Multidrug-resistant neuroblastoma cells are responsive to arsenic trioxide at both normoxia and hypoxia

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Abstract

Despite intensive treatment, the outcome of high-risk neuroblastoma patients is poor with acquired multidrug resistance as an important cause. Previously, our group has shown that arsenic trioxide (As2O3) kills multidrug-resistant neuroblastoma cells grown under normoxic as well as hypoxic (1% oxygen) conditions. At both normoxia and hypoxia, 2 and 4 μmol/L As2O3 induced evident cell death in the drug-sensitive SH-SY5Y and IMR-32 cells as well as in the multidrug-resistant SK-N-BE(2)c (with a mutated p53) and SK-N-Fl cells after 72 hours of exposure. In contrast, the conventional chemotherapeutic drug etoposide showed lowered efficiency in hypoxic IMR-32 cells. In accordance with our previously published results, although not to the same extent as in their normoxic counterparts, Bax is proteolytically cleaved also in neuroblastoma cells exposed to As2O3 at hypoxia. This suggests that similar molecular mechanisms are involved in As2O3-induced neuroblastoma cell death during hypoxia compared with normoxia. Together, our results support As2O3 as a potential candidate drug as a complement to conventional treatments for high-risk neuroblastoma patients and perhaps also for patients with other multidrug-resistant solid tumors. [Mol Cancer Ther 2005;4(7):1128–35]

Introduction

Neuroblastoma is a childhood malignancy derived from the sympathetic nervous system and accounts for 8% to 10% of all cancers in children. Intensive chemoradiotherapy supported with bone marrow transplantation and followed by 13-cis-retinoic acid treatment has improved the survival for high-risk neuroblastoma patients (1). Still, the majority of neuroblastoma patients eventually develop progressive disease, which is refractory to further therapy. A major contributing factor to chemotherapeutic treatment failure of neuroblastoma is the development of resistance to multiple and functionally unrelated cytotoxic drugs, and as a consequence, the long-term survival rate of this group of neuroblastoma patients is only about 30% (1–3).

As2O3 has been used for therapeutic purposes for centuries, and recently, it has been successfully employed in the treatment of acute promyelocytic leukemia (APL) where several reports have shown that low doses of As2O3 can with minimal toxicity induce complete remission in patients with relapsed APL (4–6). Moreover, the potency of As2O3 to induce cell death in vitro has been shown in a variety of cancer cells including non-APL leukemia and lymphoma cells (7, 8) as well as solid tumors like neuroblastoma (9–11) and tumors originating from prostate, kidney, cervix, and bladder (12). The underlying mechanisms of As2O3-induced cytotoxicity are complex and include induction of apoptosis, cellular differentiation, degradation of the t(15;17)-specific fusion protein PML/retinoic acid receptor-α seen in APL, growth inhibition, induction of autophagy, and inhibition ofangiogenesis (13–16). In different cell lines, As2O3 has been shown to induce apoptosis by down-regulation of Bcl-2 (13), activation of caspasases (17), modulation of p53 (17), generation of reactive oxygen species (ROS; ref. 18), and uncoupling of the mitochondrial potential (19).

We and others have shown that clinically relevant concentrations of As2O3 promote cell death in vitro and in vivo not only in drug-sensitive neuroblastoma cell lines but also in the multidrug-resistant p53-mutated/deleted neuroblastoma cell lines, SK-N-BE(2) and LA-N-1 (9–11). These promising results support the possible use of As2O3 in combination therapy in high-risk neuroblastoma patients, and a phase II clinical trial with As2O3 is presently done at the Memorial Sloan-Kettering Cancer Center. We have shown that the As2O3-induced neuroblastoma cell death seems not to involve induced neuronal differentiation but includes increased expression and proteolytic activation of Bax, down-regulation of Bcl-2, and to a slight extent, activation of caspase-3 (9, 10). The activation of Bax seems to be a key event because inhibition of the Bax cleavage prevented As2O3-induced cell death (10). The activation of Bax was also shown to be independent of functional p53 (10), a feature of great interest because deletions or mutations in...
p53 often are seen in high-risk neuroblastoma patients with relapsed, refractory disease (20, 21). In contrast, drugs commonly used in induction treatment of children with advanced neuroblastoma were only able to induce cell death and hence cleavage of Bax in drug-sensitive neuroblastoma cells (10), indicating that a different cytotoxic pathway is induced upon As2O3 treatment.

