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Cytotoxic Synergy between Flavopiridol (NSC 649890, L86–8275) and Various Antineoplastic Agents: The Importance of Sequence of Administration

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Abstract

Flavopiridol, the first potent cyclin-dependent kinase inhibitor to undergo clinical trials as an antineoplastic agent in the United States, has attracted considerable attention because of its unique cellular targets and its ability to kill noncycling tumor cells in vitro. To better understand how flavopiridol might be used clinically, the present study used colony-forming assays to examine the cytotoxicity resulting from combining flavopiridol with eight other antineoplastic agents in four different administration schedules in A549 human non-small cell lung carcinoma cells in vitro. Cytotoxic synergy, as assessed by the median effect method, resulted when flavopiridol was combined with seven of the eight tested antineoplastic agents but was highly dependent upon administration schedule. Cisplatin was the only agent that resulted in sequence-independent synergy when combined with flavopiridol. For paclitaxel, cytarabine, topotecan, doxorubicin, and etoposide, synergy was more pronounced when the agents were administered before flavopiridol rather than concomitant with or following flavopiridol. Examination suggested that this sequence dependence reflected arrest of cells in G1 and G2 phases of the cell cycle during and for 24 h following flavopiridol treatment. Interestingly, 48–72 h after flavopiridol removal, the fraction of surviving cells in S phase increased 2–3-fold relative to untreated controls. Consistent with these results, administration of flavopiridol for 24 h followed 3 days later by exposure to an S phase-active agent (cytarabine or 5-fluorouracil) resulted in a highly synergistic interaction. These results highlight the importance of administration schedule when combining flavopiridol with other agents and provide a starting point for examining the effect of flavopiridol in drug combinations in vivo.

Introduction

Multiple genetic alterations in neoplastic cells affect the regulation of the cell cycle machinery (1, 2). As a result, there is considerable interest in restoring normal cell cycle control in tumor cells using therapeutic approaches such as gene therapy or small molecule inhibitors of CDKs. Flavopiridol is the first potent CDK inhibitor to undergo clinical testing in the United States (3, 4). Preclinical studies have revealed that this agent binds to the ATP binding site of CDK1, CDK2, and CDK4 (5–7), resulting in CDK inhibition that is competitive with respect to ATP. In addition, this agent inhibits CDK1 phosphorylation (8), raising the possibility that the CDK-activating kinase (9) is inhibited. Further studies have revealed that flavopiridol inhibits other serine/threonine and tyrosine protein kinases as well, albeit at higher concentrations than are required to inhibit CDKs (4, 10). The net result is that flavopiridol inhibits proliferation of a variety of cell types, including prostate, breast, lung, and leukemia cell lines in vitro (10–13). In addition, flavopiridol induces apoptosis in several tumor cell lines in vitro (12, 14, 15) and demonstrates antiproliferative activity against a wide range of human tumor xenografts in vivo (11, 13).

Phase I clinical trials of flavopiridol were recently completed (3, 16); and Phase II trials of flavopiridol as a single agent are planned in a variety of tumor types. Further clinical development of this agent will likely depend on the ability to successfully combine it with other antineoplastic agents. On the one hand, recent data indicate that flavopiridol can kill noncycling cells in vitro (12), raising the possibility that flavopiridol might be profitably combined with agents that inhibit cell cycle progression. On the other hand, the observation that flavopiridol, like other CDK inhibitors, can itself induce arrest in G1 and G2 (6, 10) raises the possibility that flavopiridol might inhibit the effects of proliferation-dependent agents. Accordingly, the challenge will be to combine flavopiridol with other antineoplastic agents in such a way that the effects of the other agents are not inhibited. The present study used colony-forming assays to examine the cytotoxicity of flavopiridol in combination with eight different antineoplastic agents in vitro. Because of concern that flavopiridol-induced cell cycle arrest might profoundly affect the cytotoxic effects of these other agents, particular attention was paid to the effects of various sequences of administration.

Materials and Methods

Materials. Flavopiridol and BCNU were provided by the Pharmaceuticals Resources Branch of the National Cancer Institute (Bethesda, MD). Cytarabine, 5-fluorouracil, etoposide, doxorubicin, paclitaxel, and cisplatin were from Sigma Chemical Co. (St. Louis, MO). Topotecan was kindly provided by Dr. Randall K. Johnson (Smith Kline Beecham, King of Prussia, PA). All other reagents were obtained as described previously (17).

