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Research Paper

Antiproliferative and anti-invasive effects of inorganic and organic arsenic compounds on human and murine melanoma cells *in vitro*

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Abstract

Objectives For patients with advanced melanoma, no treatment options are available at present that provide either sufficient response rates or a significant prolongation of overall survival. The present study examines the effects of two inorganic and six organic arsenic compounds on cell proliferation and cell invasion of melanoma cells *in vitro*.

Methods The effects of arsenic compounds on proliferation of human melanoma A375 cells and murine melanoma B16F10 cells were examined by MTT assay and 5-bromo-2′-deoxyuridine (BrdU) incorporation assay, and the effects of the compounds on cell invasion were examined by the Boyden chamber invasion assay. The amounts of active matrix metalloproteinase (MMP)-2 and pro-MMP-2 in the culture supernatant of A375 cells were determined by an MMP-2 activity assay system.

Key findings Arsenate and arsenic trioxide (As_2O_3) inhibited the proliferation of A375 and B16F10 cells significantly at concentration ranges of 0.1–20 μg/ml ($P < 0.001$), while the organic compounds arsenobetaine, arsenocholine, dimethylarsinic acid, methylarsonic acid, tetramethylarsonium and trimethylarsine oxide did not show any inhibitory effects even at 20 μg/ml. Cell invasion of A375 and B16F10 cells through a layer of collagen IV was significantly inhibited by 0.1–20 μg/ml of arsenate or As_2O_3 ($P < 0.05$), while the organic compounds did not inhibit cell invasion. Arsenate or As_2O_3 at 0.2–10 μg/ml significantly inhibited the amount of active MMP-2 and pro-MMP-2 secreted into the A375 cell culture supernatant ($P < 0.05$).

Conclusions Our findings show that the inorganic arsenic compounds arsenate and As_2O_3 inhibit cell proliferation and prevent the invasive properties of melanoma cells, possibly by decreasing MMP-2 production from the cells.

Keywords arsenic compounds; cell invasion; collagen matrix; matrix metalloproteinase; melanoma cells

Introduction

In patients with metastatic melanoma, systemic therapies are known to be ineffective because of the high resistance of melanoma cells to anticancer therapies. Single-agent dacarbazine remains the standard chemotherapy,[1] with response rates ranging from 11 to 25%. However, complete responses are rare and short in duration (3–6 months). The median survival time for patients with metastatic melanoma is less than 1 year.[2] At present, no treatment options that provide either sufficient response rates or a significant prolongation of overall survival are available for patients with advanced melanoma. Because of a lack of efficacious treatments for advanced melanoma, new approaches are necessary.

As_2O_3 has recently been confirmed as an effective treatment for acute promyleocytic leukemia (APL) both in patients with newly diagnosed APL and in those with refractory and relapsed APL.[3–5] The effects of As_2O_3 have been shown not only on the parental APL NB4 cell line,[6] but also on a retinoic acid resistant APL cell line.[7] It has been reported that the effects of As_2O_3 are not only particular to APL cells but can be observed in myeloid,[8] lymphoid origin[9] or drug-resistant sublines.[10] The mechanism of action of As_2O_3 in APL and other malignancies is thought to be the inhibition of growth and induction of

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apoptosis.[8,9,11] Glutathione depletion enhances arsenic trioxide-induced apoptosis in cancer cells, suggesting that the increase in oxidative stress relates to the antineoplastic effects of As_2O_3.[12] Furthermore, the selective sensitivity of organic arsenic-containing compounds in targeting dividing endothelial cells and inhibiting angiogenesis raises the possibility of designing better-targeted antineoplastic arsenic-containing compounds with less toxicity to normal cells.[13]

Suppressive effects of As_2O_3 on melanoma cell growth have been reported,[14] and clinical trials of the agent have even been carried out in melanoma patients.[15,16] On the other hand, the antimelanoma effects of arsenate and organic arsenic compounds have yet to have been investigated. Furthermore, although mechanisms of the anticancer actions of inorganic arsenic compounds have been suggested, as described above, the inhibitory effects of organic and inorganic arsenic compounds on the cell invasive properties of melanoma cells have been little studied.

In the present study therefore we systemically investigated the antiproliferative and anti-invasive effects of two inorganic and six organic arsenic compounds on A375 human melanoma cells and B26F10 murine melanoma cells. Our findings show that the inorganic arsenic compounds arsenate and As_2O_3 inhibit cell proliferation and prevent the invasive properties of the melanoma cells, possibly by decreasing MMP-2 production from the cells. However, organic arsenic compounds have little effect on cell proliferation and invasion of melanoma cells.

Materials and Methods

Reagents

As_2O_3, sodium arsenate, sodium methylarsonate, dimethylarsinic acid, trimethylarsine oxide, arsenobetaine, arsenocholine and tetramethylarsonium were purchased from Tri Chemical Laboratories Inc. (Yamanashi, Japan). Curcumin and N-acetyl-L-cysteine were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum and ethylenediamine tetraacetic acid were purchased from Gibco BRL Co. (Grand Island, NY, USA). Stock solutions of these agents were made at a concentration of 10 mmol/l with distilled water or ethanol, and diluted to working concentrations with distilled water before use. Cultrex Cell Invasion Assay Basement Membrane Extract, Cultrex Cell Invasion Assay Collagen I and Invasion Assay Collagen IV were obtained from R&D Systems Inc. (Minneapolis, MN, USA).

