Differential augmentative effects of buthionine sulfoximine and ascorbic acid in As_2O_3-induced ovarian cancer cell death: Oxidative stress-independent and -dependent cytotoxic potentiation

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Abstract. The potential of arsenic trioxide (As_2O_3) as a novel therapy against ovarian cancer has been progressively recognized. Its prospective usefulness for treatment of this malignancy either alone or in combination with other chemotherapeutic agents has been increasingly explored. In this study, we attempted to enhance the cytotoxicity of As_2O_3 in ovarian cancer cells through manipulation of cellular glutathione (GSH) levels using either buthionine sulfoximine (BSO) or ascorbic acid (AA). Results from our studies showed that combinatorial therapies using As_2O_3 with either low dose BSO or only pharmacological doses of AA acted synergistically to enhance the cytotoxicity of As_2O_3 in ovarian tumor cells. With these regimens, therapeutic selectivity was observed with preferential killing of ovarian tumor cells over normal fibroblast controls. Furthermore, contrary to previous reports, enhancement of As_2O_3-mediated cell killing by these two agents was propagated through different effects. With BSO, apoptotic and non-apoptotic cell death enhancement were mediated through increased arsenic accumulation and GSH depletion that occurred independently of reactive oxygen species. With pharmacological doses of AA, increase in cell death proceeded through non-apoptotic routes via an oxidative stress-related pathway independent of GSH levels. Taken together, these results indicate that GSH depleting agents or pro-oxidative chemicals have capabilities of improving the utility of As_2O_3 in ovarian cancer management.

Introduction

Ovarian cancer remains the leading cause of death in gynaecological malignancies (1). Current treatment involves surgery followed by adjuvant platinum- and taxane-based therapy (1). Despite initial response to chemotherapy, most patients in advance stages relapse with the manifestation of a platinum-resistant phenotype (1). While numerous second line agents have been tried for platinum-resistant disease, response rates remain dismal, suggesting that novel therapeutic combinations are urgently needed.

Arsenic trioxide (As_2O_3) possesses remarkable potency against acute promyelocytic leukemia (APL) (2). It has also shown potential for treatment of multiple myeloma (MM) (3,4) and various solid tumors (5-14). For ovarian cancer, we previously reported that As_2O_3 induces apoptosis in both cisplatin (Cddp)-sensitive and -resistant ovarian carcinoma cells at clinically achievable concentrations of 2-8 μM (7). However, present clinical experience with As_2O_3 is mainly derived from its use in APL therapy which corresponds to *in vitro* concentrations of 0.5-2 μM (3,15). At such, a combined modality approach is an attractive strategy to enhance its efficacy in ovarian cancer cells to clinical concentrations comparable to that used in APL.

Glutathione (GSH) redox system plays a pivotal role in the detoxification of numerous chemotherapeutic drugs (16). Overexpression of GSH confers resistance to tumor cells and often limits the efficacy of chemotherapy (16). For As_2O_3, GSH is involved in its metabolism through interaction of the thiol group within the GSH molecule (17). GSH and related enzymes including GSH peroxidase (GPx) are also involved in scavenging reactive oxygen species (ROS) generated by As_2O_3 (17). In leukemic and some solid tumors, intracellular GSH levels have been reported to be inversely related to their As_2O_3 sensitivity (18,19). In some As_2O_3-resistant cells, chemoresistance has been successfully circumvented with GSH reducing agents such as buthionine sulfoximine (BSO), an inhibitor of the rate determining enzyme in GSH synthesis, and ascorbic acid (AA) which depletes GSH levels during its auto-oxidation and reduction process (3,20).

In this study, we investigated the role of intracellular GSH levels on As_2O_3 sensitivity of ovarian tumor cells and the effects of manipulating this system on As_2O_3-induced cytotoxicity in these cells. We observed a positive correlation between cellular GSH concentration and their cytotoxic susceptibility to As_2O_3. We further demonstrated that selective

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chemosensitization to clinically achievable As_2O_3 concentrations can be achieved with BSO and only pharmacological doses of AA. Interestingly, unlike other tumor cells, our results revealed that BSO and pharmacological levels of AA mediated cytotoxic enhancement proceed through different cellular effects.

Materials and methods

Reagents. All common reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Cell lines and treatment. Three human ovarian cancer cell lines (OVCAR, OVCAR-3 and JAM) were used. Lung fibroblast cell line (MRC-5) was employed as normal control and U266, multiple myeloma cells act as a positive control for experiment involving physiological concentration of AA (100 μM). OVCAR-3, U266 and MRC-5 cells were purchased from ATCC (Rockwille, MD). OVCAR and JAM cells were previously characterized by Queensland Institute of Medical Research (7). All ovarian cancer and U266 cells were maintained in RPMI-1640 under standard conditions. MRC-5 cells were grown in DMEM.

Cells were seeded at 10,000 cells/well. After overnight incubation, culture medium was replaced with either control medium or drug containing medium. For experiments involving antioxidants, cells were pre-treated with N-acetylcysteine (NAC) (2 mM), catalase (CAT) (500 U/ml) or t-butyl-hydroxyanisole (BHA) (100 μM) for 4 h before addition of As_2O_3 with or without BSO or AA. For all experiments, cell viability after 72 h of incubation was determined using fluorometric microculture cytotoxicity assay (7). Cell survival was presented as survival index (SI) (7) and IC_{50} was calculated as previously described (7).

