Low concentrations of As₂O₃ (≤1 µmol/L) induce long-lasting remission in patients with acute promyelocytic leukemia (APL) without severe toxicity.¹⁻³ Thus, in stark contrast to the carcinogenic effect of chronic exposure to high doses of arsenic compound,⁶⁻⁷ low concentrations of As₂O₃ are of therapeutic value in APL and perhaps other leukemias.⁸⁻¹¹ In vitro studies have shown that low concentrations of As₂O₃ induce apoptosis in APL-derived NB4 cells and primary cultures of APL. In other leukemic cells, As₂O₃ can induce apoptosis but only at higher concentrations that may be unacceptable in the clinic because of toxicity.⁸⁻¹¹

The major feature that distinguishes APL cells from other malignant hematopoietic cells is the expression of PML-RARα, the product of the t(15;17) translocation.¹²⁻¹⁴ PML-RARα is a transcriptional repressor with dominant negative activity over RARα.¹⁵⁻¹⁷ Studies aimed at understanding the factors underlying the unique sensitivity of NB4 cells to As₂O₃ and, ultimately, how these relate to the expression of PML-RARα form the basis of this report. Our previous work indicated that As₂O₃-induced apoptosis was modulated by the cellular glutathione redox system, with increased intracellular levels of reduced glutathione (GSH) having an inhibitory effect.¹¹ Because of the antioxidant function of GSH, we wondered whether 1 µmol/L As₂O₃ could trigger NB4 cell apoptosis through the generation of reactive oxygen species (ROS). We show here that, indeed, the induction of NB4 cell apoptosis and the refractoriness of other leukemic cells to 1 µmol/L As₂O₃ are explained, at least in part, by the accumulation of higher H₂O₂ levels in As₂O₃-treated NB4 as compared with other leukemic cells. This difference in intracellular H₂O₂ concentration is shown to be derived from a differential pattern of expression of H₂O₂-catabolizing enzymes. A causal link between H₂O₂ levels and apoptosis is supported by the coordinate regulation of these events in response to both positive and negative regulation of H₂O₂-catabolizing enzymes. Moreover, we show that, in the presence of glutathione peroxidase (GPx) and catalase inhibitors, it is possible to achieve high apoptotic rates in leukemic cells otherwise refractory to a therapeutic concentration of As₂O₃ (1 µmol/L). Finally, we demonstrate that the generation of H₂O₂ in As₂O₃-treated NB4 cells triggers apoptosis via a reduction in mitochondrial membrane potential, cytochrome c release, and caspase activation.

**NEOPLASIA**

**Arsenic Trioxide Selectively Induces Acute Promyelocytic Leukemia Cell Apoptosis Via a Hydrogen Peroxide-Dependent Pathway**

By Yongkui Jing, Jie Dai, Ruth M.E. Chalmers-Redman, William G. Tatton, and Samuel Waxman

Low concentrations of As₂O₃ (≤1 µmol/L) induce long-lasting remission in patients with acute promyelocytic leukemia (APL) without significant myelosuppressive side effects. Several groups, including ours, have shown that 0.5 to 1 µmol/L As₂O₃ induces apoptosis in APL-derived NB4 cells, whereas other leukemic cells are resistant to As₂O₃ or undergo apoptosis only in response to greater than 2 µmol/L As₂O₃. In this report, we show that the ability of As₂O₃ to induce apoptosis in leukemic cells is dependent on the activity of the enzymes that regulate cellular H₂O₂ content. Thus, NB4 cells have relatively low levels of glutathione peroxidase (GPx) and catalase and have a constitutively higher H₂O₂ content than U937 monocyctic leukemia cells. Glutathione-S-transferase γ (GSTγ), which is important for cellular efflux of As₂O₃, is also low in NB4 cells. Moreover, As₂O₃ further inhibits GPx activity and increases cellular H₂O₂ content in NB4 but not in U937 cells. Selenite pretreatment of NB4 cells increases the activity of GPx, lowers cellular H₂O₂ levels, and renders NB4 cells resistant to 1 µmol/L As₂O₃. In contrast, concentrations of As₂O₃ that alone are not capable of inducing apoptosis in NB4 cells induce apoptosis in the presence of the GPx inhibitor mercaptothecanic acid. Similar effects are observed by modulating the activity of catalase with its inhibitor, aminotriazol. More important from a therapeutic point of view, U937 and HL-60 cells, which require high concentrations of As₂O₃ to undergo apoptosis, become sensitive to low, clinically acceptable concentrations of As₂O₃ when cotreated with these GPx and catalase inhibitors. The induction of apoptosis by As₂O₃ involves an early decrease in cellular mitochondrial membrane potential and increase in H₂O₂ content, followed by cytochrome c release, caspase 3 activation, DNA fragmentation, and the classic morphologic changes of apoptosis.