Cell culture

Human melanoma cell line A375 cells and murine melanoma cell line B16F10 cells were obtained from American Type Cell Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% foetal bovine serum, 100 000 IU/l penicillin and 100 mg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2. For our experimental studies, the cells were grown to 90% confluence, harvested with 0.025% trypsin and 0.52 mM ethylenediamine tetraacetic acid in a phosphate-buffered saline (Gibco BRL Co.), plated at a desired density, and allowed to re-equilibrate for 24 h before any treatment.[17] All experiments were conducted in DMEM containing 10% foetal bovine serum, 100 000 IU/l penicillin and 100 mg/ml streptomycin.

Cell proliferation assay

Proliferation of the A375 cells and B16F10 cells was tested by MTT assay procedures (Promega, Madison, WI, USA). Briefly, the cells were plated at a density of 1×10^4 cells/well in 98 µl of medium in 96 flat-bottom wells of a microtiter plate, with the exception of the medium control wells. After incubation for 3 h at 37°C in a humidified incubator with 5% CO_2, 2 µl of distilled water containing the test agents were added to give a final agent concentration of 0.002–20 µg/ml, and the plate was incubated for an additional 24, 48 or 72 h at 37°C in a humidified incubator with 5% CO_2. A volume of 2 µl of distilled water was added to the control wells. After the culture, 10 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dissolved in phosphate-buffered saline was added to each well, and the plate was reincubated at 37°C in 5% CO_2 for a further 4 h. The plate was then centrifuged at 800*g* for 5 min to precipitate cells and formazan produced by the growing cells. Aliquots of 150 µl of the supernatant were removed from every well and 175 µl of dimethylsulfoxide was added to dissolve the formazan crystals. The plate was mixed on a microshaker for 10 s and then read on a microplate reader (Corona MT P-32, Corona, Tokyo, Japan) at 550 nm. The dose–response curve was plotted and the concentration that gave a 50% inhibition of cell growth (IC50) was calculated.

The proliferation of A375 and B16F10 cells *in vitro* was also examined by measuring the amounts of BrdU incorporated into cellular DNA[18] using the Cell Proliferation ELISA, BrdU (colorimetric). The amounts of BrdU incorporated into the cells were assayed according to the manufacturer's instructions.

Cell invasion assays

The invasion ability of melanoma cells was evaluated *in vitro* using Boyden chambers according to the manufacturer's instructions (Transwell Costar, Cambridge, MA, USA). The Boyden chamber assay uses a chamber with two medium-filled compartments separated by a microporous membrane. The upper layer of the chamber was coated with 50 µl of a coating buffer containing basement membrane extract, collagen I or collagen IV, respectively. Appropriate volume (150 µl) of DMEM medium containing 10% foetal bovine serum was added to the lower layer. A375 and B16F10 cells at cell numbers of 5×10^4 and 1×10^5 cells, respectively, were placed in the upper compartment in the presence or absence of serial concentrations of the test compounds dissolved in 50 µl of DMEM medium. The cells were allowed to migrate through the pores of the membrane into the lower compartment for 24 h at 37°C in a humidified atmosphere with 5% CO_2. At the end of the incubation period, viable cells in the bottom of the upper and the lower layer of the chamber were stained with 100 µl of calsein AM reagent dissolved in cell dissociation solution for 30 min, and then the fluorescence intensity of each well was measured by a microplate reader

(Safire, Techan Co.) with a wavelength of 485 nm/520 nm. The experiment was repeated at least three times to obtain data for statistical analysis.

Determination of active matrix metalloproteinase in melanoma cell culture

The amounts of active MMP-2 and pro-MMP-2 in the culture supernatant of A375 cells were determined with an MMP-2 Biotrak Activity Assay System (GE Healthcare Co.) according to the manufacturer's instructions. In brief, 5×10^3 cells/well in 98 µl of culture medium were placed in 96 flat-bottom wells of a microtiter plate and incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. Two microlitres of distilled water containing the test agents were added to give final agent concentrations of 0.2–10 µg/ml and the plate was incubated for an additional 24 h. After incubation, the plate was centrifuged at 800*g* and the culture supernatant was collected. The culture supernatant was diluted 10-fold with the assay buffer and stored at −80°C until assay. The 10-fold diluted culture supernatant was placed into each well of a 96 flat-bottom well plate coated with anti-MMP-2 antibody and the plate was incubated for 20 h at 2–8°C. After incubation, the supernatant was removed by pipetting and each well of the plate was washed four times with 200 µl assay buffer. Fifty microlitres of 352 mg/ml p-aminophenylmercuric acetate dissolved in dimethylsulfoxide was added to the wells for measurement of the total MMP-2, while the same amount of assay buffer was added to the wells for measurement of active MMP-2. Then, 50 µl of the detection reagent was added to each well, and the plate was sealed and mixed for 20 s with a micromixer. Subsequently, the absorbance of each well was measured by a microplate reader (Safire, Techan Co.) with a wavelength of 405 nm (time zero). Then the plate was incubated for a further 6 h at 37°C in a humidified incubator with 5% CO_2. After incubation, the plate was mixed for 20 s with a micromixer and the absorbance of each well was measured by a microplate reader with a wavelength of 405 nm. The amounts of active MMP-2 and pro-MMP-2 (total MMP-2 minus active MMP-2) were determined by reference to a standard curve made using serial concentrations of human pro-MMP-2 (GE Healthcare Co.).

