Mcl-1 Stability Determines Mitotic Cell Fate of Human Multiple Myeloma Tumor Cells Treated with the Kinesin Spindle Protein Inhibitor ARRY-520

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Abstract

Kinesin spindle protein (KSP/Eg5) inhibitors are novel anticancer agents that have thus far shown only modest activity in the clinic. Understanding how to identify patients who may be most sensitive to treatment is clearly needed to improve the development of these molecules. We studied four multiple myeloma cell lines treated with the KSP inhibitor ARRY-520 to identify factors important for initiating apoptosis while cells are arrested in mitosis. The majority (three of four) of cell lines underwent mitotic arrest, with apoptosis occurring in mitosis within 24 to 30 hours. The remaining line (NCI H929) is temporally refractory to ARRY-520 treatment, undergoing mitotic slippage and subsequently peaking in apoptotic markers after 72 hours of treatment, while most cells are in interphase. Interestingly, loss of the antiapoptotic protein myeloid cell leukemia 1 (Mcl-1) coincided with mitotic cell death. Stabilization of Mcl-1 resulted in a delayed onset of apoptosis, whereas enforced downregulation of Mcl-1 increased cell death in response to KSP inhibition. Thus, variation in responses to KSP inhibition is governed by a balance between survival proteins and spindle checkpoint integrity. Cells relying on short-lived survival proteins during mitosis are more likely to undergo apoptosis in response to KSP inhibition. We propose that patients with hematologic malignancies, which rely on Mcl-1, would therefore be good candidates for treatment with KSP inhibitors.

Introduction

Antimitotic microtubule inhibitors are among the most active and broadly used cancer drugs. These drugs, which include taxanes, epothilones, and Vinca alkaloids, directly perturb microtubule function, resulting in a defective mitotic spindle and mitotic arrest. However, as microtubules perform critical functions in postmitotic cells, these drugs also exhibit unwanted side effects, including peripheral neuropathies (1). Thus, inhibitors of mitosis-specific targets that lack side effects in postmitotic cells are desired. One such target is kinesin spindle protein (KSP, Eg5). KSP is a mitosis-specific kinesin essential for the assembly of a bipolar spindle and equal segregation of sister chromatids (2). Inhibition of KSP prevents centrosome separation, resulting in the formation of a monopolar spindle and activation of the mitotic spindle assembly checkpoint (SAC), causing metaphase arrest due to maintenance of cdc2/cyclin B activity (3). Drug-induced accumulation of mitotic cells is tightly associated with cell death (4). ARRY-520 is a selective KSP inhibitor with low nanomolar potency in solid and hematologic tumor cell lines (5) and activity in a wide range of tumor xenograft models (6).

Mitotic arrest is not permanent, but results in either death while cells are arrested in mitosis or exit from mitosis in the presence of misaligned chromosomes, a process known as adaptation or mitotic slippage, leading to multinucleated cells (7). One model for adaptation suggests that apoptosis induced by mitotic inhibitors requires the activation of the SAC followed by mitotic slippage (8, 9). Presumably, postmitotic cell death results from a failure of mitosis associated with tetraploidy, which is essential to activate a cell death program in G1 to prevent further polyploidization (10). An alternative to death after mitotic slippage is death during mitotic arrest (11, 12). One hypothesis is that because transcription is absent in mitosis, yet proteolysis persists, cell fate is largely determined by the rate of depletion of short-lived proteins that have short-lived mRNAs (13). In this model, a decline in antiapoptotic protein levels below a survival threshold, before the loss of SAC activity and subsequent degradation of cyclin B, would be expected to trigger apoptosis during mitotic arrest. Conversely, failure of the SAC before depletion of survival signals will result in adaptation and subsequent cell death in G1. The key
elements of this hypothesis have recently been verified. Treatment of HeLaM cells with microtubule poisons confirms the gradual accumulation of apoptotic signals in cells that undergo a prolonged mitotic arrest (14). Furthermore, it has been shown in multiple cell lines that cells that typically die directly in mitosis only slowly degrade cyclin B1, thereby preventing mitotic exit before death (15). By contrast, cells that undergo adaptation progressively destroy cyclin B1, leading to mitotic exit. These data suggest that depending on the differential requirement for survival protein activity and its rate of degradation, it may be possible to determine whether a tumor would succumb to mitotic cell death versus postmitotic cell death in the presence of a mitotic inhibitor.