[^{3}H]-thymidine uptake. Proliferation and cytotoxicity of U266 cells were assessed using [^{3}H]-thymidine after incubating for 72 h with either As_2O_3 or AA (100 μM) alone or in combination. [Methyl-[^{3}H]-thymidine (0.5 μCi/well, Amersham Biosciences, Piscataway, NJ) was added during the final 18 h of incubation. At the end of incubation, samples were harvested and analyzed using 1450 microbeta microplate β-scintillation counter (Perkin-Elmer, Gaithersburg, MA). Cell survival was presented as SI and was defined as mean count of drug-treated cells as a percentage of that of control untreated cells. IC_{50} was obtained as described above.

Detection of apoptosis. Cells were harvested after treatment, washed with phosphate buffered saline (PBS) and fixed in 70% (v/v) ice-cold ethanol. Subsequently, they were incubated in PI/Triton X-100 staining solution containing 0.2 mg/ml of DNAse-free RNAse A for 30 min at 37˚C before data acquisition using flow cytometry. The percentage of cells in different cell cycle phases was obtained from analysis of DNA histograms using WinMDI Software (Scripps Institute, La Jolla, CA). Cells with DNA content less than the cells in the G_1 phase (sub-G_1 phase) were considered apoptotic.

Determination of intracellular GSH content. Cells were collected, washed with PBS and lysed with 200 μl of 10 mM HCl. Forty μl of the lysate was kept for protein determination using bradford reagent. The remaining lysate was protein-precipitated using 40 μl of 5% (w/v) 5-sulfosalicylic acid, cooled on ice for 10 min and centrifuged at 8000 x g at 4˚C for 15 min. Supernatants were collected and analysed for total GSH contents using a modified 5'-dithiobis-(2-nitrobenzoic) acid (DNTB)-enzyme recycling assay (21). Twenty μl of the respective standards, samples and blanks were pipetted in triplicates into separate wells of a 96-well plate. Five ml of 1 mM DNTB, 5 ml of 1 mM nicotinamide adenine dinucleotide phosphate (NADPH), 5.75 ml of phosphate buffer (100 mM Na_3PO_4, 1 mM EDTA, pH 7.4) and 0.1 ml of GSH reductase (GR) were thoroughly mixed. One hundred μl of the reaction mixture was immediately added into each well. The absorbance generated was read at 405 nm. Concentration of GSH in the samples was determined with reference to the standard curve (GSH 0.5-20 μM) generated with each batch of samples. Intracellular GSH content was expressed as nmol/mg of total protein measured.

Assay for cellular GPx level. GPx levels were assayed using a commercially available kit from Cayman Chemical as per manufacturer's instructions (Ann Arbor, MI). GPx activity of each sample was normalized to protein concentration determined using bradford reagent. One unit of GPx was defined as the amount of enzyme causing the oxidation of 1 nmol of NADPH/min/mg of protein.

Immunoblotting. Extraction of protein was performed as previously described (22). Protein (30 μg) was separated on SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were incubated overnight with anti-CAT antibody (Calbiochem, Darmstadt, Germany) or anti-actin antibody (Sigma) after blocking, followed by incubation with secondary antibody (peroxidase-conjugated anti-rabbit IgG (Dako, Glostrup, Denmark) or anti-mouse IgG (Abcam, Clearbrook, IL) respectively). All antibody dilutions were carried out as per manufacturer's recommendation. Immunoblots were visualized using enhanced chemiluminescence detection system (Perkin-Elmer Life Sciences, Wellesley, MA). Densitometry data were normalized by actin and presented as fold-change, which is defined as a ratio of the CAT/actin ratio of the respective cell line over that of OVCAR cells.

Measurement of cellular AA uptake. OVCAR, OVCAR-3 and JAM cells were seeded into 24-well plates. After 24 h, cells were washed and equilibrated with Hank's balanced salt solution (HBSS)/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) incubation medium at 37˚C for 30 min. HBSS/HEPES incubation medium was replaced by 300 μl of HBSS/HEPES containing 100 μM of DTT, 0.1 μCi of ^{14}C AA (Perkin-Elmer, Wellesley, MA) and 100 μM of unlabelled AA and incubated for 0.5-4 h at 37˚C. At specific times, cells were lysed with 0.1 N NaOH/5% (w/v) SDS. The lysate (300 μl) was mixed with 3 ml of scintillation liquid. Intracellular ^{14}C AA was measured using scintillation counter (LS 3801, Beckman Instruments, Inc., CA). Each experiment was performed in triplicate. Parallel culture of cells was prepared and cells in each well of parallel culture plates were separately counted. Intracellular AA was expressed as pmol/10,000 cells.

Table I. IC_{50} values of As_2O_3 alone and in combination with either low dose BSO or AA.

