

# Tetraarsenic oxide–induced inhibition of malignant glioma cell invasion in vitro via a decrease in matrix metalloproteinase secretion and protein kinase B phosphorylation

## Laboratory investigation

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**Object.** Local invasiveness of malignant glioma is a major reason for the failure of current treatments including surgery and radiation therapy. Tetraarsenic oxide ( $As_4O_6$  [TAO]) is a trivalent arsenic compound that has potential anticancer and antiangiogenic effects in selected cancer cell lines at a lower concentration than arsenic trioxide ( $As_2O_3$  [ATO]), which has been more widely tested in vitro and in vivo. The authors tried to determine the cytotoxic concentration of TAO in malignant glioma cell lines and whether TAO would show anti-invasive effects under conditions independent of cell death or apoptosis.

**Methods.** The human phosphatase and tensin homolog (PTEN)-deficient malignant glioma cell lines U87MG, U251MG, and U373MG together with PTEN-functional LN428 were cultured with a range of micromolar concentrations of TAO. The invasiveness of the glioma cell lines was analyzed. The effect of TAO on matrix metalloproteinase (MMP) secretion and membrane type 1 (MT1)-MMP expression was measured using gelatin zymography and Western blot, respectively. Akt, or protein kinase B, activity, which is a downstream effector of PTEN, was assessed with a kinase assay using glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) as a substrate and Western blotting of phosphorylated Akt.

**Results.** Tetraarsenic oxide inhibited 50% of glioma cell proliferation at 6.3–12.2  $\mu$ M. Subsequent experiments were performed under the same TAO concentrations and exposure times, avoiding the direct tumoricidal effect of TAO, which was confirmed with apoptosis markers. An invasion assay revealed a dose-dependent decrease in invasiveness under the influence of TAO. Both the gelatinolytic activity of MMP-2 and MT1-MMP expression decreased in a dose-dependent manner in all cell lines, which was in accordance with the invasion assay results. The TAO decreased kinase activity of Akt on GSK-3 $\beta$  assay and inhibited Akt phosphorylation in a dose-dependent manner in all cell lines regardless of their PTEN status.

**Conclusions.** These results showed that TAO effectively inhibits proliferation of glioblastoma cell lines and also exerts an anti-invasive effect via decreased MMP-2 secretion, decreased MT1-MMP expression, and the inhibition of Akt phosphorylation under conditions devoid of cytotoxicity. Further investigations using an in vivo model are needed to evaluate the potential role of TAO as an anti-invasive agent.

(<http://thejns.org/doi/abs/10.3171/2014.8.JNS131991>)

**KEY WORDS** • glioblastoma • tetraarsenic oxide • invasion •  
matrix metalloproteinase • protein kinase B • oncology

**G**LIOMASTOMA (GBM) is notoriously refractory to all current therapeutic modalities, and the median survival of patients with this tumor is 15 months.<sup>40</sup> Neuroimaging modalities, such as CT and/or MRI, have

*Abbreviations used in this paper:* AP-1 = activator protein-1; ATO = arsenic trioxide; BBB = blood-brain barrier; ECM = extracellular matrix; EGTA = ethylene glycol tetraacetic acid; FACS = flow-activated cell sorter; GBM = glioblastoma; GSK-3 $\beta$  = glycogen synthase kinase-3 $\beta$ ; IC<sub>50</sub> = 50% inhibitory concentration; MMP = matrix metalloproteinase; MT1 = membrane type 1; PBS = phosphate-buffered saline; phospho-Akt = phosphorylated active Akt; PI3K = phosphoinositide 3-kinase; PTEN = phosphatase and tensin homolog; ROS = reactive oxygen species; SDS = sodium dodecyl sulfate; SDS-PAGE = SDS–polyacrylamide gel electrophoresis; TAO = tetraarsenic oxide.

revealed that the recurrence of GBM after surgery and radiation therapy is confined to within 2 cm of the primary site, and that only 5% of patients show distant recurrence after combined treatments including chemotherapy.<sup>1,42</sup> These results suggest that the main cause of treatment failure is local tumor invasion of surrounding brain tissue. The specific molecular mechanism facilitating the invasive behavior of malignant glioma cells largely remains unclear. However, it is necessary for glioma cells to degrade multiple elements of the extracellular matrix (ECM) to invade and spread through surrounding normal tissue. Among the various proteolytic enzymes secreted by tumor cells, matrix metalloproteinase (MMP), particularly MMP-2 and MMP-9, partially accounts for the proteolytic capacity of GBM, which in turn explains its

invasiveness. The expression and proteolytic activities of these MMPs are upregulated in more aggressive forms of brain tumors including gliomas.<sup>9,35</sup> Matrix metalloproteinase activities quantitatively increase in proportion to the invasiveness and malignancy of a brain tumor.<sup>29</sup> For example, MMP-2 is overexpressed particularly in malignant gliomas as compared with low-grade gliomas.<sup>30</sup>

Akt, or protein kinase B, a serine/threonine-protein kinase, plays a critical role in controlling the balance between cell survival and apoptosis, and the cellular oncogene *c-Akt* is overexpressed in various cancers and may be associated with tumor aggressiveness.<sup>3,11</sup> Protein kinase B is a core component of the phosphoinositide 3-kinase (PI3K) signaling pathway. Akt promotes cancer cell invasion by increasing motility and metalloproteinase production.<sup>19</sup> Specific inhibitors of PI3K, such as LY294002, decrease the invasiveness of GBM cell lines by disrupting Akt phosphorylation and MMP activation.<sup>23</sup>

