Neoplasma 2023; 70(2): 287–293

doi:10.4149/neo_2023_221217N1185

Synergistic activity and mechanism of cytarabine and MCL-1 inhibitor AZD5991 against acute myeloid leukemia

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Received December 17, 2022 / Accepted February 13, 2023

The 5-year overall survival rate of acute myeloid leukemia (AML) is less than 30%. Improving clinical outcomes is still a clinical challenge for AML treatment. Simultaneous use of chemotherapeutic drugs and targeting of apoptosis pathways has become a first-line clinical treatment for AML. Myeloid cell leukemia 1 (MCL-1) is a candidate target for AML treatment. In this study, we demonstrated that inhibition of the anti-apoptotic protein MCL-1 by AZD5991 synergistically increased chemotherapeutic agent cytarabine (Ara-C)-induced apoptosis in AML cell lines and primary patient samples. Apoptosis induced by a combination of Ara-C and AZD5991 was partially dependent on caspase activity and Bak/Bax. The downregulation of MCL-1 by Ara-C and the enhancement of Ara-C-induced DNA damage through inhibition of MCL-1 are potential mechanisms underlying the synergistic anti-AML activity between Ara-C and AZD5991. Our data support the application of MCL-1 inhibitor in combination with the conventional chemotherapeutic agent for the clinical treatment of AML.

Key words: acute myeloid leukemia; combination treatment; MCL-1; apoptosis; DNA damage

DNA damage agent cytarabine (Ara-C)-based chemotherapy remains the standard of treatment for acute myeloid leukemia (AML). The 5-year overall survival rate is less than 35% and 15% in younger patients (age <60 years) and older patients (age ≥60 years), respectively [1]. Designing a combination drug regimen based on the intrinsic properties of AML cells and the mechanism of drugs is an effective strategy to break through the current therapeutic obstruction.

Evading apoptosis is one of the recognized hallmarks of cancer cells [2]. The BCL-2 selective inhibitor venetoclax (VEN) is the only drug approved for clinical application which targets apoptosis. VEN has been approved by the U.S. Food and Drug Administration (FDA) for use in combination with low-dose cytarabine, azacytidine, or decitabine and it is for the treatment of newly diagnosed AML in adults over 75 years of age or not candidates for intensive chemotherapy. These combinations result in a median overall survival of 8.4–17.5 months [3]. Clinical application of VEN confirms the enhancement of targeting apoptosis on the anti-AML activity of chemotherapeutic drugs. However, its therapeutic effect still needs to be improved.

Myeloid cell leukemia 1 (MCL-1) is a member of the anti-apoptotic BCL-2 protein family which highly expresses in AML cells. MCL-1 plays an important role in the survival of AML cells [4]. Ewald et al. showed that inhibition of MCL-1 produced the best anti-AML effect compared with the other two main anti-apoptotic BCL-2 family members, BCL-2 and BCL-xL [5]. Our previous work demonstrated that Ara-C could downregulate the protein level of MCL-1 [6]. Notably, MCL-1 also has played an important regulatory role in repairing DNA damage [7–9]. These results inspired us to use Ara-C in combination with MCL-1 inhibition to treat AML. AZD5991 is a novel selective MCL-1 inhibitor with clinical application prospect [10, 11]. AZD5991 has been extensively studied both in preclinical models, though it combines with other drugs [12–14], and in clinical trials [11] in AML.

In this study, AZD5991 has demonstrated that it synergistically increased Ara-C-induced apoptosis in AML cell lines and primary patient samples, and its mechanism was also preliminarily explored from the perspective of MCL-1 protein level and DNA damage.

Patients and methods

Drugs. AZD5991, Ara-C, and Z-VAD-FMK were purchased from AbMole Bioscience (Shanghai branch, Shanghai, China).

Cell culture. MV4-11 and U937 AML cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and 2 mmol/l L-glutamine, plus 100 U/ml penicillin and 100 mg/l streptomycin, in a 37 °C humidified atmosphere containing 5% CO_2/95% air. The cells were tested for the presence of mycoplasma by polymerase chain reaction (PCR) method monthly [15].

Clinical samples. Clinical residual AML patient samples were obtained from the First Hospital of Jilin University in the presence of informed consent based on the Declaration of Helsinki. This study was approved and supervised by the Human Ethics Committee of the First Hospital of Jilin University. Primary AML patient cells were isolated and detected the genetic mutations as described previously [12].

