Arsenic trioxide exerts synergistic effects with cisplatin on non-small cell lung cancer cells via apoptosis induction

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Abstract

Background: Despite multidisciplinary treatment, lung cancer remains a highly lethal disease due to poor response to chemotherapy. The identification of therapeutic agents with synergistic effects with traditional drugs is an alternative for lung cancer therapy. In this study, the synergistic effects of arsenic trioxide (As2O3) with cisplatin (DDP) on A549 and H460 non-small cell lung cancer (NSCLC) cells were explored.

Methods: A549 and H460 human lung cancer cells were treated with As2O3 and/or DDP. Cell growth curves, cell proliferation, cell cycle, and apoptosis of human cancer cell lines were determined by the 3-(4,5)-dimethylthiahiazol (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) method, clonogenic assay, and flow cytometry (FCM). Apoptosis was further assessed by TUNEL staining. Cell cycle and apoptosis related protein p21, cyclin D1, Bcl-2, bax, clusterin, and caspase-3 were detected by western blot.

Results: MTT and clonogenic assay showed As2O3 within 10^{-2} μM to 10 μM exerted inhibition on the proliferation of NSCLC cells, and 2.5 μM As2O3 exerted synergistic inhibition on proliferation with 3 μg/ml DDP. The combination indices (CI) for A549 and H460 were 0.5 and 0.6, respectively, as confirmed by the synergism of As2O3 with DDP. FCM showed As2O3 did not affect the cell cycle. The G0/G1 fraction ranged from 57% to 62% for controlled A549 cells and cells treated with As2O3 and/or DDP. The G0/G1 fraction ranged from 37% to 42% for controlled H460 cells and cells treated with As2O3 and/or DDP. FCM and TUNEL staining illustrated that the combination of As2O3 and DDP provoked synergistic effects on apoptosis induction based on the analysis of the apoptosis index. Western blotting revealed that the expression of cell cycle related protein p21 and cyclin D1 were not affected by the treatments, whereas apoptosis related protein bax, Bcl-2, and clusterin were significantly regulated by As2O3 and/or DDP treatments compared with controls. The expression of caspase-3 in cells treated with the combination of As2O3 and DDP did not differ from that in cells treated with a single agent.

Conclusion: As2O3 exerted synergistic effects with DDP on NSCLC cells, and the synergistic effects were partly due to the induction of caspase-independent apoptosis.
Background
Lung cancer is the number one cause of cancer mortality in both males and females worldwide [1]. Despite multidisciplinary treatment, lung cancer is still a highly lethal disease due to late detection and resistance to chemotherapy. The identification of new therapeutic agents that exert synergistic effects in combination with traditional cytotoxic agents is an alternative strategy for the systemic treatment of lung cancer.

Recent evidence indicates that arsenic trioxide (As$_2$O$_3$) may induce clinical remission in patients with acute promyelocytic leukemia (APL), and several investigations show that As$_2$O$_3$ induced programmed cell death in APL cell lines [2-5]. DDP, a platinum-containing anticancer drug, is one of the most commonly used cytotoxic agents for the treatment of lung cancer. Due to the poor therapeutic effects of current cytotoxic-agents on lung cancer, the ability of As$_2$O$_3$ to induce apoptosis in non-small cell lung cancer cells was explored in the present study, and the synergistic effects of As$_2$O$_3$ with DDP on A549 and H460 lung cancer cells were analyzed.

Methods
Cell culture and reagents
Human lung cancer A549 and H460 cell lines were obtained from the ATCC and maintained in RPMI 1640 medium with 10% fetal bovine serum and 1% penicillin. As$_2$O$_3$ was purchased from Yida Pharmaceutical Co. (GMP, Ha'erbin, PR. China) and DDP was from Bristol-Myers Squibb Co. (Shanghai, PR. China).

MTT assay
Briefly, cells were seeded at a density of 2,000 to 5,000 cells/well in 96-well plates and incubated overnight. After treatment with As$_2$O$_3$, DDP, or their combination (described below), 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) was added (50 μL/well) for 4 hours. Solubilization of the converted purple formazan dye was accomplished by placing cells in 100 μL of 0.01 N HCl/10% SDS and incubating them overnight at 37°C. The reaction product was quantified by absorbance at 570 nm. All samples were repeated three times, and data were analyzed by Student’s t test.