Various survival proteins, such as members of the Bcl-2 family or inhibitors of apoptosis proteins (IAP), have been found to confer resistance to antimitotic chemotherapy in various tumor types (16, 17). Of these, the prosurvival Bcl-2 family member myeloid cell leukemia 1 (Mcl-1) stands out as an intriguing candidate for determining whether an arrested cell undergoes mitotic cell death. Mcl-1 has an extended PEST region, which is responsible for its relatively short half-life (18, 19). In addition, Mcl-1 becomes phosphorylated on sites within its PEST region in response to treatment with phorbol ester, oxidative stress, cytokine withdrawal, and microtubule-damaging agents, which affects its overall stability (20, 21). Lastly, Mcl-1 is predominately expressed in hematopoietic cell lineages, including multiple myelomas where it has been shown to be indispensable (22–25). We have previously shown that xenograft tumors of hematologic origin, including multiple myeloma, are often more responsive to drug treatment with ARRY-520, compared with xenograft tumors of nonhematologic origin. Here, we have examined Mcl-1 and other survival proteins in multiple myeloma cell lines following treatment with ARRY-520 to determine whether Mcl-1 plays a role in determining cell fate decisions following KSP inhibition.

Materials and Methods

Cell lines and culture
RPMI 8226, NCI H929, and U266 cells were obtained from the American Type Culture Collection. JJN3 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. Cells used for experiments were grown in culture for less than 2 months under the conditions recommended by the suppliers.

In vivo xenograft models
RPMI 8226 tumor cell lines, 2 × 10⁷ (tumor growth inhibition; Fig. 2A) or 5 × 10⁷ (pharmacodynamic analysis; Fig. 2B–D), in 100 μL of 50% Matrigel were implanted s.c. into female severe combined immunodeficient-beige mice as described previously (6). All studies were done in accordance with the Guide for Laboratory Animal Care and Use. Immunocompromised mice were purchased from Charles River. Tumor growth inhibition included eight mice per group. Pharmacodynamic analysis included three mice per group (average tumor volume of each group ~165 mm³).

Caspase-3/7 assay
Vehicle or drug-treated cells in growth medium were seeded (10,000 viable cells per well) in duplicate wells of black-walled 96-well plates and incubated at 37°C in 5% CO₂. Caspase-3/7 activity was measured using Caspase-Glo 3/7 reagent (Promega) at various time points after drug addition. Data are reported as the mean luminescence of drug-treated wells divided by the mean luminescence of vehicle control wells.

Cell viability
Inhibition of cell proliferation was measured using the Promega CellTiter-Blue assay. Cells were plated in 96-well plates at a density that allowed for logarithmic growth over the 72-hour period of the assay. Compound 65 (0.1–50 μmol/L) was added to the cells at a final DMSO concentration of 0.5%. Mcl-1 siRNA (10 nmol/L) was transfected into the cells as described below. Fluorescent signal was converted to percent of control relative to high (DMSO-treated control) and low (fully inhibited control) samples. The EC₅₀ for inhibition of viability was determined from the inflection point of a standard four-parameter logistical curve.

