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The Cyclin-Dependent Kinase Inhibitor Flavopiridol Potentiates Doxorubicin Efficacy in Advanced Sarcomas: Preclinical Investigations and Results of a Phase I Dose-Escalation Clinical Trial

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Abstract

Purpose: Dysregulated cyclin-dependent kinases are important to the growth of some sarcomas. Flavopiridol is a pan-CDK inhibitor that has been shown to potentiate chemotherapy. As such, we explored the potentiation of doxorubicin by flavopiridol in sarcoma, *in vitro* and *in vivo*, and conducted a phase I trial of flavopiridol with doxorubicin in patients with advanced sarcomas.

Experimental Design: Sarcoma cell lines and xenografts were treated with flavopiridol alone and in combination with doxorubicin. In the phase I study, doxorubicin and flavopiridol were administered on two flavopiridol schedules; a 1-hour bolus and split dosing as a 30-minute bolus followed by a 4-hour infusion.

Results: Preclinically, flavopiridol potentiated doxorubicin. *In vivo*, doxorubicin administered 1 hour before flavopiridol was more active than doxorubicin alone. Clinically, 31 patients were enrolled on protocol and flavopiridol was escalated to target dose in two schedules (90 mg/m^2 bolus; 50 mg/m^2 bolus + 40 mg/m^2 infusion) both in combination with doxorubicin (60 mg/m^2). Dose-limiting toxicities were neutropenia, leukopenia, and febrile neutropenia but no maximum tolerated dose was defined. Flavopiridol pharmacokinetics showed increasing C_{max} with increasing dose. Response Evaluation Criteria in Solid Tumors (RECIST) responses included two partial responses, however, stable disease was seen in 16 patients. Of 12 evaluable patients with progressive well- and dedifferentiated liposarcoma, eight had stable disease greater than 12 weeks.

Conclusions: The sequential combination of doxorubicin followed by flavopiridol is well tolerated on both schedules. Disease control was observed in well- and dedifferentiated liposarcoma specifically, a disease in which *CDK4* is known to be amplified. *Clin Cancer Res; 18(9); 2638–47. ©2012 AACR.*

Introduction

Cyclin-dependent kinases (CDK) are serine/threonine kinases that are activated upon association with cyclin proteins to form CDK complexes. In response to mitogenic and stress stimuli, CDK complexes phosphorylate effector protein complexes, regulating both RNA polymerase II–mediated transcription and progression through the cell cycle. CDKs are attractive targets for drug development, given that certain malignancies are dependent on dysregulated cyclin activity (1) and CDK inhibition has been observed as a potent vehicle to overcome resistance to standard chemotherapy (2–4).

Flavopiridol is a pan-CDK inhibitor that specifically inhibits CDK2, CDK4, CDK6, and CDK9 at nanomolar concentrations. Inhibition of CDKs by flavopiridol blocks cell-cycle progression at the G_1–S or G_2–M checkpoints and is associated with cell-cycle arrest and subsequent apoptosis (5, 6). Flavopiridol has been tested at various dosing levels and schedules in both hematologic (7, 8) and solid tumor malignancies (3, 9, 10). To date, the most compelling data about clinical efficacy have been observed in hematologic malignancies, such as chronic lymphocytic leukemia (11).

The therapeutic impact of single-agent flavopiridol in solid tumors has been less robust. However, preclinical

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J.J. Luke and D.R. D'Adamo share first authorship for equal work on the study.

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Translational Relevance

Doxorubicin is part of standard therapy for the treatment of soft tissue sarcomas. Flavopiridol, the pan-cyclin-dependent kinase (CDK) inhibitor, has been shown to enhance the effects of chemotherapy. We report that flavopiridol potentiates the effects of doxorubicin in soft tissue sarcoma cell lines both *in vitro* and *in vivo*. On the basis of these results, we designed and conducted a phase I clinical trial of fixed dose doxorubicin followed by escalating doses of flavopiridol on two different flavopiridol schedules. Our results indicate that the combination is well tolerated with clinical benefit at biologically active doses. These studies provide a foundation upon which to further examine the role of CDK inhibitors in augmenting doxorubicin. This is particularly applicable in diseases where CDKs are known to be oncogenic drivers and in which doxorubicin is active, including certain types of liposarcomas, lymphomas, and leukemias.

models and clinical trial results suggest a use of flavopiridol in combination with chemotherapy. For example, flavopiridol, as a pan-CDK inhibitor, blocks CDK9 leading to suppression of Rad51, an enzyme involved in homologous recombination. This defect in DNA repair then sensitizes tumor cells to p53-dependent induction of apoptosis by irinotecan (12). Flavopiridol has also been associated with the potentiation of other chemotherapies such as paclitaxel, docetaxel, gemcitabine, and doxorubicin, in which flavopiridol exposure has been shown to enhance apoptosis in tumor cells (13–17).

Advanced sarcomas are a group of heterogeneous mesenchymally derived neoplasms for which clinical treatment options are limited. After progression to metastatic or unresectable disease, systemic treatment options include chemotherapies such as doxorubicin, ifosfamide, dacarbazine, gemcitabine, and docetaxel. While the molecular characteristics of these diseases are increasingly being elucidated, cytotoxic chemotherapy remains the clinical standard-of-care. Unfortunately, response rates to these agents are low and patients rarely obtain durable clinical benefit.

