8 h, whereas the majority of NCI-H661 cells do not exit S-phase before 10–11 h. The shorter S-phase duration correlates with the absence of early apoptosis after flavopiridol exposure; A549 cells most likely undergo arrest at the G1 and G2 boundaries before the apoptosis of S-phase cells ensues.

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synchronization and treatment during S-phase. After a hydroxyurea-induced G1-S block (1H-24), cells released into DMSO for 24 h continue to cycle without significant cell death (1H/DMSO-24), whereas cells released into 300 nM flavopiridol are impeded in S-phase progression and undergo cell death by 24 h (1H/300F-24). By 48 h, the majority of the population is undergoing apoptosis with a loss of cells with S/G2 DNA content. B, cells were treated with either DMSO or 300 nM flavopiridol alone for 24, 36, or 48 h. Alternatively, cells were first treated with 1 mM hydroxyurea for 24 h, rinsed, and released into DMSO or 300 nM flavopiridol for 24, 36, or 48 h. At the respective time points, adherent and nonadherent cells were pooled, and apoptosis was quantitated by TUNEL assay. Bars, SD of six experiments. Where error bars are not shown, graphs were generated from a single experiment. C, after treatment with 300 nM flavopiridol alone or 1 mM hydroxyurea, followed by 300 nM flavopiridol, adherent and nonadherent cells were pooled, and viable cell count was determined by trypan blue exclusion, confirming the sensitivity of these cells to flavopiridol when treatment occurs during S-phase. Cell count at time 0 was designated as 100%. Data from two experiments were used to generate the graphs. D and E, A549 cells are less sensitive to flavopiridol if they are treated late after release from synchronization at the G1-S boundary. Cells were treated with 1 mM hydroxyurea for 24 h and rinsed, and medium was replaced for 0, 2 (1H + 2), 4, 8 (1H + 8), or 10 h before treatment with 300 nM flavopiridol for an additional 48 h, followed by analysis of DNA content by flow cytometry and quantitation of apoptosis by TUNEL assay. Results of three experiments were used to generate the graphs for each condition. Bars, SD.

After synchronization and release into flavopiridol, the inhibition in S-phase progression is not as marked in A549 cells as in NCI-H661 cells. At 24 h, G2 populations are detected in A549 cells, whereas NCI-H661 cells retain a broad S-phase peak (compare Fig. 2A and Fig. 4A). Over time, death in A549 cells most likely occurs from the entire S/G2 population. Nonetheless, the apoptotic population at 24 h contains cells primarily with S-phase DNA content, whereas the majority of cells in G2 are not undergoing cell death at this time point, suggesting that S-phase cells may be the most susceptible to cell death. Furthermore, A549 cells appear most sensitive to flavopiridol if they are treated during early-mid S-phase. In the experiments shown in Fig. 4, D and E, cells were exposed to flavopiridol at various times after release from the hydroxyurea-induced block at the G1-S bound-
ary. If cells are permitted to pass through S-phase and are not treated with flavopiridol until 8 or 10 h after release from hydroxyurea, less cell death occurs over 48 h compared with cells exposed to flavopiridol shortly after release into S-phase. Similar results were obtained with NCI-H661 cells.

**Sensitivity to Flavopiridol Is Enhanced following Chemotherapy-imposed S-Phase Delay.** In another set of experiments, NCI-H661 cells were treated with noncytotoxic doses of chemotherapy agents, including cisplatin and gemcitabine, capable of retarding S-phase progression (Fig. 5A, left-column panels). After 24 h of chemotherapy exposure, significant cell death did not occur, whether or not drug treatment was continued or drug was replaced with DMSO (Fig. 5A, middle-column panels). However, flavopiridol treatment after recruitment of cells to S-phase with these chemotherapy agents resulted in markedly increased apoptosis (Fig. 5A, right-column panels). Because the sub-G1 peak does not represent the entire apoptotic population, apoptosis was more accurately quantitated using the flow cytometry-based TUNEL assay. Fig. 5B shows the results of an experiment in which either saline or cisplatin preceded treatment with either DMSO or 300 nM flavopiridol, demonstrating the synergism of the sequential cisplatin/flavopiridol combination. The majority of the cell population undergoes apoptosis following the combined treatment.

