Arsenic Trioxide Inhibits the Metastasis of Small Cell Lung Cancer by Blocking Calcineurin-Nuclear Factor of Activated T Cells (NFAT) Signaling

Background: The inhibitory effect of arsenic trioxide (As$_2$O$_3$) on lung cancer has been reported in some preclinical studies. However, its effect on small cell lung cancer (SCLC) has been poorly explored. Calcineurin and its substrate, nuclear factor of activated T cells (NFAT), mediate the downstream signaling of VEGF, and is critical in the process of endothelium activation and tumor metastasis. In this study, we aimed to evaluate whether As$_2$O$_3$ had inhibitory effects on endothelial cells activation and the metastasis of SCLC, and to explore the possible mechanisms.

Material/Methods: In vitro, human umbilical vein endothelial cells (HUVECs) were used. Cell Counting Kit-8 assay and cell migration assay were performed to determine the effect of As$_2$O$_3$ on HUVECs proliferation and migration. The level of calcineurin, NFAT, downstream factors for Down syndrome candidate region 1 (DSCR1), and the endogenous inhibitor of calcineurin, were evaluated by quantitative PCR and western blotting. In vivo, SCLC metastasis models were established by injecting NCI-H446 cells into tail veins of nude mice. Tumor-bearing mice were treated with As$_2$O$_3$ or calcineurin inhibitor for 10 days, after which tumor metastasis in target organs was evaluated.

Results: As$_2$O$_3$ significantly inhibited the proliferation and migration of endothelial cells. Also, As$_2$O$_3$ inhibited the expression levels of calcineurin, NFAT, and the downstream target genes CXCR7 and RND1, while it upregulated the level of DSCR1. Both As$_2$O$_3$ and calcineurin inhibitor exhibited notable inhibitory effect on the metastasis of SCLC, without obvious side effects.

Conclusions: These findings suggested that As$_2$O$_3$ had remarkable inhibitory effects on the endothelial cell activation and SCLC metastasis, and the mechanism might be related to the blocking of calcineurin-NFAT signaling by upregulating DSCR1.

MeSH Keywords: Neoplasm Metastasis • Neovascularization, Pathologic • Small Cell Lung Carcinoma

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Background

Small cell lung cancer (SCLC) accounts for 15% to 20% of lung cancer, and is characterized by poor differentiation, rapid growth, early lymphatic metastasis, and distant metastasis through invasion of blood vessels [1,2]. As a highly malignant type of lung cancer, most cases of SCLC have no opportunity to receive surgical resection after diagnosis due to existing extrapulmonary metastases. So far, there is no effective targeting drugs for SCLC, which further limits the therapeutic options. Therefore, the treatment strategy mainly relies on chemotherapy based on cisplatin combined with etoposide. The prognosis of SCLC is the worst among all types of lung cancer, with a 2-year survival rate of less than 5% [3].

Metastasis is the most characteristic feature of this tumor, which distinguish it from other diseases. In fact, most cancer patients eventually died of tumor invasion and metastasis. From this point of view, if invasion and metastasis could be inhibited, the mortality rate for cancer patients will be greatly reduced, and their survival time will be prolonged. In current medical scenarios, the treatment of SCLC is undergoing a bottleneck, and the control of early metastasis of SCLC is possibly the way out. Angiogenesis is the key step in the pathological process of tumor invasion and metastasis, and anti-angiogenic agents have been applied in the treatment of lung cancer [4,5]. These drugs, mainly targeting vascular endothelial growth factor (VEGF), only slightly prolong progression-free survival, but do not improve overall survival [6]. Therefore, it is urgent and significant to find new targets for inhibition of tumor metastasis.

