The Cyclin-dependent Kinase Inhibitor Flavopiridol Induces Apoptosis in Multiple Myeloma Cells through Transcriptional Repression and Down-Regulation of Mcl-1

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ABSTRACT

Multiple myeloma (MM) is a B-cell malignancy characterized by the accumulation of malignant plasma cells with slow proliferative rate but enhanced survival. MM cells express multiple Bcl-2 family members, including Bcl-2, Bcl-XL, and Mcl-1, which are thought to play a key role in the survival and drug resistance of myeloma. The cyclin-dependent kinase inhibitor flavopiridol has anti-tumor activity against hematopoietic malignancies, including CLL, in which induction of apoptosis was associated with reduced expression of antiapoptotic proteins. Therefore, we sought to characterize the effect of flavopiridol on the proliferation and survival of myeloma cells and to define its mechanisms of action. Treatment of MM cell lines (8226, ANBL-6, ARP1, and OPM-2) with clinically achievable concentrations of flavopiridol resulted in rapid induction of apoptotic cell death that correlated temporally with the decline in Mcl-1 protein and mRNA levels. Levels of other antiapoptotic proteins did not change. Overexpression of Mcl-1 protected MM cells from flavopiridol-induced apoptosis. Additional analysis demonstrated that flavopiridol treatment resulted in a dose-dependent inhibition of phosphorylation of the RNA polymerase II COOH-terminal domain, thus blocking transcription elongation. These data indicate that Mcl-1 is an important target for flavopiridol-induced apoptosis of MM that occurs through inhibition of Mcl-1 mRNA transcription coupled with rapid protein degradation via the ubiquitin-proteasome pathway.

INTRODUCTION

MM is an incurable malignancy of B-cell origin characterized by the accumulation of plasma cells with a low proliferation index and an extended life span (1, 2). Approximately 14,000 new cases are diagnosed in this country each year. Glucocorticoids and alkylating agents such as melphalan are used frequently to treat MM, and despite an initial response rate of 40–50%, median survival remains 30–36 months (3–7). Treatment with high-dose chemotherapy and autologous stem cell transplant leads to improvement in event-free and long-term survival but provides no cure for most patients (8–10).

Malignant plasma cells depend on the supportive microenvironment of the BM, where multiple interactions (cell-cell, cell-stroma, and paracrine growth factor/cytokine pathways) occur that are at least partially responsible for up-regulation of antiapoptotic factors in the tumor cells (11–15). IL-6 is an important myeloma cell proliferation and survival factor that is provided in a paracrine fashion by BM stromal cells (16–19). Binding of IL-6 to its receptor on the surface of myeloma cells results in activation of the Ras/mitogen-activated protein kinase and Jak/STAT pathways (20, 21). The former is considered more important for MM cell proliferation (22), whereas STAT3 is thought to play a critical role by promoting transcription of survival genes such as Bcl-XL and Mcl-1 (23, 24). Furthermore, STAT3 was found to be constitutively activated in freshly isolated MM cells and may confer an important survival advantage in vivo (23).

Under selection from therapeutic agents, genetically unstable myeloma cells become resistant to apoptotic signals through a variety of mechanisms. The expression of antiapoptotic members of the Bcl-2 family appears to be one important factor in the acquisition of clinical resistance by MM cells. Bcl-2 has been found to be highly expressed in malignant plasma cells (25–27). Bcl-XL expression was detected more frequently in patients at relapse and was shown to correlate with resistance to chemotherapy (28, 29). Bcl-XL was reported to be induced by IL-6 in a STAT3-dependent manner and promote resistance to Fas-induced apoptosis in U266 cells (23, 30). Mcl-1 is distinct from...
Bcl-2 and Bcl-XL in size ($M_r 40,000$ compared with $M_r 26,000$), and it contains a 180 amino acid long NH$_2$-terminal segment that shares no homology with other Bcl-2 family members (31, 32). This region of Mcl-1 encodes proline, glutamic acid, serine, threonine rich sequences (PEST) that may account for its short half-life (1–2 h compared with 24 h for Bcl-2; Refs. 32, 33). Recent data from our laboratory indicate that Mcl-1 is a critical survival factor for MM cells (34). The inhibition of Mcl-1 expression by antisense oligodeoxynucleotides led to the rapid induction of apoptosis in MM cells, whereas overexpression of Mcl-1 in MM cells delayed the activation of caspases in a manner consistent with the hypothesis that a threshold level of Mcl-1 is required for MM cell survival (34). A role for Mcl-1 in survival of acute myeloid leukemia and CLL cells has also been postulated (35–39). Therefore, the development of therapeutics that alter Mcl-1 expression in malignant cells may prove useful in the treatment of a wide variety of hematologic malignancies including MM.