Preclinically, flavopiridol has been shown to potentiate the effects of doxorubicin in a bone sarcoma (osteosarcoma), especially in a retinoblastoma-null background (18). However, the ability of flavopiridol to potentiate doxorubicin in soft tissue sarcoma is unknown. Certain soft tissue sarcoma subtypes, such as well-differentiated and dedifferentiated (WD and DD) liposarcoma, are especially attractive for drug targeting by flavopiridol, as *CDK4* is amplified in 90% of these tumors (19). WD and DD liposarcoma has been shown to be sensitive to inhibition of CDKs in preclinical models, and early-phase clinical trials have hinted that targeted inhibition of CDKs may lead to impressive clinical benefit (20). Therefore, we evaluated both the *in vitro* and *in vivo* ability of flavopiridol to potentiate the effects of doxorubicin in sarcoma and conducted a phase I clinical trial of the combination in patients with advanced sarcomas.

Materials and Methods

Preclinical methods

Cell culture. LS141 primary human cell line was derived from a patient with high-grade retroperitoneal dedifferentiated liposarcoma and the malignant peripheral nerve sheath tumor (MPNST) cells were derived from a patient with a high-grade peripheral nerve sheath tumor of the thigh (graciously supplied by Jonathan Fletcher, Dana-Farber Cancer Institute, Boston, MA). These were grown in RPMI-1640 supplemented with 15% heat-inactivated FBS plus penicillin and streptomycin.

Colony assays. MPNST cells were treated with doxorubicin, flavopiridol (graciously supplied by National Cancer Institute, Bethesda, MD), or the combination of the 2 drugs together in sequence. MPNST cells were chosen given that LS141 (and other CDK4-dependent) cells are exquisitely sensitive to CDK4 inhibition *in vitro*, thus making combination studies uninterruptible. MPNST cells were plated, in triplicate, at a density of 1,000 cells per 100 mm^2 per plate. Twenty-four hours after plating, cells were treated for 24 hours with the IC_{50} of doxorubicin (D, 15 nmol/L), flavopiridol (F, 150 nmol/L), drug-free media (control), or a combination of the 2 drugs, either concomitantly or sequentially for 24 hours each. After treatment, drug-containing medium was removed and cells were allowed to grow for 10 days to form colonies. The resulting colonies were stained with 0.01% crystal violet for 30 minutes and colonies counted using an automated colony counter (ColCount, Oxford Optronix). Results are presented as the percentage of untreated controls, and the statistical significance of the experimental results was determined by the 2-sided *t* test.

Immunoblotting. MPNST cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail tablets (Complete Mini, Roche Diagnostics) and 1 mmol/L $NaVO_3$. Total protein concentration of the lysates was measured by Bio-Rad protein assay (Bio-Rad Laboratories), and equal amounts of protein were loaded on 4% to 12% PAGE gels (Invitrogen). Polyvinylidene difluoride membranes were blocked with 5% nonfat dried milk in PBS buffer containing 0.1% Tween-20 (PBST) for 1 hour and probed with antibodies for full-length and cleaved PARP (Santa Cruz Biotechnology) and tubulin (Cell Signaling).

In vivo studies. LS141 xenografts were established by directly implanting into severe combined immunodeficient (SCID) mice. Once tumors reached 100 mm^3, groups of 5 mice were treated with the maximum tolerated dose (MTD) of flavopiridol (9 mg/kg), doxorubicin (0.9 mg/kg), or doxorubicin (0.7 mg/kg) followed by flavopiridol (7 mg/kg) at selected time points (1, 4, and 7 hours). In addition, one set of animals was treated in reverse order of flavopiridol followed by doxorubicin, administered 7 hours apart. All

treatments were administered in intraperitoneal fashion, twice weekly, for a total of 5 treatments. Tumors were measured every 2 to 3 days with calipers, and tumor volumes were calculated by the formula $\pi/6 \times$ (large diameter) $\times$ (small diameter)^2. Tumor volume was compared between groups of mice at various points in time based on the experiment and the statistical significance of the experimental results was determined by the 2-sided *t* test. Given the aggressive morbidity of the tumors, animal survival data could not be estimated. Toxicity was monitored by weight loss. These studies were done in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 85-23, released 1985), under an Institutional Animal Care and Use Committee (IACUC)-approved protocol.

Clinical trial methods

Patient entry criteria. All patients (age $\geq$ 18, male, and female) were histologically confirmed to have metastatic or locally recurrent sarcoma. Prior treatment excluded anthracyclines but allowed up to 2 prior lines of therapy. PPAR-γ agonists, thalidomide or targeted therapy such as tyrosine kinase inhibitors, were not considered as prior lines of therapy. A minimum of 3 weeks from last treatment had to elapse before study entry (6 weeks for nitrosoureas and mitomycin C and 1 week for targeted agents). Patients had to have a Karnofsky performance status $\geq$ 60%, total white blood cell count $\geq$ 3,500/mm^3, absolute neutrophil count (ANC) $\geq$ 1,500/mm^3, platelets $\geq$ 100,000/mm^3, and adequate hepatic, renal, and cardiac function (including ejection fraction $\geq$ 50%). Patients with central nervous system metastases were not eligible. Patients with a history of significant heart disease or radiation to both the pelvis and spine were additionally excluded. The protocol was approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center (New York, NY) and all patients provided written informed consent.

