Flavopiridol Induces Cell Cycle Arrest and p53-independent Apoptosis in Non-Small Cell Lung Cancer Cell Lines

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ABSTRACT

Flavopiridol, a synthetic flavone that inhibits tumor growth in vitro and in vivo, is a potent cyclin-dependent kinase (cdk) inhibitor presently in clinical trials. In the present study, the effect of 100–500 nM flavopiridol on a panel of non-small cell lung cancer cell lines was examined. All express a wild-type retinoblastoma susceptibility protein and lack p16INK4A, and only A549 cells are known to express wild-type p53. During 72 h of treatment, flavopiridol was shown to be cytotoxic to all seven cell lines, as measured by trypan blue exclusion, regardless of whether cells were actively cycling. In most cycling cells, cytotoxicity was preceded or accompanied by cell cycle arrest. Cell death resulted in the appearance of cells with a sub-G1 DNA content, suggestive of apoptosis, which was confirmed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay and by demonstration of cleavage of caspase targets including poly(ADP-ribose) polymerase, p21Waf1, and p27Kip1. At doses at or below 500 nM, maximal cytotoxicity required 72 h of exposure. Although flavopiridol resulted in the accumulation of p53 in A549 cells, flavopiridol-mediated apoptosis was p53 independent because it occurred to the same degree in A549 cells in which p53 was targeted for degradation by HPV16E6 expression. The data indicate that flavopiridol has activity against non-small cell lung cancers in vitro and is worthy of continued clinical development in the treatment of this disease.

INTRODUCTION

Eukaryotic cell cycle progression is governed by cdks, a family of enzymes that are regulated by phosphorylation and activated by their association with cyclins (1). In addition, two groups of inhibitors negatively regulate cdk activity. Universal inhibitors, including p21Waf1 and p27Kip1, inhibit all known cdks; ink4 family members, including p16INK4A, inhibit only α-type cdk4 and cdk6 (2). In human cancers, including NSCLCs, cdk inhibitors are commonly absent or present at diminished levels. For example, p16INK4A is frequently lost in tumors that retain a wild-type RB-susceptibility protein (3, 4). p21Waf1 is transcriptionally regulated by p53-dependent and -independent pathways, (5–8) and is difficult to detect in tumor cells carrying a p53 mutation. p27Kip1, under control of the ubiquitin system (9), is also frequently present only at low levels in tumor cells, and patients whose tumors express the lowest levels tend to have a worse prognosis (10–13).

Ectopic expression of cdk inhibitors in tumor cell lines often causes cell cycle arrest. For example, p21Waf1 expression results in arrest in G1 and G2 (14, 15); high levels of p16INK4A expression results in G1 arrest in cells expressing wild-type RB (16, 17). The ability of cdk inhibitor expression to cause cell death has also been investigated, and the results are complex. For example, expression of p16INK4A and p21Waf1 can protect cells from apoptosis in some model systems (18–20). In contrast, infection of various cell types with adenoviruses expressing p16INK4A, p18INK4C, or p27Kip1 can result in apoptosis, frequently in a delayed, p53-independent fashion (21–23).

These observations suggest that pharmacological cdk inhibition may inhibit tumor growth by causing cell cycle arrest and perhaps apoptosis; the latter may be cell type and context dependent (24). In this regard, the cdk inhibitor olomoucine can induce apoptosis in proliferating PC12 cells, whereas cdk inhibition can protect the same cells from apoptosis if they are differentiated (25). Another cdk inhibitor, butyrolactone I, has been shown to induce abrupt apoptosis in HL60 cells (26).

