Full-length article

Efficacy of sequential treatment of HCT116 colon cancer monolayers and xenografts with docetaxel, flavopiridol, and 5-fluorouracil[1]

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Key words

Abstract

Aim: Clinical treatment of solid tumors with docetaxel, flavopiridol, or 5-fluorouracil (5-FU) often encounters undesirable side effects and drug resistance. This study aims to evaluate the potential role of combination therapy with docetaxel, flavopiridol, or 5-FU in modulating chemosensitivity and better understand how they might be used clinically. **Methods**: HCT116 colon cancer cells were treated with docetaxel, flavopiridol, and 5-FU in several different administrative schedules *in vitro*, either sequentially or simultaneously. Cell survival was measured by MTT assay. The activity of caspase-3 was determined by caspase-3 assays and the soft agar colony assay was used to test the colony formation of HCT116 cells in soft agar. We also established xenograft models to extend *in vitro* observations to an *in vivo* system. **Results**: The maximum cytotoxicity was found when human colon cancer HCT116 cells were treated with docetaxel for 1 h followed by flavopiridol for 24 h and 5-FU for another 24 h. This sequential combination therapy not only inhibits tumor cell growth more strongly compared to other combination therapies but also significantly reduces colony formation in soft agar and augments apoptosis of HCT116 cells. Sequencing of docetaxel followed 1 h later by flavopiridol, followed 24 h later by 5-FU in xenograft models, also resulted in delayed tumor growth and higher survival rate. **Conclusion**: These results highlight the importance of an administrative schedule when combining docetaxel with flavopiridol and 5-FU, providing a rationale explanation for its development in clinical trials.

Introduction

Docetaxel, which inhibits microtubule depolymerization, is a chemotherapeutic agent that inhibits tumor reassembly and has been reported to induce apoptosis and inhibit cell proliferation[1]. Presently, docetaxel has been exploited in the treatment of solid tumors, both as a single agent and combined with other chemotherapy agents[1–3]. However as a single agent, docetaxel induces response rates of 16%–24%. When combined with other chemo-drugs, such as 5-FU, it has produced response rates of 33%–56%[4]. Therefore, investigators have examined combining docetaxel with other chemotherapeutic agents to achieve better response and cure

rates[5], both in preclinical and clinical models[6–9].

Flavopiridol, the first inhibitor of multiple cyclin-dependent kinases to enter clinical trials[10–12], inhibits cell growth[13] and induces apoptosis in a variety of human cancer cell lines *in vitro* and *in vivo*[14–17]. Flavopiridol also induces cell-cycle arrest and tumor regression in the clinical settings[18], and displays sequence-dependent cytotoxic synergy with docetaxel in gastric cancer preclinical models[19,20].

Studies also suggested the feasibility of synergistic interaction between flavopiridol and 5-FU in breast cancer[21,22]. 5-FU induces apoptosis and causes cell injury of several cancer cell lines. It acts by blocking the enzyme thymidylate synthase and inhibiting the synthesis of both RNA and DNA.

5-FU is used for the treatment of cancers, but many tumors are resistant to this chemotherapeutic agent[23]. Efforts to improve response rates have led to the combination of 5-FU with other chemotherapeutic drugs such as CPT-11 and oxaliplatin to improve response rate and prolong survival[24]. Despite these improvements, more than half of patients undergo chemotherapy for advanced colon cancer without any measurable shrinkage of their disease**.** Thus, further opportunities to enhance the effectiveness of 5-FU in colon cancer are being investigated.

In mammalian models, the sequential combination of docetaxel and 5-FU has shown synergistic cytotoxicity[19], and synergistic interaction between flavopiridol and 5-FU has also been observed[21,22]. Sequential treatment of solid tumors with docetaxel, flavopiridol and 5-FU appears an attractive consideration. The purpose of this study was to define the most effective sequential treatment scheme with docetaxel, flavopiridol and 5-FU in the human colon cancer monolayers and xenografts in the hope that the present findings provide useful information for the establishment of clinical protocols.

