The Cyclin-Dependent Kinase Inhibitor Flavopiridol Potentiates γ-Irradiation-Induced Apoptosis in Colon and Gastric Cancer Cells

Christoph Jung,[1] Monica Motwani,[1] Jeremy Kortmansky,[1] Francis M. Sirotnak,[2] Yuhong She,[2] Mithat Gonen,[3] Adriana Haimovitz-Friedman,[4] and Gary K. Schwartz[1]

[1]Gastrointestinal Oncology Research Laboratory, Division of Solid Tumor Oncology, Department of Medicine, [2]Program of Molecular Pharmacology and Experimental Therapeutics, [3]Department of Epidemiology and Biostatistics, and [4]Department of Radiation Oncology, Memorial Sloan Kettering Cancer Center, New York, New York

ABSTRACT

Purpose: **Flavopiridol is a cyclin-dependent kinase inhibitor currently under development by the National Cancer Institute both as a single agent and in combination with chemotherapy. There have been numerous reports that flavopiridol potently enhances the induction of apoptosis by chemotherapy. However, the effect of flavopiridol on radiotherapy (RT)-induced apoptosis has been largely untested. RT has become the cornerstone of adjuvant treatment of colorectal and gastric cancer. In view of this, we elected to evaluate the effect of flavopiridol on potentiating RT-induced apoptosis in the human colon cancer cell line HCT-116 and the gastric cancer cell line MKN-74.**

Experimental Design: **The efficacy of combination of γ-irradiation and flavopiridol was tested *in vitro* in MKN-74 and HCT-116 cells and correlated to changes in p21 expression. HCT-116 cells were also established as tumors in nude mice and treated with γ-irradiation and flavopiridol either as single agents or in sequential combinations such that flavopiridol was either given 7 h before, concomitantly, or 3 and 7 h after γ-irradiation.**

Results: **Flavopiridol significantly enhanced the induction of apoptosis by γ-irradiation in both cell lines as measured by quantitative fluorescent microscopy, caspase-3 activation, poly(ADP-ribose) polymerase cleavage, and cytochrome *c* release. To achieve the best effect, it was important to expose the tumor cells to γ-irradiation before the flavopiridol. This sequence dependence was confirmed *in vivo*. When γ-irradiation was administered 7 h before flavopiridol, 42% of the tumor-bearing animals were rendered disease free, compared with no animals treated with either γ-irradiation or flavopiridol alone. Examination of the p21 status of HCT-116 and MKN-74 cells, after treatment with sequential γ-irradiation and flavopiridol, indicated a loss of p21 protein expression. Loss of p21 was mainly due to cleavage by caspases. HCT-116 cells that lack p21 ($p21^{-/-}$) also exhibited sensitization to γ-irradiation and showed an even greater enhancement of γ-irradiation-induced apoptosis by flavopiridol when compared with the parental HCT-116 cells.**

Conclusions: **These studies indicate that γ-irradiation followed by flavopiridol enhances apoptosis and yields significantly increased tumor regressions and cures that are not achievable with radiation alone. These results indicate that flavopiridol can potently enhance the effect of γ-radiation both *in vitro* and *in vivo* and may provide a new means to treat patients with locally advanced gastrointestinal cancers.**

INTRODUCTION

Flavopiridol is a synthetic flavone initially identified as an active agent in the National Cancer Institute 60-cell line drug screen. It has been shown *in vitro* to inhibit tumor cell growth at nanomolar concentrations through blockade of cell cycle progression at G_1 or G_2 (1). It is a potent inhibitor of CDKs[5] with respect to the ATP binding site. Inhibition of CDKs including CDK-1 (cdc-2), -2, -4, and -7 and hypophosphorylation of retinoblastoma protein have also been reported (2, 3). It has also been shown to induce apoptosis, inhibit angiogenesis, and potentiate the effects of chemotherapy (4–7). We have reported previously that flavopiridol at nanomolar concentrations significantly enhances the induction of apoptosis by mitomycin C and paclitaxel in gastric and breast cancer cells, gemcitabine in pancreatic cancer cells, and SN-38 (the active metabolite of CPT-11) in colon cancer cells (7–9). These studies indicate that combinations of chemotherapy and flavopiridol are highly sequence dependent, such that these cytotoxic agents must precede flavopiridol to achieve their maximal effect (7–9). This sequence dependency has been confirmed in several xenograft models. In the case of CPT-11, sequential CPT-11 followed by flavopiridol resulted in over a doubling of tumor regressions and a 30% cure rate that was not possible with the maximum tolerated dose of CPT-11 alone. Similar results were also re-

Received 4/15/03; revised 8/14/03; accepted 8/15/03.
Grant support: Grant R01CA67819 from the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Note: Christoph Jung and Monica Motwani contributed equally to this work.

Requests for reprints: Gary K. Schwartz, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, New York 10021. Phone: (212) 639-8324; Fax: (212) 717-3320; E-mail: schwartg@mskcc.org.

[5] The abbreviations used are: CDK, cyclin-dependent kinase; PARP, poly(ADP-ribose) polymerase; z-VAD, z-Val-Ala-Asp-fluoromethylketone; QFM, quantitative fluorescence microscopy; DAPI, 4′,6′-diamidino-2-phenylindole; RT, radiotherapy; CR, complete response.

ported *in vivo* for sequential docetaxel and flavopiridol (10). Interestingly, in all of these *in vivo* models, there was not only sequence dependence but also time interval dependence. In particular, separating the chemotherapy from the flavopiridol by 4–7 h resulted in the maximum antitumor effect. In fact, if the flavopiridol was given concomitantly with either CPT-11 or docetaxel or was delayed until 24 h later, the effect was no better than that of chemotherapy alone (9, 10).

Despite numerous publications on combining flavopiridol with chemotherapy, there are still very limited data on combining flavopiridol with radiation. However, *in vitro* treatment of two bladder cancer cell lines (UMUC-3 and 5637) with 2 Gy of γ-irradiation and flavopiridol concomitantly showed a slightly additive effect when compared with γ-irradiation alone (11). RT has become part of standard therapy in the adjuvant treatment of gastrointestinal malignancies, especially for resected rectal and stomach cancer (12, 13). It is also used in the neoadjuvant setting for locally advanced esophagus cancer (14). However, despite this approach, local regional failure still remains high, even when RT is combined with classical forms of chemotherapy (15). New approaches are needed to improve the therapeutic index of radiation. We therefore elected to test the effects of RT and flavopiridol *in vitro* on the induction of apoptosis and *in vivo* in xenografts.

MATERIALS AND METHODS

Cell Culture and Treatment. The human gastric cancer cell line MKN-74 (heterozygous for p53; Dr. E. Tahara; Hiroshima University, Hiroshima, Japan) and human colon cancer cell lines HCT-116 (p21 intact and deficient; kindly provided by Dr. Bert Vogelstein; John Hopkins Oncology Center) were maintained in MEM or RPMI 1640, respectively, supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37°C in 5% carbon dioxide. The cell lines were tested as *Mycoplasma* free. The stock solutions of the pan-caspase inhibitor z-VAD (50 mM; R&D systems) and PS-341 (10 mM; graciously supplied by Louis Grenier; ProScript) were prepared in DMSO, whereas flavopiridol (4.5 mM; graciously supplied by Dr. Edward Sausville; National Cancer Institute, Bethesda, MD) was prepared in water. The stocks were stored at –20°C, and drugs were diluted in the media before use.

QFM. The assay was performed as described previously (7, 9). MKN-74 cells were treated with 300 nM flavopiridol and 20 Gy (137 cesium irradiator; JL Shepaherd Mark I, Model 68; SN643; Glendale, CA), and HCT-116 cells were treated with 150 nM flavopiridol and 10 Gy. Cells were treated with these fixed doses of γ-irradiation and flavopiridol on different schedules, either concomitantly for 24 h or sequentially such that 24 h after γ-irradiation, the media were removed, and fresh media were added containing flavopiridol for an additional 24 h, or the treatments were done in reverse sequence. For sequential treatment, the floating cells were collected at the end of the first 24-h interval and then added back for the second drug treatment. At the end of the treatment, cells were collected, fixed, and stained with DAPI (Sigma, St. Louis, MO). The aliquots of cells were taken to prepare slides, and duplicate samples of 400 cells each were counted and scored for the incidence of apoptotic chromatin condensation.

