

# Tetraarsenic Hexoxide Demonstrates Anticancer Activities at Least in Part through Suppression of NF- $\kappa$ B-mediated Cellular Responses in Hep3B Human Hepatocellular Carcinoma Cells

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Tetraarsenic hexoxide (As<sub>4</sub>O<sub>6</sub>) has been used in Korean folk remedy for the treatment of cancer since the late 1980's, and arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) is currently used as a chemotherapeutic agent. However, evidence suggests that As<sub>4</sub>O<sub>6</sub>-induced cell death pathway was different from that of As<sub>2</sub>O<sub>3</sub>. Nuclear factor- $\kappa$  B (NF- $\kappa$  B) is a well-known transcription factor involved in cell proliferation, invasion and metastasis. Here, we investigated the effects of As<sub>4</sub>O<sub>6</sub> on NF- $\kappa$  B activity and NF- $\kappa$  B-regulated gene expressions. As<sub>4</sub>O<sub>6</sub> partially suppressed NF- $\kappa$  B activation induced by TNF- $\alpha$ , and inhibited cell proliferation induced by TNF. As<sub>4</sub>O<sub>6</sub> also suppressed the downstream NF- $\kappa$  B-regulated proteins involved in cancer anti-apoptosis, proliferation, invasion, and metastasis. This study demonstrates that As<sub>4</sub>O<sub>6</sub> have anticancer properties through suppression of NF- $\kappa$  B-mediated cellular responses. (*Cancer Prev Res* 17, 331-337, 2012)

**Key Words:** Tetraarsenic hexoxide, Nuclear factor- $\kappa$  B, Apoptosis, Hepatocellular carcinoma

## INTRODUCTION

Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) had been used in Chinese medicine for cancer treatment, and is now used as standard the treatment for refractory acute promyelocytic leukemia (APL).<sup>1,2)</sup>

Some clinical trials was performed in a certain type of solid cancers,<sup>3,4)</sup> but they failed to prove clinical efficacy due to its toxicities.<sup>5,6)</sup> Tetraarsenic hexoxide (As<sub>4</sub>O<sub>6</sub>) had been used as a Korean folk remedy for the management of cancer since the late 1980's. However, few researches regarding the anticancer effects of As<sub>4</sub>O<sub>6</sub> have been executed. A previous study showed

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that the anticancer effects of As<sub>4</sub>O<sub>6</sub> was 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>.<sup>7)</sup> We previously demonstrated that As<sub>4</sub>O<sub>6</sub> induced both caspase-dependent apoptosis and Beclin-1-induced autophagic cell death in human leukemic cell, suggesting that As<sub>4</sub>O<sub>6</sub> differ from As<sub>2</sub>O<sub>3</sub>.<sup>8)</sup> In addition, As<sub>4</sub>O<sub>6</sub> had mostly been used for solid cancer in Korea. Hepatocellular carcinoma is one of the most frequent cancers in the world.<sup>9)</sup> For the treatment, surgical resection is frequently limited due to metastasis like most of other cancers even though the hepatic reservoirs are suitable for the surgery. In addition, few chemotherapeutic drugs are available for the treatment of metastatic lesions. Therefore, the development of chemotherapeutic agents is urgent for this disease. Nuclear factor- $\kappa$  B (NF- $\kappa$  B) is a well-known transcription factor linked with carcinogenic process including oxidative and inflammatory process, cell survival, and proliferation and metastasis.<sup>10)</sup> Here, we explored the anti-cancer effects of As<sub>4</sub>O<sub>6</sub> with special focus on NF- $\kappa$  B pathway, on NF- $\kappa$  B-regulated gene products, and on NF- $\kappa$  B-mediated cellular responses.

## MATERIALS AND METHODS

### 1. Cells and reagents

Hep3B human hepatocellular carcinoma cells from the American type culture collection (Rockville, MD, USA) were cultured in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 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 from Chonjisan Institute (Seoul, Korea). Antibodies against COX-2, cyclin D1, c-Myc, Bcl-2, Bcl-xL, XIAP, cIAP-1, cIAP-2, MMP-2, MMP-9, VEGF, ICAM-1, poly (ADP-ribose) polymerase (PARP), procaspase 3, and procaspase 9, and NF- $\kappa$  B (p65) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, 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).

1) MTT assay: For the cell viability assay, the cells were

seeded onto 24-well plates at a concentration of  $5 \times 10^5$  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.

