Flavopiridol enhances human tumor cell radiosensitivity and prolongs expression of γH2AX foci

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Abstract

Flavopiridol is a cyclin-dependent kinase (CDK) inhibitor, which has recently entered clinical trials. However, when administered as a single agent against solid tumors, the antitumor actions of flavopiridol have been primarily cytostatic. Given its reported effects on cell cycle regulation, transcription, and apoptosis, flavopiridol may also influence cellular radioresponse. Thus, to evaluate the potential for combining this cyclin-dependent kinase inhibitor with radiation as a cancer treatment strategy, we have investigated the effects of flavopiridol on the radiation sensitivity of two human prostate cancer cell lines (DU145 and PC3). The data presented here indicate that exposure to flavopiridol (60–90 nM) after irradiation enhanced the radiosensitivity of both DU145 and PC3 cells. This sensitization occurred in the absence of significant reductions in cell proliferation, retinoblastoma protein phosphorylation, or P-TEFb activity. Moreover, the post-irradiation addition of flavopiridol had no effect on radiation-induced apoptosis or the activation of the G2 cell cycle checkpoint. However, flavopiridol did modify the time course of γH2AX expression in irradiated cells. Whereas there was no significant difference in radiation-induced γH2AX foci at 6 h, at 24 h after irradiation, the number of cells expressing γH2AX foci was significantly greater in the flavopiridol-treated cells. These results indicate that flavopiridol can enhance radiosensitivity of human tumor cells and suggest that this effect may involve an inhibition of DNA repair.

Introduction

Because the increased or unopposed activity of cyclin-dependent kinases (CDKs) is associated with the aberrant cell cycle regulation of neoplastic cells, these regulatory molecules have been suggested as targets for cancer chemotherapy (1). Although there are now a number of CDK inhibitors under investigation, flavopiridol was the first to progress to clinical trials (2). This semisynthetic flavonoid inhibits all cdks, but is primarily effective against cdks 1, 2, and 4 (3, 4). Consistent with this inhibitory activity, flavopiridol has been shown to induce cell cycle arrest in vitro and slow tumor growth in a number of experimental tumor models (5). More recently, flavopiridol was reported to inhibit P-TEFb activity resulting in the reduced transcriptional elongation of mRNA species with short half-lives, which includes inducible transcripts (6–8). This reduction in P-TEFb appears to play a role in the frequently reported flavopiridol-mediated decrease in cyclin D1 (9, 10). In addition to inhibiting proliferation, flavopiridol induces apoptosis in certain tumor models, which has been attributed to reduced P-TEFb activity and the resulting decrease in a number of anti-apoptotic proteins (9). Finally, flavopiridol has also been reported to bind directly to DNA, although the biological significance has not been determined (11). Thus, although initially proposed to target tumor cell proliferation via CDK inhibition, flavopiridol clearly has additional molecular and cellular actions.

In the treatment of solid tumors both in experimental models and in clinical trials, the effects of flavopiridol have been primarily cytostatic (2, 5, 12, 13). However, the ability of flavopiridol to disrupt cell cycle progression (5) as well as decrease the expression of survival-related genes (9) suggested that it might also enhance tumor cell sensitivity to cytotoxic agents. Indeed, a number of studies have reported that the combination of flavopiridol with cytotoxic chemotherapeutic agents results in an enhancement in tumor cell killing (14–19). Although a number of mechanisms responsible for the enhancement have been suggested, for the most part, the specifics remain undefined.