Treatment	IC_{50} value of As_2O_3 (μM)			
	OVCAR	OVCAR-3	JAM	MRC-5
As_2O_3	1.96±0.67[a]	1.48±0.41[a]	7.89±0.84[a]	9.01±0.10
As_2O_3 + BSO 1 μM	0.55±0.03[a,b]	0.45±0.02[a,b]	3.08±0.18[a,b]	>8.00
As_2O_3 + BSO 5 μM	0.12±0.06[a,b]	0.14±0.01[a,b]	1.18±0.25[a,b]	4.07±0.38[b]
As_2O_3 + AA 100 μM	2.31±0.12[a]	1.12±0.20[a]	>8.00	>8.00
As_2O_3 + AA 500 μM	0.88±0.02[a,b]	0.35±0.17[a,b]	7.95±0.51[a]	>8.00
As_2O_3 + AA 1000 μM	-	-	2.45±0.07[a,b]	>8.00

Data are expressed as the mean ± SD of at least three independent experiments. [a]$p<0.05$ versus MRC-5. [b]$p<0.05$ versus As_2O_3 treatment for each respective cell line.

Measurement of intracellular arsenic concentration. Cells were harvested, washed with PBS, counted and digested in 65% (v/v) nitric acid using High Pressure Asher (Aston Par, Graz, Austria) as per manufacturer's instructions. Arsenic concentrations in the digesates were determined using quadrupole ICP-MS (Thermo Electron Corp., Cheshire, UK) as per manufacturer's recommendations. Arsenic concentration in each sample was normalized to initial cell count prior to sample digestion and expressed as ng/million cells.

Statistical analyses. Results are presented as the mean ± SD of at least three independent experiments. Data were analyzed by Student's t-test or one-way ANOVA with Tukey's posthoc analysis using Graphpad Prism 4 Software (San Diego, CA). $p<0.05$ was considered statistically significant.

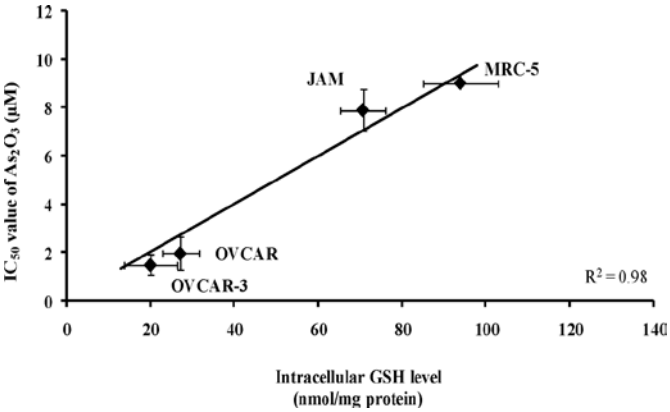

Figure 1. Basal intracellular GSH levels of ovarian cancer and normal fibroblast cells and their correlation with cellular IC_{50} values. Cellular GSH levels were expressed as the mean ± SD of three independent experiments.

Results

Positive correlation between intracellular GSH levels in ovarian cancer and normal fibroblast cells to their sensitivity to As_2O_3 treatment. The relationship between As_2O_3-induced cytotoxicity and intracellular GSH levels was examined in OVCAR, OVCAR-3, JAM and MRC-5 cells. IC_{50} values in ascending order after As_2O_3 treatment alone were: OVCAR-3 = OVCAR < JAM < MRC-5 (Table I). Similarly, basal cellular GSH levels in ascending order were: OVCAR-3 = OVCAR < JAM < MRC-5 (Fig. 1). MRC-5 and JAM cells harbored higher GSH contents and were less responsive to the effects of As_2O_3. Conversely, OVCAR and OVCAR-3 cells have lower GSH levels and were more responsive to As_2O_3 treatment. A positive correlation (Spearman's correlation coefficient, $r^2 = 0.98$) between intracellular GSH levels and As_2O_3 sensitivity of tumor and normal fibroblast cells was found (Fig. 1).

BSO but not physiological level of AA sensitized ovarian tumor cells to cytotoxicity of As_2O_3. Following this observation, we determined whether concurrent use of BSO (1 and 5 μM) or physiological level of AA (100 μM) were able to augment As_2O_3-induced cell kill. BSO (1 and 5 μM) or AA (100 μM) alone showed minimal cell toxicity (Table II). With concurrent BSO therapy, 1 μM of BSO sensitized all ovarian cancer cells, including the less As_2O_3-sensitive JAM cells to the cytotoxicity of As_2O_3, as shown by the lower IC_{50} values for all the ovarian cancer cell lines following combined therapy (Table I). With co-treatment of BSO at 5 μM, IC_{50} value of As_2O_3 for JAM cells was further lowered to 1.18±0.25 μM ($p<0.05$) (Table I), which is comparable to that in APL cells (3,15).

Contrary to previous reports (3,4), physiological level of AA (100 μM) failed to modulate the cytotoxicity of As_2O_3 in ovarian tumor cells since no significant reduction in IC_{50} values were observed after combined drug treatment (Table I). To confirm that this observation was not due to AA degradation, U266 cells was employed as a positive control. U266 cells retained high viability (SI = 85.03±14.20%) when treated with AA 100 μM alone. Upon co-treatment with AA (100 μM) and As_2O_3, viability of U266 cells was lower compared to As_2O_3 treatment alone, for concentrations of As_2O_3 at ≤2 μM ($p<0.05$) (Fig. 2A). Furthermore, percentage of apoptotic cells was greater than As_2O_3 treatment alone ($p<0.05$) (Fig. 2B), confirming that physiological level of AA was indeed active in our cellular systems.