Arsenic derivative compounds have been used as pharmaceutical agents in a variety of diseases for more than 2000 years.<sup>17</sup> These applications have received renewed attention recently given the successful clinical application of arsenic trioxide ( $As_2O_3$ , [ATO]) in treating acute promyelocytic leukemia (APL) without severe toxicity.<sup>38,39</sup> Although the mechanism of the ATO antileukemic effect has not been well defined, ATO at clinically allowable concentrations induces apoptosis in an APL cell line, a process that may be mediated through the downregulation of Bcl-2 and the modulation of the promyelocytic leukemia-retinoic acid receptor  $\alpha$  fusion protein.<sup>5</sup> Several studies have reported that ATO exerts its anticancer effect not only in hematological cancers but also in solid tumors via an antiangiogenic effect by inhibiting vascular endothelial growth factor<sup>25,28,36</sup> or via a proapoptotic effect by causing cell cycle arrest and activating the reactive oxygen species (ROS) pathway.<sup>44,45</sup> Based on these preclinical results, phase I studies of ATO in combination with radiotherapy were successfully performed in patients with malignant glioma.<sup>7,12</sup>

Tetraarsenic oxide ( $As_4O_6$ , [TAO]) is a trivalent arsenic compound with a discrete adamantyl structure, which gives it physical and chemical properties different from those of ATO, which has a divalent 2D structure.<sup>2</sup> One of the coauthors (M.J.P.) has revealed in a mouse model that TAO is less toxic than ATO and shows antiangiogenic effects and the induction of apoptosis in vitro and in vivo at lower concentrations than with ATO.<sup>34</sup> Tetraarsenic oxide is more effective at suppressing cancer cell growth in vitro and in vivo, and cells treated with TAO show more prominent features of apoptosis than those treated with ATO.<sup>4</sup> Furthermore, ATO has already shown the induction of autophagic cell death in malignant glioma cell lines at a low concentration as well as enhanced radiation-induced killing of a GBM cell line via increased intracellular ROS in vitro and in vivo in a xenograft mouse model.<sup>18,31</sup> Hence, we hypothesized that TAO could exert the same cytotoxic effect on malignant glioma at a lower concentration than ATO and could be more safely combined with radiation therapy, which takes at least 4–6 weeks of exposure to augment the cell-killing effect and/or prevent local invasion.

The purpose of this study was to determine the cytotoxic concentration of TAO in several malignant glioma cell lines and to evaluate its anti-invasive effects under conditions devoid of direct cytotoxicity.

## Methods

### *Cell Culture and Reagents*

The human GBM cell lines U87MG, U251MG, and U373MG were obtained from the American Type Culture Collection, and LN428 was kindly provided by Dr. Frank Funari (Ludwig Institute for Cancer Research, La Jolla, CA). The different *p53* and *PTEN* genetic statuses of the tested malignant glioma cell lines are summarized in Table 1.<sup>16</sup> The cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 50 U/ml of both penicillin and streptomycin in a humidified 5%  $CO_2$ /air atmosphere at 37°C. The TAO (> 99.9% purity, Chonjisan Institute, Seoul, Korea) was dissolved at  $5 \times 10^{-2}$  M concentration in 1 N of NaOH as a stock solution. The 1-N concentration of NaOH in the culture medium had no influence on the growth of the GBM cells.

### *Growth Inhibition Assay*

The in vitro growth-inhibitory effects of TAO on the GBM cells were determined by measuring the dimethyl thiazolyl diphenyl tetrazolium (MTT) dye absorbance of live cells. Cells ( $5 \times 10^3$  cells/well) were seeded in a 96-well microtiter plate (Nunc). After exposure to the drug for 48–72 hours, 15  $\mu$ l of MTT solution (Sigma; 2 mg/ml in phosphate-buffered saline [PBS]) was added to each well, and the plates were incubated for an additional 4 hours at 37°C. To solubilize the formazan crystals formed in viable cells, 100  $\mu$ l of dimethyl sulfoxide was added to each well, and absorbance was measured at 570 nm. The 50% inhibitory concentration ( $IC_{50}$ ) was determined from relative absorbance compared with that of the control, and the mean value was obtained from triplicate assays by using Prism software (GraphPad).

### *Cell Invasion Assay*

This assay was modified from a procedure described previously.<sup>47</sup> A  $10^5$  aliquot of cells per chamber was used for each invasion assay. Fifty microliters of Matrigel (1:1 vol/vol dilution in cold serum-free medium) was applied to the upper parts of a 24-well Transwell plate (8- $\mu$ m pore size, Corning) and was coated at 37°C. After rinsing with PBS, the  $10^5$  cells in 100  $\mu$ l of serum-free DMEM was added to the upper chamber. Conditioned medium containing 0.1 mg/ml of bovine serum albumin (Sigma) was added to the lower compartments. Various concentrations of TAO were added to the upper chambers. Invasion was allowed to proceed for 6 hours at 37°C. After the incubation, filters were fixed and stained with a Diff-Quick staining kit (Fisher Scientific). Cells that reached the underside of the filter were counted. The average number of cells in 10 randomly chosen microscopic fields was determined for each filter. The final values for each condition were the average from the three invasion chambers.

## Anti-invasive effect of TAO in malignant glioma cells

**TABLE 1: Genetic characteristics of tested malignant glioma cells and IC<sub>50</sub> of TAO\***

Cell Line	p53	PTEN	IC <sub>50</sub> (μM)	
			serum (+)	serum (-)
U87MG	wild-type	mutant	6.3 ± 0.3	6.0 ± 0.1
U251MG	mutant	mutant	7.0 ± 0.3	6.5 ± 0.2
U373MG	mutant	mutant	12.2 ± 1.1	11.8 ± 0.1
LN428	mutant	wild-type	6.4 ± 0.5	0.9 ± 0.3

\* Data are expressed as the mean ± standard deviation. Values represent those of three independent experiments.

