ABSTRACT

Flavopiridol, a cyclin-dependent kinase (cdk) inhibitor, can cause cell cycle arrest, induce apoptosis in cancer cells, and inhibit tumor cell growth in vivo. The present study investigated the in vitro radiosensitizing effect of flavopiridol and the underlying molecular mechanisms in a murine ovarian cancer cell line, OCA-I. Flavopiridol inhibited cell growth in a dose-dependent manner and enhanced cell radiosensitivity assessed by the clonogenic cell survival assay. A flavopiridol dose of 300 nM, given for 1 day, enhanced radiosensitivity by a factor of 2.1. Clonogenic cell survival after split-dose radiation showed that flavopiridol inhibited repair from radiation damage. In addition, flavopiridol treatment (300 nM, 1 day) resulted in decreased levels of Ku70 and Ku86 proteins that play a role in DNA repair processes, suggesting that DNA repair processes may have been disrupted by this agent. Flow cytometry analysis showed that flavopiridol (300 nM, 1 day) accumulated the cells in G1 and G2 phases, with a significant reduction in the S phase component. This cell cycle redistribution is likely another mechanism underlying flavopiridol-induced cell radiosensitivity. Flavopiridol down-regulated cyclin D1 and cyclin E protein levels and also inhibited phosphorylation of retinoblastoma protein, which is inconsistent with the observed cell cycle arrest. Among the cdks tested, cdk-9, the catalytic subunit of positive transcription elongation factor b, was significantly down-regulated by flavopiridol, suggesting that flavopiridol may modulate cellular transcription processes. Furthermore, flavopiridol on its own induced apoptosis in the OCA-I cells, whereas in combination with radiation, exerted no additional increase in apoptosis. Taken together, our data show that flavopiridol strongly augmented the response of ovarian carcinoma cells to radiation and that the underlying mechanisms included inhibition of sublethal DNA damage repair and cell cycle redistribution. At the molecular level, transcriptional regulation by flavopiridol may have been involved.

INTRODUCTION

Targeting specific molecules or molecular processes whose structures or functions are abnormal in cancer cells can potentially improve tumor response to both radiotherapy and chemotherapy. Among the potential molecular targets, cell cycle regulatory proteins, including cyclins and cdks, have been under intense investigation because their functions are well regulated in normal cells but not in tumor cells (1, 2). A novel inhibitor of cdks, flavopiridol, has been shown to exert antitumor activity in preclinical tumor models (3, 4) and undergone clinical trials as a single agent and also in combination with chemotherapeutic agents, such as paclitaxel and gemcitabine, flavopiridol has been reported to exert synergistic effects (19–22). Because flavopiridol arrests cycling cancer cells at the radiosensitive G2 phase of the cell cycle (10), it has the potential to augment cell response to radiation. In the present study, flavopiridol was investigated in combination with γ-radiation for its potential enhancing effect on the radiation response of ovarian carcinoma cells in culture.

MATERIALS AND METHODS

Cell Culture. Murine ovarian cell line designated as OCA-I was maintained in McCoy’s medium supplemented with 15% FCS and 10,000 units/ml penicillin-streptomycin.

MTT Assay. The cells were plated in a 96-well plate, and the next day, they were treated with various concentrations (25–1000 nM) of flavopiridol. After 1 day, the cells were stained with 200 μg/ml MTT and lysed in ethanol:DMSO mixture (1:1), and the absorbance was read at 540 nm using a 96-well plate reader.

Clonogenic Survival Determination. Cells in culture were exposed to flavopiridol (50, 100, or 300 nM) for 3 or 6 h or 1 day, after which they were irradiated with graded doses (1, 2, 4, or 6 Gy) of γ-rays using a 137Cs source (3.7 Gy/min). For split dose experiments after 300 nM flavopiridol treatment for 1 day, two doses of 3 Gy were given with a 4-h intertreatment interval. The cells were assayed for colony-forming ability by replating them in specified numbers in 100-mm dishes in drug-free medium. After 12 days of incubation, the cells were stained with 0.5% crystal violet in absolute ethanol, and colonies numbers in 100-mm dishes were counted under dissection microscope. Radiation survival curves were plotted after normalizing for the cytotoxicity induced by flavopiridol alone. Clonogenic survival curves were constructed from at least three independent experiments by fitting the average survival levels using least squares regression by the linear quadratic model (23).