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fetal bovine serum. Cells in logarithmic growth were seeded at \(1 \times 10^5\) cells/mL for studies performed in duplicate and repeated at least 3 times.

**Quantification of apoptotic cells.** Apoptotic cells were determined by morphology and fluorescence-activated cell sorting (FACS) analysis with propidium iodide (PI) as well as TUNEL assay. For morphologic evaluation, cells were stained with acridine orange (AO) and ethidium with propidium iodide (PI) as well as TUNEL assay. For morphologic times.

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**RESULTS**

As \(\text{As}_2\text{O}_3\)-induced apoptosis is dependent on cellular \(\text{H}_2\text{O}_2\) levels. Treatment of NB4 cells with \(1 \mu\text{mol/L As}_2\text{O}_3\) induced apoptosis, as indicated by morphologic analysis (Fig 1A), DNA distribution by FACS analysis demonstrating hypodiploid DNA (Fig 1B), and TUNEL assay (Fig 1C). In contrast, treatment of U937 cells with even \(2 \mu\text{mol/L As}_2\text{O}_3\) did not induce apoptosis (Fig 1A, B, and C). Although apoptosis was not detected in \(\text{As}_2\text{O}_3\)-treated U937 cells, cell growth was inhibited by \(\text{As}_2\text{O}_3\) both in NB4 and U937 cells, with \(\text{IC}_{50}\) of about 0.7 and 1.2 mmol/L at 3 days of treatment, respectively. The growth inhibition was not correlated with arrest in a specific cell cycle phase (Fig 1B). These data were consistent with our previous report that \(\text{As}_2\text{O}_3\) inhibited cell growth in several lymphoma cells by prolongation of the cell cycle without blocking cells in a specific phase. Thus, the selective effect of \(\text{As}_2\text{O}_3\) in NB4 cells was due to apoptosis induction and not to growth inhibition. This differential sensitivity to \(\text{As}_2\text{O}_3\)-induced apoptosis in NB4 cells was associated with differences in cellular \(\text{H}_2\text{O}_2\) levels that were determined by FACS analysis of cells labeled with DCFH-DA. The dramatic oxidation of DCFH-DA to DCF was cleaved by nonspecific esterases forming DCF, which is the nonfluorescent form and is oxidized to the fluorescent compound \(2',7'\)-dichlorofluorescein (DCF) in the presence of \(\text{H}_2\text{O}_2\). Exponentially growing cells \((1 \times 10^5 \text{ cells/mL})\) were labeled with \(0.5 \mu\text{mol/L DCFH-DA}\) for 1 hour and then incubated in the absence or presence of \(\text{As}_2\text{O}_3\) at \(37^\circ\text{C}\) for various periods of time. After washing with PBS, cells \(10,000\) per point were analyzed by FACSscan (Becton Dickinson) with excitation and emission settings of 495 and 525 nm, respectively. Arithemtic histograms analysis was used to determine the mean of the oxidized DCF peak in each group.

**Quantification of DNA fragmentation.** DNA fragmentation was quantified as described previously. Cells were harvested by centrifugation, and the pellets were suspended in lysis buffer containing \(20 \mu\text{mol/L Tris-HCl, 0.5% Triton X-100, pH 8.0}\). After 30 minutes on ice, samples were centrifuged at 14,000 rpm for 30 minutes, and cellular DNA was extracted. Electrophoresis was performed in 1% agarose gel in 40 mmol/L Tris-acetate buffer \((\text{pH 7.4})\) at 50 V. After electrophoresis, DNA was visualized by ethidium bromide staining.