Flavopiridol, presently in clinical trials, is a novel flavone, structurally related to a natural alkaloid originally purified from the extract of the stem bark of Dysoxylum binectariferum, a plant native to India (27). The drug is a potent inhibitor of several cdks, including cdk1 (cdk2), cdk2, cdk4, and cdk7, because it has been shown to directly inhibit the kinase activity of cdks from exponentially growing cells with IC50s of 100–400 nM (27–29). Structural studies have demonstrated that the drug is structurally related to a natural alkaloid, Dysoxylum binectariferum, a plant native to India (27). The drug is a potent inhibitor of several cdks, including cdk1 (cdk2), cdk2, cdk4, and cdk7, because it has been shown to directly inhibit the kinase activity of cdks from exponentially growing cells with IC50s of 100–400 nM (27–29). Structural studies have demonstrated that the
aromatic portion of flavopiridol interferes with binding of ATP to the adenine binding pocket of the cdks (30). Furthermore, inhibition of cdk7 prevents the phosphorylation events necessary for activating the other cdks; therefore, the inhibition of cdk1, cdk2, and cdk4 is both direct and indirect.

Flavopiridol can cause arrest both in G1 and G2 phases of the cell cycle, consistent with its inhibition of cdk2, cdk4, and cdk1 (31). In addition, it is active against tumor xenografts in nude mice (27, 32–34). Recently, flavopiridol has been shown not only to be cytostatic but also cytotoxic to both hematopoietic and epithelial cell lines of diverse origin, including those derived from prostate, esophageal, lung, and head and neck carcinomas (32–38). In many cases, cytotoxicity is the result of apoptosis, based on cell morphology and DNA fragmentation. In other cells, including the one NSCLC cell line examined, morphological hallmarks of apoptosis have not been observed (35).

In clinical trials, flavopiridol is presently administered by a 72-h continuous infusion, resulting in median steady-state concentrations in the 200–300 nM range (39). To assess the potential activity of flavopiridol against NSCLC, we examined the effect of 72 h of treatment with 100–500 nM flavopiridol on several NSCLC cell lines. Like the majority of NSCLCs, these express wild-type RB and lack p16INK4A, and most (except A549 cells) also lack functional p53. Here, we demonstrate that after prolonged exposure of 48–72 h, flavopiridol is cytotoxic to seven cell lines. Although some cell lines required a dose of 500 nM for significant cytotoxicity, most were sensitive at lower doses of flavopiridol, which are more easily achieved in vivo. At this dose range, shorter exposure to drug (i.e., 24 h) was insufficient to achieve equivalent cytotoxicity. In the majority of cell lines examined, cell death was preceded or accompanied by cell cycle arrest and occurred by p53-independent apoptosis.

MATERIALS AND METHODS

Cell Culture. NSCLC cell lines, including A549, Calu-1, SK-Lu-1, Calu-6, NCI-H520, NCI-H661, and SW900, were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM supplemented with 10% bovine calf serum. All express RB and lack functional p16INK4A (3, 40–44). A549 cells express wild-type p53 (45). The p53 status of SW900 cells is not documented in the literature; in the remaining five cell lines, p53 is deleted or inactivated by mutation (45–48).

Retroviral Infection. PA317 packaging cell lines expressing the LXSN and LXSN16E6 amphotropic viruses were provided by Erik Espling and Denise Galloway (49, 50). Filtered viral supernatants, containing Polybrene at a final concentration of 4 μg/ml, were used to infect subconfluent A549 cells. After 48 h, cells were trypsinized and selected in G418 at 400 μg/ml; several colonies were isolated individually, and others were subsequently pooled and maintained in G418.

Drug Treatment. Flavopiridol was provided by Dr. Edward A. Sausville of the Developmental Therapeutics Program, National Cancer Institute. A 50 mM stock of flavopiridol was prepared in DMSO and stored at −70°C. Drug was diluted in medium and used at final concentrations ranging from 100 to 2000 nM. Twenty-four to 30 h prior to treatment, cells were plated at a subconfluent density of ~1 × 10^5/100-mm dish (A549, NCI-H520, and SW900), 2 × 10^5/100-mm dish (Calu-6), or 8 × 10^5/100-mm dish (Calu-1, NCI-H661). In all cases, untreated cells behaved identically to those treated with DMSO alone.

