

The small-molecule Bcl-2 inhibitor HA14-1 interacts synergistically with flavopiridol to induce mitochondrial injury and apoptosis in human myeloma cells through a free radical-dependent and Jun NH₂-terminal kinase-dependent mechanism

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Abstract

Interactions between the cyclin-dependent kinase inhibitor flavopiridol and the small-molecule Bcl-2 antagonist HA14-1 were examined in human multiple myeloma cells. Whereas individual treatment of U266 myeloma cells with 10 μmol/L HA14-1 or 100 nmol/L flavopiridol had little effect, exposure of cells to flavopiridol (6 hours) followed by HA14-1 (18 hours) resulted in a striking increase in mitochondrial dysfunction (cytochrome *c* and Smac/DIABLO release; loss of mitochondrial membrane potential), activation of the caspase cascade, apoptosis, and diminished clonogenic survival. Similar findings were noted in other myeloma cell lines (e.g., MM.1S, RPMI8226, and NCI-H929) as well as in those resistant to dexamethasone and cytotoxic agents (e.g., MM.1R, 8226/Dox40, and 8226/LR5). Combined exposure to flavopiridol and HA14-1 was associated with down-regulation of Mcl-1 and Bcl-x_L, Bid cleavage, and mitochondrial translocation of Bax. Flavopiridol/HA14-1-treated cells also exhibited a pronounced activation of Jun NH₂-terminal kinase, a modest activation of p38 mitogen-activated protein kinase, and down-regulation of cyclin D1. Flavopiridol/HA14-1-induced apoptosis was associated with a marked increase in reactive oxygen species generation; moreover, both events were attenuated by the antioxidant *N*-acetyl-L-cysteine. Finally, in contrast to dexamethasone, flavopiridol/HA14-1-induced lethality was unaffected by exogenous interleukin-6 or insulin-like growth factor-I. Together, these findings indicate that flavopiridol and the small-molecule Bcl-2 antagonist HA14-1 cooperate to

trigger oxidant injury, mitochondrial dysfunction, caspase activation, and apoptosis in human multiple myeloma cells and suggest that this approach may warrant further evaluation as an antimyeloma strategy. [Mol Cancer Ther 2004;3(12):1513–24]

Introduction

Multiple myeloma represents a generally incurable plasma cell disorder that arises from the dysregulated growth and survival of differentiated plasma cells. Until relatively recently, treatment approaches for multiple myeloma have consisted of cytotoxic agents (e.g., melphalan, doxorubicin, and vincristine), corticosteroids, and, for those patients who are eligible, bone marrow transplantation (1). However, recent advances have increased understanding of the pathophysiology of multiple myeloma, including characterization of the role of stromal cells and growth factors such as interleukin (IL)-6 and insulin-like growth factor-I (IGF-I) in multiple myeloma pathogenesis as well as the delineation of signal transduction pathways that contribute to enhanced survival of myeloma cells (2). These efforts have prompted intense interest in novel, molecularly targeted agents for myeloma therapy, including angiogenesis inhibitors (3), small-molecule kinase inhibitors (4), histone deacetylase inhibitors (5), etc. In particular, the introduction of the proteasome inhibitor bortezomib (Velcade) into the clinic seems likely to have a significant impact on the natural history of this disease (6).

Another such agent currently being explored in multiple myeloma is the cyclin-dependent kinase (CDK) inhibitor flavopiridol (NSC 649890, L86-8275, or HMR 1275), a rohitukine alkaloid that binds tightly to the CDK ATP binding site and broadly inhibits multiple CDKs, including CDKs 1, 2, 4/6, and 7 (7). In so doing, flavopiridol induces perturbations in various proteins involved in cell cycle regulation, including E2F1, leading in some cases to tumor cell death in S phase (8). Flavopiridol is the first of the CDK inhibitors to enter the clinic (9). In preclinical studies, flavopiridol seems to be highly lethal to malignant hematopoietic cells, inducing apoptosis at submicromolar concentrations (10). In lung cancer cells, flavopiridol has been reported to trigger apoptosis via the extrinsic, receptor-related pathway (11), although in human leukemia cells flavopiridol-induced mitochondrial injury (e.g., cytochrome *c* release) seems to be the primary mode of lethality (12). Recently, attention has focused on the capacity of flavopiridol to inhibit the cyclin T/CDK9 positive transcription elongation factor b complex and in so doing to act as a transcriptional repressor (13). In fact,

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flavopiridol-induced inhibition of phosphorylation of the carboxyl-terminal domain of RNA polymerase II has been correlated with induction of apoptosis in multiple myeloma cells (14). Furthermore, this event is accompanied by down-regulation of the antiapoptotic protein Mcl-1, an important survival factor for myeloma cells (14, 15). Flavopiridol has also been reported to induce down-regulation of cyclin D1 (16), the dysregulation of which is commonly encountered in multiple myeloma (17). For each of these reasons, as well as accumulating preclinical evidence that multiple myeloma cells are highly sensitive to flavopiridol (18), phase II trials of flavopiridol in myeloma and related malignancies are currently under way.

Bcl-2 represents a member of a large number of evolutionarily conserved proteins that play critical roles in regulating the cell death process and maintaining cellular homeostasis. Bcl-2, like several of its homologous proteins, including Bcl-x_L, Mcl-1, A1, Bag, etc., blocks apoptosis (19). In contrast, various proapoptotic members, particularly those of the multidomain subfamily (e.g., Bax and Bak) and BH3-only domain subfamily (e.g., Bad and Bid), promote cell death (20, 21). Although the mechanism by which Bcl-2 blocks apoptosis has not been completely elucidated, it is thought to interfere with the ability of proapoptotic proteins such as Bax and Bak to homodimerize or heterodimerize and to form mitochondrial membrane channels through which death-promoting mitochondrial proteins such as cytochrome *c* are released (22). Bcl-2 has also been shown to protect cells from certain forms of oxidative stress (23). Significantly, increased expression of Bcl-2 can protect neoplastic cells from cytotoxic drugs (24) and correlates with poor clinical outcome in some hematologic malignancies (25). For this reason, intensive efforts have been under way to develop strategies to circumvent the cytoprotective effects of Bcl-2 in neoplastic cells. One approach involves the use of Bcl-2 antisense oligonucleotides, which have been shown to promote drug-induced lethality in transformed cells (26). However, another approach has been to use small-molecule inhibitors of Bcl-2 such as HA14-1, which binds to the Bcl-2 surface pocket and disrupts its antiapoptotic function (27). HA14-1 has been shown to sensitize breast cancer cells to the lethal effects of epothilone B (28) and, more recently, human leukemia cells to pharmacologic inhibitors of the mitogen-activated protein kinase (MAPK) kinase-1/2-extracellular signal-regulated kinase (ERK) pathway (29). Such findings suggest that the rational combination of Bcl-2 antagonists such as HA14-1 with either standard or novel agents deserves further attention.

There are several reasons why the concept of combining HA14-1 with flavopiridol is attractive, particularly in the case of multiple myeloma. For the reasons stated above, flavopiridol is currently undergoing investigation as an antimyeloma agent. Because flavopiridol induces mitochondrial injury, at least in some malignant hematopoietic cells (12), the notion that HA14-1 might amplify this process seems plausible. In addition, multiple myeloma cells are known to express relatively high levels of Bcl-2 (30); hence,

attempts to overcome the cytoprotective effects of this protein are rational. To address these issues, we have examined the effects of combining HA14-1 with flavopiridol in multiple myeloma cells, including those resistant to standard cytotoxic agents, with respect to apoptosis. Here, we report that HA14-1 and flavopiridol interact in a highly synergistic and sequence-dependent manner to promote mitochondrial injury and cell death through a process that involves generation of reactive oxygen species (ROS). Together, these findings suggest that a strategy combining small-molecule Bcl-2 inhibitors with flavopiridol deserves further investigation in multiple myeloma and related malignancies.