Stock (1000-fold concentrated) solutions of flavopiridol, cytarabine, paclitaxel, topotecan, doxorubicin, etoposide, and 5-fluorouracil were prepared in DMSO and stored at −20°C prior to use. Cisplatin and BCNU were prepared immediately before use as 1000-fold concentrated solutions in DMSO.

Colony-forming Assays. A549 cells were cultured in RPMI 1640 containing 5% (v/v) heat-inactivated fetal bovine serum and 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine (medium A). Cells were passaged twice weekly and maintained at 37°C in an atmosphere containing 5% CO2 (v/v).

Colony-forming assays were performed as described previously (17). In brief, to examine the effects of single agents, subconfluent cells were trypsinized, plated at a density of 750 cells/plate in multiple 35-mm dishes containing 2 ml of medium A, and incubated for 24 h at 37°C to allow cells to attach. Graded concentrations of each drug or equivalent volumes of DMSO (0.1%) were then added to triplicate plates. After a 24-h treatment, plates were washed twice with 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 counted. Diluent-treated control plates typically contained 200–250 colonies.

The effect of concomitant exposure to two agents was assessed by exposing cells simultaneously to graded concentrations of both drugs at a fixed ratio (typically the ratio of the IC50s from single-agent exposures) for 24 h and

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3 The abbreviations used are: CDK, cyclin-dependent kinase; CI, combination index; IC50, IC75, IC95, drug concentration that inhibits colony formation by 50, 75 or 95%, respectively; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea.

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washing as described above. To examine the effect of sequential drug exposures, cells that had been allowed to adhere for 14–16 h were exposed to graded concentrations of the first drug for 24 h, washed twice with serum-free RPMI 1640, exposed to graded concentrations of the second drug in 2 ml of medium A for 24 h, washed twice with serum-free RPMI 1640, and incubated in drug-free medium A for an additional 7 days. In each experiment, additional plates were exposed to each drug alone at the same time that plates were exposed to the drug in sequence.

To examine the effect of delayed exposure to drugs following flavopiridol exposure, cells that had been allowed to adhere for 14–16 h were exposed to graded concentrations of flavopiridol for 24 h, washed twice with serum-free RPMI 1640, incubated in medium A for 48 h, exposed to graded concentrations of the second drug in 2 ml of medium A for 24 h, washed twice with serum-free RPMI 1640, and incubated in drug-free medium A for 4 days to allow colonies to form. In each experiment, additional plates were again exposed to each drug alone at the same time that plates were exposed to the drug in sequence.

A similar approach was used to examine the effect of selected combinations in OVCAR-3 ovarian cancer cells and HCT-8 colorectal carcinoma cells. Experiments were performed in the media described previously (17).

Analysis of Combined Drug Effects. Cells were treated with serial dilutions of each drug individually and with both drugs simultaneously at a fixed ratio of doses that typically corresponded to ¼, ¼, ½, ½, ⅓, ⅓, ⅓, and 3 times the individual IC50. Fractional survival (f) was calculated by dividing the number of colonies in drug-treated plates by the number of colonies in control plates. Data were subsequently analyzed by the method of Chou and Talalay (18). In brief, \[ \log\left(\frac{1}{f} - 1\right) \] was plotted against \[ \log(\text{drug dose}) \]. From the resulting median effect lines, the X-intercept (log IC50) and slope m were calculated for each drug and for the combination by the method of least squares. These parameters were then used as described (18) to calculate the doses of the individual drugs and the combination required to produce various levels of cytotoxicity \( f = 0.95, 0.90, 0.85, \ldots, 0.05 \) according to the following equation:

\[
Dose_x = \frac{\text{Dose IC}_{50}(1 - f)^{1/m}}{f^{1/m}}
\]