### 2. DAPI staining

After treatment with the indicated concentration of As<sub>4</sub>O<sub>6</sub>, the cells were harvested, washed with phosphate-buffered saline (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 two times with PBS and analyzed under a fluorescent microscope.

### 3. Western blotting

The concentrations of cell lysate proteins were determined by Bradford protein assay (Biorad Lab., 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. 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.

### 4. Determination of caspase activity

Caspase activities were determined by colorimetric assays using caspase-3 activation kits under the manufacturer's instructions. The kits include synthetic tetrapeptides labeled with p-nitroanilide. Briefly, the cells were lysed with the provided lysis buffer. The supernatants were collected and incubated with the supplied reaction buffer containing dithiothreitol and substrates at 37°C. The caspase activity was determined by assessing the absorbance at 405 nm, using the microplate reader.

### 5. Statistics

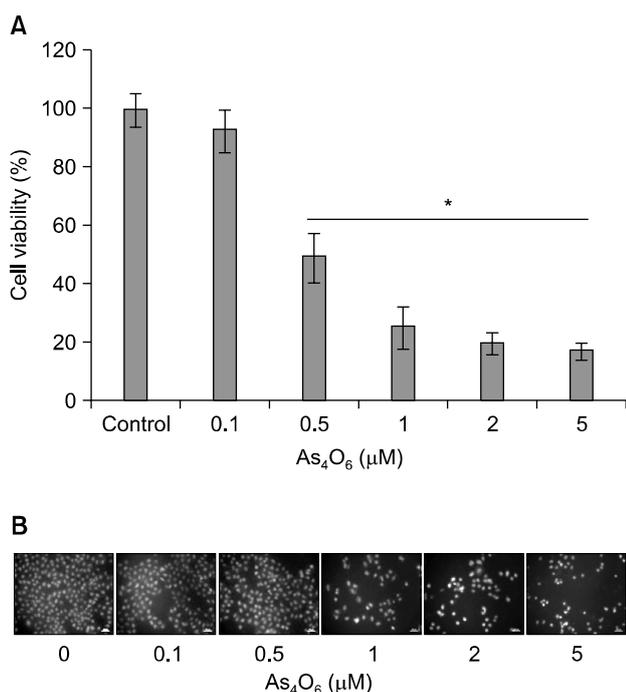
Each experiment was performed in triplicate. The results were expressed as means $\pm$ SD. Significant differences were de-

terminated using the one-way ANOVA with post-test Neuman-Keuls in the cases at least three treatment groups and Student's *t* test for two group comparison. Statistical significance was defined as  $p < 0.05$ .

## RESULTS

### 1. Effects of As<sub>4</sub>O<sub>6</sub> on cell growth in Hep3B human hepatocellular carcinoma cells

To investigate the anti-tumor activity of As<sub>4</sub>O<sub>6</sub> in Hep3B cells, cells were treated for 48 h with various concentrations of As<sub>4</sub>O<sub>6</sub> (0.1~5  $\mu$ M). The cell growth was assessed by MTT assay, which revealed that by As<sub>4</sub>O<sub>6</sub> inhibited the growth of Hep3B cells in a dose-dependent manner, and the 50% inhibition of cell growth (IC<sub>50</sub>) was less than 0.5  $\mu$ M (Fig. 1A).



**Fig. 1.** Inhibition of cell growth and induction of apoptosis by As<sub>4</sub>O<sub>6</sub> in Hep3B cells. The cells were seeded at the density of  $5 \times 10^4$  cells per ml. The inhibition of cell growth was measured by MTT assay. (A) The cells were treated with the indicated concentrations of As<sub>4</sub>O<sub>6</sub> for 48 h. The growth inhibition and cytotoxicity As<sub>4</sub>O<sub>6</sub> are shown in a dose-dependent manner. (B) After fixation, the cells were stained with DAPI solution to observe apoptotic body. Stained nuclei were then observed under fluorescent microscope using a blue filter (Magnification,  $\times 400$ ). The data are shown as means  $\pm$  SD of three independent experiments. \* $p < 0.05$  between the treated and the untreated control group.

### 2. Effects of As<sub>4</sub>O<sub>6</sub> on apoptosis

To determine whether the decrease in cell growth of Hep3B 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 the condensed and fragmented nuclei at a concentration of 1  $\mu$ M or higher. However, the amount of fragmented nuclei was substantially increased in a dose-dependent manner (Fig. 1B). This study suggests that As<sub>4</sub>O<sub>6</sub>-induced cell death is related to apoptosis.

