Arsenic trioxide and triptolide synergistically induce apoptosis in the SKM-1 human myelodysplastic syndrome cell line

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Abstract. Although certain combination therapies comprising arsenic trioxide (As_2O_3) with other agents exist for the treatment of several types of human cancer, few As_2O_3 combination therapies are clinically effective for myelodysplastic syndromes (MDS). Triptolide (TL) may be an effective therapeutic agent for the treatment of MDS. However, to date, there is no combination therapy for MDS with As_2O_3 and TL. Therefore, the aim of the present study was to investigate this combination therapy on the apoptosis of MDS SKM-1 cells. The MDS SKM-1 cells were treated with As_2O_3, TL or the two in combination at various concentrations, or were mock-treated. Cell viability, cell apoptosis, levels of reactive oxygen species (ROS) and the expression of the cell apoptosis-associated genes, B cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax) and caspase-3, were determined using an MTT assay, flow cytometric analysis of annexin V-fluorescein isothiocyanate/propidium iodide double-stained cells, flow cytometic analysis of intracellular 2',7'-dichlorodihydrofluorescein diacetate fluorescence and reverse transcription-quantitative polymerase chain reaction analysis, respectively. Combination index (CI) analysis was performed to determine whether effects were synergistic (CI<1). The combination treatment was found to synergistically inhibit MDS SKM-1 cell growth, induce cell apoptosis, increase ROS levels, upregulate the expression levels of Bax and caspase-3, and downregulate the mRNA expression of Bcl-2. In conclusion, the combination treatment of As_2O_3 and TL synergistically induced apoptosis in the MDS SKM-1 cells.

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Introduction

Myelodysplastic syndromes (MDS) are clonal stem cell disorders characterized by peripheral cytopenias with dysplasia in one or more cell lineages, including erythrocytic, granulocytic and megakaryocytic lineages, leading to the progression to acute myelogenous leukemia (AML) with a poor prognosis (1-4). At present, allogeneic hematopoietic stem-cell transplantation is the only treatment option, which can induce long-term remission (5,6). However, its use is only possible in a minority of patients with MDS due to the advanced age of presentation, limited availability of donor sources, high rate of treatment-associated mortality (~39% at 1 year), suboptimal disease-free survival rates (~29% at 5 years) and chronic graft-versus-host disease (~15% at 1 year) (6). Aberrant DNA methylation is frequently associated with MDS; therefore, demethylating agents, including as azacytidine and decitabine, are used to treat patients with MDS. However, treatment of patients with a higher risk of MDS with azacitidine (7,8) only increases the overall survival rate to 24.5 months, compared with 15.0 months with conventional care, supportive care, treatment with low-dose cytarabine or intensive chemotherapy. In addition, treatment with decitabine (9) prolongs the median duration of the progression of AML or associated mortality rates to 12 months, compared with 6.8 months following supportive care alone. In addition, the rates of complete remission (9-17%) following treatment with demethylating agents (7-9) are similar to those following conventional care with low-dose cytarabine (11-18%) (10), and substantially lower, compared with those following induction chemotherapy in patients with AML (>50%) (11). Lenalidomide, a derivative of thalidomide, reduces transfusion requirements, and reverses cytologic and cytogenetic abnormalities in patients who have MDS with the 5q31 deletion (12). However, lenalidomide increases the risk of developing other malignancies, including AML and B-cell lymphoma (13). Thus, a more effective treatment option for MDS is urgently required.

Arsenic trioxide (As_2O_3) is a traditional Chinese medicine, which is effective in the clinical management of patients with acute promyelocytic leukemia (APL) (14,15). However, in two-phase II multicenter trials, rates of hematological improvement with As_2O_3 were 20-29%, with moderate toxicity

reported (16,17). As$_2$O$_3$ induces the apoptosis of nonpromyelocytic leukemia and other types of malignant tumor cells (18-20) through the inhibition of B cell lymphoma-2 (Bcl-2) (21), and the upregulation of Bcl-2-associated X protein (Bax) (22) and caspase-3 (23).

