# Pro-angiogenesis action of arsenic and its reversal by selenium-derived compounds

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Inorganic arsenic (arsenite and arsenate) in drinking water has been associated with skin cancers and increased incidence of cardiovascular diseases. Additionally, studies have demonstrated the pro-angiogenic effect of arsenite and its potential promotion of tumor angiogenesis and tumor progression. Furthermore, recent reports demonstrated reversal of skin co-carcinogenesis by an organoselenium compound. The present study was undertaken to determine the effect and mechanism on angiogenesis of arsenite at low level and its potential reversal by various selenium-derived compounds. The pro-angiogenesis effects and mechanisms of sodium arsenite were determined using the chick chorioallantoic membrane (CAM) model over 3 days and compared with standard pro-angiogenesis factors, such as basic fibroblast growth factor (b-FGF). Additionally, the potential effect of various selenium-derived compounds—such as dimethyl selenone, diphenyl selenone, sodium selenite or *Se*-methyl selenocysteine—in reversing the pro-angiogenesis effect of arsenite or b-FGF was also determined in the CAM model. The pro-angiogenesis effect of arsenite or b-FGF was significantly ($P < 0.01$) blocked by dimethyl selenone, diphenyl selenone, sodium selenite or *Se*-methyl selenocysteine. The pro-angiogenesis effect of either sodium arsenite at 33 nM or b-FGF was blocked ($P < 0.01$) by the extracellular signal-regulated kinases 1 and 2 (ERK1/2) activation inhibitor, PD 98059. Additionally, the pro-angiogenic effect of arsenic or b-FGF was blocked as well ($P < 0.01$) by the $\alpha v \beta 3$ antagonist, XT199. These data suggest that the pro-angiogenesis effect of arsenic is initiated at the plasma membrane integrin $\alpha v \beta 3$, involves activation of the ERK1/2 pathway and is effectively reversed by various selenium-derived compounds.

## Introduction

Arsenic, an element that is found both in native and in combined form, has been used medicinally for over 2400 years. In the 19th century, it was the mainstay of the *materia medica*. A solution of potassium arsenite (Fowler's solution) was used for a variety of systemic illnesses from the 18th until the 20th century. This multipurpose solution was also the primary therapy for the treatment of chronic myelogenous leukemia until it was replaced by radiation and cytotoxic chemotherapy. The past 100 years have seen a precipitous decline in arsenic use; by the mid-1990s, the only recognized indication was the treatment of trypanosomiasis. Much of this decline was due to concerns about the toxicity and potential carcinogenicity of chronic arsenic administration (1). Chinese physicians had been using arsenic-containing medicines, including arsenic trioxide, as part of a treatment

**Abbreviations:** APL, acute promyelocytic leukemia; b-FGF, basic fibroblast growth factor; CAM, chorioallantoic membrane; ERK1/2, extracellular signal-regulated kinases 1 and 2; FGF, fibroblast growth factor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor.

for acute promyelocytic leukemia (APL). Their accumulated experience showed that a stable solution of arsenic trioxide given by intravenous infusion was remarkably effective both in patients with newly diagnosed APL and in those with refractory and relapsed APL (2). The mechanisms of action of arsenic derivatives in this disease and other malignancies are many and include induction of apoptosis, inhibition of proliferation and inhibition of angiogenesis (2). Molecular studies and ongoing clinical trials suggest that, as a chemotherapeutic agent, arsenic trioxide shows great promise in the treatment of malignant diseases (2).

Arsenic trioxide can inhibit proliferation and induce apoptosis in multiple myeloma cells *in vitro* and *in vivo* (3). However, low concentrations of arsenic trioxide stimulate vascular cell proliferation in cell culture and angiogenesis *in vivo* (4,5). Arsenite was shown to cause dose-dependent increase in vessel density in the chick chorioallantoic membrane (CAM) assay (5). The threshold arsenic trioxide concentration for this response was 0.033 µM, and inhibition of vessel growth was observed at concentrations $>1$ µM (5). Hence, arsenite might have carcinogenic effect at low levels of exposure, which might be accelerated by its pro-angiogenesis effects.