Because the two drugs were administered at a fixed ratio, the dose of the combination required to produce fractional survival \( f \) could be divided into the component doses \( (D_1) \) and \( (D_2) \) of drugs 1 and 2, respectively. For each level of cytotoxicity \( f = 0.95, 0.90, 0.85, \ldots, 0.05 \), a parameter called the CI (18) was then calculated according to the following equation:

\[
\text{CI} = \frac{(D_1)/(Df_1) + (D_2)/(Df_2) + \alpha(D_1)(D_2)/(Df_1)(Df_2)}{2}
\]

where \( (D_1) \) and \( (D_2) \) are the concentrations of the combination required to produce survival \( f \), \( (Df_1) \) and \( (Df_2) \) are the concentrations of the individual drugs required to produce \( f \), and \( \alpha = 1 \) or 0 depending on whether the drugs are assumed to be mutually nonexclusive or mutually exclusive, respectively (18). In this method, synergy is indicated by CI < 1, additivity by CI = 1, and antagonism by CI > 1 (18). The CIs presented in the text were calculated under the assumption of a mutually nonexclusive drug interaction, i.e., under the assumption that the other drugs act at targets that are different from that of flavopiridol. Experiments were repeated until three replicates yielded correlation coefficients \( r > 0.9 \) for all three median effect lines.

Flow Cytometry. After trypsinization, aliquots containing \( 1 \times 10^6 \) cells were sedimented at 200 \( \times \) g for 5 min. All further steps were performed at 4°C unless otherwise indicated. Cells were washed in PBS, resuspended in 300 \( \mu \)l of PBS and diluted with 300 \( \mu \)l of 95% ethanol by dropwise addition. After fixation for >1 h, cells were washed twice with PBS, resuspended in 300 \( \mu \)l of 0.1% (w/v) sodium citrate containing 1 mg/ml RNase A and incubated at 37°C for 15 min. Immediately prior to flow cytometry, samples were diluted with 300 \( \mu \)l of 0.1% sodium citrate containing 100 \( \mu \)g/ml propidium iodide and incubated in the dark at 21°C for an additional 15 min. Samples were examined using a Becton Dickinson FACScan (San Jose, CA) using an excitation wavelength of 488 nm and an emission wavelength of 585 ± 21 nm. Histograms were analyzed using ModFit software (Verity Software House, Topsham, ME).

Evaluation of M-Phase Entry. A549 cells were grown to 20–30% confluence on 60-mm tissue culture dishes in the presence of 5 ml of medium A. To assess the percentage of cells entering mitosis during a 24-h period of flavopiridol exposure, triplicate plates were simultaneously exposed to the indicated concentrations of flavopiridol (or the equivalent volume of diluent) and 50 ng/ml nocodazole. At the completion of the 24-h coincubation, adherent cells were released by trypsinization, washed twice with serum-free RPMI 1640, combined with floating cells, and fixed for 1 h in 4:1 (v/v) methanol:acetic acid. Aliquots of fixed cells were deposited on microscope slides, stained with 1 \( \mu \)g/ml Hoechst 33258, and examined by fluorescence microscopy to determine the percentage of metaphase cells (≥200 cells examined/sample). A similar approach was used to determine the percentage of cells entering mitosis during the 24-h period following a 24-h flavopiridol exposure, except that cells were exposed to the indicated concentrations of flavopiridol (or the diluent DMSO) for 24 h, washed twice with serum-free RPMI medium, and then incubated for an additional 24 h in fresh medium A containing 50 ng/ml nocodazole prior to fixation.

Results

Because flavopiridol is the first potent CDK inhibitor to enter clinical trials, there is relatively little information available regarding the types of effects that might be expected when this drug is combined with other antineoplastic agents. To address this issue, A549 human non-small cell lung cancer cells, which were recently shown to be killed by flavopiridol in a cell cycle-independent fashion (12), were exposed to flavopiridol alone or in combination with other agents. The 24-h exposure chosen for these experiments corresponds to one of the flavopiridol schedules (24 h continuous infusion) being investigated in the clinical setting. Because of concern that flavopiridol-induced cell cycle arrest might affect the cytotoxicity of cell cycle-dependent agents, four separate administration schedules were explored. Representative results of individual clonogenic assays are presented in Fig. 1. Results of all of these experiments are summarized in Fig. 2A, where the effect of flavopiridol on the IC50 of various agents is presented, and in Fig. 2, B–D, where the CIs of the various combinations at different levels of cytotoxicity are presented. Studies exploring the mechanisms of some of the observed interactions are presented in Figs. 3 and 4.