### Analysis of TAO-Induced Apoptosis Via Flow Cytometry

The possible apoptosis fraction of glioma cells during the invasion assay was investigated using flow-activated cell sorter (FACS) flow cytometry. Briefly, following a 6-hour treatment with various concentrations of TAO, the U87MG cells were trypsinized and washed twice with PBS. The cells were centrifuged at 12,000g for 5 minutes and washed twice with PBS. Cell suspensions of 10<sup>6</sup> cells/ml were prepared by adding an appropriate volume (300–400 μl) of binding buffer (10 mM of HEPES/NaOH [pH 7.4], 140 mM of NaCl, 2.5 mM of CaCl<sub>2</sub>). Five microliters of Annexin V-FITC (PharMingen) and 10 μl of propidium iodide were added to measure the cell fractions representing apoptosis and necrosis, respectively, and were incubated at room temperature for 30 minutes in the dark. The cells were analyzed using a FACScan flow cytometer (FACSCalibur, Becton Dickinson Immunocytometry Systems).

### Gelatin Zymography

Production of MMPs by malignant glioma cells was analyzed using gelatin zymography, as described previously.<sup>34</sup> Briefly, cells were incubated in serum-free medium overnight (12–16 hours) after drug treatment. The conditioned medium was mixed with sodium dodecyl sulfate (SDS) sample buffer without heating or reduction, and the mixture was applied to 10% polyacrylamide gels copolymerized with 1 mg/ml of gelatin. After electrophoresis, the gels were washed for 2 hours at room temperature in buffer containing 2.5% (vol/vol) Triton X-100 in 50 mM of Tris-HCl (pH 7.5). The gels were then incubated in 50 mM of Tris-HCl (pH 7.5) with 5 mM of CaCl<sub>2</sub> and 1 μM of ZnCl<sub>2</sub> for 16 hours at 37°C. After staining with Coomassie Brilliant Blue (0.5%), zones of gelatinolytic activity were detected as clear bands against a blue background.

### Akt Kinase Activity Assay

Akt kinase activity was detected using glycogen synthase kinase 3β (GSK-3β) as the substrate. Briefly, after exposure to the indicated reagents (6 hours), cells were washed twice with ice-cold PBS and lysed in a lysis buffer solution containing 20 mM of Tris-HCl (pH 7.5), 1.25 mM of β-glycerophosphate, 137 mM of NaCl, 1 mM of ethylene glycol tetraacetic acid (EGTA), 1 mM of EDTA, 2 mM of NaF, 1% NP-40, 1 mM of sodium orthovanadate, and Complete protease inhibitor mix (Roche Bio-

chemicals). The cell lysates were centrifuged, and 250 μg of supernatant was immunoprecipitated with anti-Akt antibody and protein A/G plus-agarose. The beads were washed three times with a solution containing 150 mM of NaCl, 20 mM of Tris-HCl (pH 7.5), 1 mM of EGTA, 1 mM of EDTA, and 0.5% NP-40 and once with a kinase assay buffer containing 50 mM of Tris-HCl (pH 7.5), 137 mM of NaCl, 1 mM of MgCl<sub>2</sub>, 1 mM of sodium orthovanadate, 2.5 mM of β-glycerophosphate, 2 mM of EDTA, and 5 mM of adenosine triphosphate (ATP), and then were subjected to the kinase assay. The Akt activity was measured in a reaction mixture consisting of kinase assay buffer, 1 mg of GSK-3β, and 5 mCi [γ-<sup>32</sup>P] of ATP for 20 minutes at 30°C. The reaction was terminated by adding SDS sample buffer, and the samples were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Phosphorylated GSK-3β was visualized using autoradiography.

### Western Blotting Analysis

Control and reagent-treated malignant glioma cells were washed twice with ice-cold PBS and lysed in lysis buffer (20 mM of Tris-HCl [pH 7.4], 150 mM of NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM of EDTA, 1 mM of EGTA, 2 mM of sodium orthovanadate, 2 mM of NaF, and Complete protease inhibitor mix for 20 minutes on ice). After centrifugation, the protein concentration in the cell lysates was determined using the Bio-Rad protein assay kit (Bio-Rad). Proper concentrations of proteins (50 μg for membrane type 1 [MT1]-MMP, 20 μg for Akt, and 40 μg for p-Akt) were resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in TBST (10 mM of Tris-HCl [pH 7.5], 100 mM of NaCl, and 0.05% Tween 20) for 1 hour at room temperature and then were incubated with appropriate primary antibody (MT1-MMP: IM39 L, Calbiochem; anti-Akt: N-19, Santa Cruz Biotechnology; anti-phosphorylated active Akt [anti-phospho-Akt]: Ser473, New England Biolabs) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody at 1:2000 dilution for 1 hour at room temperature. The immunoblots were visualized with an enhanced chemiluminescence plus Western blotting detection system (Amersham Pharmacia Biotech).

### Statistical Analysis

Quantitative data are expressed as the mean ± standard deviation. All measurements for each condition were performed in triplicate. The IC<sub>50</sub> values were automatically obtained using Prism software version 3.0. Significant differences were assessed using a 2-tailed unpaired Student t-test and 1-way ANOVA (SPSS version 11.0, SPSS Inc.). A p value < 0.05 was considered significant.

## Results

### Effect of TAO on the Growth of Malignant Glioma Cells

The effect of TAO on malignant glioma cell proliferation was examined in both conditioned and serum-free medium to confirm the inhibitory effect and to evaluate the reference concentration of TAO for subsequent experi-

ments. Significant dose-dependent growth inhibition was observed at 72 hours after TAO treatment, but no discernible growth inhibition was observed at concentrations < 2  $\mu\text{M}$  (Fig. 1). The  $\text{IC}_{50}$  values in conditioned medium were 6.3–12.2  $\mu\text{M}$  (Table 1). Evaluation was performed at 24 hours after drug exposure in serum-free medium because of shriveled growth. The  $\text{IC}_{50}$  values were in a range and order similar to those of the conditioned medium except that the LN428 cell line had an  $\text{IC}_{50}$  of  $0.9 \pm 0.3 \mu\text{M}$ .