Materials and methods

Cell culture and drug treatments for cell line The colon cancer adenocarcinoma cell line HCT116 was obtained from the American Tissue Culture Collection (Manassas, VA, USA). These cells were propagated and maintained in DMEM (Gibco BRL, Rockville, MD, USA) and supplemented with 10% fetal bovine serum (Gibco BRL), 1% penicillin/streptomycin and 1% L-glutamine. The cells were maintained with 5% CO_2. Docetaxel and flavopiridol were supplied by Aventis Pharmaceuticals (Bridgewater, NJ, USA) and 5-FU was purchased by INC Pharmaceuticals (Costa-mesa, CA, USA). All chemotherapy agents were diluted in DMEM (Dulbecco's Modified Eagle Medium) before use.

MTT assay Five thousand HCT116 cells were seeded in a 96-well plate. Twenty-four hours later cells were treated with: (1) no drug as control; (2) docetaxel for 1 h; (3) flavopiridol for 24 h; (4) 5-FU for 24 h; (5) docetaxel for 1 h followed by flavopiridol for 24 h; (6) docetaxel for 1 h followed by 5-FU for 24 h; (7) flavopiridol for 24 h followed by docetaxel for 1 h; (8) 5-FU for 24 h followed by docetaxel for 1 h; (9) flavopiridol for 24 h followed by 5-FU for another 24 h; (10) 5-FU for 24 h followed by flavopiridol for another 24 h; (11) docetaxel for 1 h followed by flavopiridol for 24 h and 5-FU for another 24 h; (12) docetaxel for 1 h followed by 5-FU for 24 h and flavopiridol for an additional 24 h; (13) flavopiridol for 24 h followed by 5-FU for 24 h and docetaxel for 1 more hour; (14) 5-FU for 24 h followed by flavopiridol

for 24 h and docetaxel for 1 more hour; and (15) docetaxel, flavopirdol and 5-FU simultaneously for 24 h. After each drug treatment cells were washed twice with drug-free medium. The doses of docetaxel, flavopiridol and 5-FU were 100 nmol/L, 300 nmol/L, and 20 µmol/L, respectively. As soon as drug treatment was completed 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide(MTT, 5 mg/mL in PBS) was added to each well, the plate was incubated for 4 h at 37 °C and 100 µL of 0.04 mol/L HCl in isopropanol was added. Within an hour the absorbance was measured on an ELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm. The percent of surviving cells at each concentration relative to the non-treated group was plotted.

Caspase-3 assays Induction of apoptosis was assessed by detecting caspase activity in cell lysates after drug treatment. The activity of DEVD (Asp-Glu-Val-Asp) specific proteases was measured using an EnzCheck Caspase-3 Assay Kit (Molecular Probes, Eugene, OR) as suggested by the manufacturer. The cells were treated with different combinations of the drugs as follows: (1) no drug; (2) docetaxel for 1 h; (3) flavopiridol for 24 h; (4) 5-FU for 24 h; (5) docetaxel for 1 h followed by flavopiridol for 24 h; (6) docetaxel for 1 h followed by 5-FU for 24 h; (7) flavopiridol for 24 h followed by 5-FU for 24 h; (8) docetaxel for 1 h followed by flavopiridol for 24 h and 5-FU for an additional 24 h; (9)docetaxel, flavopiridol and 5-FU administered at the same time for 24 h. After drug treatment cells were frozen at -80 °C until the time of assay. The frozen cells were resuspended in 50 µL lysis buffer, incubated on ice for 30 min and the cellular debris was pelleted. Lysates (50 µL) were transferred to 96-well plates and incubated at room temperature for 30 min with substrate (Z-DEVD-AMC) in 2× reaction buffer at a 100 µmol/L final concentration. Cell lysates alone with the caspase inhibitor Ac-DEVD-CHO at a 100 µmol/L final concentration was also included. Fluorescence was measured by a fluorometer at an excitation wavelength of 350 nm and a detection wavelength of 450 nm.

