

5-Azacytidine, a DNA methyltransferase inhibitor, induces ATR-mediated DNA double-strand break responses, apoptosis, and synergistic cytotoxicity with doxorubicin and bortezomib against multiple myeloma cells

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Abstract

In this study, we investigated the cytotoxicity of 5-azacytidine, a DNA methyltransferase inhibitor, against multiple myeloma (MM) cells, and characterized DNA damage-related mechanisms of cell death. 5-Azacytidine showed significant cytotoxicity against both conventional therapy-sensitive and therapy-resistant MM cell lines, as well as multidrug-resistant patient-derived MM cells, with IC_{50} of ~ 0.8 – $3 \mu\text{mol/L}$. Conversely, 5-azacytidine was not cytotoxic to peripheral blood mononuclear cells or patient-derived bone marrow stromal cells (BMSC) at these doses. Importantly, 5-azacytidine overcame the survival and growth advantages conferred by exogenous interleukin-6 (IL-6), insulin-like growth factor-I (IGF-I), or by adherence of MM cells to BMSCs. 5-Azacytidine treatment induced DNA double-strand break (DSB)

responses, as evidenced by H2AX, Chk2, and p53 phosphorylations, and apoptosis of MM cells. 5-Azacytidine-induced apoptosis was both caspase dependent and independent, with caspase 8 and caspase 9 cleavage; Mcl-1 cleavage; Bax, Puma, and Noxa up-regulation; as well as release of AIF and EndoG from the mitochondria. Finally, we show that 5-azacytidine-induced DNA DSB responses were mediated predominantly by ATR, and that doxorubicin, as well as bortezomib, synergistically enhanced 5-azacytidine-induced MM cell death. Taken together, these data provide the preclinical rationale for the clinical evaluation of 5-azacytidine, alone and in combination with doxorubicin and bortezomib, to improve patient outcome in MM. [Mol Cancer Ther 2007;6(6):1718–27]

Introduction

DNA methylation and related modulation of gene expression contributes to the development of malignancies (1–4). Specifically, methylation of CpG dinucleotides in promoter regions have been associated with transcriptional silencing of tumor suppressor genes, suggesting DNA methylation as a target for novel therapeutics (5, 6). 5-Azacytidine (5-AzaC) belongs to a class of cytosine analogues which were developed as inhibitors of DNA methylation (6–8) and have shown clinical efficacy in myelodysplastic syndromes (MDS) and acute myelogenous leukemia (AML; refs. 9, 10). Despite the widely accepted demethylating activity of 5-AzaC, the exact basis of its clinical efficacy and its cytotoxic mechanism still remain unclear.

The biological activity of 5-AzaC is associated with its incorporation into cellular DNA and/or RNA, with subsequent sequestration of DNA methyl transferases (DNMT) via covalent bond formation between C6 of 5-AzaC and cysteine thiolate of DNMTs. Under physiologic conditions, this enzyme-DNA/RNA adduct is formed irreversibly, thereby depleting the cells of DNMT activity and causing demethylation of cellular DNA (11–14). Based on the chemical mechanism of 5-AzaC activity, a number of non-mutually exclusive mechanisms of its tumor cytotoxicity have been proposed (15). Among these, two major ones are (a) demethylation of cellular DNA, with reactivation of silenced genes; and (b) induction of DNA damage due to the formation of irreversible, covalent enzyme-DNA adducts. Although the biological effects of methylation and demethylation have been extensively studied in various cancers (10, 16, 17), DNA damage-related sequelae of these agents in cancer cells have not been delineated (18–20).

Multiple myeloma (MM) is a B-cell malignancy characterized by proliferation of monoclonal plasma cells in the

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bone marrow (BM). Epidemiologically, MM is the second most common hematologic malignancy in the United States (21) and still remains incurable due to the development of drug resistance (22–24). Factors contributing to the development of drug resistance include those intrinsic to tumor cells, as well as the growth and survival advantages provided by the BM microenvironment (25, 26). Importantly, novel therapies such as bortezomib and lenalidomide that target not only the MM cell but also the MM cell-BM interactions can overcome conventional drug resistance in both preclinical and clinical settings (22, 27, 28). To date, the effects of 5-AzaC against MM cells in the BM milieu have not been characterized.

In this study, we investigated the cytotoxic effects of 5-AzaC against human MM cells and characterized the DNA damage-related biochemical and cellular mechanisms associated with 5-AzaC-induced tumor cell death. We show that 5-AzaC is cytotoxic against MM cells and overcomes the growth and survival advantages provided by the BM microenvironment. Mechanistic studies show that 5-AzaC induces DNA double-strand break (DSB) responses and causes apoptosis of MM cells. These responses are mediated predominantly via the PIKK family member sensor protein ATR. Importantly, 5-AzaC induces synergistic MM cell death with doxorubicin, which induces DNA DSB responses predominantly mediated via ATM, another PIKK family member. Finally, pretreatment of MM cells with bortezomib markedly enhances 5-AzaC-induced cytotoxicity. Taken together, these studies provide the preclinical rationale for the clinical evaluation of 5-AzaC, alone and in combination with doxorubicin and bortezomib, to improve patient outcome in MM.

Materials and Methods

5-AzaC and Inhibitors

5-AzaC was provided by Pharmion Corporation. Stock solutions of 5-AzaC (200 mmol/L) were prepared in DMSO and stored at -20°C . The stock solutions were further diluted in PBS (1–20 $\mu\text{mol/L}$; $<0.1\%$ DMSO in the final concentration) for cell culture experiments. Cytarabine (AraC) and doxorubicin were obtained from Sigma-Aldrich. Bortezomib was provided by Millenium Pharmaceuticals. KU-55933, wortmannin, and z-VAD-fmk were purchased from Calbiochem; caffeine was purchased from Sigma-Aldrich.

