

# Arsenic hexoxide enhances TNF- $\alpha$ -induced anticancer effects by inhibiting NF- $\kappa$ B activity at a safe dose in MCF-7 human breast cancer cells

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**Abstract.** Arsenic hexoxide (As<sub>4</sub>O<sub>6</sub>) has been used in Korean folk remedy for the treatment of cancer since the late 1980s. Evidence suggests that the anticancer effects of As<sub>4</sub>O<sub>6</sub> are different from those of As<sub>2</sub>O<sub>3</sub>. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is generally increased in advanced cancer and is closely related to cancer progression, although it has cancer-killing effects. The reason is that TNF- $\alpha$  activates nuclear factor- $\kappa$ B (NF- $\kappa$ B) that is involved in cell proliferation, invasion, drug resistance and metastasis. In the present study, we investigated the effects of As<sub>4</sub>O<sub>6</sub> on NF- $\kappa$ B activity, NF- $\kappa$ B-mediated cellular responses, and NF- $\kappa$ B-regulated gene expressions involved in metastasis at the concentrations of As<sub>4</sub>O<sub>6</sub> where no cytotoxicity was observed. As<sub>4</sub>O<sub>6</sub> suppressed NF- $\kappa$ B activation in both TNF- $\alpha$ -treated and control cells, and also suppressed I $\kappa$ B phosphorylation in a time-dependent manner, suggesting the suppression of NF- $\kappa$ B results, in part, from the inhibition of I $\kappa$ B degradation. We also confirmed the anti-NF- $\kappa$ B activity of As<sub>4</sub>O<sub>6</sub> with synergism with TNF- $\alpha$  by augmenting caspase-8 activation. As<sub>4</sub>O<sub>6</sub> also suppressed NF- $\kappa$ B activation induced by TNF- $\alpha$ , and some of the downstream NF- $\kappa$ B-regulated proteins involved in cancer proliferation, anti-apoptosis and metastasis. In conclusion, the present study demonstrated that As<sub>4</sub>O<sub>6</sub> has anticancer properties by inhibiting NF- $\kappa$ B activation and NF- $\kappa$ B-regulated proteins at least in part through the

inhibition of I $\kappa$ B phosphorylation, especially in the conditions of advanced cancer where TNF- $\alpha$  is highly secreted.

## Introduction

Breast cancer is one of the most common causes of cancer-related mortality in women, and its incidence is steadily increasing (1,2). It is considered a highly metastatic cancer as a large portion of the patients frequently relapse with systemic dissemination of cancer even after radical extensive surgery (3). In addition, many patients succumb to this disease each year worldwide although many cancer therapeutic methods have been developed for the treatment of breast cancer. Therefore, new treatment strategies are needed for this disease.

Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) was used in Chinese medicine for solid cancer treatment, and is now being used as a standard treatment for refractory acute promyelocytic leukemia (APL) (4,5). Clinical trials with As<sub>2</sub>O<sub>3</sub> were performed in a certain type of solid cancers (6,7), but failed to prove clinical efficacy due to serious toxicities (8,9). Arsenic hexoxide (As<sub>4</sub>O<sub>6</sub>) has been used as a Korean folk remedy for cancer management since the late 1980s. There were scarce toxicities at the doses where As<sub>4</sub>O<sub>6</sub> was used as a Korean folk remedy for the solid and hematologic malignancies. However, few studies regarding the anticancer effects of As<sub>4</sub>O<sub>6</sub> have been performed. Only a few reports showed that the anticancer effects of As<sub>4</sub>O<sub>6</sub> were more potent than those of As<sub>2</sub>O<sub>3</sub> in human cancer cells *in vitro*, and that signaling pathways of As<sub>4</sub>O<sub>6</sub>-induced cell death were different from those of As<sub>2</sub>O<sub>3</sub> (10). We previously demonstrated that As<sub>4</sub>O<sub>6</sub> induced both caspase-dependent apoptosis and autophagic cell death in human cancer cells (11). In addition, As<sub>4</sub>O<sub>6</sub> has mostly been used for solid cancers in Korea. The anecdotal cases show some marked responses even in very advanced cancer.

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a cytokine involved in systemic inflammation and is produced chiefly by activated macrophages. The primary role of TNF consists in the regulation of immune cells. TNF is able to induce interleukin (IL) production, apoptotic cell death and inflammation to inhibit

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tumorigenesis and viral replication. TNF- $\alpha$  induces cell death through the extrinsic pathway in some cancer cells (12). However, most cancer cells are resistant to TNF- $\alpha$ -induced cell death by activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) followed by the enhanced transcription of anti-apoptotic proteins that interfere with cell death signaling (13). High serum level of TNF- $\alpha$  is more frequently observed in patients with advanced and metastatic cancer than in those with early stage cancer (14), and is closely related to cancer progression and patient quality of life (14,15). In addition, NF- $\kappa$ B is involved in drug resistance as well as metastasis (16). Therefore, NF- $\kappa$ B is a suitable therapeutic target for cancer treatment. If NF- $\kappa$ B is suppressed by less toxic drugs, TNF- $\alpha$  induces apoptosis of cancer cells. This will be an alternative approach to treat the patients with metastatic or advanced cancer without showing serious side-effects. We also found the synergism between TNF- $\alpha$  and As<sub>4</sub>O<sub>6</sub>. In the present study, we hypothesized that As<sub>4</sub>O<sub>6</sub> induced the synergism with TNF- $\alpha$  by the inhibition of NF- $\kappa$ B. Therefore, we explored the anticancer effects of As<sub>4</sub>O<sub>6</sub> with a particular focus on NF- $\kappa$ B and NF- $\kappa$ B-regulated gene products involved in cancer metastasis, and on NF- $\kappa$ B-mediated cellular responses in breast cancer cells.