Immuoassays
ARRY-520–treated cell lines were harvested at various time points after drug addition. Cell lysates were generated by thawing cells in radiomunoprecipitation assay buffer [50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 0.05% SDS, 2 mmol/L EDTA, protease inhibitor cocktail set III, EDTA-free, phosphatase inhibitor cocktail set I; Calbiochem]. Mice bearing tumor xenografts were euthanized by CO₂ inhalation, and tumors were harvested and flash frozen in liquid nitrogen. Tumor lysates were prepared as described previously (6). Proteins were resolved on 12% Tris-glycine gels (NuPage, Invitrogen) and transferred onto nitrocellulose membranes. Membranes were probed with commercial primary antibodies: Bcl-2 (BD Biosciences), poly(ADP-ribose) polymerase (PARP), XIAP, Bcl-XLt (Cell Signaling), glyceraldehyde-3-phosphate dehydrogenase (Sigma), phosphorylated histone H3 (Ser10), cyclin B1, Mcl-1, p42/p44 mitogen-activated protein kinase (Santa Cruz Biotechnology), and NOXA (Calbiochem). Blots were then probed with secondary antibodies: IRDye 800-conjugated anti-mouse IgG (Rockland) or Alexa Fluor 680 anti-rabbit IgG (Invitrogen), and imaged using the LiCOR Odyssey infrared imaging system. Lysates from cycling NCI H929 cells used for immunoprecipitation experiments were prepared as outlined above. Protein lysates (300 μg) supplemented with DMSO or 1 μmol/L compound 65 were subjected to immunoprecipitation...
overnight at 4°C using 5 μg of rabbit IgG or Mcl-1 antibody (Santa Cruz Biotechnology) bound to protein A-Sepharose CL-4B resin (Sigma).

Electroporation of cells
RPIMI 8226, JJN3, and U266 cells were diluted to a final concentration of 1 x 10⁶ cells in 0.4 mL of OptiMEM (Invitrogen). Cells were mixed with 10 μg of pZsGreen1-N1 plasmid (Clontech) or pZsGreen1-N1 plasmid containing full-length human Mcl-1 cDNA. Cells were electroporated in a 4-mm cuvette (Bio-Rad Laboratories) by exponential decay pulse (240 V, 1,070 μF) using the Gene Pulser Xcell electroporation system (Bio-Rad Laboratories). Cells were immediately placed into growth medium following electroporation, and dead cells were removed 24 hours later by magnetic beads (Dead Cell Removal Kit; Miltenyi Biotec) before being used for drug treatment.

Transfection of siRNA
Specific Accell-modified siRNA duplexes in 1× siRNA buffer were stored at −20°C: Accell green nontargeting siRNA, human NOXA siRNA N8 (26), and human Mcl-1 siRNA duplex 34 (Dharmacon). Cells were transfected by seeding in fresh Accell delivery medium containing the appropriate siRNA for 24 or 48 hours under normal growth conditions.

Compounds
ARRY-520 and compound 65 were synthesized at Array BioPharma. For cell-based assays, ARRY-520 or compound 65 was added to growth medium, with DMSO used as a vehicle control. DMSO concentrations never exceeded 0.5% in the assay.

Results
The onset of apoptosis varies among cell lines treated with ARRY-520
We have previously shown that multiple myeloma tumor xenografts are highly sensitive to ARRY-520 in vivo (6). Thus, we decided to investigate the molecular mechanisms responsible for mitotic cell death in four different multiple myeloma cell lines (RPIMI 8226, JJN3, U266, and NCI H929). Most studies report that cell death resulting from prolonged mitotic arrest is executed by proteolytic caspase enzymes (9, 11, 15, 27, 28). The kinetics of apoptosis induction in these four cell lines was investigated by measuring the activation of caspase-3 and caspase-7 at various time points following drug addition. Cells seeded into identical 96-well plates were treated with either 10 nmol/L ARRY-520 (~5-fold above the cellular IC₅₀ for these lines; Supplementary Fig. S1A) or an equivalent volume of vehicle (DMSO). Three of the cell lines (RPIMI 8226, JJN3, and U266) exposed to ARRY-520 exhibited a rapid onset of apoptosis as defined by a peak in caspase-3/7 activity that occurred ~24 hours after compound treatment (Fig. 1A). However, whereas the NCI H929 cell line also displayed a gradual increase in caspase-3/7 activity over time, the peak in activity occurred much later (72–80 hours).