Cell Culture and Reagents

Dex-sensitive (MM.1S) and Dex-resistant (MM.1R) human MM cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). RPMI-8226 was obtained from the American Type Culture Collection. Doxorubicin-resistant (RPMI-Dox40) and melphalan-resistant (RPMI-LR5) cells were kindly provided by Dr. William Dalton (H. Lee Moffitt Cancer Center, Tampa, FL). Human bone marrow stromal cells (BMSC) lines KM104 and KM105 were kindly provided by Dr. Kenichi Harigaya (Chiba University Graduate School of Medicine,

Chiba, Japan). All MM and BM stromal cell lines were cultured in RPMI 1640 (Sigma) containing 10% fetal bovine serum, 2 mmol/L L-glutamine (Life Technologies), 100 units/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin (Life Technologies). Blood samples collected from healthy volunteers were processed by Ficoll Paque gradient to obtain peripheral blood mononuclear cells (PBMC), which were cultured in RPMI 1640 containing 20% fetal bovine serum (FBS). Patient MM and BM cells were obtained from BM samples after informed consent was obtained per the Declaration of Helsinki and approval by the Institutional Review Board of the Dana-Farber Cancer Institute (Boston, MA). BM mononuclear cells were separated using Ficoll Paque density sedimentation, and plasma cells were purified ($>95\%$ CD138⁺) by positive selection with anti-CD138 magnetic activated cell separation microbeads (Miltenyi).

Growth Inhibition and Proliferation Assays

To evaluate the growth inhibitory effect of 5-AzaC on MM cells, PBMCs, and BMSC, colorimetric MTT assay (Chemicon) was done as described previously (29). Briefly, cells were incubated in 96-well plates in the presence of increasing concentrations of 5-AzaC (or vehicle control) for 72 h. MTT was added to the cultures during the last 4 h of incubation. This was followed by the addition of isopropanol containing 0.04 N HCl to the wells and measurement of absorbance at a wavelength of 570 nm, with a reference wavelength of 630 nm.

To measure the proliferation of MM cells and BMSCs, the rate of DNA synthesis was measured as described previously (29). Briefly, cells were incubated in 96-well plates with increasing concentrations of 5-AzaC for 72 h. During the last 8 h of incubation, cells were pulsed with [³H]thymidine (0.5 μCi per well) and then harvested onto glass filters with an automatic cell harvester. Radioactivity was counted using the LKB Betaplate scintillation counter (Wallac).

To evaluate the effects of growth factors, 10 ng/mL recombinant interleukin-6 (IL-6; R&D Systems) or 50 ng/mL insulin-like growth factor-I (IGF-I; R&D Systems) was added to the wells with increasing concentrations of 5-AzaC at the beginning of the incubation. To evaluate the effects of BMSC on MM cell proliferation, BMSCs were incubated in 96-well culture plates ($\sim 5,000$ – $10,000$ BMSCs per well) for 24 h. After washing, MM cells were added to the wells (2.5×10^4 cells per well) and incubated with increasing concentrations of 5-AzaC. In each case, proliferation of MM cells was measured after 72 h as described above.

Western Blotting

MM cells were cultured with the indicated concentrations of 5-AzaC for the specified times, harvested, washed, and lysed using lysis buffer (radioimmunoprecipitation assay buffer, 2 mmol/L Na_3VO_4 , 5 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 5 mg/mL leupeptin, and 5 mg/mL aprotinin). Cell lysates were subjected to SDS-PAGE; transferred to polyvinylidene difluoride membrane; and immunoblotted with antibodies for poly(ADP-ribose)

polymerase (PARP), caspase 8, caspase 9, Bcl-2, Bcl-xl, Mcl-1, Bax, Noxa (Imgenex), Puma, AIF, EndoG (Axxora), phospho-(Ser²⁰)-p53, p53, phospho-(Ser³¹⁷)-Chk1, Chk1, phospho-(Thr⁶⁸)-Chk2, Chk2, phospho-(Ser¹³⁹)-H2AX, H2AX (Upstate Biotechnologies), ATM (Santa Cruz Biotechnologies), phospho-(Ser¹⁹⁸¹)-ATM (Rockland), ATR (Bethyl), phospho-(Ser⁴²⁸)-ATR, NBS1, phospho-(Ser³⁴³)-NBS1 (Novus), and tubulin (Santa Cruz Biotechnologies). All the antibodies were purchased from Cell Signaling or otherwise indicated.

Flow Cytometry

For detection of apoptotic cells, cell surface staining was done with FITC-labeled anti-Annexin V antibody and PI (BD Pharmingen) and were analyzed by a FACScan flow cytometer (Becton Dickinson).

Immunocytochemistry

Cytospins of MM cells on glass slides were made after treatment with 5-AzaC and fixed with 4% paraformaldehyde, followed by methanol at -20°C. The slides were blocked with 5% FBS at 37°C, followed by incubation with primary antibody for 1 h, FITC-labeled secondary antibody for 30 min, and 4',6-diamidino-2-phenylindole (Invitrogen) for 5 min. Coverslips were then mounted on glass slides with ProLong Gold antifade (Invitrogen) and analyzed using Nikon E800 fluorescence microscopy.

Isobologram Analysis

For combination treatment of 5-AzaC with doxorubicin, MTT assay data were converted to fraction of growth affected (FA) and analyzed using CalcuSyn software (Biosoft) with combination index (CI) values based on the Chou-Talalay method.

Results

5-AzaC Is Cytotoxic against MM Cell Lines and Patient MM Cells, but Not PBMCs or BMSCs

The cytotoxic effects of 5-AzaC against conventional therapy-sensitive MM cell lines (i.e., MM.1S, RPMI8226) and conventional therapy-resistant cell lines (i.e., dexamethasone-resistant MM.1R, doxorubicin-resistant RPMI-Dox40, and melphalan-resistant RPMI-LR5) were determined using MTT assay. 5-AzaC was significantly cytotoxic against all five MM cell lines tested, with IC₅₀ of ~0.7–3.2 μmol/L at 72 h (Fig. 1A; Table 1). 5-AzaC was also cytotoxic against multidrug-resistant patient MM cells with IC₅₀ of ~1.2–1.7 μmol/L at 72 h (Fig. 1B; Table 1). Importantly, 5-AzaC at these doses did not show any significant cytotoxicity against normal donor PBMCs (Fig. 1C) or in rapidly dividing KM104 and KM105 BMSC (Fig. 1C)