Flavopiridol is a semisynthetic flavonoid that was originally identified as a CDK inhibitor (40–43). Flavopiridol competes with ATP for binding in the active site of many CDKs, and can induce either cell cycle arrest or apoptosis, depending on the cell type, culture conditions, and drug concentration (40, 42, 44). Possible antitumor mechanisms of flavopiridol include a decrease in the levels of cyclin D1 and cyclin D3, and an antiangiogenic activity that blunts vascular endothelial growth factor production (40, 42, 45). Flavopiridol is now known to globally inhibit mRNA transcription by inhibition of kinases that phosphorylate the CTD of RNA polymerase II (46, 47). Phosphorylation of the CTD is required for transcription elongation; hence, flavopiridol inhibits RNA polymerase II-dependent transcription of cellular mRNA. Originally it was thought that the action of flavopiridol was mediated by inhibition of CDK7/cyclin H (48). In addition to its role as a CDK activating kinase, this kinase is a component of TFIH and as such has a positive regulatory role in transcription elongation mediated by its phosphorylation of the CTD (48, 49). Recent data demonstrate that the P-TEFb (CDK9/cyclin T1) may be the more important CTD kinase (50). Flavopiridol is a potent inhibitor of P-TEFb, and it is likely that its inhibitory effect on transcription elongation is because of inhibition of P-TEFb (51, 52).

Flavopiridol has been shown to be effective in inducing apoptosis in a variety of hematopoietic neoplasms (35, 53–57). Acute myeloid leukemia and CLL are known to express Mcl-1, and it is likely that its inhibitory effect on transcription elongation is because of inhibition of P-TEFb (51, 52).

Western Blot Analysis. Western blotting was performed as described previously (34). The following primary Abs were used: anti-Mcl-1 (rabbit polyclonal Ab S-19; band size $M_r 40,000$ from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; anti-Bcl-XL ($M_r 26,000$) rabbit polyclonal Ab (Transduction Laboratories, Lexington, KY); anti-BAG-1 ($M_r 50,000$, 46,000, and 33,000 isoforms) monoclonal Abs (3.9F1E11 + 3.10G3E2; Neomarkers, Inc., Fremont, CA); anti-XIAP ($M_r 57,000$) mouse monoclonal Ab clone 48 (BD Biosciences, San Jose, CA); anti-cyclin D2 ($M_r 34,000$) rabbit polyclonal Ab sc-181 (Santa Cruz); antihuman Bcl-2 ($M_r 26,000$) mouse monoclonal Ab.

MATERIALS AND METHODS

Cell Lines. The MM cell lines 8226, ARP1, OPM-2, and ANBL-6 were kindly provided by Dr. Guido Tricot (Arkansas Cancer Research Center, Little Rock, AR) and Dr. Diane Jelinek (Mayo Clinic, Rochester, MN). For generation of the cell lines 8226-Mcl-1 and 8226-Bcl-XL, 8226 cells were infected with the VSV-G-pseudotyped viruses encoding either pH’-HA-Mcl-1 or pH’-HA-Bcl-XL as described previously (34). The ARP1-Bcl-2 cell line has been described previously (58). Cells were cultured in RPMI 1640 supplemented with 100 IU/ml penicillin G, 100 µg/ml streptomycin, 2 mM l-glutamine, 10% FBS (all from Life Technologies, Inc., Gaithersburg, MD), and 1 ng/ml recombinant human IL-6 (R&D Systems, Minneapolis, MN). Growth characteristics and relevant genetic lesions in these cell lines were described previously (34). Flavopiridol was obtained from the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, NIH. DRB was purchased from Sigma Chemical Co. (St. Louis, MO).

TUNEL Assays and Hoechst Staining. TUNEL assays were performed using the ApopTag Plus Fluorescein In Situ Apoptosis Detection kit according to the manufacturer’s instructions (Intergen Company, Purchase, NY). After the TUNEL reaction, slides were washed in PBS, counterstained with 1 µm Hoechst 33342 (Sigma), and mounted using Vectashield Mounting Medium (Vector Laboratories, Inc., Burlingame, CA). Fluorescent micrographs were taken using a Zeiss Axiovert 10 microscope (Zeiss, Thornwood, NY) and digitally recorded using Oncor Imaging System Software (Oncor, Gaithersburg, MD).