Clinical trial design. The trial design was a nonrandomized, open-label dose-escalation of doxorubicin and flavopiridol. Groups of 3 to 6 patients were treated sequentially according to the dose-escalation in Table 1. Treatment was every 3 weeks (1 cycle), provided the ANC was $\geq$1,500/mm^3 and platelet count $\geq$100,000/mm^3. Doxorubicin was given intravenous) push over 5 to 7 minutes on day 1 of each cycle at a dose of 60 or 75 mg/m^2. On the basis of our and *in vitro* and *in vivo* preclinical model (see Results), flavopiridol was given 1 hour following doxorubicin as a 60-minute i.v. bolus (cohorts 1–6), starting at a dose of 40 mg/m^2 to a goal escalation dose of 70 mg/m^2, the approximate MTD defined in single-agent bolus schedule studies (21). This dose has also been shown to consistently achieve more than 2.0 μmol/L of flavopiridol in human plasma. In view of 90% protein binding in plasma, this achieves a therapeutically active free flavopiridol plasma level of approximately 200 nmol/L. Given the desire to continue to increase flavopiridol exposure and the success of split dosing (bolus followed by infusion) in the treatment of chronic lymphocytic leukemia (22), further cohorts were examined using a split dosing schedule. Patients in cohorts 7 and 8 received flavopiridol as a 30-minute bolus followed by a 4-hour infusion on day 1 of each cycle, beginning 1 hour after the administration of doxorubicin. The target flavopiridol dose was 90 mg/m^2 (Table 1); the single-agent MTD with divided dose flavopiridol therapy. Because of concerns for tumor lysis syndrome with the split-dose schedule, tumor lysis blood samples were obtained, including lactate dehydrogenase, calcium, magnesium, and phosphorous, on the day following therapy. Where indicated, dexrazoxane was given before each dose of doxorubicin (cumulative doxorubicin dose > 300 mg/m^2). Dexrazoxane was given at 10 times the dose of doxorubicin. Doxorubicin was given within 30 minutes of start of the dexrazoxane infusion. After 600 mg/m^2

Table 1. Clinical trial dosing cohorts

Cohort	Doxorubicin (mg/m^2) i.v. push over 5 min	Flavopiridol (mg/m^2) 60-min bolus or 30-min bolus followed by 4-h infusion	Number accrued to each level	DLT (cycle 1)
1	60	40	3	0
2	60	50	3	0
3	60	60	3	0
4	60	70	3	0
5	75	60	3	0
6	60	70 (40/30)	4[a]	0
7	60	80 (50/30)	6	1[b]
8	60	90 (50/40)	6	1[c]

[a]One patient withdrew before treatment in the fourth cohort.

[b]DLT in cohort 7 of grade IV neutropenia.

[c]DLT in cohort 8 grade III febrile neutropenia and grade IV neutropenia and leukopenia.

doxorubicin (including use of dexrazoxane), doxorubicin was discontinued and flavopiridol could be continued as a single agent until progression of disease. All treatments were administered in the outpatient setting and intrapatient dose-escalation was not permitted.

Toxicity was graded in accordance with the Common Toxicity Criteria Version 3.0 (23). Dose-limiting toxicity (DLT) was defined as the occurrence during the first cycle of grade IV hematologic toxicity 21 days after treatment, grade IV hematologic toxicity lasting 7 days or longer, grade III or IV nonhematologic toxicity including diarrhea despite antidiarrheal prophylaxis, nausea despite maximum antiemetic therapy, or any delay in treatment of more than 2 weeks. The MTD was defined as the dose one level below the dose at which 2 or more of the patients in the initial dose level experienced DLT during the first treatment course. Patients who experienced a DLT, or toxicity attributed to study medication, could continue to receive study treatment after recovery, with appropriate dose modifications as defined per protocol.

To be evaluable for response and to be assessable for determination of MTD, patients had to have received at least one full cycle of therapy. Otherwise, treatment responses were evaluated after every 2 cycles with computed tomographic scans or other diagnostic tests, as appropriate. Response Evaluation Criteria in Solid Tumors (RECIST), version 1.0, were used for response assessment and done by an independent protocol radiologist. Complete or partial responses (PR) were confirmed by repeat studies at least 4 weeks after the criteria for response were first met.

The main objective of this study was to determine the MTD or achieve a maximal identified target dose of flavopiridol, when administered in combination with a doxorubicin. Standard $3 + 3$ design was used for dose-escalation. The incidence of hematologic and nonhematologic toxicities was summarized separately by flavopiridol cohort. Secondary analyses included a pharmacokinetic (PK) analysis of flavopiridol by noncompartmental methods.

Drug supply. Flavopiridol (also known as alvocidib, HMR-1275) was supplied by Sanofi Aventis Pharmaceuticals and distributed by CTEP. Doxorubicin and dexrazoxane are commercially available.