Statistics

The data were expressed as the mean (SE) unless otherwise mentioned. The differences in the mean numbers of cells proliferated between wells treated with and without serial concentrations of the test compounds were analysed by Dunnett's test. The differences in the percentages of cell proliferation between wells treated with arsenate in the presence and absence of N-acetyl-L-cysteine were examined by unpaired *t*-tests. The differences in the mean number of cells invaded through membrane layers or the mean amounts of active- and pro-MMP-2 in culture supernatant between wells treated with and without serial concentrations of the test compounds were also analysed by Dunnett's test. These analyses were performed with Graph Pad Prism 4.0 software. In each case, two-sided *P* values less than 0.05 were considered to be significant.

Results

Effects of inorganic and organic arsenic compounds on cell proliferation of A375 and murine B16F10 melanoma cells

The effects of two inorganic and six organic arsenic compounds on the cell proliferation of human A375 melanoma cells and murine B16F10 melanoma cells *in vitro* were examined by MTT assay procedures and by measuring the amounts of BrdU incorporated into cellular DNA.[18]

The percentages of viable cells incubated in the presence of serial concentrations of the test compounds for 24, 48 and 72 h, as examined by MTT assay, are shown in Figures 1 and 2. Arsenate and As_2O_3 inhibited the proliferation of both A375 (Figure 1) and B16F10 (Figure 2) cells dose- and time-dependently. The effects of these compounds were

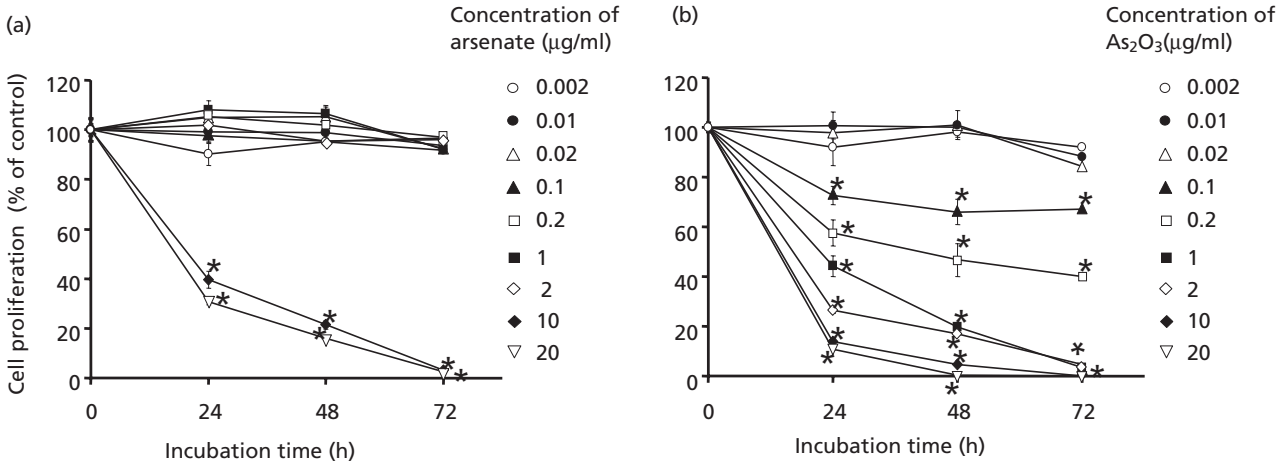

Figure 1 The time- and dose-dependent effects of (a) arsenate and (b) As_2O_3 on the proliferation of human melanoma A375 cells. Cells were treated with 0.002–20 µg/ml of arsenic compounds for 24, 48 and 72 h, respectively, and the living cells were analysed by MTT assay. The data are expressed as the mean (SE) percentage of living cells in arsenic-treated wells as compared to that of control wells ($n = 4$). The asterisks indicate significant differences between the control and agent-treated cells ($^*P < 0.01$).

