Arsenic trioxide nanoparticles inhibit acute promyelocytic leukemia cell proliferation and induce apoptosis via PTEN/AKT signalling pathway.

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Abstract

Background: Arsenic Trioxide (As₂O₃) is a FDA-approved agent for the treatment of Acute Promyelocytic Leukemia (APL). But the high-toxicity is a bottleneck of the effect of As₂O₃. The aim of the present study was to preliminary study the possible mechanisms of the antitumor effect of As₂O₃ nanoparticles on NB4 cells. We examined the proliferation and apoptosis of NB4 cells incubated with the As₂O₃ or As₂O₃ nanoparticles. Protein levels of p-PTEN, p-AKT, Bax, caspase-3, caspase-9 and AIF of NB4 cells after using As₂O₃ nanoparticles and traditional As₂O₃ were determined by Western blotting analysis.

Results: In vitro cytotoxicity test showed that the inhibition rate and apoptosis level of NB4 cells treated with As₂O₃ nanoparticles was much higher than that of traditional As₂O₃. Moreover, As₂O₃ nanoparticles resulted in a more significant increase in p-PTEN expression and a greater reduction in p-Akt expression compared with traditional As₂O₃.

Conclusions: Our findings indicated the obvious anticancer effect of As₂O₃ nanoparticles and demonstrate the possible mechanism of its therapeutic potential. The results provide a foundation for the future clinical studies of As₂O₃ nanoparticles in APL patients.

Keywords: Arsenic trioxide, Acute promyelocytic leukemia, Nanoparticles, PTEN, Akt.

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Introduction

Acute Promyelocytic Leukemia (APL) is the M3 subtype of acute myelogenous leukemia and cytogenetically is characterized by a translocation of chromosomes 15 and 17, which results in the fusion between Retinoic Acid Receptor α (RARα) and the Promyelocytic Leukemia (PML) gene. APL is always accompanied with severe bleeding tendency and disseminated intravascular coagulation. The first-line treatment of APL was chemotherapy. More than 80% of patients treated with all-trans retinoic acid along with chemotherapy can achieve a prolonged remission. Relapsed patients are often treated with arsenic trioxide successfully [1].

Arsenic Trioxide (As₂O₃) is the primary component of arsenic (pishuang), a kind of traditional Chinese medicine. In the early 1970’s, some Chinese physicians from Harbin Medical University recognized arsenic as an effective ingredient for leukemia treatment. In 1996 and 1997, notable effect was reported for the use of As₂O₃ in APL [2]. The Food and Drug Administration (FDA) approved As₂O₃ as frontline treatment for APL in 2000 [3]. Apart from APL, it was used in several kinds of diseases, such as Chronic Lymphocytic Leukemia (CLL), liver cancer, syphilis, psoriasis, rheumatism [4-7]. However, the toxicity of arsenic was recognised by people gradually. The side effect of long-term chronic exposure to arsenic include hyperpigmentation of the skin, hyperkeratosis of the palms and feet, cancers of lung, bladder, prostate, kidney, liver as well as neuropathy, leucopenia, encephalopathy, peripheral vascular diseases and diabetes. Consequently, it faded out from clinical applications owing to its toxicity and carcinogenic properties [5].

Nanoparticles are becoming an emerging treatment modality for cancer. The advantages of nanoparticles are delivering anticancer drugs to the tumor, the ability to enhance permeability, bioavailability and efficacy as well as reduction or reversion of multidrug resistance. Owing to the unique features of tumor vasculature, the “Enhanced Permeability and Retention (EPR) effect” allows nanoparticles to extravasate into extravascular spaces and increase the drug concentration inside tumor tissues more than free drugs [8]. Nowadays, most nanoparticles can selectively target on cancer cells, reducing the undesirable side effects of conventional chemotherapy, and therefore improving the efficacy of patients [9,10].