**Western blot analysis.** Protein extracts \((50 \mu\text{g})\) prepared with RIPA lysis buffer \((50 \mu\text{mol/L Tris-HCl, 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate, 1 mmol/L phenylmethyl sulfonyl fluoride (PMSF), 100 \mu\text{mol/L leupeptin, and 2 \mu/g protein}}\) were separated on 8% or 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were stained with 0.2% Ponceau S red to assure equal protein loading and transfer. After blocking with 5% nonfat milk, the membranes were incubated with polyclonal antibody to PARP (Boehringer Mannheim, Indianapolis, IN) and monoclonal antibodies to Cpp32 and Bcl-2 (Oncogene Research Products, Cambridge, MA). Immunocomplexes were visualized by chemiluminescence (ECL kit; Amersham, Arlington Heights, IL).
Fig 1. Comparison of apoptosis induction by As$_2$O$_3$ in NB4 versus U937 cells. (A) Fluorescence microscopy determination of apoptotic cells. Cells were treated with either 0 (control), 1, or 2 $\mu$mol/L As$_2$O$_3$ for up to 4 days, and the number of apoptotic cells was determined by fluorescence microscopy according to the morphology. The results are expressed as the percentage of apoptotic cells in the culture. Values shown are the mean of triplicate determinations with a standard deviation of less than 10%. (B) FACS analysis of apoptotic cells. Cells were treated for 3 days with the indicated concentrations of As$_2$O$_3$ and then evaluated for DNA content after propidium iodide staining. (C) TUNEL assay to determine apoptotic cells. Cells were treated with As$_2$O$_3$ at the indicated time for 3 days.
production or lower catabolism of H$_2$O$_2$. After treatment with 1 µmol/L As$_2$O$_3$, the mean of oxidized DCF peak increased compared with the untreated control from 1.9 to 2.4, 4.1 to 6.7, and 15.2 to 21.4 in 1, 8, and 24 hours after initial loading of DCFH-DA, respectively. In contrast, the mean of oxidized DCF peak was not increased by 1 µmol/L As$_2$O$_3$ in U937 cells even after 24 hours of treatment (Fig 2B). Thus, both the constitutive level of H$_2$O$_2$ and the ability of As$_2$O$_3$ to increase the level of H$_2$O$_2$ were correlated with the apoptotic activity of As$_2$O$_3$.

The role of H$_2$O$_2$ scavenging enzymes in As$_2$O$_3$-induced apoptosis. To determine whether the higher basal levels of H$_2$O$_2$ in NB4 cells depends on lower H$_2$O$_2$ catabolism, the major cellular scavenging enzymes GPx and catalase were measured in several leukemia cell lines with different sensitivities to As$_2$O$_3$-induced apoptosis. The data in Table 1 show that the activity of H$_2$O$_2$ scavenging enzymes, GPx and catalase, was much higher in 4 cell lines that were insensitive to As$_2$O$_3$-induced apoptosis than in NB4 cells. The only exception is represented by K562 cells, in which the GPx activity was lower than that in NB4 cells. However, the very high activity in these cells of GST-$\pi$, as compared with the low activity in NB4 cells may compensate for the low activity of GPx and catalase by greater efflux of cellular As$_2$O$_3$.\textsuperscript{23,27,28}

Based on the finding that NB4 cells have relatively low activities of GST-$\pi$, catalase, and GPx (Table 1), we hypothesized that the higher sensitivity of NB4 cells towards As$_2$O$_3$-induced apoptosis is related to their low ability to metabolize the H$_2$O$_2$ produced during As$_2$O$_3$ treatment. To test this hypothesis, we
tested the effect of selenite, an activator of GPx. To test the effect of a physiologic concentration of selenite, NB4 cells were grown for several generations in the presence of 100 nmol/L selenite. This treatment increased GPx activity by a factor of 7 while having no effect on cell growth. Under these conditions, As2O3 did not increase H2O2 levels (Fig 3), and it induced apoptosis in only 7% of the cells, as compared with 58% in cultures not pretreated with selenite (Table 2). The data also demonstrated that As2O3 inhibited the activity of GPx and that this effect was less pronounced in cells pretreated with selenite (Table 2).

