Advances in Brief

Flavopiridol: A Cytotoxic Flavone That Induces Cell Death in Noncycling A549 Human Lung Carcinoma Cells

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Abstract

Flavopiridol (NSC 649890, L86-8275), a potent inhibitor of cyclin-dependent kinase 1/2 (cdk1/cdk2) phosphorylation and kinase activity, is currently undergoing Phase I clinical testing as a potential antineoplastic agent. Previous studies have suggested that flavopiridol is cytostatic but not cytotoxic when applied to exponentially growing cells. In the present study, various human tumor cell lines were assayed for trypan blue exclusion and ability to form colonies after exposure to flavopiridol under a variety of growth conditions. When log phase A549 non-small cell lung cancer cells were examined 72 h after the start of a 24-h flavopiridol exposure, as many as 90% of the cells accumulated trypan blue. A 24-h exposure to 250—300 nM resulted in trypan blue uptake in 50% of A549 cancer cells were examined 72 h after the start of a 24-h flavopiridol exposure. Similar results were observed in HCT8 ileocecal adenocarcinoma, T98G glioblastoma, MCF-7 breast adenocarcinoma, and HL-60 leukemia cells. With A549 cells, identical results were obtained in actively growing logarithmic phase cells and growth-arrested confluent cells. Treatment with the DNA synthesis inhibitor aphidicolin only minimally affected the cytotoxicity of flavopiridol. In contrast, the RNA synthesis inhibitor 5,6-dichloro-1-b-ribofuranosyl-benzimidazole or the protein synthesis inhibitor cycloheximide reduced the cytotoxicity of flavopiridol. These results suggest that: (a) flavopiridol is not only cytostatic, but also cytotoxic to a variety of human tumor cell lines; (b) flavopiridol is equally active against cycling and noncycling A549 cells; and (c) RNA and protein synthesis appear to play a role in flavopiridol-induced cytotoxicity.

Introduction

Flavopiridol [NSC 649890, L86-8275, (–)cis-5,7-dihydroxy-2-(2-chlorophenyl)-8-[4-(3-hydroxy-1-methyl)-piperidinyl]-4H-1-benzo-pyran-4-one, Fig. 1A], a synthetic flavone closely related to a compound originally isolated from the stem bark of the native Indian plant Dyssoxylum binecarifera (1), is currently undergoing Phase I clinical trials as a potential antineoplastic agent (2). The parent compound, which is identical to flavopiridol except that a methyl group replaces the chlorophenyl moiety at the 2 position, was originally identified because of its anti-inflammatory and immunomodulatory activity (1). Flavopiridol has subsequently attracted interest because of its ability to inhibit tumor cell growth and because it is the first multitumor agent to directly target CDKs.3

Flavopiridol induces arrest in both G1 and G2 phases of the cell cycle (3, 4). It has been reported to be a potent inhibitor of the kinase activity of CDK1, CDK2, CDK4, and CDK7 (4, 5). Kinetic studies with CDK1 (6) and X-ray crystallography of CDK2 bound to a derivative of flavopiridol (7) both indicate that flavopiridol competes with ATP at the nucleotide binding site of CDKs. In addition, flavopiridol inhibits the tyrosine phosphorylation of CDK1, raising the possibility that it also inhibits the CDK-activating kinase (8). As would be expected for an agent that inhibits CDK activity, flavopiridol has been reported to cause cytostatic changes in exponentially growing cells, but not to be cytotoxic to actively growing or stationary phase cells (3). Additional preclinical data suggest that flavopiridol is particularly cytostatic in cell lines derived from melanoma and carcinomas of the breast, prostate, and lung (3, 4, 9).

In the present study, the effect of flavopiridol on a number of human tumor cell lines was examined, with particular emphasis on A549 non-small cell lung cancer cells. Trypan blue dye exclusion, colony-forming ability, and cell cycle distribution were determined after exposure to flavopiridol under a variety of growth conditions. These studies lead to the conclusion that: (a) flavopiridol is cytotoxic rather than merely cytostatic as originally suggested; and (b) flavopiridol is equally toxic to noncycling and cycling cells. These properties make flavopiridol an appealing agent for further preclinical and clinical development as an antineoplastic agent.

Materials and Methods

Materials. Flavopiridol was a generous gift from Dr. Edward Sausville (National Cancer Institute, Bethesda, MD). Reagents were purchased from the following suppliers: aphidicolin, DRB, and cycloheximide from Sigma Chemical Co. (St. Louis, MO); and apigenin, biochanin A, flavone, and α-naphthoflavone from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were obtained as described previously (10).