## Materials and methods

**Cells and reagents.** MCF-7 human breast cancer cells from the American Type Culture Collection (Rockville, MD, USA) were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA), 1 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. As<sub>4</sub>O<sub>6</sub> was provided by the Chonjisan Institute (Seoul, Korea). Antibodies against procaspase-3, procaspase-8, COX-2, cyclin D1, c-Myc, Bcl-2, Bcl-xL, XIAP, cIAP-1, cIAP-2, MMP-2, MMP-9, VEGF and NF- $\kappa$ B (p65) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies against phospho-I $\kappa$ B $\alpha$  (Ser 32/36), and I $\kappa$ B, were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against poly(ADP-ribose) polymerase (PARP), LC3 and Beclin-1 were purchased from Pharmingen (San Diego, CA, USA). An antibody against  $\beta$ -actin was from Sigma (Beverly, MA, USA). Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulins, and an enhanced chemiluminescence (ECL) kit were purchased from Amersham (Arlington Heights, IL, USA). All other chemicals not specifically cited here were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All these solutions were stored at -20°C. Stock solutions of DAPI (100  $\mu$ g/ml) and propidium iodide (PI; 1 mg/ml) were prepared in phosphate-buffered saline (PBS).

**Cell viability assays.** For the cell viability assay, the cells were seeded onto 24-well plates at a concentration of 5x10<sup>5</sup> cells/ml, and then treated with the indicated concentration of As<sub>4</sub>O<sub>6</sub> for 24 or 48 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/ml) was subsequently added to each well. After 3 h of additional incubation, 100  $\mu$ l of a solution containing 10% SDS (pH 4.8) plus 0.01 N HCl was added to dissolve the crystals. The absorption values at 570 nm were determined with an ELISA plate reader.

**Nuclear staining.** After treatment with the indicated concentration of As<sub>4</sub>O<sub>6</sub>, the cells were harvested, washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with 2.5  $\mu$ g/ml 4,6-diamidino-2-phenylindole (DAPI) solution for 10 min at room temperature. The cells were washed twice with PBS and analyzed under a fluorescent microscope.

**Flow cytometry assay.** The cells were plated at a concentration of 1x10<sup>6</sup> cells/well in 6-well plates. Reduced (sub-G<sub>1</sub>) DNA content was measured by PI staining. The DNA content in each cell nucleus was determined with a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). Three independent experiments were performed (17).

**Western blot analysis.** Total cell lysates were obtained using lysis buffer containing 0.5% SDS, 1% NP-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5) and protease inhibitors. The concentrations of cell lysate proteins were determined by Bradford protein assay (Bio-Rad Laboratories, Richmond, CA, USA) using bovine serum albumin as the standard. To determine the protein expression of NF- $\kappa$ B in the cytoplasm and the nuclei, we prepared separate extracts. The cells were washed with ice-cold PBS (pH 7.4) and lysed in buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol (DTT), 5  $\mu$ M leupeptin, 2  $\mu$ M pepstatin A, 1  $\mu$ M aprotinin and 20  $\mu$ M phenylmethylsulfonyl fluoride] by repeated freezing and thawing. Nuclear and cytoplasmic fractions were separated by centrifugation at 1,000 x g for 20 min. The cytoplasmic extract (supernatant) was obtained. The pellets were washed with buffer A, and resuspended in buffer B [10 mM Tris-Cl (pH 7.5), 0.5% deoxycholate, 1% NP-40, 5 mM EDTA, 0.5 mM DTT, 5  $\mu$ M leupeptin, 2  $\mu$ M pepstatin A, 1  $\mu$ M aprotinin and 20  $\mu$ M phenylmethylsulfonyl fluoride]. The suspension was agitated for 30 min at 4°C and centrifuged at 10,000 x g for 20 min. The supernatant fraction containing nuclear proteins was collected. Molecular mass markers for proteins were obtained from Pharmacia Biotech (Saclay, France). Thirty micrograms of the lysate proteins were resolved by electrophoresis, electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), and then incubated with primary antibodies followed by secondary antibody conjugated to peroxidase. Blots were developed with an ECL detection system.

**Transfection.** NF- $\kappa$ B-luciferase constructs (consensus NF- $\kappa$ B binding sequence was cloned into the pGL3 basic luciferase expression vector) were kindly provided by Dr G. Koretzky (University of Pennsylvania). Transient transfection was performed using Lipofectamine (Gibco-BRL) according to the manufacturer's protocol.

