MicroRNAs Contribute to Promyelocyte Apoptosis in As\textsubscript{2}O\textsubscript{3}-Treated APL Cells

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Key Words
As\textsubscript{2}O\textsubscript{3} • Acute promyelocytic leukemia • Apoptosis • microRNA • PML

Abstract
Background: Arsenic trioxide (As\textsubscript{2}O\textsubscript{3}), an ancient drug used in traditional Chinese medicine, has substantial anticancer activities, especially in the treatment of patients suffering from acute promyelocytic leukemia (APL); however the underlying mechanisms are not well understood. Methods: MTT assay was used to detect the cell viability. Flow Cytometry analysis and caspase-3 activity assay were used to measure apoptosis of APL cells. Caspase-3 and Bax levels were analyzed by western blot and let-7d and miR-766 levels were determined by real-time RT-PCR. Results: As\textsubscript{2}O\textsubscript{3} significantly inhibited cell viability and induced apoptosis in APL cells. Several microRNAs, including let-7d and miR-766, were dysregulated in APL cells treated with As\textsubscript{2}O\textsubscript{3}. The expression of caspase-3 and Bax, which are targets of let-7d and miR-766, were up-regulated in As\textsubscript{2}O\textsubscript{3} treated cells. Correspondingly, transfection of these microRNAs increased NB4 cell viability. As\textsubscript{2}O\textsubscript{3} induced degradation of promyelocytic leukemia (PML), and then induced the down-regulation of both let-7d and miR-766 in NB4 cells. Conclusions: We construct a dysregulated microRNA network involved in As\textsubscript{2}O\textsubscript{3}-induced apoptosis in APL. Targeting this network may be a new strategy for the prevention of side effects associated with APL treatment with As\textsubscript{2}O\textsubscript{3}.

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Introduction

Acute promyelocytic leukemia (APL), M3 subtype of acute myeloid leukemia (AML), is characterized by a reciprocal translocation between chromosomes 15 and 17, which results in the fusion protein production between the promyelocytic leukemia (PML) gene and the retinoic acid receptor alpha (RARα) gene, and subsequent inhibition of myeloid cell apoptosis and differentiation [1, 2]. APL accounts for approximately 10-15% of all AML cases and represents a highly malignant form of leukemia with high bleeding tendency and a fatal course of only few weeks [3, 4]. Although two-thirds of APL patients achieved complete remission after the conventional chemotherapy, such as anthracyclines and cytosine arabinoside, high frequency of early death mainly due to the exacerbation of bleeding syndrome and low 5-year disease-free survival rates dwarf them to new drugs [5].

Early in 1971, researchers at the Harbin Medical University in China made the groundbreaking finding that arsenic trioxide (As$_2$O$_3$), the main component of arsenic, has prominent efficacy in the treatment of APL [6, 7]. Since then, As$_2$O$_3$ has drawn great attention because of its superior therapeutic effects on APL and has been applied on many other tumors, such as prostate cancer, breast cancer, lung cancer, liver cancer and so on [8-10]. Clinical experiments have shown the effectiveness of As$_2$O$_3$ in inducing complete clinical remission in patients with newly diagnosed and relapsed cases of APL. The molecular mechanism of anti-leukemic effect of As$_2$O$_3$ is complex. Accumulating evidence has suggested that the therapeutic effect of As$_2$O$_3$ mainly results from inducing apoptosis of cancer cells, but the underlying mechanisms are not well understood [11].

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs, which are 18-22 nt in length. Mature miRNAs bind to the 3'-UTR of target genes and repress translation of target genes or induce degradation of target mRNA [12]. Increasing evidence supports that miRNAs play indispensable roles in many biological processes involved in the pathogenesis of cancer, such as cell proliferation, apoptosis, angiogenesis, inflammation and so on [13-16]. For example, miR-21 has been proved as an oncomiR, which targets multiple key players in cell proliferation and apoptosis. MiR-125b functions as a tumor suppressor in liver cancer and bladder cancer by inducing the abnormal cell proliferation [17-20].