Western Blot Analysis. Cells were treated with flavopiridol (300 nM, for 3 or 6 h or 1 day) and γ-radiation (6 Gy), and cell lysates were obtained and subjected to Western analysis as described (24), and the primary and secondary antibodies were bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, Texas).
CA). The immunoreaction was visualized using enhanced chemiluminescence-Plus detection system (Amersham, Arlington Heights, IL).

**Fluorescent Staining of Apoptotic Cells.** The cells were plated in chamber slides and treated with flavopiridol (300 nM, for 1 day) and 6 Gy radiation. The cells were fixed at specified time points in 4% paraformaldehyde and stained with 10 μM Hoechst 33342 (Sigma, St. Louis, MO) overnight in the dark at 4°C. Percentage of apoptotic cells was obtained by calculating the average of three independent counts (100 cells, each time) from three different areas selected randomly under a fluorescent microscope. In addition, cells were subjected to TUNEL assay after flavopiridol (50–300 nM, 3 h to 1 day) and radiation (6 Gy, assayed 3 h to 1 day after irradiation) treatments. Apo-Direct kit (PharMingen, San Diego, CA) was used following the manufacturer’s protocol to quantify the apoptotic cells.

**Cell Cycle Distribution.** The cells were plated in 100-mm plates and treated with flavopiridol. After 3 h, 6 h, and 1 day, the cells (2 x 10⁶) were washed with PBS. They were then suspended in 70% ethanol and stored at −20°C overnight or until use for flow cytometry analysis. The cells were washed with PBS and resuspended in propidium iodide/RNase A solution (0.5 ml), incubated at 37°C for 30 min, and analyzed by flow cytometry.

**RESULTS**

**Effect of Flavopiridol on Cell Radiosensitivity.** Before testing the effect of flavopiridol on radiosensitivity of OCA-I cells, the dose-dependent cytotoxicity of flavopiridol alone was determined using MTT assay. Tumor cells were incubated in the presence of various concentrations of flavopiridol, ranging from 25 to 1000 nM, for 1 day and subjected to MTT assay. As shown in Fig. 1, flavopiridol reduced cell survival, and the effect was dose dependent. Significant reduction in cell survival was observed at the dose of ≥100 nM. A dose of 100 nM flavopiridol reduced cell survival by 31 ± 5%, 300 nM by 57 ± 6%, and 1000 nM by 78 ± 4%.

To determine whether flavopiridol increases the sensitivity of tumor cells to radiation, in vitro clonogenic cell survival was assayed. OCA-I cells were exposed to flavopiridol for 3 or 6 h or 1 day and then treated with 1–6 Gy of γ-radiation. The cells were then plated and incubated for 12 days to determine the colony formation. Flavopiridol treatment (50–300 nM) for 3 or 6 h did not affect the radiosensitivity of OCA-I cells; however, the treatment for 1 day enhanced the radiosensitivity of these cells. Fig. 2 shows radiation dose response curves for the 1-day treatment with flavopiridol. The curves representing the effect of flavopiridol plus radiation were normalized, i.e., the colony number after drug alone was used as 1 (100% survival). On its own, flavopiridol treatment for 1 day reduced the number of OCA-I colonies to 67% after 50 nM, 61% after 100 nM, and 6% after 300 nM. Radiation alone caused a dose-dependent reduction in cell survival. Treatment with flavopiridol enhanced the radiation-induced cell killing, the extent of which depended on drug concentration. The effect increased as the dose of flavopiridol was increased. The enhancement factors were calculated at the surviving fraction of 0.1 by dividing radiation dose of the control curve with that of the corresponding flavopiridol plus radiation curves. Enhancement factors were 1.14, 1.29, and 2.1, respectively, after 50, 100, and 300 nM flavopiridol treatments. Treatment with 300 nM flavopiridol modified the shape of the cell survival curve by almost completely removing the “shoulder” region on the survival curve.

Inhibition of repair from sublethal radiation damage. Removal of the “shoulder” region on the radiation response curve by flavopiridol (Fig. 2) suggests that the drug inhibited repair from sublethal radiation damage (SLDR). This possibility was tested in split-dose experiments. The clonogenic cell survival was assayed after exposing OCA-I cells to a single radiation dose of 6 Gy or two radiation doses of 3 Gy each, given 4 h apart. Before the radiation was delivered, the cells were treated with 300 nM flavopiridol for 1 day (controls were left untreated). Fig. 3 shows the percentage of surviving cells after these treatments. A single dose of 6 Gy was more effective in cell killing (only 16.7% surviving cells) than the same total dose split in two equal doses (38.1% surviving cells). Thus, splitting the dose of radiation allowed SLDR to take place, producing a recovery ratio of 2.28, calculated by dividing the survival fraction after split doses of radiation (3 + 3 Gy), by the surviving fraction after a single dose of 6 Gy. Treatment with flavopiridol reduced cell survival after a single dose of 6 Gy to 9.1% and after split doses of 3 + 3 Gy to 9.7%. The recovery ratio was only 1.06, indicating that in the presence of flavopiridol, the cells were not able to recover during the 4-h time period between the split doses of radiation.