**Luciferase assay.** After experimental treatments, cells were washed twice with cold PBS, lysed in a passive lysis buffer provided in the dual luciferase kit (Promega, Madison, WI, USA), and assayed for luciferase activity using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) according to the manufacturer's protocol. Data were presented as a ratio between Firefly and *Renilla* luciferase activities.

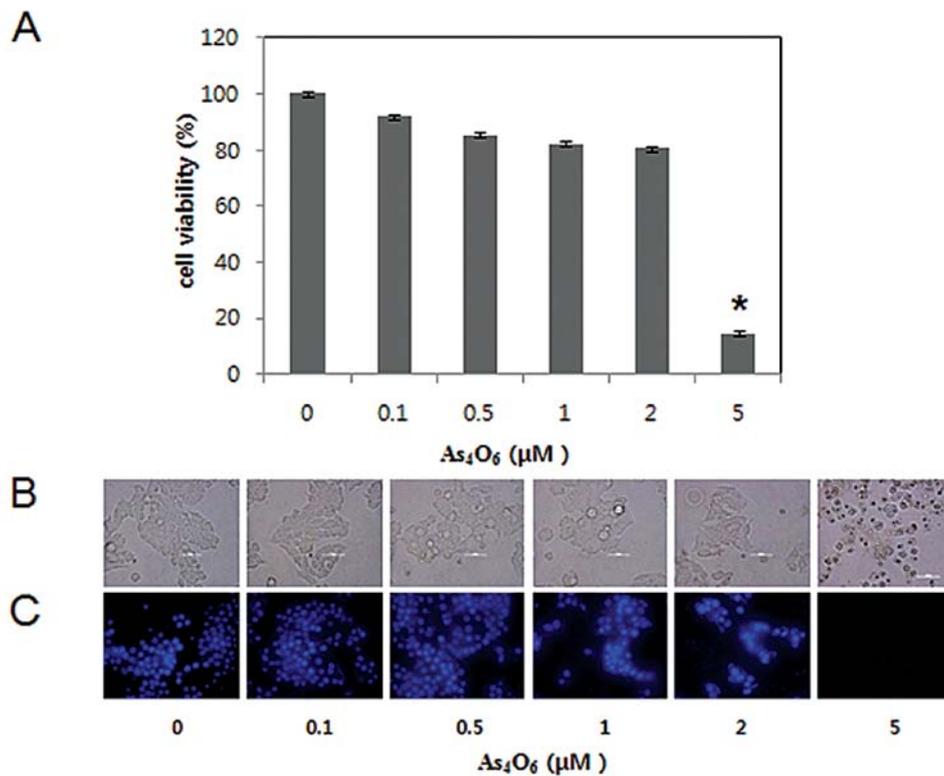


Figure 1. Inhibition of cell growth by As<sub>4</sub>O<sub>6</sub> in MCF-7 human breast cancer cells. The cells were seeded at the density of 5x10<sup>4</sup> cells/ml. The inhibition of cell growth was measured by MTT assay. The cells were treated with the indicated concentrations of As<sub>4</sub>O<sub>6</sub> for 24 h. (A) The growth inhibition and cytotoxicity of As<sub>4</sub>O<sub>6</sub> are shown at the concentration of 5 μM, and the 50% inhibition of cell growth (IC<sub>50</sub>) was >2 μM. (B) The cells were photographed by an inverted microscope (original magnification, x200). (C) After fixation, the cells were stained with DAPI solution to observe apoptotic body. Stained nuclei were then observed under a fluorescent microscope using a blue filter (original magnification, x400). The data are shown as means ± SD of three independent experiments. \*P<0.05 between the treated and the untreated control group.

**Statistical analysis.** Each experiment was performed in triplicate. The results are expressed as means ± SD. Significant differences were determined using the one-way ANOVA with post-test Neuman-Keuls in the cases of at least three treatment groups and Student's t-test for two-group comparison. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Effects of As<sub>4</sub>O<sub>6</sub> on cell growth in MCF-7 human breast cancer cells.** To investigate the antitumor activity of As<sub>4</sub>O<sub>6</sub> in MCF-7 cells, we performed MTT, light microscopic observation, and DAPI staining. Cells were treated for 48 h with various concentrations of As<sub>4</sub>O<sub>6</sub> (0.1–5 μM). The cell growth was assessed by MTT assay, which revealed that As<sub>4</sub>O<sub>6</sub> significantly inhibited the growth of MCF-7 cells at the concentration of 5 μM, and the 50% inhibition of cell growth (IC<sub>50</sub>) was >2 μM (Fig. 1A). To determine whether the decrease in cell growth of MCF-7 cells was related to induction of cell death and which type of cell death, we assessed the changes in nuclear morphology of As<sub>4</sub>O<sub>6</sub>-treated cells under microscopy with DAPI staining. The DAPI staining revealed that the condensed and fragmented nuclei observed at a concentration of ≥2 μM, and the amount of fragmented nuclei was substantially increased at the concentration of 5 μM (Fig. 1B). The present study suggests that As<sub>4</sub>O<sub>6</sub> induced cell death at the concentrations of >2 μM.