Hypoxic microenvironments are frequently found in solid tumors as a result of inefficient vascular supply and high oxygen consumption of rapidly proliferating malignant cells. Tumor hypoxia is associated with malignant progression, lower sensitivity to chemotherapy and radiotherapy, increased metastatic potential, and poor prognosis (22, 23). Many of the features in hypoxic tumors are mediated by the hypoxia-inducible factor-1α (HIF-1α) and HIF-2α, transcription factors which modulate the expression of several genes involved in both short-term adaptive mechanisms (e.g., glucose transport and switch to anaerobic metabolism) and long-term adaptive mechanisms (e.g., angiogenesis; ref. 24). In addition to these features, we recently showed that hypoxia alters the gene expression in neuroblastoma cells toward an immature phenotype (25, 26). Because there is a correlation in neuroblastoma between low stage of differentiation and poor clinical outcome, hypoxic adaptation could contribute to increased malignancy of the tumor. Hypoxia-induced dedifferentiation is not restricted to neuroblastoma cells but is also seen in other types of tumors like breast cancer (27) and prostate cancer (28). It has been shown that an As2O3-resistant APL cell line became sensitive and underwent apoptosis when the treatment included both As2O3 and all-trans retinoic acid, a combination which greatly accelerated differentiation in these cells (29). It is therefore of great interest to evaluate if hypoxia-induced adaptive mechanisms like dedifferentiation could impair the efficiency of As2O3 treatment of neuroblastoma cells.

In the present study, we investigated the cytotoxic effect of As2O3 on drug-sensitive and multidrug-resistant neuroblastoma cells, respectively. We observed an evident cytotoxic effect of As2O3 in all tested cell lines also when the cells were cultured under hypoxic growth conditions. Although As2O3 to some extent induced growth inhibition, the main effect of As2O3 was induction of cell death. These results support the idea of using As2O3 as an efficient treatment strategy also in solid tumors with hypoxic areas like neuroblastoma.

Materials and Methods

Cell Culture and Drugs

We used four neuroblastoma cell lines in this study; the SH-SY5Y and SK-N-FI cell lines are gifts from Dr. June Biedler (Memorial Sloan-Kettering Cancer Center); SK-N-BE(2)c and IMR-32 were obtained from American Type Culture Collection (Manassas, VA). SK-N-BE(2)c and IMR-32 cells have a MYCN amplification. The multidrug-resistant SK-N-BE(2)c cell line has a p53 mutation (20, 21). SK-N-BE(2)c and SH-SY5Y were grown in MEM and IMR-32 and SK-N-FI were grown in RPMI 1640. The media were supplemented with 10% FCS, penicillin (100 units/mL), and streptomycin (100 μg/mL; Invitrogen Life Technologies, Paisley, United Kingdom). The cells were cultured at 37°C in a 95% air/5% CO2 humidified incubator. Hypoxic conditions, 1% O2, were created in a Hypoxia workstation 400 (Ruskinn Technology, Leeds, United Kingdom) connected to a Ruskinn gas mixer module.

As2O3 was dissolved in 1.0 mol/L NaOH; vincristine, carboplatin, and doxorubicin were dissolved in distilled H2O and etoposide in DMSO. All cytotoxic drugs were purchased from Sigma (St. Louis, MO).

Cell Viability and Proliferation Assays

The cells were seeded at day 0 under normoxic conditions and the cytotoxic drugs were added at day 1. Thereafter, the cells were grown under normoxia or hypoxia for 72 hours. When indicated, the cells were grown under normoxia or hypoxia for 72 hours before the cytotoxic drugs were added. In these experiments, the cells were reseeded under hypoxic conditions 1 day before initiation of the drug treatments. To assess cell viability, the cells were collected and stained with trypan blue (Sigma). Approximately 200 cells per culture were counted using a light microscope. All treatments were done in triplicates and the experiments were repeated three or four times. The amount of viable cells was compared with untreated normoxic or hypoxic controls, respectively, which were both set to 100%.