These results strongly suggested that the low H2O2-metabolizing activity of NB4 cells predispose them to As2O3-induced apoptosis. As a further test of this hypothesis, we determined whether the effect of As2O3 could be enhanced by treatment with mercaptosuccinic acid (MS), an inhibitor of GPx, and aminotriazol (AT), an inhibitor of catalase. As shown in Table 3, 100 µmol/L MS had no effect on cell apoptosis by itself, whereas 20 µmol/L AT moderately increased the percentage of apoptotic cells in NB4 and HL-60 cells. However, when combined with 0.5 µmol/L As2O3, which by itself was ineffective, both inhibitors raised the percentage of apoptotic NB4 cells to nearly 50%. Remarkably, in HL-60 and U937 cells, which normally respond to only greater than 5 µmol/L As2O3, cotreatment with 25 or 100 µmol/L MS and 1 µmol/L As2O3.

Table 1. The Basal Activities of Antioxidant Enzymes and As2O3-Induced Apoptosis in Different Human Leukemia Cell Lines

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>GSTp (mU/min/mg protein)</th>
<th>GPx (µmol H2O2/min/mg protein)</th>
<th>Catalase (mU/mg protein)</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB4</td>
<td>94.0 ± 8.37</td>
<td>28.3 ± 5.4</td>
<td>25.8 ± 9.3</td>
<td>67.5 ± 5.8</td>
</tr>
<tr>
<td>HL-60</td>
<td>138.6 ± 1.9</td>
<td>55.5 ± 10.2</td>
<td>198.3 ± 10.6</td>
<td>5.8 ± 2.1</td>
</tr>
<tr>
<td>U937</td>
<td>212.1 ± 15.3</td>
<td>67.6 ± 1.5</td>
<td>170.5 ± 9.9</td>
<td>5.6 ± 3.4</td>
</tr>
<tr>
<td>KG1</td>
<td>176.2 ± 5.1</td>
<td>82.8 ± 10.6</td>
<td>108.9 ± 7.3</td>
<td>17.5 ± 4.2</td>
</tr>
<tr>
<td>K562</td>
<td>232.3 ± 6.9</td>
<td>12.3 ± 8.6</td>
<td>85.5 ± 8.9</td>
<td>6.7 ± 3.1</td>
</tr>
</tbody>
</table>

The units were nanomoles per milligram of protein per minute for glutathione-S-transferase (GSTp); milliunits per minute per milligram of protein for glutathione peroxidase (GPx); micromoles of H2O2 decomposed per milligram of protein per minute for catalase. Apoptosis (percentage of apoptotic cells) was quantitated by fluorescence microscopy in cultures treated for 2 days with 2 µmol/L As2O3. All data shown represent the mean ± SE of 3 independent experiments.

Table 2. The Effect of Selenite Pretreatment on As2O3-Induced Apoptosis and GPx Activity in NB4 Cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>As2O3 (µmol/L)</th>
<th>Apoptosis (%)</th>
<th>GPx (mU/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB4</td>
<td>0</td>
<td>2.1 ± 0.4</td>
<td>24.5 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>12.4 ± 3.8</td>
<td>13.5 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>57.8 ± 7.2</td>
<td>8.9 ± 4.2</td>
</tr>
<tr>
<td>NB4-sele</td>
<td>0</td>
<td>2.3 ± 0.8</td>
<td>166.4 ± 14.9</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>5.9 ± 3.1</td>
<td>135.2 ± 10.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>7.1 ± 2.7</td>
<td>117.4 ± 20.2</td>
</tr>
</tbody>
</table>

NB4-sele cells were pretreated with 0.1 µmol/L sodium selenite for 9 days and were then treated with As2O3 for 3 days. Apoptosis (determined by fluorescence microscopy) and GPx activity were assayed and determined after 3 days of treatment with or without As2O3. Each value is the mean ± SD of triplicate determinations.