**Effects of As<sub>4</sub>O<sub>6</sub> on NF-κB and the IκBα phosphorylation.** NF-κB comprises a heterotrimer of p50, p65 and IκBα in the cytoplasm; when activated, the heterodimer of p50 and p65 is translocated into the nucleus. Using western blot analysis, we determined whether As<sub>4</sub>O<sub>6</sub> inhibited NF-κB activation at the 1 μM where no cytotoxicity was observed. We used TNF-α as an NF-κB stimulant to clearly demonstrate the effects of As<sub>4</sub>O<sub>6</sub> on NF-κB. Western blot analysis revealed that treatment with As<sub>4</sub>O<sub>6</sub> inhibited nuclear NF-κB (p65) activity whether TNF-α was co-treated or not. This finding indicated that As<sub>4</sub>O<sub>6</sub> had clear inhibitory effects on NF-κB activation (Fig. 2A). To confirm this finding, we performed the luciferase assay for NF-κB. As shown in Fig. 2B, the NF-κB-luciferase activity was augmented by TNF-α, which indicated that the NF-κB gene was successfully transfected into the cells. The NF-κB-luciferase activity induced by TNF-α was suppressed by As<sub>4</sub>O<sub>6</sub> (Fig. 2C).

NF-κB activation is known to require the degradation of inhibitory κBα (IκBα) through phosphorylation by kinases. Next, we tested whether As<sub>4</sub>O<sub>6</sub> suppressed TNF-α-induced phosphorylation of IκBα. The degradation of IκBα through phosphorylation was observed as early as 5 min after adding TNF-α, and 1-h pretreatment with As<sub>4</sub>O<sub>6</sub> delayed the TNF-α-induced phosphorylation of IκBα in MCF-7 cells (Fig. 2D). We also observed over 48 h the effects of As<sub>4</sub>O<sub>6</sub> on IκBα phosphorylation with the TNF-α-treated cells. We found that As<sub>4</sub>O<sub>6</sub> suppressed TNF-α-induced phosphorylation of IκBα

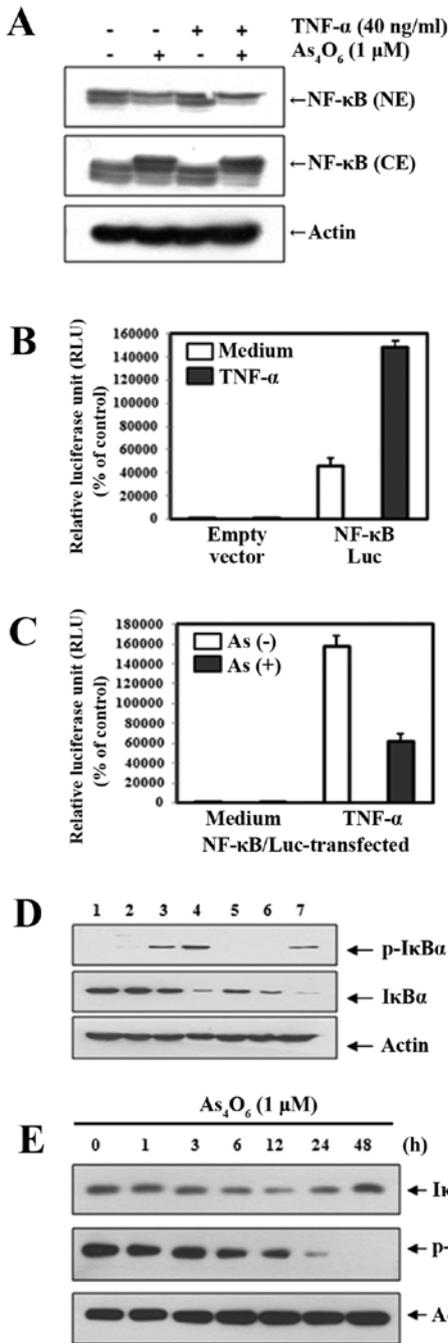


Figure 2. Effects of  $As_4O_6$  on NF- $\kappa$ B and the I $\kappa$ B $\alpha$  phosphorylation. (A) Inhibitory effects of  $As_4O_6$  on TNF- $\alpha$ -induced NF- $\kappa$ B translocation into the nucleus. Cells were pretreated with  $As_4O_6$  (1  $\mu$ M) for 1 h and then treated with TNF- $\alpha$  (10 ng/ml) for 30 min. After treatment, nuclear and cytoplasmic fractions were extracted from total cell lysates and protein levels were determined by western blot analysis. (B and C) Cells were transfected with an empty vector or 1  $\mu$ g of NF- $\kappa$ B-luciferase. (B) The cells were allowed to recover for 24 h and were then treated with 10 ng/ml of TNF- $\alpha$ . (C) The cells were allowed to recover for 24 h and were then treated with 10 ng/ml of TNF- $\alpha$  with/without 1-h pretreatment of  $As_4O_6$  (1  $\mu$ M). The cells were harvested 1 h post-treatment with TNF- $\alpha$  and luciferase activities are presented as fold activations relative to those of untreated controls. Each bar graph represents mean  $\pm$  SD of three independent experiments. \* $P$ <0.05 between the treated and the untreated control group. (D and E) Inhibitory effects of 1  $\mu$ M on I $\kappa$ B $\alpha$  phosphorylation. Cells were pretreated with  $As_4O_6$  (1  $\mu$ M) for 1 h and were then treated with TNF- $\alpha$  (10 ng/ml) for the indicated times. 1, control; 2-4, TNF- $\alpha$  alone group, 2, 5 and 10 min after TNF- $\alpha$  treatment; 5-7,  $As_4O_6$  + TNF- $\alpha$  group, 2, 5 and 10 min after TNF- $\alpha$  treatment. (E) Cells ( $5 \times 10^4$  cells) were treated with  $As_4O_6$  (1  $\mu$ M) for the indicated times in the cells pretreated with TNF- $\alpha$  (10 ng/ml) and lysed. Data are representative of three independent experiments.