Materials and Methods

Cells and Reagents

The human multiple myeloma cell lines U266, NCI-H929, and RPMI8226 were purchased from American Type Culture Collection (Rockville, MD). The dexamethasone-sensitive (MM.1S) and dexamethasone-resistant (MM.1R) human multiple myeloma cell lines were kindly provided by Dr. Steven T. Rosen (Northwestern University, Chicago, IL; ref. 31). Cells were maintained in RPMI 1640 containing 10% fetal bovine serum as reported previously (32). Doxorubicin-resistant subline (Dox40) and melphalan-resistant subline (LR5) of 8226 cells were kindly provided by Dr. William S. Dalton (University of South Florida, Tampa, FL; ref. 33) and maintained in RPMI 1640 as described above containing 400 nmol/L doxorubicin and 5 μmol/L melphalan, respectively.

The pan-CDK inhibitor flavopiridol was kindly provided by Dr. Edward Sausville (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD). A cell-permeable, low molecular weight Bcl-2 inhibitor HA14-1 was purchased from Biomol (Plymouth Meeting, PA). These reagents were dissolved in DMSO as a stock solution and stored at -80°C . *N*-acetyl-L-cysteine (L-NAC, Calbiochem, San Diego, CA) was prepared in sterile water immediately before use. Dexamethasone (Sigma, St. Louis, MO) was dissolved in DMSO, aliquoted, and stored at -20°C . Recombinant human IL-6 (Sigma) and IGF-I (R&D Systems, Minneapolis, MN) were rehydrated in PBS and 10 mmol/L acetic acid (both of which contain 0.1% bovine serum albumin), aliquoted, and stored at -80°C . Caspase inhibitor BOC-D-fmk (Enzyme System Products, Livermore, CA) was dissolved in DMSO and stored at 4°C . In all experiments, the final concentration of DMSO did not exceed 0.1%.

Experimental Format

Logarithmically growing cells ($4-6 \times 10^5/\text{mL}$) were used in all experiments. Cell suspensions were placed in sterile 25 cm² T-flasks (Corning, Corning, NY) and pretreated with flavopiridol for 6 hours at 37°C . At the end of this period, HA14-1 was added without washing the cells free of flavopiridol, and cells were further incubated at $37^{\circ}\text{C}/5\% \text{CO}_2$ at various intervals. In some studies, cells were pretreated with L-NAC for 3 hours prior to flavopiridol. BOC-D-fmk, IL-6, or IGF-I was added concurrently with

flavopiridol. Alternatively, in some experiments, cells were simultaneously exposed to these agents or to the reverse sequence (e.g., HA14-1 for 6 hours followed by flavopiridol). After drug treatment, cells were harvested and subjected to further analysis as described below.

Assessment of Apoptosis

The extent of apoptosis was evaluated by assessing Wright-Giemsa-stained cytospin slides under light microscopy and scoring the number of cells exhibiting classic morphologic features of apoptosis. For each condition, 5 to 10 randomly selected fields per slide were evaluated, encompassing at least 800 cells. To confirm the results of morphologic analysis, in some cases, apoptosis was also determined by Annexin V-FITC staining and flow cytometry as described previously (34). Annexin V positive/propidium iodide (PI) negative and Annexin V positive/PI positive reflect early and late apoptosis, respectively. In all cases, results of morphologic analysis correlated highly with results of Annexin V/PI staining ($r > 0.90$).

Cell Survival and Clonogenic Assays

For cell viability assays [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)], Cell Titer 96 Aqueous One Solution (Promega, Madison, WI) was used as per the manufacturer's instructions, and the absorbance at 490 nm was recorded using a 96-well plate reader (Molecular Devices, Sunnyvale, CA). Colony-forming ability following drug treatment was evaluated using a soft agar cloning assay as described previously (35). Briefly, cells were washed three times with serum-free RPMI. Subsequently, 500 cells per well were mixed with RPMI containing 20% fetal bovine serum and 0.3% agar and plated on 12-well plates (three wells per condition). The plates were then maintained in a 37°C/5% CO₂, fully humidified incubator. After 15 days of incubation, colonies consisting of >50 cells were scored using an inverted microscope (model CK, Olympus, Tokyo, Japan), and colony formation for each condition was calculated in relation to values obtained for untreated control cells.

Analysis of Mitochondrial Membrane Potential

Cells (2×10^5) were incubated with 40 nmol/L 3,3-dihexyloxycarbocyanine (Molecular Probes, Inc., Eugene, OR) in PBS at 37°C for 20 minutes and then analyzed by flow cytometry. The percentage of cells exhibiting decreased level of 3,3-dihexyloxycarbocyanine uptake, which reflects loss of mitochondrial membrane potential ($\Delta\Psi_m$), was determined using FACScan (Becton Dickinson, San Jose, CA).

Western Blot Analysis

Western blot samples were prepared from whole cell pellets as described previously (35). Total protein was quantified using Coomassie protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein (30 μ g) were separated by SDS-PAGE and electrotransferred onto nitrocellulose membrane. For analysis of protein phosphorylation, 1 mmol/L of each sodium vanadate and sodium pyrophosphate was added to 1 \times sample buffer, no SDS was included in the transfer buffer, and TBS was used instead of PBS throughout. The blots were probed with

primary antibodies as followed. Where indicated, the blots were reprobbed with actin antibody (BD PharMingen, San Diego, CA) or tubulin antibody (Transduction Laboratories, San Diego, CA) to ensure equal loading and transfer of proteins. The primary antibodies included poly(ADP-ribose)polymerase (PARP) antibody (Biomol), anti-caspase-8 (Alexis, San Diego, CA), anti-caspase-9 (BD PharMingen), anti-caspase-3 (Transduction Laboratories), anti-human Bcl-2 oncoprotein (DAKO, Carpinteria, CA), Bcl-x_{S/L} antibody (S-18, Santa Cruz Biotechnology, Santa Cruz, CA), Bid antibody (Cell Signaling, Beverly, MA), Bax antibody (Santa Cruz Biotechnology), Bak antibody (Santa Cruz Biotechnology), survivin antibody (R&D Systems), Mcl-1 antibody (S-19, Santa Cruz Biotechnology), phospho-p44/42 MAPK/ERK (Thr²⁰²/Tyr²⁰⁴) antibody (Santa Cruz Biotechnology), phospho-Jun NH₂-terminal kinase (JNK; Thr¹⁸³/Tyr¹⁸⁵) antibody (Santa Cruz Biotechnology), stress-activated protein kinase/JNK antibody (Cell Signaling), phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) antibody (Santa Cruz Biotechnology), p53 antibody (Santa Cruz Biotechnology), and cyclin D1 antibody (BD PharMingen).

Analysis of Cytosolic Cytochrome *c* and Smac/DIABLO

Cells (4×10^6) were lysed by incubating in digitonin lysis buffer (75 mmol/L NaCl, 8 mmol/L Na₂HPO₄, 1 mmol/L NaH₂PO₄, 1 mmol/L EDTA, and 350 μ g/mL digitonin). The lysates were centrifuged at 12,000 \times *g* for 1 minute and the supernatant, consisting of the cytosolic S-100 fraction, was collected in an equal volume of 2 \times sample buffer. The proteins were quantified, separated by 15% SDS-PAGE, and subjected to Western blot as described above. Cytochrome *c* antibody (BD PharMingen) and Smac/DIABLO antibody (Upstate Biotechnology, Lake Placid, NY) were used as primary antibodies.

Analysis of Bax Translocation

After drug treatment, cells were lysed in digitonin lysis buffer as described above. After centrifugation at 12,000 \times *g* for 1 minute, the supernatant (cytosol fraction) was collected in an equal volume of 2 \times sample buffer and the pellet was lysed by sonication in 1 \times sample buffer as described above in Western blot analysis. For both cytosol and pellet fractions, the proteins were quantified, separated by 15% SDS-PAGE, and subjected to Western blot by using Bax antibody as primary antibody.