Fig 3. FACS analysis of H2O2 accumulation in NB4 and selenite-pretreated NB4 cells. NB4 cells were pretreated without (NB4) or with 100 nmol/L sodium selenite for 9 days (NB4-sele) and then treated with the indicated concentrations of As2O3 for 24 hours. DCFH-DA was added 1 hour before the addition of As2O3, and the oxidized DCF was analyzed by FACS.
raised the percentage of apoptotic cells to 39% and 43%, respectively (Table 3). The combination of 20 mmol/L AT and 1 µmol/L As2O3 was also more effective than either agent alone in HL-60 and U937 cells.

The mechanism of As2O3-induced apoptosis. Apoptotic mechanisms are drug and cell-type–specific and are associated with the perturbation of mitochondrial functions.31 This process results in the activation of caspase and the fragmentation of DNA, coupled with characteristic morphologic changes. We tested whether As2O3-induced apoptosis is preceded by a decrease of the mitochondrial membrane potential (ΔΨM). We found that, in NB4 cells, ΔΨM decreased within 4 hours of treatment with 1 µmol/L As2O3 and decreased further with longer treatment times (Fig 4). As predicted from the decrease in ΔΨM, cytochrome c release into the cytoplasm was observed; the extent of release was slight 1 day after 1 µmol/L As2O3 treatment and complete 2 days later (Fig 5). Concomitant with the As2O3 induction of maximal cytochrome c release, Cpp32 was activated, as shown by the degradation of its precursor (32 kD) and the cleavage of PARP, a Cpp32 substrate. These events were independent of Bcl-2 degradation (Fig 6A) and were followed by DNA fragmentation (Fig 6B). As expected, high concentrations (200 µmol/L) of the general caspase inhibitor, Z-VAD-FMK, inhibited As2O3-induced apoptosis.

DISCUSSION
Recent developments suggest that a number of diverse apoptotic stimuli share a mechanistic pathway characterized by the generation of ROS and the loss of mitochondrial membrane potential, with subsequent outer mitochondrial membrane permeability changes, release of cytochrome c, and caspase activation.31-34 Thus, agents as diverse as ceramide, tumor necrosis factor-α, UV irradiation, and anthracyclines have been shown to trigger apoptosis via ROS production.24,35-38 Our findings indicate that As2O3 represents a novel apoptotic stimulus functioning via ROS, specifically H2O2, generation. This conclusion is supported by the following key observations. (1) Treatment of NB4 cells with an apoptotic concentration of As2O3 (1 µmol/L) inhibits the activity of GPx, an H2O2-catabolizing enzyme (Table 2). (2) The same treatment results in elevated intracellular H2O2 levels (Fig 2). (3) Pretreatment with selenite, a GPx activator, results in high GPx activity, lack of H2O2 accumulation, and inhibition of apoptosis (Table 2 and Fig 3). (4) Cotreatment with MS, a GPx inhibitor, and, to a lesser extent, with AT, a catalase inhibitor, potentiates the induction of apoptosis by As2O3 (Table 3).

The high sensitivity of NB4 APL cells to As2O3 appears to

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**Table 3. The Effect of GPx Inhibitor and Catalase Inhibitor on As2O3-Induced Apoptosis in NB4, HL-60, and U937 Cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NB4 (Apoptotic Cells, %)</th>
<th>HL60</th>
<th>U937</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.0 ± 0.8</td>
<td>2.5 ± 0.4</td>
<td>2.3 ± 0.7</td>
</tr>
<tr>
<td>As2O3</td>
<td>9.0 ± 3.1</td>
<td>2.5 ± 0.6</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>MS</td>
<td>6.0 ± 2.4</td>
<td>11.4 ± 1.3</td>
<td>7.3 ± 1.4</td>
</tr>
<tr>
<td>MS + As2O3</td>
<td>47.0 ± 6.7</td>
<td>39.0 ± 3.7</td>
<td>43.4 ± 5.9</td>
</tr>
<tr>
<td>AT</td>
<td>24.0 ± 8.3</td>
<td>15.4 ± 2.9</td>
<td>6.3 ± 3.1</td>
</tr>
<tr>
<td>AT + As2O3</td>
<td>49.0 ± 3.8</td>
<td>27.2 ± 5.6</td>
<td>20.5 ± 1.3</td>
</tr>
</tbody>
</table>

