The Synergistic Effects of Decitabine Combined with Arsenic Trioxide (ATO) in the Human Myelodysplastic Syndrome Cell Line SKM-1

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Abstract Despite recent advances in the treatment of myelodysplastic syndrome (MDS), single-agent clinical effects remain unsatisfactory, and decitabine monotherapy is also associated with a relatively low rate of complete remission. To study the combined effects and mechanism of decitabine (DAC) and arsenic trioxide (ATO) on the human myelodysplastic cell line SKM-1, we used the MTS assay and CalcuSyn software to determine the cytotoxicity and potential synergistic effects, respectively. Furthermore, we determined apoptosis and measured the mRNA expression level of two genes that are considered main regulators of the apoptosis process. The results showed that DAC and/or ATO can inhibit the proliferation of SKM-1 cells and demonstrated significant synergy between the two agents ($CI < 1$). Additionally, combination of 2.5 $\mu\text{mol/L}$ DAC and 5 $\mu\text{mol/L}$ ATO led to a significantly higher apoptosis rate and more significantly decreased the Bcl2/Bax ratio than either compound alone ($P < 0.001$). Based on the observations of this study, we suggest that combined administration of these two drugs might be considered a novel therapeutic regimen for treating MDS.

Keywords Decitabine · Arsenic trioxide · Myelodysplastic syndrome

Introduction

Myelodysplastic syndrome (MDS) is a highly heterogeneous group of morphologically related yet clinically and biologically distinct hematopoietic disorders that share the hallmark of variable cytopenia in the setting of dysplastic and cellular bone marrow [1]. The clinical course of the disease and response to therapy considerably vary among individuals; therefore, no single agent is expected to universally benefit all MDS patients.

Decitabine (5-aza-2'-deoxycytidine; DAC) is a nucleoside analog of cytidine in which the 5-carbon of the pyrimidine ring is replaced by nitrogen, and it has been approved by the US Food and Drug Administration (FDA) for the treatment of MDS [2, 3]. Preliminary studies have shown overall response rate (ORR) of 28–48 % with complete rate (CR) rates of 6–34 % with the use of this drug [4]. In responding patients, the median duration of response was only between 8 and 10 months, and the majority of the patients lost response or progressed despite continued therapy. These data demonstrate that the clinical effects of decitabine monotherapy in the treatment of MDS are unsatisfactory; thus, combination therapy may be a better strategy for achieving maximum benefit and delaying the development of decitabine resistance.

Several groups have attempted to increase the response rate to DAC-based therapy by developing combination treatments [5, 6]. Previous studies have suggested that patients with MDS and acute myeloid leukemia respond to the pro-apoptotic effects of arsenic trioxide (ATO) [7]. Arsenic is a trace element in the human body, which can stimulate hematopoiesis and promote the growth and reproduction of cells. Two multicenter trials have examined its use for the treatment of the MDS as a single agent, demonstrating responses in 20 % of patients [8, 9]. In

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addition, as *s*-adenosylmethionine (SAM) and DNA methyltransferase (DNMT) are consumed during ATO metabolism and SAM is a necessary activity factor, ATO consumes these required materials and reduces their quantity and activity. Tong and Fu et al. [10, 11] found re-expression of p15 and p16 through the demethylation of their promoters in the U266 and MUTZ-1 cell lines, respectively. In addition, a previous study found that arsenic trioxide combined with the demethylation drug azacitidine showed increased demethylation in *in vivo* and *in vitro* experiments [12]. The above results suggest that the combination of arsenic trioxide and decitabine may have a similar effect.

Based on data demonstrating limited efficacy using DAC and ATO as single agents in patients with MDS with similar effects, we conducted combination therapy with DAC and ATO in the MDS cell line SKM-1.

