Decitabine shows synergistic effects with arsenic trioxide against myelodysplastic syndrome cells via endoplasmic reticulum stress-related apoptosis

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ABSTRACT

Most of the International Prognostic Scoring System (IPSS) high-risk patients with myelodysplastic syndrome partly responded to hypomethylating therapy even with transient remission, while arsenic trioxide (ATO) had partial effect in patients with MDS. Therefore, we sought to investigate the effects and possible mechanisms of the combination of ATO and decitabine (DAC) in MDS cells. In our study, the MUTZ-1 and SKM-1 cells were treated with ATO, DAC or both. Cell viability, cell apoptosis, levels of reactive oxygen species (ROS) and expressions of the endoplasmicreticulum (ER) stress-associated genes and proteins were examined. Results showed the combination of ATO and DAC synergistically inhibited the proliferation and induced apoptosis of MDS cells. Through the RNA-sequence and GSEA gene function analysis, ER stress-related pathway played an important role in apoptosis of MDS cells induced by the combination of ATO and DAC. ER stress-related genes DNA damage inducible transcript 3, GRP78, and activating transcription factor-6 were significantly highly expressed in combination group than those in single agent groups; proteins were confirmed by western blot. The levels of ROS significantly increased in the combination group. Furthermore, the apoptosis of (ATO+DAC) group MDS cells could be partially reversed by antioxidant agent N-acetylcysteine, accompanied by decreased expression of intracellular ROS and ER stress-related genes. These results suggested that the combination of ATO and DAC synergistically induced the apoptosis of MDS cells by increased ROS-related ER stress in MDS cells.

Significance of this study

What is already known about this subject?

- Decitabine is effective in the treatment of patients with myelodysplastic syndrome (MDS) by decreasing levels of abnormal methylation.
- Arsenic trioxide can induce apoptosis by activating the endoplasmic reticulum (ER) stress of the cancer cells.
- Decitabine and arsenic trioxide are promising chemotherapy regimen in the treatment of hematological malignancies.

What are the new findings?

- The combination of decitabine and arsenic trioxide inhibits the proliferation and induced apoptosis of MDS cells.
- The mechanism of the combination is via ER stress-related apoptosis in vitro experiments.
- There are synergistic anti-MDS effects at a suitable drug concentration in human.

How might these results change the focus of research or clinical practice?

- Decitabine combined with arsenic trioxide at a suitable concentration shows synergistic anti-MDS effects via ER stress-related apoptosis, providing a new way for patients with MDS.

INTRODUCTION

Myelodysplastic syndrome (MDS) is a clonal stem cell-derived myeloid neoplasm that is characterized by ineffective hematopoiesis and high risk of progression to acute myeloid leukemia (AML).[1] DNA methylation is common in cancer, leading to silencing of many genes; decitabine (DAC) has been shown to decrease levels of abnormal methylation in neoplasia.[2] DAC is widely used in various hematologic disorders including MDS, AML and chronic myelomonocytic leukemia achieving good responses to a certain degree.[3 4] Hypomethylating agents can decrease malignant clonal burden, but relapse is inevitable because of the remaining transformed stem cells.[5] Major clinical trials of DAC used in patients with MDS showed that about half responded to hypomethylating therapy, whereas the majority of patients with MDS failed were International Prognostic Scoring System (IPSS) intermediate or high-risk patients.[6 7] Allogeneic hematopoietic stem cell transplantation (HSCT) is a potential curable intervention for hematopoietic diseases,[8] which is one of the curative therapies for high-risk patients with MDS, but MDS tends to occur in the elderly with potential comorbid conditions who cannot

afford the complications followed by HSCT, so combination chemotherapy with DAC was needed.

Arsenic trioxide (ATO) is used in hematologic disorders and solid tumors, including acute promyelocytic leukemia, AML, MDS, lymphoma and sarcoma.[9–13] The possible antitumor mechanisms of ATO: induced the degradation of the fusion protein PML-RARα, increased intracellular reactive oxygen species (ROS), enhanced caspase and glycogen synthase kinase 3β activity for apoptosis,[9–11] induced autophagy,[12] the stimulation of endoplasmic reticulum (ER) stress and inhibition of the ubiquitin–proteasome system.[13] However, the hematologic improvement rate of ATO in high-risk patients with MDS was less than 20% according to the multicenter studies.[14 15] Previous study had shown the synergistic effects of the combination of ATO and DAC in SKM-1 cells was partly via decreasing BCL2/BAX ratio-related apoptosis,[16] but other mechanisms involved were unclear. Recently, the low expression of DNA damage inducible transcript 3 (DDIT3) was shown in myeloid malignancies including MDS, with a higher incidence of aberrant methylation of DDIT3 in patients with MDS, which was correlated with disease progression.[17] DAC may increase the expression of DDIT3 in cells through demethylation. Meanwhile, ER stress played an important role in the ATO-induced apoptosis in solid tumors.

In our study, we try to determine whether the combination of ATO and DAC can synergistically induce the apoptosis in MDS cells and the exact molecular mechanisms involved in the combination, in order to provide evidence for the use of ATO combination with DAC in high-risk patients with MDS.

