Flavopiridol in patients with relapsed or refractory multiple myeloma: a phase 2 trial with clinical and pharmacodynamic end-points

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Flavopiridol downregulates anti-apoptotic regulators including Mcl-1, upregulates p53, globally attenuates transcription through inhibition of P-TEFb, binds to DNA, and inhibits angiogenesis. Eighteen myeloma patients were treated with 1-hour flavopiridol infusions for 3 consecutive days every 21 days. Immunoblotting for Mcl-1, Bcl-2, p53, cyclin D, phosphoRNA polymerase II and phosphoSTAT 3 was conducted on myeloma cells. *Ex vivo* flavopiridol treatment of cells resulted in cytotoxicity, but only after longer exposure times at higher flavopiridol concentrations than were anticipated to be achieved *in vivo*. No anti-myeloma activity was observed *in vivo*. As administered, flavopiridol has disappointing activity as a single agent in advanced myeloma.

Key words: therapy, cinical trial, Mcl-1.

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Malignant plasma cells in multiple myeloma (MM) are long-lived cells with a very low labeling index and a low apoptotic rate. Dysregulation of cell cycle, apoptosis, and signaling pathways have been implicated in myeloma pathogenesis. Factors including the levels of expressed genes and proteins, and phosphorylation status of cell cycle molecules may all be relevant for propagation of malignant plasma cells.[1]

Flavopiridol is a non-selective antineoplastic inhibitor of cyclin-dependent kinases that produces cell cycle arrest at both G1 and G2/M phases of the cell cycle.[2-5] Suggested mechanisms for flavopiridol-induced cytotoxicity include downregulation of anti-apoptotic regulators, disruption of transcription factor/ DNA binding, upregulation of p53, inhibition/disruption of transcription, and inhibition of angiogenesis.[2,4,6-9] In addition, flavopiridol can induce apoptosis in non-cycling human cancer cell lines,[4,10,11] myeloma cell lines (RPMI 8226, U266, ANBL-6, ARP1, and OPM-2),[12-14] chronic lymphocytic leukemia (CLL) cells treated *ex vivo* and *in vivo*,[15,16] and in squamous cell carcinoma xenografts.[17]

In addition to its potential role in affecting the cell cycle, flavopiridol dramatically and rapidly attenuates Mcl-1 levels in myeloma cell lines and kills myeloma cells *in vitro* and *ex vivo*.[6,14] Additionally, flavopiridol has been shown to reduce the levels of the STAT-3 downstream proteins Mcl-1, p21, cyclins A and D1, and Bcl-XL in the A549 cell line, putatively by attenuating their transcription. Based upon the above data, and because STAT-3 is constitutively active in some interleukin-6-independent myeloma cells,[13,18] we undertook a multicenter phase II Consortium clinical trial to explore the activity of flavopiridol in MM patients.

Design and Methods

After providing written informed consent, 18 patients with myeloma, enrolled between November 2002 and November 2003 in a phase II trial approved by the Institutional Review Board, were administered flavopiridol. All patients had disease progression/relapse as documented by standard criteria.[19] Patients were excluded if: (i) creatinine exceeded 3 mg/dL; (ii) aspartate transaminase and/or alkaline phosphatase exceeded 2.5 times the upper limits of normal; (iii) Eastern Cooperative Oncology Group performance score was 3 or higher; or (iv) neutrophils were less than 750/mm^3. Flavopiridol was administered at a dose of 50 mg/m^2 over 1 hour for 3 consecutive days every 3 weeks. Patients were continued on treatment until disease progression or prohibitive toxicities.

Data analysis was conducted in May 2004, at which time all patients had ceased flavopiridol therapy. Patients were evaluated for response prior to each cycle and were continued on study if they had disease response or stabilization according to EBMTR criteria.[19] The primary end-point was objective response (partial response or better). Toxicity was a secondary end-point. Patients were evaluated every three weeks, and adverse events were recorded according to Common Toxicity Criteria Version 2.0 (CTC 2.0) criteria. The initial design was to be a one-stage

design, testing drug efficacy in 32 patients (0.91 power to detect a hematologic response rate of 20%). However, because there were no responders in the first 18 patients accrued, an interim analysis based on a modified Fleming design (type I error rate=0.07, power=0.9) was undertaken. Under this decision rule, at least one response in the first 18 patients would have been required to justify further accrual, yet no responses were observed. Given the lack of efficacy of this treatment regimen in this population of patients, the trial was permanently closed to accrual effective on December 31, 2003.