Ovarian cancer cells are able to accumulate AA better than U266 cells. To rule out that the lack of synergism in ovarian

Table II. Survival indices of low dose BSO alone or AA alone in OVCAR, OVCAR-3, JAM and MRC-5 cells.

	Survival index (SI)			
Treatment	OVCAR	OVCAR-3	JAM	MRC-5
BSO 1 μM	97.45±8.61	102.87±0.62	104.08±11.33	86.68±3.26
BSO 5 μM	86.17±14.88	96.83±5.13	106.95±10.62	107.96±3.95
AA 100 μM	105.45±10.50	97.94±9.28	100.03±9.81	107.46±12.58
AA 500 μM	94.77±7.16	89.87±4.04	92.39±2.12	104.07±9.54
AA1000 μM	-	-	95.74±14.20	90.51±11.30

Data are expressed as the mean ± SD of at least three independent experiments.

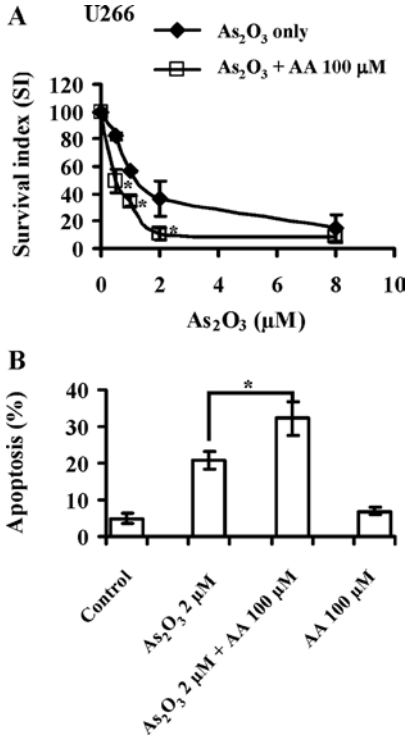

Figure 2. Effect of physiological level of AA on As_2O_3-induced cytotoxicity and apoptosis in U266 cells. (A) Cellular cytotoxicity was evaluated using [^{3}H]-thymidine assay after incubation with either As_2O_3 alone or in combination with 100 μM of AA ($p<0.05$ versus As_2O_3 only). (B) Apoptosis rates after various drug treatments were evaluated using flow cytometry of PI stained cells. Data are expressed as the mean ± SD of at three independent experiments ($^*p<0.05$).

tumor cells was not due to their inability to uptake AA, we performed AA uptake experiments. Incubation of each cell line with AA resulted in a time-dependent uptake of AA. Ovarian tumor cells showed a general trend of greater AA uptake compared to U266 cells (Fig. 3). From 2 h of AA incubation, JAM cells showed more AA accumulation than both OVCAR and OVCAR-3 cells (Fig. 3) despite being less responsive to simultaneous AA and As_2O_3 therapy. This result suggests that cell line-specific differences in response to As_2O_3 and AA co-treatment are not determined by the capacity of these cells to uptake AA.

Pharmacological doses of AA augmented cytotoxicity of As_2O_3. AA has been given at high doses to achieve pharmacological plasma concentrations of ≥0.3 mM in cancer patients (22,23). Based on these reports, the effects of pharmacological doses of AA, at 500 or 1,000 μM, on As_2O_3-induced ovarian tumor cell death were investigated. AA (500 μM) alone was non-toxic to tumor cells (Table II). AA (500 μM) in combination with As_2O_3 caused an increase in cell death with a reduction in IC_{50} values ($p<0.05$) (Table I) compared to As_2O_3 treatment alone in OVCAR and OVCAR-3 cells. At this concentration, very little cytotoxic augmentation was observed in JAM cells (Table I). When AA concentration was increased to 1,000 μM in JAM cells, AA itself was again minimally toxic to these cells (SI of 95.74±14.20%) (Table II). However at this concentration, chemosensitization of JAM cells to the cytotoxicity of As_2O_3 was observed, allowing 50% of JAM cells to be killed at about 2 μM of As_2O_3 ($p<0.05$) (Table I). Collectively, these data suggest that with careful manipulation, therapeutic augmentation of the cytotoxicity of As_2O_3 in ovarian tumor cells can be achieved using suitable concentrations of AA.

Preferential kill of ovarian cancer cells over normal fibroblast cells using clinically achievable concentrations of As_2O_3 and BSO or pharmacological doses of AA. To determine the clinical value of these drug combinations, the combined effects of BSO (1 and 5 μM) or pharmacological doses of AA (500 or 1,000 μM) with As_2O_3 were tested in normal fibroblast cells, MRC-5. MRC-5 cells were less sensitive than ovarian cancer cells to the effects of As_2O_3 and BSO or pharmacologic doses of AA as reflected by its higher IC_{50} values ($p<0.05$) (Table I). This showed that selective kill of ovarian tumor cells over normal cells can be achieved with clinically achievable concentrations of these compounds.