Extracts of the Chinese herb, *Tripterygium wilfordii* Hook F are used to treat autoimmune and/or inflammatory diseases, and triptolide (TL) is the active substance of these extracts *in vitro* and *in vivo* (24). Several studies have demonstrated that TL may be an effective therapeutic agent for the treatment of MDS (25), several types of human pancreatic (26) and adrenal (27) cancer, and T cell lymphocytic leukemia (28) via inducing cell apoptosis through the activation of caspase-3 and generation of reactive oxygen species (ROS) (25-27).

Although certain combination therapies involving As$_2$O$_3$ and other agents, are ongoing for several types of human cancer, few As$_2$O$_3$ combination therapies are clinically effective. These include combination therapy of As$_2$O$_3$ with ascorbic acid in nonrefractory APL hematologic malignancies and multiple myeloma (18), but not in other AML except nonrefractory APL, acute lymphoid leukemia (18), chronic myeloid leukemia and chronic lymphoid leukemia (18). The use of phase 2 combination therapy with As$_2$O$_3$ and gemtuzumab ozogamicin for the treatment of MDS and secondary AML has been found to have acceptable response rates and toxicity, however, the median overall survival rate was only 9.7 months (29).

The aim of the present study was to investigate the effect of As$_2$O$_3$ in combination with TL on the apoptosis of MDS SKM-1 cells by evaluating the gene expression levels of Bcl-2, Bax and caspase-3, and the generation of ROS.

Materials and methods

Reagents and cell culture. TL (purity >99.0%; Chinese Academy of Medical Sciences, Nanjing, China) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to form a 1 mM stock solution. As$_2$O$_3$ powder (Beijing Double-Crane Pharmaceutical Co., Ltd., Beijing, China) was dissolved in phosphate-buffered saline (PBS). The MDS SKM-1 cell line was obtained from the Cell Bank of the Japanese Collection of Research Bioresources (Osaka, Japan). The SKM-1 cells were cultured in RPMI 1640 medium (Life Technologies; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO$_2$. Cells in the second to fourth passages and logarithmic growth phase, with >95% viability on trypan blue staining, were used for the following experiments.

Cell treatment and cell viability assessment using an MTT assay. The cells were seeded at a density of 4-6x10^4 cells/well in 96-well plates, cultured RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin mixture at 37°C in humidified incubator with 5% CO$_2$ for 48 h and treated with various concentrations of As$_2$O$_3$ (0.25, 0.5, 2, 8 or 32 μM), TL (10, 20, 40, 80 or 160 ng/ml) or As$_2$O$_3$+TL (0.25+10 ng/ml, 0.5+20 ng/ml, 2.0+40 ng/ml, 8+80 ng/ml or 32+160 ng/ml), or were mock-treated with RPMI-1640 medium containing

0.002% DMSO. Following treatment for 48 h, cell viability was assessed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Nanjing, China), according to the manufacturer's protocol. The absorbance at 490 nm was measured using a SpectraMAX M5 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA, USA).

Flow cytometric analysis of MDS SKM-1 cell apoptosis. Following treatment of the cells for 48 h with As$_2$O$_3$, TL, As$_2$O$_3$ and TL, or mock treatment with RPMI-1640 media, the cells were collected by centrifugation at 1,300 x g for 3 min at room temperature, washed twice with PBS (BD Biosciences, Beijing, China), and resuspended in binding buffer (Novagen; EMD Millipore, Billerica, MA, USA) at 1x10^6 cells/ml. Subsequently, the cells were stained with 5 μl of annexin V-fluorescein isothiocyanate (FITC) and 5 μl of propidium iodide (PI), incubated in the dark at room temperature for 15 min, and mixed with binding buffer (400 μl). Analysis of apoptosis was then performed on a Calibur flow cytometer (BD Biosciences). Early and late apoptotic cells were calculated based on annexin V-positivity/PI-negativity and annexin V-positivity/PI-positivity, respectively.