As a secondary method of verifying apoptosis, and to characterize drug responses at a molecular level, we performed immunoblotting for survival and apoptotic proteins, and/or their posttranslational modification sites, from all four multiple myeloma cell lines. All cell lines treated with 10 nmol/L ARRY-520 exhibited rapid induction of mitotic arrest as judged by both increased phosphorylation of histone H3 (pH3) and accumulation of cyclin B1 (see Fig. 1B and C; Supplementary Fig. S2). Mitotic arrest caused by ARRY-520 was also confirmed by flow cytometry as all four cell lines exhibited an increased amount of 4 N cells after 24 hours of ARRY-520 treatment (Supplementary Fig. S1B). PARP is one of the main targets of caspase-3 (29), and its cleavage has been used as a marker for cells undergoing apoptosis (30). Using the JJN3 cell line as a representative for cell lines that undergo rapid onset of apoptosis, it was found that significant PARP cleavage occurred within 24 hours of ARRY-520 treatment, consistent with the peak in caspase activation in this cell line (Fig. 1A) and correlated with maximal mitotic arrest (Fig. 1B). However, whereas the NCI H929 cell line exhibited a slight increase in the levels of cleaved-PARP during mitotic arrest, the majority of PARP cleavage was seen after 72 hours of treatment, once the cells had exited mitosis (Fig. 1C).

Loss of Mcl-1 correlates with mitotic cell death
In an effort to identify molecular activities that have a role in regulating mitotic cell death in these cells, the levels of key antiapoptotic proteins were measured at various times following exposure to ARRY-520. In all cell lines tested, levels of Bcl-2 and Bcl-XL remained relatively stable following treatment with ARRY-520 (see Fig. 1B and C; Supplementary Fig. S2). It has been suggested that although the abundance of XIAP is not correlated with cell death, the degradation of XIAP in solid tumor lines is associated with onset of cell death in cells treated with a KSP inhibitor (28) or other chemotherapeutics (31). Although XIAP degradation indeed occurred following treatment with ARRY-520 in myeloma lines, the overall loss of XIAP protein was relatively modest and inconsistent between cell lines, suggesting that, at least in these cell lines, other proteins play a role in signaling cell death. This is compatible with the finding that cell death caused by ARRY-520 in leukemia cell lines also did not depend on XIAP (5). By contrast, we observed that the degradation of Mcl-1 coincided closely with PARP cleavage in JJN3 cells (Fig. 1B) and other cell lines that underwent rapid apoptosis in response to ARRY-520 (Supplementary Fig. S2). This effect was not seen in immunoblots of the NCI H929 cell line exposed to ARRY-520 (Fig. 1C), suggesting that Mcl-1 destabilization, promoted during prolonged mitotic arrest, may contribute to initiation of cell death. Interestingly, cells that have slipped from mitosis may not be as dependent on
Mcl-1 determines the fate of KSP-inhibited cells

Mcl-1 for survival during interphase as the levels of Mcl-1 seem to remain stable in the postmitotic time points taken from NCI H929 cells (Fig. 1C), despite the fact that these cells eventually undergo apoptosis as measured by PARP cleavage and caspase activation. RPMI 8226 tumor xenografts were particularly sensitive to low doses of ARRY-520 (12.5 mg/kg) administered on days 1 and 2 (Fig. 2A). Immunoblot analysis of tumor lysates following ARRY-520 treatment showed similar effects on Mcl-1 stability and induction of apoptosis during mitotic arrest as seen in RPMI 8226 cells treated in vitro (Fig. 2B–D). Mcl-1 levels were significantly decreased after only 24 hours of treatment with the majority of Mcl-1 expression in the tumor lost by 48 hours, corresponding with an increase in cleaved PARP at these time points. In total, these experiments suggest that Mcl-1 may be a critical factor important for multiple myeloma cell survival during prolonged mitotic arrest. Subsequent experiments test the validity of this hypothesis in multiple myeloma cell lines.

Increased expression or stabilization of Mcl-1 delays apoptosis

Our data suggest that Mcl-1 degradation in cells arrested in mitosis by ARRY-520 is a key step in the onset of apoptosis. To verify this, we stabilized Mcl-1 protein in cells through both direct overexpression of recombinant Mcl-1 protein and by knockdown of the Mcl-1 regulatory protein NOXA. In the first instance, we analyzed the effect of overexpression of Mcl1-ZsGreen1 fusion protein on the three cell lines that underwent a rapid apoptosis in mitosis following treatment with