MATERIALS AND METHODS

Cell culture and reagents

Human MDS cell lines MUTZ-1 and SKM-1 were cultured in 1640 (Gibco, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (Gibco), 100 mg/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco) at 37°C in a 5% CO_2 atmosphere. MUTZ-1, a cell line established from Childhood Myelodysplastic Syndrome with del(5q), was obtained from the German Brauschweig cell center.[18] SKM-1, a cell line established from the patient who initially possessed multiple point mutations of ras genes but lost these mutations during disease progression to myelomonocytic leukemia with the acquisition of chromosomal abnormalities involving the p53 anti-oncogene, was obtained from the Health Science Research Resources Bank in Japan.[19] For treatment with ATO (Sigma Chemical Co) and DAC (Sigma Chemical Co), fresh stock solutions were prepared before every experiment and filter sterilized using a 0.2 mm syringe filter.

Cell viability assay

The cells (2×10^5/mL) in the 100 µL complete medium were seeded in 96-well plates. After treatment with ATO and/or DAC for 72 hours, 10 µL CCK8 (Engreen, China) was added to each well and incubated for another 3 hours at 37°C in a 5% CO_2 atmosphere. The absorbance of the samples was measured against a background control at 450 nm. The experiment was repeated three times, and each sample had three duplications. Cell viability: proliferation (%)=(OD450 of the experimental group/OD450 of the control group)×100%.

Cell apoptosis assays by flow cytometry

The cells (4×10^5/well) in 6-well plates were treated with ATO, DAC and the combination for 72 hours. The 1×10^5 cells were washed and resuspended in 200 µL staining solution containing 5 µL Annexin V–FITC and 5 µL PI (FITC-Annexin V staining kit, BD) for 30 min at room temperature in the dark. Then, cells were detected by flow cytometry (Beckman).

The protocol of the RNA-sequence

The Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit) was used to do the total RNA sample QC: RNA concentration, RIN value, 28S/18S and the fragment length distribution. The experimental workflow: (1) mRNA enrichment: Oligo dT Selection or rRNA depletion; (2) RNA fragment and reverse transcription: fragment the RNA and reverse transcription to double-strand cDNA (ds-cDNA) by N6 random primer; (3) end repair, add A-tailing and adaptor ligation: the synthesized cDNA was subjected to end-repair and then was 3′ adenylated. Adaptors were ligated to the ends of these 3′adenylated cDNA fragments; (4) PCR amplification: the ligation products were purified, and many rounds of PCR amplification were performed to enrich the purified cDNA template using PCR primer; (5) denature and cyclization: denature the PCR product by heat, and the single strand DNA is cyclized by splint oligo and DNA ligase; and (6) sequencing on BGISEQ-500 platform.

ROS detection by flow cytometry

The cells (4×10^5/well) in 6-well plates were treated with ATO, DAC and the combination for 72 hours. The generation of intracellular ROS was determined using a fluorescein-labeled dye, 2′,7′-dichlorofluorescein diacetate (DCFH-DA). After treatments, cells were harvested and washed once with phosphate buffer solution (PBS). Cells were incubated with 10 µM DCFH-DA for 20 min at 37°C. Then cells were washed twice, resuspended in 300 µL PBS and immediately analyzed by flow cytometer (Beckman) to determine the ROS generation. Each group collected 10,000 individual cells.

Real-time PCR

Total RNA was extracted from treated cells using TRIzol reagent (Tiangen Biotech, China) according to manufacturer's recommendations. The first-strand cDNA was synthesized from 1 µg of total RNA using FastQuant RT Kit (with gDNase) (Tiangen Biotech, China). Subsequently, quantitative PCR (qPCR) was performed in triplicates on the IQ5 (BIO-RAD, USA) with Super Real

Table 1 Measurement of IC50 of arsenic trioxide and decitabine in MDS cell lines

	Arsenic trioxide (µM)		Decitabine (µM)	
Cell lines	IC50	95% CIs	IC50	95% CIs
MUTZ-1	0.699	0.478 to 1.022	4.182	3.689 to 4.740
SKM-1	1.457	0.939 to 2.259	1.162	0.4609 to 1.422

