Augmentation of Apoptosis and Tumor Regression by Flavopiridol in the Presence of CPT-11 in Hct116 Colon Cancer Monolayers and Xenografts

Monica Motwani, Christoph Jung, Francis M. Sirotnak, Yuhong She, Manish A. Shah, Mithat Gonen, and Gary K. Schwartz

Gastrointestinal Oncology Research Laboratory, Division of Solid Tumor Oncology, Department of Medicine [M. M., C. J., M. A. S., G. K. S.], Program of Molecular Pharmacology and Experimental Therapeutics [F. M. S., Y. S.], and Department of Epidemiology and Biostatistics [M. G.], Memorial Sloan Kettering Cancer Center, New York, New York 10021

ABSTRACT

CPT-11, a DNA topoisomerase I inhibitor, has demonstrated clinical activity in colorectal cancer. Flavopiridol, a cyclin-dependent kinase inhibitor, is rapidly emerging as a chemotherapy modulator. To enhance the therapeutic index of CPT-11 in colon cancer, we studied the combination of these two drugs in relatively resistant human colon cancer cells, Hct116. Exposure of parental Hct116 cells to clinically achievable concentrations of SN-38 (the active metabolite of CPT-11) induces p21 and a G2 arrest. However, these conditions fail to induce apoptosis. In contrast, Hct116 cells that are p21 deficient (p21−/− Hct116) readily undergo apoptosis after treatment with SN-38. In this study we show that the parental Hct116 cells can be sensitized to undergo apoptosis by the addition of flavopiridol after SN-38 treatment. The induction of apoptosis was greatest with sequential therapy consisting of SN-38 followed by flavopiridol. Clonogenic assays also showed greatest inhibition with this sequence. Sequential treatment with SN-38 followed by flavopiridol was associated with higher activation of caspase-3 and greater cleavage of both p21 and XIAP, an inhibitor of apoptosis, compared with other treatment schedules. CPT-11 induced some tumor regressions but no complete responses in the p21-intact Hct116 xenografts. CPT-11 with flavopiridol more than doubled tumor regression, compared with CPT-11 alone, and produced a 50% complete response rate. Our studies indicate that CPT-11 induces cell cycle arrest rather than cell death and that flavopiridol, by activating the caspase cascade, cleaves the inhibitors of apoptosis and sensitizes the cells to undergo cell death. Thus, flavopiridol combined with CPT-11 may provide a completely new therapeutic approach in the treatment of colon cancer.

INTRODUCTION

One of the major factors that can influence resistance to DNA-damaging agents is the balance between damage (or stress) and repair. In response to DNA damage, checkpoints are alerted in checkpoint-intact cells. This results in cell cycle arrest and initiation of DNA repair (1). However, if the damage (or stress) is beyond the capability of cell to repair, then apoptosis is induced (2, 3). Thus, a protective mechanism has been established by which checkpoint-intact cells initiate cell cycle arrest and induce a repair mechanism that protects the cell from the cytotoxic effects of DNA-damaging agents. In essence, this mechanism of self-repair prevents chemotherapy-induced apoptosis. We suspect that this plays a major role in the general failure of many conventional chemotherapeutic agents. Thus, the ability to reverse this process by agents that in themselves modulate the cell cycle would constitute a major advance in cancer therapy.

The camptothecin CPT-11 is a topoisomerase I inhibitor that induces DNA damage and transient S-phase arrest (4, 5). Increased sensitivity to this agent has been observed in cells with defective checkpoints that prevent the cells from arresting in G2 after drug exposure (6). Thus, the relative sensitivity of tumor cells to CPT-11 appears to depend, at least in part, on the absence or presence of intact checkpoints within the cell cycle.

It has been hypothesized that the damage by radiation or chemotherapy at physiological conditions does not induce sufficient stress to induce cell death. To enhance the therapeutic index, combinations of various drugs have been used in cancer therapy. For example, a synergy is observed when DC3F cells are treated with ionizing radiation and CPT-11 in a sequential manner (7). It has been suggested that “sublethal” DNA damage of CPT-11 is converted into lethal damage by radiation-induced DNA damage. A similar phenomenon has been observed when CPT-11 is combined with 5-fluorouracil (8). All of these studies have included the combination of conventional chemotherapeutic agents or radiation.