#### Effect of TAO on the Invasion of Malignant Glioma Cells

In the control study, the LN428 cell line displayed significantly decreased invasiveness compared with the other cell lines (U373MG:  $1264 \pm 115$  cells/hpf, U251MG:  $1109 \pm 312$  cells/hpf, U87MG:  $915 \pm 229$  cells/hpf, LN428:  $232 \pm 80$  cells/hpf;  $p < 0.001$ , ANOVA). The cells were suspended in serum-free medium with TAO concentrations of 1, 5, and 10  $\mu\text{M}$  for 6 hours, and the number of cells that penetrated the Matrigel-coated membrane was counted. Tetraarsenic oxide caused a significant dose-dependent reduction in invasion for all tested cell lines (Fig. 2;  $p < 0.001$ , ANOVA). Even at 1  $\mu\text{M}$  of TAO, at which no cytotoxic effect was expected, the malignant glioma cell lines showed significantly decreased invasion by 13%–37%, although invasion by U251MG decreased by only 7% ( $p < 0.05$ , unpaired t-test). All four cell lines showed a relative reduction in invasion by 40%–91% at 5  $\mu\text{M}$  of TAO, and the percentage reduction was further augmented to 72%–94% at 10  $\mu\text{M}$  of TAO.

#### Cytotoxic Effect of TAO in the Invasion Assay

To evaluate possible apoptosis caused by TAO under the invasion assay conditions, an FACS analysis of the U87MG cell line was performed 6 hours after exposure (Fig. 3). The apoptosis index (percentage of Annexin-labeled cells) was 2.2% in the control group and was not significantly different according to TAO concentration (1  $\mu\text{M}$ , 2.6%; 5  $\mu\text{M}$ , 2.9%; and 10  $\mu\text{M}$ , 1.7%). The necrosis index also revealed no differences between the control and drug-treated groups (control, 6.0%; 1  $\mu\text{M}$ , 3.9%; 5  $\mu\text{M}$ , 6.0%; and 10  $\mu\text{M}$ , 3.1%). Thus, the FACS analysis results demonstrated that exposure to TAO in the invasion assay did not result in either apoptosis or necrosis.

#### Effect of TAO on MMP-2 Activity

To determine whether the decreased invasiveness induced by TAO was associated with MMP activity, we analyzed MMP-2 (68 kD) activity using gelatin zymography in the cell culture supernatant of the serum-free condition following treatment with various TAO concentrations. Our results revealed that TAO decreased MMP-2 activity in malignant glioma cells in a dose-dependent manner (Fig. 4). The active form of MMP-2 (62 kD) was observed separately in U251MG cells, and it also decreased in proportion to the TAO concentration.

To confirm that the anti-invasive effects of TAO were mediated by decreased secretory MMP activity, we performed Western blotting to analyze MT1-MMP, an intracellular activator of latent MMP-2, in cultured cell pellets of a given TAO concentration. All four tested cell lines displayed decreased MT1-MMP expression at 5 and

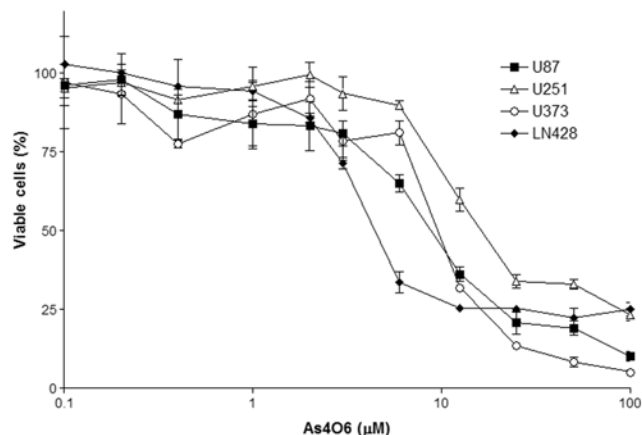


FIG. 1. The effect of TAO on the proliferation of GBM cell lines. The relative number of viable cells was determined using the MTT assay, which was performed when cells were cultured on conditioned medium with the indicated concentrations of TAO for 48–72 hours. Each bar represents the standard deviation of three independent experiments. The concentration of TAO on the x-axis is plotted on a log scale.

10  $\mu\text{M}$  of TAO, whereas the decrease in the MT1-MMP band at 1 and 2.5  $\mu\text{M}$  was marginal compared with that of the control (Fig. 5). The amount of MT1-MMP expression was different among the tested cell lines, and the difference was in accordance with the order of invasiveness measured on the invasion test. The U373MG cell line showed the most abundant expression of MT1-MMP, whereas LN428 expressed the least amount.

#### TAO-Induced Inhibition of Akt Phosphorylation

To explore the possible mechanism responsible for inhibited cell invasion, we examined whether TAO might attenuate Akt activity, as Akt requires phosphorylation to enhance cell motility by mediating the phosphorylation of its downstream effectors. Therefore, we studied Akt Ser473 phosphorylation status after exposure to TAO for 12 hours. Subsequently, whole cell extracts were probed using Western blot analysis with a Ser473-specific anti-phospho-Akt antibody. As depicted in Fig. 6, TAO decreased phospho-Akt in a dose-dependent manner, whereas total Akt remained constant in all four malignant glioma cell lines. Additionally, a 60-minute pretreatment with 10 mM of LY294002, a specific inhibitor of PI3K, markedly inhibited Akt phosphorylation as a positive control.