Stock (1000-fold-concentrated) solutions of flavopiridol, cycloheximide, DRB, and aphidicolin were prepared in DMSO and stored at −20°C. All other reagents were dissolved immediately before use.

Colony-forming Assays. Cell lines (from American Type Culture Collection, Rockville, MD, unless otherwise indicated) were cultured in the following media, which all contained 50 units/ml penicillin G, 50 μg/ml streptomycin, and 2 mM glutamine: A549 non-small cell lung cancer cells and HCT8 ileocecal adenocarcinoma cells in RPMI 1640—5% (v/v) FBS (medium A); T98G glioblastoma cells in MEM containing Earle’s salts, 10% (v/v) FBS, nonessential amino acids and 1 mM sodium pyruvate (medium B); MCF-7 breast cancer cells in medium B containing 10 μg/ml insulin; and HL-60 leukemia cells (from Robert Abraham, Mayo Clinic) in RPMI 1640—10% (v/v) FBS. Adherent cells were grown in subconfluent culture, trypsinized, fed twice weekly, and maintained at 37°C in an atmosphere of humidified 5% (v/v) CO2. Nonadherent HL-60 cells were fed three times weekly and maintained in culture between 105 and 106 cells per milliliter of medium.

To examine the effect of flavopiridol on colony formation, subconfluent A549 cells were released by trypsinization, plated (750 cells/plate) in multiple 35-mm dishes containing 2 ml of medium A, and allowed to attach for 12–16 h. Graded concentrations of drugs or equivalent volumes of diluents were then added to triplicate plates. After a 24-h incubation with drug, plates were washed twice in serum-free RPMI 1640 and incubated in drug-free medium A for an additional 7 days. The resulting colonies were stained with Coomassie blue and manually counted. Diluent-treated control plates typically contained 200—250 colonies. Similar experiments were conducted with T98G (500 cells plated, 8 days incubation time), MCF-7 (750 cells plated, 14 days incubation time), and HCT8 cells (500 cells plated, 8 days incubation time) using the
Fig. 1. Flavopiridol is cytotoxic to human cancer cell lines. A, chemical structures of flavopiridol and four other flavonoids. Colony-forming assays (performed as described in "Materials and Methods") were used to determine the IC_{50} after a 24-h exposure. B, effect of flavopiridol on colony formation in five human tumor cell lines: A549 (○), T98G (●), HCT8 (□), MCF-7 (★), and HL-60 (■). C, effect of a 24-h flavopiridol treatment on trypan blue exclusion (●) and colony formation (□) in log phase A549 cells. Bars, ±1 SD.

To examine the effect of flavopiridol on confluent (nonproliferating) cells, 35-mm plates of A549 cells were grown to confluence and incubated in a confluent state for 7 days without media replenishment. These plates were then exposed to graded concentrations of flavopiridol for the designated times. After removal of flavopiridol, cells were washed with serum-free RPMI 1640 and trypsinized. Aliquots containing 750 cells were plated in triplicate 35-mm dishes containing 2 ml of medium A, and these plates were incubated for an additional 7 days. The resulting colonies were stained with Coomassie blue and counted. Diluent-treated control plates typically contained 100–150 colonies.

Assessment of Cell Viability. As an alternative to colony-forming assays, log phase or confluent A549 cells (see above) were treated with varying concentrations of flavopiridol for the indicated times, washed twice with RPMI 1640, and incubated in medium A until 72 h after the time of flavopiridol addition. Cells attached to the plates were released by trypsinization and combined with nonadherent cells. All cells were sedimented at 200 × g for 5 min, resuspended in PBS, treated with 0.2% trypan blue, and counted using a hemocytometer.

Flow Cytometry. After trypsinization, aliquots containing ~1 × 10^6 cells were sedimented at 200 × g for 5 min. All further steps were performed at 4°C unless otherwise indicated. Cells were washed in PBS, resuspended in 300 μl of PBS, and diluted by dropwise addition of 300 μl of 95% ethanol. After
fixation for ≥ 1 h, cells were washed twice with PBS, resuspended in 300 μl of 0.1% sodium citrate containing 1 mg/ml RNAse A, and incubated at 37°C for 15 min. Immediately before flow cytometry, samples were diluted with 300 μl of 0.1% sodium citrate containing 100 μg/ml propidium iodide and incubated in the dark at room temperature for an additional 15 min. Flow cytometry was performed on a Becton Dickinson FACScan (San Jose, CA) using an excitation wavelength of 488 nm and an emission wavelength of 585 nm. Histograms were analyzed using ModFit software (Verity Software House, Topsham, ME).