Synergism with chemotherapy agents was also examined in A549 cells. As shown in Fig. 6A, the sequential treatment of gemcitabine for 24 h, followed by flavopiridol for 24–48 h (G/300F treatments), results in cell death at drug concentrations that alone produce almost no cell death in a 24- or 48-h exposure (NaCl/300F or G/DMSO treatments).

The noncytotoxic concentrations of gemcitabine used (1 or 10 μM) result in significant S-phase delay and retardation in S-phase progression. The importance of the recruitment of cells to S-phase prior to flavopiridol exposure is demonstrated in Fig. 6B. In this experiment, cells were treated with 30 μM gemcitabine for 1.5 h. Directly after this treatment, the cell cycle profile is not appreciably changed. Immediate release into flavopiridol induces only a small amount of cytotoxicity compared with release into DMSO. In contrast, if the 1.5-h treatment with 30 μM gemcitabine is followed by flavopiridol, death occurs in a large proportion of cells. B, NCI-H661 cells were treated with saline or 1 μg/ml (3.33 μM) cisplatin for 24 h. Cells were rinsed with PBS and then treated with either DMSO or 300 nM flavopiridol for an additional 24 h. Nonadherent and adherent cells were pooled and subjected to TUNEL assay. Numbers represent the percentage of the population that is fluorescein positive (i.e., apoptotic).
Because flavopiridol alone induces minimal cytotoxicity in these cells over the first 24–48 h of drug exposure, we were able to examine the sequence dependence of the gemcitabine/flavopiridol combination. Fig. 6C demonstrates that the synergism is indeed sequence dependent and requires that gemcitabine precede flavopiridol. Exposure to gemcitabine concomitantly with flavopiridol also does not result in enhanced cell death.

Treatment with a noncytotoxic concentration of cisplatin also imposes an S-phase delay in A549 cells. As shown in Fig. 6D, subsequent treatment with flavopiridol results in a diminution in this.
population with the appearance of a sub-G1 peak. Fig. 6E (left-hand columns) shows the quantitation of apoptosis achieved in experiments in which either saline or cisplatin preceded treatment with either DMSO or flavopiridol. As was the case for gemcitabine, the sequential treatment of cisplatin followed by flavopiridol results in cell death at drug concentrations that alone produce almost no cell death in a 24- or 48-h exposure. In the reverse set of experiments, in which DMSO or flavopiridol preceded treatment with saline or cisplatin (Fig. 6E, right-hand columns), cell death resulting from the flavopiridol/cisplatin sequence was not as great as when cisplatin preceded flavopiridol. With higher doses of flavopiridol (≥500 nM), the sequence dependence is less complete, which may reflect the previously reported enhanced cellular uptake of cisplatin in the presence of flavopiridol (20). Nonetheless, the sequence dependence is clear when 300 nM flavopiridol is used and is reflected in the percentage of viable cells remaining after cisplatin/flavopiridol treatment compared with flavopiridol/cisplatin (Fig. 6F).

Flavopiridol Treatment during S-Phase Is Selectively Cytotoxic to Immortalized or Transformed Cells. As discussed previously, cell lines that initially respond to flavopiridol with cell cycle arrest can undergo cell death after prolonged exposures (≥72 h) to high concentrations (≥500 nM). Fig. 7A shows that this occurs in both diploid WI38 fibroblasts and in the T-antigen transformed subclone, VA13. However, after a hydroxyurea-induced block at the G1-S boundary, release into flavopiridol only results in cell death in the transformed cells; in addition, cell death is observed at lower flavopiridol concentrations. Therefore, treatment during S-phase appears to selectively sensitize transformed cells to flavopiridol. Fig. 7B shows the quanti-
VA13 cells undergo significant cell death after the sequential combination (Fig. 7D).

These results were not restricted to fibroblasts. Fig. 8 demonstrates the results of experiments in which NHBE cells were compared with an immortalized subline generated after SV40 virus infection (BEAS-2B). Both cell lines respond to noncytotoxic concentrations of gemcitabine with S-phase accumulation (Fig. 8A). However, subsequent treatment with flavopiridol, at concentrations as low as 150 nM, results in cell death only in the immortalized population (Fig. 8B). TUNEL assay quantitation over multiple experiments confirms that only the immortalized cells are sensitized to sequential gemcitabine/flavopiridol treatment (Fig. 8C).