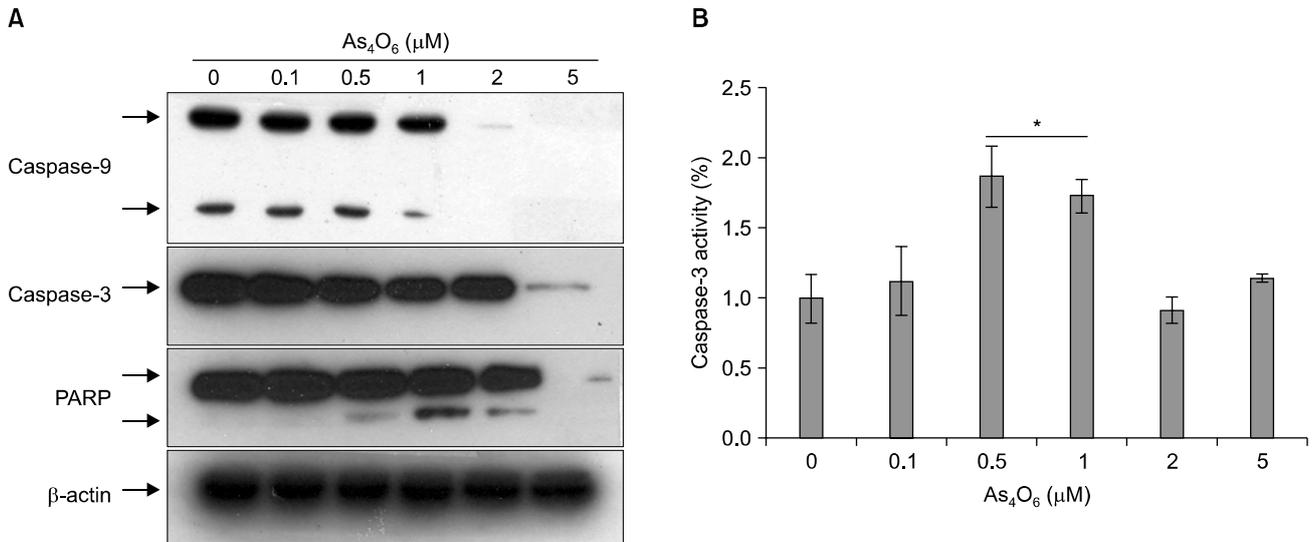
### 3. Caspases activation and subsequent cleavage of their substrates by As<sub>4</sub>O<sub>6</sub>

To determine whether As<sub>4</sub>O<sub>6</sub> induced caspase-dependent apoptosis, we assessed the effects of As<sub>4</sub>O<sub>6</sub> on caspases and their substrates (PARP). As<sub>4</sub>O<sub>6</sub> decreased the expression levels of procaspase-3, and procaspase-9, which indicated caspase activation. We also found the cleavages of PARP in a dose-dependent manner (Fig. 2A). In order to further quantify the proteolytic activation of caspases, the equalized lysates were assayed for their activity using the colorimetric assay kit for caspase-3 activity, and we found that As<sub>4</sub>O<sub>6</sub> caused substantially increases in proteolytic activities of caspase 3. This finding is consistent with data from Western blot analysis (Fig. 2B). The decrease of caspase 3 observed over the concentration of 1  $\mu$ M (IC<sub>50</sub>=0.5  $\mu$ M) may be derived from cell death. These findings suggest that As<sub>4</sub>O<sub>6</sub> may induce apoptotic death through a caspase-dependent pathway.

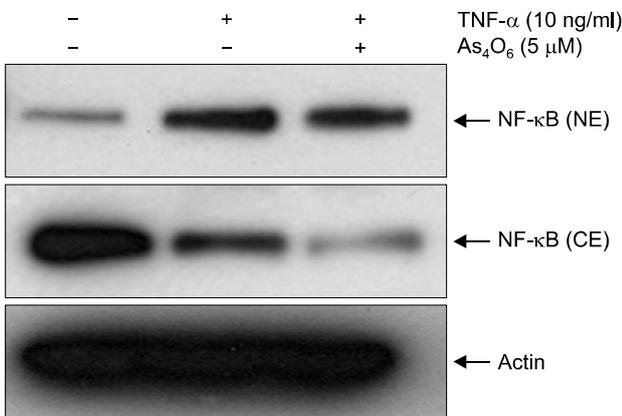
### 4. Effects of As<sub>4</sub>O<sub>6</sub> on NF- $\kappa$ B and NF- $\kappa$ B-related cellular responses.