Figure 1. Caspase-3/7 activation in multiple myeloma cell lines treated with ARRY-520 correlates with Mcl-1 degradation. A, multiple myeloma cell lines were seeded in the presence of 10 nmol/L ARRY-520 at the 0-h time point. Caspase-3/7 activity was measured at the indicated times and reported as the fold-increase above control (DMSO)-treated cells for that time point. B, immunoblot analysis of JJN3 cells in response to 10 nmol/L ARRY-520 at 11 time points starting with drug addition (0 h) to 48 hours following drug addition. C, NCI H929 cell reaction to 10 nmol/L ARRY-520. Note the longer duration of time points (80 h total) for this cell line. Full-length and cleaved forms of PARP are indicated by arrows. pH3, phosphorylated histone H3 (Ser10). The mean of at least three experiments is shown.
ARRAY-520. If multiple myeloma cells depend on Mcl-1 for mitotic survival, we hypothesized that overexpression of Mcl1-ZsGreen1 would delay apoptosis induction in these cells. Indeed, overexpression of Mcl1-ZsGreen1 caused an ~24 hours (2-fold) delay in the onset of peak caspase-3/7 activity (Fig. 3A) and PARP cleavage (Fig. 3B and C) in the JJN3 cell line following exposure to ARRY-520. Similar results were also obtained in the RPMI 8226 and U266 cell lines (Supplementary Figs. S3 and S4, respectively). Although endogenous Mcl-1 and Mcl1-ZsGreen1 were still degraded, the excess Mcl1-ZsGreen1 seemed to compensate for the loss of the native protein, thus delaying the onset of apoptosis in these cells.

It is possible that overexpression of a survival protein such as Mcl-1 could induce survival signals independent of a role in mitotic cell death responses. For this reason, we chose to investigate whether we could stabilize Mcl-1 protein by an alternative mechanism that does not directly alter physiologic levels of Mcl-1. The small, BH3-only protein NOXA functions as a specific antagonist for Mcl-1 binding to proapoptotic Bcl-2 family members, such as Bim and Bak (32–34). Binding of NOXA to Mcl-1 prohibits the formation of Mcl-1/Bak or Mcl-1/Bim dimers, thus increasing the pool of free Bak and Bim capable of promoting mitochondrial outer membrane permeabilization. In addition, NOXA binding to Mcl-1 has been suggested to promote Mcl-1 ubiquitination and subsequent degradation by the proteasome (Fig. 4A; ref. 34). Such a role for NOXA has been verified in multiple myeloma cell lines treated with a wide range of chemotherapeutics (32, 33, 35, 36). We were interested in whether the rapid decline of Mcl-1 protein in ARRY-520–arrested RPMI 8226, JJN3, or U266 cell lines was mediated through NOXA. To test this, we used NOXA-specific siRNA to diminish NOXA

Figure 2. RPMI 8226 in vivo tumor growth inhibition correlates with Mcl-1 degradation and apoptosis. Saline vehicle or 12.5 mg/kg ARRY-520 were administered i.p. to mice bearing RPMI 8226 tumor xenografts at 0 and 24 h. A, tumor growth was analyzed by measuring tumor volume on the indicated days. Tumors were excised at the indicated times, and lysates were blotted for Mcl-1 (B), pHH3 (C), or PARP (D). Band intensities were normalized to total Erk (p42/p44 mitogen-activated protein kinase) for each sample, then averages (three mice per time point) and percentages of control were calculated. Data in D are the ratio of Erk-normalized cleaved PARP to Erk-normalized full-length PARP.
protein levels before ARRY-520 treatment. Transfection of cells with NOXA siRNA 48 hours before ARRY-520 treatment resulted in significant knockdown of NOXA throughout the time course of ARRY-520 treatment as determined by immunoblotting (Fig. 4D). Caspase-3/7 activity measured in JJN3 cells transfected with control siRNA behaved very similarly to what was seen in Figs. 1 and 2, whereas cells containing reduced NOXA exhibited a significant 24-hour delay in the onset of cell death caused by ARRY-520 (Fig. 4B). Similar results were observed following knockdown of NOXA in RPMI 8226 and U266 cell lines (Supplementary Figs. S5 and S6). NOXA knockdown correlated with greater stabilization of Mcl-1 protein 24 hours after ARRY-520 treatment, and a delay in detectable levels of cleaved-PARP (Fig. 4D) compared with control-transfected cells (Fig. 4C). Taken together, these results suggest that Mcl-1 is capable of maintaining survival signals in multiple myeloma cell lines arrested in mitosis by ARRY-520, and that only once Mcl-1 levels decrease below a threshold level for survival can cell death pathways become active.