Flavopiridol, a CDK3 inhibitor, has been reported to bind to DNA and induce DNA damage (9). Furthermore, it has been
shown that the addition of flavopiridol to other “stress-inducing” agents promotes cytotoxicity and induces apoptosis (10, 11). Therefore, it stands to reason that in checkpoint-intact cells, the addition of flavopiridol to CPT-11 should produce a degree of damage that results in apoptosis rather than cell cycle arrest. In addition, we believe that any effect seen in vitro should also produce an enhanced antitumor effect in vivo that could not be achieved with CPT-11 or flavopiridol alone. Our studies indicated that the addition of flavopiridol to SN-38-treated Hct116 cells augments the induction of apoptosis and the inhibition of colony formation. Furthermore, the regression of established Hct116 xenografts obtained with CPT-11 was significantly increased with flavopiridol. Moreover, CRs were observed when CPT-11 and flavopiridol were given sequentially and separated by specific time intervals.

**MATERIALS AND METHODS**

**Cell Culture and Drug Treatments for Cell Lines.** The p21-intact and -deficient Hct116 human colon cancer cell lines were kindly provided by Dr. Bert Vogelstein (John Hopkins Oncology Center, Baltimore, MD). The cell lines were maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin at 37°C in 5% carbon dioxide. All cultures were tested as Mycoplasma free. The stock solutions of SN-38 (5 mM; supplied by Dr. Patrick McGovren, Pharmacia, Peapack, NJ), fmk-derivatized peptides of caspase-3 inhibitor z-DEVD (z-DEVD-fmk), and pan caspase inhibitor z-VAD (z-VAD-fmk; 50 mM; R&D Systems) were prepared in DMSO, whereas flavopiridol (4.5 mM; graciously supplied by Dr. Edward Sausville, National Cancer Institute, Bethesda, MD) and PS-341 (10 mM; graciously supplied by Louis Grenier, Millenium Pharmaceuticals, Inc., Cambridge, MA) were prepared in water. The stocks were stored at −80°C, and drugs were diluted in the media before use.

**Colony Formation Assays.** Hct116 cells (8 × 10^3) were plated, in duplicate, in 60-mm plates and incubated for 24 h to allow cells to adhere. Cells were treated with various doses of SN-38 and flavopiridol alone for 24 h. At the end of treatment, both floating and attached cells were collected by trypsinization and centrifuged for 5 min. Cells were plated in triplicate at the density of 1000 cells/100-mm plate containing 10 ml of drug-free medium and were allowed to grow for 10 days. The resulting colonies were stained with 0.01% crystal violet for 30 min. Control plates usually contained 300–350 colonies. Cells were also treated with various doses of SN-38 and a fixed flavopiridol dose in different schedules: concomitant treatment for 24 h; sequential treatment with SN-38 for 24 h followed by removal of the medium and the addition of medium containing flavopiridol for 24 h; or the two drugs given in reverse sequence. For sequential treatment, the floating cell were collected after treatment with the first drug and added back for treatment with the second drug. At the end of treatment, cells were trypsinized and plated to form colonies as above.

**Apoptosis Assays.** Measurement of apoptosis by quantitative fluorescence microscopy was performed as described previously (10). The appearance of oligonucleosomal DNA fragmentation characteristic of apoptosis was determined by agarose gel electrophoresis. The cells were treated as above and lysed with 2% SDS containing 10 μg/ml RNase A and incubated at 37°C for 2 h. NaCl (5 M) was added to a final concentration of 1 M, and cells were scraped and stored at 4°C for 2–24 h. The lysed cells were centrifuged for 30 min at 12,000 rpm. DNA unassociated with intact chromatin residing in the supernatant was extracted by phenol-chloroform and precipitated with ethanol. DNA (10 μg) was resolved by agarose gel electrophoresis in 1× Tris-acetate-EDTA for 1 h on 1.5% agarose gel impregnated with ethidium bromide.

**MPM-2/Propidium Iodide Bivariate Flow Cytometry.** Staining with MPM-2 antibody and propidium iodide was performed as described previously (10). In this method, MPM-2-positive (mitotic) cells show increased green fluorescence and are shifted above the baseline of the dot plot.

**Kinase Activity Assay.** Cyclin B1/cdc2 kinase assays were performed as described previously (10). In brief, cyclin B1 was immunoprecipitated from 200 μg of lysate prepared from cells treated with various treatment conditions, and the kinase assay was performed with histone H1 as substrate and [γ-32P]ATP as a phosphate donor. The activity levels on autoradiographs were quantified by a densitometric scanning system.

**Western Analysis.** Western blots were prepared as described previously (10). The membranes were probed with p53, p21 (Santa Cruz Biotechnology), PARP, and caspase-3 (PharMingen), and XIAP (Transduction Laboratories). The primary antibodies were detected with horseradish peroxidase secondary antibody (Amersham Life Sciences) and subjected to ECL Chemiluminescence reagents (DuPont NEN Life Science Products, Boston, MA). The levels of expression were quantified by a densitometric scanning system.