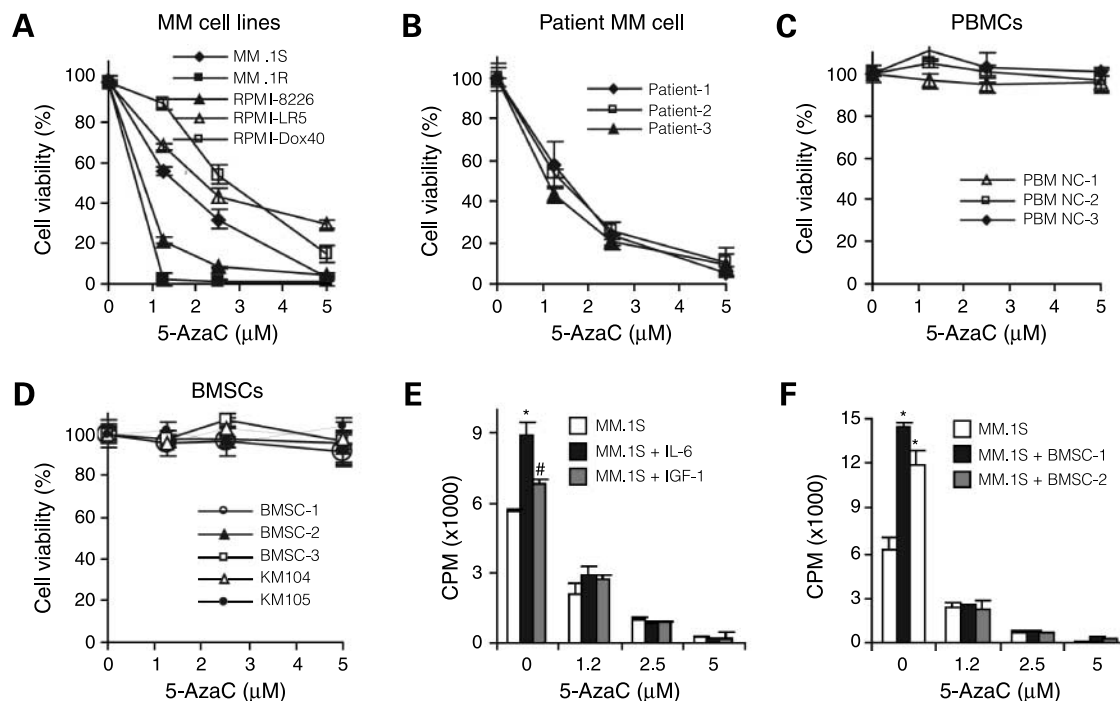


Figure 1. 5-AzaC induces cytotoxicity in MM cells, but not in PBMCs and BMSCs and overcomes the protective effects of IL-6, IGF-1, and adherence to patient BMSCs. **A**, conventional therapy-sensitive MM.1S (◆) and RPMI-8226 (■) MM cell lines, as well as conventional therapy resistant MM.1R (▲; Dex resistant), RPMI-Dox40 (□; doxorubicin resistant), and RPMI-LR5 (△; Mel resistant) MM cell lines were cultured in the presence or absence of 5-AzaC for 72 h. **B–D**, MM cells derived from three MM patients (▲, □, and ◆; **B**); PBMCs derived from three healthy subjects (□, △, and ◆; **C**); KM104 (△) and KM105 (●) BMSC lines and BMSCs isolated from three patients (■, △, and ○; **D**). Cultured with 5-AzaC for 72 h. In all cases, cell viability was assessed by MTT assay. *Points*, means of triplicate cultures; *bars*, SD. **E** and **F**, MM.1S cells were treated for 72 h with 5-AzaC (0–5 μmol/L) in the absence (□) or presence (■) of IL-6 (10 ng/mL), or (■) IGF-1 (200 ng/mL; **E**); and without (□) or with BMSCs derived from MM patients 1 (■) and 2 (■; **F**). DNA synthesis was determined by measuring [³H]-thymidine incorporation during the last 8 h of 72-h cultures. *Columns*, means of triplicate cultures, *bars*, SD. *, *P* < 0.01, and #, *P* < 0.05, as compared with MM cells only controls.

Table 1. The IC₅₀ values for 5-AzaC in MM cells

MM cells	IC ₅₀ (μmol/L)
MM.1S	1.5 ± 0.2
MM.1R	0.7 ± 0.2
RPMI-8226	1.1 ± 0.3
RPMI-LR5	2.5 ± 0.6
RPMI-Dox40	3.2 ± 0.5
Patient-derived	1.5 ± 0.3

NOTE: The IC₅₀ values (72 h) for 5-AzaC in conventional therapy-sensitive MM.1S and RPMI-8226 cell lines, conventional therapy-resistant MM.1R (Dex resistant), RPMI-Dox40 (doxorubicin resistant), and RPMI-LR5 (Mel resistant) cell lines, as well as patient-derived MM cells, are indicated. Data represent means ± SD of three independent experiments.

lines, as well as BMSCs isolated from MM patients (Fig. 1D). These data show that 5-AzaC has selective cytotoxicity against MM cells. It is also noteworthy that 5-AzaC showed enhanced cytotoxicity against MM cells when compared with AraC, another cytosine analogue that has previously been clinically tested in MM (Supplementary Fig. S1).¹

5-AzaC Overcomes the Growth and Survival Advantages Conferred by IL-6, IGF-I, and Patient-Derived BMSCs

It has previously been shown that in MM patients, the serum IL-6 concentration can reach as high as 700 pg/mL (30); with mean serum IGF-I levels of 17 ± 6 nmol/L (corresponds to 135 ± 59 ng/mL; ref. 31). These cytokines, through autocrine and paracrine mechanisms, provide growth and survival signals to MM cells and protect them against drug-induced apoptosis (32–38). Therefore, we next examined whether 5-AzaC could overcome the growth and survival advantages conferred by IL-6 or IGF-I using DNA thymidine incorporation assay. As can be seen in Fig. 1E, exogenous IL-6 (10 ng/mL) and IGF-I (200 ng/mL) triggered a ~1.6- and ~1.2-fold increase in MM.1S cell growth, respectively; nevertheless, 5-AzaC completely abrogated these growth advantages.