Calcineurin and its substrate, nuclear factor of activated T cells (NFAT), mediate the downstream signaling of VEGF, which transfers VEGF signaling into vascular endothelial cells and promotes the activation of the endothelium [7]. Down syndrome candidate region 1 (DSCR1), also known as regulator of calcineurin 1 (RCAN1), has been reported to be an endogenous inhibitor of calcineurin [8]. DSCR1 could block calcineurin-NFAT signaling and keep a balance between pro- and anti-angiogenic factors through negative feedback in physiological conditions [9]. In the environment of tumor induced angiogenesis, the markedly raised level of VEGF leads to continuous activation of calcineurin-NFAT such that endogenous DSCR1 is not enough to neutralize the effect, resulting in the formation of a large number of new blood vessels that supply oxygen and nutrients to the tumor [10]. Studies have shown that upregulation of DSCR1 could reverse the over-activation of calcineurin-NFAT signaling and thus inhibit tumor growth and metastasis [11,12]. These findings indicated that calcineurin-NFAT effect on DSCR1 might become a potential therapeutic target.

Arsenic trioxide (As$_3$O$_3$) has been commonly used in the treatment of acute promyelocytic leukemia (APL), resulting in high rates of complete remission for APL patients, with relatively low toxicity [13–15]. In solid tumors, the promising anticancer activity of As$_3$O$_3$ has also been reported in vivo and in vitro [16–20]. So far, As$_3$O$_3$ has been approved by FDA in the treatment of APL and advanced liver cancer. Our previous clinical study showed that intrapleural injection of As$_3$O$_3$ in patients with advanced lung cancer accompanied by pleural metastasis could significantly reduce the production of malignant pleural effusion (MPE) and turn it from bloody to light yellow [21]. The specific mechanism of this effect was unknown, and we suspected that it might be related to the reduced permeability of the pleural capillary. We further constructed a mouse model of lung cancer accompanied by pleural metastasis. It was found that intrapleural injection of As$_3$O$_3$ significantly inhibited the pleural vascular permeability and the microvascular density (MVD) in pleural tumor nodules, which led to the decrease of pleural metastasis and the formation of MPE [22]. In addition, we demonstrated that As$_3$O$_3$ inhibited the growth of lung cancer xenografts, and the inhibitory effect in SCLC was particularly obvious. It was also revealed that As$_3$O$_3$ inhibited angiogenesis in SCLC by downregulating VEGF; As$_3$O$_3$ also influenced the ultrastructure of the endothelial cells and the formation of neo-vascular lumen by blocking the Dll4-Notch pathway [23,24]. However, whether As$_3$O$_3$ can inhibit the metastasis of SCLC and the possible mechanism involved is still unknown.

We previously reported that As$_3$O$_3$ inhibited the proliferation and colony formation of SCLC cell line [24,25]. Other researchers also reported that As$_3$O$_3$ inhibited the migration and invasion of lung cancer and other solid tumor cells [26–28]. So, the effect of As$_3$O$_3$ on tumor cells has been well demonstrated. In this study, we focused on the effect of As$_3$O$_3$ on tumor angiogenesis. We hypothesized that As$_3$O$_3$ blocked calcineurin-NFAT signaling by upregulating DSCR1, and inhibited the proliferation and migration of vascular endothelial cells, and therefore inhibited the metastasis of SCLC. Human umbilical vein endothelial cells (HUVECs) were used in our in vitro study. SCLC metastasis models were established using NCI-H446 cells. The aim of our study was to provide further evidence for the anti-cancer activity of As$_3$O$_3$ and a basis for the application of As$_3$O$_3$ in the treatment of SCLC.

Material and Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) and human SCLC cell line NCI-H446 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HUVECs were cultured in a mixture of DMEM medium (HyClone, Logan City, UT, USA), 10% fetal bovine serum (FBS, HyClone, Logan City, UT, USA), and 1% penicillin-streptomycin (HyClone, Logan City, UT, USA). All cell cultures were maintained in an atmosphere of 5% CO$_2$ and 95% humidified air at 37°C. For cell experiments, cultured cells were prepared in a confluent condition for 5 days before use.