In vitro clonogenic assay
Human lung carcinoma cells were counted after trypsinization. Cells were serially diluted to appropriate concentrations and removed into 25-cm$^2$ flasks in 5-mL medium in triplicate per data point. After various treatments, cells were maintained for 8 days. Cells were then fixed for 15 minutes with a 3:1 ratio of methanol:acetic acid and stained for 15 minutes with 0.5% crystal violet (Sigma) in methanol. After staining, colonies were counted by the naked eye, with a cutoff of 50 viable cells. Error bars represent ± SE by pooling of the results of three independent experiments. Surviving fraction was calculated as (mean colony counts)/(cells inoculated) * (plating efficiency), where plating efficiency was defined as mean colony counts/cells inoculated for untreated controls.

Cell cycle and apoptosis analysis
Flow cytometry analysis of DNA content was performed to assess the cell cycle phase distribution as described previously [6]. Cells were harvested and stained for DNA content using propidium iodide fluorescence. The computer program Multicycle from Phoenix Flow System (San Diego, CA, USA) was used to generate histograms which were used to determine the cell cycle phase distribution and apoptosis. TUNEL staining was also used to detect apoptosis as described previously [7]. The TUNEL stained apoptotic cells were separately numbered in four randomly selected microscopic fields (400×) and graphed.

Western blot
After various treatments, cells were washed with ice-cold PBS twice before the addition of lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium NaPPi, 1 mM phenylmethylsulfonyl fluoride, and leupeptin). Protein concentrations were quantified separately by the Bio-Rad Bradford assay. Equal amounts of total protein were loaded into each well and separated by 10% SDS PAGE, followed by transfer onto nitrocellulose membranes. Membranes were blocked using 5% nonfat dry milk in PBS for 1 hour at room temperature. The blots were then incubated with anti-p21, anti-cyclin D1, anti-bax, anti-bcl-2, anti-clusterin, and anti-caspase-3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Blots were then incubated in secondary antibody conjugated with HRP (1:1000; Santa Cruz Biotechnology) for 1 hour at room temperature.

Immunoblots were developed using the enhanced chemiluminescence (ECL) detection system (Amersham, Piscataway, NJ) according to the manufacturer’s protocol and autoradiography.

Results
As$_2$O$_3$ exerted synergistic effects with DDP on the proliferation of A549 and H460
The MTT assay showed that 10$^{-2}$ μM to 10 μM inhibited the proliferation of A549 and H460 at 72 hours (Fig. 1). In vitro clonogenic assay proved 10$^{-1}$ μM to 12.5 μM As$_2$O$_3$ inhibited the proliferation of A549 and H460 cells (Fig. 2). MTT assay results showed that 2.5 μM As$_2$O$_3$ and 3 μg/ml DDP exerted synergistic inhibition effects on A549 and H460 cells at 48 hours. (Fig. 3A,B). To confirm the synergistic effects of As$_2$O$_3$ with DDP CalcuSyn™ program (Version 2.0, Biosoft, Inc., UK) was explored to make dose-effect curves and to determine the combina-
tion indices (CI) (Fig. 4A,B). The CI for A549 and H460 were 0.5 and 0.6, respectively which confirmed the synergism of As$_2$O$_3$ with DDP.

**As$_2$O$_3$ did not significantly affect the cell cycles of A549 and H460 cells**

A549 cells were treated with 2.5 μM As$_2$O$_3$ and/or 3 μg/ml DDP for 48 hours. FCM cell cycle analysis showed that the treatment of As$_2$O$_3$ and/or DDP did not significantly alter G0/G1 fractions of A549 cells compared with those of the control. The G0/G1 fraction ranged from 57% to 62% for controlled A549 cells and cells treated with As$_2$O$_3$ and/or DDP; the G0/G1 fraction ranged from 37% to 42% for controlled H460 cells and cells treated with As$_2$O$_3$ and/or DDP (Fig. 5). Western blot analysis showed that As$_2$O$_3$ and/or DDP did not affect the expression of cell cycle related protein p21 and cyclin D1 (data not shown).