In MM, tumor cells are predominantly localized in the BM where direct interaction of BMSC with MM cells provides MM cells with growth advantages as well as confers cell adhesion-mediated drug resistance (25, 26). To examine the effects of 5-AzaC on MM cells in the BM microenvironment, MM.1S cells were cocultured with patient-derived BMSC and then were treated with increasing doses of 5-AzaC. Although coculture of MM.1S cells with BMSC increased MM.1S cell growth (~2–2.5-fold), as detected by DNA thymidine incorporation, 5-AzaC completely inhibited this response (Fig. 1F). It is noteworthy that 5-AzaC treatment was not toxic to BMSCs as detected by MTT assay (Fig. 1D), indicating that the effects of 5-AzaC are selective to MM cells. Similar trends were observed in all MM cell lines tested (Supplementary Fig. S2).¹ Taken together, these results

indicate that 5-AzaC potentially overcomes the growth and survival advantages in MM cells conferred by IL-6, IGF-I, and BMSC.

5-AzaC Induces DNA DSB Responses in MM Cells

It is well established that replication-blocking DNA lesions can cause replication fork collapse and thereby lead to the formation of DNA DSBs (39–41). Because 5-AzaC forms covalent enzyme-DNA adducts with DNMTs which can cause replication fork collapse, we next explored whether 5-AzaC caused DSB formation in MM cells. An early specific cellular response to DSB in mammalian cells is the phosphorylation of the histone protein H2AX at Ser¹³⁹ (γ-H2AX), with respective foci formation (42, 43). Therefore, we next examined whether 5-AzaC-induced H2AX phosphorylation and foci formation in MM cells. Western blot and immunocytochemical analysis in MM.1S

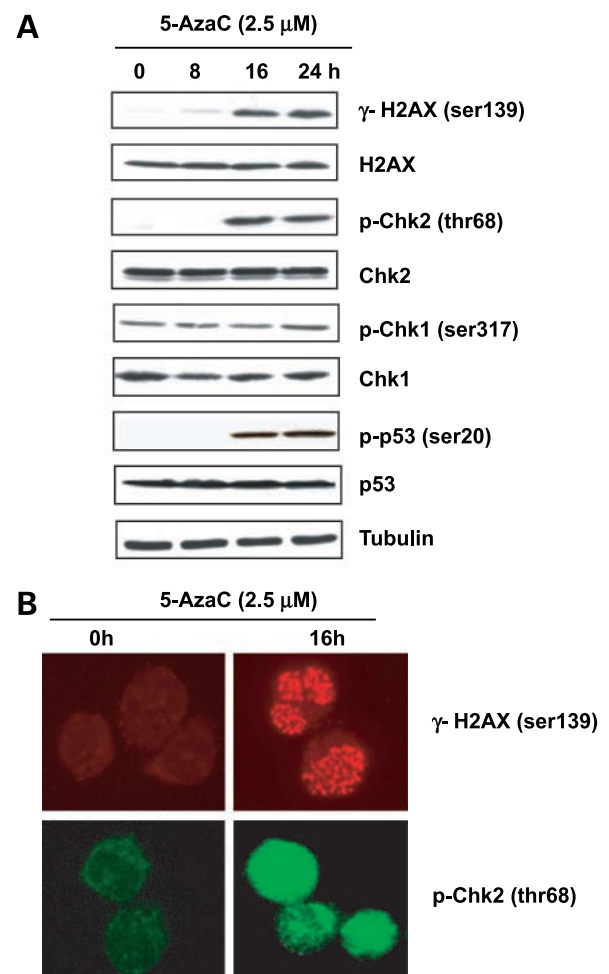


Figure 2. 5-AzaC activates DNA DSB responses in MM cells. **A**, MM.1S cells were treated with 5-AzaC (2.5 μmol/L) for 0–24 h. Phosphorylation of the DNA damage response proteins H2AX (Ser¹³⁹), Chk1 (Ser³¹⁷), Chk2 (Thr⁶⁸), and p53 (Ser²⁰) was assessed by Western blotting. **B**, representative images of the immunocytochemistry assay done with anti-phospho-(Ser¹³⁹)-H2AX and anti-phospho-(Thr⁶⁸)-Chk2 antibodies are shown at 0 and 16 h.

¹ Supplementary material for this article are available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

cells showed that 5-AzaC induces significant H2AX phosphorylation and foci formation in MM.1S cells (Fig. 2A and B). Phosphorylation of H2AX and organization of γ -H2AX into discrete foci not only indicates that 5-AzaC causes DSB formation, but also shows that DNA damage response pathways are activated in MM cells. A multitude of other proteins have been identified as mediators of DNA damage responses, including the checkpoint kinases (i.e., Chk1, Chk2) and p53 (44–46). To further explore the activation of DNA damage responses induced by 5-AzaC, we next assessed activation of Chk1 and Chk2 proteins. Western blot analysis revealed that 5-AzaC induced significant phosphorylation of Chk2 (Thr⁶⁸), without significant change in Chk1 phosphorylation or in the total protein levels of Chk1 or Chk2 (Fig. 2A). H2AX and Chk2 phosphorylation was similarly observed in all MM cell lines tested (Supplementary Fig. S3).¹ Ser²⁰ of p53 has previously been shown to be a substrate of Chk2 (47–49), and significant phosphorylation of p53 at Ser²⁰ was detected in P53 wild-type MM.1S cells after 5-AzaC exposure, with no significant changes in total p53 levels. Taken together, these results indicate that 5-AzaC activates DNA DSB response pathways in MM cells. It is noteworthy that no

cell death or caspase activation was observed until the first 24 h after 5-AzaC exposure at these doses (Fig. 3A and B); thus, the formation of DSB was not a secondary event due to internucleosomal DNA cleavage in the apoptotic process.