Culture and reagents

Culture:

- Human umbilical vein endothelial cells (HUVECs)
- Human small cell lung cancer cell line NCI-H446

Materials:

- DMEM medium
- 10% FBS
- 1% penicillin-streptomycin
- 2 mmol/L L-glutamine
- 2 mmol/L sodium pyruvate

Reagents:

- As$_3$O$_3$
- VEGF
- MTT
- Annexin V-FITC
- PI
- Annexin V-PE
- PI
- 7AAD
- Annexin V-PE
- PI
- MTT
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Utah, USA). NCI-H446 cells were cultured in RPMI 1640 medium (HyClone, Logan City, Utah, USA) supplemented with 10% FBS and the same antibiotics as described above. Cells were incubated at 37°C in a humidified incubator containing 5% CO₂.

**Cell proliferation assay**

Cells (2.5×10⁵ per well) were seeded in 96-well plates. After adhesion, cells were treated with various concentrations (0, 0.5, 1, 2, 4, and 8 μM) of As₂O₃ (Beijing Shuangli Pharmaceutical Co., Ltd., Beijing, China). After incubation for 24 hours, 48 hours, or 72 hours, cell proliferation was determined in triplicate, using a Cell Counting Kit-8 (CCK8) assay (Beyotime, Haimen, China). The absorbance was measured by a spectrophotometer at a wavelength of 450 nm. Results were expressed as relative absorbance, considering the 0 μM group as control.

**Cell migration assay**

To detect the migration ability of the cells, 24-well Transwell plates were used. HUVECs were previously treated with 2 μM or 4 μM of As₂O₃, 1 μM of cyclosporine A (CsA, Selleck Chemicals, Houston, TX, USA) or NS for 24 hours. Cells were collected and resuspended in medium without serum to a density of 2.0×10⁵/mL. Then, 100 μL of each cell suspension was placed onto the upper chamber of the well and 600 μL of complete medium was added to the lower chamber. After incubation for 24 hours, the inserts were fixed with 10% formalin and stained with crystal violet. Cells on the upper surface of the inserts were removed by swabbing with cotton swabs, and cells migrated to the lower surface were counted under microscope in 5 random fields at 200x magnification.

**Quantitative real-time PCR (qPCR)**

Cells were treated with different concentrations of As₂O₃ (2 μM or 4 μM), CsA, or NS for 72 hours. The total RNA was extracted and then reverse transcribed into cDNA. RT-PCR analysis was performed using SYBR Premix Ex Taq (Takara, Otsu, Shiga, Japan). and then reverse transcribed into cDNA. RT-PCR analysis was performed using SYBR Premix Ex Taq (Takara, Otsu, Shiga, Japan).

**Western blotting**

Cells were treated with different concentrations of As₂O₃ (2 μM or 4 μM), CsA, or NS for 72 hours. Proteins were extracted using RIPA lysis buffer, and were quantified using a bicinchoninic acid assay (BCA) protein assay kit (Thermo, Rockford, IL, USA). Equal amounts of protein (20 μg of each sample) were electrophoretically resolved on polyacrylamide gels, and then were transferred onto PVDF membranes. The membranes were blocked with a solution containing 5% nonfat dry milk for 1 hour, and were incubated with primary antibodies overnight at 4°C. After being washed 3 times with TBST (Tris buffered saline with Tween20, pH 8.0), membranes were incubated with the appropriate secondary antibody at room temperature for 1 hour, and were visualized using the enhanced chemiluminescence (ECL) detection reagents. β-actin was used as an internal control. The primary antibodies used were as follows: calcineurin A (1: 2000, Abcam, Cambridge, UK), DCR1 (calci- pressin-1) (1: 1000, Abcam, Cambridge, UK), NFKB1 (1: 500, Abcam, Cambridge, UK), RND1 (1: 500, Abcam, Cambridge, UK), CXCR7 (GPCR RDC1) (1: 250, Abcam, Cambridge, UK), and β-actin (1: 1000, Santa Cruz, Dallas, TX, USA).