**Immunoprecipitation of Cyclin/CDK Complexes.** Cyclin B1 and E immunoprecipitation was performed as described previously in kinase assays (10). The beads were washed three times with lysis buffer and separated by SDS-PAGE, and Western analysis of bound proteins was performed as described above.

**RNA Extraction and Northern Blot Analysis.** Total RNA was extracted from cells treated with different schedules of SN-38 and flavopiridol by a described previously cesium chloride method (12). Twenty μg of total RNA were electrophoresed on a 1% agarose-phosphate buffer gel and blotted onto Hybond-N nylon membranes (Amersham); the RNA was then cross-linked by UV Stratalinker (Stratagene). The membranes were hybridized with 32P-labeled p21 full-length cDNA probe (a gift from Dr. David Beach, Howard Hughes Medical Institute, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY) in Expresshyb hybridization solution (Clontech, Palo Alto, CA). The probe was labeled previously by random priming [32P]dCTP incorporation using a random-prime labeling kit (Amersham). The probe was purified by passing through Sephadex Quick Spin column (Boehringer Mannheim).

**Nuclear Run-On Assays.** Nuclei were obtained, and assays were performed by a modification of previously described procedures (12). Briefly, 5 × 10^7 cells from each treatment condition were lysed in 2 ml of NP40 buffer [10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl2, 0.5% NP40], and nuclei were harvested, resuspended in 110 μl of glycerol storage buffer [40% glycerol, 10 mM Tris (pH 7.4), 80 mM KCl, 5 mM MgCl2, 0.1 mM EDTA], and stored at −80°C. Forty μl of nuclear run-on
animals with CR divided by the total number of animals treated, counted as CR. The percentage of CR was the number of showed no microscopic evidence of residual disease were treatment and mice with small residual tumor nodules that difference between baseline and final tumor volume to the tumor regression was calculated as the percentage ratio of the volumes were calculated by the formula: $\frac{4}{3} \pi r^3$, where $r = \frac{\text{larger diameter} + \text{smaller diameter}}{4}$. The percentage of CR was the number of animals with CR divided by the total number of animals treated, multiplied by 100. These studies were performed in accordance with NIH guidelines (15).

**Biostatistical Analysis.** All *in vitro* experiments were done in duplicate and were repeated at least three times unless otherwise indicated. The statistical significance of the experimental results was determined by the two-sided $t$ test. For *in vivo* studies, the area under the time-volume curve was used as a summary measure for each mouse. This area was calculated using the trapezoidal rule. This method takes the longitudinal aspect of the data into account and does not require assumptions of linear growth (or decay), which are clearly violated for our data. Treatment groups were compared in pairs, using the exact permutation distribution of the areas under the curve (16).

**RESULTS**

**G2 Checkpoint-defective Cells Exhibit Increased Sensitivity to SN-38.** It has been shown that p21-deficient Hct116 cells have defective G1-S and G2-M checkpoint functions (17). We studied the sensitivity of parental and p21-deficient Hct116 cells to SN-38. The cells were treated with SN-38 (concentrations of 20–160 nM) for 24 h or SN-38 for 24 h followed by 24 h in drug-free medium. As shown in Fig. 1, the p21-intact cell line did not show PARP cleavage, an indicator of caspase activation and apoptosis, at the highest concentration tested (160 nM). In contrast, p21-deficient Hct116 cells, under identical treatment conditions, showed PARP cleavage at SN-38 concentrations as low as 20 nM. This was confirmed by staining of the SN-38 treated parental and p21-deficient cells with 4',6-diamidino-2-phenylindole, which stains nuclear chromatin. Only the p21-deficient cells showed condensation of nuclei, a hallmark of apoptosis, with SN-38. Other cell lines, including the colon and breast cancer cell lines HT-29 and MDA-MB-468, respectively, which are p53 defective and thus have defective G1-S and G2-M checkpoints, were also sensitive to SN-38, as indicated by PARP cleavage and caspase-3 activation at SN-38 concentrations as low as 20 nM (data not shown).

