Flavopiridol Potently Induces Small Cell Lung Cancer Apoptosis during S Phase in a Manner That Involves Early Mitochondrial Dysfunction

Julie Litz, Patricia Carlson, G. Sakuntala Warshamana-Greene, Steven Grant, and Geoffrey W. Krystal

Department of Medicine, Medical College of Virginia/Virginia Commonwealth University [S. G., G. W. K.], and McGuire Veterans Affairs Medical Center [J. L., P. C., G. S. W-G., G. W. K.], Richmond, Virginia 23249

ABSTRACT

Purpose: Accumulating evidence indicates that small cell lung cancer (SCLC) is defective in many of the regulatory mechanisms that control cell cycle progression. The purpose of this study was to determine the effects of flavopiridol, a pan-cyclin-dependent kinase inhibitor, on growth and apoptosis of SCLC cell lines.

Experimental Design: Cell growth was monitored using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and clonogenic assays. Induction of apoptosis was assessed using multiple assays, including flow cytometric determination of DNA content and mitochondrial membrane potential, terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL), and Western blot analysis of procaspase 3 and poly(ADP-ribose) polymerase cleavage.

Results: Flavopiridol induced growth inhibition and cytotoxicity in multiple SCLC cell lines, with an IC_{50} of 50–100 nM and an LD_{50} of 150–200 nM in 72-h MTT assays. The cytotoxicity seen in the MTT assay proved to be apoptosis by several criteria. Interestingly, inhibition of caspase activation with the caspase inhibitor Boc-Asp(OMe)-CH_{2}F reduced TUNEL labeling by 40% but did not have any effect on the loss of mitochondrial membrane potential (detected as early as 4 h after drug exposure) or cytotoxicity in MTT assays. These results suggest that the primary event in flavopiridol-induced apoptosis involves induction of mitochondrial dysfunction. Cells synchronized with aphidicolin at the G_{1}-S border and treated with flavopiridol during S phase showed a marked increase in apoptosis compared with an asynchronous population or a population treated during G_{1}-M. Despite the increased apoptosis, a significant proportion of synchronized cells proceeded through S, G_{2}-M, and into G_{1} phase in the presence of flavopiridol, demonstrating that a high-grade cell cycle arrest is not required for apoptosis. Cells synchronized at the G_{1}-S border treated with a short exposure to flavopiridol also showed more than a 10-fold decrease in clonogenicity compared with asynchronous cells treated identically.

Conclusions: Taken together, these data demonstrate that flavopiridol potently and selectively induces SCLC apoptosis preferentially during S phase, in a manner that involves early mitochondrial dysfunction without a requirement for a high-grade block to cell cycle progression. Furthermore, clonogenicity data suggests that prior S phase synchronization could be a highly effective way of enhancing the efficacy of bolus or short infusions of flavopiridol in the clinical setting.

INTRODUCTION

SCLC accounts for approximately 20% of all lung cancers and will cause the death of 90–95% of affected individuals (1). On the basis of the current incidence rates, this one histological subtype alone will be responsible for nearly as many deaths as breast cancer and more deaths than prostate cancer in the United States this year. Paradoxically, SCLC is a highly chemotherapy-responsive disease, with a variety of multidrug combinations producing response rates of >80%, with up to a third or more representing complete responses. Increasing the dose intensity of standard effective drugs has increased toxicity without yielding any benefit in response or survival (2). Substitution of novel cytotoxic drugs such as irinotecan may result in prolongation of median survival (3), but improvement in long-term survival will likely depend on the development of new therapeutic approaches that can prevent or overcome the development of clinical resistance to traditional cytotoxic agents. One promising approach is to use agents that are targeted against molecular abnormalities specific to SCLC.