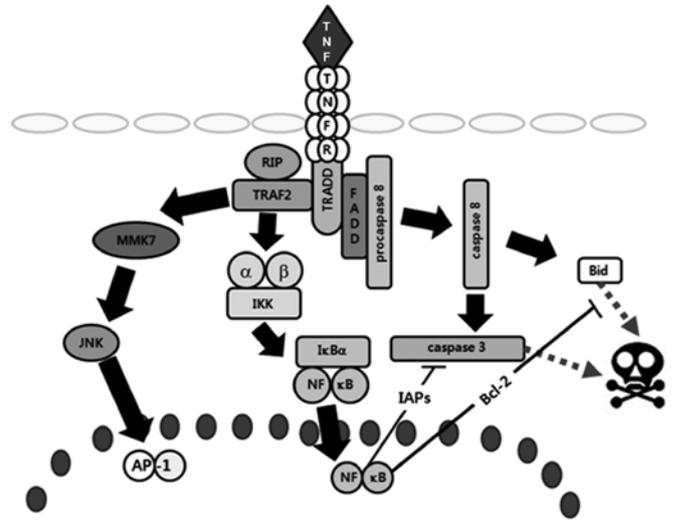


Figure 3. Schematic representation of TNF-R1 signaling pathways and the effects of  $As_4O_6$  on MCF-7 human breast cancer cells. When TNF- $\alpha$  binds TNF receptor 1 (TNF-R1), inhibitory protein SODD from the intracellular death domain is dissociated followed by the adaptor protein TRADD binding to the death domain. Subsequently, three pathways can be activated: the MAPK, the NF- $\kappa$ B and the death signaling pathway. Of the three major MAPK cascades, TNF- $\alpha$  generally induces JNK pathways involved in cell differentiation and proliferation. For activation of NF- $\kappa$ B pathways, TNF- $\alpha$  recruits TRAF2 and RIP through TRADD to activate IKK, then I $\kappa$ B $\alpha$  is phosphorylated by IKK, and finally activates NF- $\kappa$ B, which induces anti-apoptotic proteins (Bcl-2, Bcl-xL, XIAP, cIAP1 and cIAP2) to inhibit the death signaling pathways. For the induction of death signaling, FADD recruits procaspase-8 by binding to TRADD, and then procaspase-8 is activated. The activated caspase-8 leads to apoptosis through activation of caspase-3 and Bid. Data suggested that  $As_4O_6$  may augment TNF- $\alpha$ -induced death signaling pathways by suppressing I $\kappa$ B $\alpha$  phosphorylation.

and the effects became prominent 24 h after  $As_4O_6$  treatment (Fig. 2E). These findings suggest that  $As_4O_6$  inhibits NF- $\kappa$ B at least in part through suppression of the I $\kappa$ B $\alpha$  phosphorylation.

*As<sub>4</sub>O<sub>6</sub> suppresses NF- $\kappa$ B-related cellular responses.* We found that  $As_4O_6$  clearly inhibited NF- $\kappa$ B activity in both TNF- $\alpha$ -treated cells and control cells. TNF- $\alpha$  is known to be an NF- $\kappa$ B activator and to bind two receptors, TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2). Most information of TNF signaling regards TNF-R1 as TNF-R2 is only expressed in immune cells and the mechanism is barely understood. When TNF- $\alpha$  binds to TNF-R1, this binding leads to the adaptor protein TRADD to bind to the death domain. With this binding, three pathways can be initiated: NF- $\kappa$ B, MAPK and death signaling (18,19). The first one is involved in cell survival pathway and the others in pro-apoptotic or death pathway. The former pathway is related to NF- $\kappa$ B activation. Similar to all death-domain-containing members of the TNF receptor (TNFR) superfamily, TNF-R1 is also involved in death signaling at the same time (20). Therefore, the final results of TNF- $\alpha$  treatment are expressed as the sum of strength regarding TNF-induced cell death and NF- $\kappa$ B-related anti-apoptotic effects (Fig. 3). In the present study, we tested the combination effects of TNF- $\alpha$  and  $As_4O_6$  to observe whether  $As_4O_6$  augments the anticancer effects of TNF- $\alpha$  by suppressing NF- $\kappa$ B activity. As shown in Fig. 4A,  $As_4O_6$  potentiated the effects of TNF- $\alpha$  induced cell death. We then