To determine whether flavopiridol affected DNA repair proteins,
FLAVOPIRIDOL ENHANCES RADIOSENSITIVITY

Fig. 3. Clonogenic survival assay after single and split doses of radiation with and without flavopiridol (300 nM, 1 day) treatment. OCA-I cell cultures were treated with flavopiridol, 300 nM for 1 day, and then exposed to either a single radiation dose of 6 Gy or two radiation doses of 3 Gy, each given 4 h apart. Then, a known number of cells was plated in 100-mm plates for their colony-forming ability. After 12 days, the colonies were stained and counted. The percentages of surviving cell colonies were plotted with normalized values for the cytotoxicity induced by flavopiridol alone. Recovery ratios between 6 Gy single dose and 3 + 3 Gy split dose were calculated for untreated control groups and flavopiridol-treated groups. *, recovery ratio = 2.28; #, recovery ratio = 1.06.

Fig. 4. Western blot analysis of Ku proteins. The cells were plated in 100-mm plates and treated with flavopiridol/radiation. Total protein extract of the cells was obtained, and Western blot analysis was performed as described in “Materials and Methods.” Lane 1, untreated control; Lanes 2–4, flavopiridol only, 300 nM, 3 h, 6 h, and 1 day, respectively; Lane 5, 6 Gy only; Lane 6, flavopiridol, 300 nM, 1 day plus 6 Gy; cells were lysed 1 day after irradiation. Western blots shown are representative of three independent experiments.

Ku70 and Ku86 were analyzed by Western blot in cells treated with 300 nM flavopiridol for 3 or 6 h or 1 day. Flavopiridol treatment resulted in decreased levels of Ku70 and Ku86 proteins only in cells exposed to the drug for 1 day (Fig. 4). Treatment of cells with flavopiridol for 1 day followed by 6 Gy irradiation also resulted in decreased levels of Ku70 protein (Fig. 4). These observations suggest that inhibition of repair from radiation damage may be a mechanism by which flavopiridol enhances OCA-I tumor cell radiosensitivity.

Effect of Flavopiridol on Cell Cycle Distribution. As an inhibitor of cdks, flavopiridol has been shown to cause cell cycle redistribution (10), which could increase the cell killing effect of radiation if cells were positioned in the radiosensitive phases of the cell cycle at the time of radiation. To determine whether flavopiridol affected cell cycle distribution of OCA-I cells, cells were analyzed for cell cycle distribution by flow cytometry after flavopiridol treatment (50–300 nM, for 3 to 1 day) and propidium iodide staining. Flavopiridol treatment for 1 day at doses ranging from 50 to 300 nM accumulated cells at G1 and G2 phases of the cell cycle in a dose-dependent manner when compared with the untreated control cells (Fig. 5). The accumulation was dose dependent; it increased as the dose of flavopiridol increased. Flavopiridol (300 nM) increased the fraction of G1 cells from the control value of 29 to 47% and that of G2 cells from the control value of 21 to 36%. On the other hand, the same dose of flavopiridol greatly reduced the percentage of S phase cells from the control value of 50 to 14%. In contrast, no significant cell cycle redistribution was observed when OCA-I cells were treated with flavopiridol for 3 or 6 h (data not shown).

To determine whether the flavopiridol affected the target proteins that regulate the cell cycle, Western blot analysis was performed on the total proteins collected from the cells treated with flavopiridol. Although flavopiridol (300 nM, 1 day) significantly down-regulated the expression of cdk-9 protein, the protein levels of other cdks, including cdk-1, -2, and -4, were not changed (Fig. 6). Among the cyclins tested, cyclins A and B1 were not affected by flavopiridol (data not shown), but a significant reduction in cyclin D1 and cyclin E isofoms was observed (Fig. 7). In addition, this dose of flavopiridol strongly reduced the phosphorylation of Rb protein (Fig. 7). These data are consistent with the effect of flavopiridol on the cell cycle redistribution. Furthermore, combination treatment with flavopiridol and radiation (6 Gy) also down-regulated cdk-9, cyclin D1, cyclin E, and Rb proteins.