Intracellular ROS. The cells (3x10^5/well) in 6-well plates were treated with As$_2$O$_3$, TL, As$_2$O$_3$ and TL or mock treatment , cultured in RPMI 1640 medium, supplemented with 10% FCS and 1% penicillin/streptomycin mixture at 37°C in humidified incubator with 5% CO$_2$ for 48 h. Following treatment, the cells were washed once with PBS and treated with 100 nM 2',7'-dichlorodihydrofluorescein diacetate in a cell culture incubator for 30 min at 37°C with 5% CO$_2$. Following trypsinization, the cells were washed once with PBS and centrifuged at 1,300 x g for 3 min. The cell pellets were then resuspended in 1 ml PBS and analyzed on a Calibur flow cytometer (BD Biosciences).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Following the treatment of the cells for 48 h, total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RT-qPCR analysis was performed on an ABI 7900 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a qRT-PCR kit (Qiagen, Beijing, China), according to the manufacturer's protocol. The Abl gene was used as an internal control. The primer sequences were as follows: Bcl-2, forward (F) 5'-AGG ATCATGCTGTACTTAA-3' and reverse (R) 5'-ATGAGG CACGTTATTATTAG-3'; Bax, F 5'-CGAACTGGACAG TAACAT-3' and R 5'-CTCGGAAAAAGACCTCTC-3'; caspase-3, F 5'-TTGTAGAAGTCTAACTGGAA-3' and R 5'-CCATGTCATCATCAACAC-3'; Abl, F 5'-GATACGAAG GGAGGGTGTACCA-3' and R 5'-CTCGGCCAGGGTGTT GAA-3'. The 25 μl PCR reaction system included PCR mix 12.5 μl, F primer 0.5 μl, R primer 0.5 μl, probe 0.3 μl, ddH$_2$O 7.2 μl, cDNA 4 μl. The reaction parameters of Bcl-2, Bax and caspase-3 were as follows: 94°C 5 min, 94°C 40 sec, 56°C 55 sec, 72°C 1 min for 45 cycles, 72°C extension 7 min; 94°C 5 min, 94°C 40 sec, 58°C 55 sec, 72°C 1 min for 45 cycles, 72°C extension 7 min; 94°C 5 min, 94°C 40 sec, 50°C 55 sec, 72°C 1 min for 45 cycles, 72°C extension 7 min. The results
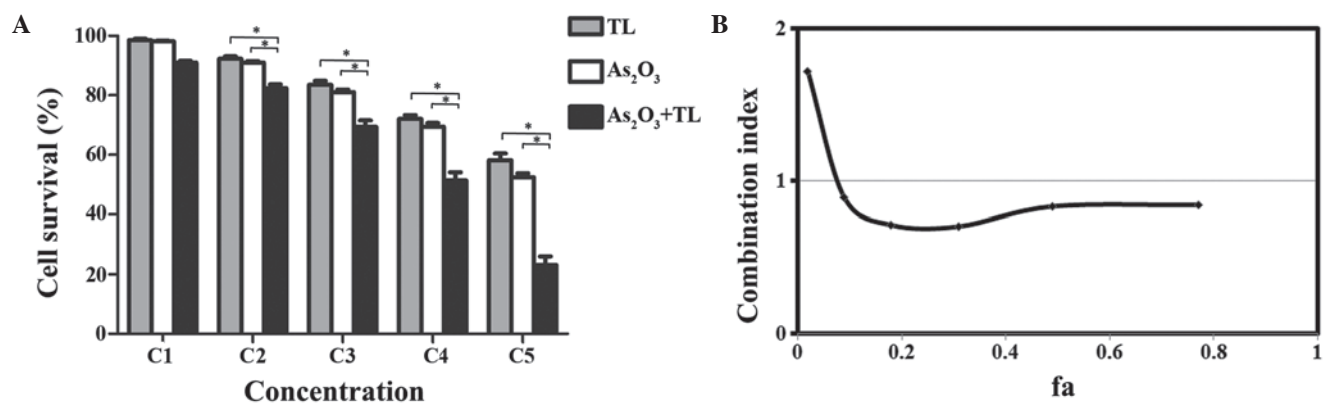
Figure 1. Synergistic effect of As$_2$O$_3$ and TL on the inhibition of MDS SKM-1 cell growth. (A) MDS SKM-1 cells treated with different concentrations of As$_2$O$_3$ (0.25, 0.50, 2, 8 or 32 μM as C1-C5) and/or TL (10, 20, 40, 80 or 160 ng/ml as C1-C5). Cell growth was measured using an MTT assay. Data are expressed as the mean ± standard error of the mean (*P<0.01; n=5). (B) Combination index of As$_2$O$_3$ with TL. The 'fa' on the x-axis denotes the fraction affected (i.e., a value of 0.2 is equivalent to a 20% reduction in cell growth). As$_2$O$_3$, arsenic trioxide; TL, triptolide; MDS, myelodysplastic syndrome.
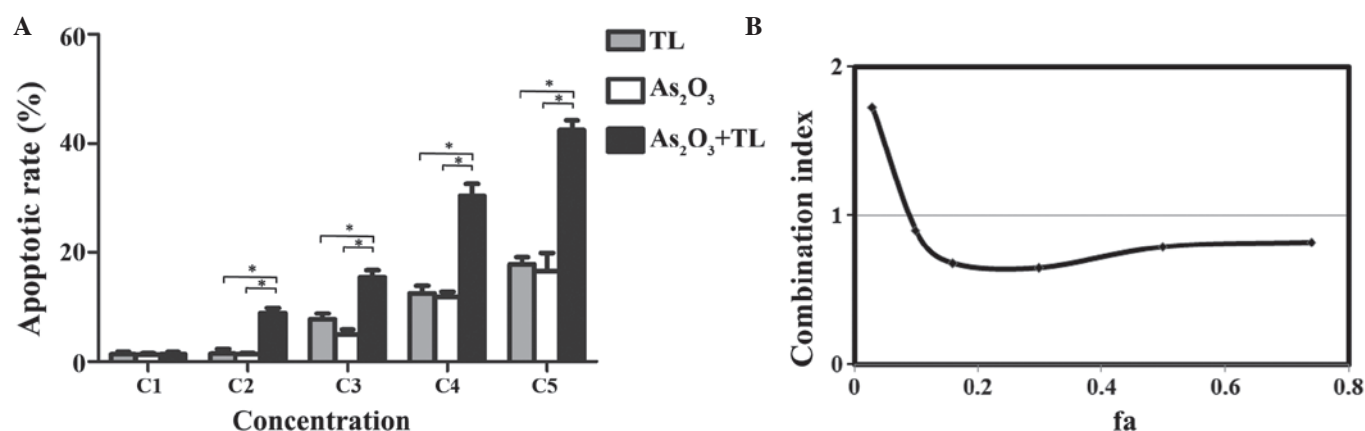