Western Blot Analysis. Nonadherent and adherent cells (collected by trypsinization) were pooled or kept separated, depending on the experimental goals. Cell lysates were prepared...
in NP40 lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1.0% NP40, and 1 mM phenylmethylsulfonyl fluoride]. Extracts were clarified by centrifugation, and protein concentrations were determined using the Bradford assay (Bio-Rad, Richmond, CA). Equivalent amounts (usually 100 μg) were subjected to SDS-PAGE. Gels were soaked in transfer buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11.0) and 15% methanol], and protein was electrophoretically transferred to Immobilon-P membranes (Millipore, Danvers, MA). Nonspecific binding sites were blocked by incubation with Tris-buffered saline (TBS)/10% nonfat dried milk. Primary antibody incubations were carried out in TBS/1% dried milk using the following antibodies: anti-PARP C2-10 (PharMingen, San Diego, CA), anti-p53 DO-1, anti-p27 N-20, and anti-p27 C-19 (all from Santa Cruz Biotechnology, Santa Cruz, CA), and anti-p21Waf1 (Oncogene Research Products, Cambridge, MA or Transduction Laboratories, Lexington, Kentucky). Primary antibody dilutions were 1:1000 for all antibodies except anti-p21Waf1 (Oncogene Research Products), which was diluted at 1:200. Filters were washed with TBS/0.1% Tween 20, incubated with horseradish peroxidase-conjugated secondary antibody (Amersham, Arlington Heights, IL), washed again, developed with enhanced chemiluminescence substrate (Amersham) according to the manufacturer’s instructions, and exposed to X-ray film. Equal loading of extracts was confirmed using anti-tubulin (Sigma Chemical Co., St. Louis, MO) or anti-PTP1D (Transduction Laboratories).

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 trypsin blue-excluding cells were counted using a hemocytometer. Viable cell counts were always compared with the number of cells/dish at time 0 of flavopiridol treatment.

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 PI 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. Both ModFit (Verity Software House, Topsham, ME) and CellQuest (Becton Dickinson, San Jose, CA) programs were used.

Detection of Apoptosis by Flow Cytometry. A fluorescence apoptosis detection system was used (Promega, 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 15-ml 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 PI and 500 μg/ml RNase A. After a 30-min room temperature incubation in the dark, cells were analyzed by two-color flow cytometry.

Histone H1 Kinase Assays. Cells were lysed for 1 h at 4°C in 50 mM HEPES (pH 7.2), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, and 0.1% Tween 20 supplemented with 10% glycerol, 1 mM NaF, 0.5 mM sodium orthovanadate, 1 μg/ml aprontin, 1 μg/ml leupeptin, 10 mM β-glycerophosphate, and 100 μg/ml phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation, and protein concentration was determined by Bradford assay. One mg of protein was preclariied with rabbit IgG prior to immune precipitation using 1 μg of rabbit antiserum against cdk2. Immune precipitates were col-