NB4 cells were treated with 0.5 µmol/L As2O3 alone or plus 100 µmol/L MS or 20 mmol/L AT for 3 days; HL-60 cells were treated with 1 µmol/L As2O3 alone or plus 25 µmol/L MS or 20 mmol/L AT for 3 days; U937 cells were treated with 1 µmol/L As2O3 alone or plus 100 µmol/L MS or 20 mmol/L AT for 3 days. Each value is the mean ± SD of triplicate determinations.
reside in a leukemic cell-specific pattern of expression of GPx and catalase, as well as GST, which represents the major As2O3 detoxifying enzyme. Thus, NB4 cells have low GST, GPx, and catalase activities relative to 4 other non-APL leukemic cell lines (Table 1), suggesting that NB4 cells detoxify As2O3 and catabolize H2O2 less efficiently.

These findings are consistent with our previous work showing that As2O3-induced apoptosis is blocked by NAC and lipoic acid, agents that increase GSH, and is enhanced by BSO, a GSH-depleting agent. Thus, high levels of GSH, which is a proton donor for the GPx-catalyzed breakdown of H2O2 and for the GST-catalyzed detoxification of As2O3, ensure rapid rates for these reactions, thus conferring a protective effect. Our findings are also consistent with the observations that 40 µmol/L arsenite induces apoptosis in hamster CHO cells with generation of ROS, that H2O2-resistant CHO cells are less responsive, and that catalase-deficient CHO cells are hypersensitive to arsenic. The ascorbic acid synergism of As2O3-induced apoptosis in NB4 is mediated by H2O2 production, because it is inhibited by catalase.

We suspect that As2O3 acts to increase H2O2 levels in NB4 cells mainly by inhibiting the activity of GPx, which is a major H2O2 scavenging thiol-enzyme. We speculate that As2O3 inhibition of GPx is mediated by the binding of arsenic to vicinal thiol group in GPx, but this remains to be tested. The low levels of GST limit detoxification of cellular As2O3, providing higher As2O3 binding ability that contributes to direct inactivation of GPx. On the other hand, it is possible that As2O3 also increases the mitochondrial production of H2O2. For instance, 20 µmol/L arsenite leads to H2O2 accumulation through a mechanism thought to depend on the activation of NADPH oxidase. It should also be pointed out that peroxidases other than GPx may also be targeted by As2O3. An example is thioredoxin peroxidase, another thiol peroxidase that protects against mitochondrial permeability transition by removing H2O2.

We have demonstrated that apoptosis in As2O3-treated NB4 cells proceeds via the classical pathway described for other ROS inductive signals. Thus, As2O3 elicited a rapid decrease in mitochondrial membrane potential (Fig 4), which preceded the release of cytochrome c, Cpp32 activation, DNA fragmentation, and morphologic evidence of apoptosis (Fig 5 and 6). However, in contrast to previous reports, we did not observe Bcl-2 degradation in NB4 cells treated with 1 to 2 µmol/L As2O3. This is consistent with a recent report showing that Bcl-2 is not degraded in NB4 cells, even when treated with 8 µmol/L As2O3. Moreover, human t(14;18) B-cell lymphoma su-DHL-4 cells overexpressing Bcl-2 are sensitive to 2 µmol/L As2O3 without degradation of Bcl-2. On the other hand, NIH3T3 and HL-60 cells with forced expression of Bcl-2 are more resistant to As2O3-induced apoptosis (Jing et al, unpublished data), consistent with the finding that cells overexpressing Bcl-2 are resistant to H2O2-induced cell death.

As2O3-induced apoptosis in NB4 cells is blocked by the caspase inhibitor Z-VAD-FMK (Fig 6). This result is consistent with the previous observation that Z-VAD-FMK blocked As2O3-induced apoptosis in lymphoma cells. Our current view of the apoptotic pathway induced by As2O3, as supported by the above-noted data, is depicted diagrammatically in Fig 7.