**As$_2$O$_3$ and/or DDP induced apoptosis of A549 and H460 cells**

A549 cells were treated with 2.5 μM As$_2$O$_3$ and/or 3 μg/ml DDP for 48 hours. FCM analysis showed the apoptotic indices (AI) for the controlled A549 cells and cells treated with As$_2$O$_3$, DDP, or the combination were 0.25 ± 0.01%, 10.6 ± 0.53%, 15.85 ± 0.79%, and 20 ± 1%, respectively. The AI for the controlled H460 cells, cells treated with As$_2$O$_3$, DDP, or the combination were 1.95 ± 0.11%, 13.6 ± 0.65%, 7.53 ± 0.43%, and 35.6 ± 1.71%, respectively (Fig. 6). Western blot analysis showed Bax expres-
Dose effect curve for A549 (A) and H460 (B) cells.
The concentration of DDP was 3 μg/ml and the concentration for As₂O₃ ranged from 0.1 μM to 12.5 μM. CalcuSyn™ (Version 2.0, Biosoft, Inc., UK) was used for dose-effect curves and to determine the combination indices (CI).

G₀/G₁ fraction analysis. FCM cell cycle analysis showed that the treatment of As₂O₃ and/or DDP did not significantly affect G₀/G₁ fractions of A549 and H460 cells compared with those of the control. The G₀/G₁ fraction ranged from 57% to 62% for control A549 cells and for A549 cells treated with As₂O₃ and/or DDP, and from 37% to 42% for control H460 cells and for H460 cells treated with As₂O₃ and/or DDP.

TUNEL staining analysis. With TUNEL staining, the AI for the control A549 cells and cells treated with As₂O₃, DDP, or the combination were 3.1 ± 0.16%, 15.41 ± 0.77%, 14 ± 0.7%, and 30 ± 1.5%, respectively; the AI for the control H460 cells and cells treated with As₂O₃, DDP, or the combination were 5.95 ± 0.25%, 18.6 ± 1.13%, 9.53 ± 0.49%, and 40.6 ± 2.11%, respectively.
Western blot analysis of Bax expression in lung cancer cell after different treatments. Bax expression was 2-fold greater in A549 cells treated with As$_2$O$_3$ and DDP than in control cells. Bax expression was 3.7-fold greater in H460 cells treated with As$_2$O$_3$ and DDP than in control cells.

Western blot analysis of Bcl-2 expression in lung cancer cells after different treatments. Bcl-2 expression was 72% less in As$_2$O$_3$ and DDP-treated A549 cells than in controls, and it 25% less in As$_2$O$_3$ and DDP-treated H460 cells than in controls.
sion increasing by 2-fold in the A549 cells treated with As$_2$O$_3$ and DDP over levels in control cells. In H460 cells treated with As$_2$O$_3$ and DDP, Bax expression was 3.7 times greater than in the control (Fig. 8). Bcl-2 expression was 72% less in the As$_2$O$_3$ and DDP treated A549 cells than in control cells, and 25% less in the As$_2$O$_3$ and DDP treated H460 cells than in control cells (Fig. 9). Expression of another tumor suppressed protein, clusterin, was 70% less in the As$_2$O$_3$ and DDP treated A549 cells than in control cells, and in H460 cells, clusterin expression was 90% less with treatment of the combination of As$_2$O$_3$ and DDP than in controls (Fig. 10). For both A549 and H460, caspase-3 expression increased with the treatment of As$_2$O$_3$ and/or DDP over control levels, but caspase-3 expression was not different in cells treated with the combination of As$_2$O$_3$ and DDP and cells treated with each single agent (Fig. 11).

Discussion and conclusion

Our in vitro study showed that As$_2$O$_3$ is an effective reagent that inhibits the proliferation of A549 and H460 lung cancer cells. As$_2$O$_3$ cytotoxicity was due to the induction of apoptosis but not cell cycle arrest. FCM and TUNEL assay analyses showed that As$_2$O$_3$ significantly induced apoptosis. When As$_2$O$_3$ and DDP were combined, a synergistic effect was found in the treatment of A549 and H460 cells. Protein assays showed that the combination of As$_2$O$_3$ and DDP affected apoptosis-related proteins such as Bcl-2, Bax, and clusterin but not caspase-3, while the use of each single agent did not. The changes in apoptosis-related protein expression partly contributed to the effect of As$_2$O$_3$ on lung cancer cells.