The ability of flavopiridol to enhance cell killing induced by DNA damaging cytotoxic drugs suggested that it might also enhance the cell killing induced by ionizing radiation. Radiotherapy remains a primary cancer treatment modality for solid tumors; the ability to enhance its efficacy is thus likely to impact on a significant number of cancer patients. With respect to its potential radiosensitizing action, Raju et al. (20) reported that flavopiridol enhanced the radiosensitivity of a murine tumor cell line. In contrast, Chien et al. (21) based on their study concluded that flavopiridol had no effect on the radiosensitivity of human bladder carcinoma cell lines. Given the conflicting results obtained from murine and human tumor cells and, moreover, recent literature suggesting that human tumor cell lines evaluated in vitro or as xenograft tumors have the best predictive value for the efficacy of cytotoxic agents in Phase II clinical trials (22), we have investigated the effects of flavopiridol on the radiosensitivity of two human prostate carcinoma cell lines. The data presented here indicate that flavopiridol

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at concentrations ranging from 60 to 90 nM significantly enhanced the radiosensitivity of the two human prostate carcinoma cell lines. This enhancement occurred in the absence of significant CDK inhibition, reduced P-TEFb activity, or abrogation of G2 arrest. However, based on the prolonged expression of γH2AX after irradiation, it appears that the flavopiridol-induced radiosensitization involves the altered metabolism of DNA double strand breaks (DSBs).

Materials and Methods

Reagents
Flavopiridol, provided by the Development Therapeutics Program of the NCI, was dissolved in DMSO to a stock concentration of 10 mM, aliquoted, and stored at −20°C. The antibodies to cyclin D1, Rb, phospho-Rb, and RNA pol II were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); RNA polymerase II, phospho-RNA polymerase II, γH2AX, and phospho-histone H3 from Upstate Biotechnology (Lake Placid, NY) and actin from Chemicon (Temecula, CA).

Cell Lines and Treatment
The human prostate carcinoma cell lines DU145 and PC3 (ATCC, Gaithersburg, MD) were used in this study. Each cell line was grown in RPMI 1640 (Life Technologies, Rockville, MD) containing glutamate (5 mM) and 5% FBS and maintained at 37°C in an atmosphere of 5% CO2 and 95% room air. Cultures were irradiated using a Pantak (Solon, OH) X-ray source at a dose rate of 1.55 Gy/min.

Clonogenic Survival
Cultures were trypsinized to generate a single cell suspension and a specified number of cells were seeded into each well of a six-well tissue culture plate. After allowing cells time to attach, cultures received graded doses of radiation with flavopiridol or DMSO (vehicle control) added 2 h before or immediately after irradiation. Twelve to 14 days after seeding, colonies were stained with crystal violet, the number of colonies containing at least 50 cells determined and surviving fractions calculated. Survival curves were then generated after normalizing for the amount of drug-induced cell death.

Apoptotic Cell Death
Apoptosis was evaluated according to cell cycle phase distribution determined using flow cytometry (FCM). The treatment protocols were essentially the same as in the clonogenic survival experiments, except that the cells were initially seeded into 10-cm dishes. All cultures were subconfluent at the time of collection. Cultures were collected for fixation, stained with propidium iodide, and analyzed using flow cytometry as previously described (23) by the Clinical Services Program at NCI-Frederick. The evaluation of apoptosis was then based on the percentage of cells with sub-G1 DNA content.

G2 Arrest
Mitotic index was used to evaluate the activation of the G2 cell cycle checkpoint. Cells were collected by trypsinization at the designated times. The flow cytometric procedure for the immunofluorescent detection of phosphorylated histone H3 and DNA content (via propidium iodide staining) was performed as described by Xu et al. (24). Mitotic cells were designated as those with 4N DNA content and staining for phospho-histone H3. Loss of mitotic cells (reduced mitotic index) then reflects the onset of G2 arrest.

Immunoblot Analysis
Cells were scraped into PBS, centrifuged, and the cell pellet resuspended in three volumes of extraction buffer [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP40, 1 mM DTT, 1 mM PMSF, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 250 mg/ml benzamide, 50 mM NaF, 1 mM NaO4V4]. Immunoblot analysis was then performed as described by Russell et al. (25). Visualization was performed using the Typhoon scanner (Molecular Dynamics, Sunnyvale, CA).