5-AzaC Induces Apoptosis Associated with Both Caspase-Dependent and Caspase-Independent Pathways

DSBs are the most cytotoxic DNA lesions and causes apoptosis of mammalian cells (50–52). Having shown that 5-AzaC induces DNA DSB responses in MM cells, we next therefore determined if 5-AzaC induced apoptosis. MM.1S cells were treated with increasing concentrations of 5-AzaC in a time course experiment, and then analyzed by flow cytometry for the apoptotic marker Annexin V. 5-AzaC treatment increased the proportion of cells positive for Annexin V after 24 h, which peaked at 72 h, in a dose-dependent manner (Fig. 3A). Similarly, 5-AzaC increased the proportion of cells positive for Annexin V in all MM cell lines tested, indicating the induction of apoptosis (Supplementary Fig. S4).¹ We further confirmed apoptosis of MM cells triggered by 5-AzaC (2.5 μ mol/L) by examining the cleavage of PARP, as well as caspases 8 and 9, in MM.1S cells by Western blotting (Fig. 3B). To further confirm the

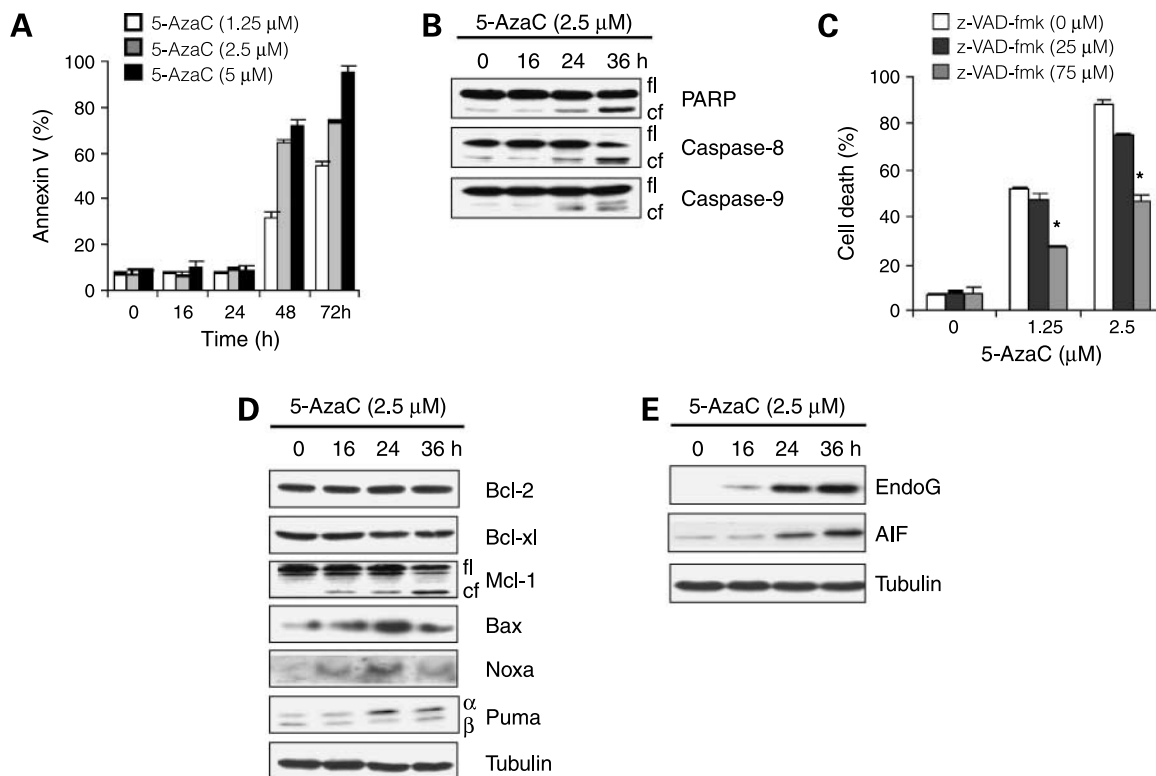
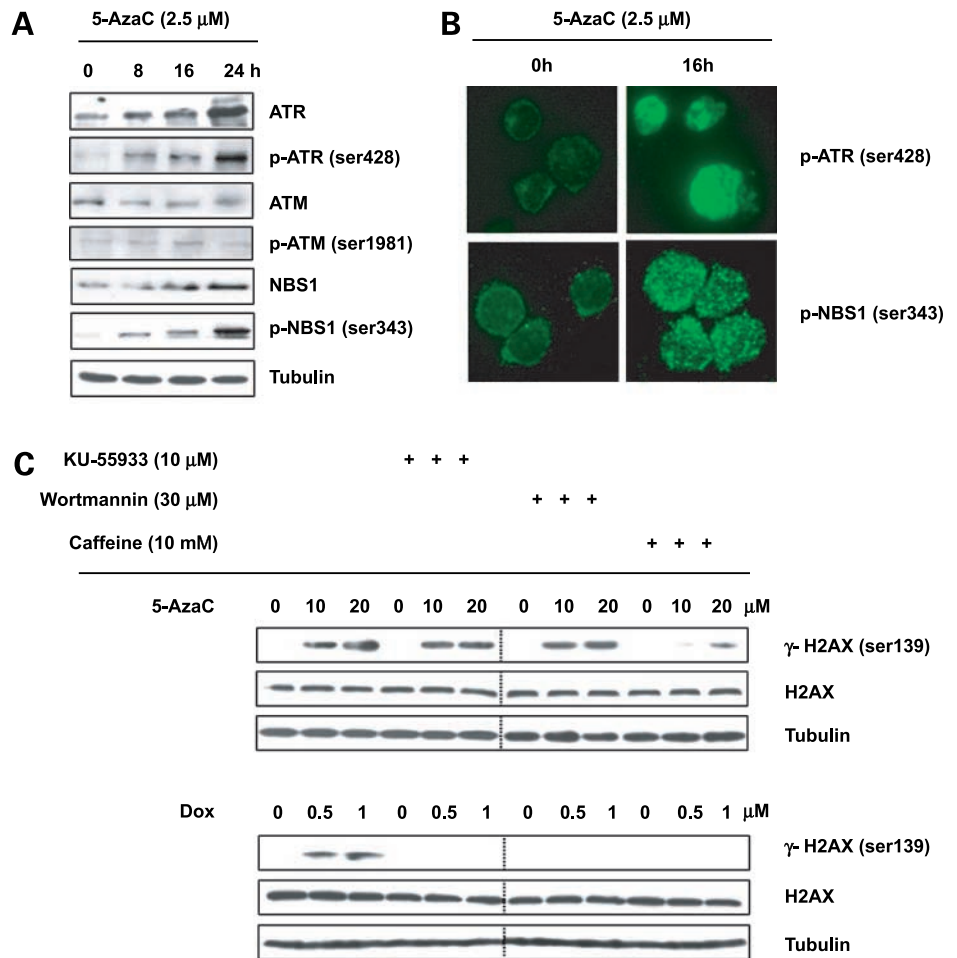