Electron Microscopy. Cells for transmission electron microscopy were washed twice with PBS, fixed for ≥ 1 h with Trump’s fixative (1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2)), treated with phosphate-buffered 1% O254, stained en bloc with 2% uranyl acetate for 30 min at 60°C and embedded in Spurr’s resin. Sections (90 nm) were cut on a Leica Ultracut E or S ultramicrotome (Leica, Inc., Vienna, Austria), collected on 200-mesh copper grids, stained with lead citrate, and examined and photographed with a JOEL 1200 EXII electron microscope (Tokyo, Japan) operating at 60 kV.

Results
Flavopiridol Inhibits Colony Formation in a Variety of Human Tumor Cell Lines. In conjunction with ongoing preclinical studies of flavopiridol, we examined the effects of this agent on the ability of human cell lines to form colonies. The results of these studies are shown in Fig. 1B. A 24-h flavopiridol treatment reduced subsequent colony formation by A549 non-small cell lung cancer cells (Δ), T98G glioblastoma cells (○), HCT8 intestinal adenocarcinoma cells (○), MCF-7 breast carcinoma cells (□), and HL-60 leukemia cells (■), with IC50s of 250, 100, 200, 250, and 100 nm, respectively.

Flavopiridol Is a More Potent Inhibitor of A549 Cell Colony Formation than Related Flavonones. Although many flavonoids inhibit tumor cell growth in vitro, flavopiridol has been reported to be much more potent in this regard (7). We examined the ability of five commercially available structurally related flavonoids to inhibit A549 colony formation in vitro (Fig. 1A). None of these compounds inhibited colony formation with the potency of flavopiridol.

To determine whether these related compounds might compete with flavopiridol at its hypothetical binding site, A549 cells were pretreated with apigenin, biochanin A, or α-naphthoflavone for 24 h before and then 24 h concomitant with flavopiridol. The presence of the flavopiridol analogues did not significantly alter the effect of flavopiridol on A549 colony formation, although apigenin, biochanin A, and α-naphthoflavone were present in vast molar excess relative to flavopiridol (data not shown).

Flavopiridol Is Cytotoxic as well as Cytostatic. Because previous studies have suggested that flavopiridol is strictly cytostatic, the dramatic inhibition of colony formation observed in Fig. 1B was unexpected. To determine whether these results reflected a prolonged cytostatic effect of flavopiridol as opposed to a cytotoxic effect, A549 cells were treated with flavopiridol for 24 h, washed, and assayed for trypan blue uptake 48 h after flavopiridol removal. Dose-response curves were similar when survival was evaluated by trypan blue exclusion (○, Fig. 1C) or colony formation (○, Fig. 1C). Trypan blue uptake likewise was demonstrated in flavopiridol-treated HL-60 and T98G cells (data not shown). These results indicate that flavopiridol is cytotoxic rather than merely cytostatic.

Flavopiridol Is Cytotoxic to Stationary Phase Cells. Additional experiments were performed to further characterize the cytotoxicity of flavopiridol. A549 cells were used for these experiments because of the ease with which their growth characteristics could be altered and their well-defined response to metabolic inhibitors (12).

To examine the effect of cell proliferation on flavopiridol cytotoxicity, log phase and confluent A549 cells were compared. Up to 95% of A549 cells could be induced to enter G0/G1 when cells were allowed to remain confluent for 1 week (Fig. 2A). Flavopiridol treatment of these stationary phase cells (○, Fig. 2B) did not result in the cell cycle alterations seen in similarly treated exponentially growing cells (■, Fig. 2B), but it nonetheless resulted in cytotoxicity as assessed by trypan blue uptake and colony formation (Fig. 2, C and D). It is interesting that the toxicity of flavopiridol to confluent A549 cells appeared greater when evaluated by colony-forming assay (□, Fig. 2D) than by trypan blue (○, Fig. 2D). This might, in part, reflect the difficulty in attempting to evaluate the nadir in cell number by trypan blue assay. However, the possibility of a flavopiridol-associated decrease in plating efficiency due to altered adheresiveness of the cells also cannot be ruled out.