Immunofluorescent Staining for γH2AX
Cells were grown and treated in chamber slides. At specified times, medium was aspirated and cells were fixed in 4% paraformaldehyde for 10 min at room temperature. Paraformaldehyde was aspirated and the cells treated with a 0.2% NP40/PBS solution for 15 min. Cells were then washed in PBS twice, and the anti-γH2AX antibody added at a dilution of 1:500 in 1% BSA and incubated overnight at 4°C. Cells were again washed twice in PBS before incubating in the dark with a FITC-labeled secondary antibody at a dilution of 1:100 in 1% BSA for 1 h. The secondary antibody solution was then aspirated and the cells washed twice in PBS. Cells were then incubated in the dark with DAPI (1 µg/ml) in PBS for 30 min, washed twice, and coverslips mounted with an anti-fade solution (Dako Corp., Carpinteria, CA). Slides were examined on a Leica DMRXA fluorescent microscope (Wetzlar, Germany). Images were captured by a Photometrics Sensys CCD camera (Roper Scientific, Tuscon, AZ) and imported into IP Labs image analysis software package (Scanalytics, Inc., Fairfax, VA) running on a Macintosh G3 computer. For each treatment condition, γH2AX foci were determined in at least 50 cells. Cells were classified as positive (i.e., containing radiation-induced γH2AX foci) when greater than five foci were detected.

Results
To determine the effects of flavopiridol on tumor cell radiosensitivity, clonogenic survival analysis was performed on the two human prostate carcinoma cell lines DU145 and PC3. For these studies, a specified number of single cells were seeded into six-well tissue culture plates and after allowing 6 h for cell attachment (but no division), cells were irradiated. This protocol was used in an attempt to eliminate any effects of trypsinization on post-irradiation signaling/recovery processes. In initial experiments, flavopiridol at concentrations of 90 and 60 nM for DU145 and PC3 cells, respectively, was added 2 h before irradiation. Flavopiridol-containing medium was not removed and was present during the entire colony forming incubation period (12 days), which did not affect colony size (data not
shown). The surviving fractions obtained after flavopiridol exposure only were 0.46 $\pm$ 0.02 and 0.59 $\pm$ 0.09 for DU145 (90 nM) and PC3 (60 nM) cells, respectively. As shown in Fig. 1, flavopiridol exposure beginning 2 h before irradiation resulted in an increase in radiation-induced cell killing for both tumor cell lines with dose enhancement factors at a surviving fraction of 0.10 of approximately 1.4 for DU145 and PC3 (Fig. 1). These data indicate that the radiosensitization induced by flavopiridol is mediated through an effect on some post-irradiation process or event. Because radiosensitivity was increased when the drug was delivered after irradiation, the possibilities that flavopiridol affected the initial level of radiation-induced DNA damage or that cells were synchronized in a radiosensitive phase of the cell cycle could be eliminated as potential mechanisms.

To begin to investigate the mechanism responsible for this radiosensitization, we focused on the cellular and biochemical effects previously reported for flavopiridol. Flavopiridol was initially proposed as an antitumor agent based on its ability to inhibit CDK2 and 4 slowing tumor cell proliferation. However, as shown by the growth curves in Fig. 2, the concentrations that enhanced radiosensitivity in radiation-induced cell killing with dose enhancement factors at a surviving fraction of 0.10 of approximately 1.4 for DU145 and PC3 (Fig. 1). These data indicate that the radiosensitization induced by flavopiridol is mediated through an effect on some post-irradiation process or event. Because radiosensitivity was increased when the drug was delivered after irradiation, the possibilities that flavopiridol affected the initial level of radiation-induced DNA damage or that cells were synchronized in a radiosensitive phase of the cell cycle could be eliminated as potential mechanisms.

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**Figure 1.** The effects of flavopiridol on tumor cell radiosensitivity. Cultures were trypsinized to generate a single cell suspension and a specified number of cells were seeded into each well of a six-well tissue culture plate. After allowing cells time to attach (6 h), cultures received graded doses of radiation. In the pre-irradiation protocol (pre), flavopiridol or DMSO (vehicle control) was added 2 h before radiation; in the post-irradiation protocol (post), flavopiridol or DMSO (vehicle control) was added immediately after irradiation. The concentrations of flavopiridol added in each protocol were 90 and 60 nM for DU145 (A) and PC3 (B), respectively. Twelve to 14 days after seeding, colonies were stained with crystal violet, the number of colonies containing at least 50 cells determined and surviving fractions calculated. Survival curves were generated after normalizing for the cytotoxicity induced by flavopiridol only. Points, mean from three independent experiments; bars, SE. Control refers to exposure to DMSO.