Figure 3. 5-AzaC induces apoptosis associated with both caspase-dependent and caspase-independent pathways. **A**, MM.1S cells were treated with 5-AzaC [1.25 μ mol/L (\square), 2.5 μ mol/L (\blacksquare), and 5 μ mol/L (\blacksquare)] for 0–72 h; apoptosis was assessed by Annexin V staining. *Columns*, means of triplicate experiments; *bars*, SD. **B**, cleavage of PARP and the initiator caspases 8 and 9 were determined by Western blotting of MM.1S cells treated with 5-AzaC (2.5 μ mol/L) for 0–36 h. **C**, MM.1S cells were treated with 5-AzaC (0–2.5 μ mol/L) for 72 h, with 25 μ mol/L (\blacksquare), 75 μ mol/L (\blacksquare), or without (\square) z-VAD-fmk; cell death was assessed by flow cytometry after PI staining. *Columns*, means of triplicate experiments; *bars*, SD. *, $P < 0.01$, as compared with no z-VAD-fmk controls. **D** and **E**, MM.1S cells were treated with 5-AzaC (2.5 μ mol/L) for 0–36 h, followed by immunoblotting for Bcl-2 family proteins (**D**) or release of the mitochondrial, caspase-independent apoptotic mediators AIF and EndoG into the cytosol (**E**).

Figure 4. 5-AzaC–induced DNA DSB responses are mediated predominantly via the sensor protein ATR in MM cells. **A**, MM.1S cells were treated with 5-AzaC (2.5 $\mu\text{mol/L}$) for 0–24 h; DNA DSB sensor proteins ATM (Ser¹⁹⁸¹), ATR (Ser⁴²⁸), and NBS1 (Ser³⁴³) were separated on a 6% gel and assessed by Western blotting of whole cell lysates. **B**, representative images of the immunocytochemical assay done with anti-phospho-(Ser⁴²⁸)-ATR and anti-phospho-(Ser³⁴³)-NBS1 antibodies are shown at 0 and 16 h. **C**, MM.1S cells were treated with 5-AzaC (0–20 $\mu\text{mol/L}$) or doxorubicin (0–1 $\mu\text{mol/L}$) for 4 h in the presence or absence of Ku-55933, wortmannin, or caffeine. Activation of the DSB responses was assessed by phosphorylation of the downstream protein H2AX (Ser¹³⁹) by Western blotting. *Dashed lines*, two gels that were run in parallel under identical experimental conditions.



role played by caspases in 5-AzaC–induced apoptosis, MM.1S cells were pretreated with the pan-caspase inhibitor, z-VAD-fmk (0–75 $\mu\text{mol/L}$), and then treated with 5-AzaC (0–2.5 $\mu\text{mol/L}$) for 72 h. z-VAD-fmk significantly, but only partially, inhibited 5-AzaC–induced cell death (Fig. 3C), suggesting that 5-AzaC–induced apoptosis is, at least in part, mediated by caspases.

Given that z-VAD-fmk only partially rescues 5-AzaC–induced cell death, we next explored whether 5-AzaC caused changes in bcl-2 family members and in particular induced the release of the caspase-independent apoptotic proteins—AIF and EndoG—from mitochondria into the cytosol. Although no significant changes were detected in the expression levels of the antiapoptotic Bcl-2 and Bcl-xl proteins, there was a significant increase in proapoptotic family members Bax, Noxa, and Puma- α , which peaked at 24 h (Fig. 3D). In addition, 5-AzaC also caused significant cleavage of the antiapoptotic protein Mcl-1 and caused the release of mitochondrial AIF and EndoG into the cytosol (Fig. 3E), suggesting the involvement of the caspase-independent pathway in 5-AzaC–induced apoptosis. Taken together, these results suggest that 5-AzaC–induced apoptosis is associated with both caspase-dependent and caspase-independent pathways in MM cells.

5-AzaC–Induced DNA DSB Responses in MM Cells Are Mediated Predominantly via ATR

The PIKK family member sensor proteins ATM and ATR are central to sensing and initiating the DNA DSB responses and are involved in signaling DNA damage to the checkpoint and apoptotic machinery in mammalian cells (44, 50). Although the activation sequence remains controversial and a multitude of proteins have been identified as substrates of ATM and ATR, the current paradigm is that ATM and ATR activation is followed by the phosphorylation of Nbs1 and recruitment of Mre11 and Rad50 to form the MRN complex to the damage site. This is then followed by the activation of downstream effector kinases, including H2AX, Chk, and p53, to signal to the checkpoint and apoptotic machinery of cells (44, 53–56). To further delineate the mechanism of 5-AzaC–induced DSB responses in MM, we next examined the effect of 5-AzaC on these upstream DNA damage signaling events, specifically on the sensor molecules ATM and ATR. Western blot analysis of MM.1S cells revealed that 5-AzaC (2.5 $\mu\text{mol/L}$) did not cause a significant increase in phospho-ATM or total ATM levels; importantly, however, a significant increase in both the phospho-ATR and total ATR, as well as phospho-NBS1 and NBS1, were evident starting at 8 h

after treatment (Fig. 4A). Immunocytochemical experiments further confirmed the phosphorylation of ATR and NBS1 triggered by 5-AzaC in MM.1S cells (Fig. 4B). The lack of any significant changes in ATM, combined with significant increases in ATR and phospho-ATR, suggests a role for ATR, but not ATM, as the sensor molecule in 5-AzaC-induced DNA damage responses.