Since lung cancer is a lethal disease due to late detection and resistance to chemotherapy, this study was conducted to determine whether As$_2$O$_3$ could exert synergistic effects in combination with traditional cytotoxic-agents on lung cancer cell death. Although As$_2$O$_3$ has been an effective treatment for the acute promyelocytic leukemia, the mechanism by which As$_2$O$_3$ induces cell death remains poorly understood. Recent reports suggest that As$_2$O$_3$ causes DNA damage, oxidative stress, and mitochondrial dysfunction [8,9]. In addition, As$_2$O$_3$ treatment results in cell-cycle arrest in MCF-7 HeLa cells [10]; however, our results demonstrate that cell cycle is not significantly affected by As$_2$O$_3$, since the G1/0 fraction and cell cycle-related protein expression did not change significantly with As$_2$O$_3$ treatment. The inconsistency between these findings may be due to different mechanisms of action by As$_2$O$_3$ in various cell lines. Our results were consistent...
with previous studies that indicated that proapoptotic Bcl-2 family members, Bcl-2 and Bax, are involved in the apoptosis of cancer cells induced by As$_2$O$_3$ [11,12]. Previous studies show that clusterin is a caspase-independent apoptosis-related protein and it is a potential target in the treatment of non-small cell lung cancer [13-15]. Here, we showed that the synergistic effects of As$_2$O$_3$ and DDP might be due, in part, to clusterin-mediated apoptosis. Depending on the cell system investigated, As$_2$O$_3$-induced cell death has been associated with caspase-dependent apoptosis, as well as caspase-independent death pathways [16-18]. In this study, the combination of As$_2$O$_3$ and DDP increased caspase-3 expression, which indicates that caspase might be involved in apoptosis induced by As$_2$O$_3$ or DDP. However, the combination of As$_2$O$_3$ and DDP did not affect caspase-3 expression compared with cells treated with a single agent, which suggests that the synergistic effects are more likely to be caspase-independent. This study showed caspase-independent death pathways that involved Bcl-2, Bax, and clusterin were the primary mechanism by which As$_2$O$_3$ exerts synergistic effects with DDP on NSCLC cells.

In conclusion, As$_2$O$_3$ exerted synergistic effects with DDP on lung cancer cells. The proliferation inhibition might be partly due to the induction of apoptosis. Based on our study, As$_2$O$_3$ may be a promising agent in the treatment of lung cancer, although further in vitro and in vivo studies are necessary to elucidate the mechanism by which As$_2$O$_3$ induces apoptosis.

**Competing interests**
The authors declare that they have no competing interests.

**Authors' contributions**
As principle investigator HL and HC had full access to all of the data in this study and take responsibility for the accuracy of the data analysis. Study concept and design: HL, XZ and JX. MTT, Clonogenic assay, Flow cytometry assay, TUNEL assay and western blot: XZ, HL. Analysis and interpretation of data: XZ, HL. Drafting of the manuscript: HL, XZ. Critical revision of the manuscript: JX, HC. Supervision: YZ, JX and HC.

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**References**
1. Landis SH, Murray T, Bolden S, Wingo PA: Cancer statistics, 1998. *CA Cancer J Clin* 1998, 48(1):6-29.
2. Soignet SL, Mslal P, Wang ZG, Jianwar S, Calleja E, Dadashri Lj, Corso D, DeBiasio A, Gabrloie J, Scheinberg DA, Pandolfi PF, Warrell RP Jr: Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. N Engl J Med 1998, 339(19):1341-1348.

3. Shao W, Fanelli M, Ferrara FF, Riccioni R, Rosenauer A, Davison K, Lammh WW, Waxman S, Pellicci PG, Lo Coco F, Avvisati G, Testa U, Peschle C, Gambacorti-Passerini C, Nervi C, Miller WH Jr: Arsenic trioxide as an inducer of apoptosis and loss of PML/RAR alpha protein in acute promyelocytic leukemia cells. J Natl Cancer Inst 1998, 90(2):124-133.

4. Look AT: Arsenic and apoptosis in the treatment of acute promyelocytic leukemia. J Natl Cancer Inst 1998, 90(2):86-88.