Unilateral iliac crest bone marrow aspirates were performed prior to and immediately after flavopiridol therapy, and on day 21 in patients treated with flavopiridol as feasible. Twenty milliliters of marrow aspirate were collected in EDTA tubes, subjected to ammonium chloride (ACK; BioWhittaker, Walkersville, MD, USA) red cell lysis and subsequently CD138 positive selection using MicroBeads and an MS magnetic bead separation column (Miltenyi Biotec, Auburn, CA, USA).

Ex vivo assessment of flavopiridol

After re-suspension of myeloma cells in MEM supplemented with 10% heat-inactivated human serum and 1 μg/mL interleukin-6, aliquots of cells were transferred to tissue culture plate wells for treatment with flavopiridol (125, 250, 500 and 1000 nM). Aliquots of cells were withdrawn at 0, 24, 48 and 72 hours and examined for viability (Trypan blue). Sham/diluent-treated myeloma cells were used as controls.

Immunoblotting

Freshly collected CD138-selected myeloma cells were washed twice with phosphate-buffered saline and solubilized in alkylation buffer [6 M guanidine hydrochloride, 250 mM Tris-HCl (pH 8.5 at 21°C) and 10 mM EDTA] and supplemented immediately before use with 150 mM β-mercaptoethanol and 1 mM α-phenylmethylsulfonyl fluoride. Resulting lysates were processed for SDS-polyacrylamide gel electrophoresis and immunoblotting as previously described.[20] Transfers were probed using the following antibodies: Mcl-1 (BD ParrMingen, San Diego, CA, USA), Bcl-2 (Dako, Glostrup, Denmark), p53 (Neomarkers, Fremont, CA, USA), cyclin D1 (Calbiochem, San Diego, CA, USA), phosphoRNA polymerase II (Covance, Cumberland, VA, USA) phospho(Tyr)STAT3 (Cell Signaling, Beverly, MA, USA), and actin (Santa Cruz, Santa Cruz, CA, USA; loading control).

Table 1. Patients' characteristics.

	*Number or median (range)**
Age, years	65 (49-81)*
Male/Female	11/7
Durie-Salmon stage II/III/missing data	4/11/3
International prognostic staging system, 1/2/ 3	4/8/6
β_2 microglobulin <3.5/3.5 - 5.5/> 5.5	5/7/6
Duration of disease, months	25.8 (7.2-213)*
Prior regimens	3 (1-5)*
Prior high dose therapy	10
Disease status	
Relapse on therapy	9
Relapse off therapy	7
Primary refractory	2
IgG/IgA/Bence Jones/Non-secretory	10/4/3/1
κ/λ/non-secretory	10/7/1
Plasma cell labeling index ≥1%	7
C-reactive protein >0.4	11
Lactate dehydrogenase > upper limit of normal	4
Bone marrow plasma cells >40%	10

Table 2. Adverse events.*

	Grade 3-4 %	*Overall %*
Hematologic		
Anemia	22	44
Leukopenia	28	83
Thrombocytopenia	28	61
Gastrointestinal°		
Diarrhea	17	83
Nausea	0	61
Vomiting	6	33
Infection	0	11
Cardiovascular		
Hypotension	0	17
Constitutional Symptoms		
Fatigue	11	61
Metabolic		
Hypercalcemia	0	11
Hyperglycemia	0	11

**For all adverse events (CTC 2.0) which affected at least 10% of patients;*
°One patient died secondary to gastrointestinal bleeding in the setting of grade 4 thrombocytopenia.

Results and Discussion

Table 1 summarizes the characteristics and disease history of the 18 patients treated on study. Patients received a median of three (range, 1-5) treatment regimens prior to enrollment Sixty-one percent of patients were refractory to prior therapy and 55% had previously undergone high dose therapy with hematopoietic stem cell support. As shown in Table 2, the most frequent adverse effects were leukopenia and diarrhea, followed by thrombocytopenia, nausea, and fatigue. One patient with grade 4 thrombocytopenia deemed not related to flavopiridol therapy died 4 days after starting flavopiridol due to gastrointestinal bleeding. One patient developed renal failure, attributed to progressive myeloma. No thrombotic episodes were observed. All patients have ended the active treatment phase. The mean number of cycles administered was 1.6 (range 1-3). Of the 18 treated patients, three discontinued therapy because of adverse events, 15 due to progression, and one due to death. No indication of anti-myeloma effects of flavopiridol was seen in any study patient.