Pharmacokinetics. Blood samples for PK studies were collected into heparin-coated tubes and analyzed per previously published methods (24). Flavopiridol levels were measured on the bolus schedule before treatment, at completion of flavopiridol (time 0) and then 0.5, 1, 2, 6, and 24 hours later. For the split dosing schedule, PK samples were collected pretreatment and at approximately 0.5, 4.5, 24, and 28 hours from initiation of flavopiridol infusion.

Results

In vitro, the doxorubicin and flavopiridol combinations were statistically superior to single-agent doxorubicin or flavopiridol. As shown in Fig. 1A, colony formation assays revealed that in comparison with doxorubicin or flavopiridol alone, observable colonies decreased significantly with all the combinations tested. For example, colony formation decreased from 59% with flavopiridol alone to 41% with concomitant therapy (combo, $P = 0.046$), 35% with doxorubicin followed by flavopiridol (D24, F24, $P = 4.4 \times 10^{-5}$), and 44% with flavopiridol followed by doxorubicin (F24, D24, $P = 3.8 \times 10^{-5}$). Although there was a trend in decrease of colony formation favoring doxorubicin followed by flavopiridol, no statistically significant differences in colony formation observed for the 3 combinations. However, when these combinations were examined for induction of apoptosis by PARP cleavage, this was predominantly observed for the cells treated with the sequential doxorubicin followed by flavopiridol combination (Fig. 1A).

In vivo, human xenograft mouse models revealed that single-agent flavopiridol decreased tumor growth relative to untreated controls and was significantly more efficacious than single-agent doxorubicin. As we have previously reported that the timing of chemotherapy relative to flavopiridol can affect the degree of tumor regressions *in vivo*, we elected to treat our tumor xenografts with doxorubicin followed by flavopiridol at 1, 4, and 7 hours, as well as with the reverse combination at a 7-hour interval. As shown in Fig. 1B, the only combination that was statistically superior to doxorubicin alone was doxorubicin followed 1 hour later by flavopiridol ($P = 0.01$ at day 38). All the other combinations were either equal to ($D \rightarrow F4$, $P = 0.19$) or inferior to ($D \rightarrow F7$, $P = 0.08$ and $F \rightarrow D7$, $P = 0.71$) doxorubicin alone. There was no significant weight loss with the combination therapy. Interestingly, when comparing flavopiridol with the $D \rightarrow F1$ combination, there appeared to be a trend toward lower tumor growth with the combination; however, this did not reach statistical significance ($P = 0.15$). These data were consistent with prior observations that dedifferentiated liposarcoma cell lines, with amplified *CDK4*, are highly sensitive to flavopiridol (data not shown) as well as the clinical observation that dedifferentiated liposarcomas are generally resistant to doxorubicin.

Given these findings, we launched a phase I dose-escalation trial of flavopiridol in combination with doxorubicin. The primary objective of this trial was to determine the MTD of flavopiridol in combination with doxorubicin in patients with advanced sarcomas. Secondary objectives were to investigate the clinical PK of flavopiridol in combination with doxorubicin and to obtain preliminary data on the therapeutic activity of this regimen.

Clinical trial results

Patient characteristics. From October 13, 2004, to January 14, 2010, 31 patients with metastatic or locally recurrent sarcoma were registered and 30 were treated. One patient was not evaluable for determining DLT as that patient did not initiate treatment after registration (withdrew consent). Three patients were not evaluable for determining response because they did not complete 2 cycles of treatment. The reasons were withdrawal of consent (2 patients) and clinical deterioration (1 patient).