Data supported a potential anti-angiogenic effect of selenium in the chemoprevention of cancer (6,7). With regard to tumor angiogenesis, the chemopreventive effect of increased selenium intake on chemically induced mammary carcinogenesis has been associated with reduced intratumoral microvessel density (6). Control of angiogenesis is a complex process involving local release of vascular growth factors, the extracellular matrix, adhesion molecules and metabolic factors (7–9). Mechanical forces within blood vessels may also play a role (7). The principal endogenous growth factors implicated in new blood vessel growth are the fibroblast growth factor (FGF) family and vascular endothelial growth factor (VEGF) (10). The mitogen-activated protein kinase (MAPK) signal transduction cascade (extracellular signal-regulated kinases 1 and 2 [ERK1/2]) is known to be involved both in VEGF gene expression and in control of proliferation of vascular endothelial cells (10). The availability of a chick CAM model of angiogenesis (10–14) allowed us to define the effect of low levels of sodium arsenite and its potential mechanism of actions.

In this report, we describe a pro-angiogenesis effect of arsenic trioxide that is comparable with that of basic FGF (b-FGF) or VEGF in the CAM model. We also provide evidence that representative selenium compounds reverse the pro-angiogenesis effect of arsenic, which is initiated at the endothelial cell plasma membrane, involves a plasma membrane integrin $\alpha v \beta 3$ receptor and is mediated by activation of the ERK1/2 signal transduction pathway.

## Materials and methods

### Reagents

Sodium arsenite and other common reagents were obtained from Sigma Chemical Company (St Louis, MO) and PD 98059 from Calbiochem (La Jolla, CA). Dimethyl selenone and diphenyl selenone were prepared by oxidation of dimethyl selenide and diphenyl selenide, as described elsewhere (15). *Se*-Methyl selenocysteine was synthesized at University at Albany, SUNY (Albany, NY), and sodium selenite was purchased from Sigma. Monoclonal antibodies to integrins $\alpha v \beta 3$ and other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Human recombinant b-FGF and VEGF were obtained from Invitrogen (Carlsbad, CA). The high-affinity small molecule $\alpha v \beta 3$ antagonist, XT199, is available at the Pharmaceutical Research Institute (Albany, NY).

### CAM model of angiogenesis

Neovascularization was examined in the CAM model, as described previously (10–14). Ten-day-old chick embryos were purchased from Spafas (Preston, CT) and incubated at 37°C with 55% relative humidity. With a hypodermic

needle, a small hole was made in the shell at the air sac, and a second hole was made on the long side of the egg, directly over an avascular portion of the embryonic membrane that was identified by candling. A false air sac was created beneath the second hole by distal application of negative pressure, so that the CAM separated from the shell. A window ~1.0 cm$^2$ was cut in the shell over the dropped CAM, allowing direct access to the underlying membrane. Sodium arsenite in comparison with standard pro-angiogenic stimuli b-FGF (1 μg/ml) and VEGF (2 μg/ml) was used. Sterile disks of #1 filter paper (Whatman International, Kent, UK) were pre-treated with 3 mg/ml cortisone acetate and air-dried under sterile conditions. Sodium arsenite, b-FGF, VEGF or control vehicle and inhibitors were then applied to the disks; the disks were allowed to dry, suspended in phosphate-buffered saline (PBS) and placed on the CAMs. Filters treated with thyroid hormone and analogs, sodium arsenite, b-FGF or VEGF were placed on the first day of the 3-day incubation, and selenium analogs were added 30 min later to selected arsenite-treated CAMs. At 24 h, the MAPK cascade inhibitor PD 98059 or the αvβ3 integrin antagonist XT199 or the various selenium-containing compounds were also added to the CAMs pre-treated with arsenite or b-FGF, by means of the filter disks.

*Microscopic analysis of CAM sections*

After incubation at 37°C with 55% relative humidity for 3 days, the CAM tissue directly beneath each filter disk was resected from each CAM sample. Tissues were washed three times with PBS, placed in 35-mm Petri dishes (Nalge Nunc, Rochester, NY) and examined under an SV6 stereomicroscope (Karl Zeiss, Thornwood, NY) at 50× magnification. Digital images of CAM sections exposed to the treatment filters were collected using a 3-CCD color video camera system (Toshiba America, New York, NY) and analyzed with Image-Pro software (Media Cybernetics, Silver Spring, MD). The number of vessel branch points contained in a circular region equal to the area of each filter disk was counted. One image was counted in each CAM preparation, and findings from eight CAM preparations were analyzed for each treatment condition. Each experiment was carried out three times. The resulting angiogenesis index is the mean ± SD of new branch points in each treatment condition.