Soft agar colony assays The cells were treated with the same drug sequencing schedule as described with the caspase-3 assays. After drug treatment the cells were mixed with tissue culture media containing 0.6% agar to result in a final agar concentration of 0.4%. Cell suspension 1 mL was immediately plated in 6-well plates coated with 1 mL/well of 0.6% agar in tissue culture media. The colonies were counted in triplicate on d 15 after plating and the number of colonies/ 3×10^4 cells was calculated.

Murine xenograft model Institutional guidelines and an Animal Research Committee (ARC)-approved protocol were

followed for mouse studies. For these studies, 4–6 week old female nude[nu/nu] mice were obtained from Charles River (Wilmington, MA,) and housed in clean specific pathogen free (SPF) rooms in groups of 5 and in cages containing micro-isolator tops. HCT116 cells were harvested, washed twice in ice-cold serum free DMEM media, and counted for viable cells by trypan blue exclusion. The cells were resuspended in the same medium and 2.5×10^6 cells in a volume of 0.25 mL were injected subcutaneously into the right flank of each mouse. Drug treatment was started on the seventh day after tumor inoculation by intraperitoneal (ip) injection. Mice received: (1) docetaxel alone (5 mg/kg) (Aventis Pharmaceuticals); (2) flavopiridol alone (2.5 mg/kg) (Aventis Pharmaceuticals); (3) 5-FU alone (70 mg/kg) (ICN Pharmaceuticals); (4) docetaxel followed by flavopiridol 1 h later; (5) docetaxel followed by 5-FU 1 h later; (6) flavopiridol followed by 5-FU 24 h later; (7) docetaxel followed by flavopiridol 1 h later and 5-FU 24 h later. All drugs were administered ip twice a week every other week until the tumor size exceeded 2 cm. The tumors were measured in three axes from d 7 onwards and the tumor volume was calculated from these measurements.

Results

HCT116 phase growth inhibition is enhanced by sequential treatment with docetaxel, flavopiridol and 5-FU In order to explore the optimal sequencing of docetaxel, flavopiridol and 5-FU, we performed MTT assay with the HCT 116 cell line using the following concentrations: docetaxel 100 nmol/L, flavopiridol 300 nmol/L, and 5-FU 20 µmol/L. The cells were exposed to docetaxel, flavopiridol and 5-FU either sequentially or simultaneously as described. Sequential treatment of HCT116 colon cancer cells with docetaxel for 1 h followed by flavopiridol for 24 h and 5-FU for an additional 24 h resulted in 87% of phase growth inhibition. However, simultaneous addition of the three drugs to the cells for 24 h was less effective with 60% of phase growth inhibition. Phase growth inhibition was only 30%–40% when cells were treated with other sequential administrative schedules (Figure 1). These results indicated that the optimal sequencing of docetaxel, flavopiridol and 5-FU enhanced cell killing.

Sequencing of docetaxel, flavopiridol and 5-FU alters the ability of these drugs to induce apoptosis To evaluate the effect on apoptosis of three drug sequencing with docetaxel, flavopiridol and 5-FU, the activity of caspase-3 and related DEVD-specific proteases was determined. The assay involved the fluorescent measurement of the proteolytic cleavage of Z-DEVD-AMC to the fluorescent molecule AMC in both the absence and presence of the caspase-3 inhibitor
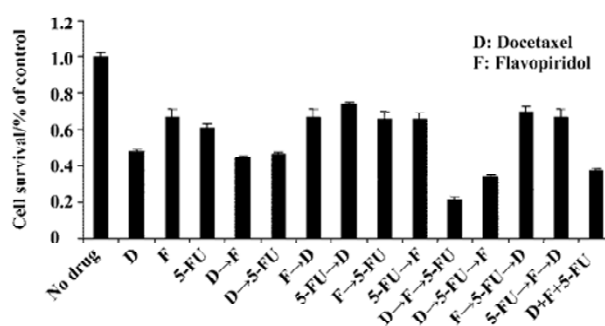