Colony Formation Assays. HCT-116 cells ($p21^{+/+}$ and $p21^{-/-}$) were plated at the density of 250–500 cells/100-mm plate. Twenty-four h later, cells were treated with γ-irradiation alone (2.5 Gy), flavopiridol alone (50 nM) for 24 h, a combination of both treatments, sequential application of the γ-irradiation followed 24 h later by flavopiridol, or the reverse sequence of flavopiridol for 24 h followed by γ-irradiation. At the end of treatment, cells were incubated in drug-free media for 10 days to form colonies. Colonies were stained with 0.5% crystal violet in 0.2 mM citric acid for 20 min. Colonies with >50 cells were counted.

Preparation of Cytosolic Fractions. MKN-74 cells were treated with γ-irradiation and flavopiridol as described above for QFM. At the end of treatment, cells were incubated for 30 min on ice with 500 μl of iso-osmolar lysis buffer B (10 mM 4-morpholinepropanesulfonic acid, 250 mM sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and homogenized using a glass Dounce homogenizer with a B pestle for 40 strokes. After centrifugation at 500 × *g* for 10 min at 4°C, the supernatant was recovered and centrifuged at 40,000 × *g* for 16 min at 4°C. The supernatant (cytosolic cell fraction) was collected and stored at –20°C.

Immunoblot Analysis. Protein lysates and Western blots were prepared as described previously (7). The Western blots were probed with mouse monoclonal antibodies specific to PARP and cytochrome *c*, rabbit polyclonal antibody to caspase-3 (BD PharMingen), or mouse monoclonal antibodies specific to p21 or p53 (Santa Cruz Biotechnology). The membranes were treated with a secondary horseradish peroxidase-conjugated antimouse or antirabbit antibody (Amersham Life Science) for 1 h at room temperature. Detection was done by enhanced chemiluminescence reagents (Dupont New England Nuclear Life Science Products, Boston, MA). Equal loading of protein was confirmed by Amido Black staining and a nonspecific protein band for each blot.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted from MKN-74 cells treated with γ-irradiation and flavopiridol by the cesium chloride method as described previously (16). Twenty μg of total RNA were then electrophoresed on a 1% agarose-phosphate buffer gel and blotted onto Hybond-N nylon membranes (Amersham), and RNA was cross-linked by UV Stratalinker (Stratagene). The membranes were hybridized with ^{32}P-labeled p21 cDNA (kindly provided by Dr. David Beach; Howard Hughes Medical Institute, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY) probe in Expresshyb hybridization solution (BD PharMingen). The probe was previously labeled by random priming [^{32}P]dCTP incorporation using random prime labeling kit (Amersham). The probe was purified by passing through Sephadex Quick Spin columns (Boehringer Mannheim).

Indirect Immunofluorescence. For indirect immunofluorescence analysis, MKN-74 cells were grown in 60-mm culture tissue plates and treated as discussed above for QFM. After the treatment, attached and floating cells were harvested and fixed in 3% paraformaldehyde in PBS for 15 min. After blocking with 5% BSA/0.5% Triton X-100 for 30 min at 37°C, cells were incubated with the primary antibody against p21(Oncogene) in 5% goat serum overnight at 4°C. Subsequently cells were incu-

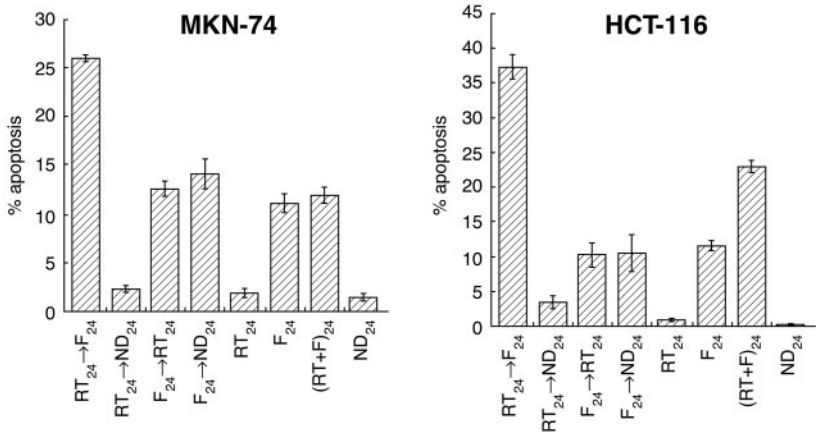

Fig. 1 Flavopiridol potentiates the irradiation-induced apoptosis in MKN-74 and HCT-116 cells. Cells were treated with γ-irradiation alone (RT_{24}), flavopiridol alone for 24 h (F_{24}), flavopiridol in combination with γ-irradiation ($RT_{24}+F_{24}$), or with sequential treatment of irradiation followed in 24 h by flavopiridol for 24 h ($RT_{24}\rightarrow F_{24}$), or with the same treatments given in reverse sequence ($F_{24}\rightarrow RT_{24}$), as described in "Materials and Methods." The fraction of apoptotic cells was assessed by fluorescence microscopy of DAPI-stained cells as described previously (7) and in "Materials and Methods." *Bars* represent the number (mean ± SD) of cells counted that undergo apoptosis as a percentage of 400 or more total cells randomly counted.

bated with the secondary antibodies in blocking buffer containing 5% goat serum/0.5% Triton X-100 for 30 min at room temperature. Cells were washed with PBS and incubated with FITC-conjugated goat antimouse antibody. For determination of nuclear morphology, cells were counterstained with 0.3 μg/ml DAPI. Subsequently, cells were mounted on glass slides and analyzed using Olympus BX60 wide-field immunofluorescence microscope with custom filters and ×400 magnification. Photomicrographs were taken with a Sony DKC-5000 3CCD camera with a T45S (×0.45) objective.

Xenograft Growth Assay. The general procedure used in the experiments has been described previously (17). Athymic-NCr-nu male mice (8–10 weeks old) were inoculated s.c. in the flank with minced p21-intact HCT-116 tumor cells mixed with Matrigel (Becton Dickinson). Treatment was started on the third day after the inoculation of tumor to allow tumor deposits to grow to a mean tumor volume of 75–78 mm^3. The mice were treated with γ-irradiation (RT) and flavopiridol in one of seven treatment arms: (*a*) control (no treatment); (*b*) RT alone; (*c*) flavopiridol alone; (*d*) RT with flavopiridol; (*e*) RT followed by flavopiridol at 3 h; (*f*) RT followed by flavopiridol at 7 h; and (*g*) flavopiridol followed by RT at 7 h. The maximal tolerated doses for RT and flavopiridol were determined in preliminary experiments. Flavopiridol was injected i.p. at a dose of 10 mg/kg. Mice received five fractions of 1.6 Gy (total, 8 Gy). The methods for delivering radiation are described below. Mice in the control group were given vehicle (PBS) alone. The single-agent or combination therapy was repeated five times on days 1, 4, 8, 11, and 15, and tumor growth was monitored for 50 days. Tumors were measured every 3–4 days with calipers, and tumor volumes were calculated by the formula $4/3 \times \pi \times r3$ (r = larger diameter + smaller diameter/4). The percentage of tumor regression was calculated as the percentage ratio of difference between baseline and final tumor volume to the baseline volume (baseline volume − final volume/baseline volume). Mice showing no palpable tumor after treatment were counted as a CR after 45 days. These studies were performed in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 85-23, released in 1985).