The effect of cytotoxic drugs under normoxic conditions was determined in 96-well plates by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (CellTiter 96, Promega, Madison, WI), according to the manufacturer’s recommendation. All experiments were done in triplicates and repeated thrice.

To evaluate the proliferative capacity of neuroblastoma cells after As2O3 treatment, SK-N-BE(2)c cells were seeded at day 0 and varying concentrations of As2O3 were added at day 1. After 72 hours of treatment, the cells were replated in 96-well plates and grown for 24 to 72 hours where 0.5 μCi [3H]thymidine (Amersham Bioscience, Bucks, United Kingdom) was added at the last 24 hours of culture. The cells were harvested and proliferation was measured by incorporation of [3H]thymidine in a beta scintillation counter.

Immunoblot Analysis

Cells were lysed in 10 mmol/L Tris-HCl (pH 7.2), 160 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L EGTA, and 1 mmol/L EDTA in the presence of Complete Protease Inhibitor (Roche Molecular Biochemicals, Mannheim, Germany). For the HIF-1α Western blot, the cells were cultured under hypoxic conditions. Equal amount of protein was separated in SDS-PAGE and blotted onto a Hybond C membrane (Amersham Bioscience) for the HIF-1α Western blot or an Immobilon-P membrane (Millipore Corp., Bedford, MA) for the Bax-Western blot. The following primary antibodies and antiserum were used: anti-actin-antibody (ICN Biochemicals, Aurora, OH) diluted 1:2,000, Bax antiserum (BD PharMingen, San Diego, CA) diluted 1:2,000, anti-caspase-3 antibody (Alexis Biochemicals, San Diego, CA) diluted 1:500, anti-glyceroldehyde-3-phosphate dehydrogenase...
antibody (Chemicon International, Inc., Temecula, CA) diluted 1:2,000, anti-HIF-1α antibody (Novus Biologicals, Littleton, CO) diluted 1:1,000, and anti-PKCα (c-20) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200. The proteins were visualized using horseradish peroxidase–conjugated anti-mouse antibody from Amersham Pharmacia Biotech (Arlington Heights, IL) and Jackson Immunoresearch Laboratories, Inc. (West Grove, PA) and anti-rabbit antibody from DAKO (Glostrup, Sweden) and a Super Signal detection system (Pierce Chemical Co., Rockford, IL).

**Immunocytochemistry**

Cell pellets were fixed with 4% buffered paraformaldehyde, paraffin embedded, and cut into 4-μm-thin sections. The sections were microwave treated with a target retrieval solution (DAKO) at pH 9.9 and the cells were stained with an anti-Ki-67 antibody (DAKO) diluted 1:500. Cell staining was done in a DAKO ChemMate 500 using the Envision protocol according to the manufacturer’s instructions.

**Results**

**Arsenic Trioxide Induces Cell Death in Hypoxic Multidrug-Resistant Neuroblastoma Cells**

We examined three neuroblastoma cell lines, grown under normoxic and hypoxic (1% oxygen) conditions, with regard to their responsiveness to As2O3. In normoxic cultures, the results confirmed our recently published data showing that the p53-mutated and multidrug-resistant neuroblastoma cell line SK-N-BE(2)c is as sensitive to As2O3 as the drug-sensitive SH-SY5Y and IMR-32 cell lines; all cell lines displaying IC50 values in the low micromolar range after 72 hours of treatment (10; Fig. 1A). In addition, As2O3 is toxic to the neuroblastoma cells under hypoxic conditions (Fig. 1A). When treated with 4 μmol/L As2O3 at normoxia and hypoxia, the cultures displayed 45% to 65% of dead cells. At both 1% and 21% oxygen, exposure of the cells to As2O3 gave rise to a concentration-dependent increase of cells with rounded morphology as well as detached floating cells (Fig. 1B). To assure that As2O3 treatment did not prevent induction of a hypoxic response, we investigated the accumulation of the hypoxia-induced transcription factor HIF-1α in normoxic and hypoxic cells treated with 4 μmol/L As2O3 for 4 hours (Fig. 1C). As expected, at normoxia, no HIF-1α protein could be detected in either untreated or As2O3-treated cells (Fig. 1C, lanes 1 and 2), whereas a marked accumulation was observed in untreated hypoxic cells (Fig. 1C, lane 3). Upon treatment with As2O3 at hypoxia, a robust HIF-1α protein stabilization was detected suggesting that an adaptation to the hypoxic environment was induced in these cells (Fig. 1C, lane 4).