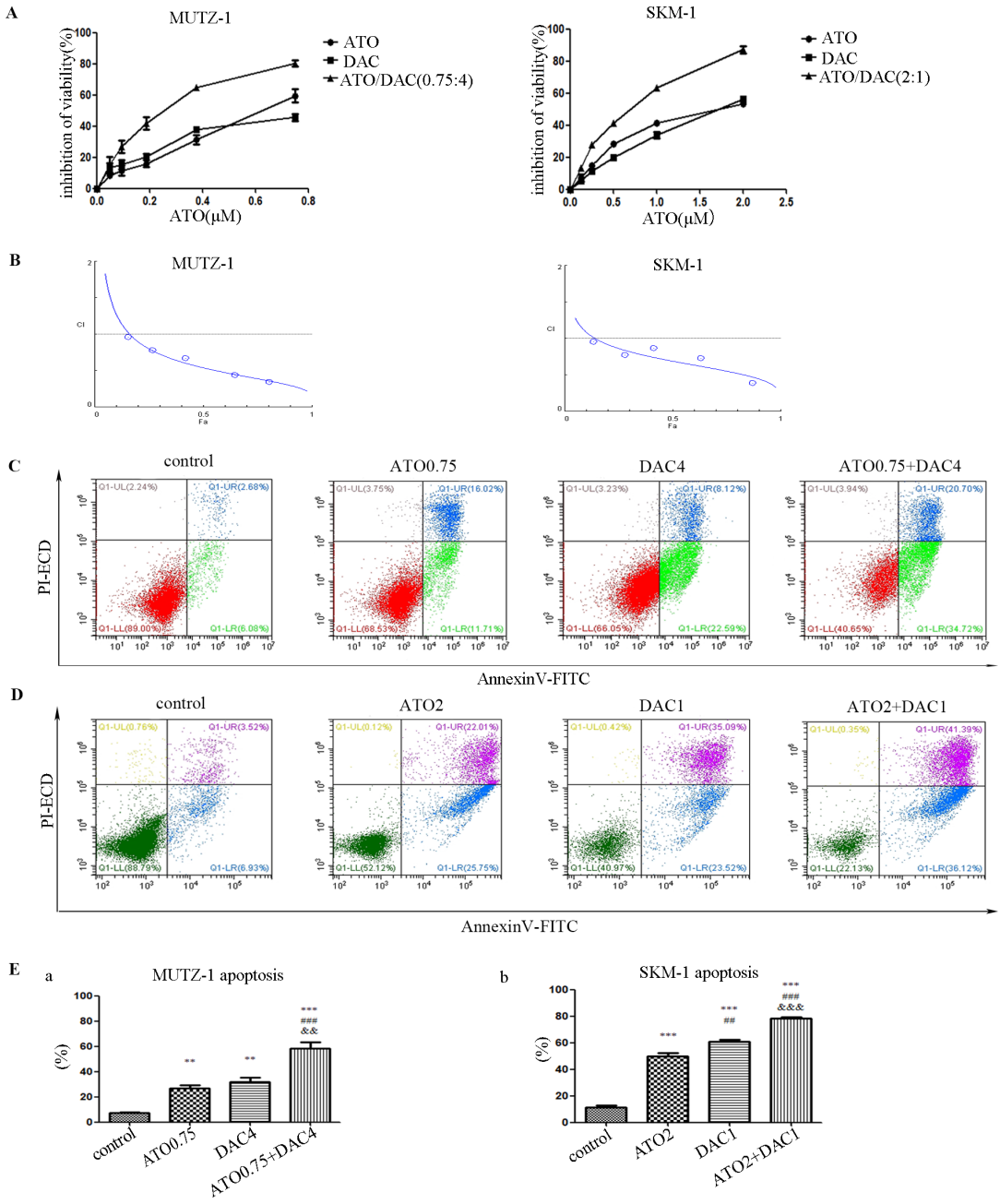

Figure 1 Arsenic trioxide (ATO) and decitabine (DAC) synergistically inhibited cell proliferation and induced apoptosis in MDS cell lines at 72 hours. (A) The combination of ATO and DAC inhibited cell proliferation. Cell inhibition rate (%)=(OD450 of treatment group−OD450 of blank)/OD450 of control group−OD450 of blank)×100%. (B) Combination index (CI) values for ATO combined with DAC in MDS cells were lower than 1. The effects of these combinations were estimated using the CompuSyn software, which was developed based on the CI equation of Chou-Talalay. CI <1 indicates synergism; CI=1 indicates additive effect in the absence of synergism or antagonism; CI >1 indicates antagonism. (C and E (a)) The apoptosis of control, ATO, DAC and the combination of ATO and DAC in MUTZ-1 at 72 hours. (D,E–b) The apoptosis of control, ATO, DAC and the combination of ATO and DAC in SKM-1 at 72 hours. Compared with control group, ** indicated p<0.01, *** indicated p<0.001; compared with ATO group, ### indicated p<0.001; compared with DAC group, && indicated p<0.01, &&& indicated p<0.001. MDS, myelodysplastic syndrome.

PreMix Plus SYBR Green (Tiangen Biotech, China) and primers (10 μM), and the amplified specific single product was validated by melt curve. The following gene-specific primer pairs were used: DDIT3: forward 5′GGAAACAG AGTGGTCATTCCC3′, reverse 5′CTGCTTGAGCCG TTCATTCTC3′; activating transcription factor-6 (ATF6): forward 5′CCCGTATTCTTCAGGGTGCTCTGG3′, reverse 5′TAGCTCACTCCCTGAGTTCCTGCT3′; GRP78: forward 5′CGAGGAGGAGGACAAGAAGG3′, reverse 5′CACCTTGAACGGCAAGAACT3′; GADD34: forward 5′ATGTATGGTGAGCGAGAGGC3′, reverse 5′ GCAGTGTCCTTATCAGAAGGC3′; Caspase-3: forward

Table 2 Combination index value of the combination of arsenic trioxide and decitabine in MDS cell lines

	Combination index				
Cell lines	ED50	ED75	ED90	ED95	Mean
MUTZ-1	0.539	0.389	0.297	0.254	0.365
SKM-1	0.688	0.551	0.443	0.382	0.516

Combination index values <1.0 are consistent with synergism, and the lower the value, the greater the synergism.
MDS, myelodysplastic syndrome.

5′TTGTAGAAGTCTAACTGGAA3′, reverse 5′CCATGTCATCATCAACAC3′; β-actin: forward 5′TGGACATC CGCAAAGACCTGT3′, reverse 5′CACACGGAGTAC TTGCGCTCA3′.