Sufficient pre-therapy fresh bone marrow plasma cells for studying the *ex vivo* cytotoxicity of flavopiridol were available from six patients. After 48 hours of continuous

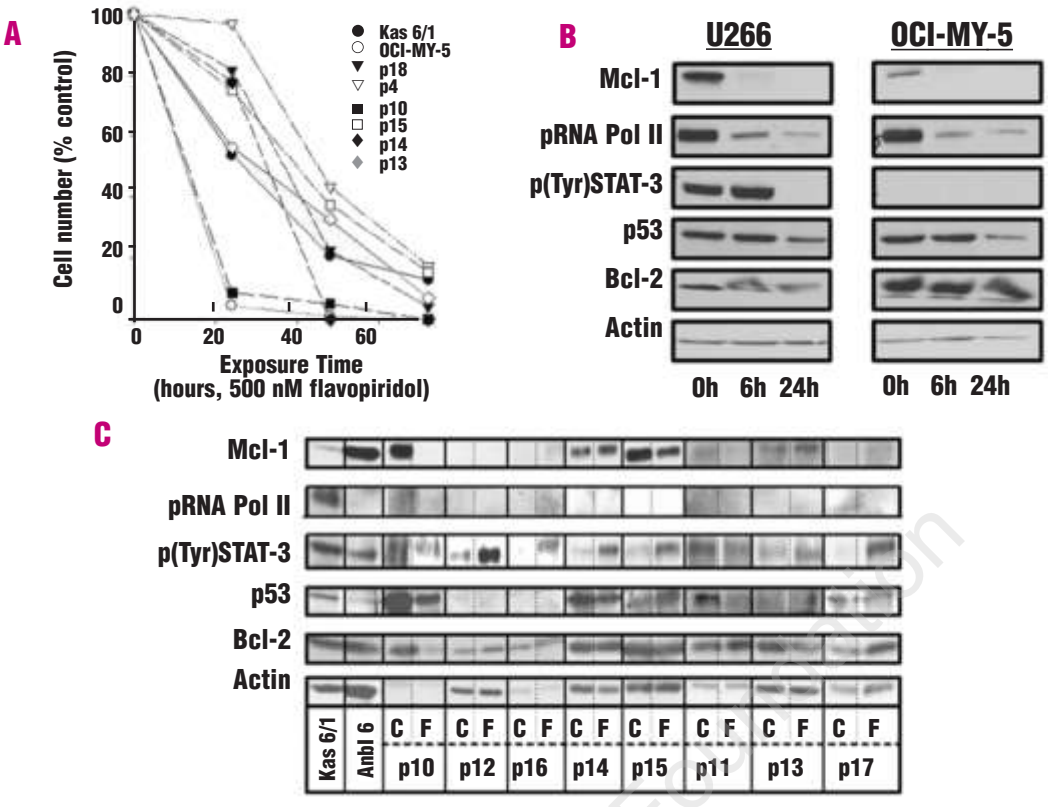

Figure 1. *In vivo* and *ex vivo* effects of flavopiridol. **A.** Flavopiridol inhibits myeloma cell proliferation in both myeloma cell lines and also freshly collected CD138-positive patient myeloma cells exposed to flavopiridol *ex vivo*. All cells were treated with continuous exposure to 500 nM flaviopiridol. Cell numbers were obtained by counting trypan blue-excluding cells at the specified time points using a hemocytometer. In *ex vivo* experiments, the numbers of corresponding sham-treated trypan blue-excluding cells at designated time points served as controls. Kas 6/1 (IL-6 dependent) and OCI-MY-5 (IL-6 independent) served as representative cell lines. With the exception of patient #4, all patients were treated with flavopiridol in the trial. Data from cell lines are representative of three independent experiments, with each data point representing an average of triplicate determinations. Data from patients' samples represent single experiments for each patient, as only limited myeloma cells were available from patients. **B.** Effects of flavopiridol therapy on levels of selected polypeptides in Kas 6/1 and OCI-MY-5 myeloma cell lines. Levels of the indicated polypeptides were assessed by immunoblotting, with 100 μL of total cellular proteins loaded per lane. **C.** Effects of flavopiridol therapy on levels of selected polypeptides in freshly collected CD138 positive cells from study patients. C indicates results in patient MM cells obtained prior to flavopiridol therapy, while F designates results obtained after the third day of three daily flavopiridol infusions for each indicated patient. Levels of indicated polypeptides were assessed by immunoblotting. SDS-PAGE gels were loaded by cell number (generally 2×10^6 cells/lane), and actin is shown as a loading control. Representative data from the Kas6/1 and Anbl 6 MM cell lines are shown on the left.