Caspase Assays. Cell lysates were prepared as reported previously (34). Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). Caspase assays were performed in 96-well plates using Ac-DEVd-AMC fluorogenic substrate (PharMingen, San Diego, CA) as described previously (34). Measurement of the AMC cleavage product was performed using a CytoFluor II Microplate Fluorescence Reader (PerSeptive Biosystems, Inc., Bedford, MA) with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Experiments were performed in triplicate, and results were expressed as the mean ± SD.
clone 124 and anti-phospho-RNA polymerase II (M, 250,000) mouse monoclonal Ab clone CTD4H8 (both from Upstate Biotechnology, Lake Placid, NY); anti-RNA polymerase II unphosphorylated (M, 210,000) rabbit polyclonal Ab sc-900 (Santa Cruz); anti-ABCG2 polyclonal Ab, 87405 (kindly provided by Dr. Douglas Ross); and anti-actin (M, 42,000) polyclonal Ab I-19 (Santa Cruz). Horseradish peroxidase-conjugated secondary antimouse or antirabbit antibodies were used, and the signal was detected by ECL (Amersham Biosciences, Corp., Piscataway, NJ).

Northern Blot Analysis. Total cell RNA was isolated using the TRIzol reagent (Life Technologies, Inc.) as suggested by the manufacturer. RNA was size fractionated on denaturing formaldehyde gels and transferred to Hybond-N+ membranes (Amersham). Mcl-1 mRNA was detected by hybridization to a full-length 32P-labeled Mcl-1 cDNA probe in Hybrisol I (Oncor). Mcl-1 mRNA was detected by hybridization to a

Isolation of CD138+ Myeloma Cells. Mononuclear cells were isolated from BM aspirates by Ficoll-Hypaque centrifugation, washed extensively, and incubated with anti-CD138-coated microbeads as per the manufacturer’s instructions (Milenyi Biotec, Auburn, CA). CD138+ cells were purified by magnetic separation, washed, and then cultured under conditions described in the text. Purified MM cells were >95% positive for clonal κ or λ light chain.

RESULTS

Flavopiridol Induces Apoptosis in MM Cell Lines. Experiments were designed to determine whether flavopiridol had antitumor activity against a panel of MM cell lines at clinically achievable concentrations. As shown in Fig. 1, when MM cells were cultured in 200 nM of flavopiridol, there was a rapid induction of apoptosis as measured by TUNEL assay. Apoptotic cells were noted at 3 h, and their number rapidly increased over a 24 h period. Almost all of the ANBL-6 and ARP1 cells were dead at 24 h, whereas 8226 cells appeared to be less sensitive than the other cell lines. Because previous data indicated an important role for Mcl-1 in MM cell survival (34), we determined whether overexpression of Mcl-1 would protect MM cells from flavopiridol-induced apoptosis. As shown in Fig. 1, overexpression of Mcl-1 in 8226 Mcl-1 cells was highly effective in blocking flavopiridol-induced apoptosis (see 8226-Mcl-1 cell data). Caspase activity was examined in a subset of the MM cell lines, was induced within the 3 h in flavopiridol-containing cultures of 8226 and ANBL-6, and reached high levels by 6 and 9 h (Fig. 2A); in 8226-Mcl-1 cells caspase activation was greatly delayed. An example of flavopiridol-induced apoptotic nuclear changes in the ARP1 cell line is shown in Fig. 2B. Taken together, these data demonstrate that flavopiridol induces the rapid apoptosis of MM cell lines and that Mcl-1 overexpression can protect against flavopiridol-induced death.

A dose-response study of flavopiridol was performed. 8226, ANBL-6, and 8226-Mcl-1 were cultured in complete medium plus IL-6 with increasing concentrations of flavopiridol, and the percentage of survival was determined on days 1, 3, and 5 (Fig. 3). Apoptotic cell death was observed beginning at 50 nM for ANBL-6 and 8226, whereas higher levels were required to kill 8226-Mcl-1 (Fig. 3, A–C). The IC50 for flavopiridol (i.e., the dose that induced 50% apoptosis at 24 h) was 100 nM for ANBL-6, between 100–200 nM for 8226, and was ≥200 nM for 8226-Mcl-1. At the 200 nM dose level, overexpression of Mcl-1 did not prevent flavopiridol-induced death but delayed its onset.