Western blot analysis

Total cell lysates were extracted using RIPA lysis buffer containing PMSF (100:1); protein concentrations were determined by BCA protein assay kit (Sangon Biotech, China). Proteins (30 µg) were separated by 10% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGEs) and transferred electronically to nitrocellulose filter membranes (NC) membranes (EASYBIO, China). NC membranes were blocked with 5% BSA for 1 hour at room temperature and then incubated overnight at 4°C with the following primary antibodies: anti-C/EBP homologous protein (CHOP) (#2895), anti-ATF6 (#65880), anti-GRP78 (#3177), anti-cleaved caspase-3 (#9661), anti-protein kinase B (AKT) (#4691S), anti-GAPDH (#5174) (Cell Signaling Technology, Beverly, Massachusetts, USA) antibody. After washing three times with TBST, each membrane was incubated with antimouse IgG (GenScript A00160) or antirabbit IgG (GenScript A00098) as a secondary antibody for 1 hour at room temperature. The expressions of the proteins of interest were detected by enhanced chemiluminescence kits (Millipore, Billerica, Massachusetts, USA).

Statistical analysis

Data are expressed as mean±SD The one-way analysis of variance was used for statistical analyses. The paired t-test was used for the data before and after the treatment of N-acetylcysteine (NAC). $P<0.05$ was considered statistically significant. The Combination Index (CI) of ATO and DAC was calculated using Calcusyn (Great Shelford, Cambridge, UK). CI<1, CI=1 and CI>1 indicated synergism, additive and antagonism effect, respectively. The immunoblots were quantified by Image-Pro Plus 6.0. SPSS V.21 was used to analyze the data, which was plotted with GraphPad Prism5.

RESULTS

The combination of ATO and DAC showed synergistically antiproliferative effects on MDS cells

To evaluate the antiproliferative effects of ATO and DAC on MDS cells, MUTZ-1 and SKM-1 cells were treated with gradient concentrations of ATO and DAC for 72 hours, and the antiproliferation effects were examined by CCK-8 assays. Both ATO and DAC showed a growth inhibitory effect by dose-dependent manner in the two cell lines. The IC50 values of ATO and DAC in MUTZ-1 at 72 hours were 0.699 µM and 4.182 µM, respectively, and those in SKM-1 were 1.457 µM and 1.162 µM, respectively (shown in table 1). The synergistic effects of the combination in MDS cells were indicated by the CI <1 (shown in figure 1A,B, table 2).

Increased apoptosis after treatments with ATO and DAC in the MDS cells

Due to the synergistic antiproliferation effects of ATO and DAC on MUTZ-1 and SKM-1, we further analyzed the apoptotic effects of the combination. After MUTZ-1 and SKM-1 cells treated with ATO, DAC or the combination for 72 hours. For MUTZ-1, the highest apoptosis rate occurred in 0.75 µmol/L ATO plus 4 µmol/L DAC group (control: 7.18%±1.39%, ATO: 27.04%±3.85%, DAC: 31.89%±6.00%, ATO and DAC: 57.75%±9.87%) (shown in figure 1C,E (a)). Meanwhile, for SKM-1, the highest apoptosis occurred in 2 µmol/L ATO plus 1 µmol/L DAC group (control: 11.64%±2.47%, ATO: 49.91%±4.12%, DAC: 60.99%±2.25%, ATO and DAC: 78.57%±1.37%) (shown in figure 1D,E (b)), which meant that the apoptotic mechanisms played an important role in the synergistic effects of ATO and DAC.

RNA-sequence analysis and ER stress-induced apoptosis-related gene expression in MDS cells

RNA-sequence was performed on the MUTZ-1 cell line after treatments with ATO, DAC or the combination. A total of 23,639 genes were detected and repeated three times for each group, among them, the number of common genes was 16 168 in the control group, 16,287 in the ATO group, 19,665 in the DAC group and 18,911 in the combination group (shown in figure 2A (a and b)). We obtained the differentially expressed genes through the comparison between the control, single agent group or the combination group and selected the genes with the q value <0.0001 among them. Compared with other groups, in combination group, 208 genes were significant upregulated, and 20 genes were significantly downregulated (shown in figure 2A (c and d)). Combination with GSEA gene function analysis (shown in figure 2A (e and f)), we compared functions of differentially expressed genes in common upregulated or downregulated genes. We found that the ER stress-related pathway played an important role in the ATO and/or DAC induced apoptosis of MDS cells. Subsequently, the expressions of ER stress-related genes DDIT3, GRP78, GADD34, and ATF6 verified by qPCR in combination group were significantly higher than those in the single agent or control groups in MUTZ-1 or SKM-1 (shown in figure 2B,C). ER stress-related proteins CHOP, GRP78, and ATF6 were significantly up-regulated in the combination group confirmed by western blot, while BCL-2 was significantly decreased and caspase-3 was significantly increased in protein level (shown in figure 2D).