Methods

Cell Culture, Reagents and Drugs Treatment

The SKM-1 cell line (graciously provided by Tongji Medical University) was grown in RPMI 1640 (Gibco-BRL, Bethesda, MD) medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Gibco-BRL, Bethesda, MD) in a humidified atmosphere of 5 % CO₂ at 37 °C, and the media was changed every 2 days. Decitabine and ATO were purchased from Sigma Chemical Co. (St. Louis, MO). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 *h*-tetrazolium, inner salt), dimethyl sulfoxide (DMSO) and PMS (phenazinemethosulfate) were purchased from Sigma (St. Louis, MO, USA) and used according to the manufacturer's instructions. DNA extraction kits, PCR master mix and the SYBR Green quantitative PCR kit were purchased from TAKARA (Dalian, China). Quantitative PCR were synthesized by Invitrogen trading (Shanghai) Co., Ltd. The Annexin V apoptosis detection kit APC (E09658-1645) was purchased from eBioscience (San Diego, USA). Dulbecco's phosphate-buffered saline (DPBS) was used to dilute the compounds to working concentrations, and they were stored away from light at −20 °C. DMSO-treated cells were used as a blank control.

Cell Viability Assay

Cells were plated at a density of 1×10^5 cells/mL in 5 mL of medium and split 24 h before intervention. Different concentrations of DAC and ATO alone or in combination were added to the medium in 96-well plates and incubated for 72 h. MTS mixed with PMS was added, and after a 1 h

incubation at 37 °C in a humidified, 5 % CO₂ atmosphere, absorbances at 490 and 690 nm were recorded using an ELISA plate reader. Doses inhibiting 50 % proliferation (IC₅₀) were analyzed by the median-effects method (CalcuSyn software, Biosoft). *In vitro* cytotoxicity was assayed under the following experimental conditions: DAC alone, ATO alone, and DAC + ATO. The effects of the combinations were estimated using CalcuSyn software, which was developed based on the median-effects method by Chou and Talalay.

PI/Annexin V Staining and Cell Cycle Analysis

Cells were washed twice in phosphate-buffered saline, resuspended in 1 mL of binding buffer containing annexin V, and they were then analyzed by flow cytometry after the addition of propidium iodide (PI). Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of cells with compromised cell membranes. This staining allows for the discrimination of live (unstained with either fluorochrome) from apoptotic (stained only with annexin V) and necrotic (stained with both annexin V and PI) cells.

RNA Extraction and cDNA Synthesis

RNA was extracted using the TRIzol reagent (Invitrogen). The RNA quality was analyzed in a 0.8 % agarose gel stained with ethidium bromide. The RNA (~1 µg) was synthesized into first-strand cDNA with random hexamer primers using the Superscript III First Strand Synthesis kit (Invitrogen, Rockville, MD).

Quantitative Reverse-Transcription PCR

We designed primers using Primer Premier five, and the primers were confirmed by searching PubMed UNISTS Primer-BLAST (Table 1). The acquired cDNA was used as a template for comparative quantitative PCR with SYBR Premix EX TaqII in 96-well plates, and each sample was assayed in triplicate. Assembled plates were

Table 1 Target gene PCR primer and probe sequences

Primer and probe	Sequence (5'–3')
Bax-F	5'-TCTGACGGCAACTTCAACTG-3'
Bax-R	5'-TTGAGGAGTCTACCCAACC-3'
Bcl2-F	5'-ATGTGTGTGGAGAGCGTCAA-3'
Bcl2-R	5'-ATGTGTGTGGAGAGCGTCAA-3'
β-actin-F	5'- CCTGGCACCCAGCACAAAT-3'
β-actin -R	5'-GCCGATCCACACGGAGTACT-3'

then covered and run using the following conditions: an initial denaturation step at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 min with a final step at 95 °C for 15 s, 60 °C for 1 min and 95 °C 15 s. The internal control gene was β -actin, and the gene expression of different samples analyzed with the target gene normalized to β -actin. The target gene PCR primers and probe sequences were designed by Express software and synthesized by Invitrogen (Table 1). The data were analyzed with an ABI 7500 Real-Time PCR System (Applied Biosystems). The resulting melting curves were examined.

Statistical Analysis

Data are presented as the mean $\pm$ SD. One-way ANOVA followed by Bonferroni multiple comparison was performed to assess differences between two groups under multiple conditions. If the data failed the normality test, Kruskal–Wallis one-way ANOVA of ranks was used. A $P < 0.05$ was considered statistically significant. CalcuSyn software (Biosoft, Ferguson, MO, USA) was used to evaluate the synergistic effects of the drug combinations and generate combination index (CI) plots. A CI value <1

indicated synergism. SPSS version 13.0 was used to analyze the data, and GraphPad Prism was used to plot the data.