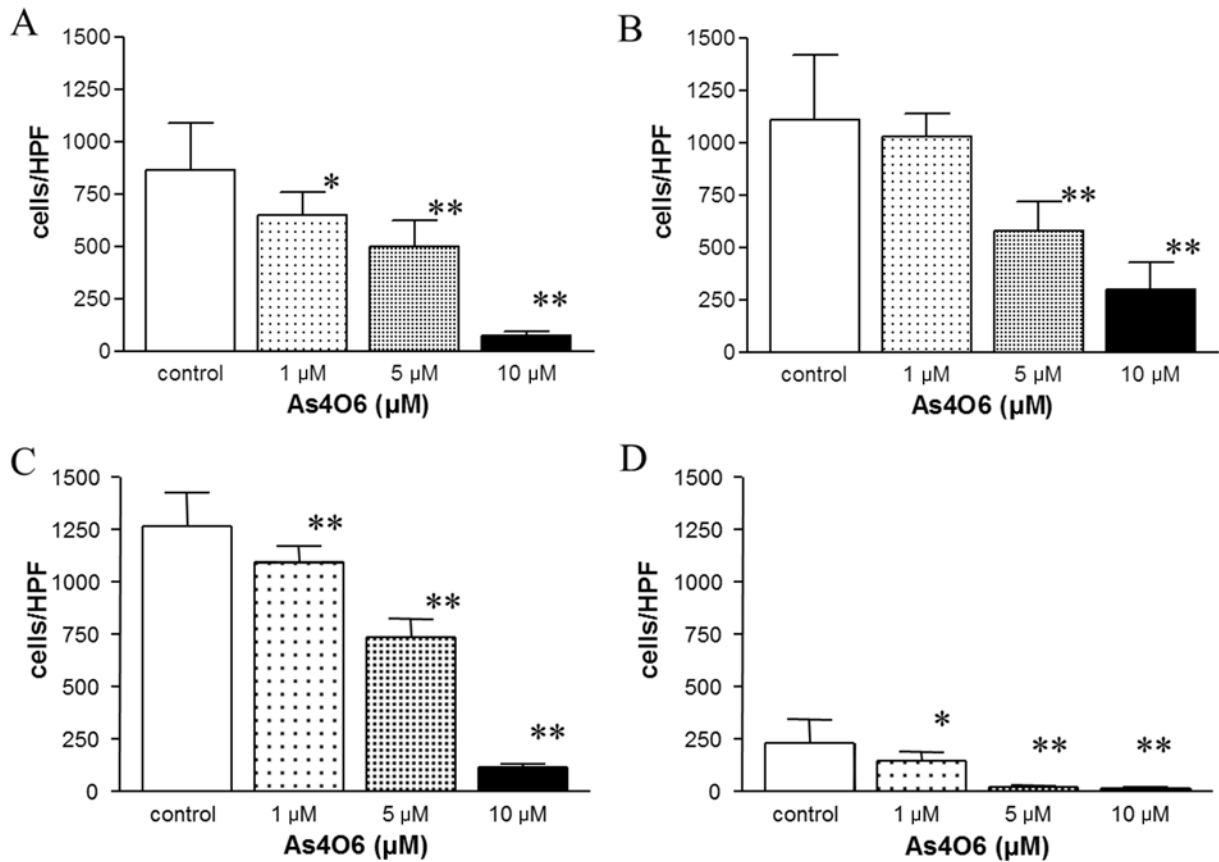
The effect of TAO on Akt activity was also tested by determining GSK-3 $\beta$  phosphorylation, which is an endogenous substrate of Akt. As shown in Fig. 7, Akt activity decreased in a dose-dependent manner in U251MG cells. In addition, phosphokinase activity of the other cell lines was inhibited in the presence of TAO at 5  $\mu\text{M}$ .

## Discussion

### Cytotoxic Effects of TAO on Malignant Glioma Cell Lines

Tetraarsenic oxide shows a mechanism of action similar to that of ATO in various cancer cell lines but acts more efficiently at lower doses. Park et al. investigated the

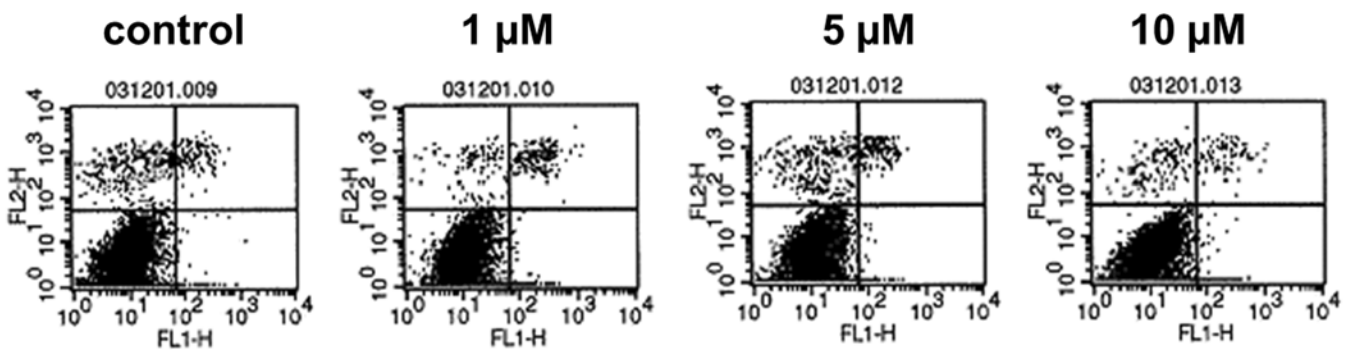
## Anti-invasive effect of TAO in malignant glioma cells