Combined As_2O_3 and BSO but not pharmacological doses of AA resulted in a significant increase in cellular apoptosis. Since we previously reported that As_2O_3 induces apoptosis in ovarian cancer cells (7), we further investigated whether augmentation of As_2O_3-induced cytotoxicity by BSO or pharmacological doses of AA was mediated by an increase in apoptosis. Concurrent As_2O_3 and BSO therapy caused an increase in cellular apoptosis in the three ovarian cancer cell types ($p<0.05$) (Fig. 4) while pharmacological doses of AA

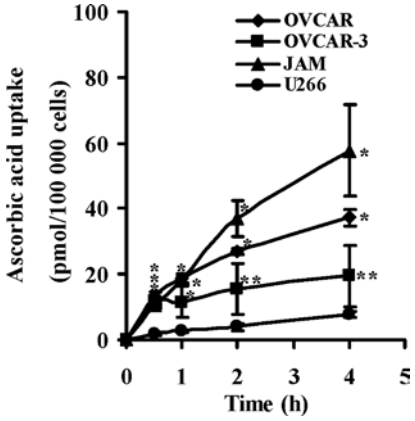

Figure 3. Intracellular accumulation of AA in ovarian cancer cells compared with U266 multiple myeloma cells. Results represent the mean ± SD of three independent experiments (*p<0.05 when compared with U266 cells; **p<0.05 when compared with JAM cells).

did not result in a significant increase in apoptosis following co-treatment (Fig. 4).

Reduction of intracellular GSH levels by BSO, but not pharmacological doses of AA in ovarian cancer cells. To better understand the cellular effects leading to the observed synergism, we analyzed intracellular GSH levels after various drug treatments. Treatment with BSO alone or simultaneously with As_2O_3 resulted in a significant reduction in GSH levels in all ovarian tumor cells ($p<0.05$) (Fig. 5). Unlike reports in APL and MM (3,4), pharmacological doses of AA either alone or in combination with As_2O_3 failed to decrease cellular GSH levels (Fig. 5). This showed that BSO and pharmacological doses of AA exhibited differential effects on GSH in these cells.

Increase in intracellular arsenic accumulation following concurrent BSO and As_2O_3 treatment. To determine whether the reduction in GSH levels with co-treatment of BSO results in increased intracellular arsenic accumulation, we examined cellular arsenic concentration after drug treatments. In OVCAR and OVCAR-3 cells, concurrent BSO treatment at 5 μM resulted in a 2-fold increase in arsenic accumulation when compared with As_2O_3 treatment alone ($p<0.05$) (Fig. 6A and B). In JAM cells, a 3-fold increase in arsenic accumulation was observed with concurrent BSO and As_2O_3 therapy ($p<0.05$) (Fig. 6C). Taken together, these results suggest that enhanced cytotoxicity to As_2O_3 with concurrent BSO use is partly contributed by increased cellular arsenic content.

Enhancement of cell death with As_2O_3 and pharmacological dose of AA is mediated by oxidative stress. GSH is known to protect cells via its antioxidant properties (16) and its depletion with BSO can impair cellular defenses, lending to ROS accumulation and increased sensitivity of cells to cytotoxic insults (17). AA on the contrary can act as a pro-oxidant, generating ROS thereby triggering cell death (24). Therefore,

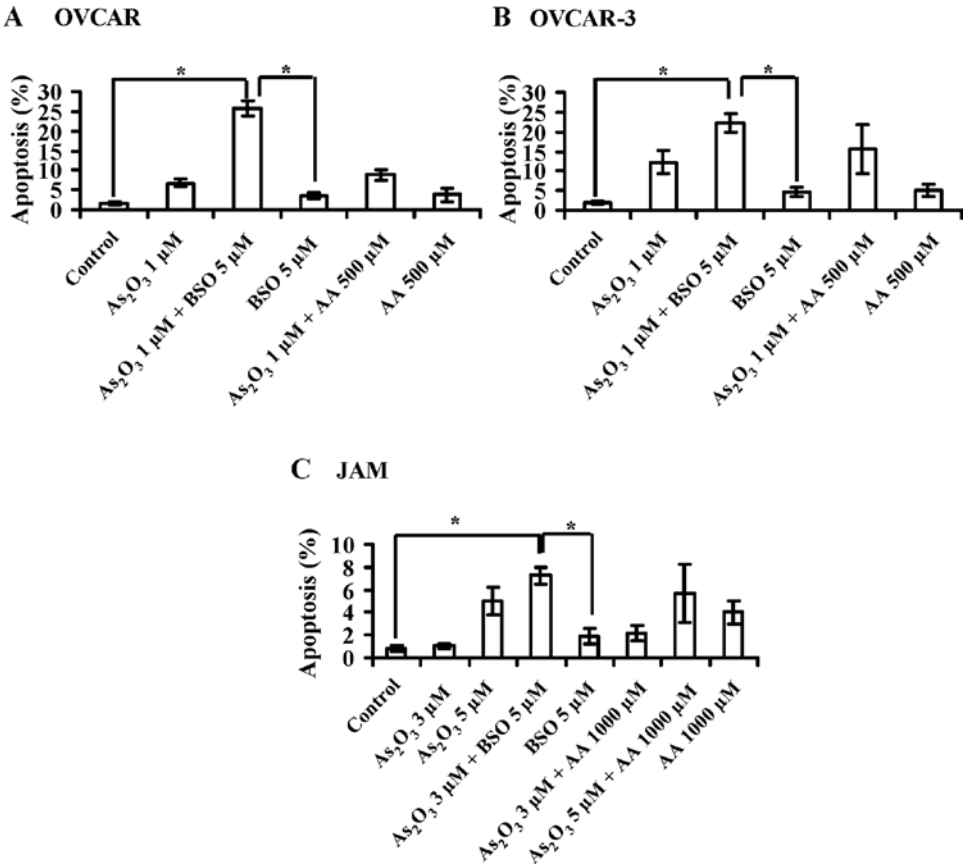

Figure 4. Cellular apoptosis rate after treatment with As_2O_3 alone or in combination with either BSO or pharmacological dose AA in (A) OVCAR, (B) OVCAR-3 and (C) JAM cells. Results are presented as the mean ± SD of three independent experiments (*p<0.05).