**DISCUSSION**

In many types of solid tumor cell lines, flavopiridol-mediated cdk inhibition results in G1 and G2 arrest. Cell death occurs only after prolonged exposure to high drug concentrations. Because the delayed cytotoxicity follows arrest and occurs in noncycling populations, it may be caused by the effect of flavopiridol on other cellular targets. For example, at high concentration, flavopiridol can bind to DNA with an affinity similar to that of known intercalating agents (21). Therefore, it is possible that cdk inhibition mediated by drugs like flavopiridol will be primarily cytostatic to the majority of solid tumors not typically predisposed to apoptotic responses.

NCI-H661 cells are representative of a small group of solid tumor cell lines that are more sensitive to flavopiridol, responding to lower concentrations of drug with apoptosis within the first 24 h of exposure. In this report, we have demonstrated the preferential death of the S-phase population of these cells at flavopiridol concentrations that correlate with cdk inhibition. Compared with A549 cells, which initially undergo cell cycle arrest after flavopiridol exposure, arrest in G1 is less complete in NCI-H661 cells, permitting passage into S-phase, during which cells are most susceptible to apoptosis.

The molecular determinants that govern whether the initial response to flavopiridol is cell cycle arrest or preferential death of the S-phase population are not yet known. Nonetheless, both types of cell lines can be sensitized to flavopiridol after recruitment to S-phase. S-phase recruitment was accomplished by synchronization or by treatment with noncytotoxic concentrations of chemotherapy agents that impose S-phase delay. Sequential combinations including gemcitabine or cisplatin, followed by flavopiridol, are synergistic. Sequence-dependent synergism has also been described when flavopiridol follows other chemotherapeutic agents, including topoisomerase I and II inhibitors and alkylating agents (22–24). These drugs are all capable of retarding S-phase progression, and a common mechanism may exist for the enhanced effects afforded by flavopiridol.

Our results demonstrate the importance of administration of flavopiridol after S-phase recruitment. When synchronized cells are allowed to pass through S-phase before flavopiridol exposure, apoptosis is dramatically reduced. The sensitivity of S-phase cells observed in vitro is in accordance with the recently reported enhanced efficacy of flavopiridol in vivo against xenografts of prostate carcinoma cell lines expressing a p27Kip1 antisense oligodeoxynucleotide. Ablation of p27Kip1 expression increases the S-phase population and sensitizes cells to flavopiridol-mediated cytotoxicity (25).

In the case of gemcitabine (Fig. 6B), when cells are treated with flavopiridol before their accumulation in S-phase, very little cell death occurs. This may explain the results of other studies in which concomitant administration of agents such as cisplatin, methotrexate, doxorubicin, and camptothecin with flavopiridol did not produce significant synergism (22, 26, 27). If the appropriate interval is not
permitted, administration of these agents with flavopiridol may be more likely to result in G1 accumulation, preventing entry into S-phase during which cell death predominantly occurs. This occurs with concomitant exposure of A549 cells to gemcitabine and flavopiridol (data not shown), perhaps accounting for the lack of synergy (Fig. 6C). Although flavopiridol-mediated G1 arrest is less complete in NCI-H661 cells, the slowing of G1-S traversal in these cells would still be expected to compromise the effects of concomitant chemotherapy/flavopiridol administration compared with flavopiridol treatment after chemotherapy-induced S-phase recruitment.

Interestingly, it has recently been reported that the concomitant administration of doxorubicin and flavopiridol is synergistic in SAOS-2 cells, which lack functional Rb, the product of the Rb susceptibility gene (27). In these Rb-negative cells, flavopiridol alone did not induce G1 arrest, and both doxorubicin alone and the concomitant administration of both drugs resulted in significant S-phase accumulation. In SAOS-2 cells engineered to express wild-type Rb, however, concomitant administration of doxorubicin and flavopiridol resulted in G1 arrest with no significant synergy, suggesting that both the absence of Rb and exposure to flavopiridol during S-phase were important for enhanced cytotoxicity. However, other Rb-negative cell lines, including MDA-MB-486 breast carcinoma cells, have been reported to undergo G1 arrest after flavopiridol exposure (9, 28). Therefore, the sequence of chemotherapy/flavopiridol combinations may be less critical in some, but not all, Rb-negative cell lines.