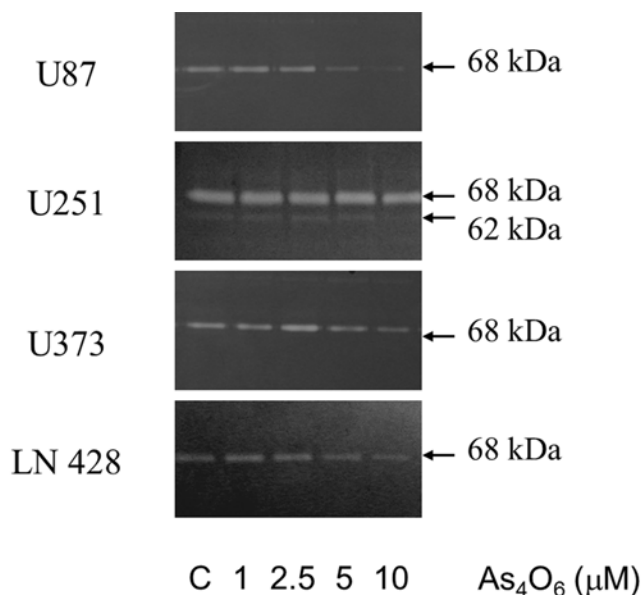
**FIG. 2.** Inhibited invasiveness of four GBM cell lines incubated with TAO: U87MG (A), U251MG (B), U373MG (C), and LN428 (D). Each bar represents the mean number of cells that migrated through Matrigel-coated membranes during 6 hours in serum-free medium. Each value for a given TAO concentration was compared with the control value, and a significant effect was observed, except at 1  $\mu$ M of U251MG (\* $p$  < 0.05, \*\* $p$  < 0.01). The values for a given cell line show a dose-dependent decrease according to the TAO concentration ( $p$  < 0.001, 1-way ANOVA) in all four cell lines. Error bars indicate standard deviations of observed cell numbers from 10 separate hpf ( $\times 150$ ).

effect of TAO on the induction of apoptosis in ATO-resistant U937 leukemic cells.<sup>32</sup> The TAO induced apoptosis in U937 leukemic cells at much lower concentrations than ATO via an early increase in cellular ROS and a decrease in cellular mitochondrial membrane potential, followed by cytochrome-c release and caspase-3 activation. In another study Park and colleagues observed that TAO inhib-

its bovine capillary endothelial (BCE) cells to proliferate in vitro and to invade through a Matrigel-coated layer in a dose-dependent manner in the nanomolar range ( $IC_{50}$  = 27.4–99.7 nM).<sup>34</sup> They attributed part of these results to the inhibited secretion of MMP-2 from BCE cells. Orally administered TAO (50 mg/kg/day) inhibited basic fibroblast growth factor-induced new-vessel formation in a rat



**FIG. 3.** Flow cytometry analysis of U87MG cells at a given concentration of TAO and under the same conditions as the invasion assay. The number of both apoptotic cells (positive for Annexin V-FITC, lower right quadrant) and necrotic cells (positive for both Annexin V-FITC and propidium iodide, upper right quadrant) remained constant despite the addition of TAO at a given concentration.



**FIG. 4.** Effect of TAO on MMP-2 secretion by the GBM cell lines. Clear bands represent gelatinolytic activity of latent MMP-2 (68 kDa) and active MMP-2 (62 kDa) secreted by each GBM cell line into the culture supernatant. The supernatant was collected after incubation with a given concentration of TAO in serum-free medium for 18 hours. A dose-dependent inhibition of gelatinolysis in response to TAO was observed. All images are representative data from three repeated experiments. C = control.

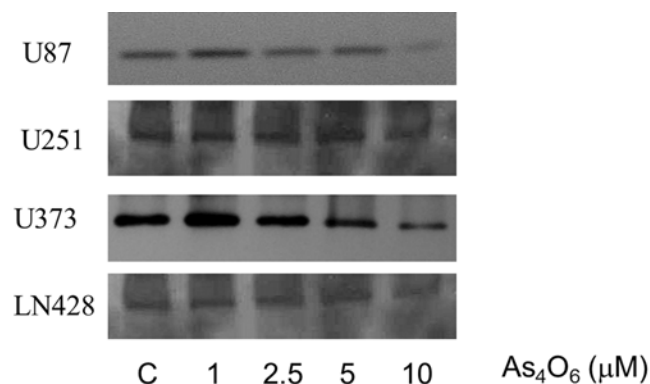
corneal micropocket assay and reduced the number of experimental pulmonary metastatic nodules by 54% in mice. Chang et al. compared the anticancer effects of ATO and TAO in cervical cancer cell lines and a xenograft model. These researchers suggested that TAO more effectively inhibited cell growth and suppressed antiapoptotic factors at a lower concentration than did ATO.<sup>4</sup>

According to reports about the inhibitory effect of ATO in solid tumors, a relatively high concentration of ATO is required to induce overt apoptotic cell death ( $IC_{50} = 10\text{--}50\ \mu\text{M}$ ).<sup>14,27</sup> Haga et al.<sup>14</sup> suggested different sensitivities to ATO according to p53 status. More than 50  $\mu\text{M}$  of ATO is required for apoptosis in the p53 wild-type T98G cell line, whereas the p53 mutant A172 cell line is susceptible at 10  $\mu\text{M}$ . We observed almost the same sensitivity to TAO despite different p53 and phosphatase and tensin homolog (PTEN) statuses. The U87MG cell line (p53 wild-type and PTEN deficient) showed an  $IC_{50}$  of 6.3  $\mu\text{M}$ , whereas U251MG (p53 mutation and PTEN deficient) and LN428 (p53 mutation and PTEN functional) were inhibited at  $IC_{50}$  values of 7.0 and 6.4  $\mu\text{M}$ , respectively.

However, Kanzawa et al. reported that ATO inhibits cell proliferation without apoptosis at a clinically safe concentration (2  $\mu\text{M}$ ) via type 2 cell death and autophagy in malignant glioma cell lines.<sup>18</sup> Although we did not observe autophagy in our experiments, the cell viability of manually counted cells that took up tryptophan blue could be different from that of manually counted functionally intact cells.