It has been confirmed that some miRNAs, such as miR-125b and miR143/145, contribute to the pathogenesis of APL and the development of drug resistance [21, 22]. Other miRNAs, such as anti-miR-21 and miR-15a/16-1, were reported to enhance the sensitivity of leukemic cells to the anti-cancer effect of As$_2$O$_3$ and all-trans retinoic acid [23-25]. However, little attention has been paid to the role of miRNAs in the process of apoptosis induced by As$_2$O$_3$ in APL patients. The aim of this study was to investigate the role of miRNAs in the process of apoptosis induced by As$_2$O$_3$ in APL cells. We expect to shed new light on the potential mechanisms of As$_2$O$_3$ on the treatment of APL and provide new molecular targets for the prevention of As$_2$O$_3$-induced side effects.

Materials and Methods

Cell Culture and Treatment

The NB4 and HL-60 cell lines, derived from the marrow of a patient with APL in relapse [26], was cultured in RPMI-1640 medium (Hycolne) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U per ml penicillin G and 100 U per ml streptomycin at 37°C in 5% CO$_2$±95% air. The cells treated with As$_2$O$_3$ were collected at 24 h and 48 h for further measurements.

Transfection

Before transfection, NB4 or HL-60 cells were grown in 25 cm$^2$ cell culture flasks with 4 ml medium. The miRNA and lipofectamine 2000 (Invitrogen, Carlsbad, CA) were separately mixed with 500 μl of Opti-MEM @ I Reduced Serum Medium (Gibco, Grand Island, NY) for 5 min. Then, the two mixtures were combined and incubated for 20 min at room temperature (RT). The lipofectamine:miRNAs mixture was added to the cells and incubated at 37°C for 36 h for further experiments.
**MTT Cell Viability Assay**

NB4 or HL-60 cells were seeded in 96-well culture plates with 1 × 10^4 cells/well, and incubated at 37°C with 5% CO₂. After treated with different concentrations of As₂O₃ or microRNAs, MTT assay (Amresco, Solon, USA) was performed. Briefly, 20 μl of MTT solution (5 mg/ml) was added to each well, and the cells were continuously incubated for 4 h. Formazan crystals were then dissolved in 150 μl DMSO. The optical density (OD) of the wells was measured with a microplate reader (BioTek, Richmond, USA) at 490 nm.

**Caspase-3 Activity Assay**

To assess the activity of caspase-3, NB4 or HL-60 cells were centrifuged at 600g for 5 min and then lysed in 50 μl of ice-cold cell lysis buffer for 30 min. The lysates were centrifuged at 16,000g for 15 min at 4°C. The supernatant was used for subsequent assay. The fluorogenic substrates for caspase-3 were labeled with the p-nitroaniline (pNA), which is released from acetyl-asp-glu-val-asp p-nitroanilide (Ac-DEVD-pNA), a substrate upon cleavage by caspase-3. The enzyme activity was determined by monitoring the fluorescence produced by free pNA using a spectrofluorophotometer (SHIMADZU Corporation, RF-5301PC, Kyoto, Japan) at 405 nm. Caspase-3 activity was expressed in micromole pNA liberated as per minute per microgram of protein [27].

**Flow Cytometry Analysis**

Apoptosis was identified by flow cytometry (BD FACSVantage SE, USA) with an apoptosis detection kit (Beijing Biosea Biotechnology Co., LTD, Beijing, China). NB4 or HL-60 cells containing about 5-10 x 10⁵ cells were centrifuged with 1000g for 10 min at 4°C and resuspended in binding buffer solution. Then, NB4 or HL-60 cells were double-stained with fluorescein isothiocyanate-labeled-Annexin V (Annexin V-FITC) and propidium iodide (PI). Annexin V-FITC is an indicator for early apoptosis, and PI is a viability dye that stains late apoptotic or necrotic cells.