Detection of apoptosis using flow cytometry. Following indicated drug treatment, the cells were stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (Bestbio, Shanghai, China), and then subjected to flow cytometry analysis. Apoptotic events are presented by the mean percentage of annexin V+/PI– (early apoptotic) and annexin V+/PI+ (late apoptotic and/or dead) cells ± the standard error of the mean (SEM) from one representative experiment. CompuSyn software (ComboSyn Inc. Paramus, NJ, USA) was used to calculate the combination index (CI). CI<1, CI=1, and CI>1 indicate synergistic, additive, and antagonistic effects, respectively.

Western blotting. Western blotting was performed as previously described [12] using anti-Bim (2819) (Cell Signaling Technologies, Danvers, MA, USA), -Bak (A5068) (Bimake.cn, Shanghai, China), -Bax (50599-2-Ig), -MCL-1 (16225-1-AP), -BCL-2 (12789-1-AP), -BCL-xL (66020-1-Ig), and -β-actin (66009-1-Ig) (Proteintech, Rosemont, IL, USA) antibodies. Protein levels were visualized and quantified using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, USA) and Odyssey V3.0 program (Li-Cor), respectively. The fold changes of protein densitometry were normalized to β-actin and then compared with the vehicle control.

shRNA knockdown and gene overexpression. The pMD-VSV-G and delta 8.2 plasmids were gifts from Dr. Dong at Tulane University. Bak (5'-TGGTACGAAGATTCTTCAAAT-3', SHCLND-NM_001188), Bax (5'-TGGTACGAAGATTCTTCAAAT-3', SHCLND-NM_004324), and non-target negative control (NTC) (SHC002V) shRNA lentiviral constructs were purchased from Sigma Aldrich (St. Louis, MO, USA). Precision LentiORF vectors for MCL-1 (OHS5897-202617926) and Red fluorescent protein (RFP) (OHS5832) were purchased from Thermo Fisher Scientific Biosciences (Lafayette, CO, USA). Lentivirus packaging and transduction were performed as previously described [16].

Alkaline comet assay. Following indicated drug treatment, the cells were subjected to an alkaline comet assay, as previously described [17]. Slides were stained using SYBR Gold (Life Technologies) and then imaged by an Olympus BX-40 microscope equipped with a DP72 microscope camera. All comets were scored on each gel using CometScore (TriTek Corp, Sumerduck, VA, USA). No less than 50 comets were included per group. The median percentage of DNA in the tail from three independent experiments was graphed as mean ± SEM.

Statistical analysis. An unpaired t-test was used to compare the differences in apoptosis between the two groups. The percent DNA present in the tail was compared by paired two-sample t-test. Statistical analyses were performed utilizing GraphPad Prism 9.0. Error bars represented SEM. Significance was set at $p<0.05$.

Results

AZD5991 synergistically enhances Ara-C-induced apoptosis in AML cells. In order to determine the interaction between Ara-C and AZD5991, AML cell lines MV4-11 and U937 were treated with various concentrations of AZD5991 and Ara-C alone or in combination for 48 h. The combination treatment of AZD5991 and Ara-C synergistically induced apoptosis in both cell lines (CI<0.7, Figure 1A, Supplementary Figure S1A). In addition, the synergistic effect of the combination on apoptosis was also prevalent in five primary AML patient samples *ex vivo* (Figure 1B, Supplementary Figure S1B, Table 1). These results show that Ara-C and AZD5991 synergistically induce apoptosis in AML cells.

Table 1. Patient characteristics of primary AML patient samples.

Patients	Gender	Age (year)	Disease status	FAB subtype	Cytogenetics	Blast purity	Gene mutation
AML#220	Male	45	Newly diagnosed	M4/M5	46, XY	73.0%	NPM1, DNMT3A, BRAF, WT1
AML#227	Male	32	Newly diagnosed	M4/M5	47, XY, +14, inv(16)(p13q22)/46, XY, inv(16)(p13q22)/46, XY	87.5%	WT1, JAK2, NRAS, PRAME
AML#235	Female	33	Newly diagnosed	M4/M5	46, XX	87%	NPM1
AML#236	Male	25	Newly diagnosed	M3	46, XY, i(17)(q10),t(15;17)(q24;21)	88%	MT1, PRAM, PML-RARa
AML#237	Male	56	Relapsed	M4/M5	46, XY	88%	FLT3-ITD, CEBPA, EC2L

