Anti-angiogenic effect of arsenic trioxide in lung cancer via inhibition of endothelial cell migration, proliferation and tube formation

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Abstract. Arsenic trioxide (As₂O₃) exhibits a remarkable effect on leukemia treatment; however, its effect on solid tumors remains poorly explored. The present study demonstrated the inhibitory effect of As₂O₃ on lung cancer and explored its possible mechanism. It was observed that As₂O₃ significantly inhibited the growth of lung cancer xenografts and tumor angiogenesis *in vivo*. The inhibitory effect of As₂O₃ on cell proliferation *in vitro* was more remarkable in vascular endothelial cells than in lung cancer cells. It was also observed that As₂O₃ inhibited the migration of vascular endothelial cells and disrupted vascular tube formation on Matrigel assays. In addition, a series of key signaling factors involved in multiple stages of angiogenesis, including matrix metalloproteinase (MMP)-2, MMP-9, platelet-derived growth factor (PDGF)-BB/PDGF receptor-β, vascular endothelial growth factor (VEGF)-A/VEGF receptor-2, basic fibroblast growth factor (FGF)/FGF receptor-1 and delta like canonical Notch ligand 4/Notch-1, were regulated by As₂O₃. These findings suggested that anti-angiogenesis may be an underlying mechanism of As₂O₃ anticancer activity in lung cancer.

Introduction

Arsenic trioxide (As₂O₃) is a common drug in traditional Chinese medicine (1). Since the remarkable therapeutic

effect of As₂O₃ on acute promyelocytic leukemia (APL) was recognized (2), an increasing number of researchers have reported its potential anticancer activity in solid tumors, including hepatocellular carcinoma (3,4), and pancreatic (5), prostate (6) and cervical cancer (7). However, the effect of As₂O₃ on lung cancer, which is the leading cause of cancer mortality worldwide (8), has been poorly explored. Furthermore, the majority of studies on As₂O₃ anticancer effects were performed on cancer cell lines, and only a few *in vivo* studies were reported (9-12). Among the known mechanisms of the anticancer action of As₂O₃, anti-angiogenesis is an important characteristic of As₂O₃. Several studies have reported that As₂O₃ could influence tumor angiogenesis (6,13,14), but the underlying mechanism remains unclear.

Angiogenesis, the process of new blood vessel formation, is the key step in solid tumor development; it is necessary in tumor growth, invasion and metastasis (15). According to the classical theory of tumor angiogenesis, tumors obtain nutrients and oxygen by diffusion in the early stage, but when the tumor size becomes larger, diffusion can no longer meet the tumor's requirement of oxygen and nutrients; thus, the angiogenesis process is initiated (16). The angiogenesis process in solid tumors can be summarized as follows: Continuing growth of a tumor promotes the so-called 'angiogenic switch' in the microenvironment, which initiates the angiogenic process (17,18). Matrix metalloproteinases (MMPs) induce the degradation and remodeling of the extracellular matrix (ECM) (19-21), and endothelial cells migrate through the remodeled ECM, induced by platelet-derived growth factor (PDGF) and chemokines (22,23). Due to the role of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), endothelial cells greatly proliferate (24-26). Meanwhile, tube-like structure formation and vascular function are achieved by delta like canonical Notch ligand 4 (Dll4)/Notch-1 signaling (27-30). Tumor angiogenesis is a complex process that includes multiple stages and is regulated by numerous signaling molecules that interact with one another (18). Currently, inhibition of angiogenesis has become an important target in the treatment of solid tumors, including lung cancer (31,32). Each stage of the angiogenic process and the relevant signal factors involved in the whole process

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of angiogenesis may become potential therapeutic targets. Several anti-angiogenic agents have been approved by the Food and Drug Administration (FDA) for cancer treatment, such as bevacizumab, a humanized anti-VEGF-A monoclonal antibody, and the tyrosine kinase inhibitors sorafenib and sunitinib, targeting VEGF receptors (VEGFRs) (31,32). These drugs may inhibit the proliferation of endothelial cells without influencing other stages of angiogenesis.