Radiation Therapy. The mice were anesthetized by injecting a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p., the animals were laid down in a specially designed plastic jig in a restraining position that only allowed the flank implanted with tumors to be exposed while the rest of the body was protected by a lead shield. Radiation was delivered using an Agfa Orthovoltage X-ray unit, operating at 250 kV and 12 mA, and filtered by 0.5 mm Cu. The field size was 15 × 15 cm at 50 cm source-to-surface distance. Control and flavopiridol-treated mice also received the same doses of anesthetics.

Biostatistical Analysis. All *in vitro* experiments were done in duplicate and repeated at least three times unless otherwise indicated, and the statistical significance of the experimental results was determined by the two-sided *t* test. For *in vivo* studies, both the Wilcoxon test and area under the time-volume curve were used to determine statistical significance. The area under the time-volume curve is a summary measure for each mouse. This area is calculated using the trapezoidal rule. This method takes the longitudinal aspect of the data into account, and it does not require assumptions of linear growth (or decay) that are clearly violated for our data. Treatment groups are compared in pairs, using the exact permutation distribution of the areas under the curve (18).

RESULTS

Enhancement of γ-Irradiation-Induced Apoptosis by Flavopiridol in MKN-74 and HCT-116 Cells. As shown in Fig. 1, treatment of MKN-74 cells with γ-irradiation alone for 24 h (RT_{24}) or flavopiridol alone for 24 h (F_{24}) induced apoptosis in 2.3 ± 0.4% and 11.1 ± 0.8% of the cells, respectively. The concomitant treatment of γ-irradiation and flavopiridol [$(RT+F)_{24}$] induced apoptosis in 12.1 ± 0.8% cells. However, when cells were treated sequentially with γ-irradiation on day 1, followed by flavopiridol for 24 h on day 2 ($RT_{24}\rightarrow F_{24}$), 26 ± 0.3% of cells underwent apoptosis. This was significantly greater than the effect of treatment of the cells with γ-irradiation on day 1 followed by incubation of cells in drug-free media on day 2 ($RT_{24}\rightarrow ND_{24}$), which resulted in only 2.3 ± 0.4% of the cells undergoing apoptosis ($P = 0.0004$ compared with $RT_{24}\rightarrow F_{24}$, $P = 0.015$ compared with F_{24}, and $P = 0.02$ compared with $(RT+F_{24})$. In contrast, the reverse sequence of flavopiridol followed by γ-irradiation ($F_{24}\rightarrow RT_{24}$) induced ap-

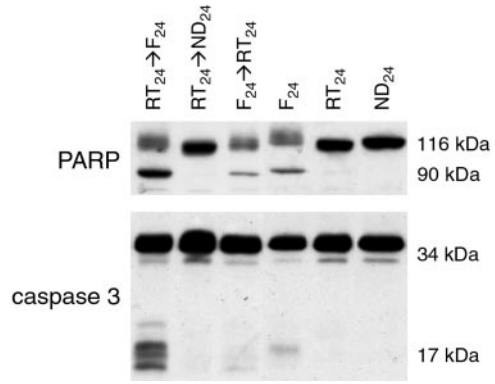

Fig. 2 Flavopiridol potentiates irradiation-induced apoptosis. Western blot analysis of PARP and caspase-3. MKN-74 cells were treated with 20 Gy of γ-irradiation and 300 nM flavopiridol with different schedules, and protein was isolated. Lysates were analyzed by immunoblotting, using antibodies specific to PARP and caspase-3. The intact PARP protein is 116 kDa, whereas the cleaved fragment is 90 kDa. Caspase-3 is 36 kDa, and the cleaved products are 24, 20, and 17 kDa. The cleaved products of PARP and caspase-3 are detected in cells undergoing apoptosis. Equal loading of protein was confirmed by Amido Black staining.

optosis in 12.6 ± 0.9% of the cells, which was no greater than $(RT+F)_{24}$ and significantly less than sequential $RT_{24}\rightarrow F_{24}$ ($P = 0.03$). As shown in Fig. 1, similar results were obtained for HCT-116 cells, showing a significant potentiation when γ-irradiation was followed by flavopiridol ($RT_{24}\rightarrow F_{24}$) as compared with $RT_{24}\rightarrow ND_{24}$ ($P = 0.01$). The only difference in the cell lines was with regard to the concomitant therapy $(RT+F)_{24}$. Under this condition in the HCT-116 cells, there was enhancement of apoptosis when compared with either γ-irradiation alone (RT_{24}) or F_{24}. This effect was not observed in the MKN-74 cells.

Next, we analyzed the activation of caspase-3 and cleavage of the PARP, a substrate cleaved by caspase-3 during apoptosis. As shown in Fig. 2, there was no PARP cleavage or caspase-3 activation observed with RT_{24} or $RT_{24}\rightarrow ND_{24}$ in MKN-74 cells. The cleavage of PARP from 116 kDa to its cleaved 90-kDa fragment was greatest with sequential $RT_{24}\rightarrow F_{24}$ treatment. F_{24} and the reverse sequence of $F_{24}\rightarrow RT_{24}$ induced similar but minor degrees of PARP cleavage. The pattern of caspase-3 activation follows that of PARP cleavage. The highest activation for caspase-3 was observed in sequential treatment of $RT_{24}\rightarrow F_{24}$, as indicated by formation of the 24-, 20-, and 17-kDa active cleaved products. Again, smaller degrees of caspase-3 activation were observed for F_{24} and the reverse sequence of $F_{24}\rightarrow RT_{24}$.

The integrity of the mitochondrial membrane and the release of cytochrome *c* from the mitochondria to the cytoplasm is an early event in the induction of apoptosis. We therefore elected to analyze cytochrome *c* release into the cytoplasm of MKN-74 cells at two time points: 24 h after γ-irradiation (RT_{24}); and 6 h after addition of flavopiridol to γ-irradiated cells ($RT_{24}\rightarrow F_6$). As shown in Fig. 3, a small amount of cytochrome *c* was released into the cytoplasm after γ-irradiation alone (RT_{24}) and $RT_{24}\rightarrow ND_6$. However, with the addition of flavopiridol ($RT_{24}\rightarrow F_6$), there was a marked increase in cytochrome *c* release when compared with either of these two conditions, as well as with flavopiridol alone (F_6).

Sequential Therapy of γ-Irradiation and Flavopiridol Augments the Tumor Regressions and Cures in Xenografts. To investigate effect of combination of γ-irradiation and flavopiridol *in vivo* and to optimize the interval between the two drugs, HCT-116 cells were established as xenografts in nude mice. Mice were treated with either γ-irradiation or flavopiridol alone or sequentially with γ-irradiation first followed by flavopiridol at 0-, 3-, and 7-h intervals or reverse order with flavopiridol first followed 7 h later with γ-irradiation. As shown in Fig. 4, flavopiridol alone had no effect on inhibition of tumor growth when compared with untreated controls. The sequence and interval between γ-irradiation and flavopiridol were important determinants of tumor regression and cures in xenografts. The greatest tumor regressions and cures were observed if the interval between γ-irradiation and flavopiridol was at least 7 h. As shown in Table 1, by day 30, there was a 64 ± 9% regression in tumor volume in mice treated with γ-irradiation alone, whereas in mice treated with γ-irradiation followed in 7 h by flavopiridol ($RT\rightarrow^{7h}F$), tumor regression was 89 ± 7% ($P = 4.34 \times 10^{-8}$). The difference between the areas under the volume-time curves (Fig. 4) for γ-irradiation alone and γ-irradiation followed by flavopiridol in 7 h was also statistically significant ($P = 0.017$). A comparison between γ-irradiation alone curves and either γ-irradiation together with flavopiridol or γ-irradiation followed 3 h later by flavopiridol did not indicate a statistically significant difference. However, the sequential combination of γ-irradiation followed in 7 h by flavopiridol was statistically superior to both concomitant therapy and γ-irradiation followed 3 h later by flavopiridol ($P = 0.0079$). In addition, if the flavopiridol was administered 7 h before γ-irradiation, the response rate was identical to that of γ-irradiation alone. Five of 12 mice treated with γ-irradiation followed in 7 h by flavopiridol had no palpable tumors, yielding a CR rate of 42%. This was in contrast to γ-irradiation alone or γ-irradiation concomitant with or followed by flavopiridol at 3 h, where no cures were found.