One molecular abnormality in SCLC that has become increasingly apparent is the loss of cell cycle regulation. It has been known for several years that the G_{1}-S restriction point is defective, largely because of loss of function of the retinoblastoma gene product, which is mutationally altered in the vast majority of SCLC tumors. One of the major mechanisms by which SCLC cells overcome the block at the G_{1}-S border is through activation of cyclin-dependent kinases (CDKs). This results in a loss of growth inhibition and an increase in proliferation of SCLC cells (4). Indeed, CDKs are the targets of increasing interest for the development of novel anti-cancer drugs. This interest is based on the fact that SCLC is a highly chemotherapy-responsive disease, with a variety of multidrug combinations producing response rates of >80%, with up to a third or more representing complete responses. Increasing the dose intensity of standard effective drugs has increased toxicity without yielding any benefit in response or survival (2). Substitution of novel cytotoxic drugs such as irinotecan may result in prolongation of median survival (3), but improvement in long-term survival will likely depend on the development of new therapeutic approaches that can prevent or overcome the development of clinical resistance to traditional cytotoxic agents. One promising approach is to use agents that are targeted against molecular abnormalities specific to SCLC. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 The abbreviations used are: SCLC, small cell lung cancer; CDK, cyclin-dependent kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PARP, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick end labeling; Boc-D-FMK, Boc-Asp(Ome)-CH_{2}F; NSCLC, non-small cell lung cancer; MTD, maximum tolerated dose; SCF, stem cell factor.
majority of SCLC (4). The G₁-S DNA damage checkpoint is also defective because of the almost universal loss of p53 function (4). Recently, it has been shown that the G₂-M and M checkpoints are also defective, attributable, in large part, to mutation and aberrant methylation of the genes encoding multiple regulators of these complex checkpoints (5, 6). The net result of these genetic and epigenetic changes is that SCLC cell cycle progression is unresponsive to normal regulatory cues, and these tumors have a high degree of genetic instability at both the nucleotide and chromosomal level. A consequence of this genetic instability could be an enhanced ability to develop resistant clones under the selective pressure of a cyclical chemotherapy regimen.

One logical approach to counteract this loss of cell cycle regulation at multiple regulatory sites would be to interfere directly with the CDK cascade that drives cell cycle progression. CDKs 4 and 6 drive progression through G₁, CDK2 is critical for entry and progression through S, and CDK1 (CDC2) is necessary for entry to and completion of mitosis (7, 8). Flavopiridol is a synthetic flavone derivative that is an effective inhibitor of all these CDKs, as well as other related enzymes that play ancillary roles in regulating cell cycle progression (9–11). An early description of its effect on tumor cell growth demonstrated a block to cell cycle progression with IC₅₀ values for inhibition of SCLC growth in the range of 25–150 nM (12). However, this study did not clearly indicate whether the end result of the block to cell cycle progression was cytostasis or apoptosis. Subsequent studies have shown that specific tumor cell types are particularly sensitive to the apoptotic effects of flavopiridol, including NSCLC (13, 14), lymphomas (15), and head and neck squamous cell carcinoma (16).

The purpose of the present study was to more rigorously define the effect of flavopiridol on SCLC cell lines, with particular reference to the induction apoptosis and the biochemical mechanisms that may regulate this process. We demonstrate that SCLC cells are uniformly highly sensitive to flavopiridol-induced cytotoxicity during prolonged 72-h exposure to clinically achievable concentrations of this drug. In addition, induction of apoptosis is correlated with mitochondrial dysfunction. Apoptosis in response to short drug exposure (8–18 h) is greatly increased by S phase synchronization, a phenomenon that is accompanied by a dramatic decline in the clonogenetic potential of S phase synchronized cells briefly exposed to flavopiridol. These observations may have translational implications for the use of flavopiridol in the treatment of SCLC and other malignancies.