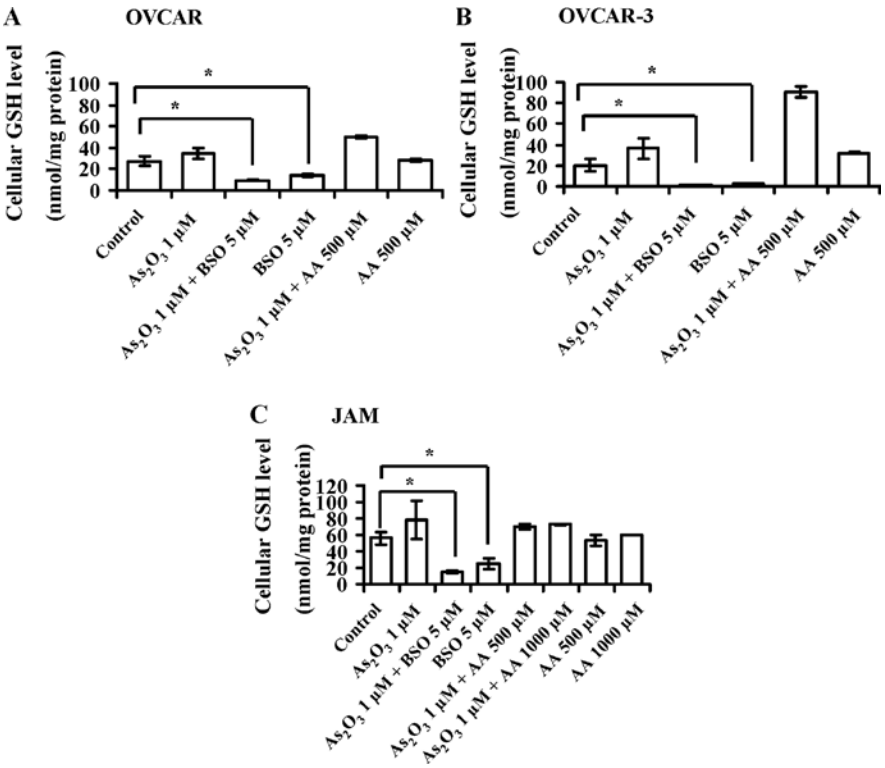

Figure 5. Total intracellular GSH levels of (A) OVCAR, (B) OVCAR-3 and (C) JAM cells after treatment with As_2O_3 alone or in combination with either BSO or pharmacological dose of AA. Results represent the mean ± SD of three independent experiments (*p<0.05).

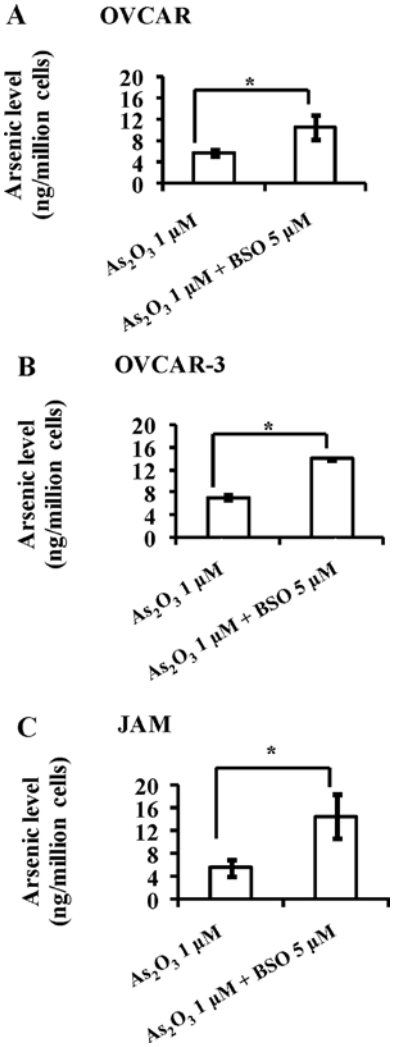

Figure 6. Intracellular As levels after treatment with As_2O_3 alone or in combination with BSO (5 μM) in ovarian tumor cells. Results are presented as the mean ± SD of three independent experiments (*p<0.05).

we employ NAC (a general free radical scavenger and GSH precursor), BHA (a lipophilic antioxidant with superoxide anion radical scavenging activity) and CAT [hydrogen peroxide (H_2O_2) degradation enzyme] to further discern whether a net increase in oxidative stress is responsible for the enhanced cytotoxicity upon BSO or AA co-treatment. For combinations of As_2O_3 and BSO, no protective effect is being conferred by NAC, CAT or BHA (Fig. 7). In contrast, NAC and CAT almost completely protected OVCAR and JAM cells and partially defended OVCAR-3 cells against cell death induced by As_2O_3 and pharmacological dose of AA ($p<0.05$) whereas BHA showed little protective effect (Fig. 7). These results indicate that augmentation of As_2O_3-induced cell death by high dose AA is largely contributed by the generation of excessive oxidative stress via H_2O_2 whereas that by BSO occurred independently of ROS.