A Decline in Mcl-1 Level Correlates with the Onset of Flavopiridol-Induced Apoptosis in MM Cells. The relative ratio of proapoptotic (e.g., Bax and Bak) and antiapoptotic (Bcl-2, Bcl-XL, and Mcl-1) Bcl-2 family members is an important determinant of cell sensitivity to a variety of noxious stimuli including chemotherapeutic agents (59, 60). Culture of primary CLL cells in flavopiridol has been shown previously to inhibit Mcl-1 protein expression in a manner that correlated temporally with the onset of apoptosis (35). Other antiapoptotic proteins such as XIAP and BAG-1 showed a similar response (35). Therefore, we determined the effects of flavopiridol on a panel of proteins known to regulate the mitochondrial pathway of apoptosis. As shown in Fig. 4A, culture of ANBL-6 in 200 nM of flavopiridol induced a rapid decline in Mcl-1 protein levels observed within 3 h, with barely detectable levels observed after 6 h. The levels of Bcl-XL, BAG-1, and XIAP did not decrease, except at 14 h when most ANBL-6 were dead (suggesting nonspecific protein degradation). For 8226 cells (Fig. 4B), there was a decrease in Mcl-1 levels at 3 and 6 h, which correlated with the early apoptosis observed in these cells; surprisingly, levels of Mcl-1 increased at 9 and 14 h. This was reproduced in numerous experiments. The levels of Bcl-XL, BAG-1, and XIAP in 8226 extracts were not affected by flavopiridol. The patterns of Mcl-1 expression observed in ARP1 (Fig. 4C) and OPM-2 (Fig. 4D) demonstrated decreased Mcl-1 levels at 3 h that were barely detectable at 6 and 9 h; this correlated with massive apoptosis observed in these cells during this time period. However, although most of the cells were dead at 14 h (i.e., 95% of ARP1 and 70–80% of OPM-2, see Fig. 1 and Fig. 2B),
an increase in Mcl-1 protein was again noted at 14 h. Bcl-XL levels decreased slightly during this time but were fairly stable. Possible mechanisms to explain the “late” increase in Mcl-1 levels will be discussed below. Note, however, that for all four of the cell lines the rapid flavopiridol-induced decrease in Mcl-1 levels (3–6 h) correlated with the induction of apoptosis.

To better understand the unusual pattern of Mcl-1 re-expression encountered in the largely apoptotic flavopiridol-treated cell populations, we examined cyclin D2 levels in the same cell extracts (Fig. 4, A–D). Cyclin D2 shares certain characteristics with Mcl-1 that made it useful for comparison. Both proteins are short-lived and, for both, the levels of expression within the cell are regulated by the ubiquitin-proteasome pathway (32, 61). In addition, D-type cyclins are thought to be a target of flavopiridol (40, 42). Interestingly, for all four of the cell lines, the levels of cyclin D2 (the most highly expressed D-family cyclin in our cell lines) followed the kinetics of Mcl-1 expression. For ANBL-6 (Fig. 4A) the levels of cyclin D2 were clearly decreased at 6 h and remained suppressed at 14 h. A
A discernible decline in cyclin D2 levels was observed in 8226 cells at 6 and 9 h followed by an increase at 14 h (Fig. 4B). For ARP1 (Fig. 4C) and OPM-2 (Fig. 4D), cyclin D2 levels initially declined but were shown to increase at 14 h. Thus, the patterns of expression of Mcl-1 and cyclin D2 were very similar and are presumed to be related mechanistically.

The hypothesis that Mcl-1 is a key target for the apoptosis-inducing activity of flavopiridol predicts that inhibition of Mcl-1 expression should occur at doses of flavopiridol that also induce apoptosis. To test this, ANBL-6 was cultured for 6 h in the presence of increasing concentrations of flavopiridol (Fig. 5). Mcl-1 protein levels were inhibited at 100 nM and were totally suppressed at 200 nM, doses that were highly effective at inducing apoptosis of this cell line (Fig. 3A). Therefore, the flavopiridol-induced decline in Mcl-1 protein levels correlated with the dose shown to induce apoptosis in MM cells.

Changes in Mcl-1 mRNA Expression Levels Induced by Flavopiridol. To determine the mechanism by which flavopiridol inhibited Mcl-1 expression, we examined Mcl-1 mRNA levels in flavopiridol-treated MM cells. ANBL-6, 8226, ARP1, and OPM-2 cells were treated with 200 nM flavopiridol for the indicated periods of time, and Northern blot analysis of Mcl-1 mRNA was performed (Fig. 6). For ANBL-6, Mcl-1 mRNA levels decreased rapidly throughout the 14 h culture period, correlating with the changes in Mcl-1 protein levels. In 8226, Mcl-1 mRNA levels followed the course of the protein as well: an initial decrease was followed by an increase apparent at 9 h. For ARP1, levels decreased dramatically but a small in-
Inhibition of Mcl-1 by Flavopiridol in MM