NF- $\kappa$ B comprises a heterotrimer of p50, p65, and I $\kappa$ B  $\alpha$  in the cytoplasm; when activated, the heterodimer of p50 and p65 is translocated into the nucleus. NF- $\kappa$ B activation pathway is involved in the cancer proliferation, invasion, and metastasis.<sup>11,12</sup> Here, we investigated whether As<sub>4</sub>O<sub>6</sub> inhibit NF- $\kappa$ B activation using Western blot analysis. We used TNF- $\alpha$  as a NF- $\kappa$ B stimulant to clearly demonstrate the effects of As<sub>4</sub>O<sub>6</sub> on NF- $\kappa$ B. Western blot analysis revealed that pre-treatment with As<sub>4</sub>O<sub>6</sub> partially inhibited NF- $\kappa$ B (p65) translocation into the nucleus, which indicated the inhibition of NF- $\kappa$ B activation caused by TNF- $\alpha$  (Fig. 3).

When TNF- $\alpha$  bind to TNF receptors, this binding lead to



**Fig. 2.** Activation of caspases and cleavage of PARP during the As<sub>4</sub>O<sub>6</sub>-induced apoptosis in Hep3B cells. (A) The cells were incubated at the indicated concentrations of As<sub>4</sub>O<sub>6</sub> for 24 h. Total cell lysates were resolved by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the anti-caspase-3, anti-caspase-9 and anti-PARP antibodies. The proteins were visualized using an ECL detection system. β-Actin was used as an internal control. (B) The cell lysates from the cells treated with As<sub>4</sub>O<sub>6</sub> were assayed for *in vitro* caspase-3 activity using DEVD-pNA as a substrate. The released fluorescent products were measured. Each bar graph represents mean±SD of three independent experiments. \*p<0.05 between the treated and the untreated control group.



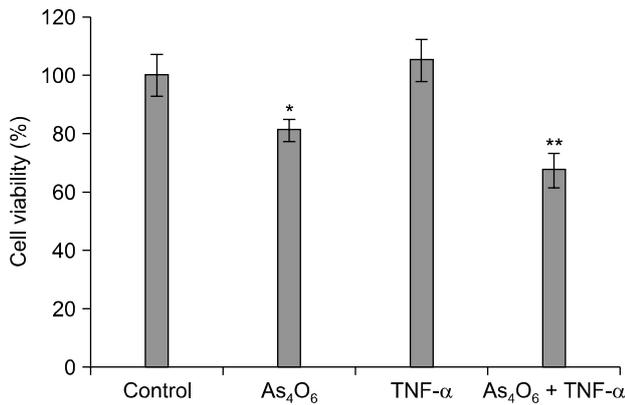
**Fig. 3.** Effects of As<sub>4</sub>O<sub>6</sub> on NF-κB. Cells (5×10<sup>4</sup> cells), either left untreated or pretreated with As<sub>4</sub>O<sub>6</sub> for 1 h, were exposed to TNF (10 nM) for 30 min. After treatment, nuclear or cytoplasmic fractions or total cell lysates were extracted, and protein levels were determined by Western blot analysis as described in Materials and Methods.

a conformational change which enables the adaptor protein TRADD to bind to the death domain. After that, three pathways can be initiated. One is survival pathway and the others are proapoptotic or death pathway. The former pathway is related to NF-κB activation. Like all death-domain-containing members of the TNFR superfamily, TNF-R1 is involved in

death signaling.<sup>13)</sup> However, TNF-induced cell death plays only a minor role compared to its overwhelming functions because its death-inducing capability is weak and often masked by the anti-apoptotic effects of NF-κB. Therefore, to confirm whether this finding is related to cancer cell proliferation, we performed MTT assay, because if As<sub>4</sub>O<sub>6</sub> suppresses NF-κB activity, TNF-α should augment the anti-cancer effects of As<sub>4</sub>O<sub>6</sub>. As shown in Fig. 4, TNF-α alone did not show anti-proliferative effects but TNF-α augmented the anti-cancer effects of As<sub>4</sub>O<sub>6</sub>. This study suggests that As<sub>4</sub>O<sub>6</sub> inhibit NF-κB activation.