Our group has previously demonstrated that As₂O₃ exhibits anti-lung cancer activity by inhibiting angiogenesis (33). It was also demonstrated that As₂O₃ could reduce malignant pleural effusion caused by the pleural metastasis of lung cancer by downregulating nuclear factor- κ B, tumor necrosis factor- α and VEGF-A (34). In the present study, the antitumor activity and anti-angiogenic effect of As₂O₃ were demonstrated on both non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) *in vivo*. The present study also revealed that As₂O₃ disrupted multiple stages of angiogenesis, including endothelial cell migration, proliferation and tube formation. In addition, a series of key signaling molecules involved in multiple stages of angiogenesis were identified to be regulated by As₂O₃. It is expected that these results would provide a basis for the application of As₂O₃ in lung cancer treatment.

Materials and methods

Cell culture. The human NSCLC cell line A549 was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The human SCLC cell line NCI-H446 and human umbilical vein endothelial cells (HUVECs), used to determine the effect of As₂O₃ on tumor angiogenesis, were obtained from the American Type Culture Collection (Manassas, VA, USA). A549 and NCI-H446 cells were cultured in a mixture of RPMI 1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 10% fetal bovine serum (FBS) (HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 μ g/ml streptomycin. HUVECs were cultured in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS and the same antibiotics as described above. All the cell lines were incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Xenograft models and drug treatment. A total of 40 male nude mice, 5-6 weeks old and ~18 g in weight, were purchased from and raised in the Experimental Animal Center of Second Military Medical University (Shanghai, China). All mice were housed at 22°C, 12-h light/12-h dark cycle and free access to clean water and food. A total of 0.2 ml A549 or NCI-H446 cell suspension at a density of 5.0 $\times$ 10⁷ cells/ml was injected subcutaneously into the right flank of the mice. At 20 days post-injection, tumor volume reached ~100 mm³. Mice were then randomly divided into four groups, and were treated with 2.5 or 5.0 mg/kg As₂O₃ (i.p.) (Beijing Shuanglu Pharmaceutical Co., Ltd., Beijing, China), 30 mg/kg sorafenib (p.o.) (LC Laboratories, Woburn, MA, USA) or normal saline (NS) (i.p.) once daily for 10 days. Tumor volume was calculated as 0.5 $\times$ a² $\times$ b², where a and b are the largest and smallest lengths of the tumor, respectively. 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.

Immunohistochemistry. Fresh tumor tissue samples were fixed in 4% paraformaldehyde solution, embedded in paraffin and cut into 5- μ m-thick sections. Sections were deparaffinized and blocked for endogenous peroxidase ablation. Then, sections were incubated with anti-cluster of differentiation CD31 primary antibody (1:75, catalog no. AF3628, R&D Systems, Inc., Minneapolis, MN, USA) overnight at 4°C and the secondary antibody (1:200, catalog no. 14-13-06, KPL, Inc., Gaithersburg, MD, USA) for 1 h at room temperature. Sections were colored with 3,3'-diaminobenzidine (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) and counterstained with hematoxylin to reveal the nuclei. The quantification of microvessels was performed by counting the number of positive CD31 signals under an inverted fluorescence microscope in five random fields at x200 magnification.

Cell proliferation assay. Cells (2 $\times$ 10³ cells/well) were seeded in triplicate in 96-well plates and incubated under the aforementioned culture conditions. VEGF-A (10 ng/ml; Shanghai Biomart Technology Co., Ltd., Shanghai, China) was added in the medium of HUVECs. Cells were then treated with various concentrations (2.0 or 4.0 μ M) of As₂O₃ (Beijing Shuanglu Pharmaceutical Co., Ltd., Beijing, China), 4.0 μ M sorafenib (LC Laboratories, Woburn, MA, USA) or NS. After additional 24 or 48 h, cell proliferation was determined in triplicate, using a Cell Counting Kit-8 (CCK-8) assay (Beyotime Institute of Biotechnology, Haimen, China). The absorbance of each well at 450 nm was measured. The results were expressed as contrast absorbance, considering the NS group as control.