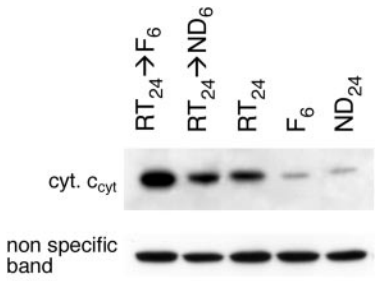

Fig. 3 Flavopiridol on irradiated cells results in increased cytochrome *c* release in cytoplasm. MKN-74 cells were treated with 20 Gy of γ-irradiation (RT_{24}), 300 nM flavopiridol for 24 h (F_{24}), or 20 Gy of γ-irradiation followed 24 h later by either flavopiridol or no drug for 6 h ($RT_{24}\rightarrow F_6$ or $RT_{24}\rightarrow ND_6$). At the end of treatment, cytosolic fractions were isolated as described in "Materials and Methods," and cytochrome *c* release was examined by Western blot analysis. Each lane contained 50 μg of proteins, and equal loading was confirmed by Amido Black staining and a nonspecific protein band.

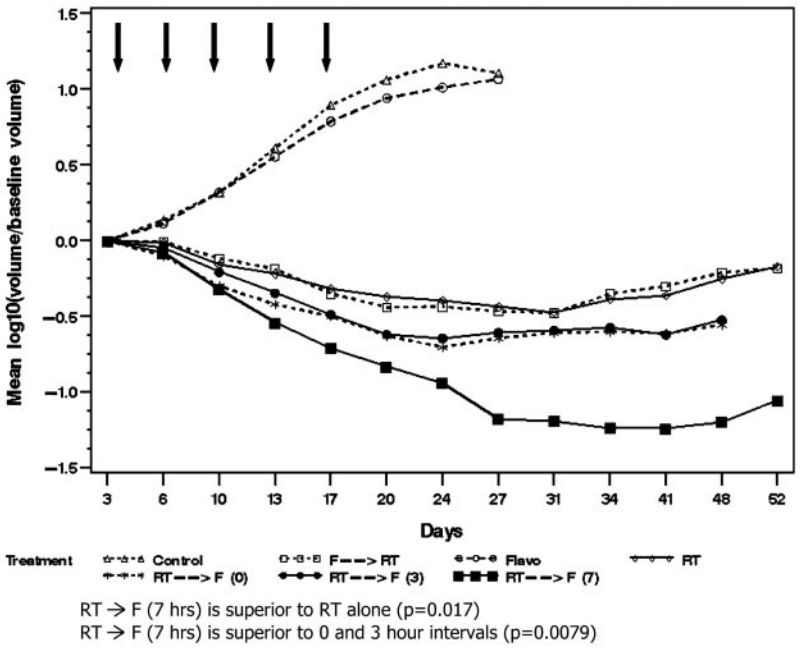

Fig. 4 Effects of γ-irradiation and flavopiridol on growth of established HCT-116 xenografts in nude mice. Mice were treated with γ-irradiation (*RT*; ◇) and flavopiridol alone (*F*; ○) or in combination as γ-irradiation followed by flavopiridol at 0 (*RT-F0*; ∗), 3 (*RT-F3*; ●), and 7 h (*RT-F7*; ■) or flavopiridol followed by γ-irradiation in 7 hours (*F-RT*; □) according to the protocol described in "Materials and Methods." Tumor volumes were measured as described in "Materials and Methods," and mean log change in volume (calculated as volume/baseline volume) was plotted against time (days). ↓ indicates the days of therapies. The control (△) and flavopiridol-treated animals were sacrificed before 6 weeks because tumor size reached the maximum allowable limits set by Memorial Sloan Kettering Cancer Center.

Mice that were treated with only γ-irradiation gained an average of 5.8 ± 3.2% in body weight, whereas mice treated with γ-irradiation followed in 7 h by flavopiridol lost an average of 2.7 ± 6.5% of body weight. After treatment, all mice gained weight, and the difference in the weight loss 2 weeks after completion of treatment was not different among all of the treatment conditions, indicating that weight loss was temporary and within the acceptable limits. This would also indicate that difference in weight loss cannot explain the significant difference in response rates for these treatment conditions. One mouse of four mice treated with γ-irradiation followed in 0 h by flavopiridol died due to toxicity during the fourth course of the experiment. One mouse of 13 mice in the γ-irradiation followed in 7 h by flavopiridol arm also died after receiving only two treatments, as did 1 mouse of 5 mice treated with only one dose, but in the reverse sequence, of flavopiridol followed in 7 h by γ-irradiation. Neither of these deaths could definitively be attributed to treatment toxicity.

Table 1 Tumor regression and CR in mouse xenografts treated with γ-irradiation (RT) and flavopiridol (F)

Tumors derived from p21-intact HCt-116 cells were established and treated with γ-irradiation alone, γ-irradiation followed by flavopiridol after 0, 3, or 7 h, and flavopiridol followed by γ-irradiation after 7 h and then measured as described in "Materials and Methods."

Treatment	Tumor regression (day 30)[a]	CR[b] (*n*)
RT alone ($n = 13$)	64 ± 9%	0% (0/13)
RT → ^{0h}F ($n = 4$)	80 ± 3%[c]	0% (0/3)
RT → ^{3h}F ($n = 4$)	77 ± 7%	0% (0/4)
RT → ^{7h}F ($n = 13$)	89 ± 7%[d]	42% (5/12)
F → ^{7h}RT ($n = 5$)	64 ± 11%[e]	0% (0/4)

[a] The tumor regression was calculated as ratio of difference between baseline and final tumor volume to the baseline volume.

[b] The CR is defined as the number of animals without tumors relative to the total number of animal in the treatment group.

[c] One mouse died after the fourth dose of treatment.

[d] One mouse died after the second dose of treatment.

[e] One mouse died after the first dose of treatment.

Enhancement of Apoptosis with Sequential Therapy of Radiation and Flavopiridol Is Associated with Suppression of p21. Radiation therapy induces DNA damage that activates the p53-mediated checkpoint (19). p21, a direct transcriptional target of p53, has been shown to play an important role in dictating the fate of the cell (cell cycle arrest *versus* cell death; Ref. 20). To understand the mechanism by which radiation-induced apoptosis is enhanced by flavopiridol, we studied p21 expression by Western blot analysis in both the HCT-116 and the MKN-74 cells. As shown in Fig. 5 (*bottom panel*), treatment of parental HCT-116 cells with γ-irradiation ($RT_{24}\rightarrow ND_{24}$ and RT_{24} alone) induced the expression of p21 protein. However, when flavopiridol was added for 24 h after γ-irradiation treatment ($RT_{24}\rightarrow F_{24}$) or when the agents were given in a concomitant fashion [$(RT+F)_{24}$], p21 protein levels were suppressed. This suppression of p21 occurred despite the induction of p53 under these treatment conditions (Fig. 5, *top panel*) and correlated with the induction of apoptosis observed with this combination in HCT-116 cells (Fig. 1). However, this correlation to apoptosis was not absolute. The greatest induction of apoptosis was seen with sequential therapy, yet the decrease in p21 expression was greater for concomitant therapy. This would suggest that factors other than loss of p21 are involved in the enhancement of apoptosis with the sequential therapy.