Measurement of Cellular ROS Production

Dichlorodihydrofluorescein, which is nonfluorescent in its dihydro form but becomes highly fluorescent on reaction with ROS, was used to monitor production of cellular ROS (29). Briefly, 2×10^5 cells were incubated with 10 μ mol/L aceto-oxyethyl ester of dichlorodihydrofluorescein (Molecular Probes) in PBS at 37°C for 30 minutes and then analyzed by flow cytometry. The production of ROS was determined by comparing increased intensity of dichlorodihydrofluorescein for drug-treated versus untreated control cells.

Statistical Analysis

For morphologic assessment of apoptosis, $\Delta\Psi_m$, ROS production, MTT, and clonogenic assays, values represent

the means \pm SD for at least three separate experiments in triplicate experiments. The significance of differences between experimental variables was determined using the Student's *t* test. Analysis of synergism was done according to median dose effect analysis (36) using a commercially available software program (Calculusyn, Biosoft, Ferguson, MO).

Results

Combined Exposure to HA14-1 and Flavopiridol Results in a Sequence-, Concentration-, and Time-Dependent Increase in Apoptosis in U266 cells

U266 myeloma cells were exposed to HA14-1 (10 μ mol/L) and flavopiridol (100 and 200 nmol/L) according to three schedules: simultaneous (24 hours, schedule 1), flavopiridol (6 hours) followed by HA14-1 for an additional 18 hours (schedule 2), and HA14-1 for 6 hours followed by flavopiridol for 18 hours (schedule 3). At the end of this period, the extent of apoptosis was assessed using flow cytometric analysis of Annexin V/PI-stained cells. As shown in Fig. 1A, whereas exposure to flavopiridol or HA14-1 alone resulted in only modest degrees of cell death, cells exposed to flavopiridol followed by HA14-1 (schedule 2) experienced a marked increase in apoptosis, reflected by an increase in Annexin V/PI positivity (i.e., \sim 75% of cells). In contrast, simultaneous administration (schedule 1) or the sequence HA14-1 followed by flavopiridol (schedule 3) resulted largely additive interactions (Fig. 1B). Consequently, for all subsequent studies, a treatment paradigm employing flavopiridol pretreatment (6 hours) followed by addition of HA14-1 (schedule 2) was employed.

Median dose effect analysis of apoptosis induction by flavopiridol and HA14-1 given at a fixed ratio (1:90) yielded combination indices of <1.0 , corresponding to a synergistic interaction (Fig. 1C). Time course studies (Fig. 1D) revealed an increase in cell death for sequential administration of flavopiridol and HA14-1 as early as 6 hours after drug administration, with further increases in apoptosis occurring over the ensuing 12 hours.

Finally, dose-response studies revealed that HA14-1 increased the lethality of flavopiridol toward U266 cells when the latter was given at concentrations as low as 50 nmol/L and had even greater effects at higher flavopiridol concentrations (Fig. 1E). HA14-1 significantly potentiated flavopiridol lethality at concentrations as low as 8 μ mol/L, although effects were most pronounced at concentrations of ≥ 10 μ mol/L (Fig. 1F).

Flavopiridol and HA14-1 Interact Synergistically to Induce Apoptosis in MM.1S, MM.1R, and H929 Multiple Myeloma Cells

Parallel studies were done in MM.1S and MM.1R cells as well as in H929 myeloma cells. As shown in Fig. 2A, exposure of each of the cell lines to flavopiridol (100 nmol/L) or HA14-1 (10 μ mol/L) individually resulted in minimal increases in apoptosis, whereas sequential exposure to flavopiridol for 6 hours followed by HA14-1 for an

additional 12 hours (MM.1S and MM.1R) or 18 hours (H929) resulted in a pronounced increase in cell death. Moreover, effects of altering the sequence of treatment (e.g., 6-hour flavopiridol followed by 12-hour HA14-1, 18-hour simultaneous exposure to flavopiridol and HA14-1, or 6-hour HA14-1 followed by 12-hour flavopiridol) were also evaluated in MM.1S cells. Consistent with results noted in U266 cells (Fig. 1A and B), the sequence of flavopiridol followed by HA14-1 (sequence 1) yielded clearly synergistic effects, whereas simultaneous treatment or the sequence HA14-1 followed by flavopiridol resulted in predominantly additive effects (Fig. 2B). Thus, sequential exposure of several multiple myeloma cell types, including those resistant to dexamethasone, to flavopiridol followed by HA14-1 resulted in a marked increase in apoptosis.

Apoptosis Induced by Flavopiridol/HA14-1, in Contrast to That Induced by Dexamethasone, Is Not Attenuated by Exogenous IL-6 or IGF-I

In view of evidence that cytokine signaling networks, particularly those involving IL-6, can protect myeloma cells from the lethal actions of conventional cytotoxic agents (37), an attempt was made to determine whether similar events occurred in cells exposed to flavopiridol in conjunction with HA14-1. As shown in Fig. 2C, 24-hour treatment of U266 myeloma cells with 100 nmol/L flavopiridol plus 10 μ mol/L HA14-1 strikingly increased apoptosis. However, cotreatment with 100 ng/mL IL-6 or 400 ng/mL IGF-I failed to protect cells from the lethal effects of these agents ($P > 0.05$). In marked contrast, coadministration IL-6 or IGF-I essentially abrogated the apoptotic response of U266 cells to 100 mol/L dexamethasone ($P < 0.01$ versus cells exposed to dexamethasone in the absence of IL-6 or IGF-I). Such findings suggest that lethality of the flavopiridol/HA14-1 regimen in myeloma cells occurs independently or downstream of IL-6-mediated or IGF-I-mediated cytoprotective signaling events.

Multiple Myeloma Cells Resistant to Melphalan or Doxorubicin Retain Their Susceptibility to the Flavopiridol/HA14-1 Regimen

To determine whether mechanisms conferring resistance to conventional cytotoxic agents would be operative in the flavopiridol/HA14-1 regimen, 8226 myeloma cells and their LR5 and Dox40 counterparts were employed. The latter cells have been shown by our group and others to be highly resistant to the lethal effects of melphalan and doxorubicin, respectively (38, 39). However, MTT assays revealed that the loss of survival (\sim 50%) in LR5 and Dox40 cells after flavopiridol/HA14-1 exposure was equivalent to that of parental 8226 cells ($P > 0.05$ in each case; Fig. 2D), suggesting the absence of cross-resistance to this regimen.

Synergistic Interactions between Flavopiridol and HA14-1 in Multiple Myeloma Cells Are Associated with Loss of $\Delta\Psi_m$ and Clonogenic Survival

Consistent with the previous findings, exposure of U266 or MM.1S myeloma cells to flavopiridol or HA14-1 individually resulted in relatively modest reductions in $\Delta\Psi_m$, whereas effects were considerably more pronounced

following sequential exposure of cells to flavopiridol (6 hours) followed by HA14-1 (U266, 18 hours; MM.1S, 12 hours; Fig. 3A). Thus, sequential exposure of multiple myeloma cells to flavopiridol followed by HA14-1 resulted in a marked increase in mitochondrial injury.

Previous studies have shown that induction of apoptosis does not necessarily correlate with loss of clonogenic potential (40). Therefore, clonogenic assays were done to determine whether combined exposure to flavopiridol and HA14-1 would diminish the self-renewal capacity of multiple myeloma cells. As shown in Fig. 3B, exposure to flavopiridol (100 nmol/L) or HA14-1 (10 μ mol/L) individually reduced colony formation by only a modest extent (i.e., 20–25%). However, sequential treatment resulted in a

substantial loss of clonogenic potential (i.e., 75–80%). These findings indicate that the flavopiridol/HA14-1 regimen displays significant toxicity toward self-renewing multiple myeloma cells.