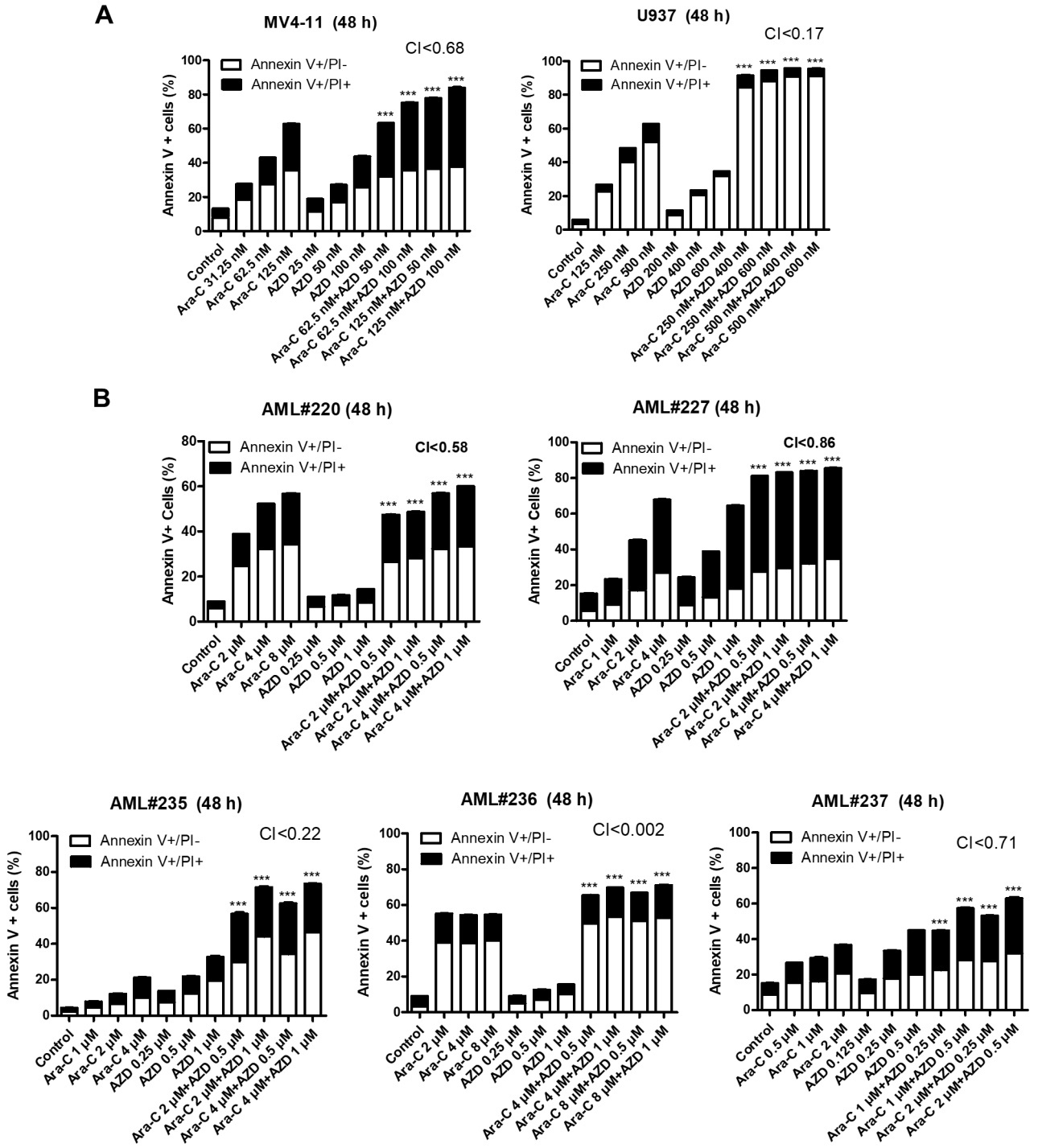

Figure 1. Ara-C and AZD5991 synergistically induced apoptosis in AML cells. MV4-11 and U937 cells (A) and primary AML patient sample cells (B) were treated with varying concentrations of AZD5991 (AZD) and Ara-C alone or in combination for 48 h, then stained with annexin V-FITC/PI, and analyzed using a flow cytometer. CI<1 indicates a synergistic effect; ***p<0.001 compared to a single drug treatment at the same concentration.