We observed similar changes in p21 expression for MKN-74 cells (Fig. 6*A*). The only differences between the cell lines were with regard to the effects of single-agent therapy on p53 expression. Although p53 protein expression was induced under all of the treatment conditions, treatment with flavopiridol

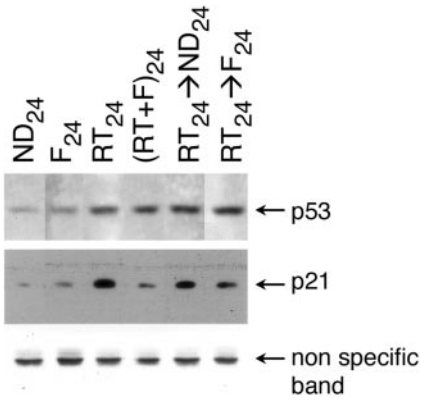

Fig. 5 γ-Irradiation induces p21, and flavopiridol on γ-irradiated HCT-116 cells down-regulates p21 protein. Western blot analysis of p53 (*top panel*) and p21 (*bottom panel*) in HCT-116 cells treated with γ-irradiation/flavopiridol combinations. Cells were treated with 10 Gy of γ-irradiation followed by either no drug or 150 nM flavopiridol. Equal loading of protein was confirmed by Amido Black staining and a nonspecific protein band.

alone (F_{24}) was associated with a greater degree of p53 expression in MKN-74 cells than in HCT-116 cells. In contrast, treatment with γ-irradiation alone (RT_{24}) resulted in a greater degree of p53 expression in HCT-116 cells than in MKN-74 cells. However, with sequential therapy, there was suppression of p21 protein expression in both cell lines, despite induction of p53.

The decrease in p21 protein levels during sequential treatment can be due to a decrease in p21 transcription or to posttranslational effects, such as increased ubiquitination or cleavage by caspases. To examine whether p21 transcription was decreased after the addition of flavopiridol, total RNA was prepared from MKN-74 cells treated with various schedules of γ-irradiation and flavopiridol, and a Northern blot was probed with radiolabeled p21 cDNA. As shown in Fig. 6*B*, p21 mRNA expression was induced under all treatment conditions when compared with no drug [flavopiridol alone (F_{24}), 1.57-fold; γ-irradiation alone (RT_{24}) or γ-irradiation followed by no drug ($RT_{24}\rightarrow F_{24}$), 1.34- and 1.29-fold, respectively; $(RT+F)_{24}$ (concurrent therapy), 1.66-fold; and sequential treatment of γ-irradiation followed by flavopiridol ($RT_{24}\rightarrow F_{24}$), 1.89-fold]. Thus, the suppression of p21 in sequential treatment could not be accounted for by a decrease in transcription.

Next, we investigated whether the changes in p21 protein levels were due to changes in posttranslational modification. Because the ubiquitin-proteasome pathway plays a critical role in maintaining the p21 levels (21, 22), we studied this pathway for inhibiting the proteasome during the combination of γ-irradiation and flavopiridol treatment. Treatment of MKN-74 cells with 20 nM PS-341, the proteasome inhibitor (23), alone for 24 h prevented the endogenous degradation of ubiquitinated p21. As shown in Fig. 6*A*, this resulted in increased levels of p21 protein. The addition of PS-341 with flavopiridol on γ-irradiated cells $[RT_{24}\rightarrow(F+PS\text{-}341)_{24}]$ increased the levels of p21 as compared with γ-irradiation followed by flavopiridol ($RT_{24}\rightarrow F_{24}$). However, the levels of p21 were also increased after addition of PS-341 on irradiated cells $[RT_{24}\rightarrow(PS\text{-}341)_{24}]$. This would indicate that PS-341 prevents the endogenous degradation of p21, irrespective of flavopiridol treatment. Unfortunately, because PS-341 also induces apoptosis as a single agent in these cell lines, we were unable to determine whether the restoration of p21 would prevent apoptosis under these experimental conditions.

It has been reported that p21 can be cleaved by caspases during apoptosis (24, 25). To analyze this further, we tested the effect of the addition of z-VAD, a pan-caspase inhibitor, during $RT_{24}\rightarrow F_{24}$ treatment. MKN-74 cells were treated with γ-irradiation and flavopiridol in the presence of 20 μM z-VAD. The cells were then analyzed by immunofluorescence microscopy for apoptosis and p21 expression. As shown in Fig. 7*A, top panel*, the addition of flavopiridol to γ-irradiated cells ($RT_{24}\rightarrow F_{24}$) resulted in an expected increase in apoptotic cells as compared with cells treated with irradiation alone ($RT_{24}\rightarrow ND_{24}$). Fig. 7*A, bottom panel*, shows the correlating fields after labeling the cells with a p21 antibody. The majority of the cells expressed p21 after γ-irradiation alone

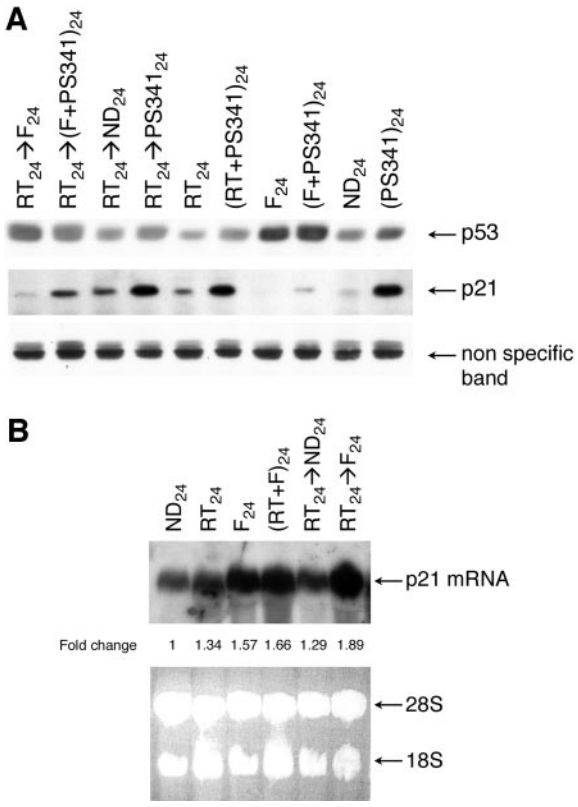

Fig. 6 γ-Irradiation induces p21, and flavopiridol, on γ-irradiated MKN-74 cells, down-regulates p21 protein but does not down-regulate p21 mRNA. Western blot analysis of p53 and p21 (*A*) and Northern blot for p21 mRNA (*B*) in MKN-74 cells treated with γ-irradiation/flavopiridol combinations with and without PS-341. Cells were treated with 20 Gy of γ-irradiation followed by either no drug or 300 nM flavopiridol in the presence or absence of 20 nM PS341. Equal loading of protein was confirmed by Amido Black staining and a nonspecific protein band. Staining of the Northern blot with ethidium bromide showed equal loading.