**Figure 2.** The effects of flavopiridol on cell proliferation and Rb phosphorylation. A, growth curves for DU145 and PC3 cells after addition of flavopiridol. Cultures were seeded at $10^4$ cells and 6 h later, flavopiridol was added at 90 and 60 nM to DU145 and PC3 cells, respectively. Cells per flask were then determined in duplicate cultures at 24-h intervals. Control cultures received corresponding concentrations of DMSO. Data are representative of two independent experiments. B, PC3 cells were exposed to flavopiridol at the indicated concentrations for 6 and/or 24 h and collected for immunoblot analysis of phospho-RB and Rb. Each blot is representative of two independent experiments with actin used as a loading control. C refers to vehicle-treated (DMSO) control.
(90 and 60 nm for DU145 and PC3, respectively) had minimal effects on the proliferation rate of these cells. The slight inhibition of DU145 cell proliferation mostly likely reflects the necessity for 90 nm as a radiosensitizing dose. As an indicator of CDK2 and 4 activities, the level of phosphorylated retinoblastoma (Rb) was determined. It had been previously shown that DU145 cells contain a mutated Rb protein that remains hypophosphorylated (26), thus the effects of flavopiridol on Rb phosphorylation were only investigated in PC3 cells. As shown in Fig. 2B, Rb phosphorylation was reduced by 600 nm flavopiridol, this concentration reduces survival by more than 2 logs (data not shown) in PC3 cells. In contrast, the radiosensitizing flavopiridol concentration (60 nm) had no detectable effect on the Rb phosphorylation status of PC3 cells, which is consistent with the minimal effects on cell proliferation shown in Fig. 2A.

In addition to CDKs 2 and 4, flavopiridol can inhibit P-TEFb activity resulting in a decrease in RNA polymerase II phosphorylation (7, 27). To investigate the potential contribution of P-TEFb inhibition to the radiosensitizing actions of flavopiridol, levels of phosphorylated RNA polymerase II were determined in DU145 and PC3 cells (Fig. 3A). After exposure to 600 nm (used as a positive control), RNA polymerase II phosphorylation was reduced in both cell lines, consistent with previous results (7). However, the radiosensitizing concentrations of 60 and 90 nm for PC3 and DU145, respectively, had no effect on RNA polymerase II phosphorylation (Fig. 3A). Finally, reductions in cyclin D1 levels have been reported to be a consistent consequence of flavopiridol exposure, which has been suggested to inhibit cell proliferation independent of CDK inhibition (10). Whereas 600 nm reduced cyclin D1 levels, the radiosensitizing concentrations had no effect (Fig. 3B). The results presented in Figs. 2 and 3 suggest that the previously identified biochemical/molecular consequences of flavopiridol exposure are not involved in the radiosensitization.

To further pursue the mechanism responsible for flavopiridol-induced radiosensitization, we then addressed a number of cellular processes that can serve as determinants of radiosensitivity. One such process is the activation of the G2 checkpoint, which is considered to protect against radiation-induced cell death. Activation of this checkpoint arrests cells in G2 allowing for the repair of DNA damage before progression into mitosis (28). To evaluate the effects of radiosensitizing concentrations of flavopiridol on the activation of the G2 checkpoint, the method of Xu et al. (29) was used, which distinguishes between G2 and mitotic cells. This assay determines the percentage of mitotic cells in the 4N population according to the flow cytometric analysis of phosphorylated histone H3, which is specifically expressed in mitotic cells. As shown in Fig. 4, irradiation of both cell lines with 2 Gy results in a decrease in mitotic cells by 1 h reaching a maximum reduction by 3 h, consistent with the rapid onset of G2 arrest and with previously published results (24). Flavopiridol alone also reduced the mitotic index (i.e., the percentage of mitotic...
cells), but not to the degree induced by radiation. The administration of 2 Gy followed by the addition of the radiosensitizing concentrations of flavopiridol resulted in essentially the same reduction in mitotic cells as detected for radiation only. Thus, these data indicate that flavopiridol does not abrogate or reduce radiation-induced G2 arrest.