Reduced Mcl-1 protein or function enhances cell death caused by KSP inhibition in NCI H929 cells

The observation that increased Mcl-1 protein can postpone cell death in M phase in most cell lines tested suggests that perhaps the NCI H929 multiple myeloma cell line could be made to undergo an early cell death in mitosis by lowering Mcl-1 activity. We reduced Mcl-1 protein function by two separate methods: the use of Mcl-1-specific siRNA and treating with a small-molecule Mcl-1 inhibitor. As the loss of Mcl-1 alone is sufficient to induce cell death in multiple myeloma cell lines (22, 25), we established conditions that resulted in knockdown of Mcl-1 without incurring substantial cell death on its own. We detected measurable Mcl-1 knockdown 24 hours posttransfection using Mcl-1 siRNA concentrations >15 nmol/L (Fig. 5A), and further determined cellular EC50 values for cell viability of 20 and 14 nmol/L at 24 and 48 hours posttransfection, respectively (Fig. 5B). Using an Mcl-1 siRNA concentration above its EC50 value (25 nmol/L), we found that addition of 10 nmol/L ARRY-520 to NCI H929 cells 24 hours posttransfection enhanced both the magnitude and time to initiation of cell death.
apoptosis as measured by activation of caspase-3 and caspase-7 (Fig. 5C). This was significantly different than the amount of cell death caused by 25 nmol/L Mcl-1 siRNA in combination with vehicle (DMSO), or through transfection of 25 nmol/L control siRNA in combination with 10 nmol/L ARRY-520 (Fig. 5C). Thus, although NCI H929 cells depend on Mcl-1 for survival, it is hypothesized that they either lack a mechanism sufficient for triggering its mitotic degradation or have developed a mechanism to promote its stabilization during a prolonged M-phase arrest caused by ARRY-520.

As an alternative method of modulating Mcl-1 function, we used a selective, small-molecule inhibitor of Mcl-1. Compound 65 (Fig. 6A) has been reported to disrupt the binding of interacting proteins, such as NOXA, to the hydrophobic pocket of Mcl-1 (37). To confirm the reported activity, we show that coimmunoprecipitation of BH3-only proteins NOXA and Bim with Mcl-1 is prevented in the presence of 1 μmol/L compound 65 (Fig. 6B), suggesting that compound 65 can disrupt the association of Mcl-1 with proapoptotic BH3-only proteins. Consistent with this, compound 65 induced rapid cell death (IC50 2 μmol/L at 48 hours; Fig. 6C) in NCI H929 cells consistent with its Mcl-1 inhibitory activity. Treatment of NCI H929 cells with 10 nmol/L ARRY-520, followed 8 hours later with the addition of 2 μmol/L compound 65, resulted in a significant increase in apoptosis by 24 hours compared with either ARRY-520 or compound 65 alone (Fig. 6D), similar to the effect of Mcl-1 knockdown (Fig. 5C). These results are consistent with a role for Mcl-1 functioning to prevent apoptosis during mitotic arrest of NCI H929 cells.

Discussion

The inhibition of mitosis-specific drug targets, such as KSP, is expected to yield improvements in clinical efficacy and safety profiles compared with current microtubule-targeted chemotherapeutics. To date, however, KSP inhibitors have shown only sporadic clinical activity in a subset of tumor types. Thus, an unresolved challenge is to determine which tumors or patients are most likely...
to be sensitive to compounds such as ARRY-520. Such translational knowledge would enable the design of better clinical trials and patient selection strategies. Previous efforts to identify the molecular sources of variation in sensitivity to apoptosis have suggested that the balance between apoptotic signals and the integrity of the SAC are key events in determining the onset of cell death in response to mitotic inhibitors (13–15, 27, 28, 38). To our knowledge, ours is the first study to successfully identify a single survival protein, Mcl-1, as a determinant controlling the decision to undergo apoptosis during mitotic arrest in multiple myeloma cell lines.