Fig. 2 Cell cycle profiles and cdk2 activity of flavopiridol-treated A549 cells. A, at the indicated times after flavopiridol treatment, adherent and nonadherent cells were pooled, fixed, and stained with PI for flow cytometry. B, adherent and nonadherent cells were pooled and used for the preparation of lysates; cdk2 immune precipitates were subjected to kinase assays using histone H1 as substrate. As a control, kinase activity of a lysate from cells treated with DMSO for 24 h was measured after immune precipitation with either normal rabbit IgG or anti-cdk2. Bands were quantitated using phosphorescence image analysis.
Cytotoxicity occurs by apoptosis in flavopiridol-treated A549 cells. A, TUNEL assay demonstrates the incorporation of fluoresceinated dUTP in the presence of TdT in treated cells. Fluorescein intensity is on the X axis; PI intensity is on the Y axis. The sub-G₁ population in cells treated with 500 nM flavopiridol shifts almost entirely from fluorescein negative to positive in the presence of TdT. NO RX, no treatment. B: left, cells were treated with 500 nM flavopiridol for the indicated times. Adherent and nonadherent cells were pooled and used for the preparation of cell lysates, which were subjected to Western blotting for PARP, demonstrating that levels of the intact Mᵣ 116,000 protein decreased with time; right, except separate lysates were prepared from adherent (A) and nonadherent (N) cells. The Mᵣ 85,000 PARP cleavage product is detectable in extracts from nonadherent cells. Western blot analysis for PTP1D was also carried out to demonstrate equal loading of the cell lysates. C, lysates were prepared from untreated A549 cells (U) or from cells treated at the indicated doses of flavopiridol (nM) for 72 h, which had been separated into nonadherent (N) and adherent (A) populations, and subjected to Western blot analysis for PARP. The experiment demonstrates a dose-dependent increase in PARP cleavage at 72 h. D, A549 cells were treated with DMSO or 500 nM flavopiridol for 24 h, fixed, and analyzed in TUNEL assays. In the middle panel, adherent and nonadherent cells were pooled (very few nonadherent cells were present); in the right panel, only adherent cells were analyzed. In the entire population, 15% of the cells are fluorescein positive; in the adherent population, 12% of the cells are fluorescein positive, indicating that apoptosis can be detected in the adherent population at early times after flavopiridol treatment.
lected by using protein A-Sepharose beads equilibrated with lysis buffer containing 4% BSA. Beads were washed four times with lysis buffer and then twice with kinase buffer containing 50 mM HEPES (pH 7.2), 10 mM MgCl₂, 5 mM MnCl₂, and 1 mM DTT. After the final wash, 25 μl of kinase mix were added, consisting of kinase buffer with 20 μM ATP, 10 μCi of [γ-32P]ATP, and 1 μg of histone H1. Samples were incubated at 37°C for 30 min with occasional mixing, boiled in SDS-PAGE sample buffer, and fractionated by electrophoresis through 10% polyacrylamide gels. Proteins were electrophoretically transferred to Immobilon-P, and phosphorylated Histone H1 was visualized by autoradiography. Bands were quantitated using phosphorescence image analysis.

**RESULTS**

**Flavopiridol Is Cytotoxic to A549 Cells.** Seventy-two h of continuous exposure to 200–500 nM flavopiridol resulted in cessation of A549 cell growth and a small amount of cell death during the first 48 h of treatment. This was followed by significant cell death during the last 24 h, when analyzed by trypan blue exclusion (Fig. 1), confirming an earlier report of the significant cell death during the last 24 h, when analyzed by trypan blue exclusion. A TUNEL assay indicated that the response of A549 cells to flavopiridol was apoptotic (Fig. 3, A). The percentage of TUNEL-positive cells was dose dependent and reached 48 ± 13% (mean ± SD of six experiments) after 72 h of treatment with 500 nM flavopiridol. In addition, time- and dose-dependent cleavage of PARP occurred, also diagnostic of apoptosis (51) in flavopiridol-treated cells (Fig. 3, B and C). Although detection of the M₆₈ 85,000 PARP cleavage product in these cells required preparation of extracts made only from the nonadherent population after flavopiridol treatment, TUNEL assays performed on adherent cells confirmed that this population was also undergoing apoptotic cell death (Fig. 3D).

**Apoptosis Is p53-independent in A549 Cells.** Unlike the majority of NSCLC cell lines, A549 cells carry a wild-type p53 gene. Interestingly, flavopiridol exposure resulted in accumulation of p53 in treated cells (Fig. 4A). In some experiments, p53 accumulation was maximal within the first 24 h. After treatment with 300 nM flavopiridol, a concomitant small increase in p21Waf1 was also seen (Fig. 4B); however, at 500 nM, p21Waf1 levels fell dramatically with time (Fig. 4, A and B). This is most likely the result of cleavage of p21Waf1 during apoptosis. With antibodies directed against the NH₂-terminal domain of p21Waf1, the expected cleavage product (52–54) was seen only in the nonadherent population (Fig. 4C). p27Kip1 cleavage during flavopiridol-mediated apoptosis was also demonstrated in these cells (Fig. 4D).