**5. As<sub>4</sub>O<sub>6</sub> suppresses NF-κB-regulated proteins involved in anti-apoptosis, proliferation, invasion and angiogenesis.**

We observed that As<sub>4</sub>O<sub>6</sub> inhibited NF-κB activation. NF-κB activation leads to activation of several genes involved in anti-apoptosis, proliferation, invasion and angiogenesis in cancer. NF-κB regulates the expression of the anti-apoptotic proteins (c-IAP1/2, XIAP, Bcl-2, Bcl-xL).<sup>14)</sup> Cyclin D1 and COX-2 are overexpressed in a variety of cancers and mediates cancer cell proliferation<sup>15)</sup> and c-Myc is also involved in cancer cell proliferation.<sup>16)</sup> The roles of MMP-2, MMP-9, ICAM-1, and VEGF



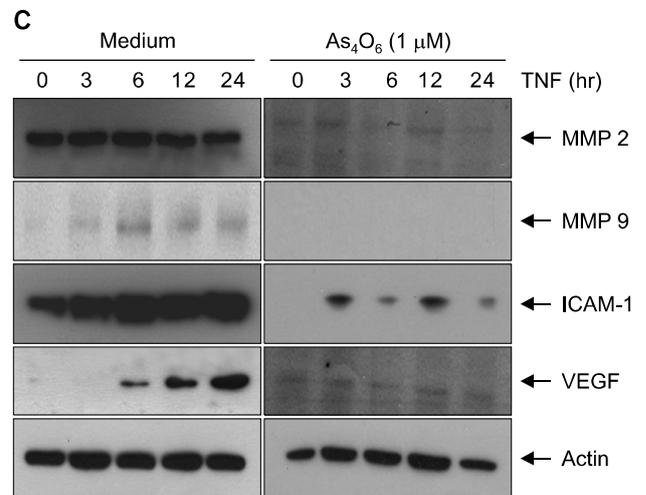
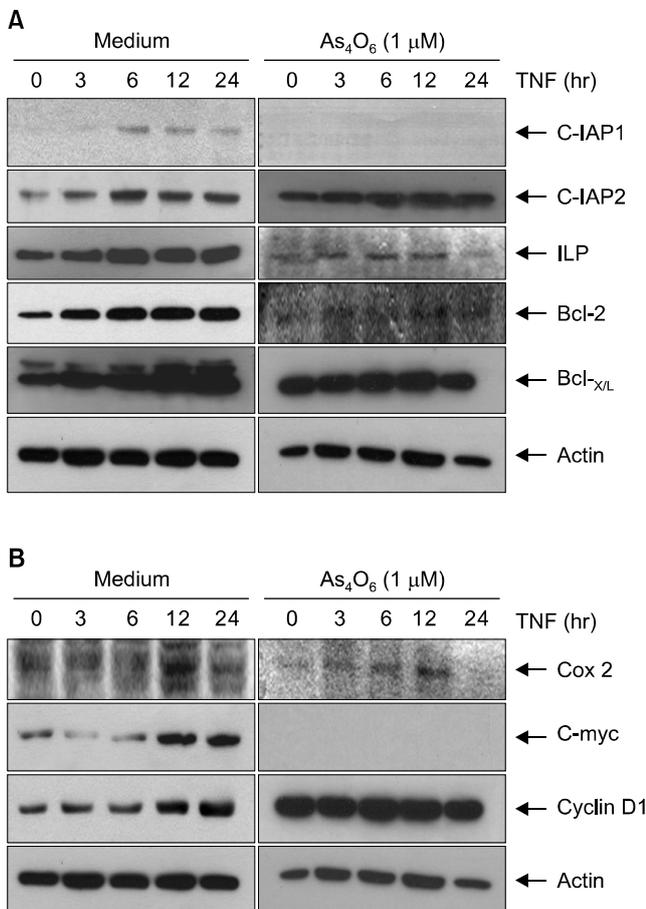
**Fig. 4.** Effects of As<sub>4</sub>O<sub>6</sub> on NF-κB-related cellular responses. Cells (5×10<sup>4</sup> cells), either left untreated or pretreated with As<sub>4</sub>O<sub>6</sub> for 1 h, were exposed to TNF-α (10 nM) for indicated times. The cells were seeded at the density of 5×10<sup>4</sup> cells per ml. The inhibition of cell growth was measured by MTT assay. Stained nuclei were then observed under fluorescent microscope using a blue filter (Magnification, ×400). 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 As<sub>4</sub>O<sub>6</sub> and As<sub>4</sub>O<sub>6</sub> +TNF group.

in invasion and angiogenesis of cancer are well known. All these gene products are known to be regulated by NF-κB,<sup>11)</sup> so we investigated the effect of As<sub>4</sub>O<sub>6</sub> on this regulation.