The combination treatment induced apoptosis partially via the intrinsic pathway. Next, the role of the intrinsic apoptotic pathway in combined anti-AML activity was investigated. MV4-11 cells were treated with the pan-caspase inhibitor Z-VAD-FMK, Ara-C, and AZD5991 alone or in combination. Z-VAD-FMK could significantly inhibit apoptosis induced by Ara-C and AZD5991 alone or in combination (Figure 2A). Consistently, Bak/Bax double

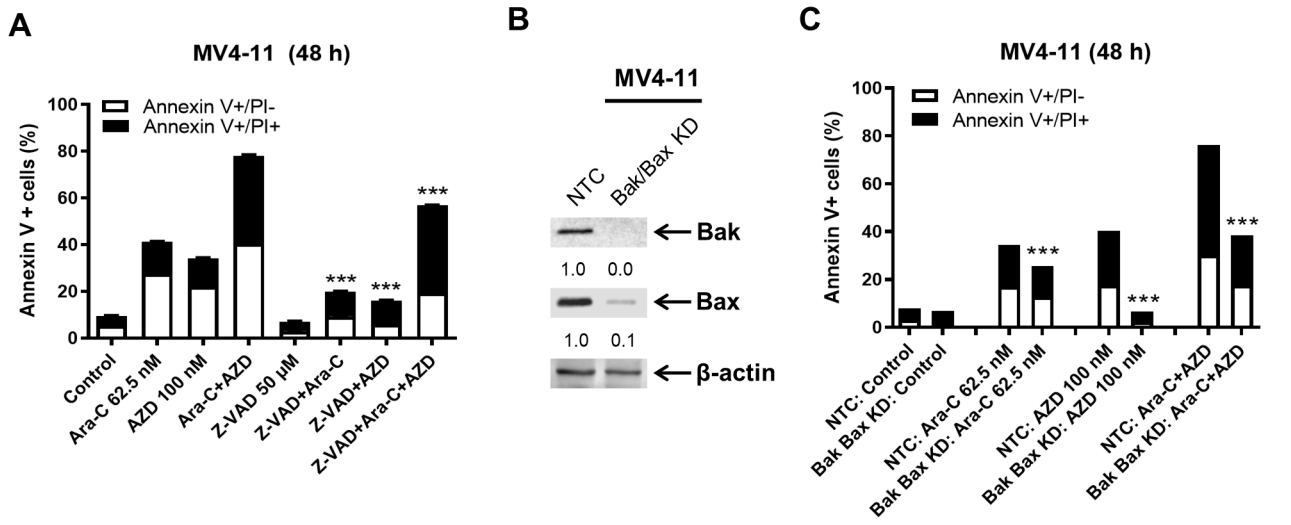

Figure 2. Apoptosis induced by the combination of Ara-C and AZD5991 is partially through the intrinsic pathway. MV4-11 cells were treated with Ara-C, AZD5991 (AZD), and Z-VAD-FMK (Z-VAD) alone or in combination for 48 hours, and then stained by annexin V-FITC/PI staining and analyzed by flow cytometry. ***p<0.001 compared to the corresponding treatment without Z-VAD (A). Bak and Bax were knocked down (KD) by shRNA in MV4-11 cells with non-template control (NTC) as the negative control. Whole-cell lysates were subjected to western blotting (B). Cells treated with Ara-C and AZD alone or in combination for 48 h were subjected to annexin V/PI staining and flow cytometry analysis. ***p<0.001 compared to the NTC group for the same drug treatment (C).