exposure to flavopiridol (500 nM), there was a variable effect on cell numbers (Figure 1A). In response to this exposure, more than 90% reduction in viability was seen in two patients (patients #10 and 14) and 50-70% reduction in three patients (patients #4, 13 and 15). In patient #18 intermediate cytotoxicity was observed. Because bolus followed by 3- hour flavopiridol infusion has been reported to be quickly cytotoxic to CLL cells *in vivo*,[15] we exposed freshly collected CD138 positive myeloma cells from patients not enrolled in this trial to 1-10 μM flavopiridol for 4, 12 and 24 hours and followed cell survival over time. Although significant cell killing was observed in myeloma cells exposed to these flavopiridol concentrations for 12 or 24 hours, no appreciable flavopiridol-induced cytotoxicity was observed in myeloma cells exposed to flavopiridol cells for only 4 hours (*data not shown*).

Previous *in vitro* studies have demonstrated that Mcl-1, cyclin D, Bcl-2, and phosphoRNA polymerase II decrease in response to flavopiridol treatment, whereas p53 and phospho(Tyr)STAT3 increase.[8,14] Overexpression of Mcl-1 in RPMI-8226 cells has been effective in blocking flavopiridol-induced apoptosis, and early reduction in Mcl-1 correlated with the induction of apoptosis.[13] In our studies of myeloma cell lines, the most consistent flavopiridol-induced changes were decreased Mcl-1 and phosphoRNA polymerase II (Figure 1B). We therefore performed immunoblotting of total cellular proteins from sorted myeloma cells pre-therapy and immediately following the third flavopiridol infusion. Cyclin D1 levels were undetectable in all patients (*data not shown*), and only minimal levels of phosphoRNA polymerase II were detected. The pattern of alterations of cellular polypeptides observed *in vitro* was not generally recapitulated *in vivo* (Figure 1C). In only one of eight assessed patients (patient #15) was decreased Mcl-1 accompanied by an increase in p53 and phospho(Tyr)STAT3 levels. Two other patients (patients #10 and 11) had decreasing levels of Mcl-1 accompanied by unexpectedly lower levels of p53 and p(Tyr)STAT-3. We can only conclude that, although the anticipated effects of flavopiridol were achieved in one patient (#15), the effects were either

short-lived or insufficient to achieve a clinical response as this patient progressed after only one cycle of flavopiridol. This is the first report of a phase II clinical trial of flavopiridol in advanced MM and of its effect on myeloma cell cellular polypeptides *in vivo*. We found that, although reasonably well tolerated, flavopiridol one-hour daily infusions for 3 consecutive days did not provide clinical benefit in patients with heavily pre-treated, refractory MM. Despite a strong rationale and good preclinical data, no cytotoxic or cytostatic activity was observed in our patients. Our *in vitro* and *ex vivo* studies, indicating that sustained exposure to micromolar concentrations of drug[4] are required to achieve significant cytotoxicity, suggest that the current treatment schedule was likely inadequate to achieve these required levels. Exposure to flavopiridol for more than 12 hours was required to achieve cytotoxicity in myeloma cells from patients even when concentrations of flavopiridol were as high as 10 μm (*data not shown*). Currently, loading infusions of flavopiridol followed by maintenance infusions – designed to achieve prolonged micromolar flavopiridol concentrations – are meeting with some success in CLL patients[5] and may be worthy of exploration in MM.

AD, MAG, MQL, SMG, and TF conceived the study. The study was performed as part of the Phase 2 Consortium group under the direction of CE; KB, RF, CI, and SZ performed all the pilot experiments and most of the analysis; KB, RF, CI, and SZ performed the benchwork. AD and KB wrote the manuscript with contributions from other authors. The authors declare that they have no potential conflicts of interest.