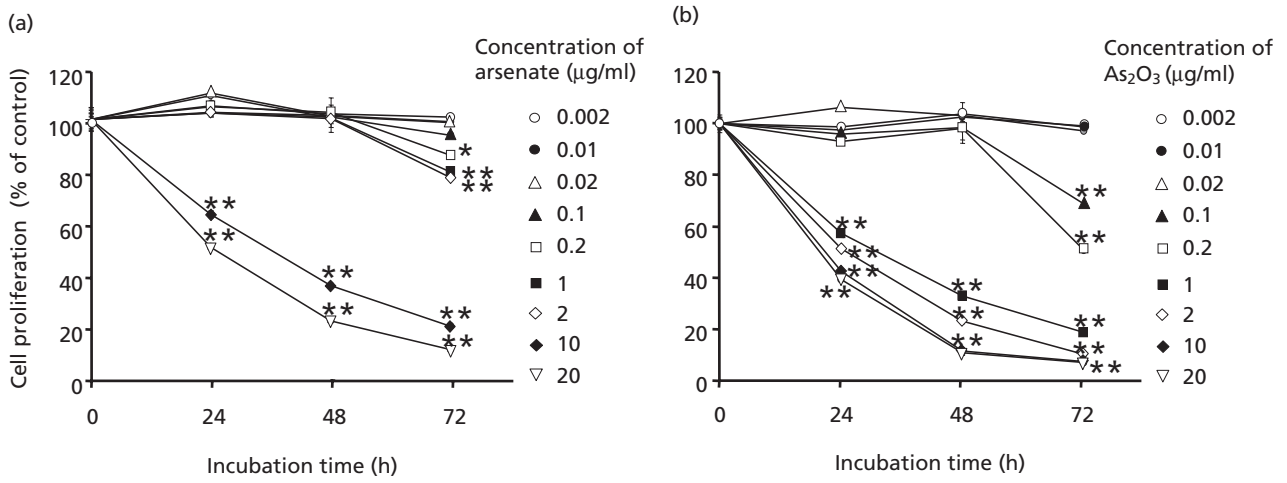

Figure 2 The time- and dose-dependent effects of (a) arsenate and (b) As_2O_3 on the proliferation of murine melanoma B16F10 cells. Cells were treated with 0.002–20 μg/ml of arsenic compounds for 24, 48 and 72 h, respectively, and the living cells were analysed by MTT assay. The data are expressed as the mean (SE) percentage of living cells in arsenic-treated wells as compared to that of control wells ($n = 4$). The asterisks indicate significant differences between the control and agent-treated cells (*$P < 0.05$, **$P < 0.01$).

statistically significant at a concentration range of 10–20 μg/ml for arsenate and 0.1–20 μg/ml for As_2O_3, respectively ($P < 0.001$). The mean IC50 values of arsenate and As_2O_3 on the proliferation of A375 cells after 48 h of culturing were 8.33 and 0.15 μg/ml, respectively, and the values on the proliferation of B16F10 cells after 48 h of culturing were 9.62 and 1.18 μg/ml, respectively. Thus the effect of As_2O_3 on melanoma cells appears to be stronger than that of arsenate. In contrast, the organic compounds arsenobetaine, arsenocholine, dimethylarsinic acid, methylarsonic acid, tetramethylarsonium and trimethylarsine oxide did not show any inhibitory effects even at high concentrations (20 μg/ml; data not shown). Dacarbazine is used clinically for the treatment of metastatic melanoma.[1] In our present assay procedures, this agent appeared to inhibit the proliferation of A375 cells *in vitro* by 20–40% at a concentration range of 25–100 μmol/l, but the effects were not statistically significant (data not shown).

The effects of arsenate and As_2O_3 on the proliferation of A375 cells and B16F10 cells were also examined by measuring the amounts of BrdU incorporated into cellular DNA with the Cell Proliferation ELISA BrdU (colorimetric). It has been shown that the cell proliferation data obtained by the Cell Proliferation ELISA assay are well correlated with those obtained by cell number.[18] We therefore used this assay procedure to show the effects of arsenic compounds on melanoma cell proliferation. The data show that both arsenate and As_2O_3 inhibit the proliferation of A375 (Figure 3, left) and B16F10 (Figure 4, left) cells dose-dependently in a concentration range of 0.1–10 μg/ml. These observations parallel those obtained by MTT assay procedures (Figures 1 and 2). We also examined the effects of N-acetyl-L-cysteine, an antioxidant, on the antiproliferative activity of arsenate and As_2O_3 by measuring the amounts of BrdU incorporated into cellular DNA. Amounts of 10 mM N-acetyl-L-cysteine significantly attenuated the suppressive effect of 1 μg/ml As_2O_3 on the proliferation of A375 cells ($P < 0.05$) (Figure 3b, right). Similarly, 10 mM N-acetyl-L-cysteine significantly attenuated the suppressive effects of 1 μg/ml arsenate (Figure 4a, right) and 0.1–10 μg/ml As_2O_3 on the proliferation of B16F10 cells (Figure 4b, right) ($P < 0.05$).

Effects of inorganic and organic arsenic compounds on the invasion ability of melanoma cells

The effects of inorganic and organic arsenic compounds on the invasive properties of A375 and B16F10 cells were examined with an in-vitro cell-invasion model using Boyden chamber methods. The percentages of the cells invading through the basement membrane layer, collagen I layer and collagen IV layer in the presence or absence of the test compounds were estimated (Figure 5). The cell invasions of A375 and B16F10 cells tended to be suppressed in the presence of 20 μg/ml of the inorganic compounds arsenate and As_2O_3. Curcumin at 100 μg/ml inhibited the invasion, and a significant effect was observed on the invasion of A375 cells through the collagen IV layer ($P < 0.01$). Arsenate and As_2O_3 significantly inhibited A375 cell invasion through the collagen IV layer ($P < 0.01$) (Figure 5c). As_2O_3 also significantly inhibited the invasion of B16F10 cells through the collagen I and IV layers ($P < 0.05$), while the effects of arsenate on B16F10 cells were not statistically significant (Figures 5b and c). However, the organic compounds arsenobetaine, arsenocholine, dimethylarsinic acid, methylarsonic acid, tetramethylarsonium and trimethylarsine oxide at 20 μg/ml did not inhibit, or most of them rather enhanced, the cell invasion of A375 and B16F10 cells through all of the three types of membrane layer (Figure 5a–c).

The dose-dependent inhibitory effects of the inorganic compounds on the cell invasions of A375 and B16F10 cells through a collagen IV layer were further evaluated with the same assay procedures (Figure 6). The effects of these inorganic compounds were dose-dependent at 0.002–20 μg/ml, and the statistically significant effects were observed at approximately 1–20 μg/ml for the A375 cell invasion and at