Results

Synergistic Effects Induced by Decitabine and Arsenic Trioxide in the Human Myelodysplastic Syndrome Cell Line SKM-1

To determine the effects of combined treatment of the DNA methyltransferase inhibitor decitabine and arsenic trioxide in human myelodysplastic syndrome cells, SKM-1 cells were exposed to different concentrations of decitabine or arsenic trioxide alone or in combination for up to 72 h followed by the examination of cell proliferation using an MTS assay. Table 2 describes the results in detail. As shown in Fig. 1, decitabine or arsenic trioxide alone could inhibit the proliferation of SKM-1 cells in a dose-dependent manner with IC₅₀ values of 1.42 μ mol/L (95 % CI 1.08–1.86 μ mol/L, $r = 0.97$) and 3.82 μ mol/L (95 % CI 3.22–4.53 μ mol/L, $r = 0.99$), respectively. Furthermore, to identify the presence of synergy, we

Table 2 Cell inhibition rate and CI values of decitabine and/or arsenic trioxide

	Group ^a	Decitabine	Arsenic trioxide	CI
Cell inhibition rate (%)	1	29.00 $\pm$ 8.34	3.78 $\pm$ 8.85	0.280
	2	39.43 $\pm$ 15.73	8.88 $\pm$ 7.38	0.441
	3	41.55 $\pm$ 13.88	20.67 $\pm$ 5.52	0.450
	4	58.19 $\pm$ 15.14	59.17 $\pm$ 6.89	0.341
	5	61.35 $\pm$ 9.46	75.44 $\pm$ 1.99	0.581

^a Group 1: decitabine 0.25 μ mol/L, arsenic trioxide 0.5 μ mol/L

Group 2: decitabine 0.5 μ mol/L, arsenic trioxide 1.0 μ mol/L

Group 3: decitabine 1.0 μ mol/L, arsenic trioxide 2.0 μ mol/L

Group 4: decitabine 2.0 μ mol/L, arsenic trioxide 4.0 μ mol/L

Group 5: decitabine 4.0 μ mol/L, arsenic trioxide 8.0 μ mol/L

Fig. 1 Effects of decitabine or arsenic trioxide alone in SKM-1 cells

Fig. 2 CI values of decitabine combined with arsenic trioxide in SKM-1 cells

treated the cells with combinations of the two agents in a constant ratio with each another and used CalcuSyn software to calculate the combination index (CI). Figure 2 shows significant synergy between the two agents ($CI < 1$) in SKM-1 cells.

Decitabine and Arsenic Trioxide Synergistically Enhance Apoptosis and Alters the Expression Level of Apoptosis Regulatory Genes in SKM-1 Cells

To identify whether the cytotoxic effects of decitabine combined with arsenic trioxide were due to the induction of apoptosis, SKM-1 cells were treated with the two compounds alone or in combination for 48 h, and then apoptosis was determined by Annexin V-PE/7-AAD and propidium iodide (PI) staining and flow cytometry analysis. The results showed that compared with the blank control, 2.5 or 5 $\mu\text{mol/L}$ decitabine alone could not influence the apoptosis rate ($4 \pm 0.69\%$ vs. $4.13 \pm 0.99\%$ vs. $3.43 \pm 0.58\%$, $P = 0.534$), but both 5 and 10 $\mu\text{mol/L}$ arsenic trioxide could increase the apoptosis rate ($12.55 \pm 0.42\%$ vs. $61.89 \pm 12.94\%$ vs. $3.43 \pm 0.58\%$, $P < 0.001$). In addition, the combination of 2.5 $\mu\text{mol/L}$ decitabine and 5 $\mu\text{mol/L}$ arsenic trioxide led to a significantly higher apoptosis rate than either compound alone ($22.71 \pm 1.41\%$ vs. $4 \pm 0.69\%$ vs. $12.55 \pm 0.42\%$, $P < 0.001$, Figs. 3 and 4).