Wound-healing assay. Cells were seeded in 6-well plates and divided into four groups: Control group, As₂O₃ 2.0 μ M group, As₂O₃ 4.0 μ M group and sorafenib 4.0 μ M group. When cells grew to confluency, a mechanical wound was created by gently scratching the cells with a pipette tip (time 0 h). Images were captured after 24 and 48 h, and the wound healing capacity was quantified by measuring the distance between the wound edges. Experiments were carried out in triplicate wells from three independent experiments.

Vascular tube formation assay *in vitro*. Plates with 24 wells were firstly coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Unpolymerized Matrigel was placed in the wells (300 μ l/well) and allowed to polymerize for 1 h at room temperature. HUVECs in 500 μ l medium were seeded onto the polymerized Matrigel at a density of 5 $\times$ 10⁴ cells/well. VEGF-A (10 ng/ml; Shanghai Biomart Technology Co., Ltd.) and basic FGF (bFGF) (10 ng/ml; Shanghai Biomart Technology Co., Ltd.) were used as angiogenic stimuli (35). After incubation at 37°C in 5% CO₂ for 18 h, images of tube formation were acquired with an inverted phase-contrast light microscope (Olympus Corporation, Tokyo, Japan) equipped with a microscope camera (Q Imaging, Surrey, BC, Canada). The degree of tube formation was quantified in five random fields from

each well at x40 magnification, using ImageJ software (version 1.48, National Institutes of Health, Bethesda, MD, USA).

Western blotting. Total proteins were extracted from cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Aliquots containing 20 μ g protein were used for western blotting. Proteins were separated by SDS-PAGE (10% gel) and transferred to polyvinylidene fluoride membranes. Membranes were blocked with a solution containing 5% nonfat milk for 1 h, and then incubated with the corresponding primary antibodies overnight at 4°C. Membranes were then washed three times with TBS containing Tween-20 and incubated with the secondary antibody (1:3,000, catalog no. ab6721, Abcam, Cambridge, UK) at room temperature for 1 h. The protein bands were detected by chemiluminescence (Western chemiluminescent horseradish peroxidase substrate, catalog no. WBKLS0500, Merck KGaA, Darmstadt, Germany). β -actin was used as an internal control. The following primary antibodies were used all at 1:1,000 dilution: Anti-MMP-2 (catalog no. ab86607, Abcam), anti-MMP-9 (catalog no. ab38898, Abcam), anti-PDGF-BB (catalog no. ab23914, Abcam), anti-PDGF receptor (PDGFR)- β (catalog no. ab111310, Abcam), anti-VEGF-A (catalog no. ab46154, Abcam), anti-VEGFR-2 (catalog no. ab39256, Abcam), anti-bFGF (catalog no. ab8880, Abcam), anti-FGF receptor (FGFR)-1 (catalog no. ab823, Abcam), anti-Dll4 (catalog no. ab7280, Abcam), anti-Notch-1 (catalog no. ab27526, Abcam) and anti- β -actin (catalog no. sc-47778, Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Statistical analysis. Data were analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The results are presented as means $\pm$ standard deviation, as analyzed by one-way analysis of variance, followed by Fisher's least significant difference t test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

As₂O₃ inhibits the growth of human lung cancer xenografts and tumor angiogenesis in vivo. To determine the effect of As₂O₃ on the growth of lung cancer, xenograft tumor models were established using the NSCLC cell line A549 and the SCLC cell line NCI-H446. When all nude mice developed tumors, drug administration was performed for 10 continuous days. As presented in Fig. 1A, the mean tumor volumes in the As₂O₃ groups were significantly smaller than those in the control groups at the end of treatment, and tumor volumes in the 5.0 mg/kg As₂O₃ group were smaller than those in the 2.5 mg/kg As₂O₃ group. These findings were observed in both types of xenograft model. Sorafenib, an anti-angiogenic, anti-tumor agent targeting VEGFR-2 (36), was used as a positive control. It was observed that the inhibitory effect of sorafenib on tumor growth was greater than that of 5.0 mg/kg As₂O₃ in the A549 xenograft model, while it was similar to that of 5.0 mg/kg As₂O₃ in the NCI-H446 xenograft model. These results suggested that As₂O₃ had an inhibitory effect on both NSCLC and SCLC tumor growth in a dose-dependent manner.