*Statistical analysis*

Statistical analysis was performed by one-way analysis of variance comparing each experimental group with its respective control group. The statistical significance was defined as $P < 0.05$.

# Results

*Effect of arsenic on angiogenesis*

Sodium arsenite at 33 nM resulted in a significant ($P < 0.01$) stimulation of angiogenesis in the CAM model that is comparable with that obtained with b-FGF or VEGF (Figure 1A and B).

*Effect of selenium-containing compounds on arsenic-induced angiogenesis*

The pro-angiogenesis effect of arsenite was significantly ($P < 0.01$) blocked by various selenium-containing compounds at 10 μmoles of either dimethyl selenone or diphenyl selenone (Figure 2A and B). Similarly, either dimethyl selenone or diphenyl selenone significantly ($P < 0.01$) blocked b-FGF-induced angiogenesis in the CAM model (Figure 3A and B). Likewise, 10 μmoles of either sodium selenite or Se-methyl selenocysteine significantly inhibited arsenite-induced angiogenesis (Figure 4A–C) or b-FGF-induced angiogenesis (Figure 5A–C) in the CAM model.

There was greater efficacy in reversing arsenite pro-angiogenesis at the same molar doses (10 μmoles) by dimethyl selenone as compared with diphenyl selenone. Additionally, sodium selenite demonstrated greater efficacy as compared with Se-methyl selenocysteine. Similar efficacy was demonstrated for these various selenium-derived compounds in reversing b-FGF-mediated angiogenesis (Figures 2–5).

*The ERK1/2 signal transduction pathway in stimulation of angiogenesis by arsenic*

Sodium arsenite at 33 nM caused a two-fold increase in blood vessel branching, a response that was effectively blocked ($P < 0.01$) by the ERK1/2 activation inhibitor, PD 98059 (Figures 6A and B). We have
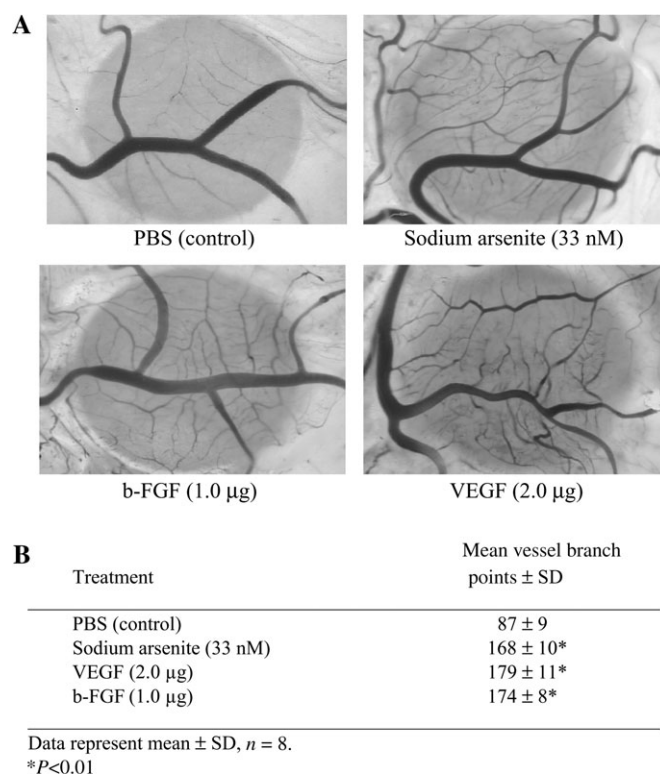


**Fig. 1.** Stimulation of angiogenesis in the chick CAM model by arsenic in comparison with b-FGF and VEGF. Membranes were treated for 3 days, as described in Materials and methods. (**A**) Results from a representative experiment are shown ($n = 8$ images/group/experiment in three different experiments). Stimulation of angiogenesis by sodium arsenite, b-FGF and VEGF is evident. (**B**) The results are summarized in the table as the angiogenesis index (mean ± SD of branch points per field) for each experimental variable.

shown previously that b-FGF stimulation of branch formation was also blocked by this inhibitor of ERK1/2 activation (14).