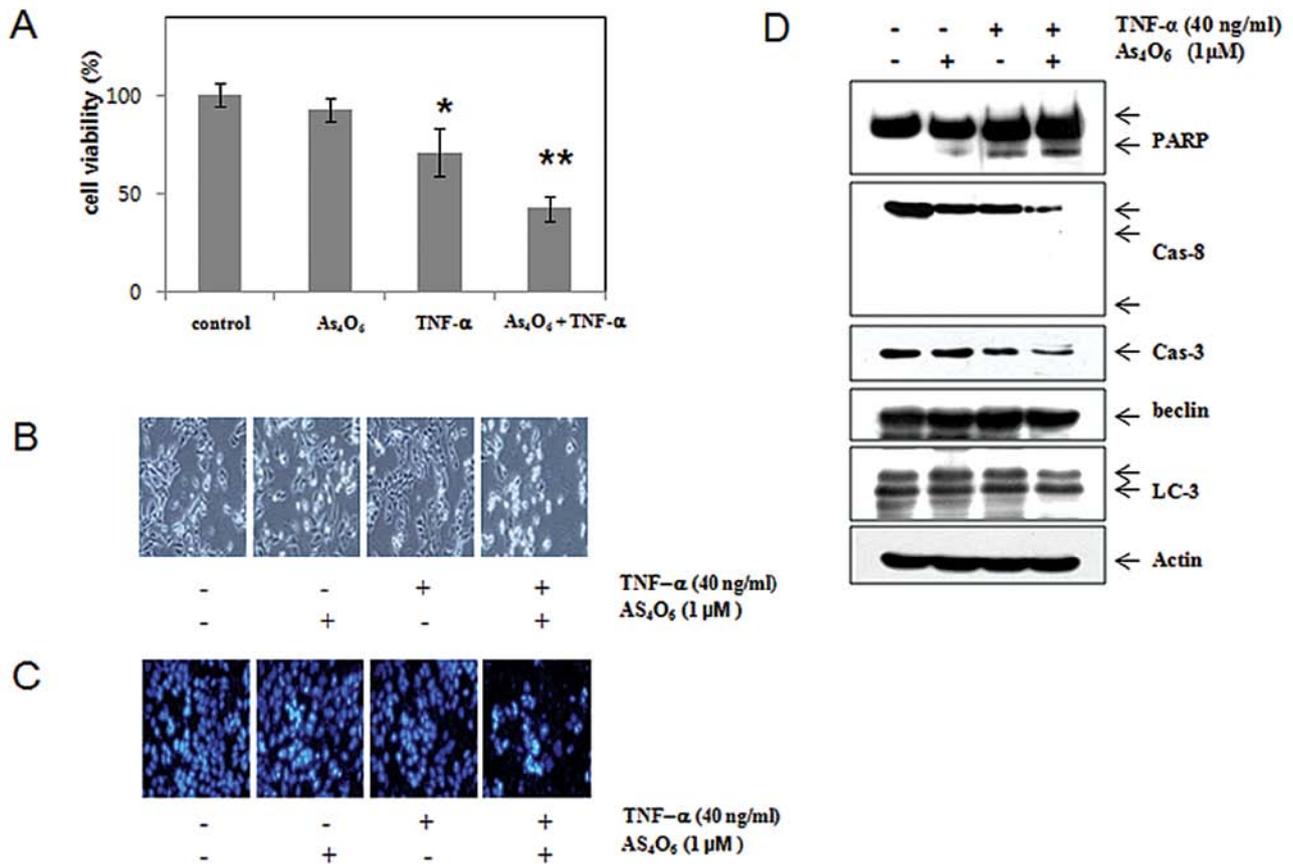


Figure 4. Effects of As<sub>4</sub>O<sub>6</sub> on NF-κB-related cellular responses. Cells (5x10<sup>4</sup> cells), either left untreated or pretreated with As<sub>4</sub>O<sub>6</sub> for 1 h, were exposed to TNF-α (10 nM) for the indicated times. (A) The cells were seeded at the density of 5x10<sup>4</sup> cells/ml. The inhibition of cell growth was measured by MTT assay. (B) Light microscopic observation. TNF in combination with As<sub>4</sub>O<sub>6</sub> significantly induced cell death. A large number of floating cells were observed (anoikis). (C) After fixation, the cells were stained with DAPI solution to observe apoptotic body. Stained nuclei were then observed under a fluorescent microscope using a blue filter (original magnification, x200). (D) Cells (5x10<sup>4</sup> cells), either left untreated or pretreated with As<sub>4</sub>O<sub>6</sub> for 1 h, were exposed to TNF-α (10 nM) for 24 h. Whole-cell extracts were prepared, and 30 μg of the whole-cell lysate was analyzed by western blot analysis using the indicated antibodies. The data are shown as means ± SD of three independent experiments. \*P<0.05 between the treated and the untreated control group. \*\*P<0.05 between the TNF-α and As<sub>4</sub>O<sub>6</sub> + TNF group.