To determine whether the accumulation of p53 was required for flavopiridol-mediated apoptosis, the HPV16E6 oncoprotein was introduced into A549 cells by retroviral transfer. E6-expressing cells were incapable of G₁ arrest after exposure to DNA-damaging agents, including γ-irradiation and Adriamycin (Fig. 5, A and B). After γ-irradiation, some cells were treated with nocodazole so that G₁ content could be analyzed without the exit of cells from mitosis (50). E6 expression prevented p53 accumulation after DNA damage, although a small p53-independent increase in p21Waf1 occurred (Fig. 5C). Taken together, the results are consistent with the abrogation of p53 function in E6-expressing cells. Although flavopiridol does not result in significant p53 accumulation in E6-expressing cells, they readily undergo apoptosis (Fig. 6), indicating that cell death is indeed p53 independent.
Apoptotic Cell Death Occurs in p53-deficient NSCLC Cell Lines. To confirm that flavopiridol-mediated apoptosis is p53 independent, we analyzed the response of several lung cancer cell lines lacking wild-type p53 to 72 h of treatment with 100–500 nM drug. In Calu-1 (Fig. 7) and SK-LU-1 cells (not shown), as in A549 cells, cell cycle arrest clearly preceded cell death. Fig. 7, A and B, shows that over the first 48 h of treatment at doses of 400 and 500 nM, there is cessation of cell growth, followed by cell death during the last 24 h of treatment. Consistent with this, Fig. 7C shows that in Calu-1 cells over the first 24 h, prior to significant cell death and at doses between 300 and 500 nM, there is an increase in cells with G1 and G2 contents and marked diminution of cells in S phase, consistent with G1 and G2 arrest. Cell cycle arrest persists and is followed by cell death at 72 h, as indicated by a dose-dependent increase in percentage of cells with a sub-G1 DNA content. The apoptotic response of these cells was confirmed by TUNEL assay and by demonstration of PARP cleavage (Fig. 7, D and E).

In contrast, in NCI-H661, Calu-6 (Fig. 8), and NCI-H520 cells (data not shown), cell death was more marked and apparent as early as 24 h at the highest doses (Fig. 8, A and B). In these cells, diminution in S phase accompanied apoptosis, without the same separation in time between cell cycle arrest and cell death, as was observed in Calu-1 cells (Fig. 8C). Once again, apoptosis was confirmed by TUNEL assay and by demonstration of PARP cleavage (Fig. 8, D–F); in these cell lines, the M, 85,000 PARP cleavage product was readily apparent in total and adherent cell extracts as well as nonadherent cell extracts. Finally, cleavage of p27Kip1 was also demonstrated, another target of caspases during apoptosis (Fig. 8G).

In summary, cell death after flavopiridol treatment was a common outcome in p53-deficient NSCLC cell lines, with a dose and time-dependent decrease in viable cell number and increase in the percentage of apoptotic cells. IC50s are shown in Table 1.

SW900 Cells Undergo Apoptosis without Antecedent Cell Cycle Arrest. In SW900 cells, both apoptosis and cell cycle arrest occurred but appeared unrelated (Fig. 9). For example, at 48 h, there is evidence of apoptosis at 500 nM flavopiridol, without apparent cell cycle arrest. The same is true at

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**Fig. 5** Characterization of LXSN and HPV16E6-expressing A549 cells. Data are shown from a pooled population of cells after infection with either the control or E6-expressing retrovirus. A, LXSN and E6-expressing A549 cells were left untreated or treated with 0.4 μg/ml nocodazole for 18 h. Alternatively, cells were subjected to 10 Gy of γ-irradiation and allowed to grow for an additional 18 h in the presence or absence of 0.4 μg/ml nocodazole. Cells were collected by trypsinization, fixed, stained with PI, and analyzed by flow cytometry. Numbers indicate the percentage of cells in G1. B, LXSN and E6-expressing A549 cells were left untreated or treated with 0.05 μg/ml Adriamycin for 24 h and analyzed by flow cytometry. Numbers indicate the percentage of cells in G1. The experiments in A and B indicate the abrogation of the p53-mediated G1 arrest checkpoint after DNA damage in E6-expressing cells. No Rx, no therapy. C, lysates from untreated cells (U) or those treated with the indicated Adriamycin concentration (μg/ml) were subjected to Western blot analysis for p53, p21Waf1, and tubulin. Accumulation of p53 and p21Waf1 is inhibited in E6-expressing cells. A small amount of p53-independent accumulation of p21Waf1 after treatment with Adriamycin has been reported in other cell types (7).
72 h at 300 nM flavopiridol. These results demonstrate that flavopiridol-mediated cell cycle arrest need not occur prior to a cytotoxic response in lung cancer cell lines.