**Western Blotting**

For western blot analysis, total protein samples were extracted from NB4 or HL-60 cells by the procedure as previously described [28]. Cells were lysed with RIPA lysis buffer (Beyotime, Jiangsu, China). Sixty μg proteins were fractionated on a 15% SDS-polyacrylamide gel. After electrophoretically transferring to a Pure Nitrocellulose Blotting membrane (Pall Life Science), the blots were probed with primary antibodies. Rabbit polyclonal antibody for caspase-3 and Bax were purchased from Santa Cruz Biotechnology. Anti-GAPDH antibody (Kangchen, Shanghai, China) was used as an internal control. The immunoreactivity was detected using Odyssey Infrared Imaging System (Gene Company Limited, Hongkong, China) and analyzed using Odyssey software (Infrared Imaging System LI-COR Biosciences). The bands were quantified by measuring the band intensity for each group [29].

**Real-Time RT-PCR**

Total RNA samples were extracted from NB4 or HL-60 cells using Trizol. Let-7d and miR-766 levels were quantified by the mirVana qRT-PCR miRNA Detection Kit (Ambion) in conjunction with Real-time PCR with SYBR Green I, as described in our previous work [30]. After circle reaction, the threshold cycle (Ct) was determined and relative miRNA levels were calculated based on the Ct values and normalized by U6 level in each sample.

**Microarray Analysis**

Microarray assay for miRNAs expression profile was performed by the Kangchen (KangChen Biotech Inc., Shanghai, China). Three samples including NB4 cells without treatment, NB4 cells treated with 2 μM As₂O₃ for 24 h and 48 h were assayed using the miRNA microarray technology miRCURY™ LNA Array (Exiqon Company, Denmark). The array output was received in Excel spreadsheets as lists of raw data and also as “simple detectable” data, which were the average of 4 signal values for each miRNA on the array. miRNAs with a DiffScore ≤-10 and ≥10, corresponding to a p-value of 0.05, were considered as significantly differentially expressed.

**Luciferase Reporter Assays**

Caspase-3/Bax 3’-UTR containing the conserved let-7d/miR-766 binding sites were synthesized by Invitrogen and amplified by PCR. The synthesized sequence and primers were: synthesized sequence: Caspase-3: CTC ATG CTC CAG AGG GTA CTT TAA GAC ATA CTC CTT CCA TCA AAT AGA ACCA CTA TGA AGC TAC
CTC AAA CTT CCA GTC AGG TAG TTG CAA TTG A; Bax: CCC CGA TTC ATC TAC CCT GCT GAC CTC CCA GTG ACC CCT GAC CTC ACT GTG ACC TTA GTG CCT TCT GCC CTC CCT GGA GCC TCC ACT GCC TCT GGA ATT GCT CA. The synthesized sequences with a mismatch in the seed sequence of the putative let-7d/miR-766 binding site were: Caspase-3: CTC ATG CTG CAG AGG GTA CTT TAA GAC ATA CTC CTT CCA TCA AAT A GTA CCA CTA TGA AGG CTC AAA CTT CCA GTG ACC TTA GTG CAA TTG A. Bax: CCC CGA TTC ATC TAC CCT GCT GAC CTC ACT GTG ACC TTA GTG CCT TCT GCC CTC CGC ACT CCC TCC ACT GCC TCT GGA ATT GCT CA. The PCR fragment was cloned downstream of the luciferase gene between the SacI and HindIII sites in pMIR-Report (Promega). 0.1 μg of the luciferase reporters containing the 3′- UTR were cotransfected with let-7d/miR-766 mimics into HEK-293 cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA). As an internal control, 10 ng of renilla luciferase reporters were also included. 48 h after transfection, the cells were collected and dual luciferase activities were measured by a luminometer according to the manufacturer’s instructions.

MiRNA Targets Databases and Apoptosis Pathway
We used miRNA-targets interactions documented in the TargetScan database [31]. The apoptosis pathway was derived from the KEGG database (http://www.genome.jp/kegg/pathway/hsa/hsa04210.html).

Statistical Assays
All data are presented as means±SEM. One way analysis of variance ANOVA followed by Dunnett’s post-hoc test was used for multiple comparisons. A two-tailed value of p<0.05 was considered statistically significant difference. Data were analyzed using the GraphPad Prism 5.0 and SPSS 14.0.