To further show the efficiency of As2O3 on hypoxic neuroblastoma cells, we wanted to include an additional multidrug-resistant neuroblastoma cell line. We have previously compared several neuroblastoma cell lines with regard to their sensitivity to vincristine, etoposide, doxorubicin, and carboplatin (9, 10), all of which are chemotherapeutic drugs commonly used in the induction treatment of high-risk neuroblastoma. In comparison with all previously tested cells, we have now identified the MYCN-amplified SK-N-FI cells to be one of the most drug-insensitive cell line (Fig. 2A), displaying resistance to all of the abovementioned drugs at clinically relevant concentrations (30–33). However, despite their multidrug-resistant phenotype and in accordance with the results obtained with the SK-N-BE(2)c cells, the amount of viable SK-N-FI cells was markedly reduced after treatment with

![Figure 1](image-url)

**Figure 1.** As2O3 is toxic to neuroblastoma cells grown under hypoxic conditions. A, the neuroblastoma cell lines SK-N-BE(2)c, SH-SY5Y, and IMR-32 were treated with indicated concentrations of As2O3 and grown under normoxic or hypoxic conditions for 72 h. The cells were harvested and stained with trypan blue and the number of living and dead cells were counted. Points, mean percentage of dead cells or percentage of viable cells compared with untreated controls (n = 9); bars, ±SD. B, phase-contrast photographs showing SK-N-BE(2)c and SH-SY5Y cell morphology after As2O3 treatments for 72 h. C, SK-N-BE(2)c cells were treated with 4 μmol/L As2O3 for 4 h under normoxic or hypoxic conditions. The cells were lysed and 100 μg of protein were subjected to SDS-PAGE and Western blotting. The filter was incubated with an anti-HIF-1α antibody and subsequently with an anti-PKCα antibody to show that proteins were present in all lanes. In vitro – translated (iv tr) GST-tagged HIF-1α was used as a positive control.
As$_2$O$_3$ for 72 hours, displaying an IC$_{50}$ value in the low micromolar range (Fig. 2A). As$_2$O$_3$ induced a pronounced cell death in SK-N-FI cells also under hypoxic growth conditions (Fig. 2B). At hypoxia after 72 hours of treatment with 4 $\mu$mol/L As$_2$O$_3$, the proportion of dead SK-N-FI cells was about 55% (Fig. 2B), a level comparable with those seen in As$_2$O$_3$-treated SH-SY5Y, IMR-32, and SK-N-BE(2)c cells (Fig. 1A).

Arsenic Trioxide Responsiveness in Hypoxic Neuroblastoma Cells Is Due to Induction of Cell Death and to Some Extent Inhibition of Proliferation