#### Anti-Invasive Effect of TAO

Our observations are the first to illustrate decreased



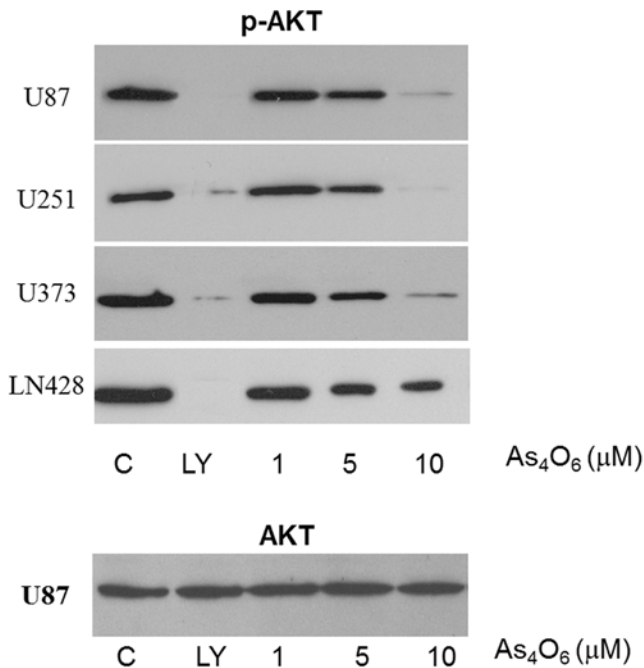
**FIG. 5.** Western blots showing MT1-MMP expression in GBM cell lines under the influence of TAO in conditioned serum. Suppression of MT1-MMP expression shows dose-dependent reduction after incubation with a given TAO concentration for 24 hours. C = control.

migration and invasion of malignant glioma cell lines under a TAO condition of not provoking direct cytotoxicity. We confirmed that the TAO concentration and exposure time in our experiment did not induce discernible apoptosis, as shown by Annexin V. The decreased invasiveness under the influence of TAO was partially mediated by the decreased secretion of MMP-2 and reduced expression of MT1-MMP. The detailed mechanism of how TAO decreases the invasiveness of glioma cell lines was difficult to determine from this experiment. However, given the more than 6 hours required to exert its effect on the above mediators, neither direct neutralization nor substrate competition was probably responsible for the observed effects. Park et al. observed that ATO inhibits migration and invasion of HT1080 cells stimulated with phorbol 12-myristate 13-acetate by blocking the promoter-stimulating and DNA-binding activity of nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ), whereas activator protein-1 (AP-1) activity was unchanged.<sup>33</sup> AP-1 inhibited cell adhesion to the collagen matrix in a concentration-dependent manner and also suppressed the expression of ECM-degrading molecules such as MMP-2, MMP-9, MT1-MMP, urokinase plasminogen activator (uPA), and the uPA receptor.

Matrix metalloproteinase expression and its relationship to malignant behavior and/or glioma grade have been reported in many studies. Among MMP families, MMP-2 is constitutively secreted as an inactive form of latent MMP-2 into the ECM surrounding a glioma, and its dense distribution to the tumor margin and perivascular area reflects its role as a main effector of glioma invasion.<sup>23</sup> Uhm et al. measured the invasiveness of various malignant glioma cell lines *in vitro* and insisted that the difference in invasiveness among cell lines was well correlated with MMP-2 activity.<sup>41</sup> In our zymography study, all glioma cell lines showed strong MMP-2 gelatinolytic activity, and MMP-2 secretion was inhibited by TAO in a dose-dependent manner.

Activation of MMP-2 *in vivo* is minimally affected by various growth factors or oncogenes because of the absence of the AP-1 unit, which has a key role in inducing transcription and amplification. Its activation is regulated not by plasmin but by MT1-MMP, and its expression is in accordance with MT1-MMP expression in both human

## Anti-invasive effect of TAO in malignant glioma cells

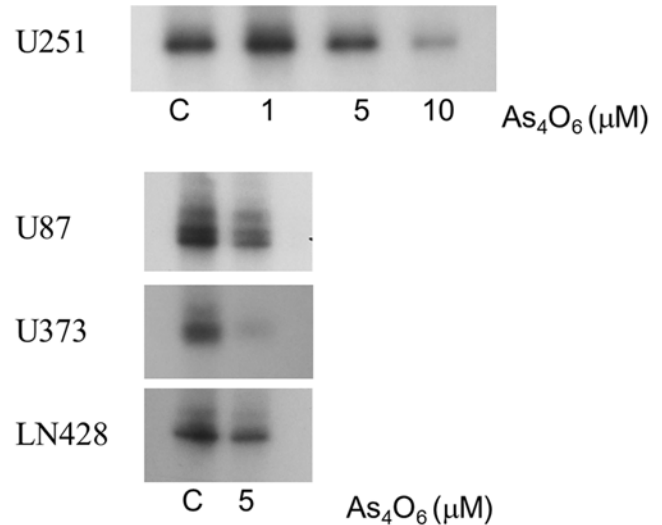


**Fig. 6.** Immunoblots showing total Akt and phosphorylated Akt (p-Akt) expression under the influence of TAO in the GBM cell lines. Phosphorylated Akt was reduced by TAO at a given concentration in a dose-dependent manner after a 12-hour incubation, whereas total Akt remained constant. LY = LY294002, a specific inhibitor of PI3K, which completely inhibits Akt phosphorylation after a 1-hour incubation at 20  $\mu$ M. C = control.

glioma tissue and glioma cell lines.<sup>15,46</sup> This observation is well supported by a trimolecular complex activation/inhibition model composed of latent MMP-2, MT1-MMP, and TIMP-2.<sup>37</sup> Deryugina et al. reported that transfection of MT1-MMP c-DNA into the U251MG cell line causes increased invasion through a Matrigel barrier and activation of MMP-2, which are abrogated by exogenous TIMP-2.<sup>8</sup> In our experiment, the inhibited expression of MT1-MMP coupled with decreased gelatinolytic activity of MMP-2 by TAO explained the decreased invasion through the Matrigel barrier.