**SN-38 Induces Cell Cycle Arrest at G2 in Hct116 Cells.** We examined cell cycle changes after SN-38 treatment in p21-intact and -deficient Hct116 cells. Our results were similar to those obtained by various other investigators with these cell lines in response to DNA-damaging agents, including radiation and etoposide (18, 19). In particular, SN-38 at 20 nM induced transient S-phase arrest in both cell lines. The S-phase population...
tion increased from 40% to 60% after 15 h of treatment with SN-38 in both cell types. After 24 h of treatment with SN-38, the cells accumulated in the S and G2 phases, and a significant decrease in the G1 population was observed (Fig. 2A). Twenty-four h after the removal of SN-38 (SN24→ND24), both cell types accumulated in G2-M with 4n DNA content. At this stage, a significant difference was observed between the two cell lines. As shown in Fig. 2A, p21-intact cells with 4n DNA showed <1% MPM-2 labeling, indicating that cells with 4n DNA content were arrested in G2. This G2 arrest was further confirmed by a dramatic decrease in cyclin B1/cdc2 kinase activity in the p21+/+ cells treated with SN-38 followed by no drug (Fig. 2B), again indicating G2 arrest. Although p21-deficient cells with 4n DNA also accumulated after treatment with SN-38 followed by no drug, these cells, in contrast, showed sustained activation of cyclin B1/cdc2 kinase activity (Fig. 2B). The MPM-2 labeling of asynchronous p21−/− Hct116 cells after treatment with SN-38 followed by no drug also showed an increase in cells in mitosis (5%; Fig. 2A).

**Flavopiridol Sensitizes SN-38-treated Cells and Induces Apoptosis.** We examined the combination of flavopiridol and SN-38 administered both concurrently and sequentially in the relatively SN-38-resistant p21-intact Hct116 cells. The cells were treated with 20 nm SN-38 and 150 nm flavopiridol in four separate schedules: individually, concurrently and sequentially with SN-38 for 24 h followed by flavopiridol for 24 h, or the same drugs given in reverse sequence. SN-38 for 24 h (SN24) or SN-38 for 24 h followed by no drug for 24 h (SN24→ND24) resulted in only 1% apoptosis. Flavopiridol for 24 h (F24) induced 12 ± 1% apoptosis. Sequential treatment of Hct116 cells with SN-38 for 24 h followed by flavopiridol for 24 h (SN24→F24) showed the highest induction of apoptosis (43 ± 0.5%; SN24→F24 versus SN24→ND24, P < 0.005). The pre-treatment of cells with flavopiridol (F24→SN24) resulted in only an additive effect (15 ± 2%) compared with SN-38 or flavopiridol alone. Concomitant therapy [(SN+F)24] induced apoptosis in 30 ± 2% of the treated cells. The percentage of induction of apoptosis with SN24→F24 was significantly greater than that obtained with either the reverse sequence (SN24→F24 versus F24→SN24, P < 0.001) or concomitant therapy [SN24→F24 versus (SN+F)24, P < 0.05].

Gel electrophoretic analysis of DNA obtained from cells treated with various schedules of SN-38 and flavopiridol revealed more intense oligonucleosomal bands in cells treated with SN24→F24 compared with those treated with other drug schedules (Fig. 3A). In keeping with these observations, higher activation of caspase-3 (i.e., formation of 24-, 20-, and 17-kDa forms) was observed in cells treated with SN24→F24 compared with those with other treatment schedules (Fig. 3B). Cells treated with a combination of SN-38 and flavopiridol together for 24 h [(SN+F)24] also showed significant activation of caspase-3, albeit to a lesser degree, when compared with cells treated with SN24→F24. PARP, the 116-kDa caspase-3 substrate, was completely degraded into its 90-kDa cleaved product in cells treated sequentially with SN-38 followed by flavopiridol, further supporting the greater induction of apoptosis in this treatment schedule. In general, the trend was SN24→F24 > (SN+F)24 > F24 for the percentage of induction of apoptosis, caspase-3 activation, and PARP cleavage.

We also studied the effect of SN-38 and flavopiridol on the ability of p21-intact Hct116 to form colonies. The colony formation in these cells was markedly inhibited by SN-38. Inhibition in colony formation by 60 and 98% was observed with 5 and 20 nm SN-38, respectively (Fig. 4). Exposure of cells to up to 75 nm flavopiridol alone did not inhibit colony formation. Increasing the concentration of flavopiridol to 150 and 300 nm inhibited colony formation by 50 and 95%, respectively (Fig. 4, inset). Cells treated sequentially with SN-38 followed by flavopiridol showed higher inhibition of colony formation at all SN-38 doses compared with SN-38 alone. Interestingly, the concurrent and reverse combination of flavopiridol followed by SN-38 showed a trend toward increased inhibition in colony formation at lower doses of SN-38, compared with single-agent
SN-38, and antagonism at higher SN-38 doses, as indicated by decreased inhibition of colony formation compared with SN-38 alone (Fig. 4, H11569). The significance of this remains unclear. Inspection of the plate for the colony assays indicated that SN-38 treated cells did not die but remained attached to the plate as permanently arrested single cells. The SN24F 24 treatment induced cell death, and cells detached from the plate. Clono-
genic assays with p21-deficient cells also showed no colony formation, and these cells completely detached from the plate with SN-38 alone (data not shown).