In some cell systems, flavopiridol has been reported to induce apoptosis and/or enhance the apoptosis induced by cytotoxic chemotherapy agents (17). Because the susceptibility to apoptosis can be a determinant of radiosensitivity, the effects of flavopiridol on radiation-induced apoptosis in the two prostate carcinoma cell lines were evaluated using flow cytometry. DU145 and PC3 cells were irradiated with 6 Gy followed by the addition of flavopiridol (90 and 60 nM, respectively); cultures were then analyzed 24 and 48 h later. Radiation alone did not induce a significant level of apoptosis nor did flavopiridol. Moreover, the combination of radiation and flavopiridol did not result in a significant increase in the sub-G1 population (apoptotic cells) at 24 or 48 h (data not shown). These data indicate that the flavopiridol-mediated increase in radiosensitivity cannot be attributed to an enhanced susceptibility to apoptosis.

A critical determinant of radiation-induced lethality is the induction and repair of DNA damage, specifically DSBs. To determine the effects of flavopiridol on DNA damage in irradiated cells, we evaluated foci of phosphorylated histone H2AX (γH2AX), which has been established as a sensitive indicator of DNA DSBs (30–32). As shown by the representative micrographs in Fig. 5, in untreated cultures, γH2AX foci were clearly detectable, which was consistent with previous studies showing increased levels of γH2AX in the S-phase cells within an asynchronous culture (33, 34). However, exposure to 2 Gy results in a definitive increase in the number of γH2AX foci in DU145 and PC3 cells. In both cell lines, the number of cells expressing γH2AX foci remained relatively constant at 1 and 6 h after irradiation; by 24 h, the percentage of positive cells began to decline in vehicle-treated cells (Fig. 6). Whereas γH2AX foci often decline between 1 and 6 h after irradiation, this decrease is dependent on cell type and dose of radiation (32, 35, 36). Flavopiridol had essentially no effect on the radiation-induced γH2AX foci at the 1 and 6 h time points. However, at the 24-h time point, the number of cells expressing γH2AX foci in cultures receiving the combined radiation/flavopiridol treatment was significantly greater as compared

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**Figure 5.** Radiation-induced γH2AX foci. Micrographs obtained from control cells and cells that had received radiation (2 Gy) 6 h earlier, (A) DU145 and (B) PC3.
to the radiation only group. Treatment with flavopiridol alone had no significant effect on γH2AX foci. The maintenance of γH2AX foci levels suggests that the flavopiridol-mediated radiosensitization involves the inhibition of the repair of DNA damage (32).

Discussion

Flavopiridol has received considerable attention as a molecularly targeted agent with selective activity against tumor cells. However, administered as a single modality against solid tumors, this CDK inhibitor has been primarily cytostatic as evaluated in both experimental models and clinical trials (2, 5, 12, 13). Whereas perhaps not effective as a single agent, preclinical data indicate that flavopiridol can enhance the cytotoxicity of several chemotherapeutic agents (14–19). The data presented here indicate that flavopiridol also enhances the radiosensitivity of human tumor cells. This conclusion is in contrast to that of Chien et al. and their study using human bladder carcinoma cell lines. Although cell-type specificity may be involved, different experimental approaches were followed in the two studies. The bladder carcinoma cells were exposed to only one dose of radiation (2 Gy) combined with increasing concentrations of flavopiridol (21). Following this experimental protocol, which did not include the generation of a complete radiation dose-response curve, moderate changes in radiation-induced cell killing may not have been detected. Whether the experimental approach or cell type specificity accounts for the conflicting results regarding flavopiridol-induced radiosensitization requires additional investigation.