The As₂O₃ nanoparticles were prepared with the so-gel method. In this study, the in vitro effect of As₂O₃ nanoparticles on NB4 cells was assessed by evaluating MTT analysis, mitochondrial membrane potential and flow cytometric
the solution was stirred at 50–60°C for 20–30 min and sonicated for another 10–20 min. Finally, distilled water was added, and the mixture were mixed and stirred for 10–30 min. Ethanol was then added, MTT analysis

Methods

Cell culture

NB4 cells were a kind gift from Dr. Jifan Hu at Stanford University Medical School (Palo Alto, CA, USA) for research in the laboratory of the First Affiliated Hospital of Jilin University (Changchun, China). NB4 cells were cultured in Iscove’s Modified Dulbecco’s Medium (Gibco-BRL, Grand Island, NY, USA) containing heat inactivated 10% Fetal Bovine Serum (FBS) (Hanzhou Sijiqing Biological Engineering Materials Co., Ltd. Hanzhou, China) along with 100 U/ml of penicillin and 0.1 mg/ml of streptomycin in an atmosphere of 5% CO2 and 100% humidity.

Preparation of As2O3 nanoparticles

The As2O3 nanoparticles were synthesized with the sol-gel method as we previously described [11]. The preparation method was as follows: As2O3 powder and hydrochloric acid were mixed and stirred for 1030 min. Ethanol was then added, the solution was stirred at 5060°C for 2030 min and sonicated for 5 min. Finally, distilled water was added, and the mixture was sonicated for another 1020 min.

MTT analysis

The inhibitory effect of As2O3 (Institute for Drug Control of the Ministry of Health of China) and As2O3 nanoparticles was estimated by MTT assay. There were 3 groups, which were control, As2O3 and As2O3 nanoparticles. NB4 cells were seeded on 96 well plates in quadruplicate at a density of 2 × 105/well in 100 μl As2O3 and As2O3 nanoparticles (4 different final concentrations: 0.5, 1, 2, 4 μmol/L) were added at the right time according to the group setting. After 24, 48 and 72 h of treatment, the cells were incubated for 3–4 h with MTT (Changchun Biotech Co., Ltd. Changchun, China) and lysed with acidified isopropanol. Absorbance was measured at 570 nm. The inhibition rate was calculated using the following formula: Inhibition rate= (absorbance value of control group-absorbance value of test group)/absorbance value of control group) × 100%. All experiments were repeated three times.

Hoechst 33342/PI staining

Cellular nuclear was stained with Hoechst 33342 (Hoechst Pharmaceuticals) and Propidium Iodide (PI) to observe the cellular apoptosis or necrosis situation by fluorescence microscopy. Briefly, 1 × 106 NB4 cells were incubated with 3 μmol/L As2O3 and As2O3 nanoparticles for 24 h. Cells were washed with PBS, suspended with new fresh medium, double stained with Hoechst 33342/PI and visualised by fluorescence microscopy.

Flow cytometric analysis

NB4 cells were treated with 1.5 μmol/L and 3.0 μmol/L As2O3, As2O3 nanoparticles against untreated control for 24 h at 37°C with 5% CO2. After treatment, 1 × 105 NB4 cells were stained with Annexin V/Propidium Iodide Apoptosis Detection Assay kit (Beyotime Institute of Biotechnology Co., Shanghai, China). The apoptosis of NB4 cells in 3 groups were examined by flow cytometry (FCM; FACSCalibur™, BD Biosciences, San Jose, CA, USA).

Mitochondrial membrane potential

NB4 cells were incubated with 1.5 and 3 μmol/L As2O3 and As2O3 nanoparticles for 24 h. 1 × 106 NB4 cells were stained with Rhodamine 123 (Sigma). Cells were washed with PBS, suspended with new fresh medium. Mean Fluorescence Intensity (MFI) was on behalf of Mitochondrial Membrane Potential (MMP) and examined the MFI in each group with Flow cytometric.