*The role of integrin αvβ3 in arsenic-mediated angiogenesis*

The integrin αvβ3 antagonist XT199 is known to inhibit b-FGF stimulation of angiogenesis in the CAM assay (14). The pro-angiogenic effect of arsenic is blocked ($P < 0.01$) by the αvβ3 antagonist, XT199 (Figures 6A and B), and thus the pro-angiogenesis effect of arsenic is initiated at the plasma membrane integrin αvβ3 and involves activation of the ERK1/2 pathway to promote b-FGF release from endothelial cells.

# Discussion

Animal studies showed that arsenite alone is not a complete carcinogen (with the exception of some transplacental studies by the Waalkes laboratory [16]). Skin tumors are the most frequent arsenic-associated tumors in humans. Arsenite (and arsenate, which is converted to arsenite) acts to enhance the carcinogencity of other agents (17). One possible way that an agent can enhance carcinogenesis without being a complete carcinogen is by enhancing angiogenesis, as demonstrated in this investigation. Inorganic arsenic (arsenite and arsenate) in drinking water has been associated with skin cancers in several countries, such as Taiwan, Chile, Argentina, Bangladesh and Mexico. This association has not been established in the USA. In addition, inorganic arsenic alone in drinking water does not cause skin cancers in animals. It was recently shown that concentrations as low as 1.25 mg/l sodium arsenite were able to enhance the tumorigenicity of solar UV irradiation in mice (17,18). In this report, we
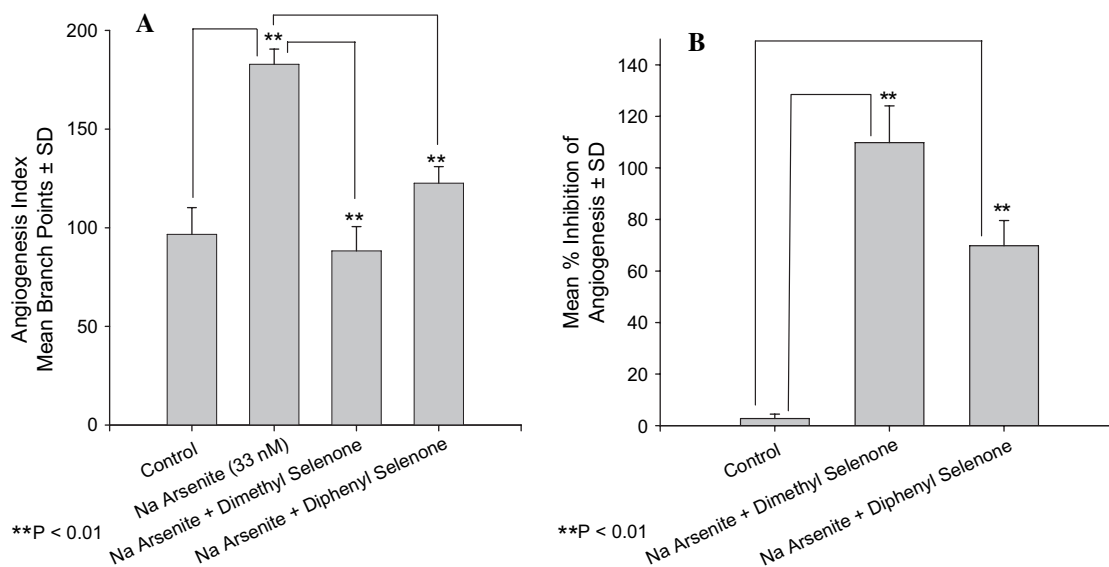
**Fig. 2.** Effect of selenium (dimethyl or diphenyl selenone at 10 μmoles each) on sodium arsenite-mediated stimulation of angiogenesis in the chick CAM model. (**A**) The results of experiments are summarized as the angiogenesis index (mean ± SD, branch points, $n = 8$ per group) for each experimental variable. (**B**) Data are summarized as mean % inhibition of angiogenesis ± SD, $n = 8$ per group.
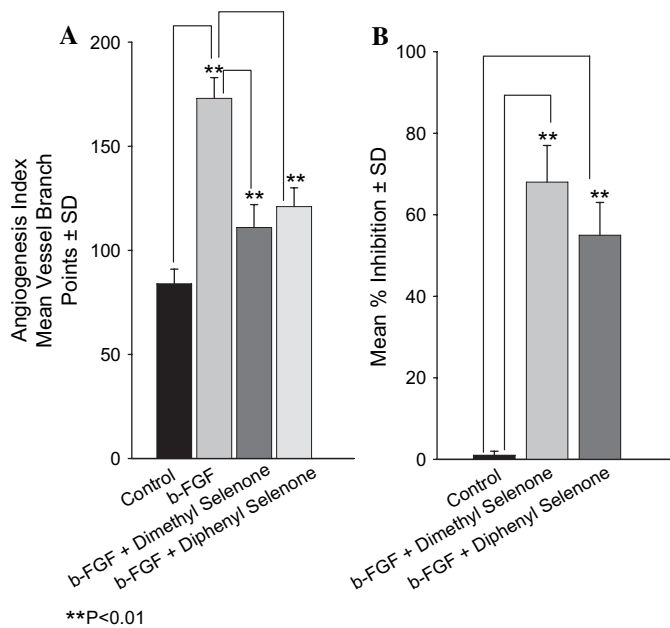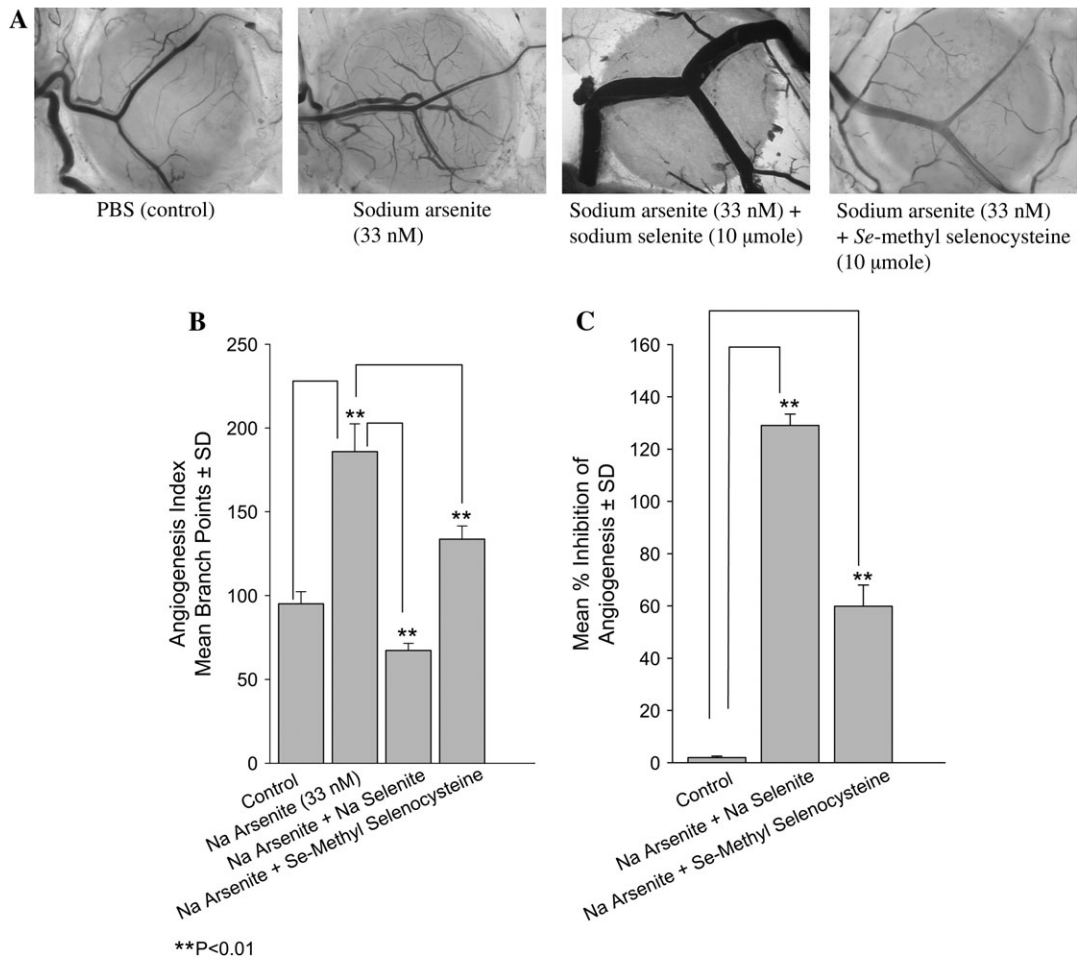