The cellular effects of flavopiridol have been primarily attributed to the reductions in the activities of CDK2 and 4 and/or P-TEFb via inhibition of CDK9 (37). In our initial studies aimed at understanding the mechanism responsible for flavopiridol-mediated radiosensitization, we focused on these putative activities. At the relatively low flavopiridol concentrations that enhance the radiosensitivity of DU145 and PC3 cells (90 and 60 nM, respectively), there were minimal reductions in cellular proliferation rate and no detectable effect on Rb phosphorylation in PC3 cells. These results are consistent with the IC50 values for flavopiridol-mediated inhibition of CDK2 and 4 ranging from 100 and 170 nM, respectively (38). In addition, at these radiosensitizing concentrations, no effects were detected on RNA polymerase II phosphorylation, which is typically used as an indicator of P-TEFb activity. This is also consistent with the previous reports in which a flavopiridol concentration of 300 nM is required to inhibit P-TEFb (8). In the absence of associating a previously established biochemical/molecular effect of flavopiridol with its radiosensitizing actions, we evaluated parameters known to influence cellular radiosensitivity. Because radiosensitization was induced when flavopiridol was added after irradiation, the potential contribution of synchronization into a radiosensitive phase of the cell cycle could be eliminated. Flavopiridol has been reported to induce apoptosis and to reduce the expression of anti-apoptotic proteins. However, according to sub-G1 content, exposure of both cell lines to flavopiridol did not induce any significant increase in apoptotic death and did not modify the already low frequency of radiation-induced apoptosis. The activation of the G2 cell cycle checkpoint, which rapidly arrests cells in G2 allowing time for DNA repair before progression into mitosis, is considered a determinant of radiosensitivity (28).

Moreover, the G2 checkpoint is considered a target for potential radiosensitizing agents. Because evaluation of this checkpoint requires distinguishing G2 cells from mitotic cells in the 4N DNA content population, the procedure of Xu et al. (24) was used to identify mitotic cells based on the expression of phosphorylated histone H3. However, as shown, flavopiridol did not inhibit the activation of the G2 checkpoint in irradiated PC3 or DU145 cells.

Figure 6. Influence of flavopiridol on radiation-induced γH2AX foci. DU145 (A) and PC3 (B) cells growing in chamber slides were irradiated (2 Gy) and flavopiridol (90 and 60 nM, respectively) added immediately afterwards. Cultures were fixed at the specified times for immunocytochemical analysis of nuclear γH2AX foci. Filled bars, data from vehicle-treated cells; open bars, data from cells exposed to flavopiridol. Foci were evaluated in 50 nuclei per treatment per experiment. Columns, mean of three independent experiments; bars, SE. Cells with greater than five foci per nucleus were classified as positive for radiation-induced γH2AX. *P < 0.05 according to Student’s t test (2 Gy versus flavopiridol/2 Gy).
Another process involved in determining cellular radiosensitivity is DNA repair, specifically the repair of DNA DSBs (30). At sites of radiation-induced DNA DSBs, the histone H2AX becomes rapidly phosphorylated (γH2AX) forming readily visible nuclear foci (30, 31). Although the specific role of γH2AX in the repair of DSBs has not been defined, recent reports indicate that the dephosphorylation of γH2AX and dispersal of γH2AX foci in irradiated cells correlates with the repair of DNA DSBs (32, 35, 36, 39). Moreover, Macphail et al. (36) in their study of 10 cell lines reported that the loss of γH2AX correlates with clonogenic survival after irradiation. The results presented here in which the expression of γH2AX in cells treated with the radiation/flavopiridol combination was similar to radiation exposure only at 1 and 6 h, but significantly greater at 24 h, are thus suggestive of an inhibition of DNA repair. Raju et al. (20) in their study of flavopiridol-induced radiosensitization of a murine tumor cell line showed that flavopiridol reduced the levels of the DNA repair proteins Ku70 and Ku86 and inhibited the repair of sublethal damage as evaluated by clonogenic assay. However, these data were generated after a 24-h exposure to 300 nm, which results in a surviving fraction of 0.06 (94% cell death). Whereas this study is consistent with an inhibition of repair of unrepaired DSBs. Clearly, further investigations are required to define the molecular processes behind these observations.

References


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