Next, sections of xenografts were stained for CD31, which was used primarily to demonstrate the presence of endothelial

cells, to detect the number and morphology of endothelial cells as a measure of tumor angiogenesis (28). Representative images of immunohistochemistry are presented in Fig. 1C, and the quantification of microvessel numbers is presented in Fig. 1B. The mean microvessel number in the two As₂O₃ groups was significantly lower than that in the control group ($P < 0.001$) in both types of xenograft model. The morphology of microvessels in the As₂O₃ groups was poorly developed compared with the normal tube-like structure in the control group. The microvessel number in the sorafenib group was also significantly lower than that of the control group ($P < 0.001$), but no poorly-developed vascular structures were observed in the sorafenib group. These data indicated that As₂O₃ could inhibit tumor angiogenesis in NSCLC and SCLC in a dose-dependent manner, but its mechanism may not be identical with that of sorafenib. As₂O₃ may decrease the number of blood vessels and delay the development of vascular structures, whereas sorafenib may only decrease the number of blood vessels.

As₂O₃ inhibits the proliferation of lung cancer cells and HUVECs. To further determine whether the anti-tumor effect of As₂O₃ *in vivo* depended on its anti-angiogenic effect or its direct cytotoxicity towards tumor cells, its effect on the proliferation of lung cancer cells and HUVECs was examined by CCK-8 assay. As presented in Fig. 2A and B, A549 and NCI-H446 cell proliferation at 24 h exhibited no significant difference between the groups, while at 48 h, cell proliferation in the As₂O₃ groups was slightly lower than that in the control and sorafenib groups. As₂O₃ significantly inhibited HUVEC proliferation compared with that of the control group at both 24 and 48 h. The inhibitory effect of sorafenib on HUVEC proliferation was also obvious (Fig. 2C). These results demonstrated that direct cytotoxicity towards tumor cells was not the main factor in the anti-lung cancer effect of As₂O₃, while inhibition of vascular endothelial cell proliferation may be important in this process.

As₂O₃ disrupts HUVEC migration and tube-like structure formation on Matrigel. To determine the effect of As₂O₃ on the migration of HUVECs, a wound-healing assay was performed. As presented in Fig. 3A, the wound-healing capacity of HUVECs was diminished by As₂O₃ at 24 h after the wounds were created, and at 48 h after scratching, this phenomenon was more obvious. According to the quantitative comparison of cell migration distances at 48 h (Fig. 3B), the distances of cell migration in the As₂O₃ 2 and 4 μ M groups were 289.52 ± 28.62 and 180.00 ± 30.90 μ m, respectively, which were significantly lower than the cell migration distance in the control group (509.52 ± 29.74 μ m; $P < 0.001$), suggesting that the migration of HUVECs was inhibited by As₂O₃ in a dose-dependent manner. However, the distance of cell migration in the sorafenib group (493.33 ± 43.74 μ m) was not significantly different from that in the control group ($P > 0.05$), which suggested that, different from As₂O₃, sorafenib did not affect the migration of HUVECs.

Next, it was examined whether As₂O₃ was able to disrupt endothelial network formation by Matrigel assay. HUVECs were plated onto Matrigel in the presence of VEGF-A and bFGF as angiogenic factors (35). Then, cells were treated with 2 or 4 μ M As₂O₃, 4 μ M sorafenib, or NS as control for 18 h,

Figure 1. As₂O₃ inhibits A549 and NCI-H446 xenograft growth and tumor angiogenesis. (A) Mean tumor volumes of the four groups in the A549 and NCI-H446 tumor models following drug treatment. (B) Quantification of microvessel numbers. Error bars indicate the standard deviation. *P<0.05, **P<0.01 and ***P<0.001. (C) As₂O₃ decreased the number of microvessels and induced poorly-developed vascular structures. Tumor sections were immunostained with an anti-cluster of differentiation 31 antibody (brown). Scale bar, 50 μm.