Molecular Regulators Associated with Enhanced Induction of Apoptosis with Sequential Treatment of SN-38 and Flavopiridol. We analyzed the molecular events of cell cycle regulation and apoptosis during SN-38 and flavopiridol treatment. The protein expression of bcl2, bax, bad, bcl-xL, and bag was unchanged in cells treated with SN-38 followed by no drug or flavopiridol (data not shown). The inhibitor of apoptosis, XIAP, inhibits cell death via direct inhibition of caspases, including caspase-9, -7, and -3 (20–22). As shown in Fig. 5, full-length XIAP was detected as a 57-kDa protein in control untreated cells (ND24). After treatment with flavopiridol (F24) or a combination of SN-38 plus flavopiridol ([SN+F]24 or SN24→F24), the levels of full-length XIAP (57 kDa) were reduced by 20–30% (Fig. 5A, shorter film exposure), and a 29-kDa fragment was detected (Fig. 5B, longer film exposure).
Flavopiridol Potentiates CPT-11-induced Apoptosis

The intensity of the cleaved product was 4-fold higher in SN24F24-treated cells compared with cells treated with flavopiridol alone, suggesting higher cleavage of XIAP.

Because p21 plays an important role in cell cycle arrest and affects the sensitivity of cells to cytotoxic agents (17, 18, 23, 24), we elected to examine the protein expression of p21 in different treatment schedules in Hct116 cells. p21 is transcriptionally regulated by p53 and p21 binds to cdc2 and inhibits the kinase activity (26), we compared with untreated cells. The addition of flavopiridol to SN-38 followed by either no drug or flavopiridol. Kinase activity was measured as in the experiment shown in Fig. 2. The experiment was repeated a minimum of three times, and representative data from one experiment are presented.

Because treatment with SN-38 followed by no drug resulted in inhibition of cyclin B1/cdc2 kinase activity (Fig. 2B) and p21 binds to cdc2 and inhibits the kinase activity (26), we analyzed the binding of p21 to cyclin B1/cdc2 in cells treated with different schedules of SN-38 and flavopiridol. The cyclin B1-associated complexes were immunoprecipitated, and Western blot analysis was performed to evaluate coimmunoprecipitated p21. As shown in Fig. 6B, a strong signal for p21 was observed in SN38ND24-treated cells. The binding of p21 to cyclin B1/cdc2 negatively regulated kinase activity, as shown by the decreased kinase activity of this complex in SN38ND24-treated cells (Fig. 6B). Flavopiridol treatment for 24 h resulted in a small decrease in kinase activity of this complex. The sequential treatment SN38ND24, which was associated with suppression of p21 expression, also inhibited kinase activity, presumably because of direct binding of flavopiridol to cdc2.

Flavopiridol Increases the Caspase-mediated Cleavage of p21 in Sequential Therapy. To further understand the decrease in p21 protein expression in cells treated sequentially with SN-38 followed by flavopiridol, we studied the transcriptional and posttranslational changes in p21 expression in cells treated sequentially with SN-38 followed by flavopiridol.

Fig. 6 p53 and p21 are induced after SN-38 treatment, and p21 (or flavopiridol) binds to cyclin B1/cdc2 complex and inhibits kinase activity. p21-intact Hct116 cells were treated with SN-38 and flavopiridol as described in the legend for Fig. 5, and protein was harvested. A, SDS-PAGE and Western blotting of p53 and p21. Fifty µg of protein were resolved on 15% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were probed with mouse monoclonal p53 or p21 antibodies. The equal loading of protein was verified initially by Amido black staining and later by tubulin expression. B, cyclin B1/cdc2 kinase activity is inhibited after both SN-38 followed by either no drug or flavopiridol. Kinase activity was measured as in the experiment shown in Fig. 2. The experiment was repeated a minimum of three times, and representative data from one experiment are presented.