Western blotting analysis

The expression level of p-PTEN, p-AKT, Bax, caspase-3, caspase-9 and AIF was determined by Western Blotting analysis in 3 groups (control, As2O3, As2O3 nanoparticles). The extracted total protein was loaded on SDS-polyacrylamide gel. The protein was separated after electrophoresis and transferred to a nitrocellulose membrane. Membrane was blocked with 5% non-fat dry milk at room temperature for 2 h and incubated with primary antibody: p-PTEN, p-AKT, caspase-3 rabbit mAb, caspase-9 mouse mAb (Cell Signaling Technology, Danvers, MA), Bax rabbit mAb (Proteintech Group, Chicago, America), AIF rabbit mAb and β-actin mouse mAb (Beyotime, Shanghai, China). The membrane was washed with PBST thrice for 10 min each time and then incubated with horseradish peroxidase (HRP)-labeled goat-anti-rabbit or goat-anti-mouse secondary antibody (Beyotime, Shanghai, China) at room temperature for 1 h. Membranes were washed for 3 times with PBST. Exposure to Kodak® X-omat LS film (Eastman Kodak Company, New Haven, CT) after incubating with enhanced chemiluminescence (ECL) substrate (Bio-Rad, Hercules, CA, USA). Densitometry was applied with Kodak® 1D image analysis software (Eastman Kodak Company).

Results

Inhibitory effect of As2O3 nanoparticles on NB4 cell proliferation

The inhibitory effect of As2O3 nanoparticles and As2O3 on NB4 cell proliferation was examined by MTT assay. As shown in Figure 1, the drugs had time and dose dependent anti-proliferative effects on NB4 cells. Furthermore, the inhibitory effect of As2O3 nanoparticles was much higher than traditional As2O3.
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Figure 1. Effect of \( \text{As}_2\text{O}_3 \) nanoparticles and \( \text{As}_2\text{O}_3 \) on NB4 cells proliferation. NB4 cells were incubated with drugs for: (A) 24 h; (B) 48 h; (C) 72 h; (D) Inhibitory effect of \( \text{As}_2\text{O}_3 \) nanoparticles on NB4 cell proliferation at 24 h, 48 h and 72 h.

Results of Hoechst 33342/PI staining

NB4 cells were incubated with 3 μmol/L \( \text{As}_2\text{O}_3 \) nanoparticles or \( \text{As}_2\text{O}_3 \) for 24 h. Hoechst 33342/PI double staining was performed to observe the cell nuclear apoptosis or necrosis situation. Apoptotic cells were stained blue by Hoechst 33342 with pyknosis and stained red by PI with chromatin condensation. As shown in Figure 2, apoptosis was present after being treated with \( \text{As}_2\text{O}_3 \) nanoparticles or \( \text{As}_2\text{O}_3 \).

As\(_2\)O\(_3\) nanoparticles induce NB4 cell apoptosis

Both \( \text{As}_2\text{O}_3 \) nanoparticles and \( \text{As}_2\text{O}_3 \) can induce NB4 cells apoptosis. Figure 3 showed a dose-dependent inducing apoptosis effect of \( \text{As}_2\text{O}_3 \) nanoparticles and \( \text{As}_2\text{O}_3 \) on NB4 cells. Furthermore, \( \text{As}_2\text{O}_3 \) nanoparticles had higher inhibition ability than \( \text{As}_2\text{O}_3 \).

Mitochondrial membrane potential

The mean fluorescence intensity level of control group is 1313.67 MFI. Meanwhile, the mean fluorescence intensity level of 1.5 μmol/L and 3.0 μmol/L \( \text{As}_2\text{O}_3 \) is 1085.09 MFI and 881.20 MFI. What’s more, the mean fluorescence intensity level of 1.5 μmol/L and 3.0 μmol/L \( \text{As}_2\text{O}_3 \) nanoparticles is 911.05 MFI and 371.17 MFI. \( \text{As}_2\text{O}_3 \) nanoparticles can significantly decrease the mean fluorescence intensity level of NB4 cells than \( \text{As}_2\text{O}_3 \) (Figure 4).