MATERIALS AND METHODS

Cell Growth. SCLC cell lines were grown in RPMI 1640 supplemented with 2 mM l-glutamine, with (complete medium) or without 10% fetal bovine serum (Life Technologies, Inc./Invitrogen, Grand Island, NY). When grown in the absence of serum, 0.1% BSA (Sigma Chemical Co., St. Louis, MO) and recombinant SCF (Peprotech, Rocky Hill, NJ) were added to the medium. The H146, H209, H510, and H526 cell lines have been characterized previously (17). MRC-5 cells were obtained from American Type Culture Collection (CCL-171; Manassas, VA). Cell growth was measured using the MTT (Sigma Chemical Co.) colorimetric dye reduction method (18). Duplicate plates containing eight replicate wells per assay condition were seeded at a density of 1 × 10⁴ cells in 0.1 ml of medium, and data were expressed as the change in absorbance at 540 nm over 72 h, relative to initial values obtained 3 h after plating. Flavopiridol was kindly provided by Dr. Edward Sausville through the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD). Etoposide, aphidicolin (Calbiochem, San Diego, CA), and flavopiridol were solubilized in DMSO; final concentration of DMSO in all cultures, including controls, was 0.1%. Synchronization of cells at the G₁-S border was accomplished by incubation of cells in medium containing 4 μg/ml aphidicolin for 24 h, followed by three washes in cold PBS.

Clonogenic Assay. H526 cells (1 × 10⁴) were synchronized with aphidicolin as described above or left unsynchronized and then treated with DMSO vehicle (control) or 150 nM flavopiridol for 14 h in complete medium. The cells were then washed and resuspended in 3 ml of 0.25% low melting point agarose (SeaKem LE; FMC Bioproducts, Rockland, ME) in complete medium (without drugs) and plated over a layer of 0.5% agarose in medium in 60-mm plates. Plates were incubated for 14 days, and viable (blue) macroscopic colonies were counted 2 h after overlay with MTT dye. Assays were performed in triplicate.

TUNEL Assay. Terminal deoxynucleotidyl transferase-mediated labeling of free DNA 3’ ends with fluorescein-conjugated dUTP (19) was accomplished using the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). Four independent ×100 fields containing a minimum of 300 cells on each of two replicate slides were evaluated for nuclear labeling by fluorescence microscopy for each treatment or condition. For calculation of the mitotic index, cytospin preparations were stained with Wright-Giemsa stain (Fisher Scientific, Pittsburgh, PA) and the number of mitotic figures in four independent ×100 fields containing a minimum of 200 cells was determined. The effect of caspase inhibition on TUNEL staining was determined by incubating cells in medium containing 25 μM Boc-i-FMK (Calbiochem).

Flow Cytometry. Nuclear DNA content was determined by propidium iodide staining using the method of Vindelov et al. (20). Fluorescence was quantitated using a Becton-Dickinson FACSscan cytofluorometer, and data were analyzed using the FACSscan Research software package. Mitochondrial membrane potential (Ψ_md) was measured using DiOC₆ (Sigma Chemical Co.; Ref. 21). For each condition, 4 × 10⁶ cells were incubated in 1 ml of 40 nM DiOC₆ at 37°C for 15 min and subsequently analyzed using the FACSscan cytofluorometer with excitation and emission settings of 488 nm and 525 nm, respectively.

Western Blotting. For preparation of whole cell lysates, cells were resuspended in ice-cold PBS, and an equal volume of 2× SDS buffer [2% SDS, 0.08 M Tris-HCl (pH 6.8), 0.1 M DTT, and 10% glycerol] was added, followed by shearing through a 25-gauge needle. Lysates were electrophoretically resolved on a 10% SDS polyacrylamide gel and transferred to Immobilon-P (Millipore, Bedford, MA) membrane using standard procedures, with detection using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL). Cytochrome c release into
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Complete inhibition of growth of MRC-5 was achieved at 200 nM flavopiridol. The IC50 for all cell lines were in the 50–100 nM range. In the top panel of Fig. 2 qualitatively shows the two apparent changes. The first is a dose-dependent decrease in the G2-M population. The second, and perhaps more dramatic, change is the progressive accumulation of cells containing a sub-G1 DNA content, characteristic of apoptotic cells. The flow cytometric results are quantitated in the bottom panel. The sub-G1 population in this representative experiment increased from 11% to 29%, whereas the G2-M population decreased from 16% to 6%, comparing the vehicle control with 200 nM flavopiridol. These data suggest that flavopiridol induces apoptosis, as measured by the increase in cells with a sub-G1 DNA content, in a dose-dependent fashion.