5. Chen GQ, Shi XG, Tang W, Xiong SM, Zhu J, Han ZG, Ni JH, Shi GY, Jia PM, Liu MM, He KL, Niu C, Ma J, Zhang P, Zhang TD, Paul P, Naoe T, Kitamura K, Miller W, Waxman S, Wang ZY, de Thé H, Chen SJ, Chen Z: Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): I. As2O3 exerts dose-dependent dual effects on APL cells. Blood 1997, 89(9):3345-3353.

6. Shao ZM, Nguyen M, Alpaugh ML, O’Connell JT, Barsky SH: The human myoepithelial cell exerts antiproliferative effects on breast carcinoma cells characterized by p21/WAF1/CIP1 induction, G2/M arrest, and apoptosis. Exp Cell Res 1998, 241(2):394-403.

7. Daigeler A, Brenzel C, Bulut D, Geisler A, Hilgert C, Lehnhardt M, Steinau HU, Flier A, Steinstraesser L, Klein-Hitpass L, Mittelkötter U, Uhl W, Chromik AM: TRAIL and Taurolidine induce apoptosis and decrease proliferation in human fibrosarcoma. J Exp Clin Cancer Res 2008, 27:82.

8. Gurr JR, Bau DT, Liu F, Lynn S, Jan KY: Dithiothreitol enhances arsenic trioxide-induced apoptosis in NB4 cells. Mol Pharmacol 1999, 56(1):102-109.

9. Dong JT, Luo XM: Arsenic-induced DNA-strand breaks associated with DNA-protein crosslinks in human fetal fibroblasts. Mutat Res 1993, 302(2):97-102.

10. Ling YH, Jiang JD, Holland JF, Perez-Soler R: Arsenic trioxide produces polymerization of microtubules and mitotic arrest before apoptosis in human tumor cell lines. Mol Pharmacol 2002, 62(3):529-538.

11. Chen GQ, Zhu J, Shi XG, Ni JH, Zhong Hj, Si GY, Jin XL, Tang W, Li XS, Xiong SM, Shen ZX, Sun GL, Ma J, Zhang P, Zhang TD, Gazin C, Naoe T, Chen SJ, Wang ZY, Chen Z: in vitro studies on cellular and molecular mechanisms of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia: As2O3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML proteins. Blood 1996, 88(3):1052-1061.

12. Kroemer G, de The H: Arsenic trioxide, a novel mitochondriotoxic anticancer agent? J Natl Cancer Inst 1999, 91(9):743-745.

13. Cao C, Shinohara ET, Li H, Niermann KJ, Kim KW, Sekhar KR, Gleave M, Freeman M, Lu B: Clusterin as a therapeutic target for radiation sensitization in a lung cancer model. Int J Radiat Oncol Biol Phys 2005, 63(4):1228-1236.

14. Scalcitri M, Santamaria A, Piacucci R, Betrucci S: Intracellular clustering induces G2-M phase arrest and cell death in PC-3 prostate cancer cells. Cancer research 2004, 64(17):6174-6182.

15. Chi KN, Siu LL, Hirte H, Hotte SJ, Knox J, Killamanderberger C, Gleave M, Guns E, Powers J, Walsh W, Du D, Eisenhauer E: A phase I study of OXG-011, a 2’-methoxethyl phosphorothioate antisense to clusterin, in combination with docetaxel in patients with advanced cancer. Clin Cancer Res 2008, 14(3):833-839.

16. McCaffrey-Grad J, Bahls NJ, Kretz N, Aguilar TM, Reis I, Lee KP, Boise LH: Arsenic trioxide uses caspase-dependent and caspase-independent death pathways in myeloma cells. Molecular cancer therapeutics 2003, 2(11):1155-1164.

17. Cai X, Shen YL, Zhu Q, Jia PM, Yu Y, Zhou L, Huang Y, Zhang JW, Xiong SM, Chen SJ, Wang ZY, Chen Z, Chen GQ: Arsenic trioxide-induced apoptosis and differentiation are associated respectively with mitochondrial transmembrane potential collapse and retinoic acid signaling pathways in acute promyelocytic leukemia. Leukemia 2000, 14(2):262-270.

18. Karlsson J, Ora I, Porn-Ares I, Pahlman S: Arsenic trioxide-induced death of neuroblastoma cells involves activation of Bax and does not require p53. Clin Cancer Res 2004, 10(9):3179-3188.