ATO plus DAC synergistically induced ER stress apoptotic pathway through the upregulation of ROS in MDS cells

According to the results of the RNA sequence about ATO, DAC and the combination of ATO and DAC on the MUTZ-1 cell line, ER stress played an important role in the

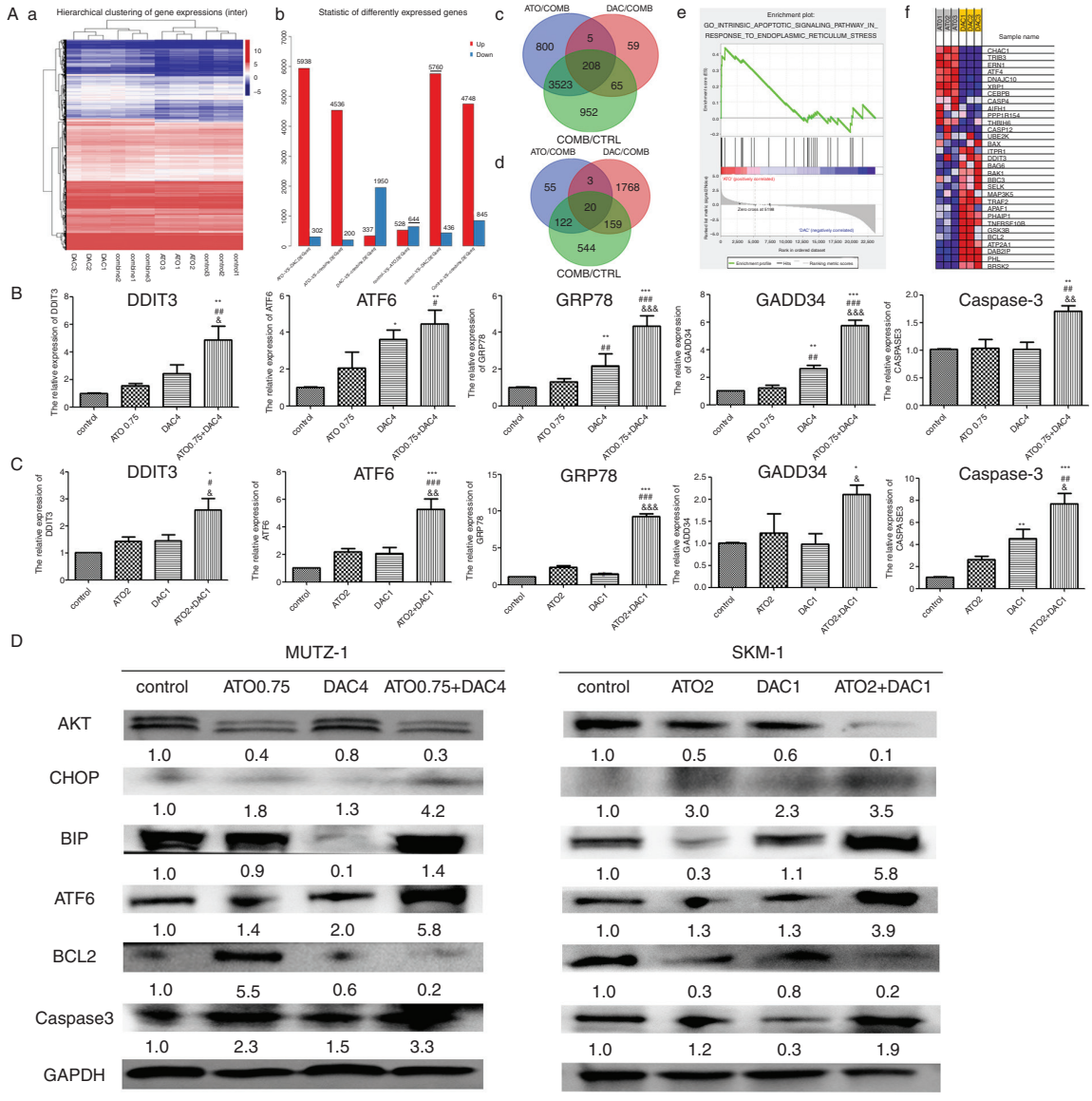

Figure 2 Arsenic trioxide (ATO) and decitabine (DAC) synergistically induced ER stress-associated apoptosis in MDS cell lines at 72 hours. (A) (a) RNA-sequence was performed on the MUTZ-1 cell line with different treatments of ATO: 0.75 μmol/L, DAC: 4 μmol/L and the combination at 72 hours. (b–d) Number of significantly different genes in each comparison group was shown. Compared with other groups, in combination group, 208 genes were upregulated, 20 genes down were regulated. (e–f) GSEA functional gene analysis showed gene enrichments in ER stress-associated apoptosis in MUTZ-1 cells. (B) The expressions of ER stress-associated genes in MUTZ-1 cells. (C) The expressions of ER stress-associated genes in SKM-1 cells. (D) The proteins associated with ER stress upregulated in the combination group, while downregulation of BCL2 with upregulation of cleaved Caspase3. Meanwhile, the AKT in the combination group was downregulated. Compared with control group, *indicated p<0.05, **indicated p<0.01, ***indicated p<0.001; compared with ATO group, # indicated p<0.05, ## indicated p<0.01, ### indicated p<0.001; compared with DAC group, & indicated p<0.05, && indicated p<0.01, and &&& indicated p<0.001. ER, endoplasmic reticulum.

apoptosis of the MDS cells. To clarify the role of ROS in the ER stress-induced cell apoptosis, we detected the levels of ROS in the MUTZ-1 and SKM-1 cell lines, the cells treated with ATO plus DAC significantly increased the level of ROS in MUTZ-1 than single agent (control: 105255±3577, ATO: 123139±5693, DAC: 91830±4858, and ATO and DAC: 140620±8265), as well as the level of ROS in SKM-1 (control: 82095±11567, ATO: 169393±14367, DAC: 147279±25315, and ATO and DAC: 204463±6751) (shown in figure 3), which was parallel with apoptosis.