Maximal Cytotoxicity Occurs with 24-h Flavopiridol Exposure Independent of Cell Cycle Length. In all of the experiments described above, cells were exposed to flavopiridol for 24 h. To examine the effect of duration of exposure, A549 cells were treated for various lengths of time, washed, and allowed to form colonies. Results of these experiments (inset, Fig. 3A) revealed that exposure times shorter than 12 h were much less toxic than exposure times of 18–24 h or longer. When the flavopiridol exposure (concentration × time) that inhibited colony formation by 50% was plotted as a function of exposure time (Fig. 3A, □), the drug exposure (concentration × time) required to produce cytotoxicity was observed to reach a minimum with an exposure of 24 h or longer.

To determine whether this time dependence was related to the 20-h population doubling time of A549 cells, A549 cells were exposed to flavopiridol in the presence of medium that contained 0.5% FBS (rather than the usual 5%). Under these conditions, the population doubling time increased from 20 h to ~40 h, but the optimum exposure time remained ~24 h (Fig. 3A, □). A similar relationship between flavopiridol cytotoxicity (assessed by trypan blue uptake) and duration of exposure was also seen in confluent (noncycling) A549 cells (data not shown). These results not only indicated that the requirement for a ~24-h exposure time is independent of cell cycle duration, but also confirmed that flavopiridol toxicity occurred independent of the rate at which cells were progressing through the cell cycle.

Cytotoxicity of Flavopiridol Is Diminished by Pretreatment with Inhibitors of RNA and Protein Synthesis. The preceding observations (Figs. 2, A–D, and 3A) raised the possibility that flavopiridol might not depend on cell cycle progression for its toxicity. To further test this hypothesis, cells were pretreated for 24 h with 10 μM aphidicolin, a treatment that inhibited DNA synthesis by 95%. Although this pretreatment markedly diminished the cytotoxicity of the S-phase-dependent agent topotecan (Fig. 3B, inset), flavopiridol cytotoxicity was only minimally affected by aphidicolin (Fig. 3B), confirming that neither actively cycling cells nor DNA synthesis was required for flavopiridol cytotoxicity. In contrast, preincubation with 94 μM DRB or 110 μM cycloheximide, concentrations that inhibited RNA and protein synthesis in A549 cells by >90%, respectively, much more markedly diminished flavopiridol cytotoxicity (Fig. 3C), suggesting that flavopiridol cytotoxicity is at least partially dependent on protein synthesis.

Flavopiridol Induces Apoptotic Cell Death in HL-60 Cells, but not A549 Cells. The observation that flavopiridol cytotoxicity is diminished by agents that inhibit protein synthesis prompted us to examine flavopiridol-induced morphological changes by electron microscopy. As is the case with many other agents (reviewed in Ref. 13), flavopiridol induces typical apoptotic morphological

*The evaluation of cytotoxicity by trypan blue exclusion underestimates the true level of cell killing because of proliferation of surviving cells as well as failure of some cells to lose membrane integrity by 72 h despite their subsequent death.
changes in HL-60 cells. Compared with control HL-60 cells (Fig. 4A), cells treated with 1.125 μM flavopiridol began to undergo apoptosis within 4 h, with nearly all cells becoming apoptotic by 6 h (Fig. 4B). Additional experiments using fluorescence microscopy after staining with Hoechst 33258 (14) revealed that 50% of the flavopiridol-treated HL-60 cells appeared apoptotic 6 h after exposure to flavopiridol at concentrations as low as 100 nM. These cells subsequently lost membrane integrity, although trypan blue uptake did not become maximal until 48–72 h after flavopiridol introduction. In contrast, treatment of A549 cells with 1.125–11.25 μM flavopiridol (4.5–45 times the IC₅₀) for 24 h resulted in cell death as assessed by trypan blue uptake at 72 h (Fig. 1C) but did not lead to apoptotic morphology when assessed serially by both electron microscopy (compare Fig. 4, C and D) and by Hoechst 33258 fluorescence microscopy (data not shown) during the 72 h after flavopiridol introduction.

Discussion

Flavopiridol is a promising antineoplastic agent that inhibits the phosphorylation and catalytic activity of multiple CDKs (see “Introduction”). In the present study, several aspects of the effect of flavopiridol on human tumor cells were examined. These studies have led to several novel observations. First, flavopiridol not only inhibits tumor cell proliferation, but is also cytotoxic. Second, flavopiridol is cytotoxic to both cycling and to noncycling cells. Third, there is a striking dependence of flavopiridol toxicity on duration of flavopiridol exposure. Fourth, the cytotoxicity of flavopiridol is diminished under conditions in which protein synthesis is inhibited. Fifth, this cytotoxicity can occur with or without typical morphological changes of apoptosis. These findings have potential implications for models of flavopiridol action and for future clinical development of this agent.