Figure 1. HCT116 cell death is enhanced by sequential treatment with docetaxel, flavopiridol and 5-FU. Cells were treated with: (1) no drug as control; (2) docetaxel for 1 h; (3) flavopiridol for 24 h; (4) 5-FU for 24 h; (5) docetaxel for 1 h followed by flavopiridol for 24 h; (6) docetaxel for 1 h followed by 5-FU for 24 h; (7) flavopiridol for 24 h followed by docetaxel for 1 h; (8) 5-FU for 24 h followed by docetaxel for 1 h; (9) flavopiridol for 24 h followed by 5-FU for another 24 h; (10) 5-FU for 24 h followed by flavopiridol for another 24 h; (11) docetaxel for 1 h followed by flavopiridol for 24 h and 5-FU for another 24 h; (12) docetaxel for 1 h followed by 5-FU for 24 h and flavopiridol for an additional 24 h; (13) flavopiridol for 24 h followed by 5-FU for 24 h and docetaxel for one more hour; (14) 5-FU for 24 h followed by flavopiridol 24 h and docetaxel for one more hour; (15) docetaxel, flavopiridol, and 5-FU simultaneously for 24 h. After each drug treatment cells were washed twice with drug-free medium. Cell survival was determined using an MTT assay and the cell number was plotted as a percentage of the control. Each value represents the mean±SD of triplicate readings.

Ac-DEVD-CHO. Sequential treatment of HCT116 cells with docetaxel for 1 h followed by flavopiridol for 24 h and 5-FU for an additional 24 h resulted in an 8-fold increase in caspase activity as compared to no drug treatment. When the cells were exposed simultaneously to docetaxel, flavopiridol, and 5-FU the caspase activity increased by 6-fold, while single or double drug treatment resulted in less than 4-fold increase (Figure 2).

Inhibition of colony formation in soft agar is dependent on the sequencing of docetaxel, flavopiridol and 5-FU We next determined the colony formation of HCT116 cells in soft agar by sequential treatment of the cells with docetaxel, flavopiridol and 5-FU. As compared to no drug treatment cells exposed to 100 nmol/L docetaxel, 300 nmol/L flavopiridol or 20 µmol/L 5-FU resulted in less than 5-fold inhibition on colony formation. Sequential treatment of the cells with any two drugs or simultaneous treatment of the cells with three drugs inhibited colony formation less than 9-fold. Colony formation was inhibited over 34-fold when cells were exposed to the three drugs sequentially, demonstrating that the combination of docetaxel, flavopiridol, and 5-FU reduced tumor cell growth in soft agar (Figure 3).
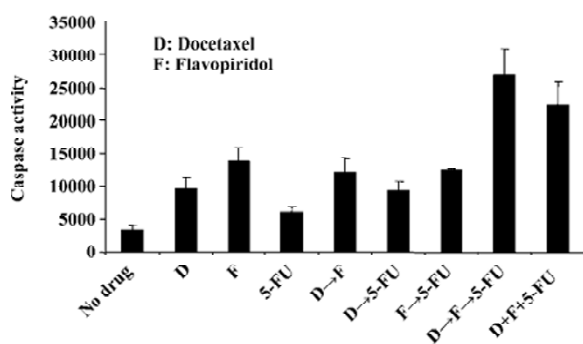
Figure 2. Apoptosis is increased by sequencing docetaxel, flavopiridol and 5-FU. The cells were treated with different combinations of the drugs as follows: (1) no drug; (2) docetaxel for 1 h; (3) flavopiridol for 24 h; (4) 5-FU for 24 h; (5) docetaxel for 1 h followed by flavo-piridol for 24 h; (6) docetaxel for 1 h followed by 5-FU for 24 h; (7) flavopiridol for 24 h followed by 5-FU for 24 h; (8) docetaxel for 1 h followed by flavopiridol for 24 h and 5-FU for an additional 24 h; (9) docetaxel, flavopiridol and 5-FU were administered at the same time for 24 h. Caspase-3 activities were determined after drug treatment.
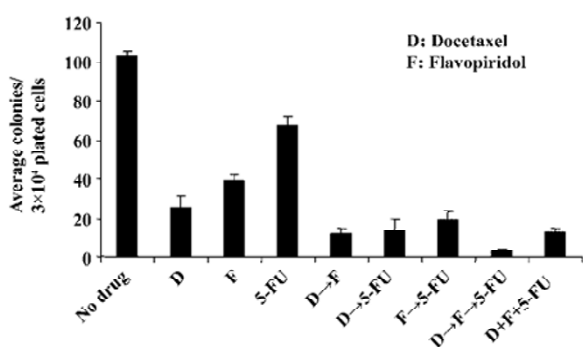