The Interaction between Paclitaxel and Flavopiridol Is Sequence Dependent. When cells were treated with paclitaxel and flavopiridol simultaneously or in the flavopiridol→paclitaxel sequence, dramatic antagonism was observed (e.g., Fig. 1, A–C). Flavopiridol inhibited the action of paclitaxel, as indicated by the shift of the dose-response curve to the right in the presence of flavopiridol (Fig. 1B). This effect is depicted in the summary graphs as an increase in the IC50 when the two agents are administered simultaneously or when flavopiridol precedes paclitaxel (Fig. 2A). The CIs calculated for these treatments (e.g., Fig. 1C) are consistently >1 (Fig. 2, B and C), indicating that the effects of the two agents are less than additive using these two schedules.

To investigate the potential mechanism of this antagonism, we examined the effect of flavopiridol on cell cycle distribution of A549 cells (Fig. 3A). A 24-h flavopiridol treatment resulted in a decrease in the percentage of S phase cells (Fig. 3A) and a marked decrease in the number of cells going through mitosis (Fig. 3B) as cells accumulated in G1 and G2. This decrease in the number of cells traversing the cell cycle during flavopiridol treatment presumably contributes to the antagonism observed between flavopiridol and paclitaxel during simultaneous exposure. The diminished cell cycle traverse induced by flavopiridol persisted for at least 24 h after removal of flavopiridol (Fig. 3B). Approaching to account for the antagonism observed when paclitaxel was administered immediately following flavopiridol as well.

A different picture emerged when paclitaxel preceded flavopiridol. The individual survival curves indicate that paclitaxel enhances the cytotoxicity of flavopiridol (Fig. 1D) and that flavopiridol enhances the cytotoxicity of paclitaxel (Fig. 1E). The apparent IC50 of paclitaxel in
Fig. 1. The effect of sequence of administration of flavopiridol and paclitaxel. A and B, A549 cells were treated with flavopiridol alone, paclitaxel alone, or a fixed 62.5:1 ratio of flavopiridol followed by paclitaxel. Colony formation from a single experiment is plotted as a function of flavopiridol concentration (A) or paclitaxel concentration (B). Bars, ±1 sample SD for a single representative experiment using triplicate plates for each drug concentration. C, CI plot for flavopiridol followed by paclitaxel calculated from data in A and B. D and E, cells were treated with flavopiridol alone, paclitaxel alone, or a fixed 62.5:1 ratio of flavopiridol preceded by paclitaxel. Colony formation was plotted as a function of flavopiridol concentration (D) or paclitaxel concentration (E). F, CI plot for paclitaxel followed by flavopiridol calculated from data in D and E. Results are representative of three experiments in each sequence.

Flavopiridol was diminished when the two agents were combined in this sequence (Fig. 2A); CIs of 0.49 ± 0.21 and 0.20 ± 0.14 were calculated at the IC50 for cytarabine and flavopiridol, respectively, with this sequence (Fig. 2D), indicating greater than additive effects (P < 0.01). Each CI is presented as a mean ± 1 sample SD. t tests (one-tailed, 2 degrees of freedom) were used to test the hypothesis that the CI calculated at the IC50 was < 1. The probability of an α type error (P) is presented for each analysis. It should be noted that two-tailed 95% confidence intervals can be calculated for all CIs using data provided in the text (e.g., 0.20 ± 0.35 at the IC50 for this combination).

Effect of Combining Flavopiridol with S Phase-selective Agents.
To determine whether similar sequence-dependent effects would be observed with other cell cycle phase-specific agents, the effect of flavopiridol in combination with cytarabine was examined. When flavopiridol and cytarabine were administered concomitantly, the IC50 for cytarabine increased slightly (Fig. 2A). Consistent with this observation, CIs of 2.1 ± 0.82 and 1.5 ± 0.25 were calculated for the IC50 for cytarabine and flavopiridol, respectively, with this sequence (Fig. 2B; P < 0.05). When cytarabine followed flavopiridol, the effects appeared to be additive (CI, 0.92 ± 0.34 and 0.70 ± 0.47 at IC50 and IC95, respectively; Fig. 2C), presumably reflecting the effect of the active metabolite cytarabine triphosphate retained in cells as they began to cycle 24 h after the removal of flavopiridol (Fig. 3A, 48–72 h). As was the case with paclitaxel, the administration of cytarabine followed by flavopiridol resulted in cytotoxic synergy. This was manifest as an apparent 3-fold decrease in the IC50 for cytarabine when it was administered with the fixed ratio of flavopiridol (Fig. 2A) and by analysis of the calculated CIs (CI, 0.38 ± 0.19 and 0.16 ± 0.02 at IC50 and IC95, respectively; Fig. 2D; P < 0.0005).