Flavopiridol Induces Procaspase 3 and PARP Cleavage and DNA Fragmentation. To confirm that flavopiridol induces SCLC apoptosis in serum-containing medium, we studied the generation of specific biochemical apoptotic markers after exposure of H526 cells to drug for 24 h. Activational cleavage of procaspase 3, resulting in cleavage of its downstream substrate PARP, occurred in a dose-dependent and proportional manner (Fig. 3A). Results were similar when the same biochemical markers were analyzed 48 h after drug exposure (data not shown). The degree of procaspase 3 activation correlated well with the percentage of cells with fragmented DNA, as measured by TUNEL assay at both 24 and 48 h after drug exposure. Taken together, the MTT assay, flow cytometric DNA content analysis, assays for procaspase 3 and PARP cleavage, and TUNEL assays all indicate that flavopiridol is a potent inducer of SCLC apoptosis at concentrations ≤200 nM.

Loss of Mitochondrial Membrane Potential Is an Early Event in Flavopiridol-induced Apoptosis. To determine whether caspase activation plays a primary role in the induction of flavopiridol-mediated apoptosis, H526 cells were exposed to 150 nM flavopiridol for 24 h in the presence or absence of Boc-γ-FMK, a broad spectrum irreversible caspase inhibitor. Fig. 3B shows that 25 μM Boc-γ-FMK efficiently inhibited flavopiridol-induced caspase 3 activation, which resulted in a 40% decrease in TUNEL-positive cells. However, a parallel 72-h MTT assay demonstrated that the caspase inhibitor had no effect on the essentially complete lethality induced by 150 nM flavopiridol. These data suggest that although caspase activation is necessary for maximal DNA fragmentation, it may not be the primary event in the induction of flavopiridol-mediated cytotoxicity. Because the MTT assay is dependent on reduction of the tetrazolium dye by functional mitochondria, we hypothesized that mitochondrial dysfunction could be an early causative event in the induction of flavopiridol-mediated apoptosis.

To determine whether flavopiridol resulted in a loss of mitochondrial membrane potential, we exposed H526 cells...
grown in serum-containing medium to increasing concentrations of flavopiridol for 24 h. Mitochondrial membrane potential (ψm) was assessed by flow cytometric determination of DiOC6 fluorescence. As shown in Fig. 4A, the number of cells with a low ψm increased in a flavopiridol dose-dependent fashion. To test the hypothesis that the loss of ψm was an early caspase-independent event, we exposed H526 cells to 150 nM flavopiridol in the presence or absence of Boc-D-FMK for 24 h and assessed the loss of ψm by measuring DiOC6 fluorescence. A 24-h exposure to flavopiridol caused a dramatic loss of mito-
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In contrast, the number of cells with an S phase DNA content could progressively be chased into those containing lower DNA content. Given the right kinetics, this could result in a progressive loss of cells containing the highest DNA content (e.g., G2-M) and a gain of cells containing the lowest (e.g., G1), with the constant flux giving the impression that the populations in the middle are relatively unaffected. Another possible explanation could be that the G2-M population is the most sensitive to the apoptotic effects of the drug, with S phase cells being the least sensitive. Alternatively, cells in S phase could be the most susceptible to flavopiridol-induced apoptosis (22), with constant progression of cells from G1 to S giving the impression that the S phase population remained constant.