Figure 3. Apoptosis of NB4 cells after being treated with \( \text{As}_2\text{O}_3 \) nanoparticles or \( \text{As}_2\text{O}_3 \). NB4 cells were treated as follows with an apoptosis rate: (A) Control, 0.95%; (B) 1.5 μmol/L \( \text{As}_2\text{O}_3 \), 1.8%; (C) 1.5 μmol/L \( \text{As}_2\text{O}_3 \) nanoparticles, 8.96%; (D) 3.0 μmol/L \( \text{As}_2\text{O}_3 \), 21.25%; (E) 3.0 μmol/L \( \text{As}_2\text{O}_3 \) nanoparticles, 37.79%.

Figure 4. Mitochondrial membrane potential of NB4 cells was examined by flow cytometry after being stained by Rhodamine123. (A) Control group; (B) 1.5 μmol/L \( \text{As}_2\text{O}_3 \); (C) 1.5 μmol/L \( \text{As}_2\text{O}_3 \) nanoparticles; (D) 3.0 μmol/L \( \text{As}_2\text{O}_3 \); (E) 3.0 μmol/L \( \text{As}_2\text{O}_3 \) nanoparticles; (F) The quantitative mitochondrial membrane potential levels of all groups; *P<0.05; **P<0.01.

Protein levels of PTEN, Akt and Bax of NB4 cells treated with \( \text{As}_2\text{O}_3 \) nanoparticles

Protein levels of PTEN, Akt and Bax of NB4 cells incubated with \( \text{As}_2\text{O}_3 \) and \( \text{As}_2\text{O}_3 \) nanoparticles were detected by Western blotting. Both \( \text{As}_2\text{O}_3 \) and \( \text{As}_2\text{O}_3 \) nanoparticles can decrease the protein expression level of PTEN and p-Akt at the concentration of 1.5 μmol/L and 3.0 μmol/L, and the drugs increase the expression of p-PTEN and Bax.

Meanwhile, the expression of PTEN and p-Akt were much lower in the \( \text{As}_2\text{O}_3 \) nanoparticles group than in the \( \text{As}_2\text{O}_3 \) group, as well as the levels of p-PTEN and Bax were much higher in the former group than the latter with a dose-dependent manner (Figure 5).
Nanobin encapsulation of As$_2$O$_3$ improve the drug concentration in tumor site but not normal tissues owing to the EPR effect and its better targeting effect. Different formulations of As$_2$O$_3$ approved as frontline treatment for APL by FDA for many years. It could also be used in other solid cancer, such as liver cancer, prostate cancer, cervical cancer and breast cancer [9,12-18]. However, its clinical application is somehow limited owing to its high toxicity to the normal tissues. Therefore, several kinds of As$_2$O$_3$ nanoparticles have been reported. Qian developed a scFvCD44v6-decorated PEG-PDLLA nanoparticle, which scFv-As-NP could target delivery of As to CD44v6-positive tumor cells PANC-1 [19]. The As$_2$O$_3$ nanoparticles with better antitumor efficacy can increase the protein level of p-PTEN and decrease the level of caspase-3 and caspase-9. We further examined the level of AIF of NB4 cells. Figure 5 showed that the AIF level was higher in the As$_2$O$_3$ nanoparticles group than the group of As$_2$O$_3$ solution.

**Figure 5.** Protein levels of PTEN, p-PTEN, p-Akt, Bax, caspase-3, caspase-9 and AIF of NB4 cells following treatment of As$_2$O$_3$ nanoparticles or As$_2$O$_3$ for 24 h. NB4 cells were treated as follows: (1) Control; (2) 1.5 μmol/L As$_2$O$_3$; (3) 1.5 μmol/L As$_2$O$_3$ nanoparticles nanoparticles; (4) 3 μmol/L As$_2$O$_3$; (5) 3 μmol/L As$_2$O$_3$ nanoparticles.