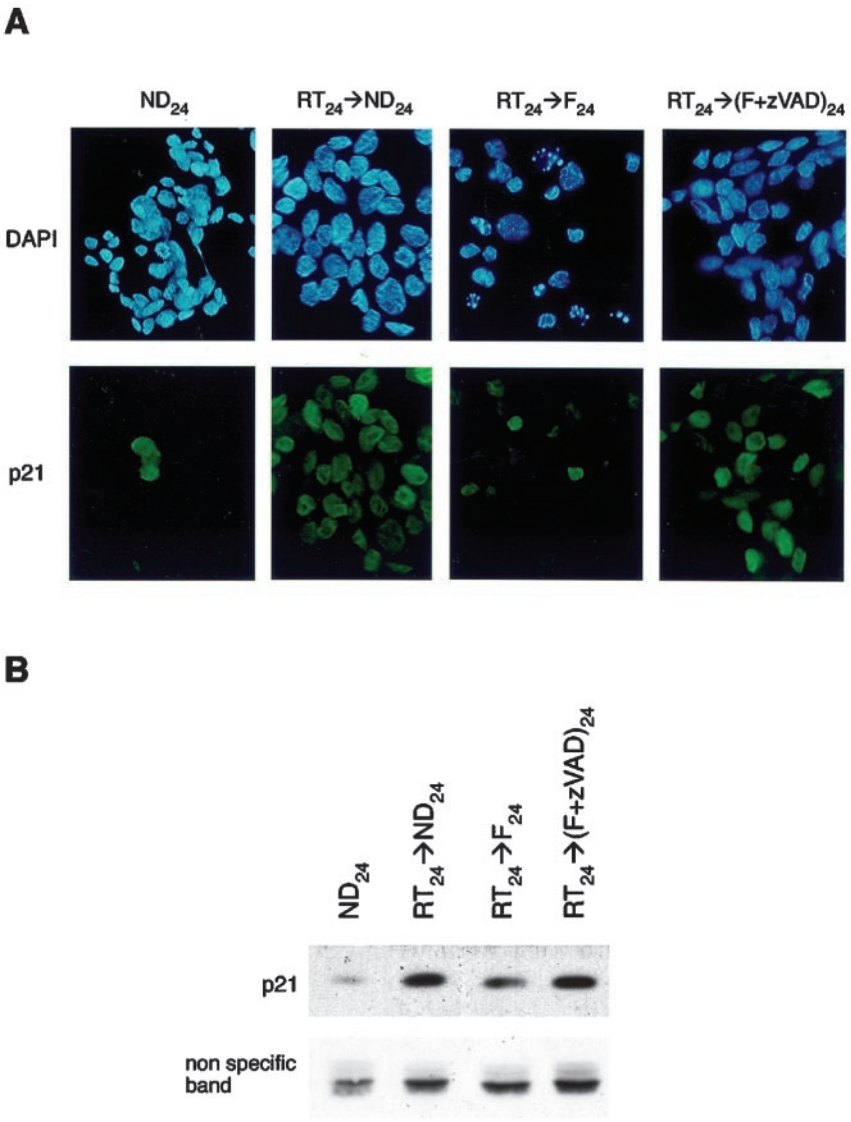

Fig. 7 Down-regulation of p21 by flavopiridol on γ-irradiated cells can be prevented by caspase inhibition. Localization of p21 in MKN-74 cells by immunofluorescence (*A*) and changes in p21 expression by Western blot (*B*). MKN-74 cells were treated with γ-irradiation (RT_{24}) and γ-irradiation followed in 24 h by drug-free media ($RT_{24}\rightarrow ND_{24}$), flavopiridol ($RT_{24}\rightarrow F_{24}$), or flavopiridol with z-VAD for an additional 24 h ($RT_{24}\rightarrow F_{24}$ + zVAD). Cells were washed, fixed, and probed with anti-p21 antibody/goat antimouse antibody conjugated with FITC as described in "Materials and Methods." Amido Black staining and a nonspecific protein band confirmed equal loading of protein on the gel.

($RT_{24}\rightarrow ND_{24}$). However, the addition of flavopiridol to γ-irradiated cells ($RT_{24}\rightarrow F_{24}$) dramatically decreased the number of cells expressing p21. The addition of z-VAD during flavopiridol treatment on γ-irradiated cells resulted in suppression of apoptosis [$RT_{24}\rightarrow(F+zVAD)_{24}$, Fig. 7*A*, *top panel*]. This also resulted in reversal of p21 expression because the majority of cells now stained positively for p21 expression [$RT_{24}\rightarrow(F+zVAD)_{24}$, Fig. 7*A*, *bottom panel*]. The changes in p21 expression, under these treatment conditions, were confirmed by Western blot analysis for the MKN-74 cells (Fig. 7*B*). Similar results were obtained with HCT-116 cells.

It has been reported that HCT-116 cells that lack $p21^{waf1/cip1}$ ($p21^{-/-}$) are more sensitive to γ-irradiation than parental HCT-116 cells ($p21^{+/+}$; Ref. 20). To further examine the role of p21 in sequential γ-irradiation and flavopiridol treatment, we compared the HCT-116 cells with deleted p21 (by homologous recombination) and parental HCT-116 cells for their ability to form colonies (Fig. 8). Fig. 8 shows the effect on colony formation in HCT-116 cells after treatment with 2.5 Gy of γ-irradiation and 50 nM flavopiridol. The $p21^{-/-}$ cells were more sensitive to γ-irradiation and to the combination therapy than the $p21^{+/+}$ cells. In fact, for all treatment conditions tested, there was a greater decrease in colony formation for HCT-116 $p21^{-/-}$ (■) as compared with parental HCT-116 cells (▨) [HCT-116 $p21^{+/+}$ *versus* HCT-116 $p21^{-/-}$ cells: RT, 62 ± 2% *versus* 34 ± 2%, $P = 0.007$; F, 87 ± 1% *versus* 57 ± 2%, $P = 0.026$; RT→F, 31 ± 1% *versus* 8 ± 0.2%, $P = 0.011$].

DISCUSSION

The findings reported here show that treatment of gastrointestinal cancer cell lines MKN-74 and HCT-116 with the CDK inhibitor flavopiridol significantly potentiates the γ-irradiation effect in a sequence-dependent manner, such that maximum apoptosis was observed when flavopiridol was administered after γ-irradiation. During apoptosis, the outer mitochondrial

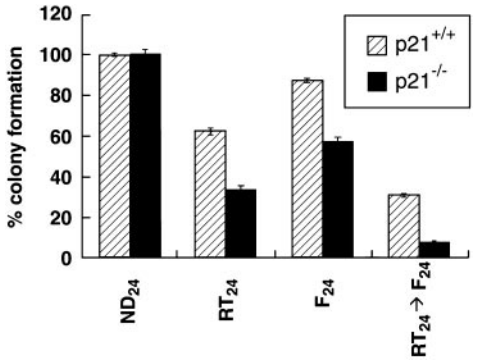

Fig. 8 HCT-116 $p21^{-/-}$ cells are more sensitive to γ-irradiation as compared with HCT-116 $p21^{+/+}$ cells, and flavopiridol potentiates the γ-irradiation effect. HCT-116 parental and $p21^{-/-}$ cells were either irradiated (RT_{24}), treated with flavopiridol for 24 h (F_{24}), or irradiated and treated 24 h later with flavopiridol for 24 h ($RT_{24}\rightarrow F_{24}$). The colony formation assay was performed as discussed in "Materials and Methods."

membrane is permeabilized, and cytochrome *c* is released into the cytosol, which can form the complex "apoptosome" that activates the caspases and orchestrates cell death. In sequential γ-irradiation followed by flavopiridol, more cytochrome *c* was released compared with single treatments, and this was associated with activation of caspase-3 and PARP cleavage. We did not observe any correlation of potentiation of apoptosis to the total protein levels of bax, bcl-2, or bcl-xl or to the bax:bcl-2 ratio under these experimental conditions (data not shown).

Under these treatment conditions, neither HCT-116 parental cells nor MKN-74 cells (which both express p21) undergo significant degrees of apoptosis with γ-irradiation treatment alone. p21 plays a significant role in dictating the sensitivity of cells to γ-irradiation because HCT-116 $p21^{-/-}$ cells were more sensitive to γ-irradiation than HCT-116 $p21^{+/+}$ parental cells, as determined by a decrease in colony formation. Similar results have been reported by Waldman *et al.* (20) using the same cell lines. There are numerous reports now supporting the hypothesis that insufficient expression of p21 during DNA damage sensitizes the cells to undergo apoptosis (20, 26–28). We observed that the induction of apoptosis with sequential $RT_{24}\rightarrow F_{24}$ correlated with a decrease in p21 protein expression in both the HCT-116 and MKN-74 cells. This decrease in p21 appears to be due to a posttranslational effect. In fact, flavopiridol induced rather than suppressed transcription of p21 under all treatment conditions. This result may appear surprising because flavopiridol has been shown to inhibit CDK-9, a component of transcription elongation factor b (P-TEFb). P-TEFb phosphorylates the COOH-terminal domain of the large subunit of RNA polymerase II, thus continuing the transcription elongation (29, 30). Our results would suggest that in the presence of flavopiridol, the expression of p21 is not affected by this transcription pathway. Rather, the induction of p21, under these treatment conditions, appears to be regulated by p53, which is induced by flavopiridol, both as a single agent and in the combination therapies. This was more striking in MKN-74 cells (with one mutated p53 allele), where we used a higher flavopiridol concentration (300 nM) compared with HCT-116 cells (150 nM).