Figure 3. Colony formation in soft agar is dependent on sequencing of docetaxel, flavopiridol and 5-FU. The cells were treated with the same drug sequencing schedule as described with the caspase-3 assays. After drug treatment the cells were mixed with media containing 0.6% agar and plated in 6 well plates coated with 1 mL/well of 0.6% agar in media. The colonies were counted in triplicate on d 15 after plating and the number of colonies/3×10^4 cells was calculated.

Combination of docetaxel, flavopiridol and 5-FU reduces *in vivo* tumor growth As sequential treatment with docetaxel, flavopiridol and 5-FU resulted in enhanced antiproliferative and apoptotic effects on HCT116 cells *in vitro*, we next tested this sequence in HCT116 xenograft models. For this experiment, 6 to 8-week-old female nude[nu/nu] mice were inoculated with 2.5×10^6 HCT116 cells subcutaneously in the right flank. One week after tumor cell implantation, mice were randomized into eight groups of 5 mice each and injected with either, 5 mg/kg docetaxel, 2.5 mg/kg flavopiridol or 70

mg/kg 5-FU alone or in different sequences as described previously. The treatment was given twice weekly every other week by ip injection. Tumor size was analyzed over a 29 d time period. At d 29, single drug treatment resulted in a 50% reduction in tumor size as compared to no drug treatment. The reduction of tumor size was 70% with two drug combination (Figure 4). However, with the three drugs sequentially, tumor growth was significantly impaired with a decrease in tumor volume by 95% as compared to control (Figure 4). These studies suggested that *in vivo* administration of docetaxel, flavopiridol and 5-FU sequentially provided significant tumor growth inhibition of HCT116 xenograft.

Sequential treatment of nude mice with docetaxel, flavopiridol and 5-FU resulted in prolonged survival A survival experiment was performed by using four groups of 6 to 8-week-old nude mice. Seven days after HCT116 cells were inoculated subcutaneously in the right flank mice received: (1) no drug treatment; (2) 5 mg/kg of docetaxel alone; (3) 5 mg/kg of docetaxel followed by 2.5 mg/kg of flavopiridol 1 h later; and (4) 5 mg/kg of docetaxel followed by 2.5 mg/kg of flavopiridol 1 h later and 70 mg/kg of 5-FU 24 h later. Mice were injected with drugs twice weekly by ip every other week until killed with a tumor size of over 2 cm at any one of the three dimensions. The tumor volumes were measured in three axes starting on d 7 after inoculation. The first two mice treated with three drugs were killed around d 53, another two were killed at d 78 and d 93. The mice treated by docetaxel only or docetaxel followed by flavopiridol were killed between d 36 to d 46. The tumor size of no-drug-treatment mice was over 2 cm in one dimension between d 33 to d 39. Treatment of xenografts with three agents sequentially doubled the survival of mice as compared to two drug combination and control (Figure 5). Thus, sequencing of the chemotherapy resulted in significant prolongation of survival in established HCT116 animal model.