Ku55933 is a highly specific ATM inhibitor at $\sim 10 \mu\text{mol/L}$ concentrations *in vivo* (57–59). Wortmannin is another potent inhibitor of ATM (at $\sim 5.8 \mu\text{mol/L}$), as well as DNA-PK (at $\sim 3.6 \mu\text{mol/L}$), and a remarkably less potent inhibitor of ATR (at $>100 \mu\text{mol/L}$; refs. 60, 61). Caffeine, on the other hand, is a potent inhibitor of both ATM and ATR (IC_{50} , 0.2 and 1.1 mmol/L, respectively), but not of DNA-PK (IC_{50} , 10 mmol/L; refs. 60, 62). To evaluate the role played by these PIKK member sensor molecules in 5-AzaC-induced DNA DSB responses, MM.1S cells were pretreated with each of these inhibitors for 1 h, which was followed by an additional 4 h in the presence of 5-AzaC, and the downstream phosphorylation of H2AX was analyzed by Western blotting. Although neither Ku55933 (10 $\mu\text{mol/L}$) nor wortmannin (30 $\mu\text{mol/L}$) significantly decreased 5-AzaC-induced H2AX phosphorylation in MM.1S cells, caffeine (10 mmol/L) showed a significant inhibitory effect (Fig. 4C). These results indicate that 5-AzaC-induced DNA DSB responses in MM cells are mediated predominantly via ATR. Interestingly, doxorubicin-induced DNA DSB

responses in human lymphoblastoid cells were previously shown to be mediated predominantly by ATM (63). Combining two agents, such as 5-AzaC and doxorubicin, whose DSBs responses are initiated via different sensor molecules, may sensitize MM cells to their cytotoxic effects. Therefore, we next investigated whether ATM played a significant role as a sensor molecule during doxorubicin-induced DNA DSB responses in MM cells. Doxorubicin-induced H2AX phosphorylation was significantly abrogated by all inhibitors, including specific ATM inhibitor Ku55933 at 4 h in MM.1S cells (Fig. 4C). This result established that doxorubicin-induced DSB signaling in MM cells is predominantly mediated by ATM, as was previously shown in human lymphoblastoid cells.

5-AzaC Is Synergistic with Doxorubicin Against Doxorubicin-Sensitive as Well as Doxorubicin-resistant MM Cells

Given that the DSB responses induced by 5-AzaC and doxorubicin are initiated and signaled by reciprocal sensor molecules, ATR and ATM, respectively, we investigated whether combining these two agents increased the sensitivity of MM cells to these agents and induced synergistic cytotoxicity. Increasing concentrations of 5-AzaC were added to conventional therapy-sensitive MM.1S cells with doxorubicin (0, 20, 40 nmol/L), as well as to doxorubicin-resistant RPMI-Dox40 cells, and cytotoxicity was assayed by MTT (Fig. 5A, *left* and *right*, respectively). Synergistic

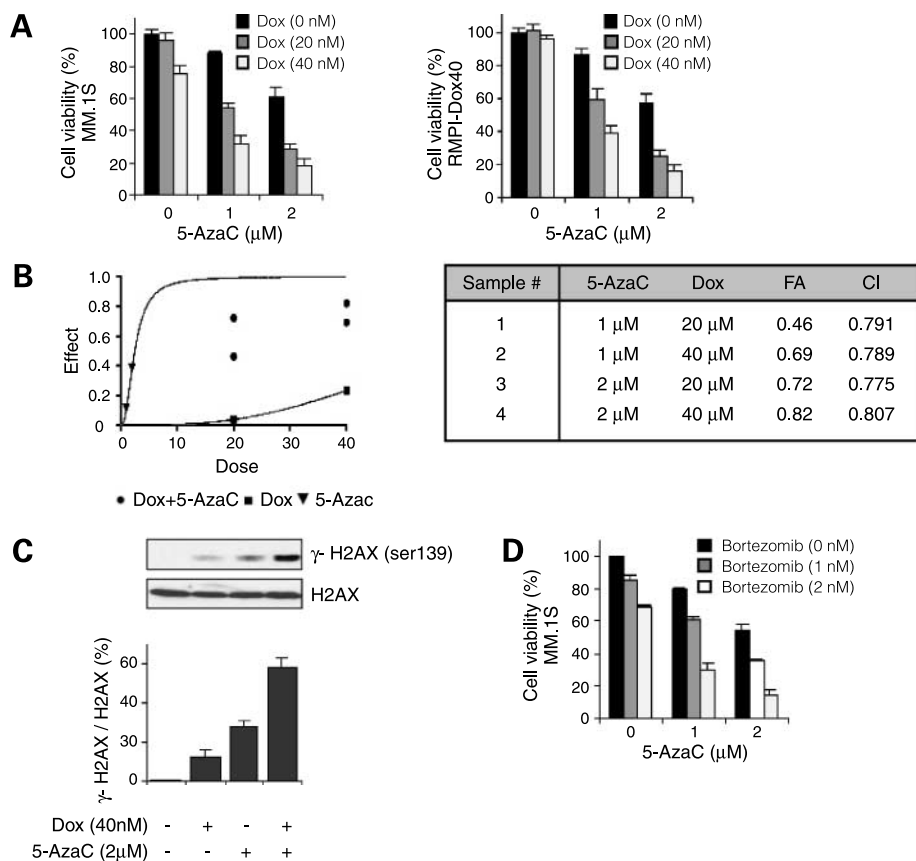


Figure 5. 5-AzaC is synergistic with doxorubicin in both doxorubicin-sensitive and doxorubicin-resistant MM cells. **A**, MM.1S (*left*) or RPMI-Dox40 (*right*) cells were treated with increasing concentrations of 5-AzaC at the indicated doses and doxorubicin [0 nmol/L (■), 20 nmol/L (▒), and 40 nmol/L (□)]; cytotoxicity was assayed by MTT after 48h. *Columns*, mean of triplicate cultures; *bars*, SD. **B**, synergistic cytotoxicity was confirmed in MM.1S cells by applying the Chou-Talalay method, with CI values <1 in all cases. The dose-effect curve and CI values for MM.1S cells are shown. **C**, representative Western blots and % quantitative densitometric values for the ratio of γ -H2AX (Ser¹³⁹)/H2AX are shown for doxorubicin alone, 5-AzaC alone, or doxorubicin and 5-AzaC in combination. *Columns*, values from two independent experiments; *bars*, SD. **D**, low doses of bortezomib sensitize MM cells to 5-AzaC. MM.1S cells were treated with bortezomib (0, 1, 2 nmol/L) for 16 h, which was followed by 5-AzaC treatment (0, 1, 2 $\mu\text{mol/L}$). Cell viability was detected by MTT assay (48 h). *Columns*, mean of triplicate cultures; *bars*, SD.

cytotoxicity was confirmed by the Chou-Talalay method to calculate the combination index (CI) values, as previously described (64, 65). Specifically, all combinations were synergistic, as can be seen in dose-effect curve (Fig. 5B, left), and all CI values were <1 (Fig. 5B, right), as presented for MM.1S cells. Importantly, densitometric analysis of Western blots revealed that phosphorylation of downstream H2AX protein was also synergistically enhanced by 5-AzaC and doxorubicin combination [13% by doxorubicin (40 nmol/L) alone; 28% by 5-AzaC (2 μ mol/L) alone; 58% by doxorubicin (40 nmol/L) and 5-AzaC (2 μ mol/L) in combination; Fig. 5C]. These results support our hypothesis that combining 5-AzaC and doxorubicin, whose DSB response signals are initiated by reciprocal sensor molecules, increases the sensitivity of the MM cells to these agents, and induces synergistic cell death.