Sequential Exposure of U266 and MM.1S Myeloma Cells to Flavopiridol and HA14-1 Results in Cytochrome *c* and Smac/DIABLO Release Accompanied by Caspase Activation

As shown in Fig. 4, exposure of U266 or MM.1S to flavopiridol (100 nmol/L) or HA14-1 (10 μ mol/L) individually had little or no effect on release of cytochrome *c* or Smac/DIABLO into the S-100 cytosolic fraction. However, a substantial increase in release of these proteins was observed in cells exposed to flavopiridol for 6 hours

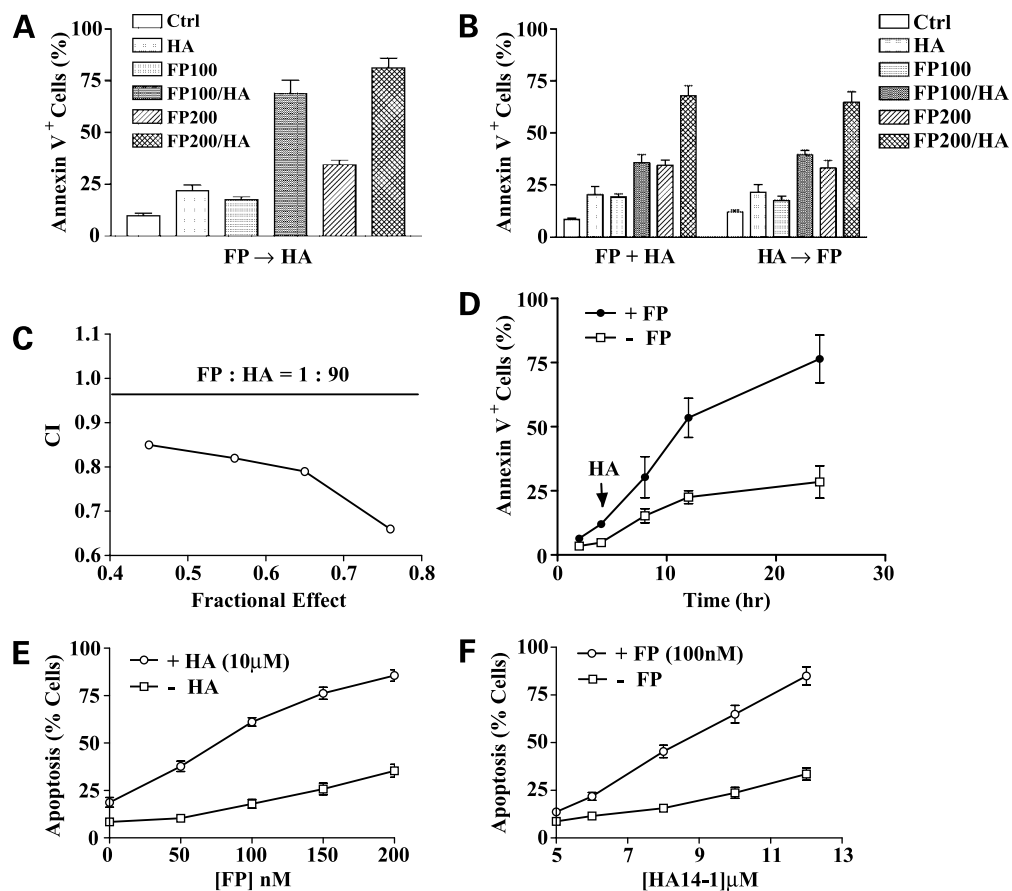


Figure 1. Sequential exposure to flavopiridol followed by HA14-1 synergistically induces apoptosis in multiple myeloma cells. **A**, U266 cells were pretreated with 100 or 200 nmol/L flavopiridol (FP) for 6 hours followed, without washing, by 10 μ mol/L HA14-1 (HA) for 18 hours, after which the percentage of apoptotic cells (Annexin V⁺) was monitored by Annexin V-FITC staining and flow cytometry as described in Materials and Methods. **B**, U266 cells were incubated simultaneously either with 100 or 200 nmol/L flavopiridol \pm 10 μ mol/L HA14-1 for 24 hours (left columns) or with 10 μ mol/L HA14-1 for 6 hours followed by 100 or 200 nmol/L flavopiridol for 18 hours (right columns), after which Annexin V assays were done as above. **A** and **B**, columns, means of three separate experiments in triplicate; bars, SD. **C**, median dose effect analysis was employed as described in Materials and Methods to characterize the nature of the interaction between flavopiridol and HA14-1 given at a fixed ratio of 1:90 in U266 cells. End points were apoptosis determined by evaluation of Wright-Giemsa-stained cytospin preparations. For each fraction affected, a combination index (CI) was calculated. Combination indices of <1.0 correspond to a synergistic interaction. Two additional studies yielded equivalent results. **D**, U266 cells were treated with HA14-1 after 6-hour pretreatment with flavopiridol. At the indicated intervals, the percentage of apoptotic cells was determined by Annexin V assay as above. **E**, U266 cells were pretreated with a range of flavopiridol concentrations for 6 hours, after which they were exposed to 10 μ mol/L HA14-1 for an additional 18 hours. **F**, alternatively, U266 cells were pretreated with 100 nmol/L flavopiridol for 6 hours followed by an exposure to the indicated concentration of HA14-1 for an additional 18 hours. In each case, the percentage of cells exhibiting apoptotic morphology was determined by evaluation of Wright-Giemsa-stained cytospin preparations. **D**, **E**, and **F**, points, means of three separate experiments in triplicate; bars, SD.

followed by HA14-1 for an additional 18 hours (U266) or 12 hours (MM.1S). Moreover, sequential treatment was associated with a pronounced increase in PARP degradation and enhanced cleavage/activation of caspase-3, caspase-9, and caspase-8. These findings are consistent with those shown in Fig. 3A and indicate that flavopiridol and HA14-1 cooperate to induce mitochondrial dysfunction and caspase activation in several multiple myeloma cell types.

Effects of Sequential Exposure of Myeloma Cells to Flavopiridol and HA14-1 on Proapoptotic and Antiapoptotic Bcl-2 Family Members

U266 and MM.1S cells were exposed to flavopiridol with or without HA14-1 as above, after which the expression of various Bcl-2 family members was monitored. For both cell types, combined but not individual exposure to these agents resulted in the formation of a Bcl-2 cleavage product (Fig. 5), which has been reported to exert proapoptotic effects (41). Treatment with flavopiridol alone or the combination of flavopiridol followed by HA14-1 was associated with down-regulation of the antiapoptotic protein Bcl-x_L. Finally, only combined treatment resulted in a slight loss of full-length Bid. No major changes were observed in levels of total Bax or Bak or in expression of the antiapoptotic protein survivin in either cell line.

The intracellular disposition of Bax was also examined. In both cell lines, HA14-1 induced a modest translocation of Bax to the pellet (mitochondrial membrane) fraction accompanied by cytoplasmic depletion, events associated with induction of apoptosis (42). However, combined treatment with flavopiridol and HA14-1 resulted in an even greater redistribution of Bax to the mitochondria.

Exposure of Myeloma Cells to Flavopiridol and HA14-1 Leads to Activation of JNK and Down-Regulation of Cyclin D1

The effects of combined treatment with flavopiridol and HA14-1 was then examined with respect to activation of signaling pathways as well as expression of cyclin D1, the dysregulation of which has been observed in multiple myeloma cells (43). Although exposure of U266 and MM.1S cells to flavopiridol or HA14-1 alone resulted in modest increases in phosphorylation/activation of the stress-related kinase JNK, combined treatment resulted in a marked increase in JNK activation (Fig. 6A). A modest increase in p38 MAPK activation was noted in U266 cells exposed to HA14-1 with or without flavopiridol, although no effects were observed in MM.1S cells. Activation of ERK and expression of p53 remained essentially unchanged for all treatment conditions. Flavopiridol alone