Downregulation of the levels of ROS and ER stress-related apoptosis after the combination treated with NAC

Due to the increased levels of ROS and ER stress-related apoptosis in the group of the combination of ATO and DAC in MDS cells, the relationship between ROS and ER stress-related apoptosis was also determined. We further detected the levels of ROS and apoptosis in the group of the combination treated with NAC. Because of the short half-life of NAC, we added NAC every 24 hours for 72 hours.

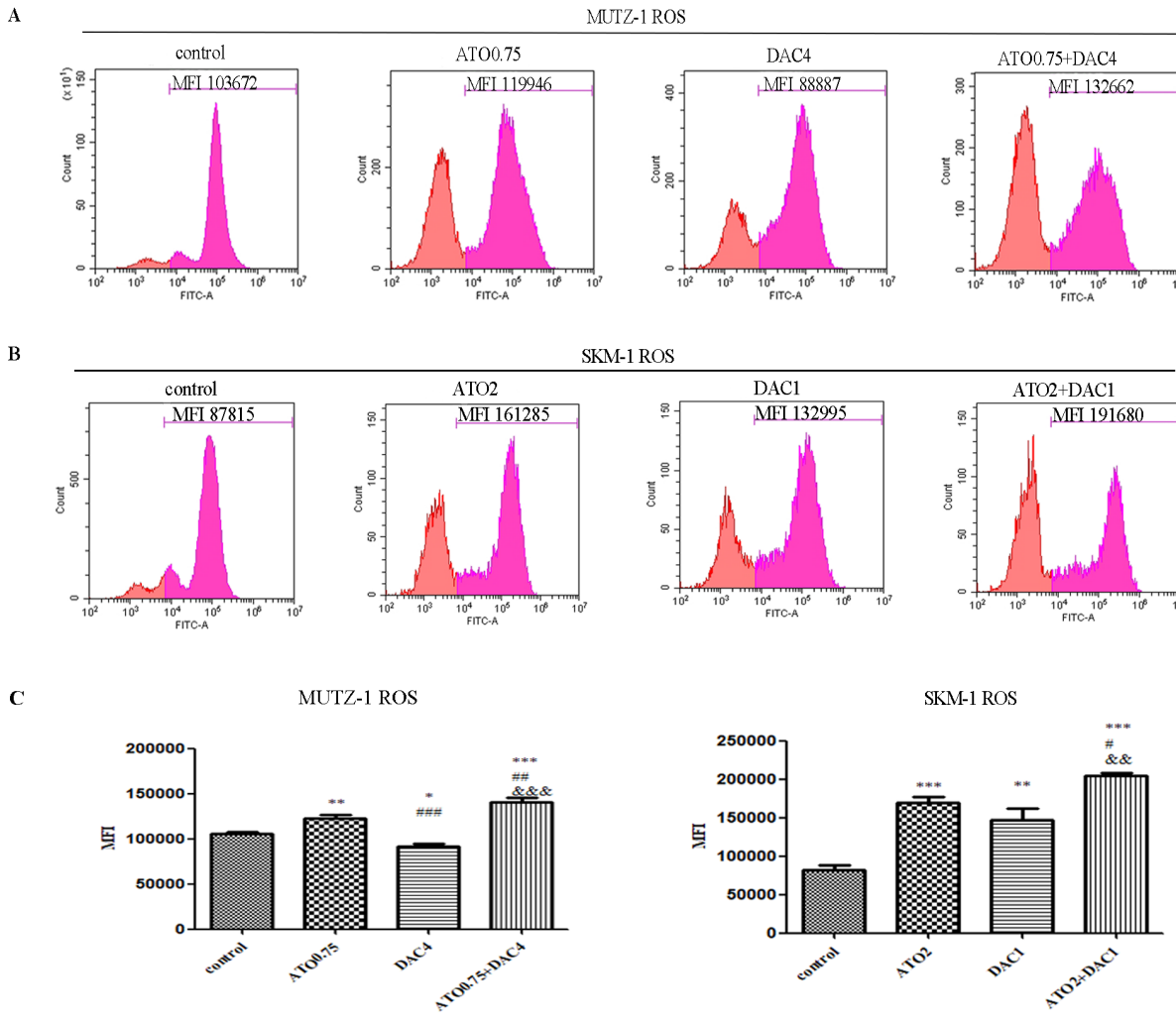

Figure 3 (A and C (a)) The cells treated with ATO and DAC significantly increased the level of ROS in MUTZ-1 than single agent at 72 hours. (B and C (b)) The cells treated with ATO and DAC significantly increased the level of ROS in SKM-1 than single agent at 72 hours. Compared with control group, * indicated p<0.05, ** indicated p<0.01, *** indicated p<0.001; compared with ATO group, # indicated p<0.05, ## indicated p<0.01, ### indicated p<0.001; compared with DAC group, & indicated p<0.05, && indicated p<0.01, &&& indicated p<0.001. ATO, arsenic trioxide; DAC, decitabine; ROS, reactive oxygen species.

We found that the levels of ROS, as well as apoptosis of MUTZ-1 and SKM-1 in the (ATO+DAC) combination group were reversed by NAC (shown in figure 4A,B). At the same time, the ER stress-related genes and proteins were examined after the treatment of NAC. The gene expressions of DDIT3, ATF6, GRP78 and GADD34 were decreased after the antioxidative treatments in both MUTZ-1 and SKM-1, especially the expression of DDIT3, GRP78 and GADD34 in MUTZ-1 and ATF6, GRP78 and GADD34 in SKM-1 (p<0.05, shown in figure 4C–E).

