Differential augmentative effects of buthionine sulfoximine and ascorbic acid in As$_2$O$_3$-induced ovarian cancer cell death: Oxidative stress-independent and -dependent cytotoxic potentiation

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Abstract. The potential of arsenic trioxide (As$_2$O$_3$) 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$_2$O$_3$ 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$_2$O$_3$ with either low dose BSO or only pharmacological doses of AA acted synergistically to enhance the cytotoxicity of As$_2$O$_3$ 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$_2$O$_3$-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$_2$O$_3$ 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$_2$O$_3$) 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$_2$O$_3$ 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$_2$O$_3$ 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$_2$O$_3$, 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$_2$O$_3$ (17). In leukemic and some solid tumors, intracellular GSH levels have been reported to be inversely related to their As$_2$O$_3$ sensitivity (18,19). In some As$_2$O$_3$-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$_2$O$_3$ sensitivity of ovarian tumor cells and the effects of manipulating this system on As$_2$O$_3$-induced cytotoxicity in these cells. We observed a positive correlation between cellular GSH concentration and their cytotoxic susceptibility to As$_2$O$_3$. We further demonstrated that selective
chemosensitization to clinically achievable \( \text{As}_2\text{O}_3 \) 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 (Rockville, 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 \( \text{As}_2\text{O}_3 \) 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).

**\[^{[H]}\]-thymidine uptake.** Proliferation and cytotoxicity of U266 cells were assessed using \(^{[H]}\)-thymidine after incubating for 72 h with either \( \text{As}_2\text{O}_3 \) or AA (100 μM) alone or in combination. [Methyl-\(^{[H]}\)-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, MD). 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 G1 phase (sub-G1 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 into 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 NaPO\(_4\), 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 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.
As BSO therapy, 1 µM of BSO sensitized all ovarian cancer cells, alone showed minimal cell toxicity (Table II). With concurrent or physiological level of AA (100 µM) were able to augment the cytotoxicity of BSO but not physiological level of AA sensitized ovarian tumor cells to cytotoxicity of As$_2$O$_3$-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$_2$O$_3$-sensitive JAM cells to the cytotoxicity of As$_2$O$_3$, 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$_2$O$_3$ 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$_2$O$_3$ 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 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.

**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.

**Results**

**Positive correlation between intracellular GSH levels in ovarian cancer and normal fibroblast cells to their sensitivity to As$_2$O$_3$ treatment.** The relationship between As$_2$O$_3$-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$_2$O$_3$ 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$_2$O$_3$. Conversely, OVCAR and OVCAR-3 cells have lower GSH levels and were more responsive to As$_2$O$_3$ treatment. A positive correlation (Spearman's correlation coefficient, $r^2$ = 0.98) between intracellular GSH levels and As$_2$O$_3$ 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$_2$O$_3$.** 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$_2$O$_3$-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, normal fibroblast cells and their correlation with cellular IC$_{50}$ values. Cellular GSH levels were expressed as the mean ± SD of three independent experiments.

![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.](image)

**Table I. IC$_{50}$ values of As$_2$O$_3$ alone and in combination with either low dose BSO or AA.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>OVCAR</th>
<th>OVCAR-3</th>
<th>JAM</th>
<th>MRC-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>As$_2$O$_3$</td>
<td>1.96±0.67$^a$</td>
<td>1.48±0.41$^a$</td>
<td>7.89±0.84$^a$</td>
<td>9.01±0.10</td>
</tr>
<tr>
<td>As$_2$O$_3$ + BSO 1 µM</td>
<td>0.55±0.03$^{a,b}$</td>
<td>0.45±0.02$^{a,b}$</td>
<td>3.08±0.18$^{a,b}$</td>
<td>&gt;8.00</td>
</tr>
<tr>
<td>As$_2$O$_3$ + BSO 5 µM</td>
<td>0.12±0.06$^{a,b}$</td>
<td>0.14±0.01$^{a,b}$</td>
<td>1.18±0.25$^{a,b}$</td>
<td>4.07±0.38$^a$</td>
</tr>
<tr>
<td>As$_2$O$_3$ + AA 100 µM</td>
<td>2.31±0.12$^a$</td>
<td>1.12±0.20$^a$</td>
<td>&gt;8.00</td>
<td>&gt;8.00</td>
</tr>
<tr>
<td>As$_2$O$_3$ + AA 500 µM</td>
<td>0.88±0.02$^{a,b}$</td>
<td>0.35±0.17$^{a,b}$</td>
<td>7.95±0.51$^a$</td>
<td>&gt;8.00</td>
</tr>
<tr>
<td>As$_2$O$_3$ + AA 1000 µM</td>
<td>-</td>
<td>-</td>
<td>2.45±0.07$^{a,b}$</td>
<td>&gt;8.00</td>
</tr>
</tbody>
</table>