**Fig. 3.** Effect of selenium (dimethyl or diphenyl selenone at 10 μmoles each) on b-FGF-mediated stimulation of angiogenesis in the chick CAM model. (**A**) The results of experiments are summarized as the angiogenesis index (mean ± SD, branch points, $n = 8$ per group) for each experimental variable. (**B**) Data are summarized as mean % inhibition of angiogenesis ± SD, $n = 8$ per group.

describe a pro-angiogenesis effect of arsenic trioxide that is comparable with that of b-FGF or VEGF in the CAM model. We also provide evidence that different selenium-derived compounds reverse the pro-angiogenesis effect of arsenic, which is initiated at the endothelial cell plasma membrane, involves a plasma membrane integrin αvβ3 receptor and is mediated by activation of the ERK1/2 signal transduction pathway.

In agreement with our results, the pro-angiogenic effects of arsenic trioxide were recently demonstrated in mouse Matrigel implants. Low and high doses of arsenic trioxide were synergistic with b-FGF in increasing vessel density in the Matrigel assay (5). Furthermore, the effects of arsenic trioxide on tumor growth implanted in nude mice after biweekly injections of 0.5–5.0 mg/kg arsenic trioxide were determined. In that study, significant tumor growth and lung metastasis were seen in all animals, with the largest tumors occurring in animals treated with lower doses of arsenic trioxide. These studies support the hypothesis and indicate that induction of angiogenesis, enhanced tumor growth and metastasis are potential dose-dependent toxic side effects of arsenic therapies (5). In contrast, the anti-angiogenic properties of tetraarsenic oxide, another trivalent arsenic compound, was shown *in vitro* and *in vivo* (19). It inhibited the proliferation, migration into the denuded area and invasion through a layer of Matrigel of b-FGF-stimulated bovine capillary endothelial cells in a dose-dependent manner (19). Further data supported the chemopreventive effect of selenium and argue against the involvement of selenoproteins (19) but rather for a key role for methylated forms of selenium metabolites (19,20).

In our current study, selenones, selenite or *Se*-methyl selenocysteine demonstrated potent anti-angiogenesis efficacy in inhibiting the pro-angiogenesis effect of either arsenite or b-FGF, with greater efficacy with dimethyl selenone and sodium arsenite as compared with diphenyl selenone and *Se*-methyl selenocysteine at the same molar doses. Previous work has suggested that anti-angiogenic activity may be a novel mechanism contributing to the cancer chemopreventive activity of selenium (21,22). Data support a potential anti-angiogenic - effect of selenium-derived compounds in the chemoprevention of cancer, as do data that contrast two pools of selenium metabolites, namely, methaneselenol versus hydrogen selenide, which differentially affect proteins and cellular processes crucial to tumor angiogenesis regulation (6,21–23). In our studies, the metabolic fate of the selenium from each compound is not known. Based on these studies and our current study, the use of selenium-derived compounds along with arsenic trioxide might lead to improved efficacy and safety of the use of arsenic alone. The exact metabolic fate of selenium derived from these various selenium-containing compounds is unknown. Further studies are needed to document such combination in various preclinical and clinical settings.