assessed the effects of As<sub>4</sub>O<sub>6</sub> on caspases and their substrates (PARP). TNF-α in combination with As<sub>4</sub>O<sub>6</sub> significantly decreased the expression levels of procaspase-3, procaspase-8. With the decrease of procaspases, the cleavages of PARP were prominent in the combination treatment group (Fig. 4D). We also assessed the expression of LC-3 (a marker for autophagy) and Beclin-1 to examine whether the augmented cell death by As<sub>4</sub>O<sub>6</sub> is involved in type II programmed cell death, autophagy. Western blotting revealed that As<sub>4</sub>O<sub>6</sub> induced LC3 conversion (increase in the ratio of LC3-II/LC3-I), but that the combination with TNF-α did not augment LC3 conversion (Fig. 4D). These findings suggest that As<sub>4</sub>O<sub>6</sub> augments TNF-α-induced apoptosis through extrinsic pathways.

*As<sub>4</sub>O<sub>6</sub> suppresses NF-κB-regulated proteins involved in anti-apoptosis, proliferation, invasion and angiogenesis.* We also observed that As<sub>4</sub>O<sub>6</sub> inhibited NF-κB-related cellular response. NF-κB also regulates several genes involved in cancer metastasis. We investigated the effects of As<sub>4</sub>O<sub>6</sub> on the NF-κB-regulated proteins involved in anti-apoptosis, proliferation, invasion and angiogenesis. We found that TNF-α stimulated the NF-κB-regulated proteins involved in anti-

apoptosis (c-IAP1, c-IAP2, XIAP, Bcl-2 and Bcl-xL) (Fig. 5A), cancer cell proliferation (COX-2, c-Myc and cyclin D1) (Fig. 5B), and invasion and angiogenesis (MMP-2, MMP-9 and VEGF) (Fig. 5C). As<sub>4</sub>O<sub>6</sub> suppressed the TNF-α-induced NF-κB-regulated proteins involved in anti-apoptosis, proliferation, invasion and angiogenesis in MCF-7 cells (Fig. 5). These findings also support that As<sub>4</sub>O<sub>6</sub> suppresses NF-κB activity, and that it has anticancer properties especially in conditions of advanced or metastatic cancer where TNF-α is highly secreted from activated macrophages or cancer cells themselves.

## Discussion

With the assumption that As<sub>4</sub>O<sub>6</sub> inhibits NF-κB at a safe concentration, showing anticancer effects without serious side-effects in advanced cancer where TNF-α is highly secreted, we investigated the anticancer effects of As<sub>4</sub>O<sub>6</sub> with a particular focus on NF-κB pathway, NF-κB-regulated gene products, and NF-κB-mediated cellular responses in human breast cancer cells. In the present study, As<sub>4</sub>O<sub>6</sub> inhibited NF-κB activity and NF-κB-regulated proteins at the concentrations where no cytotoxicity was observed, indicating that it

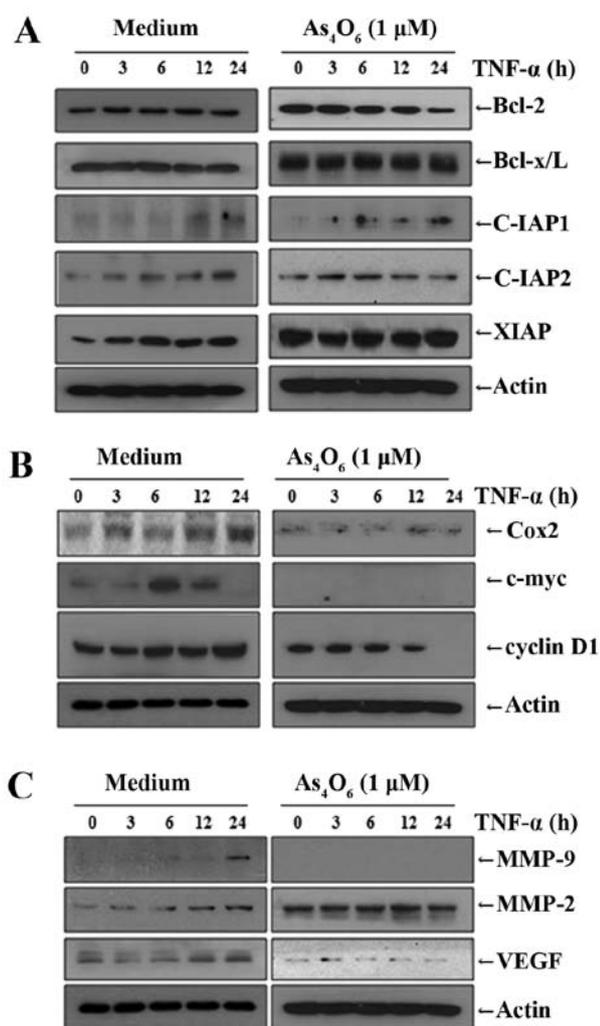


Figure 5. Effects of As<sub>4</sub>O<sub>6</sub> on NF- $\kappa$ B-regulated proteins involved in anti-apoptosis, proliferation, invasion and angiogenesis. Cells ( $5 \times 10^4$  cells), either left untreated or pretreated with As<sub>4</sub>O<sub>6</sub> for 1 h, were exposed to TNF- $\alpha$  (10 nM) for the indicated times. Whole-cell extracts were prepared, and 30  $\mu$ g of the whole-cell lysate was analyzed by western blot analysis using antibodies against various NF- $\kappa$ B-regulated proteins involved in (A) the anti-apoptosis, (B) cancer cell proliferation, and (C) invasion (MMP-2 and MMP-9), and angiogenesis (VEGF). Data are representative of two independent experiments.