Discussion

Single agent chemotherapy has clearly had an inferior clinical tumor response rate and cure rate when compared to multi-agent therapy. However, most of the planning and use of multi-agent chemotherapy has focused on the use of agents with some degree of efficacy for a given cancer where the differing toxicities of the drugs allow them to be included in the treatment plan. One might intuitively consider a sequence of drug therapy that is based on logical cellular or molecular sites of action, or bioavailability of the drugs, or ease of administration[21,25].
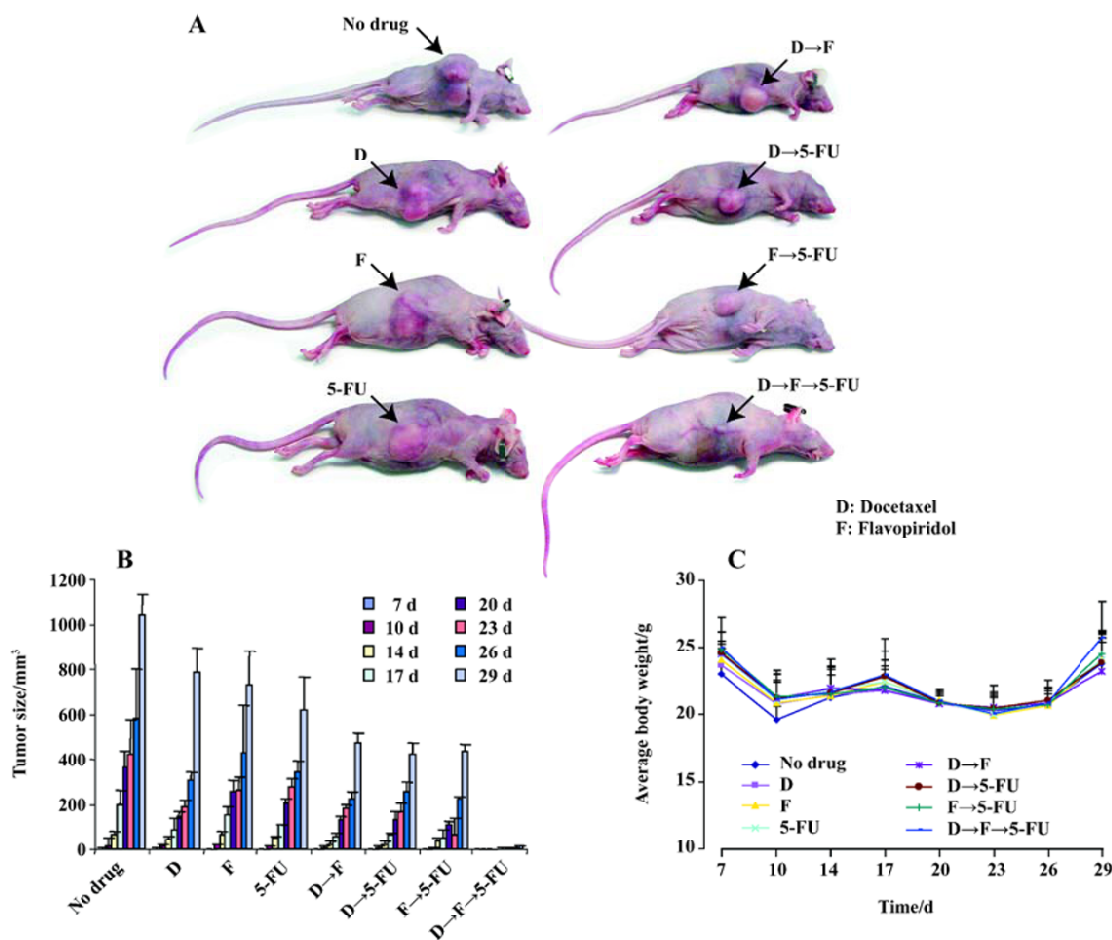
Flavopiridol has been reported to enhance the cytotoxic

Figure 4. Sequential treatment of nude mice with docetaxel, flavopiridol and 5-FU reduces *in vivo* tumor growth. Cohorts of 5 nude mice were injected with 2.5×10^6 cells subcutaneously and the tumors were allowed to grow for 7 d. Starting on d 8, the mice were treated with: (1) docetaxel; (2) flavopiridol; (3) 5-FU; (4) docetaxel followed 1 h later by flavopiridol; (5) docetaxel followed 1 h later by 5-FU; (6) flavopiridol followed 24 h later by 5-FU; or (7) docetaxel followed 1 h later by flavopiridol followed 24 h later by 5-FU. All drugs were administered ip twice a week every other week until the animals were killed because of tumor size over 2 cm at any one of three dimensions. The tumors were measured in three axes from d 7 onwards and the tumor volume was calculated from these measurements.