Other studies showed that the c-Jun N-terminal kinase (JNK) (and possibly p38) are involved with high-dose arsenite, and ERK

**Fig. 4.** Effect at 10 μmoles of either sodium selenite or *Se*-methyl selenocysteine on sodium arsenite-mediated stimulation of angiogenesis in the chick CAM model. (**A**) Representative images with sodium arsenite ± sodium selenite or *Se*-methyl selenocysteine. (**B**) The results of experiments are summarized as the angiogenesis index (mean ± SD, branch points, *n* = 8 per group) for each experimental variable. (**C**) Data are summarized as mean % inhibition of angiogenesis ± SD, *n* = 8 per group.

is involved at lower doses (24). In this report, the molecular mechanism by which MAPK pathways might differentially contribute to cell growth regulation and cell death in response to different dosages of arsenite was determined. A low level of arsenite stimulated ERK signaling pathway and enhanced cell proliferation, and this arsenite-induced ERK activity was blocked by MAPK inhibitor, PD 98059. In contrast, a high level of arsenite stimulated the JNK signaling pathway and induced cell apoptosis; this arsenite-induced JNK activity was blocked by JNK inhibitor II, SP600125. The implications of these findings are that a high concentration of arsenic exposure causes apoptosis, whereas a low concentration of arsenic exposure is carcinogenic and may result in aberrant cell accumulation (24). This carcinogenic effect of low-level arsenic might be accelerated by its pro-angiogenesis effects at low levels. Another report shows that arsenite induces ERK, but not JNK, which is required for its effects on cell transformation (25). Taking all these data together, it appears that the cellular effects of low-level arsenic is mediated via ERK, as shown in our and other reports in various cell lines (24–26).

Accumulated evidence from prospective studies, intervention trials and studies on animal models of cancer has suggested a strong inverse correlation between selenium intake and cancer incidence. Several putative mechanisms have been suggested to mediate the chemopreventive activities of selenium: of these, the inhibition of cellular proliferation and the induction of apoptosis are particularly attractive. The MAPK pathways are known to be important regulators of cell death, and signal transduction studies suggest that selenium induces several changes in the MAPK signaling pathways (27).

Interactions between arsenic and selenium at the metabolic level are multifaceted and complex. These interactions are of practical significance because populations in various parts of the world are simultaneously exposed to inorganic arsenic in drinking water and selenium mainly in the diet at varying levels. Overall, this study suggests that dietary selenium status alters arsenic metabolism and disposition (28,29). The several selenium-containing compounds showed differing degrees of effectiveness in reversing arsenic-mediated angiogenesis at equimolar doses, but the exact mechanism in term of metabolic or molecular interactions requires further investigations.