**Animal models and drug treatment**

Male nude mice (5 to 6 weeks old) were purchased from and raised in the Experimental Animal Center of Second Military Medical University (Shanghai, China). NCI-H446 cells suspended in NS were injected into the tail veins of mice (0.2 mL per mouse at a density of 1.0×10⁵ cells/mL). Mice were sacrificed and dissected 5 weeks after injection to observe metastatic nodules in possible target organs. All mice presented tumor metastasis, and the majority of metastatic nodules were found in livers. Metastatic nodules were confirmed by histopathology to be consistent with the feature of SCLC. Based on this, a total of 20 mice with the same method of construction of metastasis models were randomly divided into 4 groups (5 in each group) 5 weeks after injection, and were intraperitoneally injected with 2.5 mg/kg or 5.0 mg/kg As₂O₃, 20 mg/kg CsA, or NS, once daily for 10 days. Mice were sacrificed after drug treatment, and tissue samples were collected for histology staining and examination. Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China) and the Experimental Animal Ethical Care Guidelines of Second Military Medical University. The animal study was approved by the Committee on Ethics of Biomedicine, Second Military Medical University.

**Histology**

Tissue samples were fixed in 4% paraformaldehyde solution for 24 days, embedded in paraffin and sectioned (5 μm). Sections were stained outside this paper.
were deparaffinized, and hematoxylin and eosin (H&E) staining were performed. Tumor metastasis was measured by examining both the number and area of metastatic tumor nodules in target organ sections under the microscope in 3 representative 200× fields. When determining the area of metastatic tumor nodules, a 22×16 grid was made by a computer on the H&E stained picture, with an area of 1600 μm² for each square. The area of metastatic tumor nodules in sections was calculated as the area of one square x the number of squares covered by the tumor. The square covered less than a half could be ignored, while the square covered more than a half could be counted as one square.

**Statistical analysis**

All data were analyzed using the SPSS 22.0 software program. The measured data were presented as the means ± standard deviation (SD), and were analyzed by a one-way ANOVA, followed by LSD-t or Dunnett’s t test. A value of $P<0.05$ was considered to be statistically significant.

**Results**

**As$_2$O$_3$ inhibited proliferation of HUVECs**

CCK-8 assay was used to examine the growth inhibitory effects of As$_2$O$_3$ on HUVECs. As shown in Figure 1, HUVECs proliferation at 24 hours showed no significant difference among the groups. At 48 hours and 72 hours, each dose of As$_2$O$_3$ could inhibit HUVECs proliferation. At 72 hours, cell proliferation in the 2.0 μM, 4.0 μM, and 8.0 μM groups were significantly lower compared to the control. These data showed that As$_2$O$_3$ could inhibit the proliferation of HUVECs in a concentration- and time-dependent manner.

**As$_2$O$_3$ inhibited migration of HUVECs**

In order to determine the inhibitory effect of As$_2$O$_3$ on endothelial cell migration, Transwell assay was used. As presented
in Figure 2A, the migration capacity of HUVECs was diminished by As$_2$O$_3$, and this phenomenon was more obvious in the higher dose group. The migration of HUVECs was also suppressed by CsA, an exogenous calcineurin inhibitor. According to the quantitative comparison of cell migration (Figure 2B), the numbers for migrated cells were significantly fewer in the As$_2$O$_3$ (2 μM and 4 μM) group and CsA group than the control group (As$_2$O$_3$ 2 μM and 4 μM versus control, $P_{<0.01}$; CsA versus control, $P_{<0.05}$). The numbers of migrated cells were significantly fewer in the As$_2$O$_3$ 4 μM group than in the As$_2$O$_3$ 2 μM group ($P_{<0.01}$), which suggested that As$_2$O$_3$ inhibited HUVECs migration in a dose-dependent manner. In addition, the inhibitory effect of As$_2$O$_3$ 4 μM was more obvious than CsA ($P_{<0.01}$).