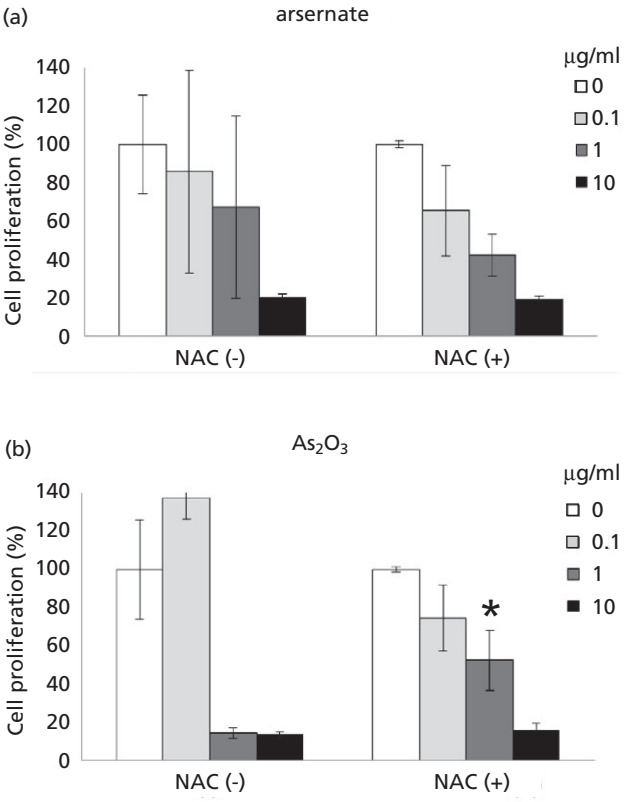

Figure 3 Effects of (a) arsenate and (b) As_2O_3 in the absence (left) or presence (right) of N-acetyl-L-cysteine (NAC) on the proliferation of human melanoma A375 cells, as examined by BrdU incorporation. Cells were treated with 0.1–10 μg/ml of arsenic compounds for 72 h, and the BrdU incorporation into cellular DNA was estimated. The data are expressed as the mean (SD) percentage of BrdU incorporation into cells in arsenic-treated wells as compared to that of control wells, in the presence or absence of 10 mM N-acetyl-L-cysteine ($n = 3$). The asterisk indicates significant difference between the wells cultured in the presence and absence of N-acetyl-L-cysteine ($P < 0.05$).

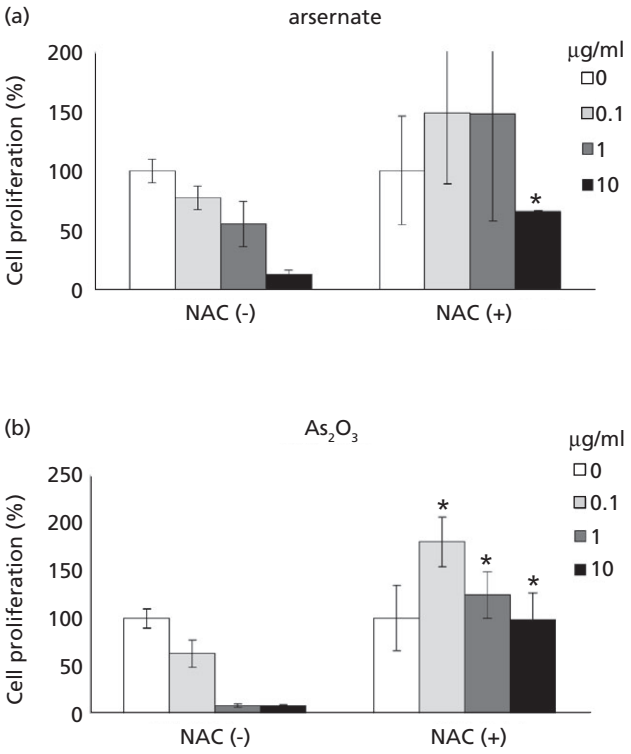

Figure 4 Effects of (a) arsenate and (b) As_2O_3 in the absence (left) or presence (right) of N-acetyl-L-cysteine (NAC) on the proliferation of human melanoma B16F10 cells, as examined by BrdU incorporation. Cells were treated with 0.1–10 μg/ml of arsenic compounds for 72 h, and the BrdU incorporation into cellular DNA was estimated. The data are expressed as the mean (SD) percentage of BrdU incorporation into cells in arsenic-treated wells as compared to that of control wells, in the presence or absence of 10 mM N-acetyl-L-cysteine ($n = 3$). The asterisks indicate significant differences between the wells cultured in the presence and absence of N-acetyl-L-cysteine ($P < 0.05$).

0.1–20 μg/ml for the B16F10 cell invasion, respectively ($P < 0.05$) (Figure 6).

Effects of inorganic arsenic compounds on the matrix metalloproteinase activity of melanoma cells

As shown in Figures 5 and 6, arsenate and As_2O_3 showed inhibitory effects on cell invasion by melanoma cells through the collagen IV layer. We also examined the effects of these inorganic arsenic compounds on the amount of active MMP-2 and pro-MMP-2 in A375 cell culture supernatant. Arsenate at 2–10 μg/ml and As_2O_3 at 1 μg/ml significantly inhibited the production of the active MMP-2 in the A375 cell culture ($P < 0.05$) (Figure 7). Similarly, arsenate at 10 μg/ml and As_2O_3 at 0.2–1 μg/ml significantly inhibited the production of pro-MMP-2 in the A375 cell culture ($P < 0.01$).