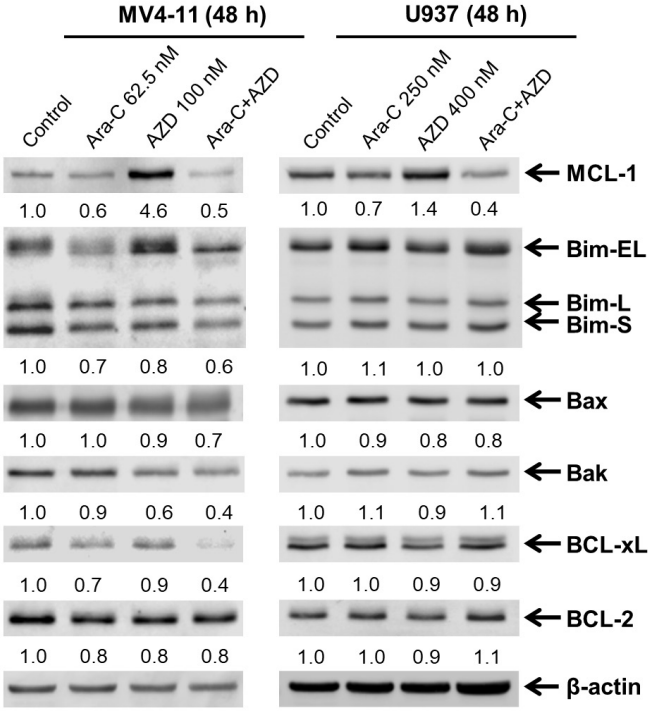

Figure 3. Ara-C abolishes AZD5991-induced upregulation of MCL-1 in AML cells. MV4-11 and U937 cells were treated with Ara-C and AZD5991 (AZD) alone or in combination for 48 h. Whole-cell lysates were subjected to western blotting and probed with the indicated antibodies. Densitometric quantification of blotting band intensity is shown below the bands.

knockdown could completely abolish AZD5991-induced apoptosis and substantially attenuate Ara-C-induced and combination-induced apoptosis in MV4-11 cells (Figure 2B). These results indicate that apoptosis induced by the combination treatment partially depends on Bak/Bax.

Ara-C suppressed the upregulation of AZD5991-induced MCL-1. Then, we investigated the effect of Ara-C and AZD5991 alone or in combination on the BCL-2 family protein levels in MV4-11 and U937 cells. Consistent with our previous results, AZD5991 increased the MCL-1 protein level [13] while Ara-C decreased it [6]. The combination treatment decreased MCL-1 protein as well (Figure 3, Supplementary Figure S2). No noticeable and consistent change was shown in the protein levels of BCL-2, BCL-XL, and pro-apoptotic BH3-only protein Bim, and pro-apoptotic pore-formers Bak and Bax in the two cell lines (Figure 3). The results indicate that Ara-C can promote the anti-AML activity of AZD5991 by downregulating MCL-1.

The level of MCL-1 plays an important role in the combination effect. Subsequently, in order to confirm the effect of MCL-1 level on the anti-AML activity of Ara-C and AZD5991, MCL-1 overexpression experiment was carried out in MV4-11 cells. Overexpression of MCL-1 significantly inhibited apoptosis induced by Ara-C and AZD5991 alone or in combination, though the sensitivity of transfected cells to Ara-C was substantially lower than that of parental cells (Figure 4). This data points out the MCL-1 targeting property of AZD5991. The above results suggest that the level of MCL-1 has a critical impact on the anti-AML activity of the drugs, and the downregulation of MCL-1 by Ara-C plays an

important role in the synergistic anti-AML activity of Ara-C and AZD5991.

AZD5991 enhances Ara-C-induced DNA damage. Based on the fact that Ara-C is an inducer of DNA damage and MCL-1 plays an important regulatory role in repairing DNA damage, the effect of drugs on DNA damage in MV4-11 and U937 cells was determined by the alkaline comet assay. It was found that AZD5991 induced DNA damage and enhanced Ara-C-induced DNA damage as early as after 4 hours, but had a limited effect on apoptosis (Figure 5). The results suggest that increased DNA damage is an important mechanism for the synergistic anti-AML activity of Ara-C and AZD5991.

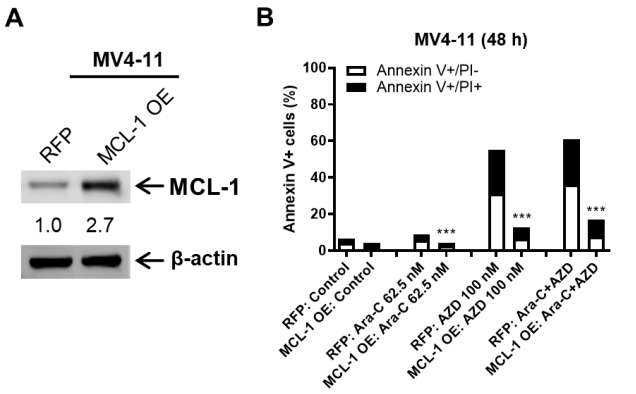

Figure 4. Overexpression of MCL-1 substantially inhibits the anti-AML activity of AZD5991. MCL-1 overexpression (OE) was performed with RFP construct as a control in MV4-11 cells. Whole-cell lysates were subjected to western blotting (A). Cells treated with Ara-C and AZD5991 (AZD) alone or in combination for 48 h were subjected to annexin V/PI staining and flow cytometry analysis. ***p<0.001 compared to the RFP group for the same drug treatment (B).