**Survival protein reliance determines cell fate**

We have observed that of the four myeloma cell lines tested, three undergo a rapid onset of apoptosis, committing to cell death within 24 hours of exposure to ARRY-520, while still arrested in mitosis. By contrast, NCI H929 cells showed a significantly delayed apoptosis following KSP inhibition compared with the other three multiple myeloma cell lines (Fig. 1). The majority of H929 cells seemed to undergo SAC failure and subsequent cell death after 72 hours when the cells were in interphase (Fig. 2B). Western analysis failed to show a significant difference in mitotic duration between these lines (Fig. 2; Supplementary Fig. S2), suggesting that this dissimilarity could not be due solely to a SAC defect. Of note, although Mcl-1 levels declined rapidly in cells that died in mitosis, Mcl-1 levels remained stable in NCI H929 throughout the course of the experiment, suggesting that Mcl-1 stability enabled survival of ARRY-520–arrested cells until SAC failure and mitotic exit. Comparable results have recently been reported whereby decreasing Mcl-1 expression in various tumor cell lines, including multiple myeloma cell lines, led to the sensitization of cells previously resistant to CDK inhibition (39). These data support a model wherein the fate of a cell once it has become arrested in mitosis is controlled by two independent clocks: the cell-specific rate of decline in survival signals below a threshold for triggering cell death and the maintenance of mitotic arrest as determined by the intrinsic rate of cyclin B1 degradation. In some tumor cells, as exemplified by JJN3, RPMI 8226, and U266, the loss of Mcl-1 during a KSP inhibitor–induced mitotic arrest is sufficient to promote the onset of apoptosis. Conversely, tumor cells that rely on Mcl-1 for survival, but are less capable of inducing Mcl-1 degradation during mitotic arrest, such as NCI H929, may exhibit a delayed onset of apoptosis and require far longer exposure to induce cell death.

**Therapeutic importance of Mcl-1 in determining cell fate**

The importance of survival proteins in determining cell fate in response to KSP inhibition has been shown by others. In a population-based analysis similar to what has been presented here, Shi et al. characterized the response of several solid tumor cell lines to three different antimitotic drugs (28). Although these cells all responded
similarly to a defective mitotic spindle, the response of the apoptotic machinery was variable between cell lines. Loss of the survival protein XIAP correlated with the onset of apoptosis. Although supporting the hypothesis that cell fate decisions in response to KSP inhibition are determined in part by modulation of survival proteins, the loss of XIAP has not yet been directly shown as being responsible for cell death. Here, we clearly show a role for Mcl-1 degradation in determining cell fate in response to KSP inhibition in several myeloma cell lines. We believe that these data can inform the identification of tumors likely to be most sensitive to KSP inhibitors clinically. KSP inhibitors are typically given over short, infusional schedules and therefore have defined periods of therapeutic exposure, with reported clinical half-lives typically ranging from 28 to 50 hours (40). Thus, we hypothesize that cells that rely on more stable survival proteins, such as Bcl-2 or Bcl-XL, may be temporally refractory to KSP inhibition. By maintaining survival signals during mitotic arrest, these cells may be able to survive transient mitotic inhibition until drug levels drop below therapeutic levels (at which time a normal spindle can be reassembled and cell cycle progression can commence). In contrast, cells that depend on Mcl-1 and rapidly degrade it during mitotic arrest are more likely to be responsive to the drug because apoptosis can be initiated within the window of exposure to the drug.