It has been shown in different types of cancer cell lines that treatment with As$_2$O$_3$ induces cell death as well as growth inhibition (34). To investigate the possible involvement of growth inhibition upon As$_2$O$_3$ treatment in neuroblastoma cells, normoxic as well as hypoxic paraffin-embedded SK-N-BE(2)c, SH-SY5Y, and SK-N-FI cells were immunostained for the proliferation marker Ki-67. All three untreated cell lines grown under normoxic conditions showed about ≥50% Ki-67-positive cells (Fig. 3A). At normoxia after 72 hours of treatment with 2 $\mu$mol/L As$_2$O$_3$, the proportions of proliferating cells were decreased in all cell lines, indicating that the reduction of viable neuroblastoma cells to some extent is due to decreased proliferation. In the SK-N-BE(2)c cells, showing the highest percentage of Ki-67-positive cells in the normoxic untreated control, the reduction of Ki-67 positivity was much more marked upon As$_2$O$_3$ treatment at normoxia (Fig. 3A). However, in all cell lines, the combination of As$_2$O$_3$ treatment and hypoxic growth conditions did not increase the growth inhibition seen with either of the treatments alone (Fig. 3A). To address the possibility that As$_2$O$_3$ induces a selection of cells with low proliferative capacity, cells treated with As$_2$O$_3$ for 72 hours were replated and grown in the absence of growth factors. The drug-resistant SK-N-FI neuroblastoma cells are sensitive to As$_2$O$_3$ under normoxic and hypoxic conditions. A, SK-N-FI cells were treated with different cytotoxic drugs for 72 h at normoxia. The amount of viable cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Points, mean percentage of viable cells compared with untreated controls ($n=9$); bars, ±SD. B, SK-N-FI cells were grown in the presence of indicated concentrations of As$_2$O$_3$ under normoxic or hypoxic conditions. The cells were collected after 72 h and stained with trypan blue. Columns, mean percentage of dead cells or percentage of viable cells compared with untreated controls ($n=9$); bars, ±SD.

Figure 2. The drug-resistant SK-N-FI neuroblastoma cells are sensitive to As$_2$O$_3$ under normoxic and hypoxic conditions. A, SK-N-FI cells were treated with different cytotoxic drugs for 72 h at normoxia. The amount of viable cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Points, mean percentage of viable cells compared with untreated controls ($n=9$); bars, ±SD. B, SK-N-FI cells were grown in the presence of indicated concentrations of As$_2$O$_3$ under normoxic or hypoxic conditions. The cells were collected after 72 h and stained with trypan blue. Columns, mean percentage of dead cells or percentage of viable cells compared with untreated controls ($n=9$); bars, ±SD.

Figure 3. The proliferating capacity of neuroblastoma cells is to some extent influenced by As$_2$O$_3$ and/or hypoxia. A, the cells were harvested after 72 h of As$_2$O$_3$ treatment at normoxia or hypoxia, fixed in paraformaldehyde, and stained immunocytochemically for the proliferation marker Ki-67. In each section, 200 cells were counted and the average percentage of Ki-67-positive cells from two separate experiments is shown. Bars, the two different measure points. B, SK-N-BE(2)c cells were treated with indicated concentrations of As$_2$O$_3$ for 72 h under normoxic conditions. Thereafter, the cells were replated and grown in absence of As$_2$O$_3$ for 24, 48, or 72 h at normoxia. $^3$H-thymidine was added to the growth medium 24 h before the cells were harvested, the incorporation was measured in a beta scintillation counter. The experiment was repeated three times in triplicates. Columns, means ($n=9$); bars, ±SD.
Arsenic Trioxide Kills Hypoxic Neuroblastoma Cells

Previously, we have shown that at normoxia As$_2$O$_3$ provoked Bax expression in neuroblastoma cells and that p21 Bax was proteolytically cleaved into a p18 Bax form (10), which is more proapoptotic (35, 36). In addition, As$_2$O$_3$ treatment only induced a slight activation of caspase-3 and inhibition of caspase-3 did not prevent cell death, whereas inhibition of Bax cleavage was associated with a decreased As$_2$O$_3$-induced cytotoxicity (10). This suggests that activation of Bax is a key event in As$_2$O$_3$-induced cell death in normoxic neuroblastoma cells and that As$_2$O$_3$ activates a cytotoxic pathway different from that induced by the conventionally used drugs. To clarify whether As$_2$O$_3$ induces a similar cell death pathway in hypoxic neuroblastoma cells, we did Western blot analyses on cell lysates from normoxic and hypoxic cells treated with 2 or 4 µmol/L As$_2$O$_3$ for 72 hours. In accordance with the normoxic samples, the proteolytic activation of Bax was induced in hypoxic SK-N-BE(2)c cells with increasing concentrations of As$_2$O$_3$ (Fig. 4). However, the proportion of cleaved Bax was not as large in the As$_2$O$_3$-treated hypoxic cells as in the normoxic cells (Fig. 4). Caspase-3 was activated to a slight extent in a dose-dependent manner upon As$_2$O$_3$ treatment and the levels of activation were comparable in the normoxic and hypoxic SK-N-BE(2)c cells (Fig. 4).