DISCUSSION

DAC was effectively used in the treatment of MDS through the demethylation of the antitumor genes,[20 21] but some of the patients poorly responded to the therapy, especially the IPSS high-risk MDS that rapidly progressed to acute leukemia.[6 7 22] Previous clinical trials showed that ATO treatment had partial effect in patients with MDS, accompanied by manageable adverse effects.[14 15] Based on the unsatisfied outcomes of single-agent ATO or DAC in the treatment of MDS, we sought to investigate whether the combination of ATO and DAC might give a synergistic effect in the treatment of high-risk MDS.

In this study, high-risk MDS cell lines MUTZ-1 and SKM-1 were treated with ATO, DAC or the combination. We found that the single agent ATO or DAC could inhibit the proliferation of MUTZ-1 and SKM-1 cells detected by CCK-8. The synergistic effect of ATO combination with DAC was obvious; the CI < 1 in both cell lines indicated synergism of them with a significant decrease of the protein expression of AKT (protein kinase B) detected by western blot. AKT was an important mediator of growth factors and involved in cell growth and survival; genetic mutations might induce the hyperactivation of AKT in hematological

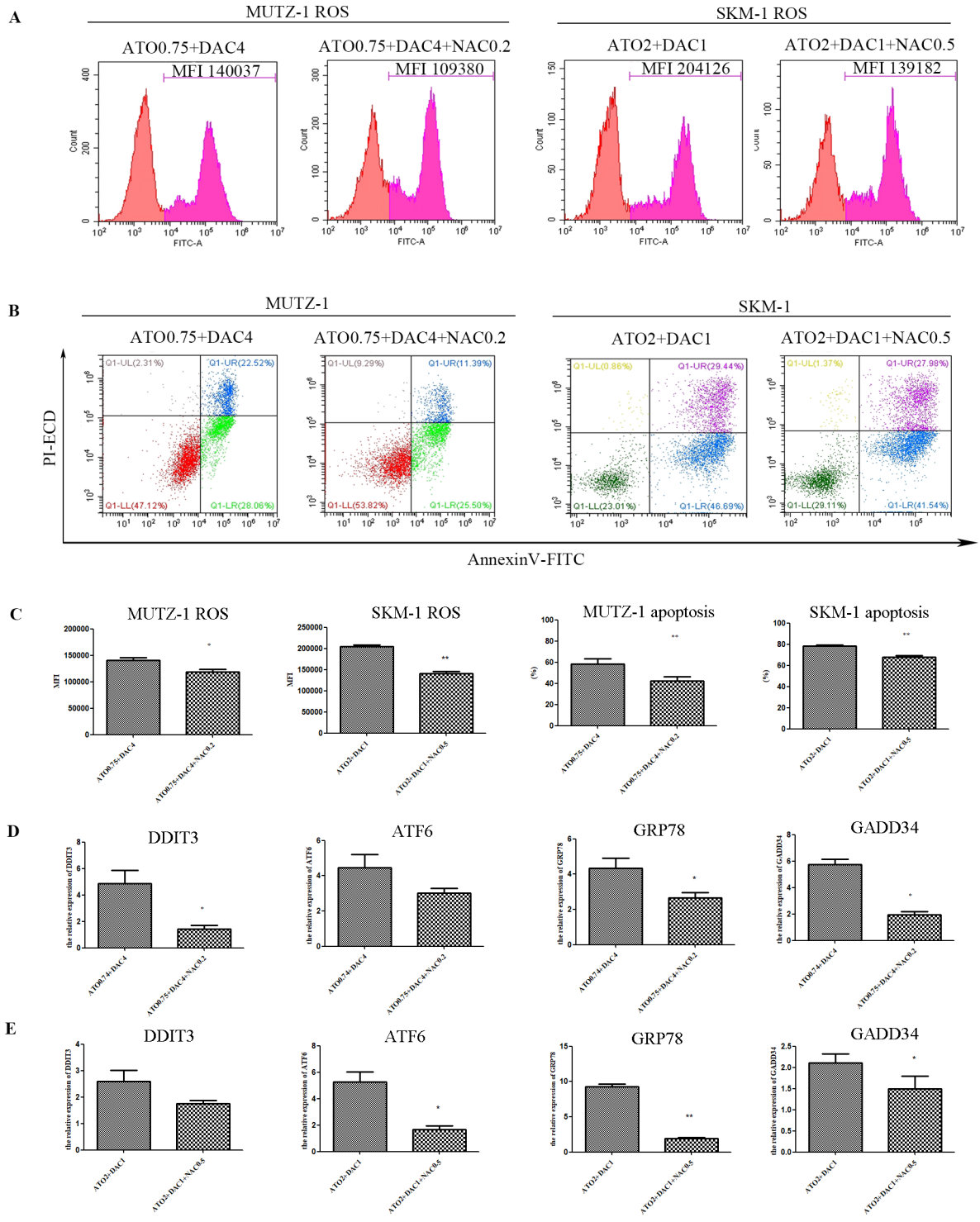

Figure 4 Antioxidant treatment with NAC partially decreased the apoptosis induced by the combination of arsenic trioxide (ATO) and decitabine (DAC) accompanied with increased ER stress in MDS cell lines at 72 hours. (A) The levels of ROS before and after the treatment with NAC in MDS cells. (B) The apoptosis before and after the treatment with NAC in MDS cells. (C) The levels of ROS and apoptosis significantly decreased after the treatment with NAC ($p<0.05$). (D and E) The gene expressions associated with ER stress decreased after the treatment with NAC in MDS cells. Compared with combination group, * indicated $p<0.05$, ** indicated $p<0.01$, *** indicated $p<0.001$. ER, endoplasmic reticulum; MDS, myelodysplastic syndrome; NAC, N-acetylcysteine; ROS, reactive oxygen species.