Although our results may be pertinent to many solid tumor cell lines, they may not apply universally to all cell types. For example, chronic lymphocytic leukemia cells, >99% in G0-G1, are killed by flavopiridol with a mean IC50 concentration of 180 nM (29). In addition, both noncycling and cycling umbilical vein endothelial cells have been found to undergo apoptosis in response to flavopiridol with similar IC50 concentrations of ~100 nM (30). Therefore, some noncycling cell types do not require exposure to high flavopiridol concentrations to undergo cell death and are killed at relatively low flavopiridol concentrations, despite the absence of significant S-phase content.

Within the context of the cell types used in our experiments, flavopiridol-induced apoptosis during S-phase is selective for immortalized or transformed cells. For example, although both diploid WI38 fibroblasts and their T-antigen-transformed counterparts can undergo cell death after 72 h of exposure to high concentrations of flavopiridol, after release from a hydroxyurea-induced block at the G1-S boundary, flavopiridol is only cytotoxic to the transformed cells. Furthermore, after recruitment of cells to S-phase, cytotoxicity mediated by flavopiridol occurs abruptly and at lower concentrations that correlate with the inhibition of cdk activity.

The mechanism of S-phase sensitivity and selectivity remains to be elucidated. However, one possibility may be related to the known consequences of cdk inhibition during S-phase. After the transition from G1 to S, E2F activity directs transcription of genes required for S-phase. Importantly, however, this transcription is activated only transiently. Appropriately timed deactivation of E2F is critical for proper S-phase progression and is in part mediated by cyclin A-cdk2 (31–33). If cyclin A-cdk2 is inhibited by flavopiridol during S-phase, the correctly timed neutralization of E2F-1 activity would be prevented. Inappropriately persistent E2F-1 activity may cause the death of cells with high enough E2F-1 activity to surpass the threshold required to induce apoptosis. Transformed cells have been shown to be more sensitive to cyclin A-cdk2 inhibition than normal cells, presumably because their baseline level of E2F activity is already high (34).

Alternatively, it is also possible that flavopiridol-mediated cdk inhibition could result in the dephosphorylation of Rb during S-phase. Dephosphorylation of Rb during S-phase has been shown to be associated with the inhibition of DNA synthesis (35). Although we have not examined the state of Rb phosphorylation when cells are exposed to flavopiridol during S-phase, it is noteworthy that DNA synthesis continues under these conditions (Fig. 2), suggesting that Rb dephosphorylation is unlikely to be complete. Nonetheless, reduced phosphorylation of Rb by flavopiridol may ultimately cause sequestration of E2F-1 and decreased transcription of target genes such as the M2 subunit of ribonucleotide reductase, enhancing cellular sensitivity to drugs such as hydroxyurea and gemcitabine (36). A decrease in ribonucleotide reductase activity has been proposed to contribute to the potentiation of apoptosis observed when gemcitabine-treated gastric cancer cells are exposed to flavopiridol (37). Additional experiments, including an analysis of the state of phosphorylation of both Rb and E2F-1, as well as direct assessment of E2F-1 activity during S-phase in the presence of flavopiridol, should help define whether either of these mechanisms is operating in the cell types we have examined.

The clinical translation of our results in solid tumor therapy may be challenging, because both the sequence and interval between an agent such as gemcitabine and flavopiridol are critical. On the other hand, the active metabolite of gemcitabine, difluorodeoxycytidine triphosphate, has a long intracellular half-life (38), which may allow maximal S-phase recruitment of cycling populations. In this regard, cdk inhibition as an anti-solid tumor strategy may hold promise if directed at cells first recruited to S-phase. Because cdk2 inhibition would be expected to produce at least some degree of G1 arrest in exponentially growing tumor cells (39), small molecule cdk2 inhibitors such as flavopiridol may be more effective if developed in sequential combinations with chemotherapy.

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REFERENCES

Selective Sensitization of Transformed Cells to Flavopiridol-induced Apoptosis following Recruitment to S-Phase

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