Figure 2. As$_2$O$_3$+TL treatment induces MDS SKM-1 cell apoptosis. The MDS SKM-1 cells were treated with different concentrations of As$_2$O$_3$ (0.25, 0.50, 2, 8 or 32 μM as C1-C5) and/or TL (10, 20, 40, 80 or 160 ng/ml as C1-C5) for 48 h. (A) Flow cytometric analysis of MDS SKM-1 cell apoptosis by double staining with annexin V/PI. Data are expressed as the mean ± standard error of the mean (*P<0.01; n=5). (B) Combination index of As$_2$O$_3$+TL. The 'fa' on the x-axis denotes the fraction affected (i.e., a value of 0.2 is equivalent to a 20% increase in apoptosis). As$_2$O$_3$, arsenic trioxide; TL, triptolide; MDS, myelodysplastic syndrome.

were reported as $2^{-\Delta\Delta Cq}$ relative to the gene expression of Abl (30).

Statistical analysis. Statistical analysis was performed with SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard error of the mean. Statistical analysis was performed using one-way analysis of variance followed by the least significant difference post-hoc test and Student's *t*-test. Factorial design analysis of variance was used to determine additive or synergistic effects. P<0.05 was considered to indicate a statistically significant difference.

Results

As$_2$O$_3$ and TL synergistically inhibit the growth of MDS SKM-1 cells. To examine whether TL enhances the chemosensitivity of MDS SKM-1 cells to As$_2$O$_3$, the present study examined the growth of MDS SKM-1 cells following treatment with As$_2$O$_3$ in combination with TL. The combination treatment of As$_2$O$_3$+TL substantially suppressed SKM-1 cell growth, compared with the cells treated with As$_2$O$_3$

or TL alone (Fig. 1A). To evaluate whether the cell growth inhibition induced by the combination of TL+As$_2$O$_3$ was additive or synergistic, the CI values were determined according to the Chou-Talalay combination index equation CI=(C)1/(CX)1+(C)2/(CX)2+(C)1(C)2/(CX)1(CX)2 (31), where CI <1 defines synergism. The CI analysis revealed that the CI values ranged between 0.70 and 0.87 (Fig. 1B). These results indicated that As$_2$O$_3$ and TL synergistically inhibited MDS SKM-1 cell growth.