Discussion

It has been reported that inhibition of MCL-1 by S63845 or siRNA enhances the anti-AML activity of Ara-C in CD157-high [18] or FMS-like tyrosine kinase 3-internal

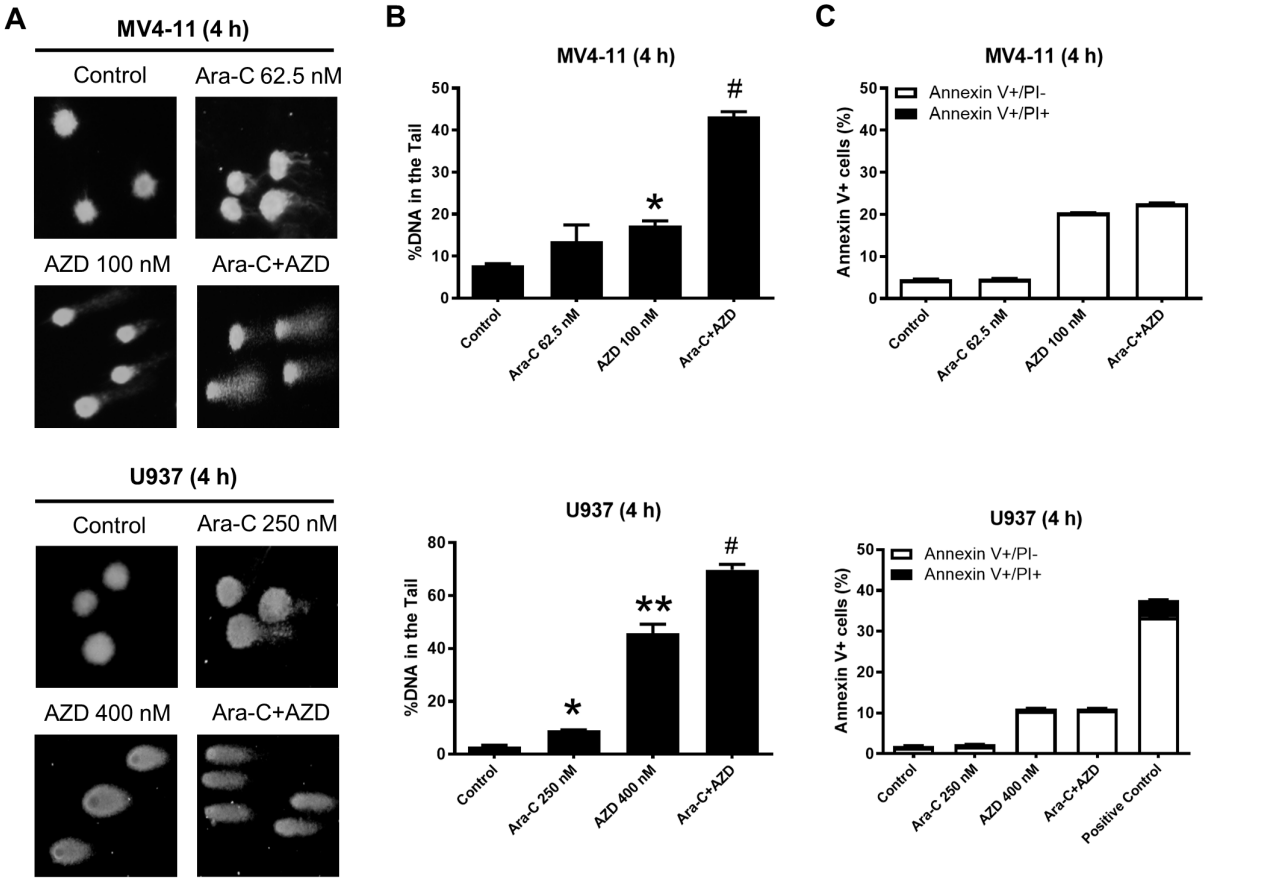

Figure 5. The combination of Ara-C and AZD5991 increases the induction of DNA damage in AML cells. MV4-11 and U937 cells were treated with Ara-C and AZD5991 (AZD) alone or in combination for 4 h, and then subjected to alkaline comet assay or flow cytometry analysis. A) Representative images from alkaline comet assay are shown. B) Alkaline comet assay results are graphed as the median percentage of DNA in the tail from three replicate gels ± SEM. *p<0.01 and **p<0.001 compared to vehicle control; #p<0.01 compared to single drug treatment. C) Annexin V-FITC/PI staining was assessed by flow cytometry analysis. Cells treated with an apoptosis inducer purchased from Beyotime Biotechnology (Shanghai, China) were used as the positive control.

tandem duplication (FLT3-ITD)-positive AML cells [19], respectively. In this study, we demonstrated that inhibition of MCL-1 by AZD5991 universally enhanced Ara-C activity in AML cells, including in an FLT3-ITD positive AML cell line (MV4-11) and primary AML patient sample with FLT3-ITD mutation (AML#237). In addition, Sean et al. proved that MCL-1 inhibitor AM-8621 could enhance the anti-AML activity of a variety of chemotherapeutics such as Ara-C, doxorubicin, and decitabine [20]. These results suggest that the combination of an MCL-1 inhibitor with a chemotherapeutic agent represents a promising strategy for AML clinical application.

It has been shown that MCL-1 inhibitors can induce a ubiquitous upregulation of MCL-1 protein level, such as AZD5991 [10, 13], S63845 [21], and AMG-176 [22]. We reproduced this phenomenon (Figure 4). In AML cells, AZD5991-induced MCL-1 upregulation is through increasing protein stability and/or gene transcription [12, 13]. At the same time, our results demonstrate that the MCL-1 level has an important influence on the anti-AML activity of AZD5991 (Figure 3). Thus, the induction of MCL-1 by AZD5991 attenuates its own anti-AML activity. On the other hand, consistent with our previous result [6], Ara-C downregulates MCL-1 (Figure 4), although the mechanism is unknown. This data points to an important contribution of the downregulation of MCL-1 by Ara-C to enhance the anti-AML activity of AZD5991 in the synergistic effect of the two drugs.