As$_2$O$_3$ downregulated calcineurin-NFAT and upregulated DSCR1 in HUVECs

We further investigated the regulatory effect of As$_2$O$_3$ on calcineurin-NFAT signaling. HUVECs were treated with different doses of As$_2$O$_3$ (2 μM or 4 μM) or CsA (1 μM) for 72 hours,
and qPCR and western blot were used to detect the expression of related factors in the level of mRNA and protein respectively. As shown in Figure 3A, mRNA level of calcineurin A, the main functional and catalytic subunit of calcineurin, was downregulated by As$_2$O$_3$, dose-dependently. The mRNA level of NFAT2 (NFATc1), the substrate of calcineurin, was also inhibited by As$_2$O$_3$, but there was no statistical significance (Figure 3B). At the protein level, both calcineurin A and NFAT2 were suppressed by As$_2$O$_3$, in a dose-dependent manner (Figure 3C). As expected, calcineurin and NFAT2 were significantly inhibited by CsA, the exogenous inhibitor of calcineurin, at both mRNA and protein level. To demonstrate the possible mechanism of the inhibitory effect of As$_2$O$_3$ on calcineurin-NFAT, we then detected the expression of DSCR1, the endogenous inhibitor of calcineurin. It was found that DSCR1 was significantly upregulated by As$_2$O$_3$ dose-dependently at both mRNA level (Figure 3D) and protein level (Figure 3E), which might suggest that As$_2$O$_3$ could inhibit calcineurin-NFAT signaling by upregulating DSCR1.

**As$_2$O$_3$ downregulated downstream factors of calcineurin-NFAT in HUVECs**

To further demonstrate the inhibitory effect of As$_2$O$_3$ on calcineurin-NFAT signaling, related downstream factors were examined. CXCR7 and RND1, the target-genes of NFAT2, are known to be involved in the regulation of angiogenesis and cell migration [29]. As shown in Figure 4A, As$_2$O$_3$ inhibited CXCR7 and RND1 mRNA levels in HUVECs dose-dependently, and a similar trend was observed at the protein level (Figure 4B). As positive control, CsA could also markedly inhibit CXCR7 and RND1 at both the mRNA level and the protein level. These data were in agreement with our results, indicating that As$_2$O$_3$ downregulated calcineurin-NFAT and its downstream factors.

**As$_2$O$_3$ inhibited the metastasis of SCLC in vivo**

To determine the effects of As$_2$O$_3$ on the metastasis of small cell lung cancer in vivo, metastasis models of SCLC were established by injecting NCI-H446 cells into tail veins of nude mice. Tumor-bearing mice were treated with 2.5 mg/kg or 5.0 mg/kg of As$_2$O$_3$, or 20 mg/kg CsA or NS, once daily for 10 days. CsA was used as a positive control. During the course of treatment, no deaths or obvious side effects were observed in all groups. At the end of drug administration, mice were sacrificed, and possible target tissue samples were collected. The vast majority of metastatic nodules were found in the liver, rather than the lungs or other organs. As shown in Figure 5A and 5B, fewer metastatic nodules were found in the liver, rather than the lungs or other organs. This work is licensed under Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0).
in the control group. To quantitatively evaluate tumor metastasis, both the number and area of metastatic nodules in liver sections were calculated. As shown in Figure 5C, the number of metastatic nodules in $\text{As}_2\text{O}_3$ 2.5 mg/kg group, $\text{As}_2\text{O}_3$ 5.0 mg/kg group, and CsA group were significantly smaller compared to the control group. Similarly, $\text{As}_2\text{O}_3$ could reduce the area of metastatic nodules in livers. The area of metastatic nodules in the $\text{As}_2\text{O}_3$ 2.5 mg/kg group was significantly smaller compared to the control group and the CsA group (Figure 5D).