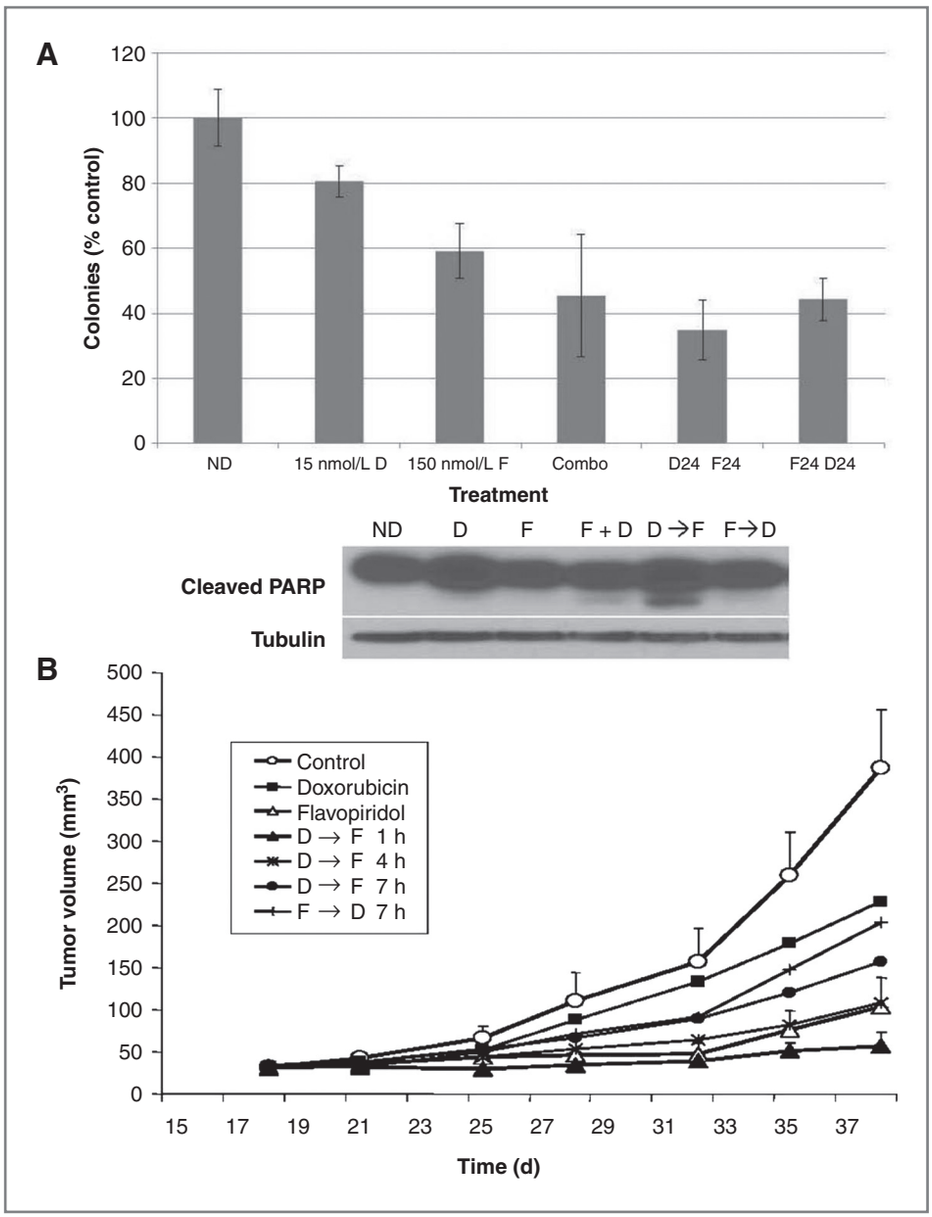

Figure 1. The effects of flavopiridol and doxorubicin on MPNST cell colony formation and dedifferentiated liposarcoma tumor xenograft growth. A, colony formation after treatment of MPNST cells with doxorubicin and flavopiridol (individual, in combination or in sequence). MPNST cells were treated with doxorubicin (D) for 24 hours, flavopiridol (F) for 24 hours, concomitantly for 24 hours (combo). or sequentially such that cells were treated with D for 24 hours followed by F for 24 hours, or the reverse combination. After treatment, drug-containing media were removed and colony formation was assayed 10 days later. Results are presented as percentages of untreated controls. Immunoblot analysis after treatment under these same conditions using antibody for cleaved PARP. α-Tubulin is shown to confirm equal loading of protein. B, treatment of dedifferentiated liposarcoma xenografts with doxorubicin and flavopiridol, as single agents or in sequence (separated by 1, 4, or 7 hours). LS141 xenografts (in groups of 5) were treated with doxorubicin, flavopiridol, or sequentially separated by 1, 4, or 7 hours or the reverse sequence. ND, no drug.

Table 2 lists the patient characteristics of the 31 patients who were accrued to the protocol. The median age was 57 years (range, 31–72 years) and the median Karnofsky performance status was 90% (range, 70%–100%). There were 20 men and 11 women. The sarcomas treated and patient numbers were liposarcoma (16: 15 of which were WD/DD, 1 pleomorphic), leiomyosarcoma ($n = 5$), fibrosarcoma ($n = 3$), MPNST ($n = 2$), undifferentiated pleomorphic sarcoma ($n = 1$), osteosarcoma (extraosseous; $n = 1$), rhabdomyosarcoma (pleomorphic; $n = 1$), gastrointestinal stromal tumor ($n = 1$), and solitary fibrous tumor ($n = 1$). The majority of patients had not received prior chemotherapy (16% pretreated) with an overall range of 0 to 2 prior treatments. No patients had received prior doxorubicin and all had progressive disease at the time of enrollment with an indication to begin systemic treatment.

Toxicity. Table 3 lists the most common grade II to IV hematologic toxicities for the first cycle of therapy (no relevant nonhematologic toxicities were observed in cycle 1). Combination treatment with 60 mg/m^2 of doxorubicin and flavopiridol as a 1-hour bolus was documented to be well tolerated without DLT to the protocol-specified levels. Starting at 60 mg/m^2 of flavopiridol, grade III lymphopenia was also observed, a common non–dose-limiting hematologic toxicity of flavopiridol (21). A single cohort of patients was treated with 75 mg/m^2 of doxorubicin and 60 mg/m^2 of flavopiridol without DLT (cohort 5). However, there was grade III neutropenia in all 3 patients treated at this dose

Table 2. Patient characteristics

Characteristic	Number of patients
Total	31
Assessable for response	28
Male	20
Female	11
Age, y	
Median	57
Range	31–72
Karnofsky performance status, %	
Median	90
Range	70–100
Prior chemotherapy	16%
Number of prior lines	
Median	0
Range	0–2
Prior doxorubicin	0
Sarcoma subtype	
Liposarcoma (15 WD/DD, 1 pleomorphic)	16
Leiomyosarcoma	5
Fibrosarcoma	3
MPNST	2
Undifferentiated pleomorphic sarcoma	1
Osteosarcoma	1
Rhabdomyosarcoma (pleomorphic)	1
Gastrointestinal stromal tumor	1
Solitary fibrous tumor	1

level, suggesting that the increased doxorubicin dose resulted in increased neutropenia.