Effect of Flavopiridol on the Induction of Apoptosis. To determine whether flavopiridol induced apoptosis in OCA-I cells, the cells

Fig. 5. Cell cycle redistribution. The cells were treated with various concentrations of flavopiridol for 1 day. Then, they were trypsinized and fixed in 70% ethanol. After washing, the cells were exposed to propidium iodide/RNase solution before performing flow cytometry analysis. Data shown are representative of two independent experiments.

Fig. 6. Western blot analysis of cdk proteins. The cells were plated in 100-mm plates and treated with flavopiridol/radiation. Total protein extract of the cells was obtained, and Western blot analysis was performed as described in “Materials and Methods.” Lane 1, untreated control; Lane 2, flavopiridol, 100 nM, 1 day; Lane 3, flavopiridol, 300 nM, 1 day; Lane 4, flavopiridol, 300 nM, 2 days; Lane 5, 6 Gy only; Lane 6, flavopiridol, 300 nM, 1 day plus 6 Gy; cells were lysed 1 day after irradiation. Western blots shown are representative of three independent experiments. *, cdk-9 was down-regulated by flavopiridol and flavopiridol/radiation.
were stained with Hoechst-33342 for fragmented DNA after 1 day with flavopiridol treatment. Cells with blue stain were counted. Although untreated cells had only 1.3 ± 0.4% apoptosis, flavopiridol-treated cells showed a 22.5 ± 0.5% apoptosis. Radiation alone at a dose of 6 Gy did not induce any measurable amount of apoptosis in these cells when analyzed 1 day after irradiation. The cells that were exposed to flavopiridol and radiation showed 23 ± 2% apoptosis, indicating that flavopiridol did not render tumor cells more susceptible to radiation-induced apoptosis. That flavopiridol did not increase the sensitivity of OCA-I cells to radiation-induced apoptosis was shown by an additional experiment, using TUNEL assay. OCA-I cells were treated with 300 nM flavopiridol for 3 or 6 h and analyzed for apoptosis. Only 1-day treatment with flavopiridol induced apoptosis: 21.2% apoptotic cells compared with 2.8% of apoptotic cells in control. Although 6 Gy radiation alone induced 4.9% apoptosis in untreated cells, it resulted in 16.9% apoptosis in cells treated with flavopiridol for 1 day.

To determine whether flavopiridol induced apoptosis via caspase-3 and poly(ADP-ribose) polymerase activation, the levels of these proteins were analyzed by Western blot using an antibody that recognizes only the active form of caspase-3. Fig. 8 shows that flavopiridol increased the levels of the active form of caspase-3 and also increased the cleavage of poly(ADP-ribose) polymerase protein to its M, 85,000 form, which is consistent with the induction of apoptosis by flavopiridol. There was no additional increase in the levels of these apoptotic proteins when flavopiridol was combined with 6 Gy irradiation, which is consistent with the Hoechst stain TUNEL data on percentage of apoptotic cells mentioned above.

DISCUSSION

The results of the present study show that flavopiridol is a potent enhancer of cellular radiosresponse and that the effect is mediated by a number of mechanisms. Flavopiridol significantly enhanced in vitro radiosensitivity of murine OCA-I cancer cells by factors ≥2.1, but the degree of the radioenhancement greatly depended on drug dose between the range of 50 and 300 nM (Fig. 2). Higher concentrations of flavopiridol resulted in higher radioenhancement. This enhancement of radiosensitivity was observed when flavopiridol was present with tumor cells for 1 day. Shorter treatment times with flavopiridol, i.e., 3 or 6 h, caused no change in cell radiosensitization. Our results are in concordance with those reported previously, where 25 nM flavopiridol enhanced radiosensitivity of human U251 glioma and PC-3 prostate cancer cell lines (25). In another study, only an additive effect of flavopiridol at a concentration of 200 nM and radiation was observed on two bladder cancer cell lines (26).

Our experiments identified a number of mechanisms that could be responsible for the observed flavopiridol-induced enhancement of cell radiosensitivity. The cell clonogenic survival curve after treatment with flavopiridol (300 nM) was completely devoid of the shoulder, suggesting that flavopiridol may have enhanced cell radiosensitivity by inhibiting SLDR. This mechanism was confirmed by the results of the split-dose experiments. Although the recovery ratio of cells exposed to radiation alone was 2.28, for cells pretreated with flavopiridol, it was 1.06, indicating that flavopiridol blocked the cellular recovery from radiation damage. The drug down-regulated Ku70 and Ku86 proteins that are known to be involved in DNA repair. Ku proteins are the subunits of DNA-dependent protein kinase (DNA-PK) complex that are required for the DNA-binding activity of the complex on DNA breakage to facilitate DNA repair process (27, 28). Down-regulation of Ku proteins by flavopiridol suggests that flavopiridol treatment may have blocked the formation of DNA-PK complex, thus inhibiting DNA repair after irradiation.