The observation that flavopiridol is cytotoxic rather than merely...
The demonstration that flavopiridol is equally toxic to both cycling and noncycling cells is potentially important from mechanistic and therapeutic standpoints. Results presented above indicate that flavopiridol can kill confluent noncycling cells (Fig. 2, C and D) as well as cells arrested in G1 by aphidicolin treatment (Fig. 3B). In addition, cells can be killed without any alteration in their cell cycle distribution (Fig. 2, A—D). Notwithstanding the ability of flavopiridol to competitively inhibit CDK1 phosphorylation (8), bind to the ATP binding site of CDK2 (7), and inhibit CDK kinase activity (5, 6), these observations raise the question of whether the mechanism of flavopiridol cytotoxicity is related to its cell cycle and CDK effects. It is possible, of course, that CDKs play a heretofore unappreciated role in cell survival independent of their role in cell cycle regulation. However, the possibility that flavopiridol-induced cell death involves another intracellular target must also be considered.

Recent studies have indicated that several flavonoids trap topoisomerase I in a covalent complex with DNA (15). From a mechanistic standpoint, the cytotoxicity of flavopiridol in noncycling cells distinguishes this agent from topoisomerase I-directed agents such as topotecan (Fig. 3B). Additional experiments have indicated that flavopiridol also fails to inhibit topoisomerase I catalytic activity, distinguishing it further from topoisomerase I-directed flavonoids.

The demonstration that inhibitors of RNA and protein synthesis diminish the cytotoxicity of flavopiridol (Fig. 3C) raises the question of whether flavopiridol might be killing cells by inducing apoptosis. The present study provides mixed results in this regard. In HL-60 cells, flavopiridol induces prompt apoptosis (within 4—6 h, Fig. 4B), and this seems to be the principal mode of flavopiridol-induced death in this cell line. While the present work was in progress, two recent abstracts also reported apoptosis in SUDHL-4 lymphoma cells, PC-3 prostate cancer cells, and MKN-74 gastric carcinoma cells after flavopiridol treatment (16, 17). In contrast, apoptosis was not observed in flavopiridol-treated A549 cells (Fig. 4D), although they were clearly dying 72 h after the initiation of treatment. Topotecan and etoposide likewise produced typical apoptotic changes in HL-60 cells but not A549 cells, indicating that the failure to induce apoptosis in A549 cells was not unique to flavopiridol. These results indicate that the classical changes of apoptosis are not a requirement for flavopir-
dol-induced cell death, although apoptosis may be prominent in some cell lines.

The present observations also provide information that might be useful in the design of new clinical trials involving flavopiridol. The exquisite sensitivity of HL-60 cells to this agent (Fig. 1B) raises the possibility that flavopiridol might be particularly active in hematological malignancies. In addition, the striking dependence of flavopiridol cytotoxicity on exposure time (Fig. 3A) is of potential importance. The observation that exposures shorter than 18–24 h are less cytotoxic even when corrected for total drug exposure (concentration × time, Fig. 3A) and the independence of this effect from cell doubling time raises the possibility that a threshold exposure of 18–24 h might be required for maximum efficacy of this drug. Whether there will be an advantage (or disadvantage) to the use of infusion durations longer than 24 h remains uncertain, but infusions shorter than 24 h would seem to be too brief to exploit the maximum cytotoxicity of this agent.

Finally, the observation that flavopiridol kills noncycling cells has practical implications as well. The low percentage of cycling cells in many human solid tumors limits the potential of many widely used antineoplastic agents to kill tumor cells in vivo (18). Few antineoplastic agents other than the alkylating agents and platinum compounds are cytotoxic to noncycling cells (19). The observation that flavopiridol kills noncycling cells makes it an appealing agent to combine with other antineoplastic agents that might cause cells to arrest in S or G2 phases of the cell cycle. Additional experiments to examine this possibility are currently under way.

Acknowledgments

We are grateful to Edward Sausville (Developmental Therapeutics Program, National Cancer Institute, NIH, Bethesda, MD) for providing flavopiridol. Technical assistance and helpful discussions provided by Phyllis Svingen, Imawati Budihardjo, Tim Kotke, Peter Messner, and by the staff of the Core Electron Microscopy and Flow Cytometry Laboratories at the Mayo Clinic are also acknowledged. Secretarial assistance was also kindly provided by Deb Strauss.

References

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