Results
Growth Inhibition and Apoptosis of APL Cells Induced by As$_2$O$_3$
As$_2$O$_3$ is one of the most highly effective agents for APL therapy [32]. It was suggested that the therapeutic plasma concentration for treating APL was 1–2 μM [33]. In this study, we found that As$_2$O$_3$ decreased the viability of NB4 and HL-60 cells in a dose- and time-dependent manner using MTT assay. As shown in Fig. 1A, the IC$_{50}$ of As$_2$O$_3$ was 1.673 μM in NB4 cells and 1.857 μM in HL-60 cells after 48h of treatment; thus, 2 μM As$_2$O$_3$ was used in the subsequent experiments. To investigate whether the observed reduction of viability was associated with cell apoptosis, flow cytometry assay was applied to detect the apoptotic rate of NB4 and HL-60 cells treated with As$_2$O$_3$. As shown in Fig. 1B, compared with the control group, apoptosis was markedly increased in NB4 and HL-60 cells after treatment with 2 μM As$_2$O$_3$ for 48h.

Both caspase-dependent and caspase-independent pathways are known to be involved in the process of apoptosis. We investigated whether the apoptosis of APL cells caused by As$_2$O$_3$ was associated with the activation of caspase-3. Caspase-3 activity was significantly increased in the NB4 and HL-60 cells treated with As$_2$O$_3$. As shown in Fig. 1C, compared with the control group, apoptosis was markedly increased in NB4 and HL-60 cells after treatment with 2 μM As$_2$O$_3$ for 48h.

Down-Regulation of Let-7d and MiR-766 Expression by As$_2$O$_3$
Our miRNA microarray analysis revealed the differential expressions of miRNAs between the NB4 cells with and without 2 μM As$_2$O$_3$ treatment for 48h. Let-7d, miR-128a, and miR-766 etc. were significantly down-regulated in As$_2$O$_3$-treated NB4 cells compared with control NB4 cells without As$_2$O$_3$ treatment (Fig. 2A). The down-regulation of let-7d and miR-766 were further verified in NB4 and HL-60 cells by qRT-PCR (Fig. 2B). By bioinformatics analysis, we identified several candidate targets for these miRNAs, which based on the KEGG database are relevant to apoptosis such as caspase-3 and Bax. We therefore analyzed their protein levels using western blot. As illustrated in Fig. 2C, caspase-3 and Bax genes contain the putative binding sites for let-7d and miR-766, respectively. Western Blot showed that the expression of caspase-3 and Bax proteins were up-regulated in NB4 and HL-60 cells treated with As$_2$O$_3$, which is negatively correlated with the levels of let-7d and miR-766 (Fig. 2D).
Liang et al.: As$_2$O$_3$-Induced Apoptosis via microRNAs

Fig. 1. As$_2$O$_3$ reduces cell viability and induces apoptosis in APL cells. (A) As$_2$O$_3$ decreased the viability of NB4 and HL-60 cells in a time- and dose-dependent manner, as assessed by MTT. (B) As$_2$O$_3$ increased the apoptosis of NB4 and HL-60 cells, as assessed by flow cytometry. (C) As$_2$O$_3$ enhances caspase-3 activity in NB4 and HL-60 cells. n=6, ** p<0.01 vs control.

Fig. 2. Dysregulation of miRNAs induced by As$_2$O$_3$ in APL cells. (A) miRNA microarray analysis revealed the differential expression of miRNAs in NB4 cells with As$_2$O$_3$ treatment, compared with control group. (B) Validation of differentially expressed miRNAs by real-time PCR. (C) The sequences showed the unique sites of miRNA:mRNA complementary between let-7d and caspase-3, and between miR-766 and Bax for human genes. The Genbank accession numbers of the genes are indicated in the brackets, and the positions of the target sites are numbered. (D) Increased expression of cleaved-caspase-3 and Bax in NB4 and HL-60 cells treated with As$_2$O$_3$ compared with control group by Western blot. n=4, ** p <0.01 vs control.
Liang et al.: As2O3-Induced Apoptosis via microRNAs