To distinguish between the alternative possibilities, H526 cells were synchronized at the G1-S boundary using a 24-h treatment with the DNA polymerase inhibitor aphidicolin. After release of the block, H526 cells incorporate [3H]thymidine for 9 h and reach a peak mitotic index (as determined by Giemsa staining) between 14 and 16 h, defining the length of the S and G2 phases (data not shown). Table 1 shows that treatment of synchronized H526 cells with flavopiridol through the period of S-M enhances apoptosis >10-fold, relative to unsynchronized cells treated for the equivalent 18 h. To more specifically define whether the most sensitive period was in S or G2-M, we also limited flavopiridol treatment to the 8-h period immediately after release of the aphidicolin block (S), or to the 10- to 18-h period after release (G2-M). Treatment during G2-M did not induce significantly more apoptosis than the controls treated with flavopiridol or aphidicolin alone. Flavopiridol treatment confined to S phase did dramatically increase apoptosis, although not to the extent that treatment for the entire S-M period did. It was unclear whether this difference was a function of a longer exposure interval or of treatment of cells capable of exiting S in the presence of flavopiridol during both S and G2-M phases.

To determine the extent of cell cycle arrest imposed by flavopiridol treatment of synchronized cells, [3H]thymidine incorporation as well as cellular DNA content was monitored at 2-h intervals after release of the aphidicolin block. [3H]thymidine incorporation leveled off between 8 and 10 h in both control and flavopiridol-treated cells, indicating that a 150-nM concentration did not inhibit progression of S phase (Fig. 5A). Total thymidine incorporation was approximately a third less in flavopiridol-treated cultures. This decrease was most likely caused by the induction of apoptosis, indicated by the progressive appearance of cells with a sub-G1 DNA content, characteristic of apoptosis, populations containing higher DNA content could progressively be

Table 1  Treatment during S phase maximizes flavopiridol-induced apoptosis

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<th>Aphidicolin&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Flavopiridol&lt;sup&gt;b&lt;/sup&gt; (0–8 h, S)</th>
<th>Flavopiridol&lt;sup&gt;c&lt;/sup&gt; (10–18 h, G2-M)</th>
<th>Apoptosis (% TUNEL&lt;sup&gt;d&lt;/sup&gt;)</th>
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<td></td>
<td>–</td>
<td>+</td>
<td>1.6 ± 0.9</td>
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<td>4.4 ± 1.8&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>21.6 ± 3.4</td>
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<td>6.2 ± 1.4</td>
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<sup>a</sup> H526 cells were treated with 4 μg/ml aphidicolin for 24 h and then washed free of drug.
<sup>b</sup> Cells were treated with 150 nm flavopiridol for 8 h, followed by a drug-free interval of 10 h.
<sup>c</sup> After removal of aphidicolin, cells were incubated for 10 h in drug-free medium, followed by treatment with 150 nm flavopiridol for 8 h.
<sup>d</sup> Unsynchronized cells were treated with 150 nm flavopiridol continuously for 18 h.
<sup>e</sup> Synchronized cells were treated with 150 nm flavopiridol continuously for 18 h.
cytochrome c release relative to controls. Therefore, treatment of SCLC cells with flavopiridol during S phase appears to induce a greater degree of mitochondrial dysfunction than treatment of unsynchronized cells.

**Synchronization of H526 Cells at the G1-S Boundary Markedly Enhances the Efficacy of Short-Term Flavopiridol Exposure in Clonogenic Assays.** Fig. 5B shows that despite a marked increase in apoptosis after S phase synchronization, a significant fraction of cells continue to cycle 18 h after flavopiridol exposure. To determine whether the cells still in cycle after a short-term flavopiridol exposure would continue to proliferate or were destined to eventually die, we performed clonogenic assays using either unsynchronized cells or cells synchronized with aphidicolin and treated with 150 nM flavopiridol for 14 h. Fig. 6 shows that synchronization resulted in a >90% reduction in the number of colonies obtained after 14 days, in marked contrast to the limited effects of exposure of asynchronous cells to an identical concentration of flavopiridol. These results confirm that the effects of short-term exposure to flavopiridol can be markedly enhanced by treatment of cells during S phase. In addition, they demonstrate that the effects on clonogenic potential are greater than that observed when apoptosis is assessed 14–18 h after flavopiridol exposure.