Discussion

The data presented above show that the inorganic arsenic compounds arsenate and As_2O_3 inhibit proliferation of both human and murine melanoma cells at concentration ranges of 0.1–20 μg/ml, whereas organic arsenic compounds show no inhibitory effects even at 20 μg/ml. The data also show that cell invasion of the melanoma cells through a layer of collagen IV is inhibited by arsenate and As_2O_3, while organic compounds do not inhibit the cell invasion. Our findings suggest that inorganic arsenate and As_2O_3 attenuate the invasive properties of melanoma cells by decreasing MMP-2 production from the cells. Studies on the clinical pharmacokinetics of As_2O_3 have shown that the peak concentration of plasma As_2O_3 under a general treatment schedule was approximately 1.1–1.4 μg/ml.[6] According to the data of the present study, cell proliferation, cell invasion through a collagen IV layer and active MMM-P production from melanoma cells were inhibited by approximately 1–5 μg/ml of As_2O_3, a concentration that is achievable clinically.[6] As_2O_3 has been reported to be metabolised in the liver to an organic arsenical methylarsonic acid, and then to dimethylarsinic acid.[19,20] Thus our data suggest that in humans the antiproliferative and anti-invasive efficacies of As_2O_3 attenuate after its metabolic conversion to organic arsenicals by the liver.

Dacarbazine is used as a standard chemotherapy for the treatment of patients with metastatic melanoma. However,

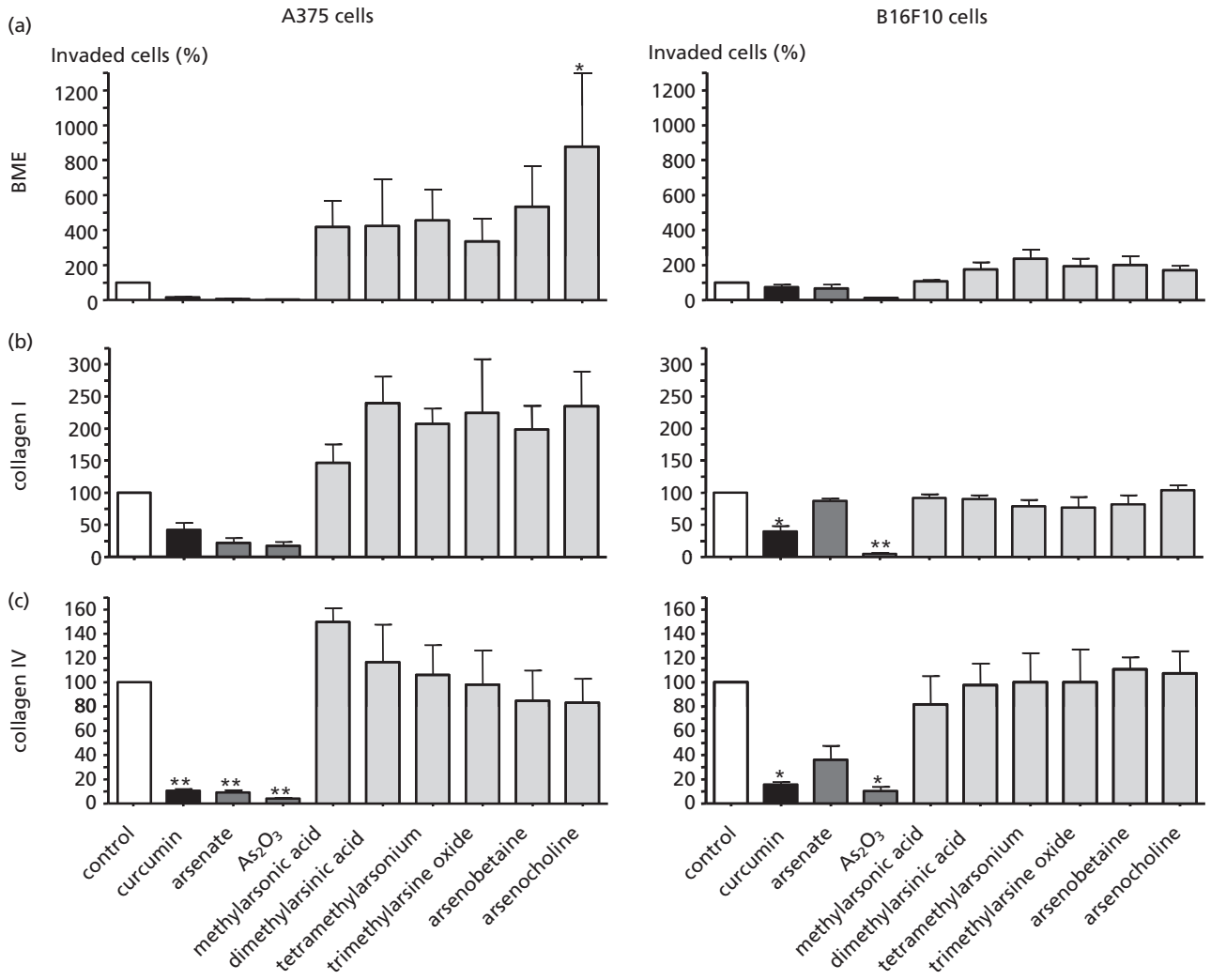

Figure 5 Effects of arsenic compounds on the invasive properties of A375 cells (left) and B16F10 cells (right) through layers of basement membrane (a), collagen type I (b) and collagen type IV (c), respectively. Cell invasion was assayed by the Boyden chamber method. Cells invaded in the absence of the arsenic compounds were used as a control. The data are expressed as the percentage of invaded cells in the presence of the arsenic compounds as compared to the control. Each bar shows the mean (SE) of the percentages of invaded cells, as compared to control ($n = 3$). The asterisks indicate significant differences between the control and agent-treated cells (*$P < 0.05$, **$P < 0.01$).