Flavopiridol Inhibits the Phosphorylation of the RNA Polymerase II CTD in MM Cells. We were interested in determining the mechanism by which flavopiridol, a CDK inhibitor, could inhibit Mcl-1 mRNA transcription. RNA polymerase II exists in two “phosphorylation states” within the cell, termed IIA and IIO, which differ in the extent of phosphorylation of the CTD (48–50). The CTD contains 52 tandem repeats of a heptapeptide with the sequence Tyr-Ser-Pro-Thr-Ser-Pro-Thr, which contains multiple potential targets for CDK kinases (48–50, 62). RNA polymerase II with an unphosphorylated CTD binds to promoters as a part of the preinitiation complex but cannot elongate mRNA >50 bp from the initiation site; transcript elongation requires the activity of kinases that phosphorylate CTD at the multiple sites in the repeat sequences (48, 49, 62). Proteins with CTD kinase activity include CDK7/cyclin H (CDK activating kinase), CDK9/cyclin T1, casein kinase II, DNA-dependent protein kinase, and others (48, 49). Most recently, it has been suggested that one of the mechanisms responsible for flavopiridol-induced cell death is a block in transcription elongation related to its ability to inhibit CDK7/cyclin His (48, 49, 50). ANBL-6 and ARP1 (Fig. 4, arrowhead) and a broad band of lower molecular weight, partially phosphorylated species. Treatment of ANBL-6 (Fig. 4A) resulted in loss of fully phosphorylated CTD; this occurred within 90 min and persisted throughout the course of the experiment. For 8226 cells (Fig. 4B), the fully phosphorylated form of CTD initially declined, but it was never fully inhibited, and increased at 14 h. For ARP1 (Fig. 4C) and OPM-2 (Fig. 4D), levels of fully phosphorylated CTD decreased significantly during the first 6 h, but there is a trend toward a more highly phosphorylated form at 14 h. The unexpected increase in more highly phosphorylated forms of the RNA polymerase II CTD observed at the 14 h culture period correlated with the increase in expression levels of Mcl-1 mRNA and protein observed in three of the MM cell lines.

Flavopiridol inhibits the phosphorylation of Mcl-1 protein level is already observed at 3 h, and progresses was noted at 14 h. In OPM-2 cells, Mcl-1 mRNA levels nadired at 6 h but showed an increase at 9 and 14 h. Therefore, the flavopiridol-induced changes in Mcl-1 protein levels observed in Fig. 4 correlate with the changes in mRNA levels. Note that by 14 h most of the cells of all four cell lines were apoptotic (especially ANBL-6 and ARP1), suggesting that the observed increases in Mcl-1 mRNA level may be a paradoxical manifestation of the apoptotic process altering rates of Mcl-1 gene transcription and mRNA half-life in the few remaining viable cells (discussed below).

Overexpression of Bcl-XL or Bcl-2 Inhibits Flavopiridol-induced Apoptosis. The previous data suggest that inhibition of Mcl-1 expression by flavopiridol results in cell death by activation of the mitochondrial pathway of apoptosis. This can be inhibited by overexpression of Mcl-1 as shown in Fig. 1 and Fig. 2A. This hypothesis additionally predicts that overexpression of other antiapoptotic Bcl-2 family members could replace lost Mcl-1 at the mitochondrial outer membrane and prevent apoptosis. To test this, we compared the sensitivity to flavopiridol of the 8226-Bcl-XL and ARP1-Bcl-2 cell lines (which overexpress Bcl-XL and Bcl-2 respectively, see Fig. 7B) to the parental cell lines. As shown in Fig. 7A, apoptosis is significantly reduced by overexpression of either protein. These data support the hypothesis that flavopiridol targets the mitochondrial apoptosis pathway and that antiapoptotic Bcl-2 family members inhibits its action. However, Bcl-XL and Bcl-2 protein levels are not affected by flavopiridol, suggesting that it is the loss of the Mcl-1 expression that lowers the apoptotic threshold and induces the mitochondrial events.

Dose effect of flavopiridol on Mcl-1 expression and on phosphorylation status of the RNA polymerase II CTD. ANBL-6 cells were cultured in complete medium containing IL-6 and flavopiridol at the concentrations indicated. Extracts from each culture were prepared at two time points: for analysis of Mcl-1 and actin protein levels, cells were harvested at 6 h; for evaluation of the level of RNA polymerase II CTD phosphorylation (a much earlier effect of flavopiridol), cells were harvested after 90 min. Western blot analysis was performed using antibodies directed against Mcl-1, phosphorylated CTD, and unphosphorylated CTD. Actin levels are included as a control for equal protein loading. The arrowhead indicates the position of the most highly phosphorylated species of RNA polymerase II.
to undetectable levels at 14 h. 8226 cells showed a similar pattern, with a continuous drop in Mcl-1 levels with no "late" increase in levels. The levels of Bcl-XL remained unaffected by DRB. Furthermore, for both ANBL-6 and 8226, the decline in the highly phosphorylated form of CTD correlated with observed decrease in Mcl-1 protein; the levels declined rapidly and remained inhibited at 14 h. Therefore, whereas early effects of DRB and flavopiridol may be the same, the observed increase in CTD phosphorylation and Mcl-1 protein levels at the late time point in flavopiridol-treated cells appears to be unique to this drug.