TNF-α is known to be linked to the activation of these genes. To investigate the effects of As<sub>4</sub>O<sub>6</sub> on the NF-κB-regulated proteins, we performed Western blot analysis. Here, we also use TNF-α as a stimulator. 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), cancer cell proliferation (COX-2, c-Myc and cyclin D1), and invasion & angiogenesis (MMP-2, MMP-9, ICAM-1, and VEGF) (Fig 5). 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 Hep3B cells (Fig. 5). These finding also support that As<sub>4</sub>O<sub>6</sub> NF-κB-mediated cellular responses.

### DISCUSSION

This study was designed to investigate the anti-cancer effects



**Fig. 5.** Effects of As<sub>4</sub>O<sub>6</sub> on NF-κB-regulated proteins involved in anti-apoptosis, proliferation, invasion, and angiogenesis. Cells (5×10<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 indicated times. Whole-cell extracts were prepared, and 30 μg of the whole-cell lysate was analyzed by Western blot using antibodies against various NF-κB-regulated proteins involved in (A) cancer cell proliferation, (B) the anti-apoptosis and (C) invasion & angiogenesis

of As<sub>4</sub>O<sub>6</sub> with special focus on NF- $\kappa$ B pathway, on NF- $\kappa$ B-regulated gene products, and on NF- $\kappa$ B-mediated cellular responses. We found that As<sub>4</sub>O<sub>6</sub> inhibited NF- $\kappa$ B activity and NF- $\kappa$ B-regulated proteins involved in anti-apoptosis, cell proliferation, invasion, and angiogenesis. Even though this finding is novel for As<sub>4</sub>O<sub>6</sub>, and there are previous supporting evidence showing arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) could suppress NF- $\kappa$ B activation.<sup>17)</sup> TNF- $\alpha$  induces cell death through extrinsic pathway in some cells,<sup>18)</sup> but most of cancer cell lines are resistant to TNF- $\alpha$ -induced cell death by activation of NF- $\kappa$ B followed by the enhanced transcription of anti-apoptotic proteins like cIAP1/cIAP2, C-FLIP, and Bcl-2 that can interfere with death signaling.<sup>19)</sup> Therefore, like Hep3B cells, TNF- $\alpha$  indeed augments cell proliferation in most of cancer cells.<sup>16,19)</sup> In addition, TNF- $\alpha$  is usually increased in patients with advanced cancers.<sup>20)</sup> Therefore, pathophysiological relevance of TNF- $\alpha$ -induced NF- $\kappa$ B activation is underlined in NF- $\kappa$ B-related study in cancer field. Here, we clearly demonstrate the inhibitory effects of As<sub>4</sub>O<sub>6</sub> by using TNF- $\alpha$  on the molecules involved in metastasis, especially on the molecules related to NF- $\kappa$ B.

NF- $\kappa$ B is a well known transcription factor involved in cancer metastasis. We found that As<sub>4</sub>O<sub>6</sub> suppressed MMP-2 and MMP-9 activity. MMP-2 and MMP-9 are key molecules in cancer cell invasion<sup>21,22)</sup> which have been targets for drug development against cancer invasion.<sup>23)</sup> We also found that As<sub>4</sub>O<sub>6</sub> suppressed COX-2, Cyclin D1, and c-Myc. COX-2 are well-known to be overexpressed in a variety of cancers and mediates cancer cell proliferation<sup>14,15,24)</sup> and c-Myc is also involved in cancer cell proliferation.<sup>16)</sup> In addition the roles of ICAM-1, and VEGF in angiogenesis of cancer are well known.<sup>14)</sup> All these gene products are known to be regulated by NF- $\kappa$ B.<sup>14,24)</sup> In conclusion, this study suggested that As<sub>4</sub>O<sub>6</sub> should exert anticancer effects by suppressing NF- $\kappa$ B and NF- $\kappa$ B-regulated genes involved in anti-apoptosis, proliferation, and invasion & angiogenesis in cancer. This study provides evidence that As<sub>4</sub>O<sub>6</sub> might have anticancer effects on human hepatocellular carcinoma.

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