This sequence-dependent interaction was not limited to cytarabine. Antagonism was also observed when flavopiridol was administered with or preceding the S phase-specific (19) topoisomerase I poison topotecan (Fig. 2, A–C). When topotecan preceded flavopiridol, however, greater than additive cytotoxicity was again observed, with CIs of 0.79 ± 0.14 and 0.33 ± 0.09 at the IC50 doses of the combination, respectively (Fig. 2D; P < 0.0005). The effect of combining flavopiridol with 5-fluorouracil was somewhat different. Additive effects were observed when the two agents were administered simultaneously (Fig. 2B), and greater than additive effects were observed at high levels of cytotoxicity when 5-fluorouracil preceded flavopiridol (Fig. 2D). In contrast to the combination of cytarabine and flavopiridol, however, there was also striking synergy when flavopiridol preceded 5-fluorouracil, with CIs of 0.50 ± 0.11 and 0.13 ± 0.03 at IC50 and IC95, respectively (Fig. 2C; P < 0.0005).

Effect of Delayed Exposure to Antimetabolites after Flavopiridol. Although it is possible to neatly sequence drugs in vitro, variations in clearance of parent drugs and their intracellular metabolites make this a potentially difficult strategy in the clinic. Accordingly, we examined an alternative strategy of combining flavopiridol with S phase-selective agents. As illustrated in Fig. 3A, the treatment of A549 cells with 1125 nm flavopiridol results in growth arrest of cells for...
Fig. 2. The effect of sequence of administration on the cytotoxic interaction between flavopiridol and eight other antineoplastic agents. A, alterations in IC_{50} concentrations resulting from various combinations of flavopiridol and other antineoplastic agents. Triplicate or quadruplicate experiments were performed to produce IC_{50}s for the drugs administered separately and administered in fixed-ratio combination. The relative IC_{50}s were obtained by dividing the IC_{50} for each drug resulting from the combined flavopiridol-drug administration by the corresponding single-drug IC_{50} for the same experiment. The final value represents the higher of the means of the two resulting ratios (one resulting from flavopiridol, one from the other agent). Values less than 1 represent reductions in IC_{50} resulting from the drug combination, whereas values greater than 1 represent increases in IC_{50} resulting from the drug combination. Bars, ±1 sample SD. B, CIs for combinations involving 24-h simultaneous exposure to flavopiridol and each of the eight drugs. C, CIs for combinations involving 24-h exposure to flavopiridol followed by 24-h exposure to each of the indicated drugs. D, CIs for combinations involving 24-h exposure to the indicated drug followed by 24-h exposure to flavopiridol. Columns, means of triplicate or quadruplicate experiments (each consisting of triplicate plates); bars, ±1 sample SD.

~48–72 h. Thereafter, surviving cells begin to proliferate again, causing a transient 2-fold increase in the fraction of cells in S phase (Fig. 3A). This observation prompted us to examine the possibility that sequential administration of flavopiridol followed by cytarabine might be more effective if a 48-h drug-free interval were interposed. Results of these experiments are shown in Fig. 4. When administered on this schedule, each agent markedly enhanced the cytotoxicity of the other (Fig. 4, A and B). Consistent with these results, the CI was calculated to be less than 1 at all levels of cytotoxicity (CI, 0.48 ± 0.12, 0.40 ± 0.26, and 0.42 ± 0.37 at IC_{50}, IC_{75}, and IC_{95} of this combination, respectively; Fig. 4E; P < 0.05). Similar results were observed when 5-fluorouracil was administered on day 0 following day 1 flavopiridol exposure (Fig. 4, C and D). In this sequence, CIs of 0.51 ± 0.24, 0.31 ± 0.28, and 0.17 ± 0.23 were calculated at IC_{50}, IC_{75}, and IC_{95}, respectively (Fig. 4E; P < 0.025). The synergy observed with delayed administration of the antimetabolites after flavopiridol was among the strongest (lowest CIs) observed with any of the drug combinations.