As$_2$O$_3$ and TL synergistically induce apoptosis in MDS SKM-1 cells. To examine whether As$_2$O$_3$ and TL synergistically inhibit MDS SKM-1 cell growth through the induction of cell apoptosis by treatment with As$_2$O$_3$ in combination with TL, cell apoptosis was assessed using flow cytometry with annexin V-FITC/PI double staining. The combination treatment of As$_2$O$_3$+TL substantially induced SKM-1 cell apoptosis, compared with either As$_2$O$_3$ or TL alone (Fig. 2A). CI analysis revealed that the CI values ranged between 0.65 and 0.85 (Fig. 2B). The results indicated that As$_2$O$_3$ and TL synergistically induced MDS SKM-1 cell apoptosis.
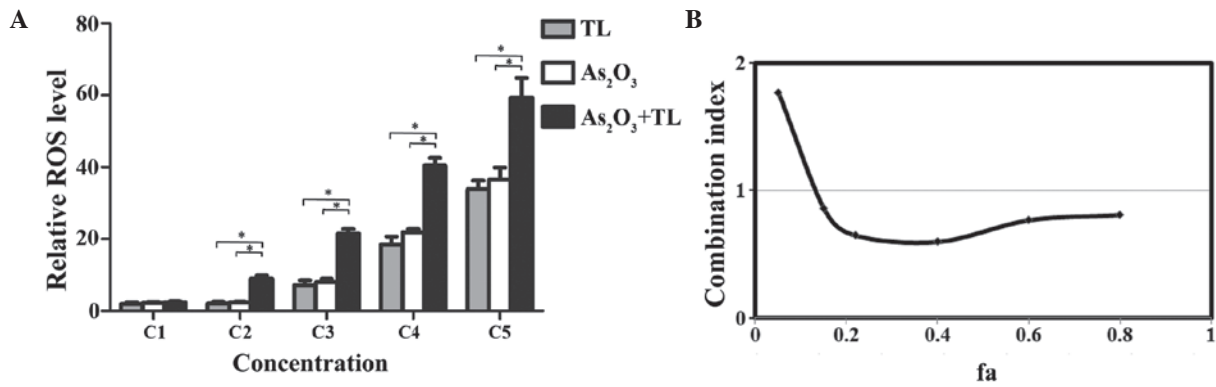