**Discussion**

Early metastasis has been recognized as an important feature of SCLC and a main challenge in the therapy of SCLC. However, there is still a lack of satisfactory results both in the research of metastasis mechanism and in the clinical methods of metastasis inhibition [30]. This calls for further exploration of potential targets, as well as new drugs to effectively prevent the metastasis of SCLC. It is well known that endothelial activation and angiogenesis VEGF signaling are strongly associated with tumor metastasis. In the current study, we observed that $\text{As}_2\text{O}_3$ dramatically inhibited the proliferation and migration of endothelial cells, which are both key steps in tumor angiogenesis. In the cell proliferation assays, HUVECs were
treated with various doses of As$_3$O$_3$ for 24 hours, 48 hours, or 72 hours, and it was shown that As$_3$O$_3$ inhibited the proliferation of HUVECs in a dose- and time-dependent manner. In the cell migration assays, we found that As$_3$O$_3$ diminished the migration ability of HUVECs dose-dependently, and the inhibitory effect in the As$_3$O$_3$ 4 μM group was more obvious than in the positive control group (CsA group). Combined with our previous in vivo results, we believed that the anti-angiogenic activity of As$_3$O$_3$ is attributed to its direct inhibitory effect on vascular endothelial cells.

Most of previous studies on tumor angiogenesis focused on the VEGF pathway and its upstream factors, but fewer studies were concerned about the phase after VEGF signaling in endothelial cells [31–33]. It has been confirmed that the activating signal of VEGF is taken over by calcineurin-NFAT within endothelial cells [34,35]. When VEGF binds to its receptor on the endothelial cell membrane, phospholipase C-γ (PLC-γ) is activated. With the rapid increase of intracellular calcium levels and the following activation of calcineurin, NFAT is activated by dephosphorylation and is induced to aggregate into the nucleus, which will trigger the transcription of angiogenic genes and promote angiogenesis [29,36–38]. Fuentes et al. first found a new group of genes located on chromosome 21 when they studied Down syndrome related genes and name it this group of genes the Down syndrome candidate region 1 [39]. Subsequently, DSCR1 was proven to be an endogenous inhibitor of calcineurin, which could inhibit the dephosphorylation and intranuclear aggregation of the substrate (NFAT) and play a negative feedback role in calcineurin-NFAT signaling [40,41]. In a physiological state, DSCR1 maintains the balance between pro- and anti-angiogenesis through this feedback mechanism [42]. However, in the process of tumor development, endogenous DSCR1 is not enough to inhibit continuous activation of calcineurin-NFAT induced by markedly elevated level of VEGF. Two epidemiological studies showed that the incidence and mortality of some solid tumors in patients with Down syndrome were significantly lower than those in the general population, which was possibly attributed to the fact that the extra chromosome 21 carried a higher-than-normal level of DSCR1, which inhibited tumor angiogenesis [43,44]. In vivo studies showed that overexpression of DSCR1 suppressed the activation of calcineurin-NFAT induced by VEGF, and significantly inhibited angiogenesis, tumor growth, and metastasis in melanoma and lung cancer [45–47]. These facts suggest that DSCR1 and calcineurin-NFAT could be potential therapeutic targets.

Our data showed that, in vascular endothelial cells, As$_3$O$_3$ downregulated calcineurin and NFAT at both the mRNA level and the protein level, similar to the effect of CsA, the exogenous inhibitor of calcineurin. Several angiogenesis-related genes have been reported to be activated by calcineurin-NFAT signaling, including CXCR7 and RND1. These downstream target genes have been shown to be crucial for endothelial cell activation. Knockdown of CXCR7 could abrogate VEGF-mediated endothelial cell migration and tube formation, and siRNA treatment of RND1 could impaired vascular barrier function [29]. To further demonstrate the inhibitory effect of As$_3$O$_3$ on calcineurin-NFAT signaling, the downstream factors were also detected in our study. It was found that both the mRNA level and the protein level of CXCR7 and RND1 were decreased by As$_3$O$_3$ dose-dependently, which further demonstrated our hypothesis that As$_3$O$_3$ inhibited calcineurin-NFAT signaling. We next looked at whether As$_3$O$_3$ inhibited calcineurin-NFAT by directly suppressing the enzymatic activity of calcineurin just like CsA, or by increasing its endogenous inhibitor. Of note, the expression of DSCR1 mRNA and protein was significantly increased under the treatment of As$_3$O$_3$ in a dose-dependent manner. This implied that DSCR1 might be the direct target of As$_3$O$_3$, and As$_3$O$_3$ suppressed calcineurin activity by upregulating its endogenous inhibitor.