Effect of Combining Flavopiridol with Doxorubicin or Etoposide. In contrast to topoisomerase I inhibitors like topotecan, the anthracyclines and epipodophyllotoxins exhibit some degree of cytotoxicity in G_{1} and G_{2}, as well as S phase. In addition, the widespread use of these classes of agents makes the effect of combining them with flavopiridol a matter of substantial interest.

When flavopiridol was administered concomitant with or preceding doxorubicin, the strong antagonism described above with cell cycle phase-specific agents like paclitaxel and cytarabine was not observed (Fig. 2, B and C). Instead, the IC_{50}s for doxorubicin were unchanged or diminished (Fig. 2A). However, the greatest reduction in IC_{50}s was observed when flavopiridol followed doxorubicin (Fig. 2A). Consist-

Fig. 3. Effects of flavopiridol on cell cycle traverse. A, time dependence of the cell cycle distribution for A549 cells exposed to 1125 nM flavopiridol for 24 h. Cell cycle distributions were determined by flow cytometry using propidium iodide staining of fixed cells and represent surviving cells only. Bar (lower left), time of flavopiridol exposure. A single representative experiment is shown. Similar results were obtained using 250 nM flavopiridol. B, effect of various flavopiridol concentrations on the percentage of cells entering mitosis during 24 h flavopiridol exposure and during the 24-h period immediately following 24-h flavopiridol exposure. Data points, mean ± 1 sample SD of triplicate samples for each drug concentration.
ent with these observations, effects were essentially additive when the two drugs were administered simultaneously (CI, 0.88 ± 0.34 and 0.57 ± 0.54 at IC75 and IC95, respectively; Fig. 2B; P > 0.1) or when flavopiridol preceded doxorubicin (CI, 1.6 ± 0.4 and 0.92 ± 0.33 at IC50 and IC95, respectively; Fig. 2C). In contrast, cytotoxic synergy was seen at high levels of cytotoxicity when doxorubicin preceded flavopiridol (CI, 0.46 ± 0.18 at IC95 of the combination; Fig. 2D; P < 0.025).

Similar results were obtained with etoposide. The effects were indistinguishable from additivity when flavopiridol and etoposide were administered concomitantly (CI, 1.6 ± 0.83 and 2.1 ± 1.3 at IC75 and IC95, respectively; Fig. 2B) or when flavopiridol was administered before etoposide (CI, 0.91 ± 0.42 and 0.99 ± 0.04 at IC75 and IC95, respectively; Fig. 2C). However, when etoposide was administered before flavopiridol, cytotoxic synergy was observed at higher levels of cytotoxicity (CI, 0.42 ± 0.35 at IC95; Fig. 2D; P = 0.05). Examination of survival curves and reductions in IC50 suggested that cytotoxicity was greatest when etoposide preceded flavopiridol (Fig. 2A).

Effect of Combining Flavopiridol with BCNU. When flavopiridol was combined with BCNU, the results were not distinguishable from an additive interaction in either sequence (Fig. 2, A–D). For example, when BCNU was administered following flavopiridol, CIs of 0.96 ± 0.1 and 0.97 ± 0.27 were observed at IC50 and IC95, respectively (Fig. 2C).

Sequence-independent Synergy between Cisplatin and Flavopiridol. In contrast to the strictly additive results observed with BCNU, synergy was demonstrated for the combination of flavopiridol and cisplatin when cisplatin was administered preceding (CI, 1.2 ± 0.14 and 0.58 ± 0.14 at IC75 and IC95, respectively; Fig. 2D; P < 0.025), following (CI, 0.89 ± 0.16 and 0.515 ± 0.19 at IC75 and IC95, respectively; Fig. 2C; P < 0.025), or concomitantly with (CI, 0.80 ± 0.23 and 0.41 ± 0.15 at IC75 and IC95, respectively; Fig. 2B; P < 0.025) flavopiridol. Cisplatin was the only drug examined that exhibited sequence-independent cytotoxic synergy with flavopiridol. However, examination of the reductions in drug IC50 obtained directly from the survival curves (Fig. 2A) suggests that the most pronounced effect occurs when cisplatin is administered before flavopiridol.