Sensitivity of ovarian cancer cells to pharmacological doses of AA and As_2O_3 treatment is related to cellular antioxidant enzyme levels. Since JAM cells more effectively accumulate AA but require higher pharmacological doses of AA for As_2O_3-induced cytotoxic enhancement and ROS play an important role in mediating AA-induced cytotoxic augmentation, we postulated that these cells may harbor higher cellular anti-oxidant enzyme levels. Indeed, intracellular levels of both GPx ($p<0.05$) (Fig. 8A) and CAT (Fig. 8B) were higher in

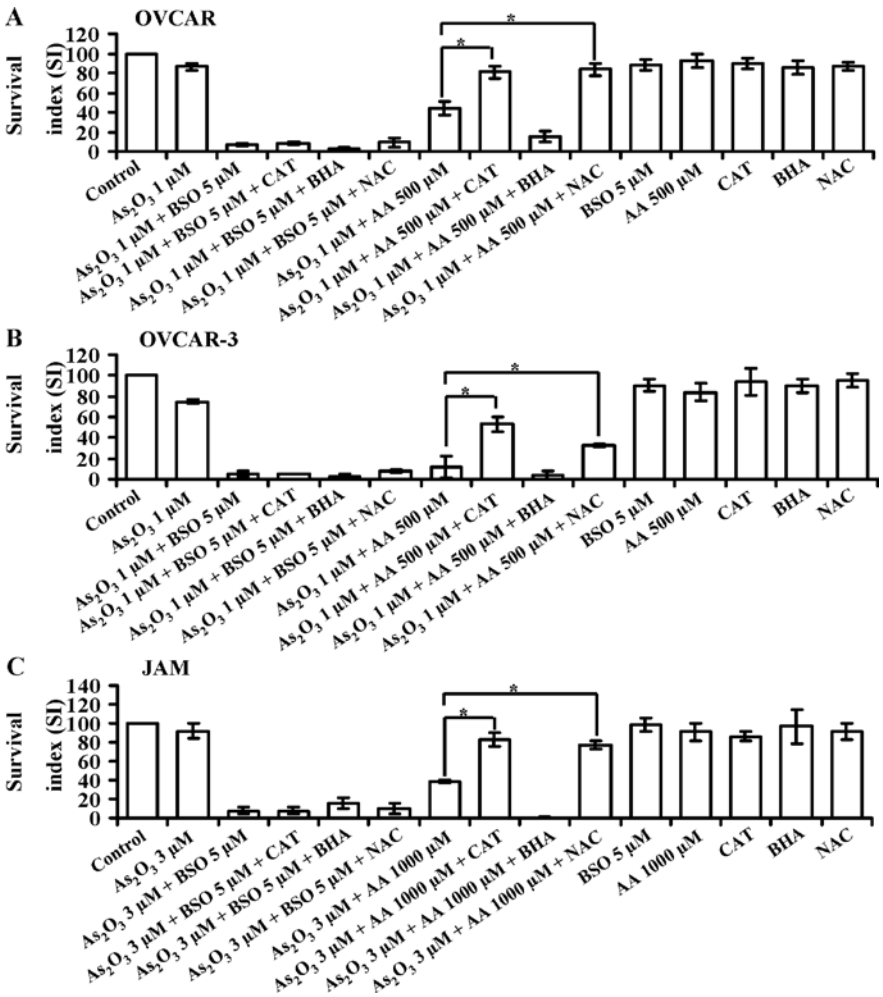

Figure 7. Effects of antioxidants on cell death mediated with As_2O_3 alone or in combination with either BSO or pharmacological dose of AA in (A) OVCAR, (B) OVCAR-3 and (C) JAM cells. Results represent the mean ± SD of three independent experiments (*p<0.05).

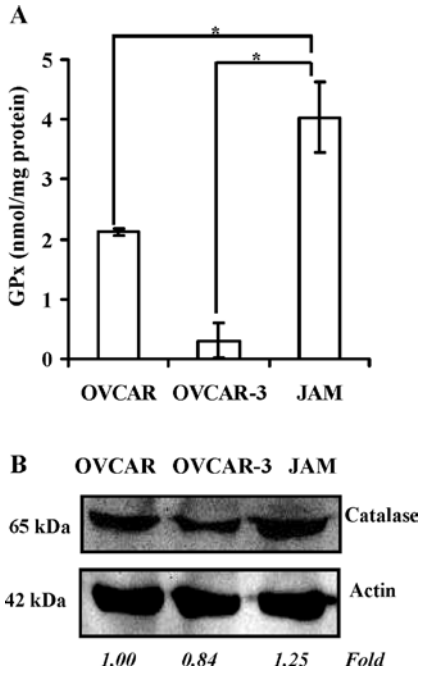

Figure 8. Intracellular (A) glutathione peroxidase and (B) catalase levels in ovarian tumor cells. Results represent the mean ± SD of three independent experiments for GPx levels (*p<0.05). For immunoblotting, a representative result of 3 independent experiments is shown.