**Decreased Sensitivity of 8226 to Flavopiridol Is Not Mediated by ABCG2 (BCRP).** The reversal of flavopiridol-induced inhibition of CTD phosphorylation and subsequent increase in Mcl-1 mRNA and protein levels was most pronounced in the 8226 cell line. Furthermore, no such reversal of drug effect was noted using DRB, a mechanistically related compound. One possible explanation for this would be de novo or induced expression of drug efflux pumps in the flavopiridol-treated 8226 cells. Recent studies suggest that neither multidrug resistance protein nor P-glycoprotein play a role in the resistance to flavopiridol; however, an ATP-binding cassette half-transporter ABCG2 (BCRP) may be important (66–68). Culture of the human breast cancer cell line MCF-7 in incremental doses of flavopiridol resulted in the development of resistant subline MCF-7 FLV1000 that overexpressed ABCG2 but not other drug transporters (66). Therefore, we performed experiments to determine whether decreased sensitivity of 8226 cells could be related to ABCG2 overexpression and drug efflux. No expression of ABCG2 was detected in untreated 8226 cells, and its expression was not induced by culture in flavopiridol (data not shown). Therefore, these data do not indicate a role for ABCG2-mediated drug efflux as a mechanism to explain the apparent reversal of flavopiridol effects in 8226 cells.

**Effects of Flavopiridol on Primary Patient MM Samples.** Experiments were designed to determine the effects of flavopiridol on primary MM cells. BM samples from patients in relapse after autologous stem cell transplant were collected and CD138/H11001 cells isolated. All such samples were >95% CD138/H11001 and were light-chain restricted. Although in some cases the number of purified MM cells limited a full analysis, we attempted to culture CD138+ cells with and without IL-6 (in the absence of drug), and in the presence of actinomycin D (1 μg/ml) or flavopiridol (200 nM). The data from 4 such patients are shown in Fig. 9. MM cells from patient 1 express easily detectable levels of Mcl-1 and Bcl-2 (but undetectable Bcl-XL, not shown; Fig. 9A). After culture in actinomycin D or flavopiridol for 20 h, there was a near complete disappearance of Mcl-1.

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**Fig. 6** Decreased transcription of Mcl-1 mRNA in MM cell lines treated with flavopiridol. 8226, ANBL-6, ARP1, and OPM-2 were cultured in complete medium containing IL-6 plus flavopiridol (200 nM). At the times indicated, RNA was isolated and Northern blot analysis performed using an Mcl-1 cDNA probe. Arrowheads indicate the position of the two Mcl-1 mRNA species. The bottom panels show ethidium bromide-stained gels that demonstrate equivalent RNA loading.

**Fig. 7** Overexpression of Bcl-XL or Bcl-2 inhibits flavopiridol-induced apoptosis. A, 8226, 8226-Bcl-XL, ARP1, and ARP1-Bcl-2 cell lines were cultured in RPMI 1640/10% FBS + IL-6 (1 ng/ml) and flavopiridol (200 nM). Cytospins were prepared at time 0 and after 24 h culture in flavopiridol and TUNEL assays were performed. For each time point, >200 cells were analyzed for determination of percentage of apoptosis. B, protein extracts from each of the cell lines were prepared and Western blot analysis was performed. The target of the primary Ab is indicated along the left margin. Actin levels are included as a control for equal protein loading; bars, ±SD.
Mcl-1 levels in MM cells of patient 2 also decreased after a 6-h incubation in either drug, whereas levels of Bcl-2 and Bcl-XL did not change (Fig. 9B). Cells from patient 3 also demonstrated a rapid decline in Mcl-1 levels after a 6-h incubation in either drug, without changes in Bcl-2 or Bcl-XL. Aliquots of these cultures were analyzed for apoptosis by TUNEL assays of cytospin preparations, and as shown in Fig. 9C, drug treatment resulted in high levels of apoptosis that correlated with Mcl-1 decline. Similar induction of apoptosis and loss of Mcl-1 expression were noted for patient 4 (Fig. 9D). These patient samples are representative of all of the samples with adequate numbers of CD138-H11001 cells. We conclude that the mechanism of flavopiridol-induced apoptosis that we have defined for MM cell lines also functions in primary MM samples.