malignancies.[23] Meanwhile, single ATO or DAC could induce the apoptosis of MUTZ-1 and SKM-1 cells, and the apoptosis in even higher combinations of ATO and DAC, determined by flow cytometry using an annexin V-FITC/PI double staining kit. The combination of ATO and DAC significantly inhibited proliferation and induced apoptosis in MDS cells, which inspired us to study the potential synergistic mechanisms.

To explore the synergistic mechanisms of ATO combined with DAC more effective in MDS cells, we examined the gene expressions in MUTZ-1 with different groups (control, ATO, DAC and combination) through the RNA-sequence. According to the result of RNA-sequence, we found that single DAC significantly increases the gene expressions in MUTZ-1 cells through the gene demethylation compared with single ATO group and control. In the combination group, DAC and ATO synergistically increased the proliferation and apoptosis-related gene expressions in MUTZ-1.

Compared with other groups, in combination group, 208 genes were significant upregulated and 20 genes were significantly downregulated. From gene screening and functional analysis of GSEA, the gene expressions of ER stress were significantly enriched in apoptosis-related signaling pathways in the combination group of ATO and DAC. ER was the main machinery for synthesizing, folding and transporting proteins. Imbalance of homeostasis in ER led to the accumulation of unfolded or misfolded proteins in the ER, known as ER stress, which activated the unfolded protein response (UPR). UPR activation can promote cell survival, or cell death if ER stress is chronic or severe.[24] ER stress could lead to cell apoptosis in pathophysiological processes through CHOP, inositol requiring protein-1α and integration among UPR branch signal pathways.[25] Prolonged UPR activation induced apoptosis through the activation of CHOP, encoded by the gene of DDIT3, and verified by the reduced oxidative stress and apoptosis after deleting gene of DDIT3.[26] ATF6 had a major role in chaperone induction; after the dissociation of GRP78 (glucose-regulated protein 78), active ATF6 moved to the nucleus and induced the transcription factor CHOP and X box-binding protein.[27] In our study, the gene expressions of ER stress-related signal pathways were verified by qPCR; the gene expressions of DDIT3, ATF6, GRP78, GADD34, and Caspase-3 were significantly higher in combination group than in single-agent group or control, which were consistent with the RNA-sequence results. ATO might induce the production of ROS in tumor cells, activation of the signal pathway of ER stress and induction of DNA and protein damages, resulting in cell apoptosis.[13 28] DAC might induce delayed ROS accumulation in leukemia cells resulting in cell death, independent of its demethylation effects.[29] We further detected the level of intracellular ROS in groups of control, ATO, DAC, and the combination by flow cytometry. In combination group, the levels of ROS in MDS cell lines were significantly higher than that in single-agent groups. The ER stress-related proteins were examined by western blot. It showed a significant increase in the protein expressions of CHOP, ATF6, GRP78, while a significant decrease in expression of BCL2, which played important roles in the ER stress-associated apoptosis pathway, suggesting that the ER stress-related proteins might participate in the apoptosis of MDS cell lines MUTZ-1 and SKM-1 induced by the combination of ATO and DAC.

We further confirmed the effects of ER stress in the apoptosis induced by the combination of ATO and DAC on MDS cells by adding antioxidant NAC that might block the production of ROS. Our current study showed that NAC could reduce the production of ROS induced by the combination of ATO and DAC as well as the apoptosis in both MUTZ-1 and SKM-1 cells. At the same time, we compared the gene expressions of DDIT3, GRP78, ATF6, and GADD34 after the treatment of adding NAC in combination of ATO and DAC. The decreased expressions of ER stress-related genes after the antioxidant treatment showed that the combination of ATO and DAC could induce the apoptosis of MDS cell lines MUTZ-1 and SKM-1 through the activation of ER stress in cells, and antioxidant treatment could partially relieve the ER stress-related cell damage. Previous studies also showed that ATO might induce apoptosis through ROS-induced ER stress in myoblasts and tumors cells, which were reversed by antioxidant NAC to a certain degree.[15 30]

In conclusion, our study demonstrated that the treatment of ATO in combination with DAC synergistically induced apoptosis of MDS cells via the induction of intracellular ROS-related ER stress, which upregulated the expressions of DDIT3, GRP78, ATF6, and caspase3 and downregulated the expression of Bcl-2, and antioxidant treatment would partially relieve the oxidative stress-induced ER stress-associated apoptosis of the MDS cells in the combination of ATO and DAC. These findings may provide a strategy to develop a novel chemotherapy regimen for patients with MDS.

Contributors ZL carried out the molecular genetic studies. HJ and LL participated in the cell culture and performed the statistical analysis. RF conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Patient consent for publication Not required.

Provenance and peer review Not commissioned; externally peer reviewed.

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