Fig. 7 Transcriptional regulation of p21 in SN-38- and flavopiridol-treated p21-intact Hct116 cells. A, Northern blot analysis of p21. Cells were treated with SN-38 and flavopiridol as described in the legend for Fig. 5, and total RNA was isolated as described in “Materials and Methods.” Twenty µg of total RNA were denatured and separated on a 1% agarose gel. The RNA was transferred to a nylon membrane and was probed with 32P-labeled p21 cDNA. Staining of the gel with ethidium bromide showed equal loading. B, analysis of p21 transcription by nuclear run-on assay. Cells were treated with SN-38 and flavopiridol as described in the legend for Fig. 5, and nuclear run-on assay was performed as described in “Materials and Methods.” GAPDH was used as control gene for each treatment condition.
Previous studies have indicated that p21 can be degraded by the proteasome-ubiquitination pathway (27, 28) or cleaved by caspases, particularly by caspase-3 (29, 30). To investigate whether flavopiridol activated the ubiquitin-proteasome pathway for p21 degradation, p21-intact Hct116 cells were treated with the different schedules of SN-38 and flavopiridol in the presence or absence of 20 nM PS-341, a proteasome inhibitor (31). As shown in Fig. 8A, PS-341 stabilized the p21 protein by preventing its degradation, and treatment of cells with PS-341 for 24 h [(PS)24] resulted in an increase in p21 protein levels compared with untreated control (ND24). Addition of PS-341 together with flavopiridol to SN-38-treated cells [SN24→(F+PS)24] prevented degradation of the p21 protein, and increased p21 protein levels were observed compared with SN24→F24-treated cells. However, the addition of PS-341 to SN-38-treated cells (SN24→PS24) also resulted in a similar increase in p21 protein levels (compare SN24→ND24 and SN24→PS24 in Fig. 8A). Because PS-341 also induces apoptosis and PARP cleavage in this cell line, we were unable to show reversal of apoptosis in cells treated with PS-341 in combination with SN-38 followed by flavopiridol, despite the restoration of p21 (data not shown).

We next treated the cells with SN-38 and flavopiridol in the presence of 20 μM each of the caspase-3 inhibitor z-DEVD-fmk or the pan caspase inhibitor z-VAD-fmk. As shown in Fig. 8B, the addition of z-DEVD-fmk or z-VAD-fmk with flavopiridol to SN-38-treated cells [SN24→(F+Z-DEVD)24 and SN24→(F+z-VAD)24] restored the protein expression of p21. z-VAD-fmk alone or z-VAD-fmk added to SN-38-treated cells (z-VAD24 and SN24→z-VAD24, respectively) did not change the levels of p21, when compared, respectively, with ND24 and SN24→ND24. More than 95% of PARP cleavage with SN-38 followed by flavopiridol was reversed by z-DEVD-fmk, indicating that PARP is preferentially cleaved by caspase-3 (Fig. 8C). The addition of z-DEVD-fmk also resulted in the disappearance of the 20- and 17-kDa cleaved fragments and the accumulation of the 24-kDa fragment of caspase-3. The pan caspase inhibitor z-VAD-fmk reversed PARP cleavage and caspase-3 activation (e.g., disappearance of 24-, 20-, and 17-kDa fragments) during sequential therapy with SN-38 followed by flavopiridol [SN24→(F+z-VAD)24; Fig. 8C].

Sequential Therapy with CPT-11 and Flavopiridol Augments Tumor Regression and CR in Xenografts. The clonogenic and apoptosis assays indicated that sequential treatment with SN-38 followed by flavopiridol achieved the best results. To investigate whether these in vitro observations are also reflected in vivo and to optimize the time interval between the two drugs, we established p21-intact Hct116 cells as xenografts in nude mice. Mice were treated with either each drug alone or sequentially with CPT-11 followed by flavopiridol at 4-, 7-, and 16-h intervals. As shown in Table 1 and Fig. 9, the interval between CPT-11 and flavopiridol was an important determinant of the percentage of tumor regressions in xenografts. The greatest tumor regression (the percentage of decrease in tumor volume) was observed when the interval between CPT-11 and flavopiridol was at least 7–16 h. Two weeks after the end of treatment (day 30), there was a 40 ± 25% regression of tumors in mice treated with CPT-11 alone, whereas in mice treated with CPT-11 followed by flavopiridol 7 and 16 h later, tumor regression was 86 ± 9% and 82 ± 5%, respectively. There was no statistically significant difference in response in mice treated with CPT-11 alone and CPT-11 followed by flavopiridol after 4 h. If the interval between CPT-11 and flavopiridol was increased to 24 h, the response rate was similar to that for CPT-11 alone (data not shown). Two of 10 mice and 1 of 7 mice treated with CPT-11 followed by flavopiridol...
Flavopiridol Potentiates CPT-11-induced Apoptosis

CPT-11 followed by flavopiridol was observed for at least 4–7, or 16 h and measured as described in “Materials and Methods.”