The suppression of p21 protein by flavopiridol on cells treated with γ-irradiation is a posttranslational event. Our data show that flavopiridol on γ-irradiated cells does not induce the ubiquitin-proteasome pathway. Instead, it appears that loss of p21 is due to cleavage of p21 by caspases in sequential therapy. The addition of z-VAD to flavopiridol in γ-irradiated MKN-74 cells [$RT_{24}\rightarrow(F+zVAD)_{24}$] inhibited the induction of apoptosis and prevented the loss of p21 protein expression. We have observed a similar effect in HCT-116 cells treated with sequential SN-38 (the active metabolite of CPT-11) and flavopiridol (9).

We therefore believe that caspase-mediated cleavage of p21 is the dominant mechanism for the loss of p21. It is unclear whether the cleavage of p21 in the sequential treatment of γ-irradiation followed by flavopiridol is simply an effect of the induction of apoptosis, or whether the cleaved product is a proapoptotic element and amplifies the apoptotic cascade. Nevertheless, this is not the only mechanism that is required to explain this potentiation. HCT-116 cells in which the p21 gene is deleted by homologous recombination ($p21^{-/-}$) are sensitive to γ-irradiation. However, these cells are further sensitized to γ-irradiation by flavopiridol. If suppression of p21 was the only mechanism for potentiation of apoptosis with this combination therapy, then this would not have been the case. This is also suggested by the results indicating greater suppression of p21 protein expression with concomitant rather than sequential therapy in the HCT-116 cells. However, the induction of apoptosis always remained greatest with the sequential treatments. These issues remain the subject of ongoing investigations.

The *in vivo* studies confirm the strong sequence dependence for the combination observed in *vitro*. The correlation between the *in vitro* and *in vivo* studies, however, was also not absolute. For example, in HCT-116 cells, the concomitant therapy of $(RT+F)_{24}$ resulted in enhanced apoptosis and suppression of p21 *in vitro*, but *in vivo* concomitant therapy (RT-F0) was no better than γ-irradiation alone. Still, the sequence of γ-irradiation followed by flavopiridol ($RT_{24}\rightarrow F_{24}$) both *in vitro* and *in vivo* clearly achieved the best results. It has recently been reported that the response to radiation is regulated in part by the occurrence of endothelial induced apoptosis in the tumor microvasculature (31). Flavopiridol has been shown to prevent the induction of hypoxia-induced mRNA expression of vascular endothelial growth factor (5). Thus, the effects of flavopiridol in combination with γ-irradiation may be mediated by dual mechanisms, induction of tumor cell apoptosis and suppression of angiogenesis. The contribution of each pathway to the significant tumor regression and cures we have observed with the combination therapy *in vivo* will require further investigation.

The timing of flavopiridol administration relative to the initial γ-irradiation appears to be an especially important factor in inducing the greatest degree of tumor regressions and CRs in the xenografts. We suspect that damage from ionizing radiation is a cumulative effect, and a 7-h delay may allow time for maximal, potentially lethal DNA damage to occur. In addition, as a pan-CDK inhibitor, flavopiridol before or too soon after γ-irradiation may induce a state of prolonged G_1 and G_2 arrest, both of which have been associated with cellular radioresistance (32, 33). This time dependence is consistent with other *in vivo* combinations (9, 10). With each of these *in vivo* combinations,

the 7-h interval resulted in the greatest degree of tumor regressions and cures in both the HCT-116 tumor xenografts with sequential CPT-11 and flavopiridol and the MKN-74 tumor xenografts with sequential docetaxel and flavopiridol (9, 10). The fact that we have observed this 7-h interval to be the most effective in two different xenograft models would indicate that this is not a cell line-specific phenomenon. Alternatively, this phenomenon may be related to the pharmacokinetics of flavopiridol under these treatment conditions. Still, the exact mechanism for this 7-h interval remains unknown.

With the *in vivo* studies, we did observe three animal deaths among 26 animals treated with different γ-irradiation and flavopiridol combinations. This included one animal death with γ-irradiation followed by flavopiridol at 0 h, one animal death with γ-irradiation followed by flavopiridol at 7 h, and a third animal death with the reverse sequence. The basis for this remains unclear. Animal deaths were not observed in any of our other *in vivo* studies combining chemotherapy with flavopiridol. Nor can the deaths be attributed to increased sensitization by flavopiridol because the deaths were observed under conditions (concomitant therapy) in which there was no significant potentiation of the γ-irradiation effect. One difference between this study and our other *in vivo* studies is the dose of flavopiridol tested in combination with γ-irradiation. When flavopiridol was administered with chemotherapy (CPT-11 or docetaxel), the dose of flavopiridol was reduced from 10 mg/kg/dose (the single-agent maximum tolerated dose) to 2.5 mg/kg/dose (9, 10). However, with these γ-irradiation studies, flavopiridol was maintained at full dose (10 mg/kg) with each fraction of γ-irradiation. Ultimately, the issue of toxicity will need to be addressed in Phase I trials with this combination therapy.

These results support the hypothesis that flavopiridol potentiates the effect of γ-irradiation in gastrointestinal cell lines both *in vitro* and *in vivo*. This effect has now been confirmed *in vitro* in both ovarian and lung cancer cell lines (34, 35). The latter study confirmed that the greatest effects were observed with sequential therapy of γ-irradiation followed by flavopiridol. This was also in association with suppression of p21 protein expression (34). Thus, flavopiridol in combination with γ-irradiation may have broad-reaching clinical applications that extend beyond the treatment of gastrointestinal cancers.

RT remains a major treatment modality for the therapy of gastrointestinal cancers. It is used as both an adjuvant treatment (rectal, gastric, and pancreatic cancer) and neoadjuvant treatment (*e.g.*, locally advanced esophagus cancer and anal canal cancer) of locally advanced but unresectable disease (*e.g.*, pancreatic cancer) and for palliation of all of these diseases. However, despite this approach, local-regional failure remains a major medical problem. Thus, new treatment options, which can increase the therapeutic threshold of radiation therapy, are needed. Flavopiridol may represent such an agent. To move this concept forward, a Phase I trial of sequential γ-irradiation and escalating doses of flavopiridol is now underway at our institution for patients with locally advanced but unresectable pancreatic cancer. Similar to our *in vivo* studies, patients are treated with fixed daily fractions of γ-irradiation followed sequentially by biweekly doses of flavopiridol, administered over 1 h. Our hope is to successfully translate these preclinical studies into a new treatment approach in clinical cancer therapy.

REFERENCES

1. Kaur, G., Stetler-Stevenson, M., Sebers, S., Worland, P., Sedlacek, H., Myers, C., Czech, J., Naik, R., and Sausville, E. A. Growth inhibition with reversible cell cycle arrest of carcinoma cells by flavone L86-8275. J. Natl. Cancer Inst. (Bethesda), *84:* 1736–1740, 1992.

2. Losiewicz, M. D., Carlson, B. A., Kaur, G., Sausville, E. A., and Worland, P. J. Potent inhibition of cdc2 kinase activity by the flavonoid L86-8275. Biochem. Biophys. Res. Commun., *201:* 589–595, 1994.

3. Carlson, B. A., Dubay, M. M., Sausville, E. A., Brizuela, L., and Worland, P. J. Flavopiridol induces G_1 arrest with inhibition of cyclin-dependent kinase CDK2 and CDK4 in human breast carcinoma cells. Cancer Res., *56:* 2973–2978, 1996.