### Inhibition of Akt Phosphorylation by TAO

Akt is highlighted in the field of glioma research, as the deletion or mutation of its regulator gene, *PTEN*, is one of the most frequent genetic alterations in many GBMs.<sup>16,26</sup> *PTEN* modulates Akt activity by dephosphorylating phosphatidylinositol phosphate (PIP). Thus, *PTEN*-deficient tumor cell lines exhibit high basal levels of PIP and Akt phosphorylation, which in turn affect the generation or maintenance of the glioma phenotype.<sup>13</sup> The role of Akt in glioma cell lines has also been investigated by many authors. Li and Sun genetically introduced *PTEN* into a *PTEN*-deficient U87MG cell line and observed that *PTEN* induces G<sub>1</sub> cell cycle arrest by inhibiting G<sub>1</sub> cyclin-dependent kinase and inhibits tumorigenicity and proliferation by suppressing the PI3K/Akt pathway.<sup>26</sup> Koul et al. reported that introducing *PTEN* to *PTEN*-deficient U251/U87 cell lines decreases MMP-2 activity and inhibits in-



**Fig. 7.** Akt phosphokinase assay under the influence of TAO in the GBM cell lines using recombinant human GSK-3 $\beta$  as a substrate. Akt phosphokinase activity was decreased by TAO at a given concentration in a dose-dependent manner after 6 hours of exposure in the U251MG cell line. The other cell lines show reduced Akt phosphokinase activity at the designated concentration of TAO.

vasion.<sup>22</sup> By introducing myristoylated Akt and dominant negative Akt into the U87MG cell line, Lee et al. observed that Akt phosphorylation is related to MMP-2 promoter and enzyme activity.<sup>24</sup>

Our observation of decreased Akt phosphorylation under the influence of TAO was in accordance with previous reports stating that Akt plays a critical role in glioma migration and invasion. Among the tested cell lines in our experiment, LN428 was a *PTEN*-functional type and showed obviously weak invasiveness in the assay with the Matrigel-coated Transwell plate compared with other *PTEN*-deficient glioma cell lines. However, the invasiveness of LN428 was also inhibited by TAO in a dose-dependent manner. The above observations could be attributed to decreased MT1-MMP expression.

### Limitations of In Vitro Study and Possibility for a Clinical Trial

The anti-invasive effect of TAO at a noncytotoxic low concentration makes it a candidate for long-term adjuvant therapy with mainstream cytotoxic therapies such as radiation. However, to be effective against glioma, TAO should penetrate the blood-brain barrier (BBB) and reach a therapeutic concentration at a dosage that avoids systemic toxicities.

A phase I clinical trial and pharmacokinetic study in patients with leukemia indicated that 0.2 mg/kg/day of ATO showed dose-limiting toxicity to the heart (QT prolongation) or increased the risk of pancreatitis and that 0.15 mg/kg/day achieved the plasma maximum concentration ( $C_{max}$ ) of 0.28  $\mu$ M.<sup>10</sup> Arsenic trioxide shows synergistic effects in cervical cancer cell lines when combined with radiation and inhibits radiation-promoted migration and invasiveness at a low concentration (1  $\mu$ M) in vitro by inhibiting MMP-9 and down-regulating NF- $\kappa$ B.<sup>6,43</sup>

It is difficult to estimate if the drug could accumulate at an effective concentration across the BBB for the treatment of glioma. Although the molecular weight of ATO is relatively small, its BBB permeability has been evaluated in neither an animal glioma model nor a clinical setting. Ning and Knox<sup>31</sup> reported the effectiveness of ATO as a radiation sensitizer on the U87 cell line in vitro and increased the cure rate from an SNB75 glioma cell line xenograft in nude mice by combining 5 mg/kg ATO with radiation.<sup>31</sup> Kim et al. demonstrated that 8 mg/kg of ATO enhances the radiation response of 9L glioma in an orthotropic rat brain tumor model but had not measured the ATO concentration reaching the glioma.<sup>20</sup>

Our observation of the decreased invasion of glioma cell lines via the inhibition of MMP-2, MT1-MMP, and Akt phosphorylation suggests a possible role for TAO as long-term therapy when combined with radiation. However, it is necessary to determine if TAO meets two conditions to adopt it in glioma treatment: 1) Does it have lower toxicity profiles than ATO at a comparable concentrations in vivo, and 2) can it cross the BBB despite the fact that it is double the molecular weight of ATO? As yet, no study has revealed that TAO can meet these conditions. Kim et al. evaluated the synergistic antitumor effect of TAO at a concentration of 3  $\mu$ M combined with photodynamic therapy in cervical cancer cell lines in a mice xenograft model.<sup>21</sup> They injected 7.5 mg/kg of TAO, and this amount was comparable to that used in an animal study with ATO.<sup>20,31</sup> For future steps toward a clinical trial, we should evaluate both the systemic toxicities of TAO at its maximal tolerable dose and actuarial data for proving that TAO crosses the BBB.

### Conclusions

Tetraarsenic oxide inhibited the proliferation of glioma cells at a concentration thought to be equal to or lower than a similar dose of ATO. Moreover, TAO revealed an anti-invasive property at a concentration and time exposure that did not cause direct toxicity. Although we could suggest the anti-invasive use of TAO on glioma cells based on our results, we should provide both the systemic toxicity profiles and an orthotropic invasive glioma model before launching a clinical trial.

### Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: all authors. Acquisition of data: Gwak, MJ Park, Woo, Jin. Analysis and interpretation of data: all authors. Drafting the article: Gwak. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Rhee. Statistical analysis: Gwak. Administrative/technical/material support: Rhee, IC Park, Jung. Study supervision: Jung.

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Manuscript submitted September 14, 2013.

Accepted August 5, 2014.

Please include this information when citing this paper: published online October 10, 2014; DOI: 10.3171/2014.8.JNS131991.

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