Low-Dose Bortezomib Sensitizes MM Cells to 5-AzaC

It has previously been shown that bortezomib induces down-regulation (66) as well as cleavage of DNA-PK (67), which is an important DNA DSB repair enzyme. We therefore hypothesized that pretreatment of MM cells with bortezomib would inhibit the repair of 5-AzaC-induced DSBs and therefore enhance its cytotoxicity. Importantly, pretreatment of MM cells with low doses of bortezomib (1–2 nmol/L for 16 h) significantly enhanced 5-AzaC-induced cytotoxicity (Fig. 5D). CI values for the combinations shown were <1, indicating synergy (data not shown). It is noteworthy that bortezomib has previously been shown to have synergistic cytotoxicity with doxorubicin (66), which we have here shown to be synergistic with 5-AzaC. Taken together, these results provide the framework for combination trials of 5-AzaC with bortezomib and doxorubicin to increase therapeutic efficacy.

Discussion

In this report, we show for the first time that 5-AzaC, a DNMT inhibitor with clinical efficacy in MDS and AML, has significant *in vitro* cytotoxicity against MM cells. Our data in both conventional therapy-sensitive and therapy-resistant MM cell lines, as well as multidrug-resistant patient MM cells, show an IC_{50} of ~ 0.8 – 3μ mol/L at 72 h, which is physiologically readily achievable because pharmacokinetic studies of 5-AzaC in humans show C_{max} of 4.8 ± 1.4 , with AUC of $6.6 \pm 2.6 \mu$ mol/L at 75 mg/m²/day (68). Importantly, we observed no significant cytotoxicity of 5-AzaC against PBMCs or BMSCs at these concentrations, indicating selective cytotoxicity against MM cells. Taken together, these data suggest a favorable therapeutic index for 5-AzaC in MM.

It has previously been shown that the BM microenvironment confers conventional drug resistance in MM cells (69, 70). Novel therapies such as bortezomib and lenalidomide, which target not only MM cells, but also MM cell-BM interactions, can overcome conventional drug resistance in preclinical and clinical studies (22, 27, 28). Importantly, in this study, 5-AzaC completely abrogated the survival

and growth advantages provided both by the micro-environmental growth factors IL-6, IGF-I, as well as adherence of MM cells to BMSCs, indicating that 5-AzaC can induce cytotoxicity in MM cells in the BM milieu and overcome conventional drug resistance.

Drug resistance in MM cells can also be attributed to defective apoptotic signaling (71). Importantly, here we show that 5-AzaC induces apoptosis in MM cells via both caspase-dependent and caspase-independent pathways. Given the genetic and molecular heterogeneity of MM, the ability of 5-AzaC to induce two independent apoptotic pathways further supports its promise to overcome drug resistance.

DNA DSBs are the most cytotoxic DNA lesions. One-ended DSBs can be formed via collapse of a replication fork at the site of a blocking DNA lesion (39–41, 72). Given that 5-AzaC forms irreversible covalent DNA-enzyme adducts which can cause replication fork collapse, in this study, we investigated whether 5-AzaC causes DNA DSB formation as one possible mechanism associated with its cytotoxicity in MM cells. We showed that 5-AzaC induces DNA DSB responses in MM cells, evidenced by H2AX phosphorylation and foci formation, which is one of the most specific DNA DSB responses in mammalian cells (42, 43). In addition, we showed that 5-AzaC induces DNA damage downstream effector responses, such as Chk2 and p53 phosphorylation, and apoptosis. DNA damage can induce apoptosis by both p53-dependent and p53-independent pathways, depending on the agent and cell type (47, 73–76). Our studies revealed that 5-AzaC is cytotoxic against both p53 wild-type (MM.1S; ref. 29) and p53 mutant (RPMI-8266; ref. 77) MM cells (Fig. 1A), suggesting that p53 function does not require 5-AzaC-induced apoptosis in MM. Moreover, we showed that the 5-AzaC-mediated apoptosis is associated with the up-regulation of Bax, Puma, and Noxa, which were previously shown as mediators of p53-independent DNA damage-induced apoptosis (54).

Signaling of the DNA damage responses for DSB is initiated by PIKK family member sensor proteins ATM and/or ATR (44, 54). Our mechanistic studies showed that ATR is the predominant sensor molecule that initiates DNA DSB response signaling induced by 5-AzaC. In contrast, previous studies in human lymphoblastoid cell lines showed that ATM is the major sensor molecule mediating doxorubicin-induced DNA DSB signaling cascade (63). Given that the DSB responses induced by 5-AzaC and doxorubicin engage distinct upstream signaling pathways, we hypothesized that combining these two agents would increase MM cell sensitivity to DSBs induced by these agents and induce synergistic tumor cell death. Importantly, we show that the combination of 5-AzaC with doxorubicin induces synergistic MM cell death, including both doxorubicin-sensitive MM.1S as well as doxorubicin-resistant RPMI-Dox40 cells. Finally, pretreatment with bortezomib, which has previously shown to inhibit DNA-PK activity and thereby inhibit repair of DNA DSBs (66, 67), synergistically sensitized MM cells to 5-AzaC-induced cell death.

In conclusion, our results show for the first time that 5-AzaC, a cytosine analogue designed to inhibit DNMTs, induces apoptosis in MM cells via the formation of DNA DSBs; overcomes the protective effects of IL-6, IGF-I, and BMSCs; and is synergistic with doxorubicin and bortezomib. Together, these results provide the preclinical rationale for the clinical evaluation of 5-AzaC, alone and in combination with doxorubicin and bortezomib, to improve patient outcome in MM.

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