Neuroblastoma Cells Preexposed to Hypoxia Are Sensitive to As$_2$O$_3$

In the experiments described in Figs. 1 and 2, As$_2$O$_3$ treatment was initiated at the same time as the hypoxic growth condition was induced. However, in tumor environments, there are most likely areas exposed to hypoxia for varying periods of time. Experimental systems where the cells already have been adapted to a hypoxic environment mimics tumor areas exposed to hypoxia for longer periods and allow hypoxia-mediated dedifferentiation (25, 26) and stabilization of proapoptotic as well as antiapoptotic proteins (37) to occur before drug exposure. To further evaluate the potency of As$_2$O$_3$ in hypoxic cells, the multidrug resistant SK-N-BE(2)c cells and the drug-sensitive IMR-32 cells were thus preexposed to normoxic or hypoxic conditions for 72 hours before induction of As$_2$O$_3$ treatment under normoxia or hypoxia for 72 hours. In accordance with the results presented in Fig. 1, both the SK-N-BE(2)c cells and the IMR-32 cells showed a sustained sensitivity to As$_2$O$_3$ under hypoxic growth conditions, although the cells already had adapted to hypoxia when the As$_2$O$_3$ treatment was initiated (Fig. 5). In both cell lines, the percentage of dead cells reached about 45% after treatment with 4 µmol/L As$_2$O$_3$ at normoxia as well as hypoxia and the cell death was consistent with the cell viability data.

Influence of Hypoxia on Cell Death Induced by Etoposide in Drug-Sensitive Neuroblastoma Cells

Several reports indicate that some cytotoxic drugs are less efficient in a hypoxic environment (38, 39), but the results described above suggest that As$_2$O$_3$ does not belong to that group of substances, at least not for treatment of
neuroblastoma cells. To compare the efficiency of As2O3 with other drugs, we examined IMR-32 cells, grown under both normoxic and hypoxic (1% O2) conditions, with regard to its responsiveness to the conventional chemotherapeutic drug etoposide, a drug previously shown to have decreased efficiency under hypoxic conditions (22, 39). The effects of etoposide were tested both without (Fig. 6A) and with (Fig. 6B) preexposure to hypoxia. In agreement with our previous results (10), 72 hours of treatment with 0.5 μmol/L etoposide induced an evident cell death in IMR-32 cells grown under normoxia (Fig. 6). However, a significant difference in cell death as well as amount of viable cells was shown in the hypoxic IMR-32 cells compared with their normoxic counterparts upon etoposide treatment (Fig. 6). At 0.2 μmol/L etoposide, the cytotoxic effect was markedly reduced at hypoxia, which was seen both when the etoposide treatment was initiated at the same time as the hypoxic growth condition was induced (Fig. 6A) and even more pronounced when the cells were preexposed to hypoxia 3 days before the addition of etoposide (Fig. 6B). These results show that in contrast to As2O3, etoposide exhibits a clearly reduced efficacy under hypoxic conditions in IMR-32 cells.

Discussion
In this study, we show a potent cytotoxicity of As2O3 in two drug-sensitive as well as two multidrug-resistant neuroblastoma cell lines both under normoxic and hypoxic growth conditions. We also show that the As2O3 responsiveness seen in hypoxic neuroblastoma cells is only to some extent due to hypoxia- or As2O3-induced growth inhibition but mainly depends on induction of cell death. These results together show that the cytotoxic efficiency of As2O3 is sustained under low oxygen pressures, which is important from a clinical point of view; that is, As2O3 is a potential candidate drug in treatment of solid tumors with hypoxic areas like neuroblastoma. The fact that As2O3 seems to activate a cytotoxic pathway different from that induced by the conventionally used drugs during induction treatment of neuroblastoma and as a result is able to kill multidrug-resistant neuroblastoma cells (10) lends support to the ongoing efforts to introduce the use of As2O3 in combination with existing treatment protocols for patients with multidrug-resistant neuroblastomas.