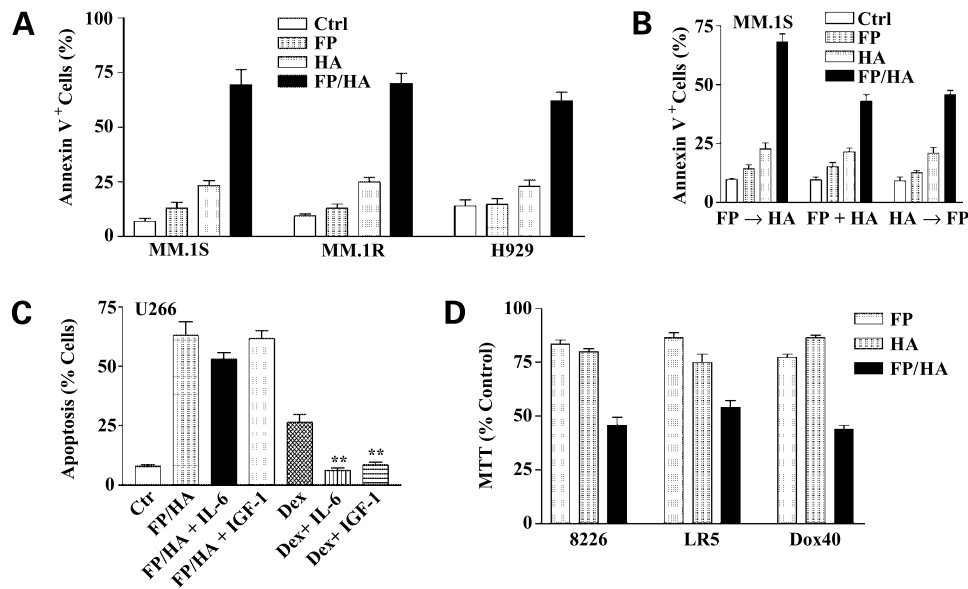


Figure 2. Flavopiridol/HA14-1 regimen effectively induces apoptosis in several multiple myeloma cell lines, including drug-resistant cells. **A**, MM.1S, MM.1R, and H929 multiple myeloma cells were sequentially treated for 6 hours with 100 nmol/L flavopiridol followed by 10 μ mol/L HA14-1 for 12 hours (MM.1S and MM.1R) or 18 hours (H929), after which the percentage of apoptotic cells was evaluated by Annexin V-FITC staining and flow cytometry. **B**, MM.1S cells were treated with 100 nmol/L flavopiridol for 6 hours followed by 10 μ mol/L HA14-1 for 12 hours (*left columns*), simultaneously with flavopiridol and HA14-1 for 18 hours (*middle bars*), or with HA14-1 for 6 hours followed by flavopiridol for 12 hours (*right columns*), after which Annexin V assays were done as above. **C**, U266 cells were incubated with 10 μ mol/L HA14-1 for 18 hours following a 6-hour pretreatment of 100 nmol/L flavopiridol in the presence or absence of 100 ng/mL IL-6 or 400 ng/mL IGF-I. At the end of this period, the percentage of cells exhibiting apoptotic morphology was determined by evaluating Wright-Giemsa-stained cytospin preparations. For comparison, U266 cells were exposed to 100 μ mol/L dexamethasone (*Dex*) for 24 hours in the presence or absence of IL-6 or IGF-I. **, $P < 0.05$, significantly lower than the values for dexamethasone-treated cells in the absence of IL-6 or IGF-I. **D**, 8226 multiple myeloma cells and their LR5 and Dox40 counterparts were incubated for 6 hours with 150 nmol/L flavopiridol followed by an exposure to 7.5 μ mol/L HA14-1 for 20 hours, after which the MTT assay was used to monitor cell survival and proliferation. **A** to **D**, *columns*, means of three separate experiments in triplicate; *bars*, SD.

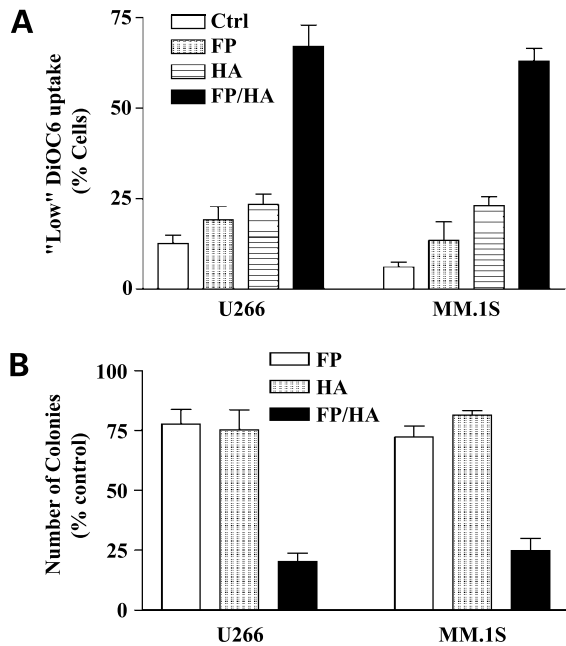


Figure 3. Coadministration of flavopiridol and HA14-1 results in mitochondrial damage and diminished clonogenic potential in multiple myeloma cells. **A**, U266 and MM.1S cells were pretreated with 100 nmol/L flavopiridol for 6 hours followed by an incubation with 10 μ mol/L HA14-1 for 18 hours (U266) or 12 hours (MM.1S), after which the percentage of cells exhibiting loss of $\Delta\Psi_m$ was determined by 3,3-dihexyloxycarbocyanine (*DiOC₆*) staining and flow cytometric analysis as described in Materials and Methods. **B**, alternatively, cells were treated as above. Cells were washed free of drug and plated in soft agar as described in Materials and Methods. After 15 days of incubation, colonies (consisting of groups of >50 cells) were scored and colony formation for each condition was expressed relative to untreated control cells. **A** and **B**, columns, means of three separate experiments in triplicate; bars, SD.

or in combination with HA14-1 induced a slight but discernible decrease in phosphorylation (activation) of carboxyl-terminal domain of RNA polymerase II, which is mediated by cyclin T/CDK9 (positive transcription elongation factor b), consistent with previous reports (13, 14). It is noteworthy that combined treatment also resulted in a modest decline in total levels of polymerase II. In addition, the combination of flavopiridol with HA14-1 resulted in cleavage as well as in a slight reduction in expression of Mcl-1, a key survival protein in myeloma cells (15). Finally, flavopiridol-treated cells displayed a modest reduction in expression of cyclin D1 as described previously in breast cancer cells (16). Moreover, this effect was slightly more pronounced in cells exposed to flavopiridol and HA14-1. Together, these findings indicate that exposure of myeloma cells to flavopiridol and HA14-1 results in activation of the stress-related kinases JNK and p38 MAPK accompanied by cyclin D1 down-regulation.

Flavopiridol/HA14-1-Induced Down-Regulation of Mcl-1, Bcl-x_L, and Cyclin D1 in Myeloma Cells Proceeds through a Caspase-Dependent Mechanism

To determine whether down-regulation of several of the proteins described above might reflect caspase-dependent

phenomena, U266 cells were exposed to flavopiridol (100 nmol/L, 6 hours) followed by HA14-1 (10 μ mol/L, 18 hours) in the presence or absence of the pan-caspase inhibitor BOC-D-fmk (20 μ mol/L). As shown in Fig. 6B, coadministration of BOC-D-fmk significantly attenuated flavopiridol/HA14-1-induced apoptosis ($P < 0.02$ compared with controls). It also markedly blocked PARP degradation as well as formation of a Bcl-2 cleavage fragment (Fig. 6C). In addition, BOC-D-fmk substantially reversed flavopiridol/HA14-1-mediated down-regulation of Bcl-x_L and cyclin D1, indicating that these events were at least in part secondary to activation of the apoptotic caspase cascade. It is noteworthy that the pan-caspase inhibitor BOC-D-fmk almost completely blocked cleavage of Mcl-1, whereas the decline in total levels of Mcl-1 was only partially reversed. Taken in conjunction with evidence of reduced carboxyl-terminal domain phosphorylation (Fig. 6A), such findings suggest that down-regulation of Mcl-1 may involve both transcriptional inhibition and caspase-dependent cleavage.