Let-7d and miR-766 Directly Regulate the Expression of Caspase-3 and Bax respectively in NB4 cells

To evaluate whether let-7d/miR-766 could interfere with caspase-3/Bax’s 3’-UTR, we utilized the pMIR-Reporter containing the binding sites of caspase-3/Bax for let-7d/miR-766. As illustrated in Fig. 3A & 3B, overexpression of let-7d/miR-766 resulted in significant decreases of caspase-3 (C) and Bax (D) at protein levels, respectively. Co-treatment with AMO-let-7d and AMO-766 alleviated the decreases of caspase-3 and Bax, whereas NC showed no effects. (E and F) Real-time PCR shows that let-7d/miR-766 has no effects on the expression of caspase-3/Bax mRNA. n=3, p <0.05, ** p <0.01 vs control; # p <0.05, ## p <0.01 vs let-7d or miR-766. AMO-let-7d and AMO-766: let-7d inhibitor and miR-766 inhibitor; NC: negative control.

Degradation of PML Gene Induced by As2O3

If the reduction of let-7d and miR-766 mediated the As2O3-induced apoptosis in NB4 cells, transfection of let-7d antisense (AMO-let-7d) or miR-766 antisense (AMO-766) alone
Fig. 4. Inhibition of let-7d and miR-766 induced apoptosis in APL cells. AMO-let-7d or AMO-766 significantly reduced cell viability (A), and enhanced caspase-3 activity (B) respectively. n=5, * p<0.05, ** p<0.01 vs control; # p<0.05, ## p<0.01 vs AMO.

Fig. 5. The effect of let-7d/miR-766 on down-regulated cell viability induced by As$_2$O$_3$. n=6, ** p<0.01 vs control; # p<0.05 vs As$_2$O$_3$.

into NB4 cells should be able to cause apoptosis. Indeed, as illustrated in Fig. 4A, AMO-let-7d or AMO-766 significantly reduced APL cell viability, and co-transfection of let-7d/miR-766 and AMO-let-7d/AMO-766 abolished the reduction of cell viability. Moreover, transfection of AMO-let-7d or AMO-766 into NB4 and HL-60 cells also enhanced caspase-3 activity and the elevation was abolished by their miRNA mimics respectively, which is consist with the results of cell viability (Fig. 4B).
To examine whether over-expression of let-7d or miR-766 was able to decrease cell apoptosis in NB4 cells treated with As$_2$O$_3$. We transfected let-7d/miR-766 or both of them into NB4 cells before treated with As$_2$O$_3$. As shown in Fig. 5, let-7d or miR-766 alone has no effect on the down-regulated cell viability induced by As$_2$O$_3$, whereas co-transfection of let-7d and miR-766 could alleviate the reduction of cell viability by As$_2$O$_3$, indicating that As$_2$O$_3$ stimulates the expression imbalance of many miRNAs rather than a single miRNA, and then activates the apoptosis pathway.

As$_2$O$_3$ was found to exert its therapeutic effect by promoting degradation of PML gene, which could regulate the expression of several miRNAs [34, 35]. We assumed that As$_2$O$_3$ might inhibit the expression of let-7d and miR-766 through degradation of PML. We found that NB4 cells treated with 2 μM As$_2$O$_3$ for 48h down-regulated the level of PML (Fig. 6A). After transfecting the PML plasmid into NB4 cells, PML was markedly increased both at protein and mRNA levels (Fig. 6B & 6C). Meanwhile, Fig. 6D showed that PML could increase the level of let-7d and miR-766, but the empty vector failed to do so. These results indicated that As$_2$O$_3$ inhibited let-7d and miR-766 by regulating PML gene.