Figure 3. Analysis of ROS in SKM-1 cells using flow cytometry. (A) MDS SKM-1 cells were treated with different concentrations of As$_2$O$_3$ (0.25, 0.50, 2, 8 or 32 μM as C1-C5, respectively) and/or TL (10, 20, 40, 80 or 160 ng/ml as C1-C5, respectively) for 48 h. The ROS levels were then determined by counting the cells with 2',7'-dichlorodihydrofluorescein diacetate fluorescence using flow cytometry. Data are expressed as the mean ± standard error of the mean (*P<0.01; n=5). (B) Combination index of As$_2$O$_3$ with TL. The 'fa' on the x-axis denotes the fraction affected (i.e., a value of 0.2 is equivalent to a 20% increase in intracellular ROS levels). As$_2$O$_3$, arsenic trioxide; TL, triptolide; MDS, myelodysplastic syndrome.
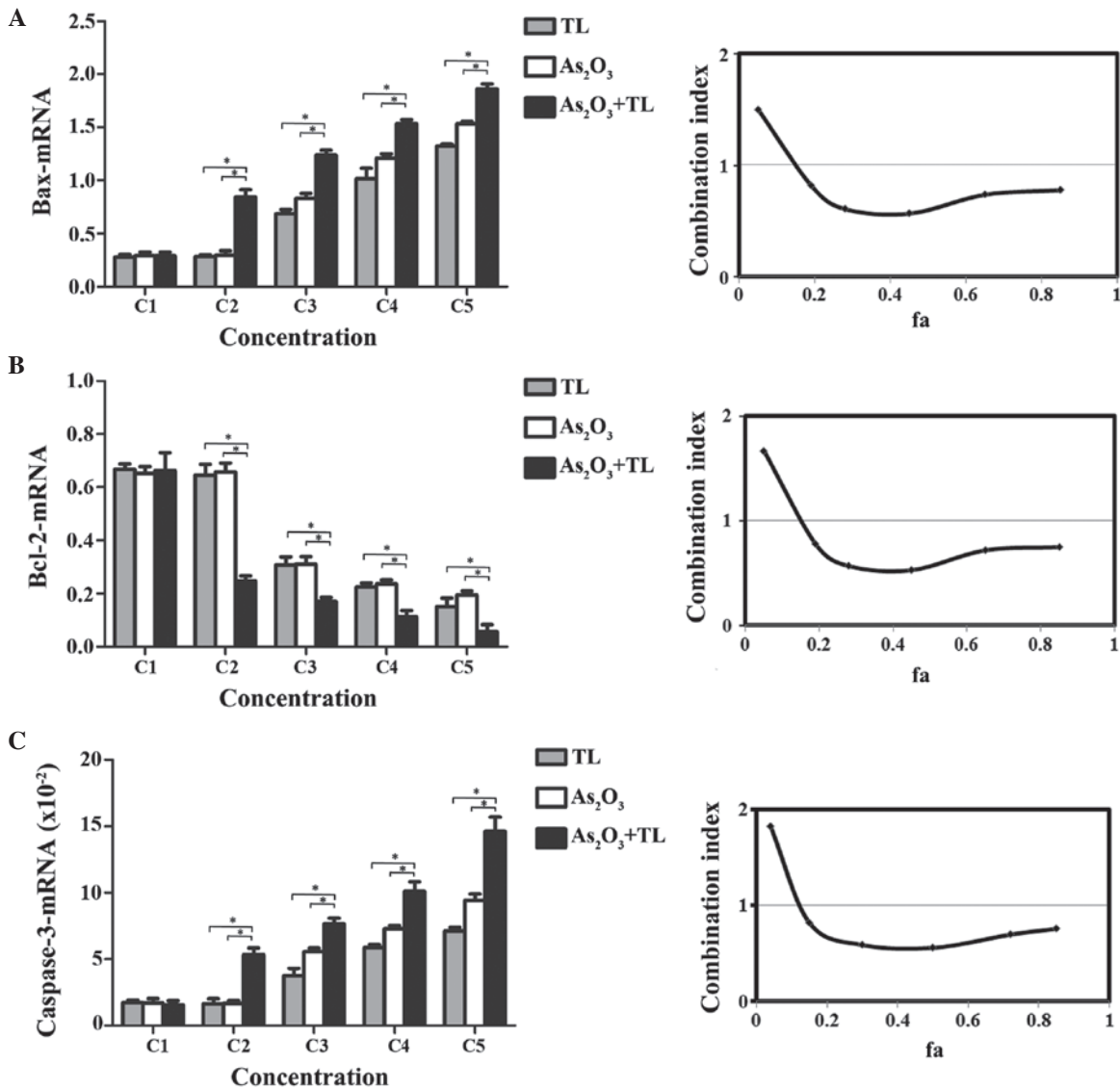