can induce anticancer effects without definite side-effects and may be used for maintenance therapy. In addition, As<sub>4</sub>O<sub>6</sub> can be used in combination with conventional chemotherapeutics without increasing toxicity since the activation of NF- $\kappa$ B is one of the drug resistance mechanisms. Bortezomib is a good example; it is a proteasome inhibitor that has inhibitory effects on NF- $\kappa$ B by suppressing I $\kappa$ B $\alpha$  degradation (21); it can be used in combination with conventional agents and for maintenance therapy.

As<sub>4</sub>O<sub>6</sub> also augmented the TNF- $\alpha$ -induced cell death in MCF-7 cells. The evidence supports that arsenic compounds could suppress NF- $\kappa$ B activation (22,23). However, As<sub>4</sub>O<sub>6</sub> showed the inhibitory effect on NF- $\kappa$ B at a concentration showing no cytotoxicity. This is the first report regarding anti-NF- $\kappa$ B effects of As<sub>4</sub>O<sub>6</sub>.

TNF- $\alpha$  induces cell death through the extrinsic pathway in some cells, such as MCF-7 cells (12), but most cancer cell lines

are resistant to TNF- $\alpha$ -induced cell death as its death-inducing ability is weak and often masked by NF- $\kappa$ B activation followed by the enhanced transcription of anti-apoptotic proteins (13). In the present study, we demonstrated that As<sub>4</sub>O<sub>6</sub> enhanced the anticancer effects of TNF- $\alpha$  by inhibiting NF- $\kappa$ B. In TNF- $\alpha$ -resistant cells, As<sub>4</sub>O<sub>6</sub> in combination with TNF- $\alpha$  showed synergism (data not shown). TNF- $\alpha$  is generally increased in patients with advanced and metastatic cancer and is associated with cancer progression and patient quality of life (14,15). Furthermore, TNF- $\alpha$  is abundantly released in chronic inflammatory disorders including rheumatoid arthritis as well, and a TNF- $\alpha$  inhibitor is used to control the chronic inflammatory disorders (24). However, the direct inhibition of TNF- $\alpha$  raises some suspicion that it may cause cancer development in chronic inflammatory disorders (24). The present study suggests that TNF- $\alpha$  can be used as a therapeutic tool by inhibiting NF- $\kappa$ B activation in advanced and metastatic cancers.

In the present study, we also investigated the inhibitory effects of As<sub>4</sub>O<sub>6</sub> on MMP-2 and MMP-9 expression in TNF- $\alpha$ -treated cells. MMP-2 and MMP-9 are key molecules in cancer cell invasion (25,26) which have been targets for drug development against cancer invasion (27). We also found that As<sub>4</sub>O<sub>6</sub> suppressed COX-2, cyclin D1 and c-Myc involved in cell proliferation. COX-2 is overexpressed in a variety of cancers and mediates cancer cell proliferation (13,28,29) and c-Myc is also involved in cancer cell proliferation (30). In addition, VEGF is an angiogenic factor (13). Both are important in metastasis and are regulated by NF- $\kappa$ B (13,28). The I $\kappa$ B family consists of I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\epsilon$  and Bcl-3. Among them, I $\kappa$ B $\alpha$  is the most extensively studied and major I $\kappa$ B protein. NF- $\kappa$ B activation is initiated by the degradation of I $\kappa$ B $\alpha$  protein which is an inhibitor of NF- $\kappa$ B. The degradation of I $\kappa$ B $\alpha$  occurs through the activation of I $\kappa$ B kinase (IKK). When activated by signals, the I $\kappa$ B kinase phosphorylates two serine residues located in an I $\kappa$ B $\alpha$  regulatory domain. When phosphorylated I $\kappa$ B $\alpha$  at serines 32 and 36, the I $\kappa$ B $\alpha$  is degraded by ubiquitination (31). Here, we found that As<sub>4</sub>O<sub>6</sub> suppressed phosphorylation of I $\kappa$ B $\alpha$ . This finding suggests that the anti-NF- $\kappa$ B activities of As<sub>4</sub>O<sub>6</sub> are contributed by suppression of I $\kappa$ B $\alpha$  phosphorylation.

In conclusion, the present study demonstrated that As<sub>4</sub>O<sub>6</sub> has anticancer properties by inhibiting NF- $\kappa$ B activation and NF- $\kappa$ B-regulated proteins at least in part through the inhibition of I $\kappa$ B phosphorylation, especially in conditions of advanced or metastatic cancer where TNF- $\alpha$  is highly secreted (Fig. 3). The present study provides evidence that As<sub>4</sub>O<sub>6</sub> may have anticancer effects through inhibiting NF- $\kappa$ B activity in human breast cancer.

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