Table 1 Tumor regression and CR in mouse xenografts treated with CPT-11 and flavopiridol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor regression (day 30)</th>
<th>CR, % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPT-11</td>
<td>40 ± 25</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>CPT-11&lt;sup&gt;a&lt;/sup&gt;F</td>
<td>60 ± 17</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>CPT-11&lt;sup&gt;b&lt;/sup&gt;F</td>
<td>86 ± 9</td>
<td>30% (3/10)</td>
</tr>
<tr>
<td>CPT-11&lt;sup&gt;c&lt;/sup&gt;F</td>
<td>82 ± 5</td>
<td>29% (2/7)</td>
</tr>
</tbody>
</table>

<sup>a</sup>The tumor regression was calculated as ratio of difference between baseline and final tumor volume to the baseline volume.

<sup>b</sup>The CR is defined as the number of animals without tumors relative to the total number of animals in the treatment group.

Flavopiridol at 7- and 16-h intervals, respectively, had no palpable tumors. We also examined the histology of any residual tissue mass remaining at the end of the treatment in different treatment groups. Despite the presence of a small palpable mass, one animal from each treatment group of CPT-11 followed by flavopiridol at 7 and 16 h showed no evidence of any tumor cells, indicating the complete pathological absence of tumor in these mice (Fig. 10). Thus, the CR rates for CPT-11 followed by flavopiridol at 7 and 16 h were 30% (3 of 10) and 29% (2 of 7), respectively. This was in contrast to CPT-11 alone and CPT-11 followed by flavopiridol at 4 h, where no CRs were found (Table 1).

The tumor growth in mice treated with CPT-11 alone or CPT-11 followed by flavopiridol was observed for at least 4–5 weeks after completion of treatment (i.e., 6–7 weeks after the beginning of treatment). The growth kinetics of tumors treated with different schedules is shown in Fig. 9. Mice that received only flavopiridol showed an increase in tumor growth that was similar to that in untreated controls. After CPT-11 treatment, the tumor growth was decreased, but administration of flavopiridol after CPT-11 caused increased tumor shrinkage. The maximum tumor shrinkage, in all treatment schedules, was observed between 29 and 32 days after the start of treatment. The differences between the areas under the volume-time curves of CPT-11 alone and CPT-11 followed by flavopiridol at 7 and 16 h were statistically significant ($P = 0.0002$ and 0.0005, respectively).

Mice that were treated with only CPT-11 lost an average of 10 ± 1% of body weight, whereas mice treated with CPT-11 followed by flavopiridol at 7 and 16 h lost an average of 15 ± 2% and 11 ± 1% of body weight, respectively, suggesting that the 7-h interval was more toxic. Nevertheless, the weight loss by mice treated with CPT-11 alone and with CPT-11 followed by flavopiridol at 16 h was essentially identical. This would indicate that the difference in weight loss could not explain the significant difference in response rates for these treatment conditions. Furthermore, all mice gained weight after treatment, and the difference in the weight loss 2 weeks after completion of treatment was not different among all treatment conditions, indicating that the toxicity with the combination was temporary and within acceptable limits. None of the mice died as a result of the toxicity of either single-agent or combination therapy during the course of the experiment.