Figure 2. As₂O₃ inhibits the proliferation of lung cancer cells and HUVECs. The proliferation of (A) A549 cells, (B) NCI-H446 cells and (C) HUVECs in each group at 24 or 48 h was examined by Cell Counting Kit-8 assay, and the relative absorbance was compared with that of the control group. Error bars indicate the standard deviation. *P<0.05, **P<0.01 and ***P<0.001. HUVEC, human umbilical vein endothelial cell.

and microphotographs were then obtained (Fig. 3C). Pictures of cords of interconnecting cells were generated by ImageJ 1.48v software (Fig. 3D), and the number of cord formations was quantitatively analyzed (Fig. 3E). As presented in Fig. 3E, a significant decrease in tube formation in the As₂O₃ groups was observed (P<0.001), where normal tube structures were destroyed with interrupted alignments and cords. Sorafenib also reduced the number of cord formations (P<0.01), but the

tube structures observed in this group were as regular as those in the control group.

As₂O₃ inhibits angiogenesis-associated factors in lung cancer cells and HUVECs. Based on the aforementioned results, As₂O₃ displayed effective anti-angiogenic activity both *in vivo* and *in vitro*. As₂O₃ could disrupt multiple stages of angiogenesis, including endothelial cell migration,

Figure 3. As₂O₃ disrupts HUVEC migration and vascular tube formation *in vitro*. (A) As₂O₃ diminished the wound-healing capacity of HUVECs at 24 and 48 h after scratching. Scale bars, 200 μm. (B) Quantification of cell migration distances at 48 h after scratching. (C) HUVECs plated onto Matrigel to form tube-like structures in the four groups were observed under an inverted phase-contrast microscope. Scale bars, 200 μm. (D) Images of cords of interconnecting cells were generated by ImageJ software (version 1.48). (E) Quantification of cord formations. Error bars indicate the standard deviation. **P<0.01 and ***P<0.001. HUVEC, human umbilical vein endothelial cell.

Figure 4. As₂O₃ inhibits angiogenesis-associated factors. As₂O₃ reduced MMP-2, MMP-9, PDGF-BB, VEGF-A, bFGF and DII4 protein levels in (A) A549 and (B) NCI-H446 cells. (C) As₂O₃ reduced MMP-2, MMP-9, PDGFR-β, VEGFR-2, FGFR-1, DII4 and Notch-1 protein levels in HUVECs. The target protein levels were evaluated by western blotting. HUVEC, human umbilical vein endothelial cell; MMP, matrix metalloproteinase; PDGFR, platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; DII4, delta like canonical Notch ligand 4.

proliferation and tube formation. The present study next examined angiogenesis-associated factors involved in these stages at the protein level by western blotting. As presented in Fig. 4A and B, As₂O₃ reduced the expression of MMP-2, MMP-9,

PDGF-BB, VEGF-A, bFGF and DII4 in A549 and NCI-H446 cells. As₂O₃ also reduced the expression of MMP-2, MMP-9, PDGFR-β, VEGFR-2, FGFR-1, DII4 and Notch-1 in HUVECs (Fig. 4C), in a concentration-dependent manner. Sorafenib

only downregulated VEGFR-2 and PDGFR- β expression in HUVECs, but had no marked effect on the expression of the other factors (Fig. 4C), verifying that sorafenib inhibited endothelial cell proliferation mainly by targeting VEGF signaling.

Discussion

As₂O₃ was firstly introduced as an effective agent with low toxicity for APL treatment by Chinese researchers (37). Since its approval by the FDA for use in leukemia therapy, As₂O₃ has also been applied to numerous solid tumors (38,39). However, the mechanism of its anticancer activity in solid tumors is not yet fully understood.