**Discussion**

As$_2$O$_3$, a kind of traditional Chinese medicine, had been approved as frontline treatment for APL by FDA for many years. It could also be used in other solid cancer, such as liver cancer, prostate cancer, cervical cancer and breast cancer [9,12-18]. However, its clinical application is somehow limited owing to its high toxicity to the normal tissues. Therefore, different formulations of As$_2$O$_3$ with higher antitumor efficacy and lower toxicity are needed to be further studied.

Several kinds of As$_2$O$_3$ nanoparticles have been reported. Qian developed a scFvCD44v6-decorated PEG-PDLLA nanoparticle, which scFv-As-NP could target delivery of As to CD44v6-positive tumor cells PANC-1 [19]. The As$_2$O$_3$ nanoparticles with better antitumor efficacy can highly improve the drug concentration in tumor site but not normal tissues owing to the EPR effect and its better targeting effect. Nanobin encapsulation of As$_2$O$_3$ improves the pharmacokinetics and antitumor efficacy of As$_2$O$_3$ in a murine model of breast cancer and lymphoma [20,21].

In our previous study, we prepared small-sized (<10 nm and ~40 nm) As$_2$O$_3$ nanoparticles with sol-gel method [11]. Herein, the superiority efficacy of proliferation/inhibition and apoptosis induction effects in vitro of As$_2$O$_3$ nanoparticles than As$_2$O$_3$ were further confirmed by evaluating MTT assay, apoptosis detection with flow cytometric assay, mitochondrial membrane potential, p-PTEN, p-AKT, Bax, caspase-3, caspase-9 and AIF protein levels. A much smaller dose of As$_2$O$_3$ nanoparticles can achieve a good antitumor effect, which would reduce the systemic toxic side effects with a reduction of toxicity.

As$_2$O$_3$ has a property of proliferation inhibition of APL cell, such as NB4 cell lines. As$_2$O$_3$ nanoparticles, even at a low concentration of 2 μmol/L (Figure 1), can significantly inhibit the growth of NB4 cells. As$_2$O$_3$ nanoparticles can also induce apoptosis of NB4 cells in a dose-dependent manner (Figure 3). PTEN/Akt signalling pathway is involved in antitumor process [22,23]. PTEN is closely associated with PI3K/Akt pathway. It is a negative regulator of PI3K/Akt pathway owing to its IP3 phosphatase activity, which influences the cell proliferation and apoptosis [24,25]. PI3K/Akt pathway has been proposed to inhibit Bax translocation from cytoplasm to mitochondria [26]. The inactivation of Akt can induce Bax translocate to mitochondria, which can decrease the mitochondrial membrane potential to induce apoptosis. In our present study, both As$_2$O$_3$ nanoparticles and As$_2$O$_3$ can increase the protein level of p-PTEN and decrease the level of caspase-3 and caspase-9 protein expression. However, the 3 μmol/L As$_2$O$_3$ nanoparticles could improve the protein expression of AIF but not caspase-3 and caspase-9. We demonstrate that the apoptosis of As$_2$O$_3$ and As$_2$O$_3$ nanoparticles in a lower concentration is related with a caspase-dependent mitochondrial apoptosis pathway, but higher concentration of As$_2$O$_3$ nanoparticles induce NB4 cells apoptosis by a caspase-independent mitochondrial apoptosis pathway.

**Conclusions**

In conclusion, As$_2$O$_3$ nanoparticles can inhibit proliferation and induce apoptosis of APL cells, and the induce-apoptosis effect was correlated with PTEN/Akt signaling pathway. As$_2$O$_3$ nanoparticles are selective and potential anti-APL agents which could inhibit proliferation and induce apoptosis of APL cells with high efficacy.

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**Conflicts of Interest**

The authors have no conflicts of interest to declare.

**Ethical Statement**

This article does not contain any studies with human participants or animals performed by any of the authors.

**References**

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