Mammalian cells exhibit significant variation in their radiosensitivity as they move through the division cycle, with cells in the G2-M phase being most radiosensitive and S phase cells most radioresistant (29). Earlier studies have shown that flavopiridol accumulated cells in either or both G1 and G2 phases of the cell cycle (10). Our results (Fig. 5) showed that treatment with flavopiridol for 1 day resulted in significant accumulation of OCA-I cells in both G1 and G2 phases of the cycle, which are radiosensitive phases of the cell cycle, whereas the proportion of cells in the S phase, the most radioresistant phase of the cell cycle, was reduced. This cell cycle redistribution at the time of cell irradiation is likely to be another mechanism by which flavopiridol augmented OCA-I cell radiosensitivity. The involvement of this mechanism is further suggested by the findings that treatment of OCA-I cells with flavopiridol for 3 or 6 h resulted in neither tumor cell radiosensitization nor cell cycle redistribution.

Flavopiridol caused cell cycle arrest by inhibiting several molecular processes regulating the cell cycle. To allow transition of cells at the G1-S checkpoint, the D and E type cyclins form complexes with cdk-2 and cdk-4 or cdk-6, respectively (30, 31). These cyclin/cdk complexes are responsible for the phosphorylation of Rb protein, which on phosphorylation, dissociates itself from E2F transcription factor, al-
lowing it to a complex with dimerization protein, followed by transcriptional activation by the E2F/dimerization protein complex for the cell cycle progression (32, 33). Our results showed that flavopiridol treatment attenuated the phosphorylated form of Rb, leaving it hypophosphorylated by which E2F/Rb complex remained intact, thus blocking the cell cycle progression. In addition, flavopiridol treatment resulted in decreased expression of cyclin D1 and E levels. The mechanism by which flavopiridol decreased cyclin D1 and E remains to be elucidated. More recently, transcriptional repression of cyclin D1 by flavopiridol in a breast cancer cell line was reported (11). Furthermore, Bible et al. (34) showed that flavopiridol binds to duplex DNA, suggesting that DNA might be another target of flavopiridol to kill noncycling cancer cells. More interestingly, our results show that flavopiridol may repress the transcription machinery by inhibiting cdk-9 expression (Fig. 6). Cdk-9 has been reported to pair with flavopiridol may repress the transcription machinery by inhibiting cdk-9 expression (Fig. 6). Cdk-9 has been reported to pair with different forms of cyclin T (35) and also with cyclin K (36). The cdk-9/cyclin complex, known as P-TEFb, regulates the activity of RNA polymerase II (35). Flavopiridol has been shown to bind to cdk-9 (37) at a relatively low concentration (Ki = 3 nM), inhibiting P-TEFb activity in vitro (13) and in vivo (38). These data show that flavopiridol may influence the transcription processes by blocking cdk-9 and also by its DNA-binding activity.

Because flavopiridol has been reported to induce apoptosis in a variety of tumor cells (10), we tested whether flavopiridol rendered tumor cells more susceptible to radiation-induced apoptosis. Although flavopiridol on its own was effective in inducing apoptosis, it had no effect on the ability of radiation to cause apoptotic cell death. OCA-I cells were also insensitive to apoptosis induction when exposed to radiation as a single agent. Thus, rendering cells more susceptible to apoptotic death was eliminated as a possible mechanism in the flavopiridol-induced enhancement of OCA-I cell radiosensitivity. In conclusion, treatment of ovarian carcinoma cells with flavopiridol augmented their radiosensitivity most likely by accumulating cells in radiosensitive phases of the cell cycle and by blocking sublethal DNA damage repair processes. Furthermore, cdk-9 and Ku proteins may play a role in flavopiridol-mediated radiosensitization. These in vitro data suggest that flavopiridol has potential to increase tumor response to radiotherapy and warrants further investigation using in vivo tumor models.

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Flavopiridol, a Cyclin-dependent Kinase Inhibitor, Enhances Radiosensitivity of Ovarian Carcinoma Cells

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