**DISCUSSION**

Defects in cell cycle checkpoint regulation, caused by both genetic and epigenetic mechanisms, are among the most prevalent functional alterations in tumor cells. Such defects are not only responsible for a lack of growth control but also are intimately associated with the rapid accumulation of genetic defects in tumor cells, as well as being a determinate of sensitivity to various cytotoxic therapies of a tumor (23, 24). Defects in both G1-S and G2-M checkpoint regulation have been identified in SCLC (4, 5). We became particularly interested in the loss of checkpoint regulation after observations that although various inhibitors of the Kit receptor tyrosine kinase slowed SCLC growth, a definable block to cell cycle progression was not evident (25, 26, 27). Given this observation, we postulated that a combination of imatinib mesylate (STI571), a Kit inhibitor, with flavopiridol, previously shown to block SCLC cell cycle progression (12), could be a potentially synergistic combination. In this regard, the combination of imatinib mesylate and flavopiridol proved to be highly synergistic in inducing apoptosis of chronic myelogenous leukemia cell lines (28). However, it rapidly became apparent in parallel studies using

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<th>Table 2</th>
<th>Active DNA synthesis is required for maximal flavopiridol-induced apoptosis</th>
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<tr>
<td>Aphidicolin (24 h)</td>
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<td>Aphidicolin (38 h)</td>
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<tr>
<td>Flavopiridol (14 h)</td>
<td>–</td>
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<tr>
<td>Mitotic index (%)</td>
<td>3</td>
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<td>Apoptosis (% TUNEL+)</td>
<td>1.1 ± 0.7</td>
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*H526 cells were treated with 4 μg/ml aphidicolin for 24 h, washed free of drug, and then treated with 150 nM flavopiridol for 14 h. Cells were treated with 4 μg/ml aphidicolin for 38 h, and 150 nM flavopiridol were added during the last 14 h.*

![Fig. 5](https://example.com/fig5.png)

**Fig. 5** Flavopiridol-mediated apoptosis does not require a high-grade cell cycle arrest, with treatment during S phase resulting in an enhanced mitochondrial cytochrome c release. H526 cells were synchronized using aphidicolin and then exposed to 150 nM flavopiridol or DMSO vehicle for the indicated times. A, incorporation of [3H]thymidine was monitored in control and flavopiridol-treated cells after aphidicolin release. B, flow cytometric profiles of flavopiridol-treated cells stained with propidium iodide and analyzed at the indicated times after aphidicolin release. C, H526 cells were treated with 150 nM flavopiridol for 13 h. Cytosolic extracts were analyzed for the presence of cytochrome c and actin, a loading control.

To determine whether S phase synchronization, followed by treatment with flavopiridol, leads to enhanced mitochondrial dysfunction, we also studied the extent of release of cytochrome c from mitochondria into the cytosol after a 13-h treatment with 150 nM flavopiridol. As shown in Fig. 5C, synchronization with aphidicolin caused a marked increase in cytosolic cytochrome c, relative to unsynchronized cells, that did not show significant

vopiridol-induced apoptosis (data not shown). Taken together, the data demonstrate that after S phase synchronization, flavopiridol progressively induces apoptosis without inducing a high-grade cell cycle arrest. It appears that active DNA synthesis is required for maximal flavopiridol-induced apoptosis.