**Discussion**

As$_2$O$_3$ was introduced as a new and efficient treatment alternative for APL patients [36, 37]. miRNAs play important roles in the initiation and progression of APL. Some APL-related miRNAs have been confirmed, such as miR-143/145 and miR-125b [21, 22, 38]. However, the role of miRNAs in the process of As$_2$O$_3$-induced apoptosis is not well understood. The present study demonstrated that miRNAs are dysregulated in APL cells treatment with As$_2$O$_3$. Our results revealed that 2 μM As$_2$O$_3$, a clinically relevant dosage, induced aberrant expression of several miRNAs including let-7d and miR-766. Overexpression of let-7d or miR-766 significantly inhibited the expression of proapoptotic genes caspase-3 and Bax. Furthermore, transfection of AMO-let-7d or AMO-766 into APL cells reduced cell viability and up-regulated caspase-3 activity.
Some studies found that the degree of target down-regulation by miRNAs is quantitatively modest and even an over-expressed miRNA typically down-regulates most of its endogenous targets by less than 50%, but most proteins should remain effective over this degree of inhibition \cite{39, 40}. Therefore, we speculate that As\(_2\)O\(_3\) disturbs the homeostasis of miRNAs and induces alteration of many miRNAs. Our study showed that As\(_2\)O\(_3\) induced apoptosis via disrupting multiple miRNAs rather than a single miRNA in NB4 and HL-60 cells. As shown in Figure 7, these dysregulated miRNAs may have cross-talks with their targets and form a connected network involved in the apoptosis process.

Studies have shown that As\(_2\)O\(_3\) exerts dual-effects on the differentiation of APL cells in a dose-dependent manner. Low dose (<0.5 μM) of As\(_2\)O\(_3\) facilitates the differentiation of APL cells, which may be mediated by the PKA-cAMP signaling pathway and RAR/RXR signaling pathway to promote histone acetylation. On the contrary, higher dose (>0.5 μM) of As\(_2\)O\(_3\) initiates apoptosis via the degradation of PML/RARα oncoprotein, disruption of the balance between anti-apoptotic and pro-apoptotic proteins \cite{5}, down-regulation of mitochondrial transmembrane potentials, opening of mitochondrial permeability transition pore, and release of cytochrome C and other pro-apoptotic cytokines. In addition, As\(_2\)O\(_3\) induced apoptosis through activation of JNK pathway, modulation of ROS production in APL cells, and interference of cell cycle.

The presence of t(15;17) with the PML–RARA fusion gene is considered to be the hallmark of APL, also known as AML type M3 \cite{41}. APL patients with the PML–RARA fusion gene aberration have been shown to be highly sensitive to retinoid differentiating agents, such as all-trans retinoic acid. Zhang et al. found that As\(_2\)O\(_3\) directly binds to PML protein and induces its degradation \cite{34}. A following study by Läng et al. \cite{42} showed that As\(_2\)O\(_3\) promotes cytoplasmic sequestration of PML and PML/RARα through the inhibition of PML body recycling. PML/RARα fusion protein has been confirmed as a transcription factor that
modulates the transcription of many miRNAs [35]. In our study, 2 μM \text{As}_2\text{O}_3\text{ induced the degradation of PML in NB4 cells. Thus, we assumed that As}_2\text{O}_3\text{ reduces the expression of let-7d and miR-766 through the degradation of PML and the inhibition of PML body recycling, which deserves our future detailed research.}

Taken together, the present study found that \text{As}_2\text{O}_3\text{ induced dysregulation of some apoptosis-related miRNAs in APL cells and the miRNA-target interactions formed a network in the process of apoptosis induced by As}_2\text{O}_3\text{ in APL cells. Moreover, our results indicated that As}_2\text{O}_3\text{ reduced the expression of let-7d and miR-766 through the degradation of PML and the inhibition of PML body recycling. Our future study will focus on the investigation of the miRNAs that are differentially expressed in patients suffering from APL compared with patients cured with As}_2\text{O}_3\text{. We expect to develop strategies to prevent side effects associated with As}_2\text{O}_3\text{ treatment of APL patients using combination of miRNAs and As}_2\text{O}_3\text{, which warrants our further detailed research.}

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\textbf{Conflict of Interests}

The authors state no competing interests.

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Liang et al.: As₂O₃-Induced Apoptosis via microRNAs

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Liang et al.: As2O3- Induced Apoptosis via microRNAs

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