Discussion

Flavopiridol has attracted considerable interest as a potential antineoplastic agent because of its unique ability to target CDKs (5, 7, 8) and its ability to kill noncycling tumor cells at clinically achievable concentrations (12, 16). The present study provides an initial attempt to assess the effect of flavopiridol when combined with eight widely used antineoplastic agents. Because of the pronounced cell cycle effects of flavopiridol, special emphasis was placed upon examining various schedules of the drug combinations. These studies not only have demonstrated marked sequence dependence in the effects of combining flavopiridol with most other agents but also have demonstrated that the cell cycle effects of flavopiridol can be exploited in certain sequences of flavopiridol combined with S phase-selective agents. Additionally, these results suggest that the sequence of drug administration may be as important as the identity of the other agent in designing combinations containing flavopiridol. These observa-
tions have potential implications for the design of further Phase I and Phase II trials of flavopiridol-containing drug combinations.

The importance of sequence of administration is strikingly illustrated by the combination of paclitaxel and flavopiridol. Flavopiridol and paclitaxel result in strong cytotoxic antagonism when administered concomitantly or in the flavopiridol—paclitaxel sequence (Figs. 1, A—C, and 2, A—C). In contrast, the same agents cause greater than additive cytotoxicity when paclitaxel is administered before flavopiridol (Figs. 1, D—F, and 2D). These effects were not limited to A549 lung cancer cells but were also observed in OVCAR-3 human ovarian carcinoma cells. Although these effects were most pronounced when flavopiridol was combined with paclitaxel, the effects were not limited to this combination. For most drugs, less cytotoxicity resulted when flavopiridol was administered first in a two-drug sequence than when flavopiridol was administered second. This striking sequence dependence appears to result from the fact that flavopiridol treatment produces cell cycle arrest in surviving cells (Ref. 10 and Fig. 3), thereby making them more resistant to the effects of cell cycle-dependent antineoplastic agents administered immediately following flavopiridol exposure.

Of eight drugs combined with flavopiridol, only 5-fluorouracil produced greater cytotoxic synergy when administered immediately following, rather than preceding, flavopiridol. Although this result is contrary to predictions based upon cell cycle considerations alone, preliminary results suggest that it can be explained by a marked decrease in thymidylate synthase protein that occurs 24—48 h after the start of flavopiridol treatment.4

The observation that tumor cells are recruited into S phase 48—72 h after the end of flavopiridol exposure (Fig. 3A) led to in vitro experiments attempting to heighten cytotoxic synergy between flavopiridol and S phase-specific agents. Cytotoxicity was markedly enhanced when the S phase-dependent antineoplastic agents 5-fluorouracil and cytarabine were administered in a delayed fashion following flavopiridol exposure, i.e., at a time when surviving cells were entering S phase after flavopiridol treatment. Because most of the cells in clinical tumors are not actively traversing the cell cycle, the prospect of administering a drug that can potentially enhance the sensitivity of tumor cells to antimitabolites by recruiting them into S phase has special appeal. Further preclinical studies are required to determine whether this strategy will be feasible in vivo.

The combination of flavopiridol and cisplatin also resulted in unique effects. In contrast to other DNA-damaging agents, which exerted additive effects when combined with flavopiridol, cisplatin caused greater than additive effects (Fig. 2). Greater than additive effects were also observed with this combination in HCT-8 human ileocecal adenocarcinoma cells. Furthermore, the combination of cisplatin and flavopiridol did not exhibit the strong sequence dependence observed with several other combinations. Given the nonoverlapping toxicities of these two agents [neurotoxicity and nephrotoxicity for cisplatin (20) and gastrointestinal toxicity for flavopiridol (3, 16)], the combination of cisplatin and flavopiridol might warrant further investigation.

In summary, the present studies indicate that flavopiridol exhibits marked sequence-dependent effects when combined with most other antineoplastic agents. Whether these sequence-dependent effects will be more pronounced in neoplastic cells than in normal tissue and whether these effects will be important in the clinical development of this agent remain to be determined.

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