Aphidicolin-treated cells stained with propidium iodide and analyzed at the indicated times after aphidicolin release. H526 cells were synchronized with aphidicolin and treated with 150 nM flavopiridol for 14 h. Fig. 6 shows that synchronization resulted in a >90% reduction in the number of colonies obtained after 14 days, in marked contrast to the limited effects of exposure of asynchronous cells to an identical concentration of flavopiridol. These results confirm that the effects of short-term exposure to flavopiridol can be markedly enhanced by treatment of cells during S phase. In addition, they demonstrate that the effects on clonogenic potential are greater than that observed when apoptosis is assessed 14–18 h after flavopiridol exposure.

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SCLC cell lines (data not shown) that flavopiridol cytotoxicity was so dominant and the dose response was so steep that it was impossible to demonstrate synergy with imatinib, which is cytostatic for SCLC in the presence of serum.

Flavopiridol potently inhibited the growth of multiple SCLC cell lines with an IC₅₀ in the 50–100 nM range. Between 100–200 nM, apoptosis was selectively induced in these cell lines relative to the MRC-5 pulmonary fibroblast cell line, which was growth inhibited but did not undergo apoptosis at concentrations approximating a clinically achievable drug concentration (400 nM). Flavopiridol-induced apoptosis clearly involved caspase activation and DNA fragmentation as measured by the accumulation of cells with a sub-G₁ DNA content, as well as by TUNEL staining. However, although inhibition of caspase activation partially inhibited DNA fragmentation, it had no effect on the early loss of mitochondrial membrane potential seen after flavopiridol exposure or the loss of cell viability measured by a 72-h MTT assay. These observations suggested that the primary event in flavopiridol-mediated cytotoxicity was early mitochondrial dysfunction and not caspase activation. The inability of caspase inhibition to protect cells from mitochondrial damage and nonapoptotic forms of cell death has been described previously (29).

The mechanism of flavopiridol-induced apoptosis is of particular interest given the profound potentiation of apoptosis after synchronization of cells at the G₁-S border with aphidicolin. Matranga and Shapiro (22) first made this observation, noting that most NSCLC cell lines were resistant to flavopiridol-induced apoptosis, unless they were treated during S phase. By comparing the response of the one sensitive cell line (NCI-H661) to several resistant cell lines, they speculated that one of the mechanisms of flavopiridol cytotoxicity is the degree of flavopiridol-induced cell cycle arrest. In cells that displayed a high-grade G₁ arrest in the presence of drug, the sensitive S phase would not be reached and the response would be cytostatic and not apoptotic. This may explain the highly variable cytostatic versus apoptotic response seen when many tumor types were studied (11) and appears to be consistent with our data. The detection of a relatively low rate of TUNEL staining (approximately 5–8%) after a 24-h exposure of unsynchronized H526 cells to 150 nM flavopiridol, along with the extensive cytotoxicity seen in 72-h MTT assays, suggested that cells continued to cycle and eventually were exposed to drug during S phase. Data presented in Fig. 5 and Table 2 clearly show that cells treated with flavopiridol continue to progress through the cell cycle if they do not undergo apoptosis. Because of the enhanced flavopiridol sensitivity of SCLC cell lines relative to NSCLC, rather than treating for 24 h (22), we were able to limit drug exposure to specific phases of the cell cycle and, therefore, more clearly elucidate the cell cycle dependence of flavopiridol-induced apoptosis. Exposure of synchronized H526 cells to flavopiridol for 8 h, designed to limit exposure solely to S phase, enhanced apoptosis 5-fold relative to controls (Table 1). Exposure to drug for 18 h was required for the maximal 10-fold enhancement of apoptosis, and it was clear that some cells underwent apoptosis during mitosis. However, it was unclear whether the increased exposure interval or continued exposure during G₂-M was the most important factor in producing maximal apoptosis, although exposure solely during G₂-M did not enhance apoptosis relative to controls. Taken together, our data clearly demonstrate that short exposure solely during S phase is necessary and sufficient to markedly enhance flavopiridol-induced apoptosis, but longer drug exposures may further enhance the apoptotic rate.