In view of reports that IL-6 up-regulates Mcl-1 in myeloma cells (44), studies were done to assess the effects of flavopiridol/HA14-1 on Mcl-1 in the presence of IL-6. As shown in Fig. 6D, IL-6 failed to block PARP cleavage, consistent with the results shown in Fig. 2B. Furthermore, although IL-6 alone modestly up-regulated Mcl-1, addition of this cytokine was unable to prevent down-regulation/cleavage of Mcl-1 in cells exposed to flavopiridol and HA14-1.

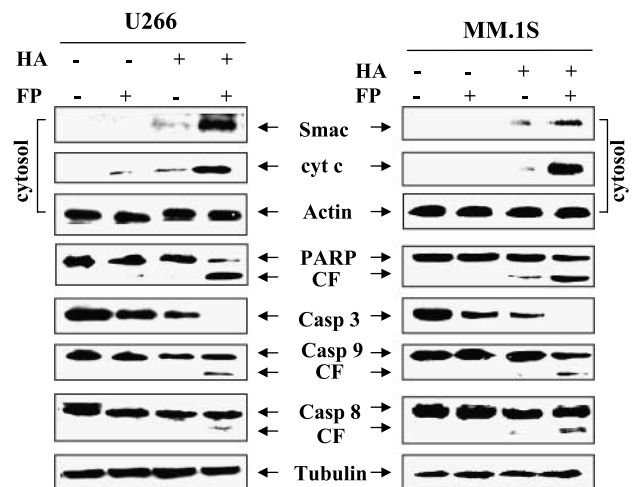


Figure 4. Flavopiridol/HA14-1 regimen triggers activation of the mitochondrial pathway and caspase cascade in multiple myeloma cells. Following 6-hour preincubation with 100 nmol/L flavopiridol, U266 (left) and MM.1S (right) cells were treated with 10 μ mol/L HA14-1 for 18 hours (U266) or 12 hours (MM.1S). At the end of this period, the cells were lysed and subjected to Western blot analysis using the indicated primary antibodies. CF, cleavage fragment; cyt c, cytochrome c. Alternatively, cells were treated as described above, after which cytosolic (S-100) fractions were prepared as described in Materials and Methods, and expression of cytochrome c and Smac/DIABLO in cytosol was monitored by Western blot. Each lane was loaded with 30 μ g of protein; the blots were stripped and reprobbed with anti-actin or anti-tubulin antibody to ensure equal loading and transfer. Representative of one experiment; two additional studies yielded equivalent results.

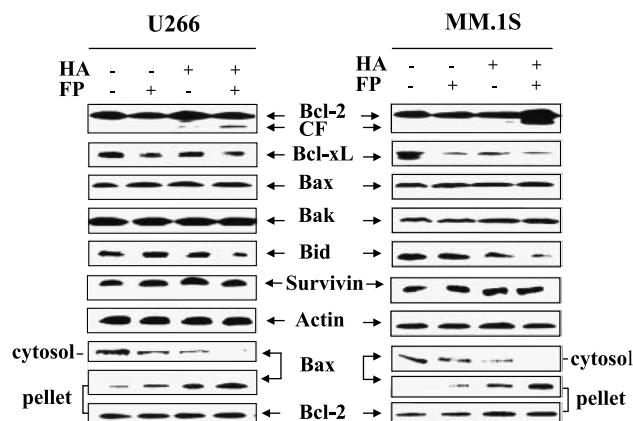


Figure 5. Combined treatment with flavopiridol/HA14-1 leads to multiple perturbations of Bcl-2 family members in multiple myeloma cells. U266 (left) and MM.1S (right) cells were incubated with 10 $\mu\text{mol/L}$ HA14-1 for 18 hours (U266) or 12 hours (MM.1S) after 6-hour preincubation with 100 nmol/L flavopiridol. At the end of this period, expression of Bcl-2 family proteins and survivin were monitored by Western blot. Alternatively, S-100 (cytosolic) and pellet (mitochondrial membrane-rich) fractions were prepared as described in Materials and Methods, and subjected to Western blot. Each lane was loaded with 30 μg of protein; the blots were stripped and reprobbed with anti-actin antibody to ensure equal loading and transfer. Representative of three separate experiments.

Flavopiridol/HA14-1-Mediated JNK Activation Plays a Functional Role in Lethality in U266 Multiple Myeloma Cells

To assess the functional role of JNK activation in these events, U266 cells were exposed to flavopiridol and HA14-1 as above in the presence or absence of the JNK inhibitor SP600125 (45). As shown in Fig. 6B, addition of SP600125 modestly but significantly attenuated flavopiridol/HA14-1-mediated apoptosis ($P < 0.05$ versus cells exposed to drugs in the absence of SP600125). Western blot analysis confirmed that SP600125 did in fact attenuate flavopiridol/HA14-1-induced JNK phosphorylation and also reduced PARP cleavage (Fig. 6E). In separate studies, coadministration of the p38 MAPK inhibitors SB203580 (5 mol/L) or SB202190 (10 mol/L) failed to attenuate flavopiridol/HA14-1-mediated apoptosis (data not shown). Together, these findings indicate that activation of the JNK pathway plays a relatively modest yet functional role in enhanced apoptosis resulting from flavopiridol/HA14-1 exposure.

Flavopiridol/HA14-1-Induced Apoptosis in Myeloma Cells Involves the Formation of ROS

Previous studies from our laboratory indicated that in human leukemia cells flavopiridol-induced apoptosis involved mitochondrial injury and formation of ROS (12). Moreover, the antiapoptotic activity of Bcl-2 has been related to protection of cells from oxidative injury (23), whereas Bcl-2 inhibitors (e.g., HA14-1) trigger ROS production (46). Therefore, studies were undertaken to determine whether the lethality of the flavopiridol/HA14-1 regimen depended on ROS generation. To this end, U266 cells were exposed to flavopiridol followed by HA14-1 in the presence or absence of the antioxidant

L-NAC (15 mmol/L), after which ROS generation and apoptosis were assessed. As shown in Fig. 7A, exposure of cells to flavopiridol/HA14-1 resulted in a significant increase in ROS formation, and this effect was largely blocked by addition of L-NAC. Similar results were observed in MM.1S cells (data not shown). Furthermore,