effects of various chemotherapeutic drugs. In several pre-clinical studies, sequence-dependent synergistic cytotoxic effects were observed when flavopiridol was administered after a variety of chemotherapeutic agents[26–28]. It has also been reported that flavopiridol enhanced docetaxel-induced apoptosis in gastric cancer cells. This effect was highly sequence dependent, such that docetaxel needed to precede flavopiridol treatment to achieve this effect[20]. Our cell proliferation assay data also clearly showed that sequencing the agents with docetaxel preceding flavopiridol decreased tumor volumes as compared to the reverse sequence. The addition of 5-FU to docetaxel and flavopiridol-treated cells increased cell death further in a sequence-dependent manner. Therefore, choosing the optimum administrative schedule may lead to a more effective chemotherapy regimen with the

combinations of docetaxel, flavopiridol and 5-FU. When this administrative schedule was followed, there was an 8-fold increase in caspase activity as compared to no drug treatment, while other administrative schedules resulted a less than 4-fold increase in caspase activity. This sequential treatment also induced the highest percentage of cell death, apoptosis and resulted in the greatest reduction of HCT116 colony formation in soft agar.

The projected goal in this study is to aim for maximum therapeutic efficacy without increasing or compounding toxicity. When we extended *in vitro* observations to an *in vivo* system, tumor regression was increased and survival was prolonged with the sequential treatment of docetaxel, flavopiridol and 5-FU as compared to single drug treatment. Thus the sequence of administrating the chemotherapy
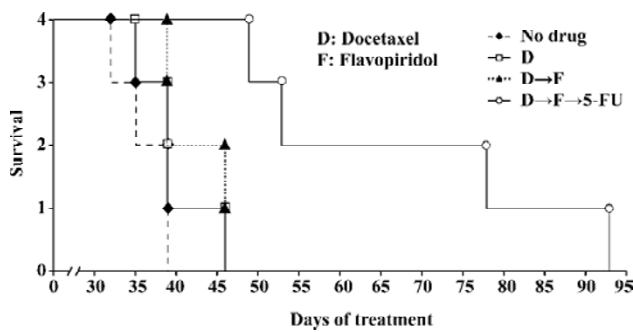
Figure 5. Sequential treatment of nude mice with docetaxel, flavopiridol and 5-FU results in prolonged survival. Cohorts of 5 nude mice were injected with 2.5×10^6 cells subcutaneously and the tumors were allowed to grow for 7 d. Starting on d 8, the mice were treated with: (1) no drug; (2) docetaxel; (3) docetaxel followed 1 h later by flavopiridol; and (4) docetaxel followed 1 h later by flavopiridol followed 24 h later by 5-FU. Mice were injected with drug twice weekly every other week until killed either because of tumor size (over 2 cm at any one of three dimensions) or appearance of ulcer at the tumor inoculation site. The tumor volumes were measured in three axes starting on d 7 after inoculation.

agents affects the degree of cytotoxicity and our data showed that the sequential treatment of colon cancer cells with docetaxel followed by sequential flavopiridol and 5-FU had a significant inhibitory effect on tumor proliferation both *in vitro* and *in vivo*.

Ever increasing evidence continues to define cancer chemotherapy efficacy in terms of the ability to achieve significant effects on checkpoints of cell-cycle control with resultant induction of programmed cell death. Although, for many chemotherapy drugs, one can preclude or identify a reasonably specific molecular site of action, the clear identification and delineation of the actual events have been difficult to prove. For many drugs the absolute and singular site of action is often difficult to recognize *in vivo* and the relationship to the microenvironment and potential release of correlate cytokines or related regulatory proteins further compounds precision. In our current studies we have attempted to overcome these obstacles by developing the data in a series of *in vitro* studies and then examining the findings in the *in vivo* circumstances. These results strongly suggest that this type of sequential administrative schedule may have important therapeutic implications and a defined construct now exists for translation to early phase clinical trials.

Acknowledgements

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November 30–December 2, 2006

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