Starting with 70 mg/m^2 of total flavopiridol in cohort 6, the combination of doxorubicin and split dosing flavopiridol was also found to be tolerable. However, DLTs were observed in the seventh and eighth cohorts. In the seventh cohort, one patient experienced DLT including both grade IV neutropenia and leukopenia leading to expansion of the cohort to 6 patients. In cohort 8, one patient experienced DLT including grade IV neutropenia and leukopenia and grade III febrile neutropenia. This cohort was also expanded without observance of further significant toxicity. Beyond these, no other DLTs were observed throughout the study. Dose-escalation beyond cohort 8 was not pursued, as the prespecified maximal flavopiridol dose of 90 mg/m^2 was achieved. As such, no MTD was formally established. However, it should be noted that there was substantial grade III neutropenia in cohort 7 (3 of 6 patients) and cohort 8 (5 of 6 patients) with 2 episodes of grade III thrombocytopenia. Therefore, it is unlikely that further dose-escalation could have been safely achieved. Notably, all DLTs were hematologic in nature and tumor lysis syndrome was not observed.

Supplementary Table S1 lists the most common grade II to IV cumulative hematologic toxicities for all cycles of treatment. The cumulative pattern of toxicity was similar to that in cycle 1 of treatment and was principally limited to hematologic effects. For the whole study population, the most common grade III/IV toxicities were neutropenia (45%), leukopenia (26%), thrombocytopenia (9%), and lymphopenia (6%). Grade III/IV neutropenia occurred in 14% of patients on the bolus schedule versus 33% of those on the bolus/infusional schedule. Similarly, grade III/IV leukopenia occurred in 4% and 23% for the bolus-only and split dosing schedules, respectively. Grade III/IV thrombocytopenia occurred in 0% and 9% of patients on the bolus-only and split dosing schedules, respectively. The rates of lymphopenia and anemia were not impressively different between the 2 schedules.

Several significant nonhematologic toxicities occurred after cycle 1, however, were generally not attributed to study treatment and were not considered dose-limiting for the protocol. The majority of these occurred with the split dosing flavopiridol schedules. The exception to this was the one death on protocol (cohort 3). This was due to a small bowel perforation and was attributed to treatment. The other toxicities (attributed as not related to treatment) included the development of grade IV central nervous system ischemia and a grade III pulmonary embolus in 2 different patients in dose cohort 6, a grade III pulmonary embolus with left ventricular dysfunction (following 2 cycles of therapy), a grade IV small bowel perforation in 2 separate patients in dose cohort 7, and both a grade III psychosis and grade IV confusion in a patient in dose cohort 8.

Pharmacokinetics. Blood samples from 31 patients were obtained to conduct PK analyses of flavopiridol. Table 4 summarizes maximum observed plasma concentration (C_{max}) across all subjects in a cohort. Flavopiridol C_{max} was observed at the first time point analyzed in each schedule (0.5 hours) and ranged from 2.22 μmol/L (SD, 0.07) at a flavopiridol dose of 50 mg/m^2, up to a maximum of 3.33 μmol/L (SD, 1.38) at a flavopiridol dose 90 (50/40) mg/m^2. Use of the split dosing schedule did not appear to increase the C_{max} of flavopiridol. There was significant interpatient variability within some cohorts (3, 7, and 8). The dose of doxorubicin was held constant for all but one dose level thus making evaluation of any potential interaction between the 2 drugs difficult. Notably however, the C_{max} range noted in this trial is similar to that seen in previous clinical trials combining flavopiridol with other chemotherapies (24, 25), suggesting that flavopiridol exposure is unlikely to be significantly altered when combined with doxorubicin. In cohorts 7 and 8, there was higher C_{max} in patients who experienced DLT as opposed to those who did not (data not shown), but this difference did not meet statistical significance. There were insufficient PK time points available to formally evaluate the area under the curve (AUC) for either schedule.

Clinical activity. Twenty-eight patients were evaluable for response assessment (Table 5). There were 2 RECIST 1.0 partial responses and 14 patients had RECIST stable disease (SD) as best response (median, 15 weeks; range, 3–99 weeks). The disease control rate [complete response (CR) + PR + SD for >3 months) was 57% (16 of 28).

Table 3. Grade II or greater hematologic toxicity observed in cycle 1

Cohort (evaluable patients)	Doxorubicin	Flavopiridol	ANC			Lymphopenia			Leukocytes			Hemoglobin			Platelets			Febrile neutropenia		
			2	3	4	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4
1 (3)	60	40	1	1					1											
2 (3)	60	50		1					1											
3 (3)	60	60	1	1			1		2	2		1								
4 (3)	60	70	1	1			1		1	1		1								
5 (3)	75	60		3			1		1											
6 (3)	60	70 (40/30)	2							1						1				
7 (5)[a]	60	80 (50/30)	1	3	1[b]				3	4	1[b]									
8 (5)[a]	60	90 (50/40)	2	5	1[c]				3	2	1[c]	1			2	2				1[c]

[a]All DLTs were hematologically related.