**Maximal Cytotoxicity Requires Continued Exposure at Low Doses of Flavopiridol.** To determine whether prolonged continuous treatment was necessary to achieve apoptosis in NSCLC cell lines, cells were treated for 24 h only and collected after an additional 48 h (i.e., at the 72-h time point) and compared with those treated continuously for 72 h. Table 2 demonstrates that in A549 cells, after 24 h of treatment at doses <500 nM, recovery occurs such that cytotoxicity is markedly diminished compared with a 72-h continuous exposure to drug. At higher doses, including 1000 nM and above, cell death at the 72-h time point is similar whether cells have been treated continuously or only for 24 h. In contrast, in NCI-H661 (Table 3) and Calu-1 cells (data not shown), cytotoxicity was greater at all doses after 72 h of continuous exposure.

These experiments were extended to analyze even longer exposures to flavopiridol. At 72 h, flavopiridol was either continued, or drug was removed and replaced with drug-free medium for an additional 48 h. Analysis of samples at 120 h indicated that removal of drug at 72 h did not reverse the lethal effect of flavopiridol, and cells continued to die. Overall, 72 and 120 h of exposure produced comparable cytotoxicity when cells were analyzed at 72 h of continuous exposure.

**Cytotoxicity Occurs in Noncycling Cells.** In some cell lines, apoptosis clearly followed cell cycle arrest, suggesting that noncycling cells could undergo apoptosis. To further investigate this, A549 cells were arrested by starvation in 0.1% bovine calf serum and were either left untreated or treated with flavopiridol for an additional 72 h in low serum. Fig. 10 demonstrates that cell death can occur in cells arrested in G1 prior to treatment. The IC50 at 72 h in starved A549 cells was 195 nM, representing a 1.5-fold increase over that seen in exponentially growing cells.

**DISCUSSION**

We have shown that flavopiridol causes apoptotic cytotoxicity in a panel of NSCLC cell lines. This was demonstrated by time- and dose-dependent decreases in viable cell count by trypan blue exclusion and concomitant increases in the percentage of cells with a sub-G1 DNA content. Apoptosis was confirmed by TUNEL assays and by the demonstration of cleavage of known caspase targets, including PARP, p21Waf1, and p27Kip1. Flavopiridol-mediated cytotoxicity had been demonstrated previously in A549 cells. In that study, morphological hallmarks of apoptosis were not observed (35). In contrast, our data clearly indicate that both DNA fragmentation and caspase-mediated events occur in response to flavopiridol, indicating activation of an apoptotic pathway in these cells.

In contrast to flavopiridol, the flavonoid flavone does not result in p53 accumulation in A549 cells, although G1 arrest occurs because p21Waf1 is transcriptionally induced in a p53-independent manner (55). Other flavonoids have been shown to activate wild-type p53 in nontransformed cells, including apigenin, luteolin, and quercetin (56), and relatively low doses of apigenin result in p53-dependent increases in p21Waf1 (57). p53 activation by flavonoids may be secondary to the formation of oxygen radicals or the inhibition of topoisomerase activity (56). Although flavopiridol does not inhibit topoisomerase activity (35, 36), p53 reproducibly accumulated after treatment of A549 cells with 500 nM flavopiridol for 72 h.
Fig. 7  Response of Calu-1 cells to flavopiridol. A, a representative experiment is shown in which cells were treated with the indicated doses of flavopiridol. At 24, 48, and 72 h after treatment, adherent cells were collected by trypsinization and combined with nonadherent cells. Viable cell number was determined by trypan blue exclusion. B, viable cell counts from a minimum of two and a maximum of four experiments were used to generate the data for each condition. Percentages represent the number of viable cells remaining compared with the number of cells/dish at time 0 of flavopiridol treatment. Bars, SD. C, cell cycle profiles of untreated Calu-1 cells and of cells treated at the indicated doses. The data demonstrate cell cycle arrest followed by the dose-dependent appearance of populations of cells with sub-G1 DNA content. D, TUNEL assays demonstrate that cytotoxicity occurs by apoptosis in Calu-1 cells. PI is on the Y axis and fluorescein is on the X axis. In the presence of TdT, the sub-G1 population is almost entirely fluorescein positive. In this experiment, 57% of the cells were fluorescein positive after treatment with 500 nM flavopiridol for 72 h. No Rx, no therapy. E, Calu-1 cells were left untreated or treated with 500 nM flavopiridol for 72 h. Extracts were prepared from untreated cells (U), adherent treated cells (A), or nonadherent treated cells (N). Lysates were subjected to Western blot analysis for PARP and PTP1D. In treated cells, the M, 85,000 PARP cleavage product is detectable.