The importance of Mcl-1 in determining apoptosis in mitosis in hematologic cell lines makes particular sense, given the fact that hematologic cells typically rely on this short-lived survival protein. Thus, we suggest that hematologic malignancies, such as multiple myeloma, leukemia, and lymphoma, are particularly good candidates to investigate the clinical activity of KSP inhibitors. Of note, the KSP inhibitor SB-743921 has shown clinical activity in Hodgkin’s and non–Hodgkin’s lymphomas, although a relationship between Mcl-1 and response has not been investigated (41). Many solid tumor cell

![Figure 6](https://example.com/fig6.png)

**Figure 6.** Inhibition of Mcl-1 binding to NOXA in NCI H929 cells confers increased sensitivity to ARRY-520. A, chemical structure of compound 65. B, coimmunoprecipitation of NOXA with endogenous Mcl-1 is blocked in the presence of 1 μmol/L compound 65 in NCI H929 cell extracts. Rabbit IgG was used as a control for immunoprecipitation. C, cell viability of NCI H929 cells exposed to the indicated concentrations of compound 65 for 24 or 48 h. Data are reported as the percentage of viable NCI H929 cells following compound 65 treatment compared with DMSO control–treated NCI H929 cells. D, NCI H929 cells treated with DMSO or 2 μmol/L compound 65 were analyzed for caspase-3/7 activity at various time points following the addition of DMSO or 10 nmol/L ARRY-520. Total DMSO concentration of the control did not exceed 0.5% (v/v). Fold caspase-3/7 activity is reported as the mean of three separate experiments; error bars, SD.
lines do not express or depend on Mcl-1. Here, it is possible that other mechanisms, such as degradation of XIAP (28), may determine cell fate following KSP inhibitor treatment. Alternatively, it has been reported that subsets of nonhematologic tumor types, such as non–small cell lung carcinomas, melanomas, and biliary cancers, may gain the ability to express Mcl-1 during tumorigenesis (42–44), and these Mcl-1–expressing solid tumors may also be expected to be sensitive to shorter drug exposures of ARRY-520. These findings would be expected to have significant value in selecting potentially sensitive patient populations in solid tumor indications.

**Temporal resistance to ARRY-520: predictive value of Mcl-1 regulation by NOXA**

The fact that NCI H929 cells rely on Mcl-1 but are unable to significantly degrade Mcl-1 during mitotic arrest, and therefore avoid mitotic cell death, indicates that Mcl-1 stabilization may represent a mechanism of resistance to KSP inhibitors. One possible mechanism for Mcl-1 stabilization in the NCI H929 cell line may be mediated through the proapoptotic BH3-only protein NOXA. NOXA antagonizes the survival function of Mcl-1 and has been found to be sufficient to promote its degradation by the proteasome (34, 45, 46). Interestingly, NOXA protein levels are lowest in the NCI H929 cell line, which may help explain why Mcl-1 levels are more stable during a prolonged mitotic arrest (Supplementary Fig. S7). Reducing NOXA levels in the other three cell lines delayed Mcl-1 degradation and prolonged M-phase arrest and survival (Fig. 4; Supplementary Figs. S5 and S6). Thus, tumor cells with higher expression of NOXA protein may be more sensitive to antimitotics, such as ARRY-520, by promoting the rapid degradation of Mcl-1 by the proteasome during mitotic arrest. As Mcl-1 has also been reported to be cleaved by active caspases (47), we hypothesize that the NOXA-driven decline in Mcl-1 levels below a death threshold would trigger apoptosis and a further decline in Mcl-1 protein levels to cement the commitment to apoptosis. Interestingly, a recent genome-wide siRNA screen of HeLa cells exposed to KSP inhibitor identified NOXA (also known as PMAIP1) knockdown as a potential suppressor of cell death caused by low concentrations of K5 (48). Further investigation of the role of NOXA in modulating cell fate in response to KSP inhibition is needed to confirm these hypotheses.

In summary, our data suggest that tumors that express and rely on Mcl-1 for survival may be more sensitive to the KSP inhibitor ARRY-520. However, this observation is dependent on the ability of the tumor to degrade Mcl-1 during mitotic arrest. Mcl-1 is a key antiapoptotic protein in a wide variety of hematologic tumor types, such as multiple myeloma (22, 25), leukemia (49), and lymphoma (50). These data therefore support further investigation of ARRY-520 in hematologic malignancies, which rely on Mcl-1 for survival. To test this hypothesis clinically, ARRY-520 is being explored in clinical trials in both acute myelogenous leukemia and multiple myeloma.

**Disclosure of Potential Conflicts of Interest**

The authors are employees and shareholders of Array BioPharma, Inc., Boulder, CO, USA. This company has patent rights on some reagents used in this study.

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