**DISCUSSION**

Flavopiridol, a novel antineoplastic agent with CDK-inhibitory activity, induced the rapid apoptosis of a panel of MM cell lines, which correlated with a decline in Mcl-1 protein levels. This is consistent with data reported for CLL and MM cells in which flavopiridol-induced death was associated with reduced Mcl-1 expression (35, 69). Certain properties of Mcl-1, such as the short half-life of its mRNA and protein (32, 70), appear to be unique among Bcl-2 family members. The levels of other antiapoptotic Bcl-2 family members known to be expressed in myeloma cells, such as Bcl-2 and Bcl-XL, were not affected by treatment of these cells with flavopiridol. Similarly, in other hematopoietic cell lines Bcl-2 protein levels were not affected by treatment with flavopiridol, perhaps because of its long half-life (40, 57, 71). However, even when down-regulation of Bcl-2 was observed, no correlation between the cytotoxic action of flavopiridol and its potency to reduce expression of Bcl-2 was observed (35, 40, 72, 73).

It has been postulated that other short-lived proteins involved in apoptotic processes such as XIAP and BAG-1 could be down-regulated by flavopiridol and as such could be at least
 partially responsible for flavopiridol-induced death in CLL cells \(35\). XIAP is a member of the IAP family that directly inhibits selected caspase family members \(74, 75\), whereas BAG-1 is a regulator of the heat shock protein 70 family that appears to control a distal apoptosis checkpoint downstream of Bcl-2 \(76, 77\). However, in MM cells treated with flavopiridol, XIAP and BAG-1 levels remained stable and, therefore, are unlikely to play a direct role in flavopiridol-induced MM cell death.

The importance of Mcl-1 for the survival of MM cells was reported recently by our group \(34\). The specific depletion of Mcl-1 by antisense oligodeoxynucleotides resulted in rapid myeloma cell death, whereas Mcl-1 overexpression provided protection against transcription inhibitors such as actinomycin D or DRB, which otherwise induce rapid apoptosis of MM cells \(34\). Interestingly, the degree of resistance to actinomycin D correlated with the level of Mcl-1 overexpression and is consistent with a model in which a critical threshold of Mcl-1 is required for viability of MM cells. In the current study, we show that overexpression of Mcl-1 in 8226 cells delays flavopiridol-induced caspase activation and DNA degradation from 3 h in parental 8226 cells to 14 h in 8226-Mcl-1 cells (Fig. 1 and Fig. 24). These data support the hypothesis that Mcl-1 is a critical survival factor for MM \(34\) and a key apoptosis-inducing target of flavopiridol. Flavopiridol treatment of MM cell lines did not affect Bcl-2 or Bcl-XL protein levels and, therefore, these proteins are unlikely to play a role in the rapid apoptotic cell death observed. However, overexpression of either Bcl-2 or Bcl-XL in MM cell lines inhibited flavopiridol-induced apoptosis. This suggests that overexpression of other antiapoptotic Bcl-2 family members can replace the lost Mcl-1 at the outer mitochondrial membrane and increase the threshold for flavopiridol-induced cell death.

Flavopiridol is known to inhibit a variety of CDKs; however, the specific mechanism by which flavopiridol induces apoptosis has been unclear \(40, 42, 53\). The cytotoxic activity of flavopiridol is not limited to cycling cells but includes resting cells as well \(78, 79\). Therefore, the inhibition of CDKs involved in the control of cell cycle cannot be the sole mechanism of flavopiridol activity, and inhibition of CDKs with additional functions must contribute to the antitumor effect. It now appears that flavopiridol targets RNA polymerase II-mediated transcription elongation by inhibiting the kinases required for the phosphorylation of the CTD \(45, 51, 52\). Two kinases have been implicated, namely TFIIH-associated CDK7/cyclin H and P-TEFb composed of CDK9 and cyclin T1 and P-TEFb. Although these kinases may act sequentially to regulate phosphorylation of the CTD, P-TEFb appears to be the critical CTD kinase based on its inhibition by DRB, its ability to reconstitute full CTD phosphorylation by addition of CDK9 and cyclin T1 to in vitro transcription assays, and the loss of CTD-kinase activity when P-TEFb is immunodepleted from these reactions \(50, 80, 81\). Furthermore, recent data indicate that P-TEFb is negatively regulated by association with the small nuclear RNA 7SK, and that this complex is a key regulator of CTD phosphorylation and of global mRNA synthesis in response to cellular stress \(82, 83\). Taken together, these observations suggest that the transcriptional effects of flavopiridol may result from a CDK-related mechanism and that our understanding of CDK action must now be broadened to include this possibility.