DISCUSSION

In this study we show that as a single agent in Hct116 cells in vitro, SN-38 induces cell cycle arrest without cell death. This correlates to the absence of CRs observed in vivo with CPT-11 alone. The addition of flavopiridol to SN-38-treated Hct116 cells caused cell death in vitro, and in vivo this translated into greater tumor regression as well as CRs. With clonogenic assays, we observed that SN-38-treated cells remained attached to the plate as viable single cells. These cells did not form colonies because cell cycle arrest by SN-38, and this resulted in the inhibition of colony formation. However, SN-38 alone did not induce apoptosis; therefore, we did not observe PARP cleavage or caspase-3 activation. In contrast, flavopiridol added to SN-38-treated Hct116 cells induced significant apoptosis as indicated by quantitative fluorescence microscopy assays, PARP cleavage, and caspase-3 activation. We observed neither single viable cells nor colonies by clonogenic assays. In essence, flavopiridol converted the cytostatic effect of SN-38 into a cytotoxic effect such that the cells arrested in G2 by SN-38, and then underwent apoptosis. We believe this conversion from cell cycle arrest to cell death could account for the increase in tumor regression and CRs observed with the CPT-11-flavopiridol combination. There is certainly precedent for this concept. A previous report by Waldman et al. (18) indicated that cell cycle arrest by DNA-damaging agents results in single cells during clonogenic assays but that this does not result in CRs in tumor xenografts. However, the induction of apoptosis (which they report similarly as detachment of the Hct116 cells from the plate...
Thus, the presence of p21 (and p53) exerts a stronger G2 arrest, transcriptionally up-regulated after SN-38 treatment. The decreased cleavage of XIAP and p21 proteins. XIAP is an inhibitor of caspases and can be cleaved by caspases during apoptosis, thus rendering cells more sensitive to cell death (21, 22). p21 is of caspases and can be cleaved by caspases during apoptosis, thus rendering cells more sensitive to cell death (21, 22). p21 is selectively cleaved during the combination of SN-38 and flavopiridol can be explained. Our results with CPT-11 alone and CPT-11 followed by flavopiridol after DNA damage response may cause apoptosis (17–19). It has been shown that caspase-mediated cleavage of p21 into p14 is crucial in G-Rh2-induced apoptosis in SK-HEP-1 cells (37). Whether cleavage of p21 in sequential treatment with SN-38 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 is unclear. Nevertheless, these studies do suggest a dual role for p21: G2 arrest and inhibition of apoptosis. Thus, p21 may serve as a critical checkpoint target for both cell cycle arrest and apoptosis during treatment with SN-38 alone or SN-38 followed by flavopiridol, respectively. Manipulation of p21 expression by flavopiridol after DNA damage response may thus provide a novel strategy for cancer therapy. Additional studies to define the role of p21 in the...
induction of apoptosis with SN-38 followed by flavopiridol are under way in our laboratory.

Because SN-38 followed by flavopiridol showed higher inhibition of colony formation and more than an additive effect when compared with single agents in apoptosis assays, we elected to test the sequential combination of CPT-11 followed by flavopiridol in vivo. It is intriguing that the timing of the administration of flavopiridol after CPT-11 had such a significant effect on tumor regression and CR. CPT-11 causes transient accumulation of cells in S-phase, after which cells exit and arrest at the G2 phase of the cell cycle. Flavopiridol is a CDK inhibitor. It is possible that administration of flavopiridol too soon after CPT-11 will block cells in G1 and prevent the cells from entering S-phase. This would then prevent the enhancement of the CPT-11 effect observed with the combination treatment. In vitro, when cells were treated with SN-38 for shorter periods of time (4–7 h) and then were exposed to flavopiridol for an additional 24 h, the induction of apoptosis was lower than the levels of induction observed when cells were treated with SN-38 for 24 h followed by flavopiridol for 24 h (data not shown). In contrast, in vivo, if flavopiridol was administered too late (i.e., 24 h after CPT-11), then the percentage of tumor regression was decreased relative to that observed with CPT-11 alone. However, the in vitro studies were carried out in monolayers, and treatment of three-dimensional in vivo tumor masses may yield completely different results. This could explain the difference in the response to the timing of flavopiridol administration after CPT-11 in both the in vitro and in vivo studies.

The introduction of CPT-11 into the armamentarium of cancer drugs has lead to significant improvements in the treatment of patients with colon cancer. However, in the metastatic setting, responses to CPT-11 remain only 30% (38). As we improve on our understanding of the molecular basis for the response to camptothecin, we should be able to considerably improve on the therapeutic index. This study and others have shown that cells with defective G2-M checkpoints are more sensitive to camptothecin. However, the challenge ahead is for the checkpoint-intact, camptothecin-resistant tumors, as represented by Hct116 parental cells. To make this agent more attractive in cancer treatment, combining it with chemotherapeutic modulators, such as flavopiridol, appears to be a logical choice. The addition of flavopiridol after SN-38 treatment appears to be necessary to activate the caspase cascade and induce apoptosis. Furthermore, we show for the first time that flavopiridol can enhance the effects of a DNA-damaging agent (i.e., CPT-11) in a corresponding in vivo model. Therefore, flavopiridol represents a new agent for manipulating cellular mechanisms of apoptosis to shift the balance from cell cycle arrest and DNA repair to irreparable DNA damage and apoptosis. On the basis of these studies, a Phase I clinical trial of sequential CPT-11 followed by flavopiridol is now underway.

ACKNOWLEDGMENTS

We thank Dr. David S. Klimstra (Department of Pathology, Memorial Sloan Kettering Cancer Center) for histological evaluation of tumors from xenografts.

REFERENCES

IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J., 17: 2215–2223, 1998.


Augmentation of Apoptosis and Tumor Regression by Flavopiridol in the Presence of CPT-11 in Hct116 Colon Cancer Monolayers and Xenografts

Monica Motwani, Christoph Jung, Francis M. Sirotnak, et al.