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References

1. Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC. Advances in biology of multiple myeloma: clinical applications. Blood 2004;104:607-18.
2. Senderowicz AM. Development of cyclin-dependent kinase modulators as novel therapeutic approaches for hematological malignancies. Leukemia 2001;15:1-9.
3. Kaur G, Stetler-Stevenson M, Sebers S, Worland P, Sedlacek H, Myers C, et al. Growth inhibition with reversible cell cycle arrest of carcinoma cells by flavone L86-8275. J Natl Cancer Inst 1992;84:1736-40.
4. Bible KC, Kaufmann SH. Flavopiridol: a cytotoxic flavone that induces cell death in noncycling A549 human lung carcinoma cells. Cancer Res 1996; 56: 4856-61.
5. Shapiro GI. Preclinical and clinical development of the cyclin-dependent kinase inhibitor flavopiridol. Clin Cancer Res 2004;10:4270S-5S.
6. Kitada S, Zapata JM, Andreeff M, Reed JC. Protein kinase inhibitors flavopiridol and 7-hydroxy-staurosporine down-regulate antiapoptosis proteins in B-cell chronic lymphocytic leukemia. Blood 2000;96:393-7.
7. Bible KC, Bible RH Jr., Kottke TJ, Svingen PA, Xu K, Pang YP, et al. Flavopiridol binds to duplex DNA. Cancer Res 2000;60:2419-28.
8. Lee Y, Kaufmann S, Bible K. Flavopiridol disrupts transcription factor/DNA binding. Proceedings of the American Association for Cancer Research 2001; 42:4876a[Abstract].
9. Chao SH, Price DH. Flavopiridol inactivates P-TEFb and blocks most RNA polymerase transcription in vivo. J Biol Chem 2001;276:31793-9.
10. Parker BW, Kaur G, Nieves-Neira W, Taimi M, Kohlhagen G, Shimizu T, et al. Early induction of apoptosis in hematopoietic cell lines after exposure to flavopiridol. Blood 1998;91:458-65.
11. Schwartz GK, Farsi K, Maslak P, Kelsen DP, Spriggs D. Potentiation of apoptosis by flavopiridol in mitomycin-C-treated gastric and breast cancer cells. Clin Cancer Res 1997;3:1467-72.
12. Akyuz C, Semenov II, Roginskaya VV, Corey S. Flavopiridol-induced growth inhibition and apoptosis of myeloid and myeloma cell lines and blasts. Exp Hematol 2000;28:1497.
13. Gojo I, Zhang B, Fenton RG. The cyclin-dependent kinase inhibitor flavopiridol induces apoptosis in multiple myeloma cells through transcriptional repression and down-regulation of Mcl-1. Clin Cancer Res 2002;8:3527-38.
14. Semenov I, Akyuz C, Roginskaya V, Chauhan D, Corey SJ. Growth inhibition and apoptosis of myeloma cells by the CDK inhibitor flavopiridol. Leuk Res 2002;26:271-80.
15. Byrd JC, Peterson BL, Gabrilove J, Odenike OM, Grever MR, Rai K, et al. Treatment of relapsed chronic lymphocytic leukemia by 72-hour continuous infusion or 1-hour bolus infusion of flavopiridol: results from Cancer and Leukemia Group B study 19805. Clin Cancer Res 2005;11:4176-81.
16. Arguello F, Alexander M, Sterry JA, Tudor G, Smith EM, Kalavar NT, et al. Flavopiridol induces apoptosis of normal lymphoid cells, causes immunosuppression, and has potent antitumor activity in vivo against human leukemia and lymphoma xenografts. Blood 1998;91:2482-90.
17. Patel V, Senderowicz AM, Pinto D Jr, Igishi T, Raffeld M, Quintanilla-Martinez L, et al. Flavopiridol, a novel cyclin-dependent kinase inhibitor, suppresses the growth of head and neck squamous cell carcinomas by inducing apoptosis. J Clin Invest 1998;102:1674-81.
18. Lam LT, Pickeral OK, Peng AC, Rosenwald A, Hurt EM, Giltnane JM, et al. Genomic-scale measurement of mRNA turnover and the mechanisms of action of the anti-cancer drug flavopiridol. Genome Biol 2001;2: RESEARCH0041.
19. Blade J, Samson D, Reece D, Apperley J, Bjorkstrand B, Gahrton G, et al. Criteria for evaluating disease response and progression in patients with multiple myeloma treated by high-dose therapy and haemopoietic stem cell transplantation. Myeloma Subcommittee of the EBMT. European Group for Blood and Marrow Transplant. Br J Haematol 1998; 102:1115-23.
20. Bible KC, Boerner SA, Kirkland K, Anderl KL, Bartelt D Jr, Svingen PA, et al. Characterization of an ovarian carcinoma cell line resistant to cisplatin and flavopiridol. Clin Cancer Res 2000; 6: 661-70.