The role of MCL-1 in DNA damage repair has been widely established. Inhibition of MCL-1 can enhance the antitumor activity of chemotherapeutic drugs by mediating DNA damage repair in prostate cancer [23] and lung cancer [24]. Our previous study demonstrated that overexpression of MCL-1 reduced Ara-C-induced DNA damage [25]. Correspondingly, the result of this study shows that inhibition of MCL-1 by AZD5991 enhances Ara-C-induced DNA damage (Figure 5). And this data also suggests that the amplification of DNA damage induction is another potential mechanism for the synergistic effect of Ara-C and AZD5991. Interaction with NBS1 [7] and IEX-1 [8] and promotion of CHK1 activation [26] are known mechanisms by which MCL-1 facilitates DNA damage repair. DNA damage can induce mitotic catastrophe mediating through CHK1 [27]. Mitotic catastrophe can lead to caspase-independent cell death with annexin V-positive [28] and Bak/Bax-independent cell death [29]. Thus, AZD5991 enhances Ara-C-induced apoptosis potentially via interfering with CHK1 and causing mitotic catastrophe by a caspase and/or Bak/Bax independent mechanism. This might explain why the inhibition of caspase or Bak/Bax only partially rescues the combination treatment-induced apoptosis (Figure 2).

In summary, we demonstrated that the combination of Ara-C and AZD5991 has a synergistic anti-AML activity. Downregulating of MCL-1 by Ara-C and enhancing DNA damage induction by AZD5991 are the underlying molecular mechanisms. This study supports the clinical evaluation of Ara-C combined with AZD5991 for the treatment of AML.

Supplementary information is available in the online version of the paper.

Acknowledgments: This study was supported by grants from the National Natural Science Foundation of China (NSGC81800154 to Guan Wang) and the Projects of Jilin Province Science and Technology Development Plan (20220101284JC to Guan Wang). The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or the in decision to publish the results.

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