This current study demonstrated a similar pro-angiogenesis mechanism for As(III) and b-FGF that is mediated via the activation of MAPK (ERK1/2) (as evident by the blockade with MAPK inhibitor) as well as via the αvβ3 integrin (as evident by the blockade with specific αvβ3 antagonist). Both pro-angiogenic effects of As(III) and b-FGF are blocked by selenium-derived compounds. Additionally, this investigation showed that selenium supplementation provided a potential solution to the risk of environmental exposure to low-level As(III).
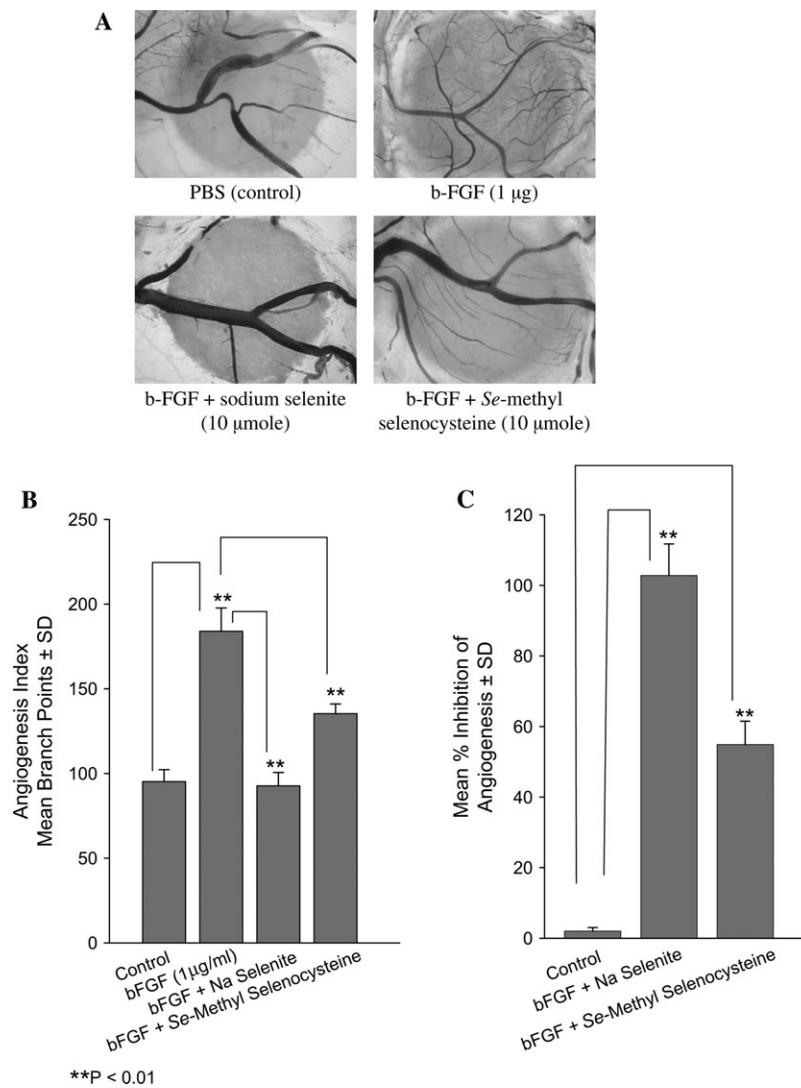
**Fig. 5.** Effect at 10 μmoles of either sodium selenite or *Se*-methyl selenocysteine on b-FGF-mediated stimulation of angiogenesis in the chick CAM model. (**A**) Representative images with b-FGF ± sodium selenite or *Se*-methyl selenocysteine. (**B**) The results of experiments are summarized as the angiogenesis index (mean ± SD, branch points, *n* = 8 per group) for each experimental variable. (**C**) Data are summarized as mean % inhibition of angiogenesis ± SD, *n* = 8 per group.
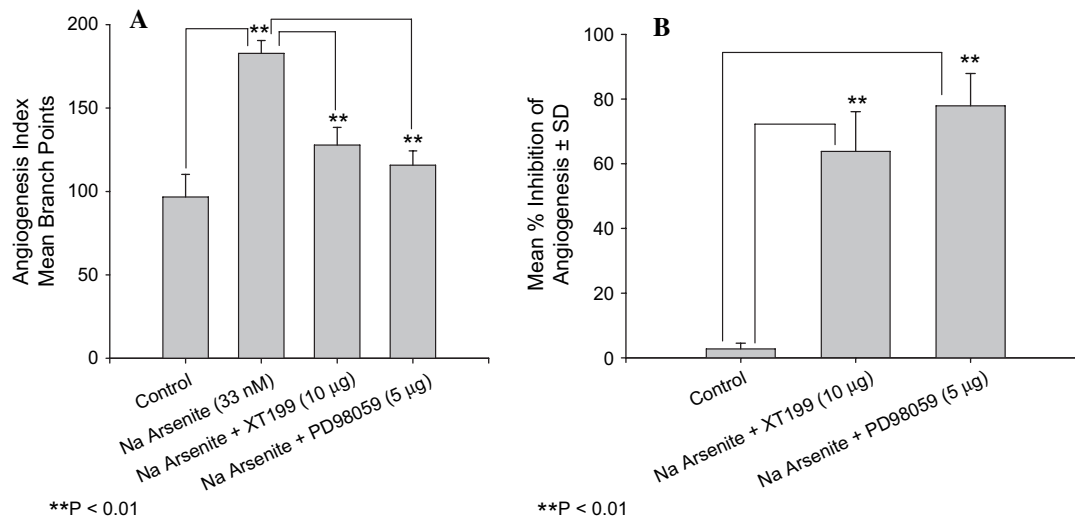


**Fig. 6.** Effect of PD 98059 or XT199 at 10 μmoles each on sodium arsenite-mediated stimulation of angiogenesis in the chick CAM model. (**A**) A two-fold increase in blood vessel branch formation exposed to 33 nM arsenic for 3 days, an effect that is inhibited by PD 98059, an MAPK (ERK1/2) signal transduction cascade inhibitor. Angiogenesis induced by arsenic was also inhibited by the anti-integrin αvβ3, XT199. (**B**) The results are summarized as the % inhibition of angiogenesis (mean ± SD, *n* = 8 per group) for each experimental variable.

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