systemic therapies with dacarbazine alone are known to be ineffective in many cases because of the high resistance of melanoma cells to various anticancer therapies, including dacarbazine.[1,2] Thus at present there are no treatment options available for patients with advanced melanoma that provide either a sufficient response rate or a significant prolongation of overall survival. It has been reported that the IC50 value of dacarbazine against the growth of A375 cells *in vitro* is higher than 548 μmol/l.[21] In the present study, we also examined the effect of dacarbazine on the proliferation of A375 cells *in vitro* and found that the suppressive effect was not enough, even at a concentration of 100 μmol/l. Thus, A375 cells appear to be resistant to the suppressive effects of dacarbazine. According to the data of the present study, the suppressive effects of As_2O_3 against the proliferation of A375 cells *in vitro* are apparent at 0.2 μg/ml (1 μmol/l), and thus the effect, together with the observations of other researchers,[21] appears to be more than 100 times stronger than that of dacarbazine.

Previous reports based on experiments with an APL cell line, NB4 cells, have suggested that the inorganic arsenic compound As_2O_3 causes apoptosis directly through down-regulation of bcl-2.[22] Apoptosis enhancement by As_2O_3 has been also reported in melanoma cells[23] and thus apoptosis induction may be implicated in the suppressive effects of arsenate and As_2O_3 on the proliferation of A375 cells and B16F10 cells that we observed in the present study. On the other hand, the glutathione redox system is known to modulate the growth-inhibitory effect of arsenicals.[24] Glutathione exerts antioxidant effects and it can conjugate and thereby inactivate molecules that generate free radicals. In the present study, the effect of As_2O_3 on both A375 and B16F10 cells was attenuated by N-acetyl-L-cysteine, which promotes synthesis of glutathione. It was found that the sensitivity to As_2O_3-induced apoptosis was inversely related to intracellular glutathione content and that pharmacological modulation of intracellular glutathione contents influences sensitivity to

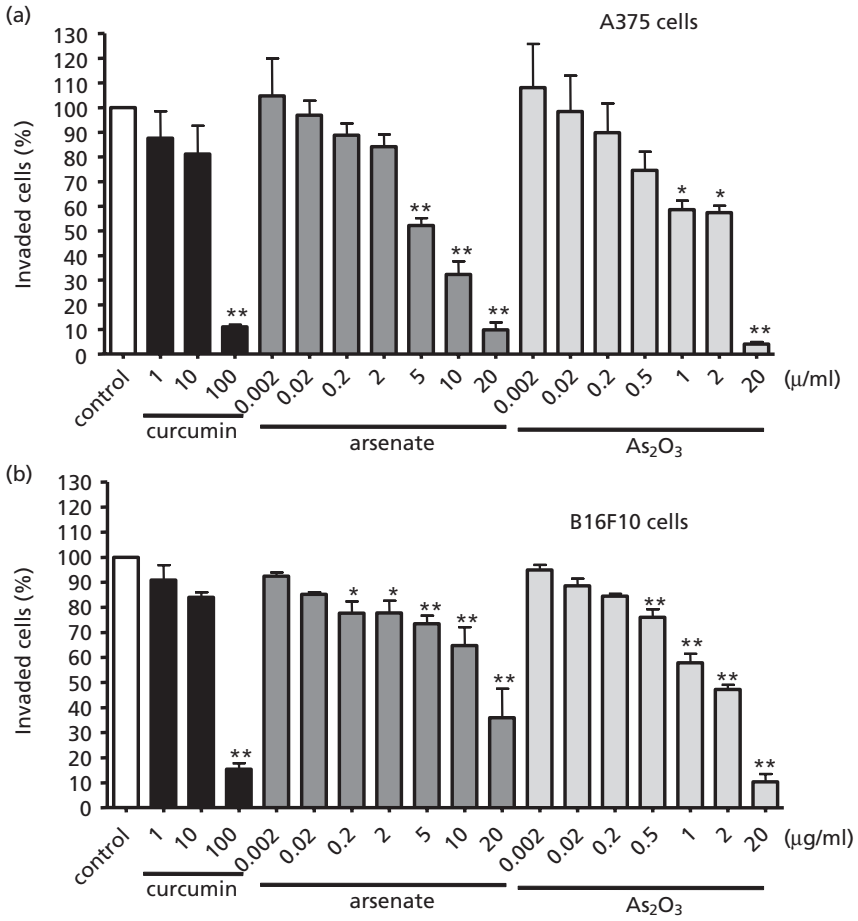

Figure 6 Dose-dependent effects of arsenate and As_2O_3 on the invasive properties of (a) A375 cells and (b) B16F10 cells through layers of collagen type IV. Cell invasion was assayed by the Boyden chamber method. Cells invaded in the absence of the arsenic compounds were used as a control. The data are expressed as the percentage of invaded cells in the presence of the arsenic compounds as compared to the control. Each bar shows the mean (SE) of the percentages of invaded cells, as compared to the control ($n = 3$). The asterisks indicate significant differences between the control and agent-treated cells (*$P < 0.05$, **$P < 0.01$).