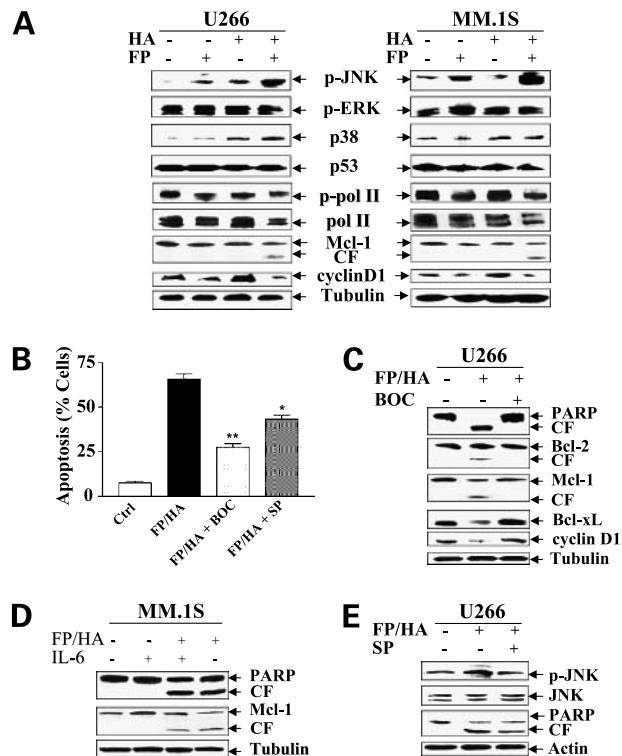


Figure 6. Lethality of the flavopiridol/HA14-1 regimen is associated with activation of stress-activated protein kinase/JNK and caspase-dependent down-regulation of several antiapoptotic proteins in multiple myeloma cells. **A**, U266 (left) and MM.1S (right) cells were incubated with 10 $\mu\text{mol/L}$ HA14-1 for 18 hours (U266) or 12 hours (MM.1S) following 6-hour pretreatment with 100 nmol/L flavopiridol, after which Western blot analysis was employed to monitor phosphorylation status of JNK, ERK, p38, and carboxyl-terminal domain of RNA polymerase II (*pol II*) and expression of p53, polymerase II, Mcl-1, and cyclin D1. **B**, U266 cells were sequentially treated with 100 nmol/L flavopiridol for 6 hours followed by 10 $\mu\text{mol/L}$ HA14-1 for 18 hours in the presence or absence of 20 $\mu\text{mol/L}$ BOC-D-fmk or 10 $\mu\text{mol/L}$ SP600125, after which the percentage of cells exhibiting apoptotic morphology was determined by evaluating Wright-Giemsa-stained cytopsin preparations. Columns, means of three separate experiments in triplicate; bars, SD. **, $P < 0.01$; *, $P < 0.05$, significantly lower than the values for cells treated with flavopiridol/HA14-1 in the absence of BOC-D-fmk (BOC) or SP600125 (SP). **C**, U266 cells were treated with flavopiridol/HA14-1 with or without BOC-D-fmk as described in **B**, after which cells were lysed and subjected to Western blot analysis using the indicated primary antibodies. **D**, MM.1S cells were incubated with 10 $\mu\text{mol/L}$ HA14-1 for 12 hours following 6-hour pretreatment with 100 nmol/L flavopiridol in the presence or absence of 100 ng/mL IL-6, after which Western blot analysis was done to evaluate cleavage of PARP and expression of Mcl-1. **E**, U266 cells were treated with flavopiridol/HA14-1 with or without SP600125 as described in **B**, after which Western blot analysis was employed to monitor expression of phospho-JNK and total JNK and cleavage of PARP. For **A**, **C**, **D**, and **E**, each lane was loaded with 30 μg of protein; blots were stripped and reprobbed with anti-actin or anti-tubulin antibody to ensure equal loading and transfer. Two additional studies yielded equivalent results.

coadministration of L-NAC significantly reduced flavopiridol/HA14-1-induced apoptosis in both cell lines ($P < 0.01$ in each case; Fig. 7B). This suggests that ROS generation is requisite for apoptosis induction by this combination.

Western analysis of whole cell lysates showed that administration of L-NAC blocked flavopiridol/HA14-1-induced PARP degradation as well as JNK activation in both U266 and MM.1S cells (Fig. 7C). Furthermore, analysis of S-100 cytosolic extracts indicated that coadministration of L-NAC also attenuated mitochondrial injury induced by these agents, manifested by diminished release of cytochrome *c* and Smac/DIABLO. Collectively, these findings indicate that ROS generation represents a primary event in the response of myeloma cells to the flavopiridol/HA14-1 regimen, responsible for activation of the stress-related JNK pathway, mitochondrial damage, activation of the caspase cascade, and apoptosis.

Finally, ROS generation was monitored in cells exposed to flavopiridol and HA14-1 according to various schedules (e.g., simultaneous, flavopiridol followed by HA14-1, and HA14-1 followed by flavopiridol) as described previously. Exposure to the optimal sequence (i.e., flavopiridol followed by HA14-1) resulted in a significantly greater increase in ROS generation at equivalent intervals compared with effects of the alternative schedules (i.e., simultaneous or the sequence HA14-1 followed by flavopiridol; 30.3 ± 2.2 versus 22.4 ± 1.1 or $19.5 \pm 0.9\%$ of cells; $P < 0.05$ in each case; data not shown).

Discussion

The present results indicate that combining flavopiridol with the small-molecule Bcl-2 inhibitor HA14-1 leads to a marked increase in mitochondrial injury, caspase activation, and apoptosis in diverse multiple myeloma cell lines, including those resistant to conventional cytotoxic drugs or dexamethasone. Although flavopiridol was originally developed as a CDK inhibitor, its capacity to induce apoptosis in malignant hematopoietic cells when given at low (e.g., nanomolar) concentrations has been documented (10). The mechanism underlying this phenomenon is not known with certainty, but evidence suggests that it is not necessarily related to CDK inhibition. In this regard, the identification of other flavopiridol functions (i.e., inhibition of positive transcription elongation factor *b*; ref. 13) may be particularly relevant. Although in epithelial tumor cells (e.g., lung cancer cells) flavopiridol has been reported to trigger apoptosis through activation of the extrinsic pathway (11), subsequent studies in leukemia cells showed that flavopiridol was a potent inducer of mitochondrial injury and activation of the intrinsic mitochondrial apoptotic pathway (12). HA14-1, by antagonizing Bcl-2 function, has been shown to potentiate the lethal effects of conventional cytotoxic drugs (e.g., 1- β -D-arabinofuranosylcytosine) in human leukemia cells (47). The present findings indicate that HA14-1 also lowers the apoptotic threshold for more novel agents, particularly those, like flavopiridol, that act through induction of mitochondrial injury.

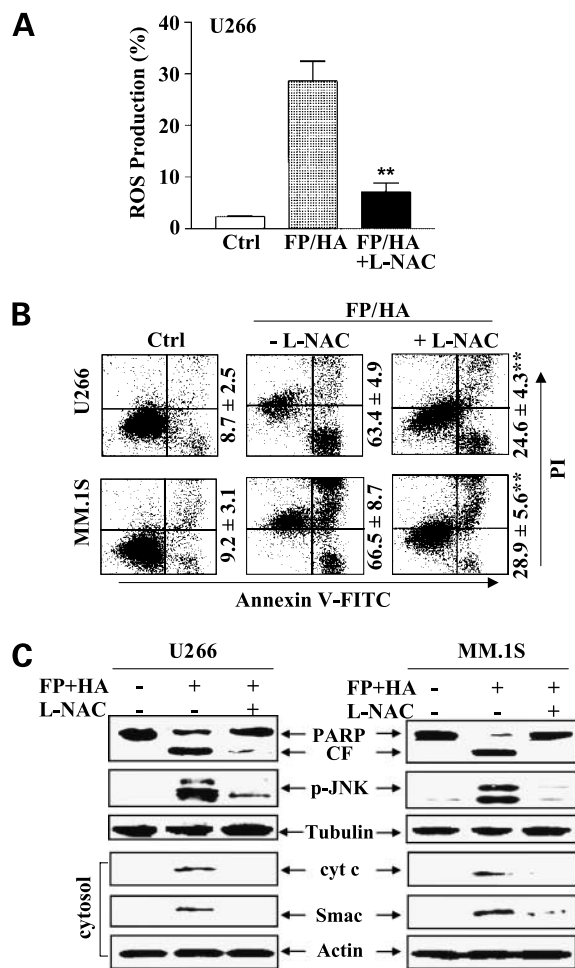


Figure 7. Flavopiridol/HA14-1 regimen induces ROS generation, which results in JNK activation, mitochondrial dysfunction, and apoptosis in multiple myeloma cells. **A**, U266 cells were treated with 10 $\mu\text{mol/L}$ HA14-1 for 6 hours following a 6-hour pretreatment with 100 nmol/L flavopiridol in the presence or absence of a 3-hour preincubation with 15 mmol/L L-NAC. At the end of this period, the percentage of cells displaying increased ROS production was determined by monitoring dichlorodihydrofluorescein staining by flow cytometry as described in Materials and Methods. **B**, U266 (*top*) and MM.1S (*bottom*) cells were treated with flavopiridol/HA14-1 with or without L-NAC as described above (15 mmol/L L-NAC, 3 hours; 100 nmol/L flavopiridol, 6 hours; 10 $\mu\text{mol/L}$ HA14-1, 18 hours in U266 or 12 hours in MM.1S), after which Annexin V assays were done to monitor apoptosis. Values are percentages of Annexin V-positive cells. *columns*, means of three separate experiments in triplicate; *bars*, SD. **, $P < 0.01$, significantly lower than the values for flavopiridol/HA14-1-treated cells in the absence of L-NAC. **C**, alternatively, cells were treated as described in **B**, after which whole cell lysates and S-100 fractions were prepared and subjected to Western blot analysis using indicated primary antibodies. Each lane was loaded with 30 μg of protein; the blots were stripped and reprobed with anti-actin or anti-tubulin antibody to ensure equal loading and transfer. Representative of three separate experiments.