A fundamental question is why are NB4 APL cells uniquely sensitive to As2O3? In other words, how does the PML-RAR fusion protein that represents the molecular signature of APL confer sensitivity to As2O3? As already discussed, our data point to the low GST, GPx, and catalase activities of NB4 cells as a logical explanation for their sensitivity to As2O3. What then is the relationship between PML-RAR and the activity of these enzymes? We suspect that the answer lies partly in the facts that GST is an RA-inducible gene and that PML-RAR is a dominant repressor of RAR function. Thus, it seems likely that PML-RAR inhibits the RAR activation of GST in NB4 cells, leading to reduced enzyme expression and activity. RA has also been shown to upregulate GPx and catalase expression in some cell types, but whether this is a transcriptional effect remains to be demonstrated. Thus, it is conceivable that PML-RAR also interferes with GPx and catalase expression. These are testable hypotheses that we are currently pursuing. For instance, one prediction is that, if PML-RAR expression is decreased by pretreatment with RA, then NB4 cells may be less sensitive to As2O3-induced apoptosis. Our preliminary experiments suggest that this is in fact the case (Jing et al, unpublished data). In addition, cotreatment of NB4 cells with tRA and As2O3 has been shown to result in decreased apoptosis.

Whatever the mechanism through which PML-RAR confers sensitivity to As2O3, it is clear that PML-RAR is indeed required for the therapeutic effect of As2O3: this is true both in vitro, as shown by the sensitization of U937 cells to As2O3 by ectopic PML-RAR expression, and in vivo, as shown by the finding that only 2 of 53 APL patients that did not experience clinical remission during As2O3 therapy had PML-RAR–negative disease.

Interestingly, we have found that P388 lymphoma cells are growth inhibited by As2O3 with an IC50 of 1.7 µmol/L, and that P388/ad cells with multidrug resistance (MDR1) to adriamycin (482-fold) and taxol (111-fold) remain responsive to As2O3 (IC50 of 2.0 µmol/L). This suggests that As2O3 is not a substrate
for the MDR1 drug efflux pathway, which is consistent with other reports\(^{40,58}\) and our proposal that GST\(\pi\)-mediated efflux represents the major As\(_2\)O\(_3\) efflux pathway in NB4 cells. Furthermore, the lack of cross-resistance between adriamycin and As\(_2\)O\(_3\) is consistent with the clinical observation that anthracycline-resistant APL is responsive to As\(_2\)O\(_3\) therapy.\(^{2,4,5}\)

It has been recently reported that the clinical remission induced by As\(_2\)O\(_3\) in APL patients and in transgenic mice is accompanied in part by the induction of cell differentiation.\(^{5,59}\) It remains to be seen to what extent the H\(_2\)O\(_2\)-dependent pathway we have unmasked here contributes to the induction and/or maintenance of clinical remission. It will also be of interest to pursue the feasibility of extending As\(_2\)O\(_3\) therapy to other leukemias or lymphomas that have shown variable sensitivity to As\(_2\)O\(_3\)\(^{51,60}\) by appropriate cotreatment with drugs capable of altering the ROS balance in favor of cell death activation, as exemplified by our in vitro findings of apoptosis induction in HL-60 and U937 cells by As\(_2\)O\(_3\) and MS.

**ACKNOWLEDGMENT**

The authors appreciate the advice of Dr George Acs throughout these studies and critical reading by Dr Rafael Mira-y-Lopez.

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**Fig 7.** Diagrammatic representation of the proposed apoptotic pathway induced by As\(_2\)O\(_3\) in NB4 cells.

**Fig 6.** As\(_2\)O\(_3\) treatment induces caspase-3 activation but not Bcl-2 degradation. (A) Activation of CPP32 and lack of Bcl-2 modulation after 3 days of treatment with the indicated doses of As\(_2\)O\(_3\). (B) Effect of the same As\(_2\)O\(_3\) treatment on DNA fragmentation. (C) Caspase inhibitor Z-VAD-FMK blocked As\(_2\)O\(_3\)-induced apoptosis. NB4 cells were treated for 3 days with 1 \(\mu\)mol/L As\(_2\)O\(_3\) or 200 \(\mu\)mol/L Z-VAD-FMK alone or in combination; Z-VAD-FMK was added 4 hours before the addition of As\(_2\)O\(_3\).
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