In the present study, *in vivo* experiments demonstrated that As₂O₃ significantly inhibited the growth of NSCLC and SCLC xenografts in a dose-dependent manner. As₂O₃ also exhibited a marked anti-angiogenic effect in animal models of lung cancer. *In vitro* cell proliferation assays were conducted in the present study, which revealed that the inhibitory effect of As₂O₃ on NSCLC and SCLC cell growth was not so remarkable as that observed *in vivo*. In addition, As₂O₃ significantly suppressed endothelial cell proliferation. These data suggested that anti-angiogenesis, rather than direct cytotoxicity towards tumor cells, was the main mechanism of the anticancer effect of As₂O₃.

The anti-angiogenic effect of As₂O₃ has been reported in various studies, both *in vivo* and *in vitro*. A previous study reported that 0.5 and 5.0 μ M As₂O₃ caused inhibition of VEGF in leukemic cells, and prevented capillary tube formation in an endothelial cell-differentiation assay (40). It was also reported that As₂O₃ delayed gastric cancer xenograft growth, decreased microvessel density, and downregulated VEGFR-1 and VEGFR-2 expression (14). Besides reducing the number of vessels, As₂O₃ also influences the vascular morphology and function. Adding As₂O₃ into the drinking water for mice induced significant vascular remodeling, with increased sinusoidal endothelial cell capillarization, resulting in decreased permeability and transport function (41). In another study, As₂O₃ (10 mg/kg i.p.) produced a preferential vascular 'shutdown' in the tumor tissue in a model of methylcholanthrene-induced fibrosarcoma in BALB/c mice, leading to extensive necrosis in the central part of the tumor (42). In addition, ^{99m}Tc clearance and ⁸⁶Rb uptake in the tumor tissue were decreased, suggesting declined tumor perfusion (42).

In the present study, As₂O₃ decreased the number of microvessels in lung cancer xenografts, and it also inhibited HUVEC proliferation significantly at both 24 and 48 h. In addition, As₂O₃ reduced the expression of VEGF-A and bFGF in lung cancer cells, as well as that of VEGFR-2 and FGFR-1 in HUVECs. VEGF-A/VEGFR-2 and bFGF/FGFR-1 are both potent stimulating factors of endothelial cell proliferation, which have been previously validated (43,44).

The present results also indicated that As₂O₃ disrupted endothelial cell migration, and downregulated MMP-2, MMP-9 and PDGF-BB in lung cancer cells, and PDGFR- β in HUVECs. MMPs such as MMP-2 and MMP-9 contribute to basement membrane degradation and ECM remodeling, which allow endothelial cell migration and sprouting (20). PDGF signaling promotes endothelial cell migration through the remodeled ECM and the formation of new blood vessels (45).

The present results confirmed that As₂O₃ could inhibit this signaling pathway and thereby disturb HUVEC migration.

Furthermore, the present data revealed that As₂O₃ influenced the morphology of microvessels by inducing poorly developed vascular structures *in vivo*. Tube formation assays on Matrigel were performed to explore the effect of As₂O₃ on the number and shape of newly formed microvessels upon stimulation with VEGF-A and bFGF. It was observed that As₂O₃ reduced the number of cord formations and destroyed the normal tube structures. This effect helped to distinguish As₂O₃ from the well-known angiogenesis-targeted inhibitor sorafenib. *In vivo*, formation of vascular lumen structures is promoted by Dll4/Notch signaling (46). It has been reported that blockade of Dll4/Notch signaling induced defective maturation of blood vessels with poor perfusion (28,47). According to the present results, As₂O₃ reduced the level of Dll4 in lung cancer cells and HUVECs, and reduced the level of Notch-1 in HUVECs. These findings implied that As₂O₃ influenced the morphology and function of new vessels in lung cancer, possibly by downregulating Dll4/Notch signaling.

In summary, the present study demonstrated that As₂O₃ could inhibit lung cancer xenograft growth and tumor angiogenesis. It was also observed that As₂O₃ could disrupt multiple stages of angiogenesis, including endothelial cell migration, proliferation and network formation, and could regulate the expression of the key signaling molecules involved in these processes. The present findings may provide the experimental basis to extend the indications of As₂O₃ and to identify novel therapeutic approaches for lung cancer.

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