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$_2$O$_3$ treatment for each respective cell line.
Table II. Survival indices of low dose BSO alone or AA alone in OVCAR, OVCAR-3, JAM and MRC-5 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>OVCAR</th>
<th>OVCAR-3</th>
<th>JAM</th>
<th>MRC-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSO 1 µM</td>
<td>97.45±8.61</td>
<td>102.87±0.62</td>
<td>104.08±11.33</td>
<td>86.68±3.26</td>
</tr>
<tr>
<td>BSO 5 µM</td>
<td>86.17±14.88</td>
<td>96.83±5.13</td>
<td>106.95±10.62</td>
<td>107.96±3.95</td>
</tr>
<tr>
<td>AA 100 µM</td>
<td>105.45±10.50</td>
<td>97.94±9.28</td>
<td>100.03±9.81</td>
<td>107.46±12.58</td>
</tr>
<tr>
<td>AA 500 µM</td>
<td>94.77±7.16</td>
<td>89.87±4.04</td>
<td>92.39±2.12</td>
<td>104.07±9.54</td>
</tr>
<tr>
<td>AA 1000 µM</td>
<td>-</td>
<td>-</td>
<td>95.74±14.20</td>
<td>90.51±11.30</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD of at least three independent experiments.
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 $\text{As}_2\text{O}_3$ 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 $\text{As}_2\text{O}_3$ 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 $\text{As}_2\text{O}_3$ 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 $\mu$M resulted in a 2-fold increase in arsenic accumulation when compared with $\text{As}_2\text{O}_3$ 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 $\text{As}_2\text{O}_3$ therapy ($p<0.05$) (Fig. 6C). Taken together, these results suggest that enhanced cytotoxicity to $\text{As}_2\text{O}_3$ with concurrent BSO use is partly contributed by increased cellular arsenic content.

Enhancement of cell death with $\text{As}_2\text{O}_3$ 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,
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$_2$O$_2$) 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$_2$O$_3$ 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$_2$O$_3$ and pharmacological dose of AA (p<0.05) whereas BHA showed little protective effect (Fig. 7). These results indicate that augmentation of As$_2$O$_3$-induced cell death by high dose AA is largely contributed by the generation of excessive oxidative stress via H$_2$O$_2$ whereas that by BSO occurred independently of ROS.

Sensitivity of ovarian cancer cells to pharmacological doses of AA and As$_2$O$_3$ treatment is related to cellular antioxidant enzyme levels. Since JAM cells more effectively accumulate AA but require higher pharmacological doses of AA for As$_2$O$_3$-induced cytotoxic enhancement and ROS play an important role in mediating AA-induced cytotoxic augmentation, we postulated that these cells may harbor higher cellular antioxidant enzyme levels. Indeed, intracellular levels of both GPx (p<0.05) (Fig. 8A) and CAT (Fig. 8B) were higher in

Figure 5. Total intracellular GSH levels of (A) OvCAR, (B) OvCAR-3 and (C) JAM cells after treatment with As$_2$O$_3$ 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$_2$O$_3$ 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).
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$_2$O$_3$ 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$_2$O$_3$ in ovarian carcinoma cells. At these clinically achievable concentrations of BSO, AA and As$_2$O$_3$, 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$_2$O$_3$. 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$_2$O$_3$-induced cytotoxicity in ovarian carcinoma cells is unlikely meditated 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$_2$O$_3$, human multidrug resistance protein 1 (MRPI) 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$_2$O$_3$ 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$_2$O$_3$ 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$_2$O$_3$. At these concentrations, AA enhancement As$_2$O$_3$-induced cytotoxicity differed from that of BSO and was not meditated 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$_2$O$_2$-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$_2$O$_2$ 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$_2$O$_2$ 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 enhance-