Apoptosis is a programmed physiological process regulated by genes, and new therapeutic strategies in oncohematology are based on apoptosis modulation. Previous studies have demonstrated that Bcl-2 family member genes are main regulators of this process, and the pro-apoptotic gene Bax and the anti-apoptotic gene Bcl-2 play an important role in this family. Thus, we used quantitative real-time PCR to measure the mRNA expression level of these two

Fig. 3 Apoptosis rate for 2.5 and/or 5 $\mu\text{mol/L}$ arsenic trioxide in SKM cells

genes in SKM-1 cells, which were exposed to different concentrations of decitabine or arsenic trioxide alone or in combination for up to 48 h. The results showed that with an increase in the decitabine concentration alone from 4 to 8 $\mu\text{mol/L}$, the Bcl2/Bax ratio significantly decreased (0.3065 ± 0.0142 vs. 0.1686 ± 0.0014 , $P < 0.001$, Table 3). Similarly, increasing arsenic trioxide concentrations from 1.6 to 3.2 $\mu\text{mol/L}$ also significantly decreased the Bcl2/Bax ratio (0.5615 ± 0.0256 vs. 0.2798 ± 0.0166 , $P < 0.001$, Table 3). In addition, the combination of decitabine and arsenic trioxide decreased the Bcl2/Bax ratio even more significantly ($P < 0.001$, Table 3).

Discussion

It is well known that MDS is a highly heterogeneous disease with a number of complex mechanisms, and gene methylation is just one of those mechanism. Decitabine (DAC) and arsenic trioxide (ATO) are two drugs that have been approved by the US Food and Drug Administration for the treatment of myelodysplastic syndrome (MDS) as single agents, but their clinical efficacy is unsatisfactory. Though DAC has advantages over best supportive care (BSC), it cannot prolong OS [13]. These observations make combination of the two drugs an attractive clinical proposition particularly for patients who have already failed one of these disease-modifying therapies, specifically including the at-risk population of patients with sAML arising from MDS [14].

A previous study has shown that chronic low-dose exposure to arsenic can induce the hypomethylation of genomic DNA and hypomethylation of oncogene

Fig. 4 Apoptosis rate for 2.5 $\mu\text{mol/L}$ decitabine (DAC) and/or 5 $\mu\text{mol/L}$ arsenic trioxide (ATO) in SKM cells

Table 3 Bcl2/Bax ratios

Group ^a	DAC	ATO	DAC + ATO	<i>P</i>
1	0.3065 ± 0.0142	0.5615 ± 0.0256	0.2629 ± 0.0050	<0.001
2	0.1686 ± 0.0014	0.2798 ± 0.0166	0.1160 ± 0.0154	<0.001
<i>P</i>	<0.001	<0.001	<0.001	–

^a Group 1: DAC 4 $\mu\text{mol/L}$ and/or ATO 1.6 $\mu\text{mol/L}$

Group 2: DAC 8 $\mu\text{mol/L}$ and/or ATO 3.2 $\mu\text{mol/L}$

promoters in mice, ATO can moderately down-regulate the global DNA methylation level in HL-60 cells, and there is a synergistic interaction between DAC and ATO in HL-60 cells [15]. In the leukemia cell lines U266 and MUTZ-1, ATO treatment was reported to restore the expression of p15 and p16 via promoter demethylation [10, 11]; however, in MDS cell lines, its mechanism remained unclear.

Thus, we studied the combination of these two drugs in MDS cell lines in vitro and found that decitabine and/or arsenic trioxide can inhibit the proliferation of SKM-1 cells, and we demonstrated significant synergy between the two agents ($CI < 1$). In addition, the combination of 2.5 $\mu\text{mol/L}$ decitabine and 5 $\mu\text{mol/L}$ arsenic trioxide led to a significantly higher apoptosis rate than either compound alone ($P < 0.001$). To determine the mechanism of the synergistic effects, we chose to examine two genes related to apoptosis that are considered the main regulators of the apoptosis process: the pro-apoptotic gene Bax and the anti-apoptotic gene Bcl-2. Moreover, these drugs induce

apoptosis via a caspase-3-dependent mechanism involving Bax up-regulation and Bcl-x1 down-regulation; thus, the Bcl2/Bax ratio indicates the anti-apoptosis ability where smaller values indicate stronger pro-apoptosis induction capacity. Our results demonstrated that the combination of DAC and ATO more significantly decreases the Bcl2/Bax ratio, indicating the combination of DAC and ATO played a synergistic effect in SKM-1 cells.

In conclusion, we found a synergistic interaction between DAC and ATO in the human myelodysplastic syndrome cell line SKM-1, which may prove to be a valuable clinical strategy that is useful in the future.

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