[b]DLT including ANC and leukocytes was experienced by the same patient in cohort 7.

[c]DLT including ANC, leukocytes, and febrile neutropenia was experienced by one patient in cohort 8.

There was no apparent difference by flavopiridol schedule. Of the 12 evaluable patients with WD and DD liposarcoma, 5 had progression of disease at first evaluation whereas 7 had stable disease for at least 3 months as best response (median, 20 weeks; range, 12–99 weeks). One notable patient (WD and DD liposarcoma) was maintained on study for 99 weeks. This patient, treated in cohort 1, initially received the maximum allowable doxorubicin dose (including use of dexrazoxane) and was thereafter continued on single-agent flavopiridol. The best response was stable disease, which was maintained through 83 weeks. At that point, the patient decided to withdraw consent for further treatment. Thirty-three weeks later, the patient was noted to have progression of disease and under a special protocol amendment was reinitiated on single-agent flavopiridol. Disease stability was recovered and the patient continued on treatment for further 16 weeks before eventually progressing.

Discussion

We examined the use of flavopiridol in potentiating the effects of doxorubicin on the growth of malignant sarcoma both *in vitro* and *in vivo*. Preclinically, we documented in MPNST cells that flavopiridol potentiates doxorubicin, when compared with single agents alone. Furthermore, we showed that flavopiridol is active *in vivo* both as a single agent and in combination with doxorubicin in liposarcoma xenograft with amplified *CDK4*.

Given these findings, we conducted a phase I dose-escalation clinical trial of flavopiridol plus doxorubicin in patients with advanced sarcomas. Biologically active and therapeutic doses of flavopiridol (90 mg/m^2; 50 mg/m^2 bolus followed by 40 mg/m^2 infusion) and doxorubicin (60 mg/m^2) were combined without reaching an MTD. The achieved dose of flavopiridol was similar to that shown to be tolerable in combination with other chemotherapies and the PK at most of the dose levels tested were in the active range based on preclinical data (13, 26). Hematologic DLTs, constituted by neutropenia, leukopenia, lymphopenia, and thrombocytopenia, were observed by the combination of flavopiridol and anthracycline chemotherapy. Adverse events were generally tolerable, with the appearance of febrile neutropenia in only one instance. We conclude that flavopiridol can be combined with doxorubicin safely at biologically active doses.

Table 4. Flavopiridol PK by dose level during cycle 1

Cohort	Doxorubicin	Flavopiridol	*n*	C_{max}, μmol/L (mean ± SD)
1	60	40	3	2.27 ± 0.49
2	60	50	3	2.23 ± 0.52
3	60	60	3	3.29 ± 1.39
4	60	70	3	2.22 ± 0.07
5	75	60	3	2.46 ± 0.67
6	60	70 (40/30)	4	2.56 ± 0.31
7	60	80 (50/30)	6	3.03 ± 1.29
8	60	90 (50/40)	4	3.33 ± 1.38

Table 5. Time on study and response by individual patient and cohort

Patient no. (Cohort)	Histology	Best response	Reason off-study	Time on study, wk	PFS ≥ 12 wk	PFS ≥ 24 wk	Total cycles
1 (1)	Leiomyosarcoma	POD	POD	7	No	No	2
2 (1)	Liposarcoma (WD/DD)	**SD**	POD	25	**YES**	**YES**	9
3 (1)	Liposarcoma (WD/DD)	**SD**	POD	99	**YES**	**YES**	31
4 (2)	Liposarcoma (pleomorphic)	**SD**	POD	7	No	No	2
5 (2)	Leiomyosarcoma	**SD**	Clinical POD	47	**YES**	**YES**	15
6 (2)	MPNST	POD	POD	7	No	No	2
7 (3)	Liposarcoma (WD/DD)	POD	POD	6	No	No	2
8 (3)	MPNST	**SD**	Clinical POD	7	No	No	3
9 (3)	Leiomyosarcoma	**PR**	POD	45	**YES**	**YES**	15
10 (4)	Fibrosarcoma	**SD**	Clinical POD	6	No	No	2
11 (4)	Leiomyosarcoma	**SD**	POD	47	**YES**	**YES**	2
12 (4)	Osteosarcoma	**SD**	POD	12	**YES**	No	4
13 (5)	Liposarcoma (WD/DD)	**SD**	Withdrew consent	12	**YES**	No	4
14 (5)	Fibrosarcoma	**SD**	POD	23	**YES**	**No**	8
15 (5)	Liposarcoma (WD/DD)	POD	POD	6	No	No	2
16 (6)	Liposarcoma (WD/DD)	**SD**	Withdrew consent	15	**YES**	No	5
17 (6)	Leiomyosarcoma	**SD**	POD	25	**YES**	**YES**	8
18 (6)	Liposarcoma (WD/DD)	Unevaluable	Intercurrent illness	3	No	**No**	1
19 (6)	Liposarcoma (WD/DD)	POD	POD	6	No	No	2
20 (7)	Liposarcoma (WD/DD)	Unevaluable	Toxicity	4	No	No	2
21 (7)	Fibrosarcoma	**SD**	POD	73	**YES**	**YES**	24
22 (7)	Liposarcoma (WD/DD)	**SD**	Withdrew consent	25	**YES**	**YES**	7
23 (7)	Solitary fibrous tumor	**SD**	Intercurrent illness	12	**YES**	No	3
24 (7)	Liposarcoma (WD/DD)	**SD**	Withdrew consent	14	**YES**	**No**	4
25 (7)	Liposarcoma (WD/DD)	POD	POD	6	No	No	2
26 (8)	Liposarcoma (WD/DD)	POD	POD	6	No	No	2
27 (8)	Rhabdomyosarcoma	**PR**	POD	38	**YES**	**YES**	12
28 (8)	Liposarcoma (WD/DD)	Unevaluable	Not treated	0	No	No	0
29 (8)	Gastrointestinal stromal tumor	**SD**	Toxicity	3	No	No	1
30 (8)	Liposarcoma (WD/DD)	**SD**	POD	15	**YES**	**No**	4
31 (8)	Undifferentiated pleomorphic sarcoma	POD	POD	6	No	No	2