Fig. 8  Response of NCI-H661 and Calu-6 cells to flavopiridol. A, a representative experiment is shown in which NCI-H661 cells were treated with the indicated doses of flavopiridol. At 24, 48, and 72 h after treatment, adherent cells were collected by trypsinization and combined with nonadherent cells. Viable cell number was determined by trypan blue exclusion. B, NCI-H661 viable cell counts from a minimum of three and a maximum of eight experiments were used to generate the data for each condition. Percentages represent the number of viable cells remaining compared with the number of cells/dish at time 0 of flavopiridol treatment. Bars, SD. C, cell cycle profiles of untreated NCI-H661 cells and of cells treated at the indicated doses. D and E, TUNEL assays demonstrate that cytotoxicity occurs by apoptosis in NCI-H661 and Calu-6 cells. PI is on the Y axis, and fluorescein is on the X axis. In the presence of TdT, the sub-G1 population is almost entirely fluorescein positive. In the NCI-H661 experiment, the percentage of fluorescein-positive cells at 48 and 72 h are 59 and 72%, respectively; in the Calu-6 experiment, 24% of the cells are fluorescein positive at 48 h. F, cells were either untreated or treated with 500 nM flavopiridol for 72 h. Extracts were prepared from untreated cells (U), adherent treated cells (A), or nonadherent treated cells (N). Lysates were subjected to Western blot analysis for PARP and PTP1D. The M, 85,000 PARP cleavage product is detectable in all treated populations. G, similar experiment in NCI-H661 cells in which cleavage of p27Kip1 was analyzed with an antibody directed at the NH3 terminus.
cells. In addition, at 300 nM, a small increase in the level of p21Waf1 was observed. The accumulation of p53 after flavopiridol treatment may be cell type specific, because, for example, it was not observed in SUDHL-4 B-cell lymphoma cells, although this cell line had high baseline levels and was only treated for 24 h (36).

At high doses of other flavonoids, apoptosis was observed during 72 h of treatment; in the nontransformed cell types studied, apoptosis appeared to be p53 dependent (56), but among transformed cells, this was not necessarily the case (58). Despite the accumulation of p53 observed in A549 cells, the apoptosis mediated by flavopiridol is independent of p53 by two criteria: (a) HPV16E6-expressing and control wild-type p53-expressing A549 cells have a similar apoptotic response; and (b) apoptosis readily occurs in cell lines in which p53 is deleted or inactivated by mutation.

Several studies have now demonstrated the association of caspase-mediated cleavage of p21Waf1 and p27Kip1 with apoptosis (52–54). In several systems, it has been proposed that this would facilitate apoptosis by releasing cells from growth arrest or allowing cdk2 activation. Such a mechanism is most likely not operating after flavopiridol treatment, because cell death can follow cell cycle arrest, and continued cdk2 inhibition occurs during flavopiridol exposure. It may be that cleavage of p21Waf1 and p27Kip1 is a consequence, rather than a cause, of apoptosis.