We previously found that As$_3$O$_3$ restrained tumor growth by inhibiting angiogenesis and cancer stem-like cells in lung cancer both in vitro and in vivo [23–25]. In our current study, we focused on the regulatory effect of As$_3$O$_3$ on the metastasis of SCLC and the possible mechanism, which have been rarely discussed in the past. We established a SCLC metastasis model by injecting human SCLC cells into nude mice’s tail veins; this method of model construction had preferably coherence and high metastatic rates. We found that the main target organ of this metastasis model was the liver. Our data showed that As$_3$O$_3$ significantly inhibited the metastasis of SCLC. Both the number and area of metastatic nodules in target organs were decreased by As$_3$O$_3$, and a higher dose exhibited a stronger inhibitory effect. In addition, the exogenous inhibitor of calcineurin, chosen as the positive control, could also inhibit SCLC metastasis.

As to the safety of As$_3$O$_3$, it has been approved for clinical use in hematological malignancies and advanced liver cancer, thus, the safety of its application in vivo has been extensively demonstrated. It has been reported that As$_3$O$_3$ used as a single agent, causes only minimal side effects in a small proportion of cases; these side effects include gastrointestinal reactions, hepatotoxicity, neurotoxicity, and mildly abnormal ECGs [48,49]. In our study, during the course of drug treatment, no abnormal deaths or obvious side effects, such as vomit, diarrhea, liver injuries or neurologic abnormalities, were observed, which implied that the doses of As$_3$O$_3$ we used for the in vivo assays were in a safe range.

Taking the in vitro and in vivo results together, our findings implied that the inhibitory effect of As$_3$O$_3$ on SCLC metastasis should be related to the blocking of calcineurin-NFAT signaling by upregulating the endogenous inhibitor of calcineurin, DSCR1.
Previous studies reported that upregulation of DSCR1 inhibited tumor growth and metastasis through anti-angiogenesis, and improved survival of tumor-bearing mice, and these studies pointed out that upregulation of DSCR1 suppressed tumor growth in a tumor cell-extrinsic but not a tumor cell-intrinsic manner, especially targeting angiogenesis in the tumor microenvironment [46–47]. Therefore, we believed that As$_3$O$_3$ could inhibit the metastasis of SCLC in a tumor cell-extrinsic manner, too. However, tumorigenesis is a complex process; the weight of anti-angiogenic and cytotoxic effect of As$_3$O$_3$ in SCLC needs to be further explored. In addition, the clinical benefit of As$_3$O$_3$ in the treatment of SCLC is still uncertain. Based on our preclinical evidences, further clinical studies are needed to evaluate the efficacy of As$_3$O$_3$.

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Conclusions

In summary, our study demonstrated that As$_3$O$_3$ inhibited the angiogenic activity of endothelial cells, including cell proliferation and migration. We also found that As$_3$O$_3$ inhibited calcineurin-NFAT signaling and its downstream target genes CXCR7 and RND1. Moreover, As$_3$O$_3$ raised the level of DSCR1, the endogenous inhibitor of calcineurin. In vivo, we found that As$_3$O$_3$ significantly inhibited the metastasis of SCLC, and the inhibitor of calcineurin exhibited a similar effect. Taken together, we suggest that role of As$_3$O$_3$ in suppressing SCLC metastasis might be associated with the blocking of endothelial cell activation mediated by calcineurin-NFAT, and the possible mechanism is that As$_3$O$_3$ upregulates the endogenous inhibitor of calcineurin, DSCR1. The present findings may provide a foundation for the application of As$_3$O$_3$ in the treatment of SCLC.

Conflict of interest

None.
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