4. Patel, V., Senderowicz, A. M., and Pinto, D., Jr. Flavopiridol, a novel cyclin-dependent kinase inhibitor, suppresses the growth of head and neck squamous cell carcinomas by inducing apoptosis. J. Clin. Investig., *102:* 1674–1681, 1998.

5. Melillo, G., Sausville, E. A., Cloud, K., Lahusen, T., Varesio, L., and Senderowicz, A. M. Flavopiridol, a protein kinase inhibitor, down-regulates hypoxic induction of vascular endothelial growth factor expression in human monocytes. Cancer Res., *59:* 5433–5437, 1999.

6. Bible, K. C., and Kaufmann, S. H. Cytotoxic synergy between Flavopiridol (NSC 649890, L86-8275) and various antineoplastic agents: the importance of sequence of administration. Cancer Res., *57:* 3375–3380, 1997.

7. Motwani, M., Delohery, T. M., and Schwartz, G. K. Sequential dependent enhancement of caspase activation and apoptosis by flavopiridol on paclitaxel-treated human gastric and breast cancer cells. Clin. Cancer Res., *5:* 1876–1883, 1999.

8. Schwartz, G. K., Farsi, K., Maslak, P., Kelsen, D. P., and Spriggs, D. Potentiation of apoptosis by flavopiridol in mitomycin-C-treated gastric and breast cancer cells. Clin. Cancer Res., *3:* 1467–1472, 1997.

9. Motwani, M., Jung, C. P., Gonen, M., Sirotnak, F., and Schwartz, G. K. Augmentation of apoptosis and tumor regressions by flavopiridol in the presence of CPT-11 in HCT-116 colon cancer monolayers and xenografts. Clin. Cancer Res., *7:* 4209–4219, 2001.

10. Motwani, M., Rizzo, C., Sirotnak, F., She, Y., and Schwartz, G. K. Flavopiridol enhances the effect of docetaxel both *in vitro* and *in vivo* in human gastric cancer cells. Mol. Cancer Ther., *2:* 549–555, 2003.

11. Chien, M., Astumian, M., Liebowitz, D., Rinker-Schaeffer, C., and Stadler, W. M. *In vitro* evaluation of flavopiridol, a novel cell cycle inhibitor, in bladder cancer. Cancer Chemother. Pharmacol., *44:* 81–87, 1999.

12. Saltz, L. B., and Minsky, B. Adjuvant therapy of cancers of the colon and rectum. Surg. Clin. N. Am., *82:* 1035–1058, 2002.

13. Shah, M. Recent developments in the treatment of gastric carcinoma. Curr. Oncol. Rep., *4:* 193–201, 2002.

14. Bains, M. S., Stojadinovic, A., Minsky, B., Rusch, V., Turnbull, A., Korst, R., Ginsberg, R., Kelsen, D. P., and Ilson, D. H. A Phase II trial of preoperative combined-modality therapy for localized esophageal carcinoma: initial results. J. Thorac. Cardiovasc. Surg., *124:* 270–277, 2002.

15. Minsky, B. D., Pajak, T. F., Ginsberg, R. J., Pisansky, T. M., Martenson, J., Komaki, R., Okawara, G., Rosenthal, S. A., and Kelsen, D. P. INT 0123 (Radiation Therapy Oncology Group 94-05) Phase III trial of combined-modality therapy for esophageal cancer: high-dose *versus* standard-dose radiation therapy. J. Clin. Oncol., *20:* 1167–1174, 2002.

16. Greenberg, M. E. Identification of newly transcribed RNA. *In:* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (eds.), Current Protocols in Molecular Biology,pp. 4.10.1–4.10.11. New York: John Wiley & Sons, Inc., 1998.

17. Sirotnak, F. M., DeGraw, J. I., Colwell, W. T., and Piper, J. R. A new analogue of 10-deazaaminopterin with markedly enhanced curative effects against human tumor xenografts in mice. Cancer Chemother. Pharmacol., *42:* 313–318, 1998.

18. Pratt, J. W., and Gibbons, J. D. Concepts of Nonparametric Theory. NY: Springer-Verlag, 1981.

19. Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R. W. Participation of p53 protein in the cellular response to DNA damage. Cancer Res., *51:* 6304–6311, 1991.

20. Waldman, T., Zhang, Y., Dillehay, L., Yu, J., Kinzler, K., Vogelstein, B., and Williams, J. Cell cycle arrest *versus* cell death in cancer therapy. Nat. Med., *3:* 1034–1036, 1997.

21. Cayrol, C., and Ducommun, B. Interaction with cyclin-dependent kinases and PCNA modulates proteosome-dependent degradation of p21. Oncogene, *12:* 2437–2444, 1998.

22. Maki, C. G., and Howley, P. M. Ubiquitination of p53 and p21 is differentially affected by ionizing and UV radiation. Mol. Cell. Biol., *17:* 355–363, 1997.

23. Adams, J., Palombella, V. J., Sausville, E. A., Johnson, J., Destree, A., Lazarus, D. D., Maas, J., Pien, C. S., Prakash, S., and Elliott, P. J. Proteosome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res., *59:* 2615–2622, 1999.

24. Zhang, Y., Fujita, N., and Tsuruo, T. Caspase-mediated cleavage of p21Waf1/Cip1 converts cancer cells from growth arrest to undergoing apoptosis. Oncogene, *18:* 1131–1138, 1999.

25. Gervais, J. L. M., Seth, P., and Zhang, H. Cleavage of CDK inhibitor $p21^{WAF1/CIP1}$ by caspases is an early event during DNA damage-induced apoptosis. J. Biol. Chem., *273:* 19207–19212, 1998.

26. Stewart, Z. A., Mays, D., and Pietenpol, J. A. Defective G_1-S cell cycle checkpoint function sensitizes cells to microtubule inhibitor-induced apoptosis. Cancer Res., *59:* 3831–3837, 1999.

27. Wouters, B. G., Giaccia, A. J., Denko, N. C., and Martin-Brown, J. Loss of $p21^{WAF1/CIP1}$ sensitizes tumors to radiation by an apoptosis-independent mechanism. Cancer Res., *57:* 4703–4706, 1997.

28. Wang, Z., Van Tuyle, G., Conrad, D., Fisher, P. B., Dent, P., and Grant, S. Dysregulation of the cyclin-dependent kinase inhibitor p21WAF1/CIP1/MDA6 increases the susceptibility of human leukemia cells (U937) to 1-β-D-arabinofuranosylcytosine-mediated mitochondrial dysfunction and apoptosis. Cancer Res., *59:* 1259–1267, 1999.

29. Chao, S. H., and Price, D. H. Flavopiridol inactivates P-TEFb and blocks most RNA polymerase II transcription *in vivo*. J. Biol. Chem., *276:* 31793–31799, 2001.

30. Price, D. H. P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. Mol. Cell. Biol., *20:* 2629–2634, 2000.

31. Garcia-Barros, M., Paris, F., Cordon-Cardo, C., Lyden, D., Rafii, S., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R. Tumor response to radiotherapy regulated by endothelial cell apoptosis. Science (Wash. DC), *300:* 1155–1159, 2003.

32. Bernhard, E. J., McKenna, W. G., and Muschel, R. J. Radiosensitivity and the cell cycle. Cancer J. Sci. Am., *5:* 194–204, 1999.

33. Maity, A., Kao, G. D., Muschel, R. J., and McKenna, W. G. Potential molecular targets for manipulating the radiation response. Int. J. Radiat. Oncol. Biol. Phys, *37:* 639–653, 1997.

34. Raju, U., Nakata, E., Mason, K. A., Ang, K. K., and Milas, L. Flavopiridol, a cyclin-dependent kinase inhibitor, enhances radiosensitivity of ovarian carcinoma cells. Cancer Res., *63:* 3263–3267, 2003.

35. Kim, J., Shah, D., Cao, Q., and Hak, C. Enhancement of radiation effects by combined docetaxel and flavopiridol treatment in lung cancer cells. Proc. Am. Assoc. Cancer Res., *44:* 32, 2003.