Figure 4. mRNA levels of apoptosis-associated genes in MDS SKM-1 cells. MDS SKM-1 cells were treated with different concentrations of As$_2$O$_3$ (0.25, 0.50, 2, 8 or 32 μM as C1-C5) and/or TL (10, 20, 40, 80 or 160 ng/ml as C1-C5) for 48 h. The mRNA expression levels were determined by reverse transcription-quantitative polymerase chain reaction analysis, and quantified using the 2$^{-\Delta\Delta Cq}$ method relative to Abl. Combination treatment led to a significant (A) increase in the mRNA expression of Bax (*P<0.01), decrease in the mRNA expression of (B) Bcl-2 (*P<0.01) and (C) increase in the mRNA expression of caspase-3 (*P<0.01). Data are expressed as the mean ± standard error of the mean (n=5; *P<0.01). The graphs on the right show the combination index of As$_2$O$_3$+TL. The 'fa' on the x-axis denotes the fraction affected (i.e., 0.2 is equivalent to a 20% change in mRNA expression). As$_2$O$_3$, arsenic trioxide; TL, triptolide; MDS, myelodysplastic syndrome; Bcl-2, B cell lymphoma-2; Bax, Bcl-2-associated X protein.
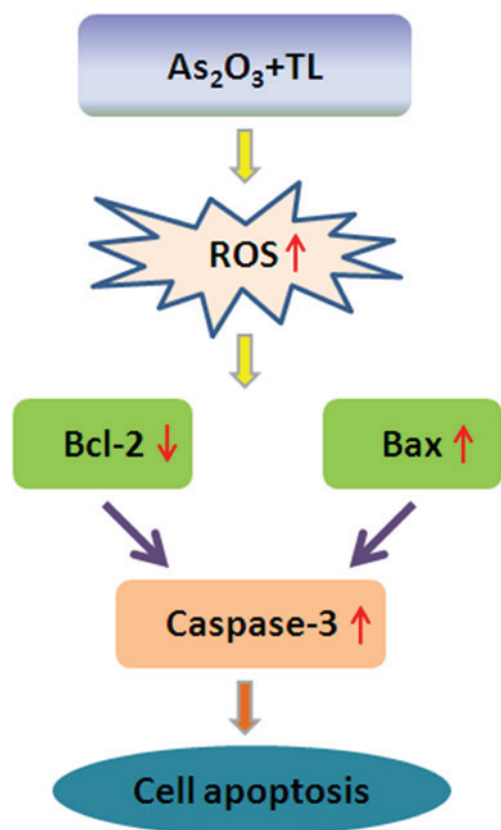
Figure 5. Schematic diagram of the potential mechanism of As_2O_3 and TL synergistically inducing MDS SKM-1 cell apoptosis. As2O3 + TL synergistically increased MDS SKM-1 intracellular ROS levels, which upregulated Bax expression, downregulated Bcl-2 expression, upregulated caspase-3 expression and induced cell apoptosis. As_2O_3, arsenic trioxide; TL, triptolide; MDS, myelodysplastic syndrome; ROS, reactive oxygen species; Bcl-2, B cell lymphoma-2; Bax, Bcl-2-associated X protein.

As_2O_3 and TL synergistically induce apoptosis via the generation of ROS in MDS SKM-1 cells. Treatment with As_2O_3 in combination with TL substantially increased the intracellular ROS levels, compared with either As_2O_3 or TL alone (Fig. 3A; $P<0.01$). CI analysis revealed that the CI values ranged between 0.60 and 0.86 (Fig. 3B). The results indicated that As_2O_3 and TL synergistically induced MDS SKM-1 cell apoptosis via the generation of ROS.

As_2O_3 and TL synergistically regulate the expression of apoptosis-associated genes in MDS SKM-1 cells. To determine whether As_2O_3 in combination with TL synergistically regulates the expression of apoptosis-associated genes, the mRNA expression levels of Bax, Bcl-2 and caspase-3 were measured in the cells treated with As_2O_3, TL or As_2O_3+TL for 48 h. As shown in Fig. 4, treatment with As_2O_3+TL led to significant increases in the expression levels of Bax and caspase-3, and a significant decrease in the mRNA expression of Bcl-2, compared with either As_2O_3 or TL alone ($P<0.01$; Fig. 4A-C). These results demonstrated that the combination of As_2O_3 and TL significantly induced apoptotic activity via inhibiting Bcl-2 and promoting the expression of Bax and caspase-3. CI analysis revealed that the CI values were 0.57-0.82 for Bax (Fig. 4A), 0.53-0.78 for Bcl-2 (Fig. 4B) and 0.56-0.82 for caspase-3 (Fig. 4C). These results indicated

that As_2O_3 and TL synergistically induced MDS SKM-1 cell apoptosis via increasing the mRNA expression levels of Bax and caspase-3 and decreasing the mRNA expression of Bcl-2.