The precise molecular mechanisms by which As2O3 induces neuroblastoma cell death are yet to be determined. Our previously published results suggest that proteolytically activation of the proapoptotic protein Bax is an important mechanism in normoxic neuroblastoma cells (10). Here we show that cleavage of Bax also occurred in As2O3-treated hypoxic cells but was not as pronounced as in the normoxic cells. This may indicate that induction of p18 Bax is less important for As2O3-induced cell death under hypoxic conditions and that complementary cell death mechanisms may be activated. Further analyses are required to be able to establish to what extent the mechanisms involved in As2O3-induced cell death differ in neuroblastoma cells grown under normoxic or hypoxic conditions. In addition to oxygen pressure, other differences like genotypically changes may influence the mechanisms of As2O3-induced cytotoxicity. For instance, published data indicate that As2O3 induces cell death of myeloma cells by two different mechanisms depending on the p53 status of the cell (40, 41). It is not known whether this is true also for neuroblastoma cells. However, we do show here and in our previous study (10) that As2O3 is a potent cytotoxic drug in wild-type p53 expressing as well as in p53-mutated/deleted multidrug-resistant neuroblastoma cells and that Bax is proteolytically activated by As2O3 treatment in all these different cells.

Pronounced As2O3-induced cell death is still seen when the neuroblastoma cells were preexposed to hypoxia for 3 days before induction of treatment, which shows that the potency of As2O3 is still intact in cells with a well-developed hypoxic phenotype (25, 26). It is possible that the hypoxic environment contributes to cellular conditions supporting the cytotoxic effect of As2O3. For instance, oxidative stress with generation of ROS is believed to be one of the major pathways for arsenic-mediated cytotoxicity (18, 42, 43), although the mechanism(s) and pathway(s) whereby these ROS are generated remain largely unknown. There is also growing evidence that mitochondrial ROS are produced during hypoxia (44). Thus, the combination of hypoxia and As2O3-derived ROS may contribute to the induced cell death seen in hypoxic conditions. For instance, reactive oxygen species (ROS) generated under hypoxia were shown to be important for induction of cell death within neuroblastoma cells grown under hypoxia (44). This adds further support to the ongoing efforts to introduce the use of As2O3 in combination with existing treatment protocols for patients with multidrug-resistant neuroblastomas.
neuroblastoma cells. Based on the literature, there is a formal possibility that hypoxic growth conditions may lead to cellular adaptations, which impair the efficiency of cytotoxic drugs (22, 38, 39). For instance, growth at hypoxia dedifferentiates neuroblastoma cells (25, 26) and a low differentiation stage is correlated to more aggressive neuroblastoma tumors and a poor response to treatment. In contrast, it has been shown that a resistant APL cell line underwent apoptosis upon combined treatment of As$_2$O$_3$ and all-trans retinoic acid, which greatly accelerated differentiation in the cells (29). However, our group has previously shown that As$_2$O$_3$, alone or in combination with retinoic acid, does not induce neuronal differentiation in neuroblastoma cells (9), and although we have not investigated the differentiation stage of the hypoxic As$_2$O$_3$-treated cells, we show that the cytotoxic potency of As$_2$O$_3$ is sustained at hypoxia, despite that hypoxia promotes a stem cell–like phenotype in neuroblastoma cells (25, 26).

When the efficiency of As$_2$O$_3$ and the conventionally used drug etoposide was compared at hypoxia, it became clear that their different mechanisms of action influenced their ability to kill hypoxic neuroblastoma cells. In contrast to As$_2$O$_3$, etoposide showed a reduced cytotoxicity in hypoxic neuroblastoma cells, results that are in keeping with those reported by Unruh et al., which show that cells with no or low levels of HIF-1α are more sensitive to etoposide treatment than cells with higher HIF-1α proteins levels (22). These observations and the successful use of As$_2$O$_3$ in treating refractory APL with minimal toxicity encourage attempts to broaden the application of As$_2$O$_3$ to treatment of other cancer forms as well. The results presented in this study indicate that As$_2$O$_3$ also could be a drug with high potential for efficient treatment of neuroblastoma patients with aggressive tumors, which invariably display areas of severe hypoxia.

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References


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