JAM cells than in OVCAR and OVCAR-3 cells. Therefore, a larger dose of AA is necessary to generate sufficient oxidative stress to overcome a higher antioxidant threshold in JAM cells for enhanced cytotoxicity.

Discussion

Here we demonstrated a positive association between cellular GSH levels and As_2O_3 sensitivity of ovarian tumor cells. We established that co-treatment with low doses of BSO or pharmacological doses of AA enhanced the cytotoxicity of As_2O_3 in ovarian carcinoma cells. At these clinically achievable concentrations of BSO, AA and As_2O_3, selective cytotoxicity of malignant cells over normal fibroblast controls were achieved, thereby establishing the potential clinical relevance of these data. More importantly, we found that contrary to previous reports in other cellular systems (3,4,20,26), distinct differences exist in the mode in which these two agents act to bring about the observed synergism.

With BSO, low concentrations of this compound depleted intracellular GSH levels and resulted in an increase in tumor apoptosis and an even greater increase in overall cellular cytotoxicity when combined with As_2O_3. The later is likely attributed to the enhanced effects on other non-apoptotic cell

death processes with combination therapy. This net increase in cell death is consistent with previous reports (3,27-32). Notably different from most cells (4,27-28,30-32), the overall enhancement of As_2O_3-induced cytotoxicity in ovarian carcinoma cells is unlikely mediated by ROS generated following GSH depletion due to the inability of common non-thiol antioxidants in reversing the increased cell death. Similar observation was made by Wu *et al* in renal carcinoma cells (29). Until recently, GSH depletion has been frequently associated with ROS generation due to its role as a ubiquitous cellular antioxidant. However, emerging evidence showed that cell death following GSH depletion can occur independently of ROS (33,34), suggesting a direct role of GSH in regulating cell death that is independent of oxidative stress as observed in the ovarian tumor cells.

GSH is also known to eliminate carcinogens by direct conjugation to carcinogens and subsequent export of the carcinogen-GSH conjugates through transporters (16). For As_2O_3, human multidrug resistance protein 1 (MRP1) mediated As-GSH efflux has been reported in lung cancer cells (35). In ovarian tumor cells we noted that the increase in As concentration was only about 3-fold while the overall IC_{50} values were reduced by at least 6-fold. This suggests that inhibition of drug transporter-mediated efflux of As-GSH adduct may play partial role in the cytotoxic enhancement observed.

When AA was used as the enhancing agent, we found that physiological level of AA was unable to enhance the cytotoxicity of As_2O_3 in ovarian tumor cells. This result deviates from our results in U266 cells and that of previous reports in leukemia (3,36,37), MM (4) and cutaneous T cell lymphoma (38). It implies possible differences in AA utilization pathways among different cells and further indicates that physiological concentration of AA used in conjunction with As_2O_3 for chemotherapy of MM (4) would not be useful if directly extrapolated for ovarian cancer treatment.

In ovarian cancer cells, we provided the first experimental evidence that AA would only be effective when used at pharmacological concentrations with As_2O_3. At these concentrations, AA enhancement As_2O_3-induced cytotoxicity differed from that of BSO and was not mediated by a reduction in intracellular GSH levels. This again deviates from reports of AA being an effective GSH modulator in other tumors (3,4) and may again indicate cell specific differences in AA utilization. Rather, we found that high dose ascorbate likely mediated its effects through a H_2O_2-dependent process. This is further corroborated by data showing an increased ascorbate requirement of JAM cells for cell kill enhancement as they harbored higher intracellular levels of H_2O_2 detoxifying enzymes compared to OVCAR and OVCAR-3 cells. Indeed, *in vitro* results showed that high dose ascorbate inhibited cancer but not normal cell proliferation by a free radical mechanism involving ROS and ascorbate radical formation (39,40). More recently, Chen *et al* confirmed that pharmacological ascorbate concentration acted as a prodrug for formation of ascorbate radical and H_2O_2 in the extracellular space, leading to reduced tumor growth *in vivo* (41-43). Such effects were consistent with our findings.

Current literature further highlighted that the mechanisms of cell death following ascorbate-mediated cytotoxic enhancement to be complex, cell type and drug combination specific (44-48). While an increase in apoptosis has been observed (45), other novel and complex mechanism such as autoschizis has been reported (46-48). In AA mediated augmentation of menadione-induced ovarian cancer cell death, a mixture of cell death phenotypes comprising of 43% autoschizis, 3% apoptosis and 1.9% onosis was reported (47). The modes of non-apoptotic cell death in our system with AA and As_2O_3 combination are presently under investigation.

In conclusion, our findings showed that BSO and only pharmacological doses of AA can enhance the chemotherapeutic effects of As_2O_3 in ovarian tumor cells. We further highlighted the distinct differences with which these two agents act to bring about the observed synergism. This also emphasized the importance of understanding the cell type specific effects of these agents when used in combinatorial therapies. Such understanding will facilitate the optimal use of these and related compounds that act in a similar fashion, as potentially useful synergistic agents with As_2O_3 for ovarian cancer treatment.

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