Discussion

To investigate whether TL enhances the chemosensitivity of MDS SKM-1 cells to As_2O_3, the present study treated MDS SKM-1 cells with As_2O_3, TL or the two in combination. It was found that As_2O_3/TL synergistically inhibited SKM-1 cell growth through upregulation of ROS levels and cell apoptosis, as evidenced by synergistically increased expression levels of Bax and caspase-3, and decreased mRNA expression of Bcl-2.

The present study found that As_2O_3+ TL synergistically induced MDS SKM-1 cell apoptosis, determined from analysis of annexin V-FITC/PI double staining using flow cytometry. Of note, As_2O_3 in combination with a mitogen-activated protein kinase kinase or proteinase (32) inhibitor, has been shown experimentally to have a synergistic effect on the induction of AML cell apoptosis. The present study also found that the combination treatment with As_2O_3 and TL resulted in a significant increase in the mRNA expression levels of Bax and caspase-3, and a significant decrease in the mRNA expression of Bcl-2, compared with the cells treated with either As_2O_3 or TL alone. To evaluate whether the combination of TL and As_2O_3 increased the mRNA expression levels of Bax and caspase-3 and decreased the mRNA expression of Bcl-2 in an additive or synergistic manner, the CI values were determined. The results indicated that As_2O_3+TL synergistically induced MDS SKM-1 cell apoptosis via increasing the mRNA expression levels of Bax and caspase-3 and decreasing the expression of Bcl-2 (CI<1). These results suggested that the synergistic cell apoptosis induced by the combination treatment resulted from inhibiting the mRNA expression of Bcl-2 and promoting the mRNA expression levels of Bax and caspase-3. It has been reported previously that As_2O_3 induces cell apoptosis via the upregulation of Bax (21) and the Bax/Bcl-2 ratio (22), and the downregulation of Bcl-2 (18). Caspase-3 is a member of the cysteine-aspartic acid protease family (33), and sequential activation of caspase proteins is central to the apoptosis of a variety of cancer cells (21-23,26). TL induces human breast and prostate cancer cell apoptosis (33), and TL in combination with tumor-necrosis factor-related apoptosis-inducing ligand enhances the apoptosis of cholangiocarcinoma cells by increasing the activity caspase-3 (34). In addition, the combination treatment of low-dose 1,25-dihydroxyvitamin D(3) combined with As_2O_3 synergistically inhibits AML cell proliferation via cell apoptosis mediated by the increased expression levels of Bax and caspase-3, and decreased expression of Bcl-2 (34).

The present study also found that the combination treatment of As_2O_3 with TL synergistically increased the generation of ROS in the cells. Therefore, it was hypothesized that the induction of cell apoptosis by the combination treatment in the present study was mediated by the generation of ROS. It is well known that the presence of increased intracellular ROS in the mitochondria is involved in the induction of apoptosis in cancer cells, and that an increased intracellular ROS concentration has been shown to cause an increase in the Bax/Bcl-2 ratio and activation of caspase-3 (35,36). In the present study, it was

found that, compared with the cells treated with either As$_2$O$_3$ or TL alone, the generation of intracellular ROS was significantly increased following exposure to As$_2$O$_3$ and TL in combination. TL has been found to induce human adrenal cancer NCI-H295 cell apoptosis through the ROS pathway (27), and treatments involving the combination of As$_2$O$_3$ and sulindac (34) or phytosphingosine (37) have been shown to enhance apoptotic cell death via increasing intracellular ROS.

In conclusion, the present study demonstrated that treatment with As$_2$O$_3$ in combination with TL synergistically induced MDS SKM-1 cell apoptosis via the induction of intracellular ROS, which upregulated the expression of Bax, downregulated the expression of Bcl-2 and upregulated the expression of caspase-3 (Fig. 5). These findings may provide a strategy to develop a novel combination therapy against MDS.

Acknowledgements

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