The mechanism of p53-independent apoptosis mediated by flavopiridol remains to be elucidated. Although cdks are the known primary targets at the doses studied, other kinases, including protein kinase C and epidermal growth factor receptor tyrosine kinase, are inhibited at higher doses of flavopiridol (27). Although inhibition of cdks clearly mediates cell cycle arrest by flavopiridol, it is not yet proved whether cdk inhibition is the cause of the apoptotic response. In this regard, our data in SW900 cells suggest that cell cycle arrest and apoptosis need not be intimately linked, as has been reported in a B-cell lymphoma cell line (36). In addition, if cdks are the critical targets, it will be important to determine whether inhibition of one or several is necessary for apoptosis. The answer to these questions will require the generation of flavopiridol-resistant cell lines as well as cell lines overexpressing various wild-type or mutant cdks.

We have confirmed the ability of flavopiridol to induce

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### Table 1

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ᵃnm ± SD. Values without SD are the result of one experiment. Other values represent the average of two or three experiments.
cytotoxicity in noncycling cells. The ability of arrested cells to undergo cell death during flavopiridol exposure was evident in experiments in which cell cycle arrest (both in G$_1$ and G$_2$) clearly preceded the time at which maximal cell death occurred. In addition, TUNEL assays suggested that cell death was not confined to a particular phase of the cell cycle. Flavopiridol-mediated cytotoxicity of noncycling cells has been investigated previously in density-arrested A549 cells by two groups of investigators (35, 59). Although both groups found that density-arrested cells were not resistant to flavopiridol, one group reported that the IC$_{50}$ in arrested cells was significantly higher. In our experiments, serum-starved cells could also undergo cell death in response to flavopiridol, with a 1.5-fold higher IC$_{50}$. Experiments in both serum-starved NCI-H661 cells and density-arrested Calu-1 cells yielded similar results. Although these cells were clearly killed by flavopiridol, there was a trend suggesting decreased apoptosis (up to 2-fold) in the noncycling populations (data not shown).

In other cells, including NCI-H661, NCI-H520, and Calu-6 cells, a diminution in S phase accompanied the onset of apoptosis. Although cell cycle arrest and apoptosis could be occurring concomitantly, our data do not rule out the possibility that S-phase cells are preferentially dying and are more sensitive to flavopiridol-mediated cell death. Additional experiments, including simultaneous bromodeoxyuridine/TUNEL analyses, will be necessary to determine whether this is the case. In this regard, one lung cancer cell line, NCI-H23, has been reported to undergo a delay in S-phase traversal after flavopiridol, with more abrupt apoptosis, evident earlier than 24 h (60). The sensitivity of S-phase cells might explain why flavopiridol potentiates the activity of chemotherapeutic drugs capable of causing S-phase delay (61, 62).

Our data have implications for the clinical use of flavopiridol. The apoptotic response of NSCLC cells suggests promise for the efficacy of flavopiridol in lung cancer. However, it is noteworthy that significant apoptosis in vitro frequently requires doses of 300–500 nm, which are higher than median levels achieved in vivo when a 72-h continuous infusion is used at the maximal tolerated dose (200–300 nm; Ref. 39). Our data suggest that if higher levels are achieved (i.e., >500 nm), a 24-h treatment may be acceptable. It is possible that higher doses of flavopiridol may be tolerated if the exposure is shorter. However, the data indicate that if levels >500 nm are not easily achieved, the 72-h continuous infusion is preferable to a shorter period of treatment. This schedule is presently in use in a Phase II trial for patients with advanced NSCLC.

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Flavopiridol Induces p53-independent Apoptosis in NSCLC

A549 Starved in 0.1% BCS

No Rx

300 nM Flavopiridol

Fig. 10 Flavopiridol is cytotoxic to serum-starved A549 cells. A549 cells were starved in 0.1% bovine calf serum for 72 h. Cells were then left untreated (left) or treated with 300 nM flavopiridol (right) in 0.1% bovine calf serum for an additional 72 h. At the conclusion of the experiment, adherent and nonadherent cells were pooled and analyzed by flow cytometry.

REFERENCES


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