The mechanism for the enhancement of flavopiridol-induced apoptosis by S phase synchronization is unclear. Although it is interesting to speculate that inhibition of the cyclin A-CDK2 complex may be particularly lethal (22), it is unlikely to be the sole explanation for several reasons. For example, Fig. 5 shows that flavopiridol-treated cells progress through S phase with timing similar to controls, suggesting that cyclin A-CDK2 inhibition is incomplete. In addition, continued incubation in aphidicolin, beyond the period of synchronization, markedly blunts the enhancement of flavopiridol-induced apoptosis by S phase synchronization. In the continued presence of aphidicolin, it is expected that cyclin A-CDK2 activity would remain high (30). Thus, it seems release from the aphidicolin block and, therefore, the process of active DNA synthesis is required for enhancement of flavopiridol-mediated apoptosis rather than inhibition of high cyclin A-CDK2 activity alone.

Whereas partial inhibition of cyclin A-CDK2 activity in the presence of active DNA synthesis may trigger lethal events, it is also likely that the early mitochondrial dysfunction is involved. Exposure of asynchronous cells to flavopiridol induces loss of mitochondrial membrane potential without enhancing cytochrome c release (Fig. 5), confirming an observation made by Achenbach et al. (31). They also showed that despite absence of cytochrome c release, cellular ATP content dropped significantly. Fig. 5C shows that flavopiridol treatment after S phase synchronization markedly increased cytochrome c release, suggesting that, in addition to the potential for amplification of caspase activation by released cytochrome c, the degree of mitochondrial dysfunction was enhanced. DNA synthesis is a highly energy-dependent process, and it may be that in the presence of severe mitochondrial dysfunction the tumor cell is unable to maintain an adequate energy supply for both DNA synthesis and viability. Flavopiridol is competitive for ATP binding to its known target enzymes, and, thus, diminishing
ATP levels could also markedly affect both its activity and specificity.

Given these findings, the mechanism of enhancement of flavopiridol-mediated apoptosis during S phase is likely to be complex and requires further detailed studies for its elucidation. However, regardless of the underlying mechanism, this phenomenon could be clinically exploitable. For example, a single brief treatment with 150 nm flavopiridol after S phase synchronization produced more than a 10 fold reduction in colonies in a clonogenic assay relative to flavopiridol alone and an ~95% reduction compared with the vehicle control. This observation could directly address one of the major problems in the clinical development of flavopiridol, the selection of an appropriate dose and schedule (11). The original Phase I studies were designed using a 72-h continuous infusion based on the assumption that the drug would have a cytostatic effect (32, 33). These studies yielded a MTD of 78 mg/m²/day with diarrheal prophylaxis, resulting in a steady-state concentration of 344 nM (32), and a MTD of 40 mg/m²/day without diarrheal prophylaxis, resulting in a steady-state concentration of 416 nM (33). This concentration range should be adequate to produce significant cytotoxicity in SCLC, based on the results of our 72-h MTT assays (Fig. 1), and, thus, should elicit serious consideration of a clinical trial of flavopiridol in SCLC. However, preclinical data indicated that bolus administration, which yields higher peak levels, may be more efficacious, and a new Phase I trial using bolus administration was performed (34). This trial demonstrated that a single 1-h infusion at the MTD (62.5 mg/m²) produced mean plasma levels of 195 nM 10 h after the infusion. Both the concentration and time interval are comparable with those we have used in conjunction with S phase synchronization. The results of our clonogenic assays would suggest that a strategy to induce S phase synchronization, such as treatment with hydroxyurea or gemcitabine (22), followed by a single bolus or short infusion of flavopiridol, could be a practical and more effective method of administering flavopiridol to patients with SCLC. Given the observation that non-neoplastic cells are apparently not sensitized to flavopiridol by S phase synchronization (22), such a strategy may also be less toxic and may, therefore, deserve further exploration in the clinical setting.

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Julie Litz, Patricia Carlson, G. Sakuntala Warshamana-Greene, et al.


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