Our data are consistent with a model in which flavopiridol-induced apoptosis of MM cells is mediated by inhibition of phosphorylation of the CTD. Flavopiridol induced a rapid, dose-dependent inhibition of CTD phosphorylation that correlated with the induction of caspase activity, the appearance of TUNEL-positive MM cells, and a decline in Mcl-1 levels. These data are consistent with a recent study of flavopiridol-induced changes in the global pattern of gene transcription in the large cell lymphoma cell line OCI-Ly3 \(47\). Using microarray technology, these investigators identified many short half-life mRNAs and were able to categorize these into groups of genes encoding proteins that regulate the cell cycle or apoptosis, and a group of transcriptionally induced genes that encode transcription factors, cytokines, chemokines, and their receptors \(47\). Mcl-1 was identified in this screen, as were other antiapoptotic proteins including A1 and members of the IAP family. Given our previous data demonstrating the importance of Mcl-1 for MM cell survival \(34\), the rapid, dose-dependent decline in Mcl-1 levels induced by flavopiridol, and protection from flavopiridol-induced apoptosis by its overexpression, we suggest that Mcl-1 is a critical target for flavopiridol-induced apoptosis in MM cells. Clearly, it is possible that other short-lived targets of flavopiridol-mediated transcription repression may also play a role in the observed antitumor effects.

An unexpected finding was the increase in Mcl-1 mRNA and protein levels in three MM cell lines observed 9–14 h after treatment with flavopiridol, a time when the majority of cells \(e.g.,\) ARP1 and OPM-2) were apoptotic by TUNEL assay. DRB, a known inhibitor of P-TEFb, also blocked CTD phosphorylation, inhibited Mcl-1 expression, and induced apoptosis of MM cells, yet did not demonstrate any reversal of CTD phosphorylation or Mcl-1 expression at any time during culture. Thus, the latter phenomenon appears to be unique to flavopiridol. We speculate that there was a population of MM cells that were not yet apoptotic (or in its early stages) in which there was a significant increase in steady-state levels of Mcl-1 mRNA. This must include de novo synthesis of Mcl-1 mRNA, because the levels actually increase (changes in mRNA half-life cannot account for the increase observed). This finding suggests that there must be relief from inhibition of transcription elongation caused by the effects of flavopiridol on P-TEFb. Consistent with this, there was an increase in the level of CTD phosphorylation in the 8226, ARP1, and OPM-2 cell lines, but not ANBL-6. That this occurs is perplexing, because it has been reported that flavopiridol binding to P-TEFb is of very high affinity with a very slow off rate (not competitive with ATP), suggesting that once bound, significant P-TEFb activity would be unlikely to result from drug dissociation \(52\). We also considered the possibility that resistance to flavopiridol could have been because of up-regulation of the ABC-transporter (BCRP; Ref. 66), but such was not the case. Experiments are in progress to directly assay P-TEFb kinase activity during culture in flavopiridol to determine whether kinase activity is reactivated in the 9–14 h period. Although we cannot at this time define the molecular events responsible for Mcl-1 up-regulation, two points are critical. First, the vast majority of cell death correlates temporally with the decline in Mcl-1 to nearly undetectable levels after 6 h of culture. Secondly, in the 5-day cultures, no cells remain viable, and resistant clones do not appear. We
Inhibition of Mcl-1 by Flavopiridol in MM

Rajkumar, S. V., and Greipp, P. R. Prognostic factors in multiple myeloma and the positive control cell line T8. "suggest that late up-regulation of Mcl-1 occurs as the result of the apoptotic process because of an "unbalanced" destruction of cell regulatory proteins and does not represent selection of a flavopiridol-resistant population of tumor cells.

In conclusion, this study indicates that flavopiridol may be an effective drug in the treatment of myeloma. MM cells are known to use multiple survival pathways to avoid apoptosis (2, 84–86), which renders them resistant to a variety of therapeutic interventions. However, it appears that inhibition of Mcl-1 may overcome one or more of these protective mechanisms, making Mcl-1 a particularly attractive target for the development of new therapeutics. The data in this report are in agreement with this hypothesis and support clinical trials of flavopiridol for treatment of MM. Furthermore, our data provide a basis for the laboratory monitoring of key potential drug targets, including Mcl-1 and RNA polymerase II CTD phosphorylation state as possible in vitro correlates of response of MM to flavopiridol.

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REFERENCES

The Cyclin-dependent Kinase Inhibitor Flavopiridol Induces Apoptosis in Multiple Myeloma Cells through Transcriptional Repression and Down-Regulation of Mcl-1

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