NOTE: Patients are listed in order of accrual to the protocol and treatment cohorts are designated by shading (i.e., cohort 1 includes patients 1–3 whereas cohort 8 includes patients 26–31).
Abbreviation: POD, progression of disease.

On the basis of the results of the clinical study, it is not possible to make a definite determination whether the bolus schedule or the split dosing schedule is preferred for future clinical development of flavopiridol in combination with doxorubicin or more generally in the treatment of sarcoma. Regarding safety, no MTD was reached. Dose-limiting hematologic toxicity was increased with the split dosing regimen and this became more evident with cumulative dosing. Nonhematologic toxicity also became more apparent with cumulative dosing on the divided dose flavopiridol schedule. Unlike studies using a split-dose schedule for the treatment of hematologic malignancies, no evidence of tumor lysis syndrome was observed in this study.

In regard to efficacy, there were 2 partial responses, as well as stable disease as long as 99 weeks. Disease control (PR + SD > 3 months) was documented at various dose levels and was independent of dosing schedules of flavopiridol. Inter-patient variability, especially in dose levels 3, 7, and 8, somewhat confounds the use of PK to determine the most efficacious dose and schedule. The flavopiridol C_{max} generally increased with increasing total flavopiridol dose with all dose cohorts having C_{max} greater than 2 μmol/L. Given this, it also does not appear that continuous flavopiridol exposure via infusion added significant clinical benefit. In view of the overall increased toxicity with the divided dose schedule, the bolus schedule would seem to be preferred for future development of flavopiridol in combination with doxorubicin for the ambulatory treatment of sarcoma.

The combination of flavopiridol and doxorubicin provided a substantial disease control in this study, with 68% (19 of 28) achieving PR or SD as best result. This is especially interesting given that 12 of the 28 evaluable patients had a diagnosis of WD and DD liposarcoma, a disease that is

generally nonchemotherapy responsive but in which CDK4 is frequently amplified (27). Within this subpopulation, the disease control rate was 67%. For the entire study, the progression-free survival rate at 12 weeks ($PFS_{12weeks}$) was 57% (16 of 28) and progression-free survival rate at 24 weeks ($PFS_{24weeks}$) was 32% (9 of 28). Given the heterogeneity of soft tissue sarcomas, the European Organization for Research and Treatment of Cancer (EORTC) has developed standards for evaluating new treatments for soft tissue sarcoma in the phase II setting (28). These standards incorporate progression-free survival and clinical benefit in evaluation of new agents. Notably, in this study, the $PFS_{12weeks}$ and $PFS_{24weeks}$ compare favorably with these reference standards and suggest that this regimen may be worth further exploration in this patient population.

While the benefit of flavopiridol-based therapy in the treatment of WD and DD liposarcoma could be hypothesized to be a function of its *CDK4* amplification, other sarcoma types are not as clearly linked to dysregulated apoptosis. In this study, we note prolonged SD in various tumor types such as leiomyosarcoma, fibrosarcoma, and pleomorphic rhabdomyosarcoma. While these tumors are also associated with chemotherapy responsiveness to anthracyclines, it is possible that doxorubicin was potentiated by flavopiridol. Recent literature has suggested that the predominant mechanism of flavopiridol efficacy is through inhibition of CDK9 (29). This results in suppression of critical antiapoptotic molecules and may lead to the potentiation of chemotherapy. Considering this, a rational mechanism for chemotherapy potentiation by flavopiridol would appear to be promotion of a death response in tumor cells after insult by chemotherapy. Given these observations, further study of flavopiridol in the treatment of WD and DD liposarcomas, and soft tissue sarcomas more generally, is warranted.

Disclosure of Potential Conflicts of Interest

M.A. Dickson and S. Singer are consultants/advisory board members for Pfizer. No potential conflicts of interests were disclosed by other authors.

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