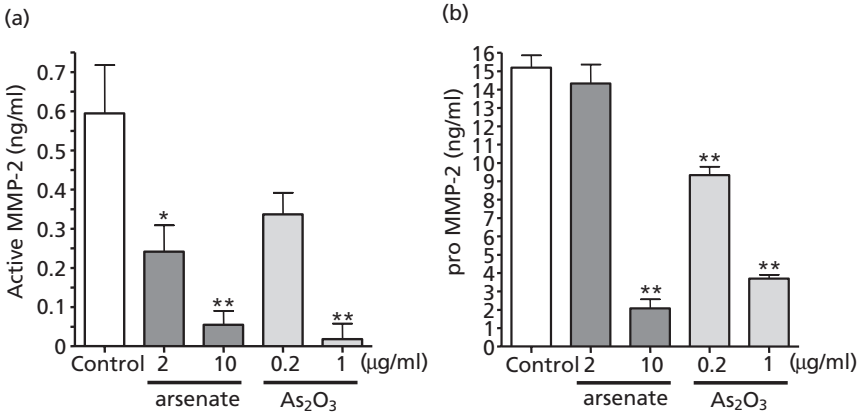

Figure 7 Effects of arsenate and As_2O_3 on the production of (a) active MMP-2 and (b) pro-MMP-2 in A375 cell culture. The concentration of active MMP-2 and pro-MMP-2 in the cell culture supernatant was determined using a Biotrak activity kit. Each bar indicates the mean (SE) of concentrations (ng/ml) of the enzymes ($n = 3$). The asterisks indicate significant differences between the control and agent-treated cells (*$P < 0.05$, **$P < 0.01$).

As_2O_3.[12,24] Thus the ability of As_2O_3 to inhibit growth in melanoma cells may possibly be related to the regulation of suppressor gene(s), particularly bcl-2, and the glutathione redox system. It has also been suggested that As_2O_3-dependent down-regulation of specificity protein (Sp) and Sp-dependent genes is due to decreased mitochondrial membrane potential and induction of reactive oxygen species, which results in inhibition of bladder cancer growth.[25] The antiproliferative mechanism of dacarbazine against cancer cells is mainly related to the methylation of O-guanine,[6] mediated by a methyldiazonium ion, a highly reactive derivative of the compound.[26] Thus the mechanisms of action of dacarbazine and inorganic arsenic compounds are suggested to be different, and therefore concomitant use may result in a synergistic efficacy against melanoma.

As_2O_3 has been reported to show anti-invasive action against tumour cells of cervical cancer,[27] ovarian carcinoma[28] and fibrosarcoma[29] in some cell-invasion assay systems. For the examination of the effects of arsenic compounds against melanoma cell invasion, we carried out a cell invasion assay using the Boyden chamber.[30] The basement membrane is a thin extracellular matrix that underlies the epithelia and endothelia, and separates them from the stroma. Melanoma cells must cross this membrane to invade stroma and establish distant metastases. Tumor cells, including melanoma cells, invade stroma by producing proteases that degrade the matrix. Of several in-vitro models, those using Matrigel are known to be the most reliable, reproducible and representative of in-vivo invasion.[30] Our data show that inorganic arsenic compounds, but not organic arsenic compounds, efficiently suppress the invasion of A375 and B16F10 melanoma cells through a collagen IV layer. Curcumin has been reported to inhibit the invasion of B16F10 melanoma cells across the collagen matrix of the Boyden chamber.[31] Curcumin at 100 μg/ml also inhibited the invasion in the present study. Collagen types I and IV constitute important components of the extracellular matrix and vascular basement membrane, respectively.[32] It has been suggested that collagen receptors are implicated in melanoma cell metastasis, and thus collagen receptors may be potential targets for the development of new antimetastatic therapies.[33] Our present data may therefore give new insights into antimelanoma therapy targeting collagen IV receptors, such as collagen-binding integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$, with inorganic arsenic compounds.

MMPs-2 and -9 have been implicated in malignant tumour progression, partly because they degrade collagen type IV, a major component of basement membranes.[34] Thus MMP-2 expression is suggested to be associated with the progression of melanoma.[34] We showed here that As_2O_3 inhibits the production of active MMP-2 into the culture medium of human melanoma cells, suggesting that the compound attenuates the cell invasion of the melanoma cells through the collagen IV layer via the inhibition of the production of active MMP-2 from the cells.

In summary, the present study showed that the inorganic arsenic compounds arsenate and As_2O_3 inhibit cell proliferation, cell invasion through the collagen IV layer and active MMP-2 production from melanoma cells. The current observations warrant further in-vivo study to investigate the antimetastatic efficacies of these compounds for use in melanoma therapies.

Conclusion

The effects of two inorganic and six organic arsenic compounds on cell proliferation and cell invasion of human melanoma A375 cells and murine melanoma B16F10 cells were investigated *in vitro*. Arsenate and As_2O_3 inhibit the proliferation of A375 and B16F10 cells at concentration ranges of 0.1–20 μg/ml, while the organic compounds arsenobetaine, arsenocholine, dimethylarsinic acid, methylarsonic acid, tetramethylarsonium and trimethylarsine oxide do not show any inhibitory effects. Cell invasion of A375 and B16F10 cells through a layer of collagen IV was significantly inhibited by 0.1–20 μg/ml of arsenate or As_2O_3, while the organic compounds did not inhibit cell invasion. Arsenate or As_2O_3, at 0.2–10 μg/ml, significantly inhibit the amount of active MMP-2 and pro-MMP-2 secreted into A375 cell culture supernatant. Thus, our data show that the inorganic arsenic compounds arsenate and As_2O_3 inhibit cell proliferation and prevent the invasive properties of melanoma cells by decreasing MMP-2 production from the cells. We also concluded that organic arsenic compounds have little effect on cell proliferation and cell invasion of melanoma cells.

Declarations

Conflict of interest

The Authors declare that there are no conflicts of interest.

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