Combined exposure of myeloma cells to flavopiridol and HA14-1 was associated enhanced redistribution of Bax to the mitochondria. Bax exists primarily as a cytosolic protein until cells are exposed to an apoptotic stimulus, at which time it translocates to the mitochondria where it undergoes

a conformational change (48). Bax can homodimerize either with other Bax molecules or with Bak, another multidomain proapoptotic Bcl-2 family member (20). Such events may facilitate pore formation within the mitochondrial membrane through which proapoptotic mitochondrial proteins can be released into the cytoplasm (49). Consistent with this notion, Bax translocation was associated with release of cytochrome *c* and Smac/DIABLO into the cytoplasm. Furthermore, it has been shown that antiapoptotic proteins such as Bcl-2 and Bcl-x_L act at least in part by sequestering BH3-only domain molecules (e.g., Bid, Bad, Bim, and Noxa) from interactions with multidomain proapoptotic Bcl-2 family members (e.g., Bax and Bak), thus preventing the activation of multidomain molecules leading to engagement of the mitochondrial pathway (i.e., through release of cytochrome *c* into the cytosol; ref. 22). HA14-1 acts by binding to the Bcl-2 surface pocket, which is required for Bcl-2 cytoprotective functions (27). It is therefore tempting to postulate that HA14-1, by interfering with Bcl-2 function, promoted flavopiridol-mediated mitochondrial dysfunction.

The present findings suggest that prior, rather than simultaneous or subsequent, exposure of myeloma cells to flavopiridol maximizes apoptosis in HA14-1-treated cells, although the mechanism underlying the sequence dependence of phenomenon is not entirely clear. In this context, we have recently described synergistic interactions between the proteasome inhibitor bortezomib and HA14-1 in multiple myeloma cells (34). Interestingly, the sequence bortezomib followed by HA14-1 also resulted in the greatest degree of apoptosis induction for this combination. Such findings suggest that perturbations induced by flavopiridol (or bortezomib) must first be established (or initiated) for disruption of Bcl-2 function to exert maximal impact. Of the events associated with flavopiridol administration, transcriptional repression of certain proteins (e.g., Mcl-1; ref. 14) or induction of mitochondrial injury and/or oxidative damage (12) represents plausible candidates. Alternatively, it is known that proteasome inhibitors promote cell death at least in part by disrupting the cytoprotective nuclear factor- κ B pathway (50). Moreover, flavopiridol has recently been shown to inhibit the κ B α kinase and, by extension, nuclear factor- κ B function (51). Such findings raise the possibility that initial disruption of the nuclear factor- κ B cascade may potentiate the lethal actions of subsequently given Bcl-2 antagonists.

In addition to the pronounced increase in apoptosis, combined exposure to flavopiridol and HA14-1 also resulted in a marked reduction in myeloma cell clonogenic potential. Previous studies have shown that the extent of apoptosis induced by a particular cytotoxic agent does not necessarily correlate with loss of clonogenic survival (40). Furthermore, increased expression of Bcl-2 may delay but not prevent mitochondrial injury and thus fail to restore leukemic cell self-renewal capacity (52). Alternatively, the response of clonogenic cells to an apoptotic stimulus may differ from that of the population as a whole. The finding

that the flavopiridol/HA14-1 regimen markedly reduced the clonogenic potential of myeloma cells suggests that self-renewing myeloma cells are also vulnerable to the lethal effects of this drug combination.

It is noteworthy that myeloma cells resistant to dexamethasone, doxorubicin, and melphalan, all agents that are commonly employed in multiple myeloma, did not exhibit cross-resistance to the flavopiridol/HA14-1 regimen. Similarly, growth factors such as IL-6 and IGF-I have been implicated both in the enhanced survival of myeloma cells (53, 54) and in resistance to cytotoxic agents (55). The inability of IL-6 or IGF-I to attenuate flavopiridol/HA14-1-mediated lethality suggests that this regimen triggers apoptosis downstream or independently of IL-6-related or IGF-I-related survival signaling cascades.

The lethal effects of the flavopiridol/HA14-1 regimen in myeloma cells were associated with several perturbations in signaling, survival, and cell cycle regulatory proteins. Of these, particularly notable was the marked increase in JNK activation, which has been associated with proapoptotic effects in diverse systems (56). The finding that the pharmacologic JNK inhibitor SP600125 partially but significantly attenuated flavopiridol/HA14-1-mediated lethality indicates that JNK activation plays at least some functional role in apoptosis induction. The mechanism by which JNK activation exerts proapoptotic actions is not known with certainty but may be related to promotion of cytochrome *c* release (57) as well as phosphorylation and inactivation of antiapoptotic proteins such as Bcl-2 (58) and Mcl-1 (59). Because Mcl-1 has been shown to play an important role in myeloma cell survival (15, 44), it is conceivable that JNK-mediated phosphorylation in conjunction with down-regulation of this protein may contribute to the marked increase in apoptosis. Analogously, the formation of a putatively proapoptotic Bcl-2 cleavage protein (41) in flavopiridol/HA14-1-treated cells may, in combination with inhibition of Bcl-2 function by HA14-1, further lower the cell death threshold. Finally, treatment of myeloma cells with flavopiridol/HA14-1 was associated with cyclin D1 down-regulation, a phenomenon observed previously in breast cancer cells undergoing flavopiridol-mediated apoptosis (16). It should be noted, however, that flavopiridol-mediated down-regulation of Mcl-1, Bcl-x_L, and cyclin D1 were all reversed at least in part by the pan-caspase inhibitor BOC-D-fmk, indicating that these are caspase-dependent events and unlikely to represent a primary cause of cell death. Nevertheless, they may serve to amplify the apoptotic process and thereby potentiate the extent of myeloma cell death. Furthermore, the possible contribution of transcriptional repression of these proteins (via CDK9 inhibition) in cells exposed to flavopiridol cannot be excluded.

The present results argue strongly that enhanced oxidative injury (e.g., the formation of ROS) plays an important functional role in the lethality of the flavopiridol/HA14-1 regimen. Specifically, exposure of multiple myeloma cells to flavopiridol/HA14-1 resulted in a pronounced increase in ROS generation; moreover, both the

increase in ROS and lethality were reversed by the antioxidant L-NAC. Significantly, L-NAC also attenuated release of the proapoptotic mitochondrial proteins cytochrome *c* and Smac/DIABLO, placing ROS generation proximally in the cell death hierarchy. Moreover, L-NAC also attenuated flavopiridol/HA14-1-mediated activation of JNK, an event closely linked to the apoptotic response of cells to oxidative stress (60). It is worth noting that flavopiridol lethality has been related to mitochondrial injury and ROS generation in human leukemia cells (12), and Bcl-2 has been shown to protect cells from oxidative injury possibly by regulating the intracellular distribution of reduced glutathione (61). Thus, it is tempting to speculate that HA14-1, by interfering with the antioxidant function of Bcl-2, may promote flavopiridol-induced oxidative injury. Alternatively, it is conceivable that flavopiridol, by interfering with the cytoprotective nuclear factor- κ B pathway, may potentiate the lethal actions of Bcl-2 antagonists.

In summary, the present findings indicate that a strategy combining the CDK inhibitor flavopiridol with HA14-1, a small-molecule inhibitor of Bcl-2, effectively triggers oxidative injury, mitochondrial dysfunction, caspase activation, and apoptosis in multiple myeloma cells, including those resistant to conventional therapeutic agents such as dexamethasone, melphalan, and doxorubicin. For various reasons, the therapeutic potential of flavopiridol is currently being investigated in multiple myeloma and related hematopoietic malignancies. Moreover, there is extensive interest in the therapeutic development of small-molecule inhibitors of various antiapoptotic proteins, including Bcl-2, Bcl- κ , and X-linked inhibitor of apoptosis (62, 63). Based on the present findings, the concept of combining such agents in disorders such as multiple myeloma seems worthy of further investigation.

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